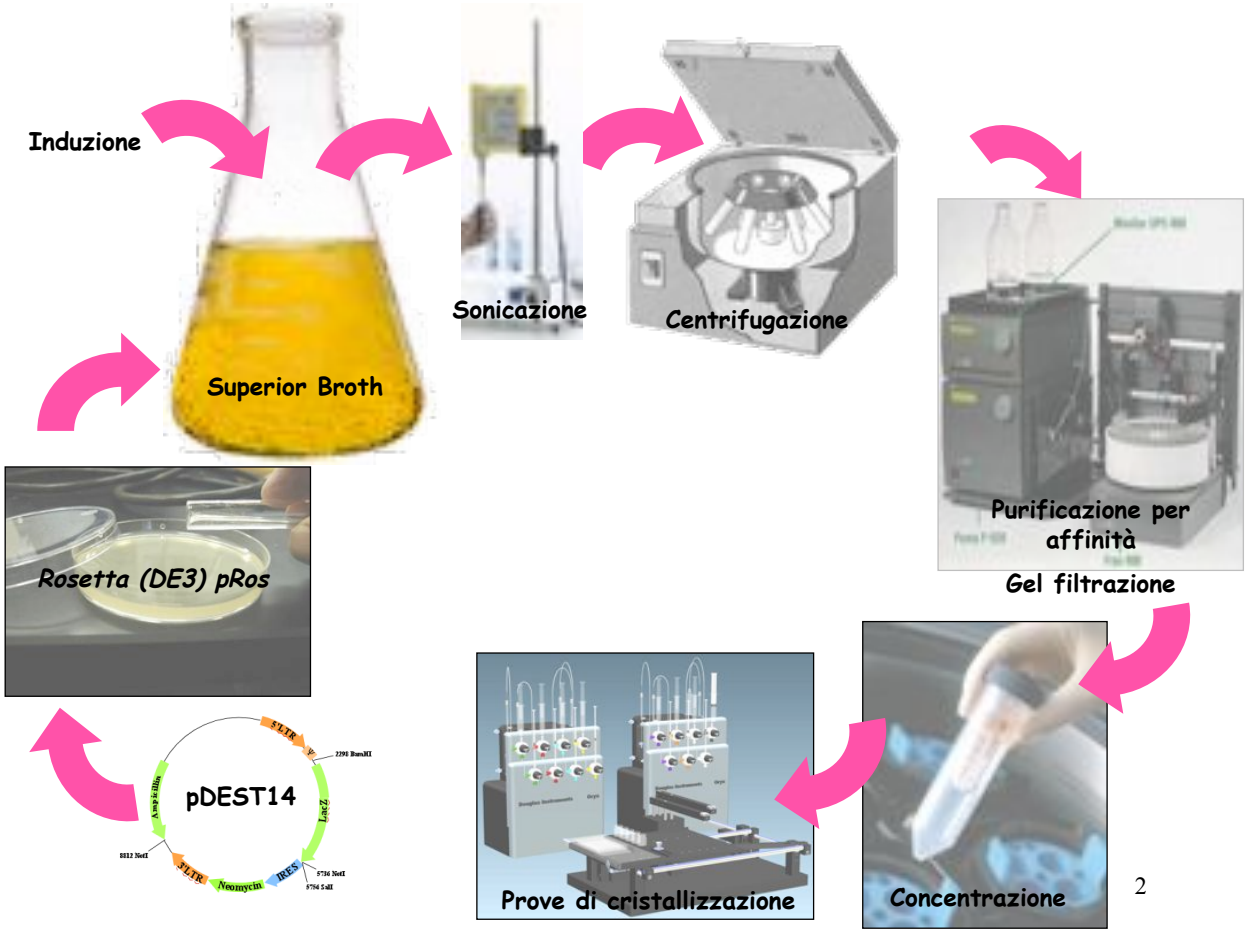
Espressione e purificazionedi proteine ricombinanti

