第五章 離子交換法

離子交換法乃利用分子的帶電性質進行分離，解析力好且具多樣性，是重要而應用極廣的純化方法。

壹、 原理概述

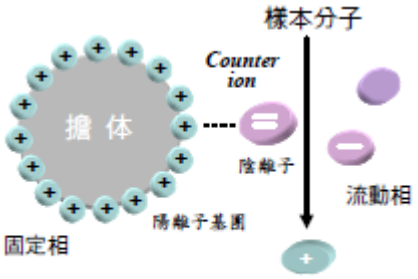


圖 3.6 離子交換法原理

(壹). 離子交換法：
是一種 adsorption 層析法，流動相為溶離緩衝液，固定相為介質擔体表面的帶電基團。 樣本中各種離子，與介質表面帶電基團間的親和力強弱不同，吸附上去之後，可使用不同離子濃度的緩衝液，分別溶離出這些成分 (圖 3.6)。

(貳) 兩大類離子交換介質：
由介質帶電基團的不同，可分為兩大類： 介質-帶電基團 (counter ion)
一、陽離子交換介質 (cation exchanger)： 擔体-陰離子基團 ..... 陽離子
二、陰離子交換介質 (anion exchanger)： 擔体-陽離子基團 ..... 陰離子

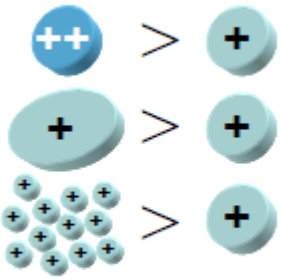


圖 3.7 離子取代順序

(參) Pecking order：
離子交換的進行，可視為各種 counter ions 間，對擔体介質上帶電基團的爭奪戰，離子 (包括蛋白質) 競爭著佔到固体介質上；其競爭優勢順序如下 (圖 3.7)：
一、 帶電荷高者取代帶電荷低者。
二、 電荷相同時，原子序 (或離子体積) 大者優勢。
三、 濃度 可克服以上兩種優勢，高濃度氫離子可取代其它陽離子。

(肆) 離子取代優先順序例：
陽離子： 兩價陽離子 > $NH^{4+}$ > $K^+$ > $Na^+$ > $H^+$ > $Li^+$
陰離子： I- > Br- > Cl- > $HCO_3$- > $CH_3COO$-, OH-

貳、 離子交換介質

(壹) 介質種類：

一、離子交換介質的種類很多，歸納起來分為陰離子及陽離子兩大類；每一類又依其帶電基團的強弱，分為強、中、弱三種。

二、另外依介質的材質不同，略分為合成樹脂 (resin) 及 聚醣 (glycan) 兩種，前者對蛋白質的純化並不適用，只用在小分子樣本的分離上。

三、聚醣多使用 Sephadex, Sepharose, cellulose 等為擔体，在糖分子加上帶電基團；而 cellulose 又有做成結晶球形的 Sephacel，可增加膠柱的流率。

表 3.2 各種離子交換介質

| 陰陽強弱分類 | | Resin / Polystyrene | | Glycan / Cellulose (= X) | |
|---|---|---|---|---|---|
| Anion Exchanger | Strong | Dowex-1<br>Dowex-2 | $-\overset{+}{N}R_3$ | TEAE-X<br>(QAE-X) | $-\overset{+}{N}R_3$ |
| | Weak | Dowex-3<br>IR-45 | $-\overset{+}{N}HR_2$ | DEAE-X | $-OCH_2CH_2\overset{+}{N}HR_2$ |
| Cation Exchanger | Strong | Dowex-50 | $-SO_3^-$ | Phospho-X | $-PO_4^{-2}$ |
| | Weak | IRC-150 | $-COO^-$ | CM-X | $-CH_2COO^-$ |

X = Sephadex, Sepharose, Sephacel or cellulose

(貳) 選擇交換介質：

一、若已知樣本蛋白質的 pI，則先選擇適當緩衝液的 pH，使蛋白質帶有正 (或負) 電，而採用陽 (或陰) 離子交換介質。

二、若尚不知樣本的 pI 時，則可用試管裝少量介質，在各種緩衝液 pH 下(2-10)，加入樣本蛋白質，然後測上清中有無酵素活性，即得知樣本蛋白質有無吸附上去，可選得適當的介質及緩衝液 pH。

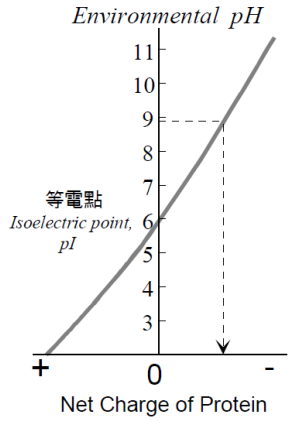



圖 3.1 環境 pH 的影響

(參) 一般使用：

一、 通常在純化蛋白質時，都使用較弱的離子交換介質，如 DEAE 或 CM(carboxymethyl)；介質則用聚醣類為材料，多使用 Sepharose CL-6B 或 Sephacel，而不用体積變化太劇烈的 Sephadex，或流率較差的 cellulose。

二、 Sepharose 本來有膠体過濾的作用，應用在離子交換時，作用並不明顯；但在分離異構酶時，因各個異構酶的分子量相近，不要使用。

三、 DEAE 型膠體使用的 pH 不能高過 9，CM 者不能低於 pH 6，否則介質會失去原先帶有的電荷。

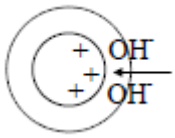
(肆) 介質容量有限：

一、 離子交換介質與蛋白質的結合量有一定限度，稱為該交換介質的 容量 (capacity)；若超過此一容量，多出的樣本會直接流出。

二、 交換介質的結合容量大小，受層析條件不同、蛋白質種類不同、緩衝液不同、pH 或離子濃度不同等，有很大的差異。 如 DEAE-Sepharose CL-6B 每 100 mL 可結合 11 克白蛋白，

但對 ferritin 只有 0.4 克。

(伍) 介質表面的微環境：




一、 由於交換介質的帶電性，其微視環境中的 pH，並不成為一均勻的狀態。

二、. 緊靠近介質表面的 pH，要比外圍緩衝液的 pH 相差一個 pH 單位左右： 陰離子交換介質高一個單位，陽離子者低一個單位 (稱為 Donnan effect)。

(陸) 氫氧基磷灰石(Hydroxylapatite)-Bio-Rad：

一、 可與 DNA 或 RNA(蛋白質也可)結合，原本用在分離單股與雙股 DNA，是一種結晶型的磷酸鈣，其作用機制不很清楚，但顯然與其帶電性質有關。

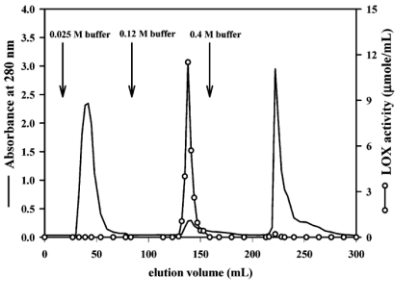



**Figure 2.** Elution profile of banana leaf LOX on hydroxyapatite column (2.6 × 15 cm). Column was equilibrated with 25 mM phosphate buffer (pH 6.3) containing 0.1% Triton X-100 and eluted stepwise with 0.12 and 0.4 M potassium phosphate buffer at a flow rate of 1 mL/min. Fractions of 3 mL were collected and assayed for protein as absorbance at 280 nm and for LOX activity. (banana leaf LOX)

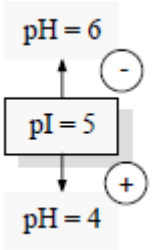
二、 操作法與離子交換法類似，在低離子濃度(0.02M 磷酸 buffer)時使蛋白質結合上去，再以高濃度溶離下(如 0.2M 磷酸 buffer)，但較複雜；不同的鹽類 (如 磷酸鹽, NaCl 或 CaCl2)，會有不同的溶離結果，要以實驗嘗試求得。

參、 緩衝液與層析系統

(壹) 緩衝液種類：

可能會影響離子交換結果，例如 DEAE 介質若使用磷酸緩衝液，則其中的磷酸根離子 (帶兩個負電) 與交換介質的結合力相當強，會影響樣本蛋白質的結合。但反過來說，此時能夠結合上去的離子，一定有相當的強度。(故一般 DEAE 介質會用 Tris-buffer)

(貳) 緩衝液的 pH：




一、 可定在樣本蛋白質 pI 的上或下一個 pH 單位，使樣本分子帶有正確電荷，能夠結合到所選用的介質上去，但又不會太強，以免難以溶離下來。

二、 用酸鹼度溶離時，當緩衝液的 pH 趨近樣本分子的 pI 在 0.5 pH 單位以內，蛋白質會開始溶離出來。

三、 所用緩衝液的離子濃度，在不影響蛋白質與介質的結合能力下，儘量採稍高的濃度，以降低非必要性的吸附，通常在 10~100 mM (NaCl) 之間。

(參) 膠体 pH 要平衡好：

決定緩衝液的 pH 與離子濃度後，交換介質要先平衡在此緩衝液中，如膠体過濾法一樣，可在玻璃漏斗中進行。為加速平衡達成，交換介質可先用 10×濃度緩衝液浸泡(10V)沖洗，然後再用 1×者澈底洗過。

(肆) 管柱系統：

離子交換法所用的管柱系統，其要求比膠体過濾法嚴格，最好使用附有 adaptor 的 Pharmacia 管柱 (K 或 C column)，可降低無效空間，避免梯度破壞。與膠体過濾相反，多使用矮胖型的管柱，太長並無必要。

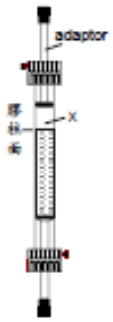
(伍) 膠体裝填：

裝填方法與膠体過濾法一樣，但要求反較不嚴格；裝填完成後，要以緩衝液洗過數個体積後方可使用。 不能使用 Blue Dextran，只用手電筒檢查有無氣泡。Sepharose 或 Sephacel 介質可耐高流速的壓力，但流速過快可能影響解析力。

伍、 管柱操作方法

(壹) 樣品蛋白質液：

樣品必須平衡在管柱所使用的緩衝液中，否則要先對緩衝液透析。樣品溶液的体積並無限制，因蛋白質會結合到交換介質上，溶離下來時有濃縮效果；若目標蛋白質不吸附到介質，而直接通過交換介質，則其條件同膠体過濾法。




(貳) 溶離方法：

一、 可用 pH 或鹽梯度；溶離方式有連續梯度 (continuous gradient)及階段梯度 (stepwise gradient)_如右圖。

二、 但 pH 的連續梯度不容易拉得好，因此一般較少使用，可改用階段梯度，或色層焦集法。

三、 由於是以濃度的梯度來溶離，因此在離子交換管柱中，膠体上方不能積有緩衝液層 (無效空間，如左圖 X 所指)，否則做好的梯度會在此處破壞，失去梯度的連續性；因此離子交換管柱最好能使用 adaptor。

(參) 梯度体積：

會影響結果，通常溶離体積較大時，解析度較佳；但体積太大時，會使溶離出來的蛋白質濃度變稀。 而梯度的上下範圍 (如 0~0.3 M NaCl) 也要適當，範圍太寬或太窄，均會降低解析度；都要以實驗試出最佳條件。

(肆) 膠体再生：

一、蛋白質全部溶離出來後，交換介質要經過再生(regeneration) 後，才能再次使用。

二、以 1~2 M NaCl 即可洗去雜蛋白，澈底清洗可用 0.1 M NaOH 流洗；陰離子交換介質可用 1 M 醋酸鈉 (pH 3.0) 洗 1.5 個体積；再以緩衝液平衡完全，才能再度使用。

三、可測流出液的 pH 或離子濃度，是否與加入的緩衝液一樣。也可把膠体取出，在燒杯或漏斗中澈底清洗。大多數失敗是因於再生不良！

(伍) 批次法：

離子交換法不一定要在管柱中進行，也可在燒杯中以批次法(batch)吸附並溶離蛋白質，一般

應用在工業上的大量純化，其效果較差。

伍、 色層焦集法

(壹). 也是一種離子交換法：

一、 若非使用 pH 梯度進行溶離不可，則可改用 Pharmacia 發展的色層焦集法。 此法使用類似 DEAE-Sepharose 的陰離子交換介質 (polyethyleneimine agarose)，在管柱中以特殊的緩衝液 (Polybuffer) 流洗以形成 pH 梯度。

二、 Polybuffer 中含有如同 等電焦集法所使用的 ampholyte，以較低的 pH 通入

管柱，與介質上面的鹼性基團中和，由酸 (上方進入管柱處) 漸鹼 (出口處)，直接在管柱中形成 pH 梯度。

(貳) 作用機制：

樣本蛋白質進入色層焦集管柱後，先遇到較高 pH 環境 (介質)，通常高於其 pI 而帶負電，因此會結合到介質上。 當 Polybuffer 開始注入管柱，降低環境 pH，使樣本分子失去負電荷而溶離下來；蛋白質便依 pI 大小順序，pI 高的先溶離出來；同時會集中在其 pI 的地方，成為一條極細色帶，故稱為焦集法。

(參) 注意發生沉澱：

有些酵素若處在其 pI 的環境，會發生不可逆的沉澱而失去活性，則不適用以 pI 為分離基礎的純化方法。一般較少使用色層焦集法，除非一定要以 pI 或 pH 梯度來作分離，否則儘量採用其他方法。

## 陸、離子交換(ion exchange, IEX)實務

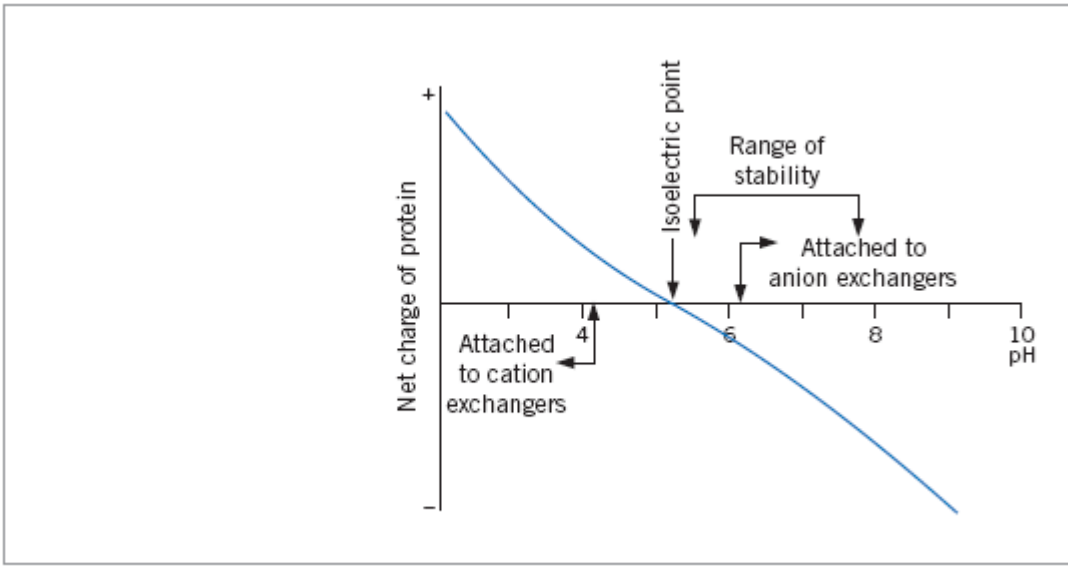



**Fig. 18.** Considerations when selecting a suitable IEX medium.

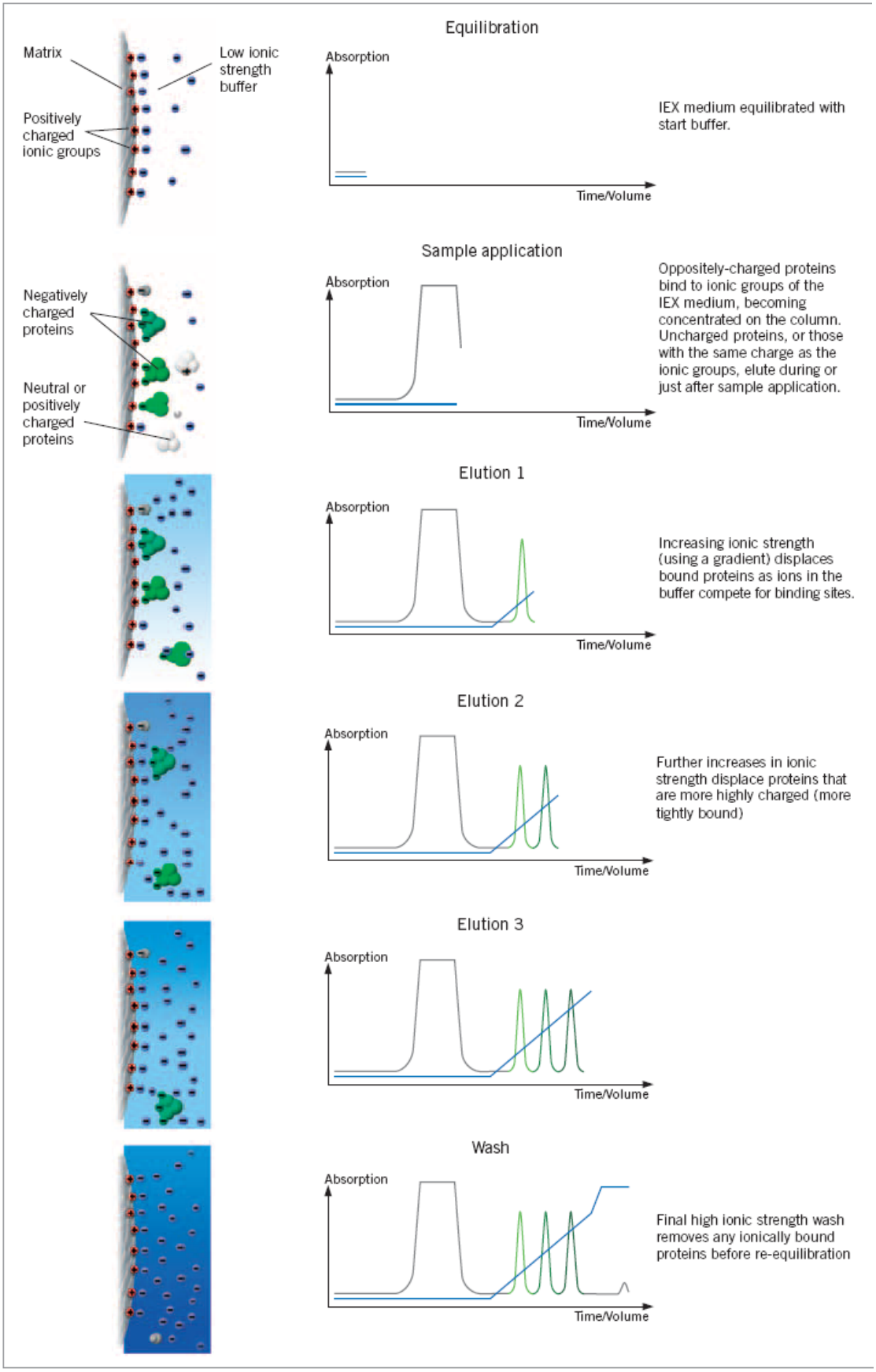



**Fig. 3.** Principles of an anion exchange separation.

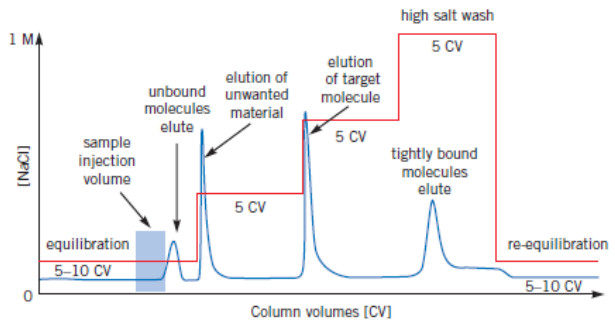



**Fig. 10b.** Typical IEX separation using step elution (25–30 column volumes).

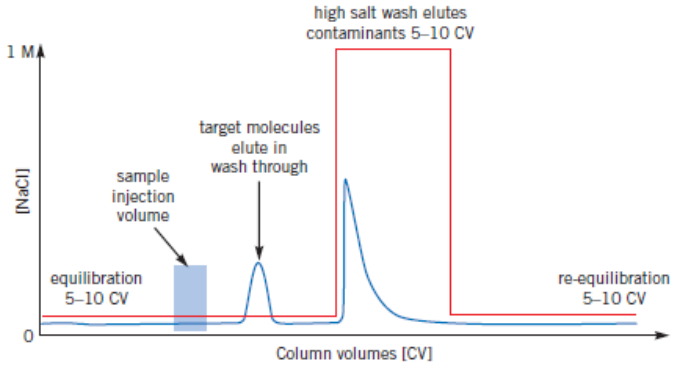



**Fig. 10d.** Contaminant removal: target protein(s) elute in the wash through, contaminants bind to the column.

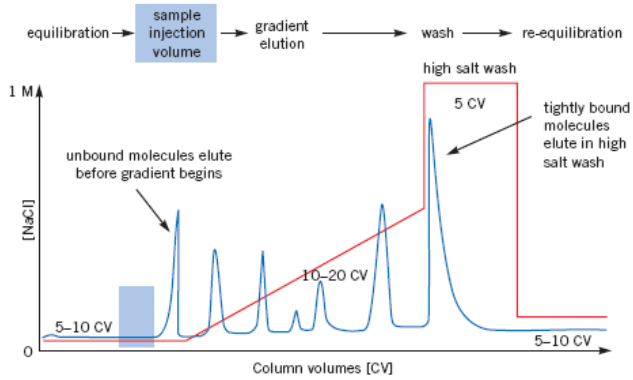



**Fig. 10a.** Typical high resolution IEX separation using linear gradient elution (25–45 column volumes).

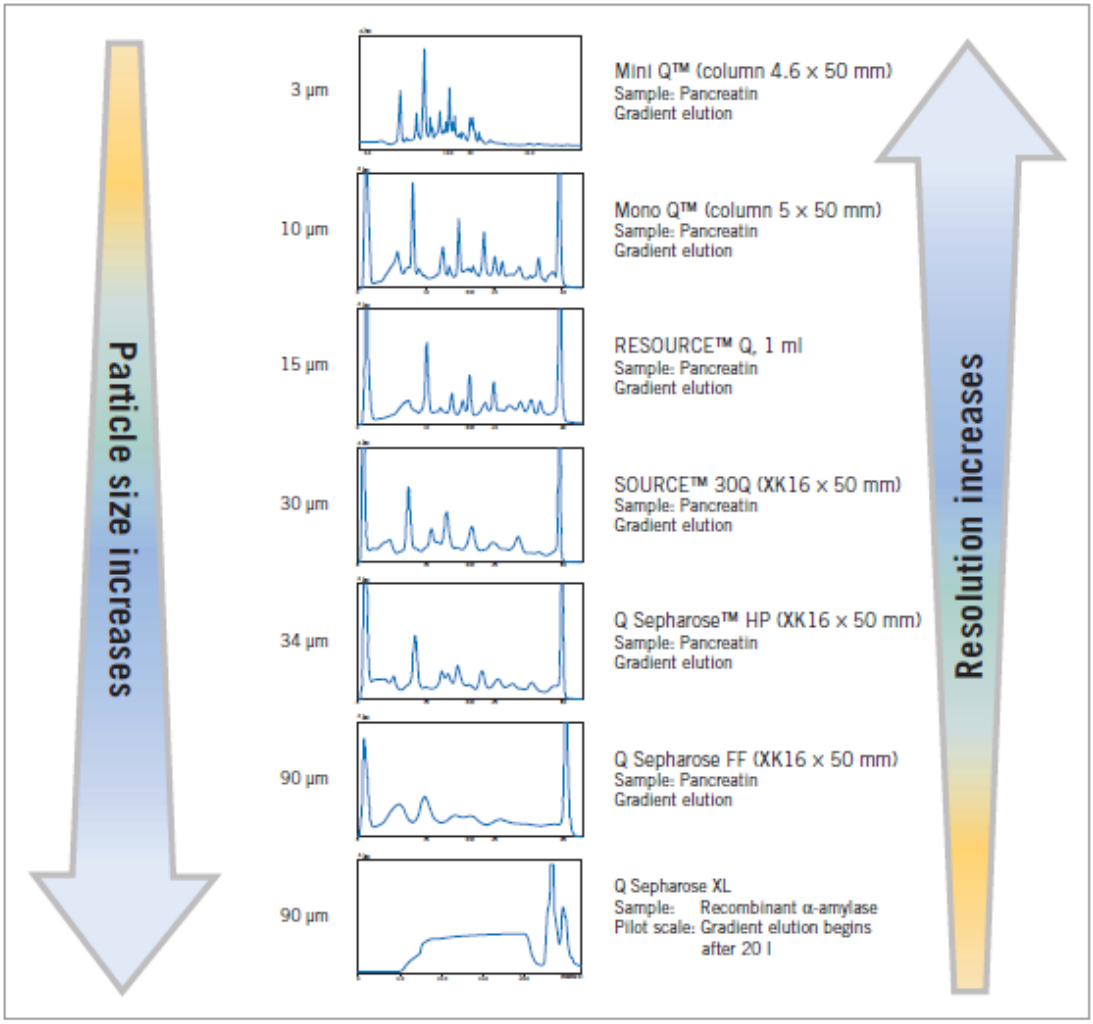


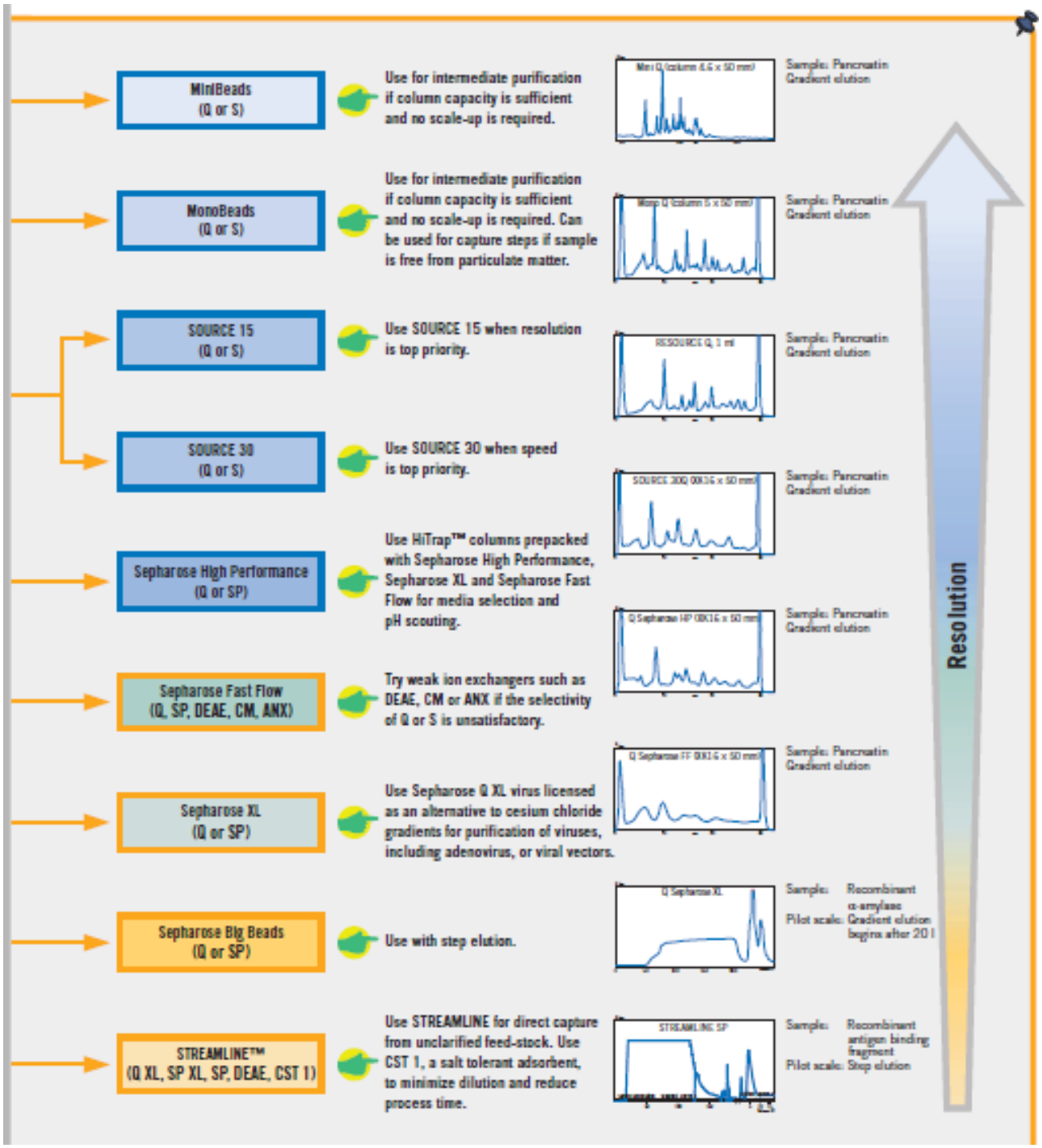

Fig. 7. The influence of particle size and selectivity on final resolution.

Modern IEX media use either polymer or agarose-based matrices to fulfil not only the requirements for high binding capacity, chemical and physical stability, but to generate media with suitable particle sizes for a range of applications (Table 1).

MiniBeads is a matrix made from polystyrene, with divinyl benzene as cross-linker, to produce highly spherical (monodispersed), very small (3 µm), non-porous particles that facilitate micropreparative or analytical separations when extremely high resolution is more important than high binding capacity or high flow rates.

Table 1. Ion exchange matrices.

| | Form | Mean particle size |
|---|---|---|
| MiniBeads™ | Polystyrene/divinyl benzene | 3 µm |
| MonoBeads™ | Polystyrene/divinyl benzene | 10 µm |
| SOURCE 15 | Polystyrene/divinyl benzene | 15 µm |
| SOURCE 30 | Polystyrene/divinyl benzene | 30 µm |
| Sepharose High Performance | Agarose 6% | 34 µm |
| Sepharose Fast Flow | Agarose 6% | 90 µm |
| Sepharose 4 Fast Flow | Agarose 4% | 90 µm |
| Sepharose XL | Agarose 6%, dextran chains coupled to agarose | 90 µm |
| Sepharose Big Beads | Agarose 6% | 200 µm |




Functional groups The functional groups substituted onto a chromatographic matrix (Table 2) determine the charge of an IEX medium i.e. a positively-charged anion exchanger or a negatively-charged cation exchanger.

Table 2. Functional groups used on ion exchangers.

| Anion exchangers | | Functional group |
|---|---|---|
| Quaternary ammonium (Q) | strong | $-O-CH_2N^+(CH_3)_3$ |
| Diethylaminoethyl (DEAE)* | weak | $-O-CH_2CH_2N^+H(CH_2CH_3)_2$ |
| Diethylaminopropyl (ANX)* | weak | $-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$ |
| **Cation exchangers** | | **Functional group** |
| Sulfopropyl (SP) | strong | $-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO_3^-$ |
| Methyl sulfonate (S) | strong | $-O-CH_2CHOHCH_2OCH_2CHOHCH_2SO_3^-$ |
| Carboxymethyl (CM) | weak | $-O-CH_2COO^-$ |