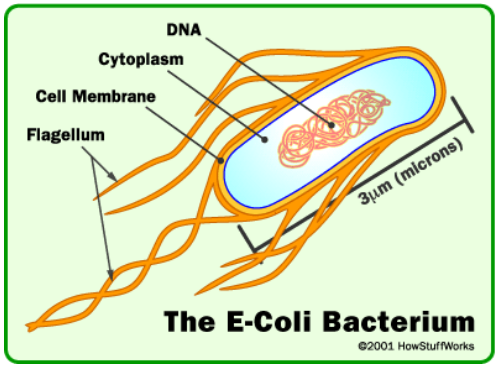
- Clonaggio
- Crescita delle cellule
- Induzione (produzione della proteina)
- Raccolta delle cellule e lisi
- Purificazione
- Analisi



Clonaggio

Il DNA che codifica per la proteina 'NEST' viene inserito nel plasmide pET-21b:

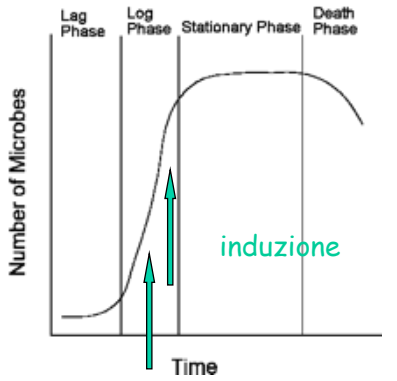
His-tag: coda di 6 His utile a purificare la proteina.



Il plasmide viene inserito in e. coli

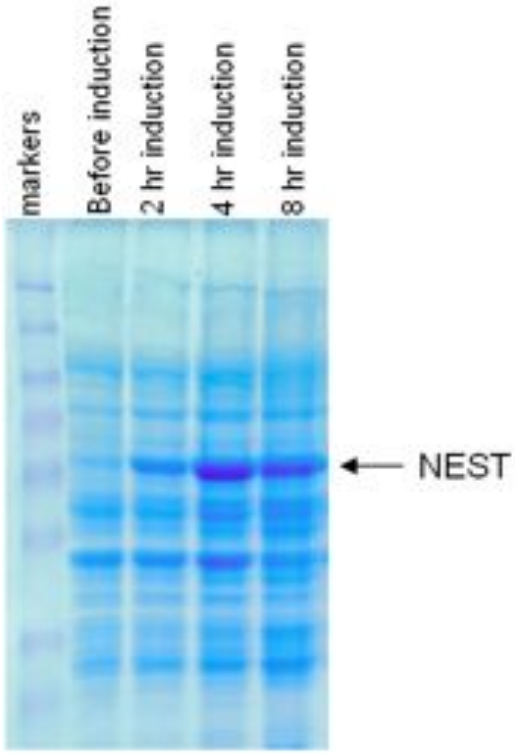
Crescita dei batteri

Bacterial Growth Curve



Assorbanza a 600 nm

espressione della proteina dopo l'induzione



SDS elettroforesi

Purificazione di proteine nella loro *forma nativa* (struttura 3D intatta)

si sfruttano:	carica dimensioni affinità con molecole specifiche idrofobicità

Le proteine naturali esistono in basse concentrazioni in una soluzione che contiene molte altre proteine. Al contrario, le proteine ricombinanti esistono in grosse quantità e le impurezze derivano dalla cellula ospite, come altre proteine, lipidi, DNA.

Separazioni cromatografiche

1 Cromatografia di scambio ionico

2 Cromatografia per gel filtrazione

3 Cromatografia per affinità

4 Cromatografia per interazione idrofobica

Protein property	Technique
Size	Gel filtration (GF)
Charge	Ion exchange (IEX)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)

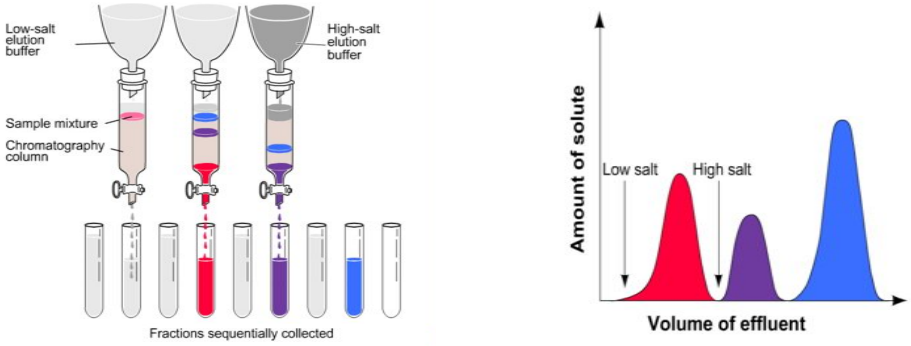
Principi della cromatografia

Nel 1903 il botanico russo Mikhail Tswett ha descritto la separazione dei pigmenti delle foglie delle piante in soluzione attraverso l'uso di un mezzo assorbente solido. A questo processo diede il nome di "cromatografia" (dal greco CHROMA, colore + GRAPHEIN, scrittura) a causa delle bande colorate che si formarono sul mezzo assorbente, dovute alla separazione dei pigmenti

Fase mobile: miscela di sostanze da separare disciolte in un liquido o in un gas

Fase stazionaria: matrice solida porosa posta in una colonna nella quale viene fatta passare la fase mobile

La separazione è dovuta alle interazioni della miscela con la fase stazionaria. Tali interazioni ritardano l'avanzamento dei componenti della miscela attraverso la matrice secondo le proprietà di ogni singolo componente.



3 step principali: 1 caricamento del campione, 2 eluizione (a gradiente o a step), 3 lavaggio/rigenerazione

Table 6. Protein properties used during purification

Protein property	Technique
Size	Gel filtration (GF)
Charge	Ion exchange (IEX)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)

Every technique offers a balance between resolution, capacity, speed and recovery.

Capacity refers to the amount of target protein loaded during purification.

Speed is most important at the beginning of purification where contaminants, such as proteases, must be removed as quickly as possible.

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product.

Resolution is achieved by the **selectivity** of the technique and the **efficiency** of the chromatography matrix in producing narrow peaks.

Risoluzione

The resolution (R_s) is determined from the chromatogram as shown in Figure 3.

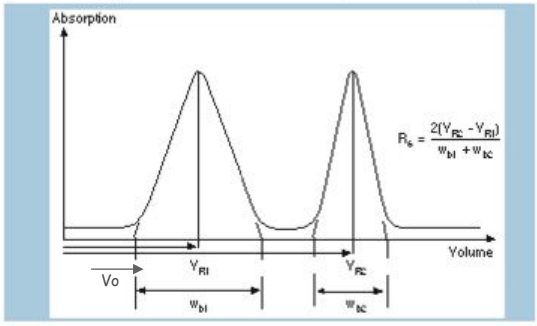


Fig. 3. Hypothetical chromatogram. V_0 = void volume, V_{R1} = elution volume for peak 1, V_{R2} = elution volume for peak 2, V_t = total volume, w_{b1} = peak width for peak 1, w_{b2} = peak width for peak 2.

Good selectivity is a more important factor than **high efficiency** in determining resolution