** The active end of the charged group is the same for DEAE and ANX. The difference between them is in the length of the carbon chain of the charged group. DEAE has a diethylaminoethyl-group bound to the agarose. ANX has a diethylaminopropyl-group attached which prevents the formation of quaternary groups, giving a different selectivity compared to DEAE.*

## Purification options

Fig. 27. Mini Q and Mini S™ media are available prepacked in T ricorn™ (4.6/50 PE) and Precision (PC 3.2/3) columns.

| Product, column volume | Binding capacity per column | Maximum flow | Recommended working flow | Working pH range* | Maximum operating back pressure ** (MPa/psi) *1 MPa=10 bar* |
|---|---|---|---|---|---|
| **Strong anion exchangers** | | | | | |
| Mini Q PC 3.2/3, 0.24 ml*** | 1.44 mg (α-amylase, $M_r$ 49 000)<br>1.44 mg (trypsin inhibitor, $M_r$ 20 100) | 1 ml/min | 0.1–1.0 ml/min | 3–11 | 10/1450 |
| Mini Q 4.6/50 PE, 0.8 ml | 4.8 mg (α-amylase, $M_r$ 49 000)<br>4.8 mg (trypsin inhibitor, $M_r$ 20 100) | 2 ml/min | 0.5–2.0 ml/min | 3–11 | 18/2600 |
| **Strong cation exchangers** | | | | | |
| Mini S PC 3.2/3, 0.24 ml*** | 1.2 mg (ribonuclease, $M_r$ 13 700)<br>1.2 mg (lysozyme, $M_r$ 14 300) | 1 ml/min | 0.1–1.0 ml/min | 3–11 | 10/1450 |
| Mini S 4.6/50 PE, 0.8 ml | 4 mg (ribonuclease, $M_r$ 13 700)<br>4 mg (lysozyme, $M_r$ 14 300) | 2 ml/min | 0.5–2.0 ml/min | 3–11 | 18/2600 |

## Purification examples

### Fast separations at high resolution

*Column:* Mini S 4.6/50 PE
*Sample:* α-chymotrypsinogen A (25 µg/ml)
ribonuclease A (75 µg/ml)
lysozyme (25 µg/ml)
*Sample volume:* 200 µl
*Start buffer:* 20 mM sodium acetate, pH 5.0
*Elution buffer:* 20 mM sodium acetate, 0.4 M NaCl, pH 5.0
*Flow:* 0.83 ml/min
*Gradient:* 0–100% elution buffer in 12 CV

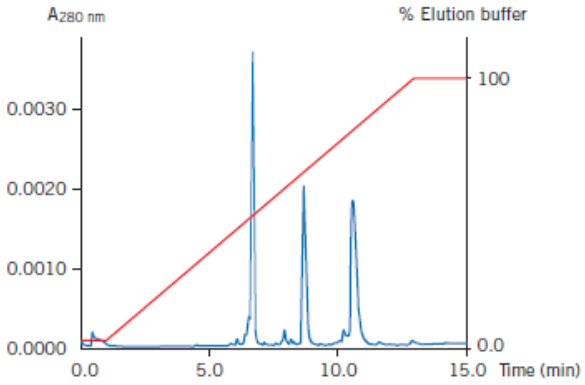



**Fig. 28.** Separation of a protein mixture on Mini S 4.6/50.

### Purity check

*Column:* Mini Q 4.6/50 PE
*Start buffer:* 10 mM NaOH
*Elution buffer:* 10 mM NaOH, 2 M NaCl
*Flow:* 1.0 ml/min

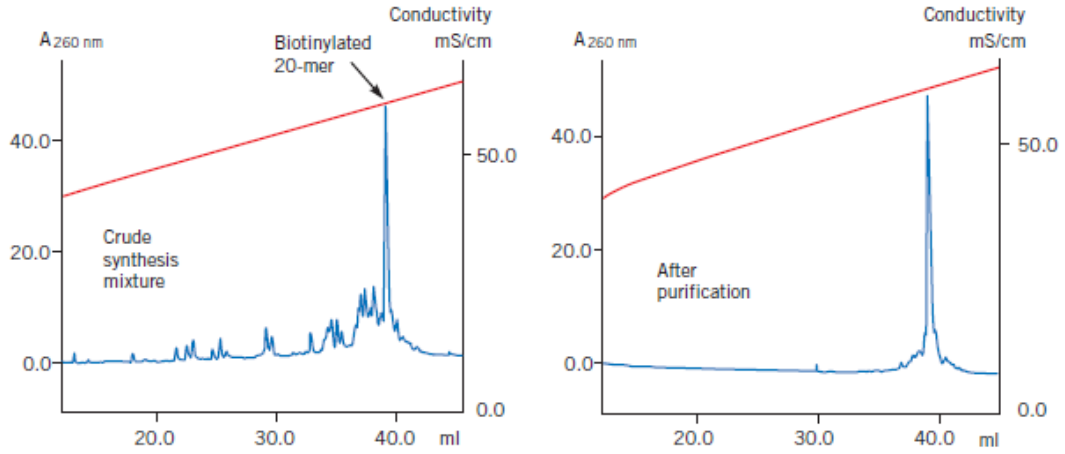



**Fig. 30.** Purity check of 5'-biotinylated synthetic oligonucleotide 20-mer on Mini Q 4.6/50 PE before and after purification on a RESOURCE RPC column.

## 管柱清洗

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

## 移除管柱汙染物常用的處理法

1. Wash with 2 column volumes of 2 M NaCl at 0.2 ml/min.
2. Wash with 4 column volumes of 1 M NaOH at 0.2 ml/min.
3. Wash with 2 column volumes of 2 M NaCl at 0.2 ml/min.
4. Rinse with at least 2 column volumes of distilled water at 0.2 ml/min until the UV-baseline and elutent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer at 0.2 ml/min until pH and conductivity values have reached the required values.

## 化學穩定性

For daily use, MiniBeads are stable in all common aqueous buffers in the range pH 3–11 and in the

presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride), non-ionic or ionic detergents and up to 30% acetonitrile in aqueous buffers. Note that aqueous solutions of urea, ethylene glycol and similar compounds will increase the back-pressure due to increased viscosity. MiniBeads can be used with organic solutions such as dimethylsulfoxide, dimethylformamide or formic acid, but the separation properties of the media will change. Avoid anionic detergents with Mini Q. Avoid cationic detergents with Mini S. Avoid oxidizing agents.

## 貯藏

For column storage, wash with 4 column volumes of distilled water followed by 4 column volumes of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Ensure that the column is sealed well to avoid drying out. Do not freeze.

| Product | Functional group | pH stability* | Mean particle size |
|---|---|---|---|
| Mini Q | $-CH_2N^+(CH_3)_3$ | Long term: 3–11<br>Short term: 1–14 | 3 µm (monosized) |
| Mini S | $-CH_2SO_3^-$ | Long term: 3–11<br>Short term: 1–14 | 3 µm (monosized) |

## Purification options

Fig. 34. MonoBeads (Q and S) are available prepacked in Tricorn PE (PEEK) and Tricorn GL (glass) columns.

| Product, column volume | Binding capacity per column | Recommended working flow | Maximum flow | Working pH range* | Maximum operating back pressure ** (MPa/psi) *1 MPa=10 bar* |
|---|---|---|---|---|---|
| **Strong anion exchangers** | | | | | |
| Mono Q 5/50 GL, 1 ml | 25 mg (thyroglobulin, $M_r$ 669 000)<br>65 mg (HSA, $M_r$ 68 000)<br>80 mg (α-lactalbumin, $M_r$ 14 300) | 0.5–3.0 ml/min | 3 ml/min | 2–12 | 4/580 |
| Mono Q 4.6/100 PE, 1.7 ml | 40 mg (thyroglobulin, $M_r$ 669 000)<br>110 mg (HSA, $M_r$ 68 000)<br>140 mg (α-lactalbumin, $M_r$ 14 300) | 0.5–3.0 ml/min | 3 ml/min | 2–12 | 4/580 |
| Mono Q 10/100 GL, 8 ml | 200 mg (thyroglobulin, $M_r$ 669 000)<br>520 mg (HSA, $M_r$ 68 000)<br>640 mg (α-lactalbumin, $M_r$ 14 300) | 2.0–6.0 ml/min | 10 ml/min | 2–12 | 4/580 |
| Mono Q HR 16/10, 20 ml | 500 mg (thyroglobulin, $M_r$ 669 000)<br>1300 mg (HSA, $M_r$ 68 000)<br>1600 mg (α-lactalbumin, $M_r$ 14 300) | up to 10 ml/min | 10 ml/min | 2–12 | 3/435 |
| **Strong cation exchangers** | | | | | |
| Mono S 5/50 GL, 1 ml | 75 mg (human IgG, $M_r$ 160 000)<br>75 mg (ribonuclease, $M_r$ 13 700) | 0.5–3.0 ml/min | 3 ml/min | 2–12 | 4/580 |
| Mono S 4.6/100 PE, 1.7 ml | 130 mg (human IgG, $M_r$ 160 000)<br>130 mg (ribonuclease, $M_r$ 13 700) | 0.5–3.0 ml/min | 3 ml/min | 2–12 | 4/580 |
| Mono S 10/100 GL, 8 ml | 600 mg (human IgG, $M_r$ 160 000)<br>600 mg (ribonuclease, $M_r$ 13 700) | 2.0–6.0 ml/min | 10 ml/min | 2–12 | 4/580 |
| Mono S HR 16/10, 20 ml | 1500 mg (human IgG, $M_r$ 160 000)<br>1500 mg (ribonuclease, $M_r$ 13 700) | up to 10 ml/min | 10 ml/min | 2–12 | 3/435 |

## Purification examples

### Two step purification using complementary selectivities

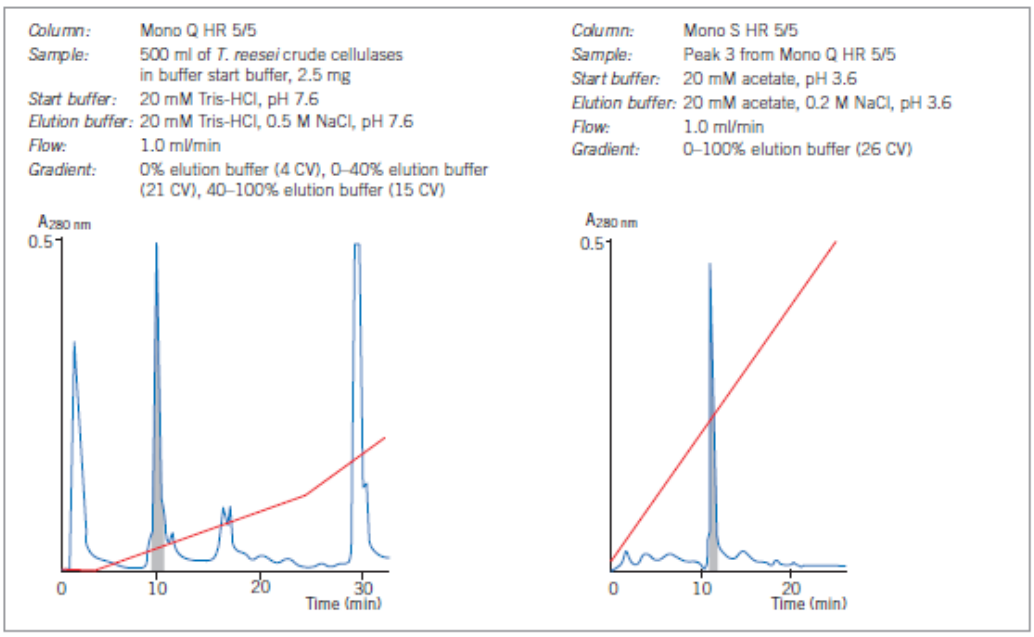



Fig. 35. Purification of cellulose on Mono Q and Mono S HR 5/5 columns (now available as Mono Q 5/50 GL and Mono S 5/50 GL).

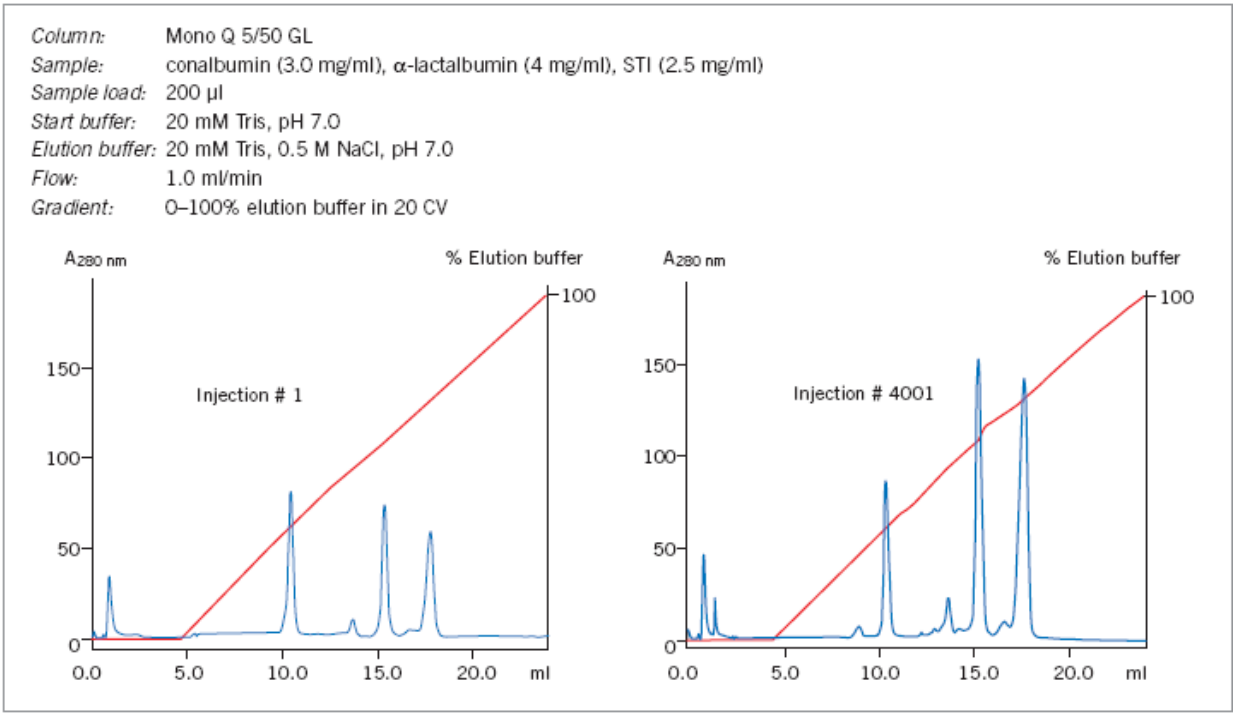



Fig. 37. Chromatograms illustrating run to run reproducibility for Mono Q 5/50 GL (Tricorn). Runs 1, 1000 and 2000 are shown.

| Product | Functional group | pH stability* | Mean particle size |
|---|---|---|---|
| Mono Q | $-CH_2N^+(CH_3)_3$ | Long term: 2–12<br>Short term: 2–14 | 10 µm (monosized) |
| Mono S | $-CH_2SO_3^-$ | Long term: 2–12<br>Short term: 2–14 | 10 µm (monosized) |

## 化學穩定性

For daily use, MonoBeads are stable in all common, aqueous buffers in the range pH 2–12, and in the presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride), non-ionic or ionic detergents and up to 20% acetonitrile in aqueous buffers. Note that aqueous solutions of urea, ethylene glycol and similar compounds will increase the back-pressure due to increased viscosity. MonoBeads can be used with organic solutions such as dimethylsulfoxide, dimethylformamide or formic acid, but the separation properties of the media will change. Avoid anionic detergents with Mono Q. Avoid cationic detergents with Mono S. Avoid oxidizing agents.

## 貯藏

For column storage, wash with 5 column volumes of distilled water followed by 5 column volumes of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Do not freeze.

**SOURCE**: purification at high throughput with high resolution and easy scale-up

## Purification options

Fig. 40. SOURCE is available in media packs and prepacked in Tricorn or RESOURCE columns.

## Purification examples

### Fast, high resolution separations

| | |
|---|---|
| *Column:* | RESOURCE Q 1 ml |
| *Sample:* | pancreatin 5 mg/ml |
| *Sample volume:* | 200 µl |
| *Start buffer:* | 20 mM bis-Tris-propane, pH 7.5 |
| *Elution buffer:* | 20 mM bis-Tris-propane, 0.5 M NaCl, pH 7.5 |
| *Flow:* | 9.6 ml/min, 1800 cm/h |
| *Gradient:* | 0–80% elution buffer in 20 CV |

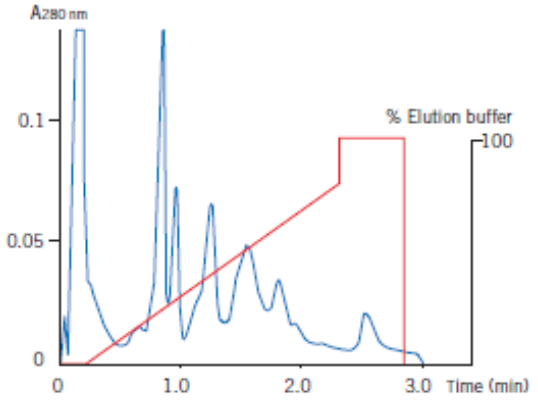



Fig. 41. Separation of pancreatin on RESOURCE Q, 1 ml within 3 minutes.

| | |
|---|---|
| *Column:* | RESOURCE S 1 ml |
| *Sample:* | snake venom, 4 mg/ml |
| *Sample volume:* | 100 µl |
| *Start buffer:* | 20 mM sodium phosphate, pH 6.8 |
| *Elution buffer:* | 20 mM sodium phosphate, 0.4 M NaCl, pH 6.8 |
| *Flow:* | 1 ml/min, 180 cm/h |
| *Gradient:* | 0–100% elution buffer in 20 CV |

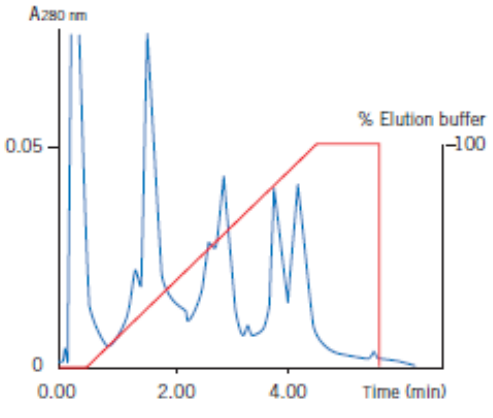



Fig. 42. Separation of snake venom on RESOURCE S, 1 ml within 4 minutes.