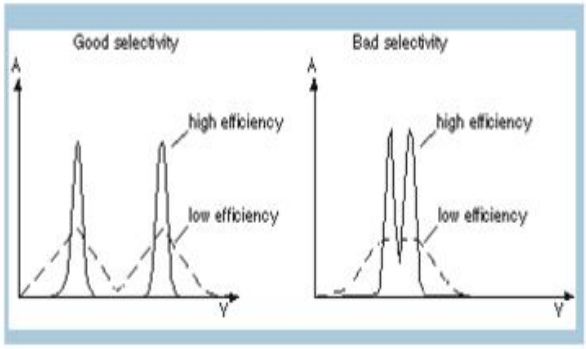
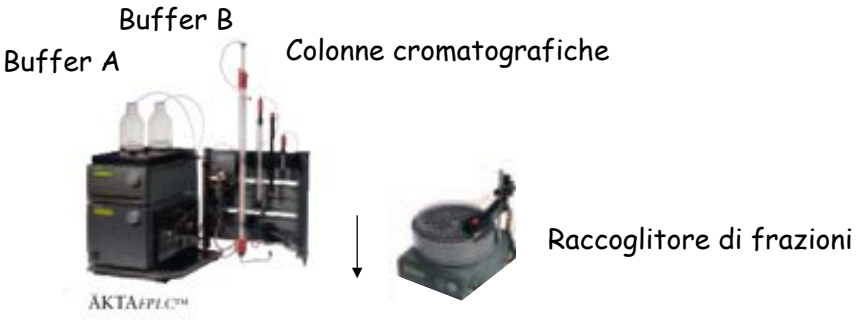


Fig. 6. The effect of selectivity and efficiency on resolution.

$$R = \frac{2(V_2 - V_1)}{w_1 + w_2} \qquad \begin{cases} V_2 - V_1 \geq w \Rightarrow R \geq 1 \\ V_2 - V_1 \leq w \Rightarrow R \leq 1 \end{cases}$$

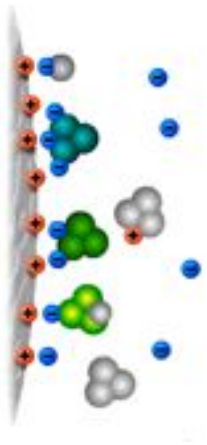
Es. di strumentazione per la purificazione di proteine

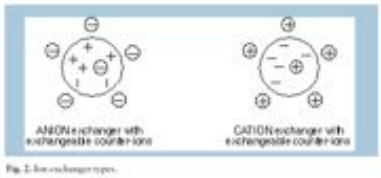


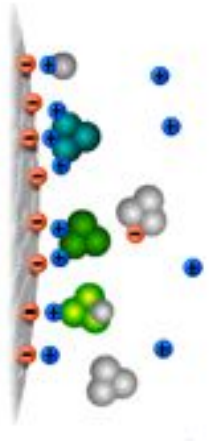
FPLC: Fast Protein Liquid Chromatography

Cromatografia per scambio ionico (IEC)

Ion exchange is probably the most frequently used chromatographic technique for the separation and purification of proteins, polypeptides, nucleic acids, polynucle-otides, and other charged biomolecules. Separation in ion exchange chromatography depends upon the reversible adsorption of **charged** solute molecules to immobilized ion exchange groups of opposite charge.



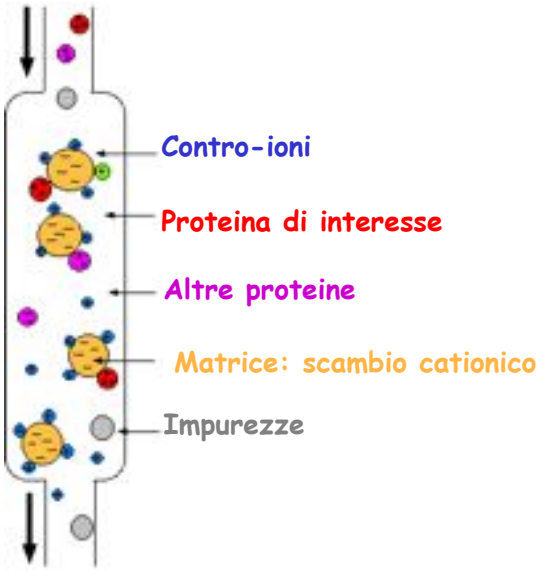


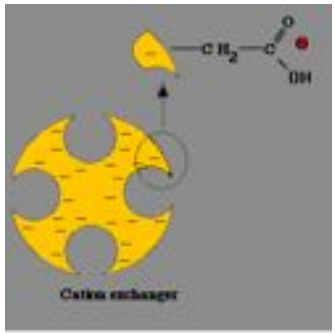


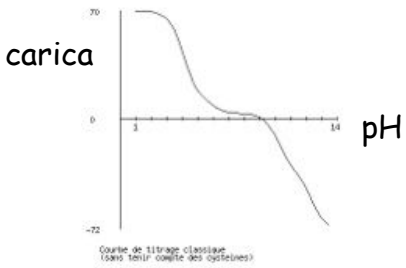
Cromatografia a scambio anionico (-)