| Product, column volume | Binding capacity per column or per ml medium | Recommended working flow* | Maximum flow* | Working pH range** | Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar* |
|---|---|---|---|---|---|
| **Strong anion exchangers** | | | | | |
| RESOURCE Q 1 ml | 45 mg (BSA, $M_r$ 67 000) | 1.0–10 ml/min | 10 ml/min | 2–12 | 1.5/220 |
| RESOURCE Q 6 ml | 270 mg (BSA, $M_r$ 67 000) | 1.0–60 ml/min | 60 ml/min | 2–12 | 0.6/87 |
| SOURCE 15Q 4.6/100 PE, 1.7 ml | 75 mg (BSA, $M_r$ 67 000) | 0.5–2.5 ml/min | 5 ml/min | 2–12 | 4/580 |
| SOURCE 15Q | 45 mg/ml (BSA, $M_r$ 67 000) | 150–900 cm/h | 1800 cm/h | 2–12 | 0.5/72 |
| SOURCE 30Q | 40 mg/ml (BSA, $M_r$ 67 000) | 300–1000 cm/h | 2000 cm/h | 2–12 | 0.5/72 |
| **Strong cation exchangers** | | | | | |
| RESOURCE S 1 ml | 80 mg (lysozyme, $M_r$ 14 500) | 1.0–10 ml/min | 10 ml/min | 2–13 | 1.5/220 |
| RESOURCE S 6 ml | 480 mg (lysozyme, $M_r$ 14 500) | 1.0–60 ml/min | 60 ml/min | 2–13 | 0.6/87 |
| SOURCE 15S 4.6/100 PE, 1.7 ml | 140 mg (lysozyme, $M_r$ 14 500) | 0.5–2.5 ml/min | 5 ml/min | 2–13 | 4/580 |
| SOURCE 15S | 80 mg/ml (lysozyme, $M_r$ 14 500) | 150–900 cm/h | 1800 cm/h | 2–13 | 0.5/72 |
| SOURCE 30S | 80 mg/ml (lysozyme, $M_r$ 14 500) | 300–1000 cm/h | 2000 cm/h | 2–13 | 0.5/72 |

| Product | Functional group | pH stability* | Mean particle size |
|---|---|---|---|
| SOURCE 15Q | $-CH_2N^+(CH_3)_3$ | Long term: 2–12<br>Short term: 1–14 | 15 µm (monosized) |
| SOURCE 30Q | $-CH_2N^+(CH_3)_3$ | Long term: 2–12<br>Short term: 1–14 | 30 µm (monosized) |
| SOURCE 15S | $-CH_2SO_3^-$ | Long term: 2–13<br>Short term: 1–14 | 15 µm (monosized) |
| SOURCE 30S | $-CH_2SO_3^-$ | Long term: 2–13<br>Short term: 1–14 | 30 µm (monosized) |

## 管柱清洗

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants. It is recommended to reverse the direction of flow during column cleaning so that contaminatants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

## 移除管柱汙染物常用的處理法

1. Wash with at least 2 column volumes of 2 M NaCl at 0.2 ml/min (SOURCE 15 4.6/100 PE), 1 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml) or 40 cm/h with a contact time of 1–2 h for SOURCE packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as in step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as in step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as in step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as in step 1) until eluent pH and conductivity have reached the required values.

## 化學穩定性

For daily use, SOURCE media are stable in all common, aqueous buffers pH 2–12, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 75% acetic acid, 1 M NaOH, 1 M HCl, 70% ethanol, 30% acetonitrile and with additives such as non-ionic detergents. Avoid cationic detergents with SOURCE S. Avoid anionic

detergents with SOURCE Q. Avoid oxidizing agents.

貯藏

For column storage, wash with 5 column volumes of distilled water followed by 5 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for SOURCE S. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C +30° C in 20% ethanol. Do not freeze.

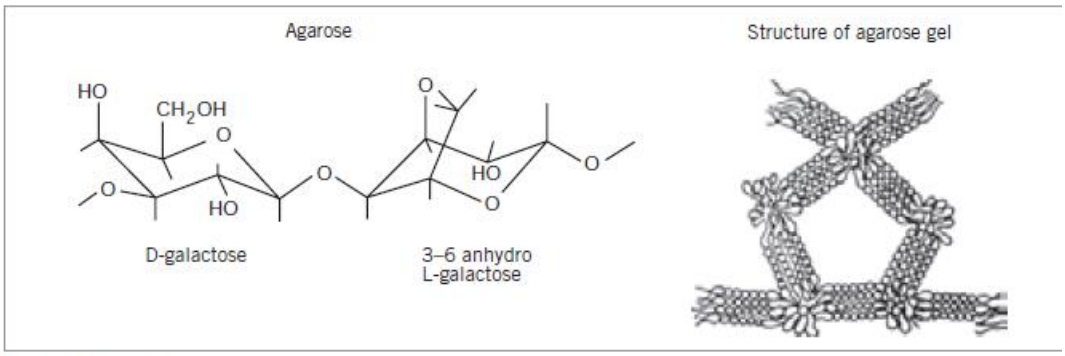



Fig. 55. Partial structure of agarose.

Purification options

Fast media selection and method development

Fig. 16. IEX Selection Kit.

Fig. 49. Q and S Sepharose High Performance media are available prepacked in HiT rap and HiLoad columns or in media packs.

| Product | Binding capacity per column or per ml medium | Recommended working flow* | Maximum flow* | Working pH range** | Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar* |
|---|---|---|---|---|---|
| **Strong anion exchangers** | | | | | |
| HiTrap Q HP, 1 ml | 50 mg (HSA, $M_r$ 68 000) | up to 1 ml/min | 4 ml/min | 2–12 | 0.3/43 |
| HiTrap Q HP, 5 ml | 250 mg (HSA, $M_r$ 68 000) | up to 5 ml/min | 20 ml/min | 2–12 | 0.3/43 |
| HiLoad 16/10 Q Sepharose High Performance, 20 ml | <1200 mg (BSA, $M_r$ 67 000) | up to 5 ml/min | 5 ml/min | 2–12 | 0.3/43 |
| HiLoad 26/10 Q Sepharose High Performance, 53 ml | <3000 mg (BSA, $M_r$ 67 000) | up to 13 ml/min | 13 ml/min | 2–12 | 0.3/43 |
| Q Sepharose High Performance | 70 mg/ml (HSA, $M_r$ 68 000) | 30–150 cm/h | 150 cm/h | 2–12 | 0.5/72 |
| **Strong cation exchangers** | | | | | |
| HiTrap SP HP, 1 ml | 55 mg (ribonuclease, $M_r$ 13 700) | up to 1 ml/min | 4 ml/min | 4–13 | 0.3/43 |
| HiTrap SP HP, 5 ml | 275 mg (ribonuclease, $M_r$ 13 700) | up to 5 ml/min | 20 ml/min | 4–13 | 0.3/43 |
| HiLoad 16/10 SP Sepharose High Performance, 20 ml | <1000 mg (ribonuclease, $M_r$ 13 700) | up to 5 ml/min | 5 ml/min | 4–13 | 0.3/43 |
| HiLoad 26/10 SP Sepharose High Performance, 53 ml | <3000 mg (ribonuclease, $M_r$ 13 700) | up to 13 ml/min | 13 ml/min | 4–13 | 0.3/43 |
| SP Sepharose High Performance | 55 mg/ml (ribonuclease, $M_r$ 13 700) | 30–150 cm/h | 150 cm/h | 4–13 | 0.5/72 |

# **Sepharose** Fast Flow (FF): purification with good resolution and easy scale-up

## Purification options

Fig. 56. Sepharose Fast Flow media, with a range of selectivities, are available prepacked in HiT rap and HiPrep columns and in media packs.

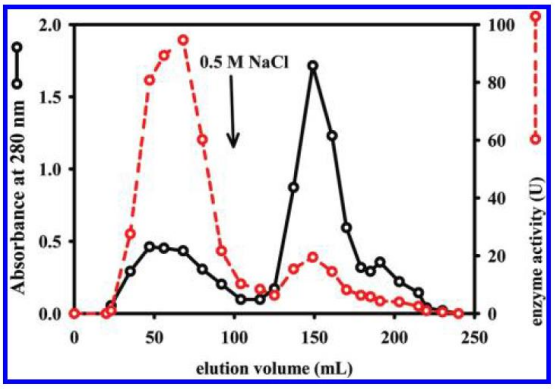



**Figure 2.** Anion exchange chromatography of the enzyme from *Vogesella* sp. 7307-1 on a Q FF column (1.6 × 10 cm) pre-equilibrated with 25 mM Tris buffer (pH 7.5) at a flow rate of 0.5 mL/min. The enzyme was eluted with 25 mM tris buffer (pH 7.5) and the same buffer containing 0.5 M NaCl. Fractions of 3 mL were collected and assayed for protein content and protease activity.

*Columns:* a) HiTrap ANX Sepharose 4 Fast Flow (high sub) 1 ml
b) HiTrap Q XL 1 ml
c) HiTrap Q Sepharose Fast Flow 1 ml
d) HiTrap DEAE Sepharose Fast Flow 1 ml

*Sample:* 0.4 mg conalbumin (pI=6.3),
0.8 mg α-lactoglobulin (pI=5.8),
1.2 mg soya bean trypsin inhibitor (pI=4.5)
dissolved in 2 ml start buffer

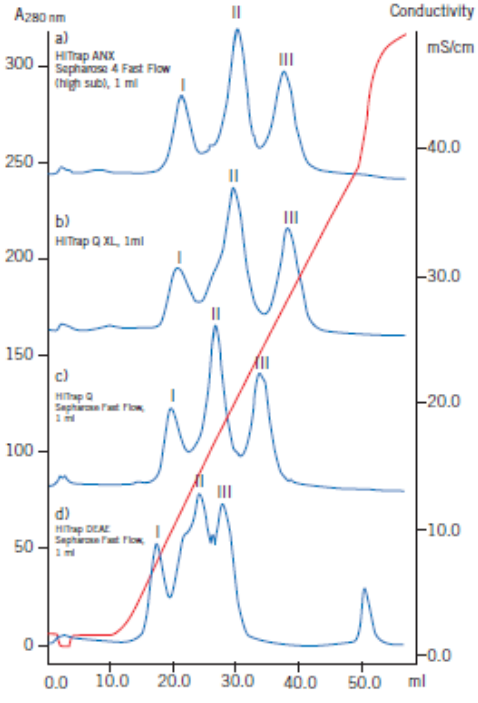
*Sample volume:* 2 ml

*Start buffer:* 20 mM Tris-HCl pH 7.4

*Elution buffer:* 20 mM Tris-HCl, 0.5 M NaCl pH 7.4

*Flow:* 1 ml/min (150 cm/h)

*Gradient:* 0% elution buffer (25 CV), 0-80% elution buffer (40 CV)
Wash: 5 ml start buffer
Elution: 40 ml, linear gradient, 0–80 % elution buffer



| Product | Binding capacity per column or per ml medium | Recommended working flow* | Maximum flow* | Working pH range** | Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar* |
|---|---|---|---|---|---|
| **Strong anion exchangers** | | | | | |
| HiTrap Q FF, 1 ml | 3 mg (thyroglobulin, $M_r$ 669 000)<br>120 mg (HSA, $M_r$ 68 000)<br>110 mg (α–lactalbumin, $M_r$ 14 300) | up to 1 ml/min | 4 ml/min | 2–12 | 0.3/43 |
| HiTrap Q FF, 5 ml | 15 mg (thyroglobulin, $M_r$ 669 000)<br>600 mg (HSA, $M_r$ 68 000)<br>550 mg (α–lactalbumin, $M_r$ 14 300) | up to 5 ml/min | 20 ml/min | 2–12 | 0.3/43 |
| HiPrep 16/10 Q FF, 20 ml | 60 mg (thyroglobulin, $M_r$ 669 000)<br>2400 mg (HSA, $M_r$ 68 000)<br>2200 mg (α–lactalbumin, $M_r$ 14 300) | 2–10 ml/min | 10 ml/min | 2–12 | 0.15/22 |
| Q Sepharose Fast Flow | 3 mg/ml (thyroglobulin, $M_r$ 669 000)<br>120 mg/ml (HSA, $M_r$ 68 000)<br>110 mg/ml, (α–lactalbumin, $M_r$ 14 300) | 50–400 cm/h | 750 cm/h | 2–12 | 0.3/43 |
| **Strong cation exchangers** | | | | | |
| HiTrap SP FF, 1 ml | 50 mg (bovine COHb, $M_r$ 69 000)<br>50 mg (human IgG, $M_r$ 160 000)<br>70 mg (ribonuclease A, $M_r$ 13 700) | up to 1 ml/min | 4 ml/min | 4–13 | 0.3/43 |
| HiTrap SP FF, 5 ml | 250 mg (bovine COHb, $M_r$ 69 000)<br>250 mg (human IgG, $M_r$ 160 000)<br>350 mg (ribonuclease A, $M_r$ 13 700) | up to 5 ml/min | 20 ml/min | 4–13 | 0.3/43 |
| HiPrep 16/10 SP FF, 20 ml | 1000 mg (bovine COHb, $M_r$ 69 000)<br>1000 mg (human IgG, $M_r$ 160 000)<br>1400 mg (ribonuclease A, $M_r$ 13 700) | 2–10 ml/min | 10 ml/min | 4–13 | 0.15/22 |
| SP Sepharose Fast Flow | 50 mg/ml (bovine COHb, $M_r$ 69 000)<br>50 mg/ml (human IgG, $M_r$ 160 000)<br>70 mg/ml (ribonuclease A, $M_r$ 13 700) | 50–400 cm/h | 750 cm/h | 4–13 | 0.3/43 |
| **Weak anion exchangers** | | | | | |
| HiTrap DEAE FF, 1 ml | 100 mg (α–lactalbumin, $M_r$14 300)<br>110 mg (HSA, $M_r$ 68 000) | up to 1 ml/min | 4 ml/min | 2–9 | 0.3/43 |

| Product | Binding capacity per column or per ml medium | Recommended working flow* | Maximum flow* | Working pH range** | Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar* |
|---|---|---|---|---|---|
| HiTrap DEAE FF, 5 ml | 500 mg (α–lactalbumin, $M_r$ 14 300)<br>550 mg (HSA, $M_r$ 68 000) | up to 5 ml/min | 20 ml/min | 2–9 | 0.3/43 |
| HiPrep 16/10 DEAE FF, 20 ml | 2000 mg (α–lactalbumin, $M_r$ 14 300)<br>2200 mg (HSA, $M_r$ 68 000) | 2–10 ml/min | 10 ml/min | 2–9 | 0.15/22 |
| DEAE Sepharose Fast Flow | 100 mg/ml (α–lactalbumin, $M_r$ 14 300)<br>110 mg/ml (HSA, $M_r$ 68 000) | 50–400 cm/h | 750 cm/h | 2–9 | 0.3/43 |
| HiTrap ANX FF (high sub), 1 ml | 43 mg (BSA, $M_r$ 67 000)<br>5 mg (thyroglobulin, $M_r$ 669 000) | up to 1 ml/min | 4 ml/min | 2–9 | 0.3/43 |
| HiTrap ANX FF (high sub), 5 ml | 215 mg (BSA, $M_r$ 67 000)<br>25 mg (thyroglobulin, $M_r$ 669 000) | up to 5 ml/min | 20 ml/min | 2–9 | 0.3/43 |
| HiPrep 16/10 ANX FF (high sub), 20 ml | 860 mg (BSA, $M_r$ 67 000)<br>100 mg (thyroglobulin, $M_r$ 669 000) | 2–10 ml/min | 10 ml/min | 2–9 | 0.15/22 |
| ANX Sepharose 4 Fast Flow (high sub) | 43 mg/ml (BSA, $M_r$ 67 000)<br>5 mg/ml (thyroglobulin, $M_r$ 669 000) | 50–300 cm/h | 400 cm/h | 2–9 | 0.1/14 |
| **Weak cation exchangers** | | | | | |
| HiTrap CM FF, 1 ml | 50 mg (ribonuclease A, $M_r$ 13 700) | up to 1 ml/min | 4 ml/min | 6–10 | 0.3/43 |
| HiTrap CM FF, 5 ml | 250 mg (ribonuclease A, $M_r$ 13 700) | up to 5 ml/min | 20 ml/min | 6–10 | 0.3/43 |
| HiPrep 16/10 CM FF, 20 ml | 1000 mg (ribonuclease A, $M_r$ 13 700) | 2–10 ml/min | 10 ml/min | 6–10 | 0.15/22 |
| CM Sepharose Fast Flow | 50 mg/ml medium (ribonuclease A, $M_r$ 13 700) | 50–400 cm/h | 750 cm/h | 6–10 | 0.3/43 |

## 化學穩定性

For daily use, Sepharose Fast Flow media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as non-ionic detergents, 70% ethanol, 1 M acetic acid and 30% isopropanol. Sepharose Fast Flow can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix. Avoid cationic detergents with SP or CM Sepharose Fast Flow. Avoid anionic detergents with Q, DEAE or ANX Sepharose Fast Flow. Avoid oxidizing agents.

## 貯藏

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for SP Sepharose Fast Flow. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

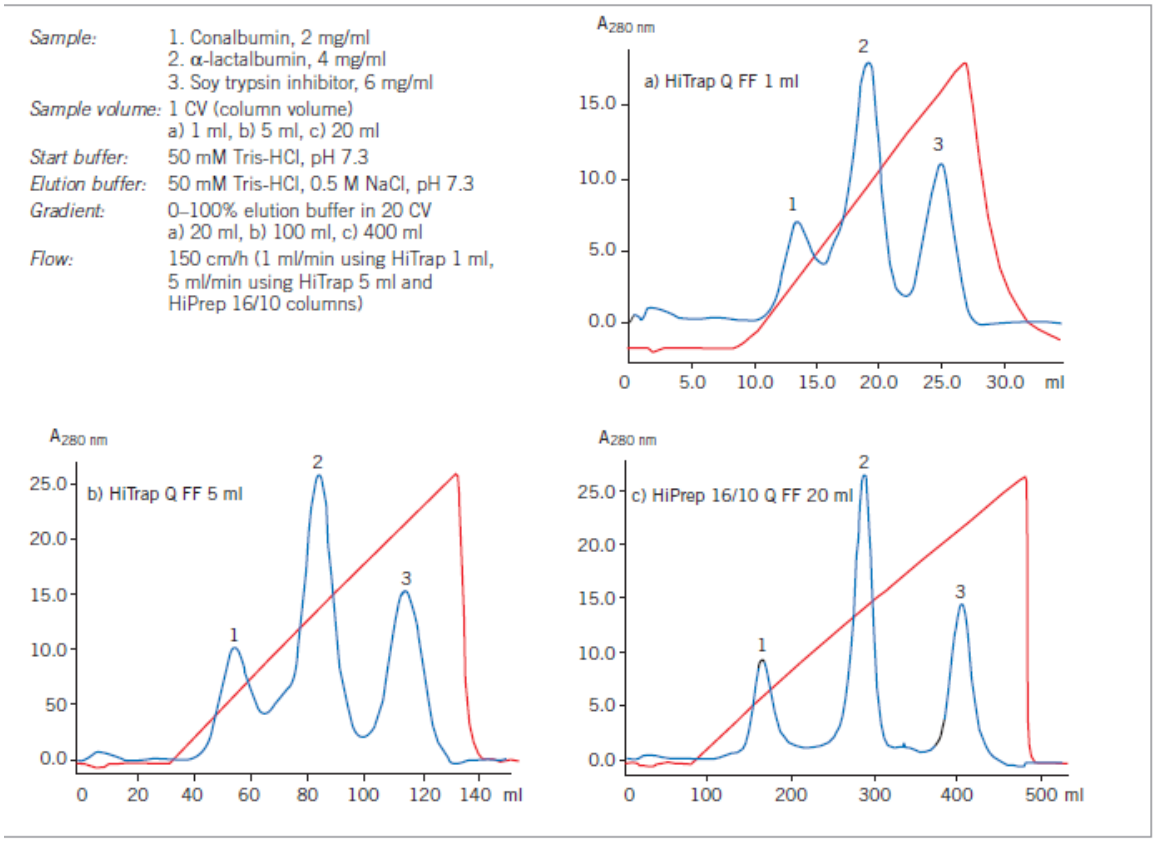
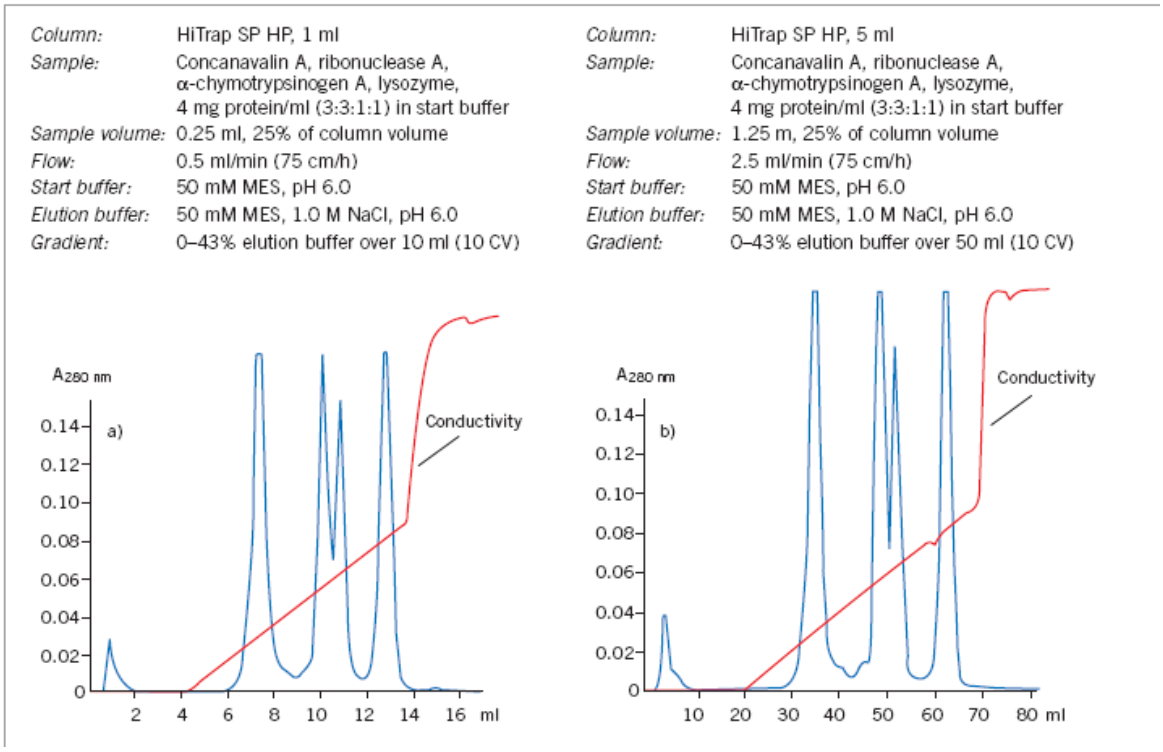



**Fig. 59.** 5-fold and 20-fold scale-up using prepacked Q Sepharose Fast Flow columns.




## 管柱清洗

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants. It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere

with performance.

## 移除管柱汙染物常用的處理法

1. Wash with at least 2 column volumes of 2 M NaCl at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 40 cm/h with a contact time of 1–2 hours for Sepharose Fast Flow packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

## 離子交換樹脂的組成分:

- sulfopropyl (SP), carboxymethyl (CM), quaternary amino (Q) or diethylaminoethyl (DEAE) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds.
- diethylaminopropyl (ANX) group coupled to highly cross-linked 4% agarose via chemically stable ether bonds.

| Product | Functional group | pH stability* | Mean particle size |
|---|---|---|---|
| Q Sepharose Fast Flow | $-CH_2N^+(CH_3)_3$ | Long term: 2–12<br>Short term: 1–14 | 90 µm |
| SP Sepharose Fast Flow | $-CH_2CH_2CH_2SO_3^-$ | Long term: 4–13<br>Short term: 3–14 | 90 µm |
| DEAE Sepharose Fast Flow | $-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$ | Long term: 2–13<br>Short term: 1–14 | 90 µm |
| ANX Sepharose 4 Fast Flow | $-OCH_2CHOHCH_2OCH_2CHOHCH_2N^+H(CH_2CH_3)_2$ | Long term: 3–13<br>Short term: 2–14 | 90 µm |
| CM Sepharose Fast Flow | $-O-CH_2COO^-$ | Long term: 4–13<br>Short term: 2–14 | 90 µm |

表 3.2 各種離子交換介質

| 陰陽強弱分類 | | Resin / Polystyrene | | Glycan / Cellulose (= X) | |
|---|---|---|---|---|---|
| Anion Exchanger | Strong | Dowex-1<br>Dowex-2 | $-\overset{+}{N}R_3$ | TEAE-X<br>(QAE-X) | $-\overset{+}{N}R_3$ |
| | Weak | Dowex-3<br>IR-45 | $-\overset{+}{N}HR_2$ | DEAE-X | $-OCH_2CH_2\overset{+}{N}HR_2$ |
| Cation Exchanger | Strong | Dowex-50 | $-SO_3^-$ | Phospho-X | $-PO_4^{-2}$ |
| | Weak | IRC-150 | $-COO^-$ | CM-X | $-CH_2COO^-$ |