Cromatografia a scambio cationico (+)

Cromatografia a scambio cationico





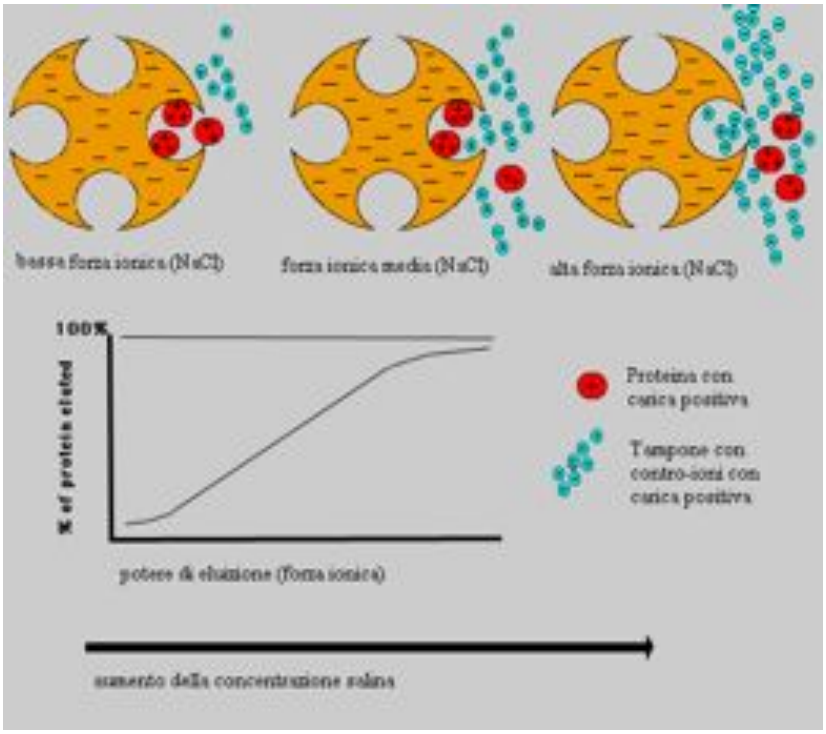


Condizionamento: buffer a pH acido

Caricamento del campione e lavaggio

Eluizione: buffer a pH basico e/o a più elevata forza ionica

Eluizione delle proteine per aumento della forza ionica



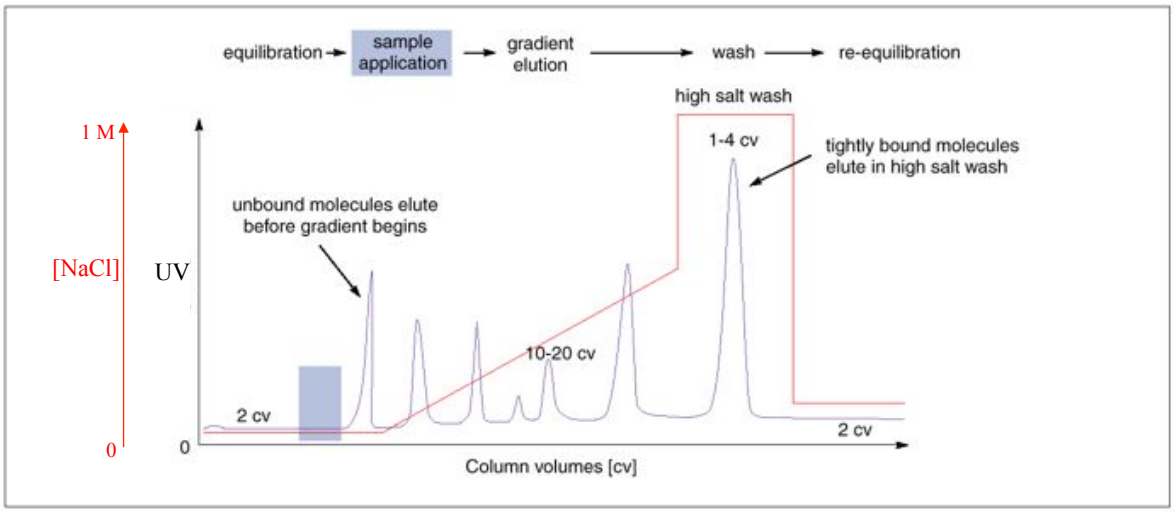


Fig. 31. Typical IEX gradient elution.

Principali resine usate

Functional groups used on ion exchangers.

Anion exchangers	**Functional group**
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(C_2H_5)_2$
Quaternary aminoethyl (QAE)	$-O-CH_2-CH_2-N^+(C_2H_5)_2-CH_2-CHOH-CH_3$
Quaternary ammonium (Q)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$

Cation exchangers	**Functional group**
Carboxymethyl (CM)	$-O-CH_2-COO^-$
Sulphopropyl(SP)	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$
Methyl sulphonate (S)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$

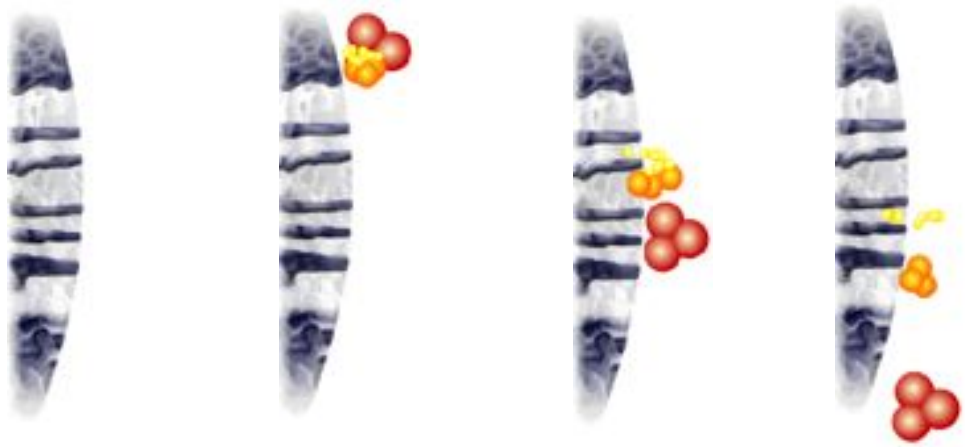
Sulphonic and quaternary amino groups are used to form strong ion exchangers;

the other groups form weak ion exchangers.

The terms strong and weak refer to the extent of variation of ionization with pH and not the strength of binding. Strong ion exchangers are completely ionized over a wide pH range whereas with weak ion exchangers, the degree of dissociation and thus exchange capacity varies much more markedly with pH.

Cromatografia di gel filtrazione (GF)

For more than forty years since the introduction of Sephade, gel filtration has played a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins and other biological macromolecules. Gel filtration is the simplest and mildest of all the chromatography techniques and separates molecules on the basis of differences in **size**.