X = Sephadex, Sepharose, Sephacel or cellulose

## 人工合成樹脂之陽離子交換樹脂活化方法

1. 取 100 克陽離子交換樹脂加 1.5 公升的 4% NaOH 洗 60-120 分鐘
2. 將 NaOH 移出，以 RO 水水洗至中性左右
3. 1.5 公升 5%的 HCl 洗 60-120 分鐘 (濃鹽酸一般為 37%)
4. 將 HCl 移出，以 RO 水水洗至中性左右
5. 離子交換樹脂加入 RO 水，冰箱中保存備用

## 陽離子交換樹脂再生方法

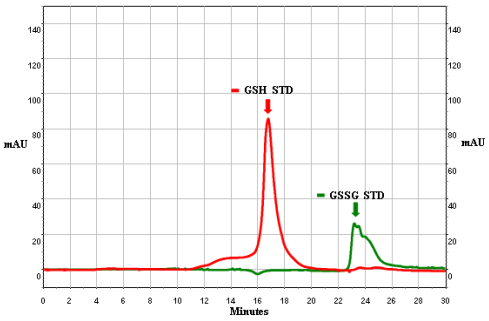
1. 以 15 倍體積 5%的 HCl 洗 60-120 分鐘
2. 將 HCl 移出，以 RO 水水洗至中性左右
3. 離子交換樹脂加入 RO 水，冰箱中保存備用
4. 若裝在管柱中則以 5%的 HCl 洗 2-3CV 後再以 RO 水洗 2-3CV 即可

## GSH(glutathione，麩胱甘肽)純化分離法

1. 配製 6000 ppm GSH 5 mL 以及 pH 3、4、5 緩衝液各 10 毫升
2. GSH 6000 ppm 取 0.5mL +緩衝液(pH 3、4、5) 1mL 得 2000ppm GSH
3. +0.3 克活化過陽離子交換樹脂(Amberlite IR120)於 GSH (2000 ppm)中，150rpm 震盪 2 小時
4. 5000 rpm 離心 2 分鐘分光光度計檢測上清液中 GSH 含量
5. 可知陽離子交換樹脂於何種 pH 下會吸附 GSH
6. 對照組為不加 0.3 克過陽離子交換樹脂

## 陰離子交換樹脂活化方法

1. 取 100 克陰離子交換樹脂加 1.5 公升的 5% HCl 洗 60-120 分鐘
2. 將 HCl 移出，以 RO 水水洗至中性左右
3. 1.5 公升 4%的 NaOH 洗 60-120 分鐘
4. 將 NaOH 移出，以 RO 水水洗至中性左右
5. 離子交換樹脂加入 RO 水，冰箱中保存備用




## 陰離子交換樹脂再生方法

1. 以 15 倍體積 4%的 NaOH 洗 60-120 分鐘
2. 將 NaOH 移出，以 RO 水水洗至中性左右
3. 離子交換樹脂加入 RO 水，冰箱中保存備用
4. 若裝在管柱中則以 4%的 NaOH 洗 2-3CV 後再以 RO 水洗 2-3CV 即可

## 樣品澄清處理

Centrifugation and filtration are standard laboratory techniques for sample clarification and are used routinely when handling small samples. It is highly recommended to centrifuge and filter any sample immediately before chromatographic purification.

## 離心

Centrifugation removes lipids and particulate matter, such as cell debris. If the sample is still not clear after centrifugation, use filter paper or a 5 µm filter as a first step and one of the filters below as a second step filter.

- For small sample volumes or proteins that adsorb to filters, centrifuge at 10 000 g for 15 minutes.
- For cell lysates, centrifuge at 40 000–50 000 g for 30 minutes.
- Serum samples can be filtered through glass wool after centrifugation to remove any remaining lipids.

## 過濾

Filtration removes particulate matter. Membrane filters that give the least amount of nonspecific binding of proteins are composed of cellulose acetate or PVDF.
For sample preparation before chromatography, select a filter pore size in relation to the bead size of the chromatographic medium.

| Nominal pore size of filter | Particle size of chromatographic medium |
|---|---|
| 1 µm | 90 µm and upwards |
| 0.45 µm | 30 or 34 µm |
| 0.22 µm | 3, 10, 15 µm or when extra clean samples or sterile filtration is required |

## 離子交換膠體的篩選及其操作 pH 條件

1. Start buffers: set up a series of buffers with pH values in the range 4–8 (SP, CM) or 5–9 (Q, DEAE, ANX) and with 0.5–1 pH unit intervals between each buffer. See Appendix 2 for recommended buffers.
2. Elution buffers: set up a second series of buffers with the same pH values, but including 1 M NaCl.
3. Equilibrate the column (s) with 5 ml start buffer at 1 ml/min. Wash with 5 ml elution buffer.
4. Re-equilibrate with 5–10 ml start buffer.
5. Adjust the sample to the pH of the start buffer and apply a known amount of the sample at 1 ml/min.Collect eluate.
6. Wash with at least 5 ml of start buffer or until no material appears in eluent. Collect eluate.

7. Elute bound material with elution buffer (3–5 ml is usually sufficient, but other volumes may be required dependent on the exact experimental conditions). Collect eluate.
8. Analyze all eluates (for example by an activity assay) and determine purity and the amount bound to the column.
9. Perform steps 3–8 for the next buffer pH.
10. Select medium and pH: the most suitable pH should allow the protein(s) of interest to bind, but should be as close to their point of release as possible.

## 離子強度操作條件的篩選

1. Using the selected medium, start buffer and pH from the previous protocol, set up a series of elution buffers at the same pH, but vary the salt concentration from 0–0.5 M with intervals of 0.05–0.1 M salt between each buffer.
2. Repeat steps 3–8 from the previous protocol for each salt concentration.
3. Determine the maximum ionic strength which permits binding of the protein(s) of interest and the minimum ionic strength required for complete elution.

## 最適化

1. If gradient making equipment is available, determine the steepest gradient that gives acceptable resolution at the selected pH. Begin with a gradient of 10 column volumes over an ionic strength range based on the maximum and minimum values determined when screening. Alternatively, begin with a gradient of 0–50% elution buffer that contains 1 M NaCl and a gradient volume of 10–20 column volumes.
2. Determine the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
3. Determine the maximum sample load that can be applied while maintaining satisfactory resolution. In general, loading 20–30% of the total binding capacity of the column gives optimal resolution with gradient elution. Sample loads can often be increased if resolution is satisfactory or when using a step elution.

## 離子交換層析操作實務

This section covers detailed aspects of each step in an IEX separation, together with practical hints and tips to improve resolution and overall performance. In practice a separation can be summarized as follows:

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes with an increasing ionic strength up to 0.5 M NaCl (50%B). Alternatively (if gradient-making equipment is not available) elute bound proteins with 5 column volumes of start buffer + NaCl at chosen ionic strength. Repeat at higher ionic strengths until the target protein(s) has been eluted.
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values

For samples with unknown charge properties, try the following:
– anion exchange (Q, DEAE or ANX) start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0
– cation exchange (S, SP, CM) start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0
--When using a weak exchanger, work within the pH values given below to minimize variations in performance: DEAE: pH 2–9 ANX: pH 2–9 CM: pH 6–10

## IEX media for capture steps should offer high speed and high capacity.

1. Sepharose Fast Flow (90 µm particle size) – good resolution for crude mixtures at any scale using flows up to 300 cm/h and offering a wide range of selectivities.
2. Sepharose XL (90 µm particle size) – high capacity, good resolution for capture of selected proteins at laboratory and process scale using flows up to 300 cm/h.
3. Sepharose Big Beads (200 µm particle size) – for viscous samples that preclude the use of IEX media

with smaller particle size, using flows up to 300 cm/h, or for fast separations of very large sample volumes when resolution is of less importance, using flows up to 1000 cm/h.

Use a technique with a selectivity that is complementary to that used in the capture step. IEX media for intermediate purification should offer high capacity and high resolution with a range of complementary selectivities:

1. Sepharose High Performance (34 µm particle size) – high resolution using flows up to 150 cm/h.
2. SOURCE 15 (15 µm particle size) – high throughput, high resolution for laboratory or large-scale applications using flows up to 1800 cm/h.
3. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large-scale applications when flows up to 2000 cm/h can be used.
4. Sepharose Fast Flow (90 µm particle size) – fast separations, good resolution using flows up to 300 cm/h, broad range of selectivities. If only milligram quantities are required and the intermediate purification step will not be scaled-up, use MonoBeads or MiniBeads according to the capacity required.

IEX media for polishing steps should offer highest resolution:

1. MiniBeads (3 µm particle size) – polishing at microscale when highest resolution is essential.
2. MonoBeads (10 µm particle size) – polishing at laboratory scale when highest resolution is essential and a higher capacity than MiniBeads is required.
3. SOURCE 15 (15 µm particle size) – rapid, high resolution polishing for laboratory or large scale applications using flows up to 1800 cm/h.
4. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large scale applications when flows up to 2000 cm/h can be used.

## 陰離子交換層析 **anion exchange chromatography** 常用的緩衝液

| pH interval | Substance | Conc. (mM) | Counter-ion | pKa (25 °C)[1] | d(pKa)/dT (°C) |
|---|---|---|---|---|---|
| 4.3–5.3 | N-Methylpiperazine | 20 | $Cl^-$ | 4.75 | -0.015 |
| 4.8–5.8 | Piperazine | 20 | $Cl^-$ or $HCOO^-$ | 5.33 | -0.015 |
| 5.5–6.5 | L-Histidine | 20 | $Cl^-$ | 6.04 | |
| 6.0–7.0 | bis-Tris | 20 | $Cl^-$ | 6.48 | -0.017 |
| 6.2–7.2; 8.6–9.6 | bis-Tris propane | 20 | $Cl^-$ | 6.65; 9.10 | |
| 7.3–8.3 | Triethanolamine | 20 | $Cl^-$ or $CH_3COO^-$ | 7.76 | -0.020 |
| 7.6–8.6 | Tris | 20 | $Cl^-$ | 8.07 | -0.028 |
| 8.0–9.0 | N-Methyldiethanolamine | 20 | $SO_4^{2-}$ | 8.52 | -0.028 |
| 8.0–9.0 | N-Methyldiethanolamine | 50 | $Cl^-$ or $CH_3COO^-$ | 8.52 | -0.028 |
| 8.4–9.4 | Diethanolamine | 20 at pH 8.4 | $Cl^-$ | 8.88 | -0.025 |
| | | 50 at pH 8.8 | | | |
| 8.4–9.4 | Propane 1,3-Diamino | 20 | $Cl^-$ | 8.88 | -0.031 |
| 9.0–10.0 | Ethanolamine | 20 | $Cl^-$ | 9.50 | -0.029 |
| 9.2–10.2 | Piperazine | 20 | $Cl^-$ | 9.73 | -0.026 |
| 10.0–11.0 | Propane 1,3-Diamino | 20 | $Cl^-$ | 10.55 | -0.026 |
| 10.6–11.6 | Piperidine | 20 | $Cl^-$ | 11.12 | -0.031 |

## 陽離子交換層析 **anion exchange chromatography** 常用的緩衝液

| pH interval | Substance | Conc. (mM) | Counter-ion | pKa (25 °C)[1] | d(pKa)/dT (°C) |
|---|---|---|---|---|---|
| 1.4–2.4 | Maleic acid | 20 | $Na^+$ | 1.92 | |
| 2.6–3.6 | Methyl malonic acid | 20 | $Na^+$ or $Li^+$ | 3.07 | |
| 2.6–3.6 | Citric acid | 20 | $Na^+$ | 3.13 | -0.0024 |
| 3.3–4.3 | Lactic acid | 50 | $Na^+$ | 3.86 | |
| 3.3–4.3 | Formic acid | 50 | $Na^+$ or $Li^+$ | 3.75 | +0.0002 |
| 3.7–4.7; 5.1–6.1 | Succinic acid | 50 | $Na^+$ | 4.21; 5.64 | -0.0018 |
| 4.3–5.3 | Acetic acid | 50 | $Na^+$ or $Li^+$ | 4.75 | +0.0002 |
| 5.2–6.2 | Methyl malonic acid | 50 | $Na^+$ or $Li^+$ | 5.76 | |
| 5.6–6.6 | MES | 50 | $Na^+$ or $Li^+$ | 6.27 | -0.0110 |
| 6.7–7.7 | Phosphate | 50 | $Na^+$ | 7.20 | -0.0028 |
| 7.0–8.0 | HEPES | 50 | $Na^+$ or $Li^+$ | 7.56 | -0.0140 |
| 7.8–8.8 | BICINE | 50 | $Na^+$ | 8.33 | -0.0180 |

## 壓力單位(MPa)的轉換

**Pressure units may be expressed in megaPascals, bar or pounds per square inch and can be converted as follows: 1MPa = 10 bar = 145 psi**

## 線性流速 linear flow (cm/hour) 與一般流速 volumetric flow rates (ml/min)的互轉

It is convenient when comparing results for columns of different sizes to express flow as linear flow (cm/hour). However, flow is usually measured in volumetric flow rate (ml/min). To convert between linear flow and volumetric flow rate use one of the formulae below.