Mezzo cromatografico

Applicazione del campione

Eluizione

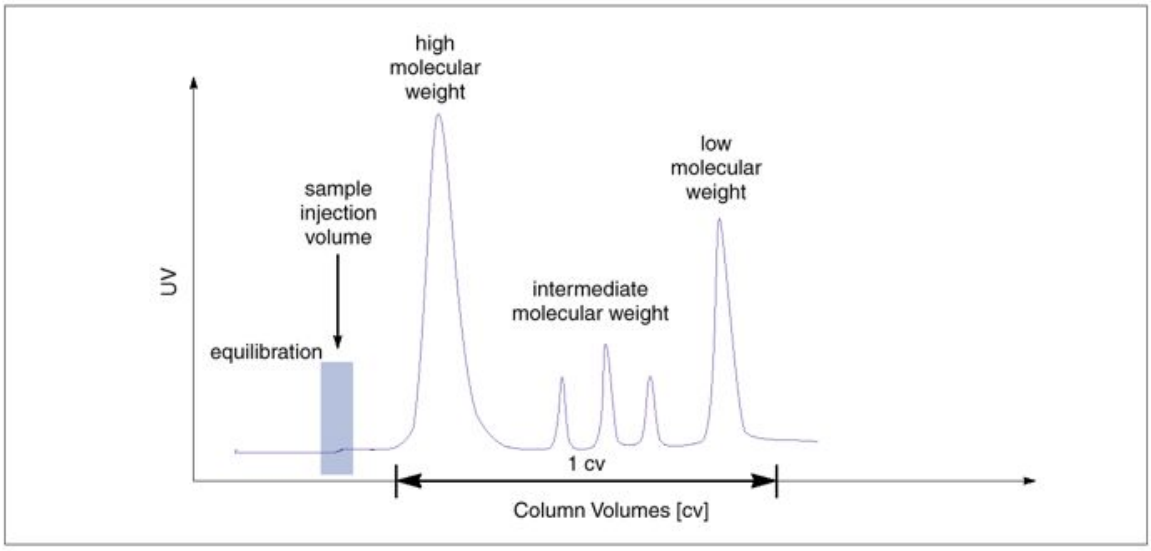


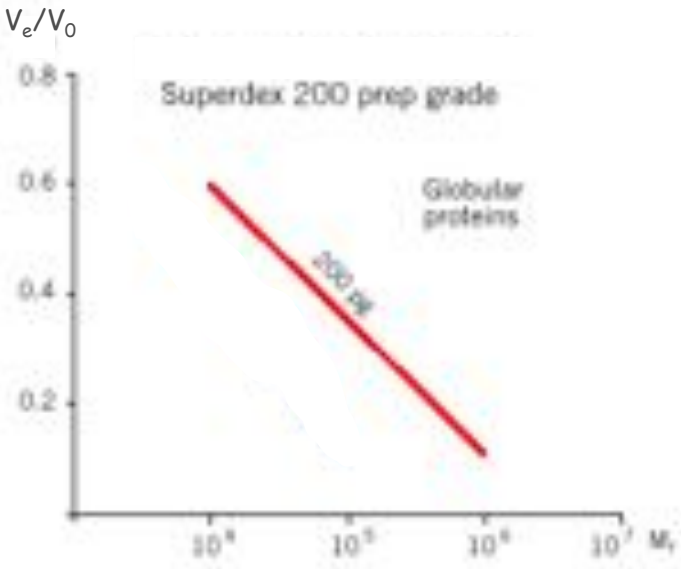
Fig. 41. Typical GF elution.

Determinazione dei pesi molecolari

Gel filtration provides a means of determining the molecular weight or size of proteins.

There is a linear relationship between the relative elution volume (V_e/V_0) of a protein and the logarithm of its molecular weights over a considerable molecular mass range.

A calibration curve is prepared by measuring the elution volumes of several standards, calculating their corresponding V_e/V_0 values (or similar parameter), and plotting their V_e/V_0 values versus the logarithm of their molecular weight.