### From linear flow (cm/hour) to volumetric flow rate (ml/min)

Volumetric flow rate (ml/min) = column cross sectional area (cm2) x Linear flow (cm/h)/60

$$(Y/60)\ x[\ (\pi\ x\ d^2)/4]$$

where

Y = linear flow in cm/h  d = column inner diameter in cm

Example 1:

What is the volumetric flow rate in an XK 16/70 column (i.d. 1.6 cm) when the linear flow is 150 cm/hour?

Y = linear flow = 150 cm/h  d = inner diameter of the column = 1.6 cm

Volumetric flow rate (ml/min) =(150 x π x 1.6 x 1.6)/( 60 x 4)= 5.03

### From volumetric flow rate (ml/min) to linear flow (cm/hour)

Linear flow (cm/h)= [Volumetric flow rate (ml/min) x 60]/ [column cross sectional area (cm2)]

$$=(Z\ x\ 60\ x4)/(\ \pi\ x\ d^2)$$

where

Z = volumetric flow rate in ml/min  d = column inner diameter in cm

Example 2:

What is the linear flow in an HR 5/5 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

Z = Volumetric flow rate = 1 ml/min  d = column inner diameter = 0.5 cm

Linear flow (cm/h) = 1 x 60 x4/ (π x 0.5 x 0.5)= 305.6

**From ml/min to using a syringe**

1 ml/min = approximately 30 drops/min on a HiTrap 1 ml column

5 ml/min = approximately 120 drops/min on a HiTrap 5 ml column

## 蛋白質沉澱物的可溶化處理

Many proteins are easily resolubilized in a small amount of the buffer to be used in the next chromatographic step. However, a denaturing agent may be required for less soluble proteins. Specific

conditions will depend upon the specific protein. These agents must always be removed to allow complete refolding of the protein and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table 17 gives examples of common denaturing agents.

**Table 17.**

| Denaturing agent | Typical conditions for use | Removal/comment |
|---|---|---|
| Urea | 2 M–8 M | Remove using Sephadex G-25. |
| Guanidine hydrochloride | 3 M–6 M | Remove using Sephadex G-25 or during IEX. |
| Triton X-100 | 2% | Remove using Sephadex G-25 or during IEX. |
| Sarcosyl | 1.5% | Remove using Sephadex G-25 or during IEX. |
| N-octyl glucoside | 2% | Remove using Sephadex G-25 or during IEX. |
| Sodium dodecyl sulphate | 0.1%–0.5% | Exchange for non-ionic detergent during first chromatographic step, avoid anion exchange chromatography. |
| Alkaline pH | > pH 9, NaOH | May need to adjust pH during chromatography to maintain solubility. |

**Table 6.** Troubleshooting.

| Situation | Cause | Remedy |
|---|---|---|
| Reduced or no flow through the column. | Outlet closed or pumps not working. | Open outlet. Check pumps for signs of leakage (if using a peristaltic pump, check tubing also). |
| | Blocked filter, end-piece, adaptor or tubing. | Remove and clean or replace if possible. Always filter samples and buffer before use. |
| | Lipoproteins or protein aggregates have precipitated. | Remove lipoproteins and aggregates during sample preparation, (see Appendix 1). Follow cleaning procedures, Appendix 10. |
| | Protein precipitation in the column. | Modify buffer, pH and/or salt conditions during the run to maintain stability. Follow cleaning procedures, Appendix 10. |
| | Protein precipitation in the column caused by removal of stabilizing agents during separation. | Modify eluent to maintain stability. |
| | Microbial growth has occurred in the column. | Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10. |
| Peak of interest is poorly resolved from other major peaks. | Sample applied incorrectly. | Check bed surface and top filter for possible contamination. |
| | Large mixing spaces at top of or after column. | Adjust top adaptor to surface of medium if necessary. Reduce all post-column volumes. |
| | Incorrect buffer pH and/or ionic strength. | Check pH and ionic strength to ensure that column was re-equilibrated after previous run. Check conditions required. Prepare new solutions. |
| | Sub-optimal elution conditions e.g. incorrect pH, gradient too steep, flow rate too high. | Alter elution conditions: alter pH, use shallower gradient, reduce flow rate (listed in priority order). |
| | Sample is too viscous. | Dilute with buffer. Maintain protein concentration below 50 mg/ml. |

| | | |
|---|---|---|
| | Column is poorly packed. | Check column efficiency (see Appendix 3). Repack if needed. Use prepacked columns. |
| | Column overloaded. | Decrease sample load. |
| | Lipoproteins or protein aggregates have precipitated. | Remove lipoproteins and aggregates during sample preparation (see Appendix 1). |
| | Precipitation of proteins in the column. | Modify buffer, pH and/or salt conditions during the run to maintain stability. |
| | Microbial growth has occurred in the column. | Store in the presence of 20% ethanol to prevent microbial growth. Always filter buffers. Follow cleaning procedures, Appendix 10. |
| Proteins do not bind or elute as expected. | Proteins or lipids have precipitated on the column or column filter. | Clean the column and exchange or clean the filter. Check pH and salt stability of sample. |
| | Sample not filtered properly. | Clean the column, filter the sample and repeat. |
| | Sample has changed during storage. | Prepare fresh samples. |
| | Protein may be unstable or inactive in the elution buffer. | Determine the pH and salt stability of the protein. |
| | Column equilibration incomplete. | Repeat or prolong the equilibration step until conductivity and pH are constant. |
| | Incorrect buffer pH and/or ionic strength. | Check conditions required. Prepare new solutions. |
| | Proteins are forming aggregates and binding strongly to the medium. | Use urea or zwitterions, betaine up to 10%, taurine up to 4%. |
| | Sample or buffer conditions are different from previous runs. | Check sample and buffer conditions. |
| | Microbial growth has occurred in the column. | Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10. |

| Situation | Cause | Remedy |
|---|---|---|
| Protein elutes later than expected or not at all. | Incorrect buffer pH. | Check pH meter calibration. Use a buffer pH closer to the pI of the protein. |
| | Ionic strength too low. | Increase salt concentration in elution buffer. |
| | Ionic interactions between protein and matrix. | Maintain ionic strength of buffers above 0.05 M. |
| | Hydrophobic interactions between protein and matrix. | Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent, e.g. 5% isopropanol. |
| Protein elutes earlier than expected (during the wash phase). | Ionic strength of sample or buffer is too high. | Decrease ionic strength of sample or buffer. |
| | Incorrect pH conditions. | Increase pH (anion exchanger).<br>Decrease pH (cation exchanger). |
| | Column equilibration incomplete. | Repeat or prolong the equilibration step until conductivity and pH are constant |
| Leading or very rounded peaks in chromatogram. | Channeling in the column. | Repack column using a thinner slurry of medium. Check column packing (see Appendix 3). |
| | Column overloaded. | Decrease sample load and repeat. |
| | Column contaminated. | Clean using recommended procedures. |
| Peaks are tailing. | Incorrect start buffer conditions, sample is not binding to column. | Adjust pH. Check salt concentration in start buffer. |
| | Sample too viscous. | Dilute in application buffer. |
| | Column packing too loose. | Check column efficiency (see Appendix 3).<br>Repack using a higher flow rate.<br>Use prepacked columns. |
| Peaks have a leading edge. | Column packing compressed. | Check column efficiency (see Appendix 3).<br>Repack using a lower flow rate.<br>Use prepacked columns. |
| Medium/beads appears in eluent. | Column packing compressed. | Check column efficiency (see Appendix 3).<br>Repack using a slower flow rate.<br>Use prepacked columns. |
| | Bed support end piece is loose or broken. | Replace or tighten. |
| | Column operated at too high pressure. | Do not exceed recommended operating pressure for medium or column. |
| | Medium has been damaged during column packing. | Do not use magnetic stirrers when equilibrating loose medium |
| Low recovery of activity, but normal recovery of protein. | Protein may be unstable or inactive in the buffer. | Determine the pH and salt stability of the protein. |
| | Enzyme separated from co-factor or similar. | Test by pooling aliquots from the fractions and repeating the assay. |
| Protein yield lower than expected. | Protein may have been degraded by proteases. | Add protease inhibitors to the sample and buffers to prevent proteolytic digestion.<br>Run sample through a medium such as Benzamidine 4 Fast Flow (high sub) to remove trypsin-like serine proteases. |
| | Adsorption to filter during sample preparation. | Use another type of filter. |
| | Sample precipitates. | Check pH and salt conditions, adjust to improve sample solubility. |
| | Hydrophobic proteins. | Add denaturing agents, polarity reducing agents or detergents. Add 10% ethylene glycol to running buffer to prevent hydrophobic interactions. |
| | Non-specific adsorption. | Reduce salt concentration to minimize hydrophobic interaction. Add suitable detergent or organic solvent e.g. 5% isopropanol. If necessary, add 10% ethylene glycol to running buffer to prevent hydrophobic interactions. |

| Situation | Cause | Remedy |
|---|---|---|
| Peaks too small. | Sample absorbs poorly at chosen wavelength. | If appropriate, check absorbance range on monitor. If satisfactory, use a different wavelength, e.g. 214 nm instead of 280 nm. |
| | Different assay conditions have been used before and after the chromatographic step. | Use same assay conditions for all assays. |
| | Excessive band broadening. | Check column packing. Repack if necessary. |
| More sample is recovered than expected. | Protein co-eluting with other substances. | Optimize conditions to improve resolution. Check buffer conditions used for assay before and after the run. Check selection of medium. |
| More activity is recovered than was applied to the column. | Different assay conditions have been used before and after the chromatography step. | Use same assay conditions for all assays. |
| | Removal of inhibitors during separation. | |
| Back pressure increases during a run or during successive runs. | Bed compressed. | If possible repack the column or use a new column. Check sample preparation. |
| | Microbial growth. | Store in the presence of 20% ethanol to prevent microbial growth. Always filter buffers. Follow cleaning procedures, Appendix 10. |
| | Turbid sample. | Improve sample preparation (see Appendix 1). Improve sample solubility: add betaine (max. 10% w/v at 25°C), taurine (max. 4% w/v at 25°C, below pH 8.5) or glycerol (1–2 %). For hydrophobic samples, add ethylene glycol, urea, detergents or organic solvents. |
| | Precipitation of protein in the column filter and/or at the top of the bed. | Clean using recommended methods. If possible, exchange or clean filter or use a new column. Include any additives that were used for initial sample solubilization in the running buffer. |
| | Incorrect pH is causing precipitation. | Calibrate pH meter, prepare new solutions and try again. Change pH. |
| | Precipitation of lipoproteins at increased ionic strength. | Lipoproteins may be removed prior to chromatography by the addition of 10% dextran sulfate (final 0.2%) and 1 M calcium chloride (final 0.5 M). |
| Air bubbles in the bed. | Buffers not properly degassed. | Degas buffers thoroughly. |
| | Column packed or stored at cool temperature and then warmed up. | Remove small bubbles by passing degassed buffer through the column. Take special care if buffers are used after storage in a fridge or cold-room. Do not allow column to warm up due to sunshine or heating system. Repack column, if possible (see Appendix 3). |
| Cracks in the bed. | Large air leak in column. | Check all connections for leaks. Repack the column if possible (see Appendix 3). |
| Negative peaks at solvent front. | Refractive index effects. | Exchange the sample into start buffer. |
| Unexpected peaks in chromatogram. | Buffer impurities. | Clean the buffer by running it through a precolumn. Use high quality reagents. |
| Peaks appear on gradients. | Incomplete elution of previous sample. | Wash the column according to recommended blank methods. |
| Spikes in chromatogram. | Air bubble trapped in UV monitor flow cell. | Always use degassed buffers. |
| UV baseline rises with gradient. | Micelle formation as salt concentration changes. | Work below or above the critical micelle concentration of any detergents being used or change the gradient so that the increase in UV absorption does not occur while the samples are eluting. |
| | Buffer impurities. | Use high quality reagents. |

**Polar organic solvents such as methanol, ethanol, isopropanol and acetonitrile can be used at concentrations from 0–20%, but remember that some proteins may irreversibly lose their biological activity in the presence of organic solvents. Check sample and buffer solubility, buffer pH and chemical stability of the medium before running a column. Note that back pressure may increase when working with organic solutions.*