The molecular weight of an unknown substance can be determined from the calibration curve once its V_e/V_0 value is calculated from its measured elution volume.

the FEBS Journal

Evidence for self-association of the alternative sigma factor σ^{54}

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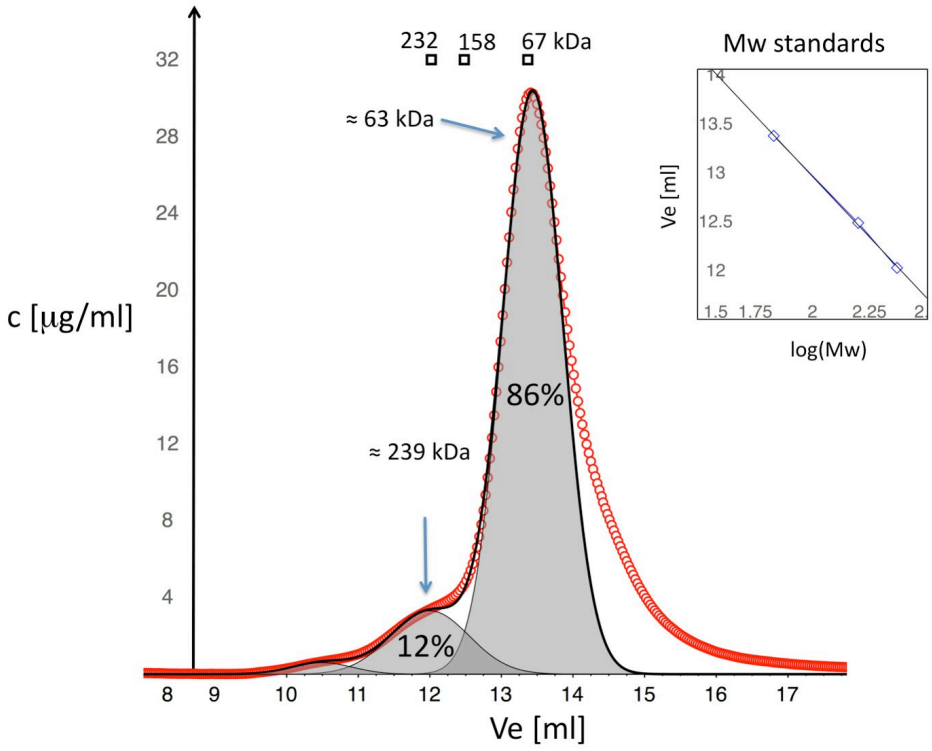
1 Department of Life Sciences, Università degli Studi di Milano, Milan, Italy
2 Consiglio Nazionale delle Ricerche-Istituto di Biofisica, Università degli Studi di Milano, Milan, Italy



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Principali resine usate

The earliest gel filtration matrices were formed by cross-linking polymers to form a three-dimensional network, for example ***Sephadex*** is formed by cross-linking **dextran**. Controlling the degree of cross-linking and particle size made it possible to produce a broad range of media, each one having a high selectivity over a narrow range of molecular weight values.

However, to increase the speed of a separation the medium must withstand higher flow rates and so alternative polymers such as **agarose** were investigated. This resulted in gel filtration media based on ***Sepharose*** and, later, the more highly cross-linked ***Superose***. Matrices based on agarose are, in general, more porous than those based on dextran so that, although the speed of a separation could be increased there was less selectivity when compared to *Sephadex*.

A major advance in gel filtration technology occurred when composite gels could be prepared by grafting a second polymer onto a pre-formed matrix, for example ***Sephacryl*** (cross-linking allyl **dextran** with **N,N'-methylene bisacrylamide**) and the most recent, ***Superdex*** (**dextran** chains covalently bonded to a highly cross-linked **agarose** matrix): it has been possible to create a range of media with the same high selectivity as Sephadex, but with the mechanical strength of a highly cross-linked agarose-based matrix.

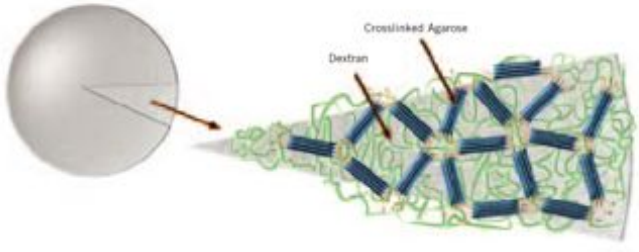

Fig. 20. In Superdex the dextran chains are covalently linked to a highly cross-linked agarose matrix. The figure shows a schematic of a section through a Superdex particle.

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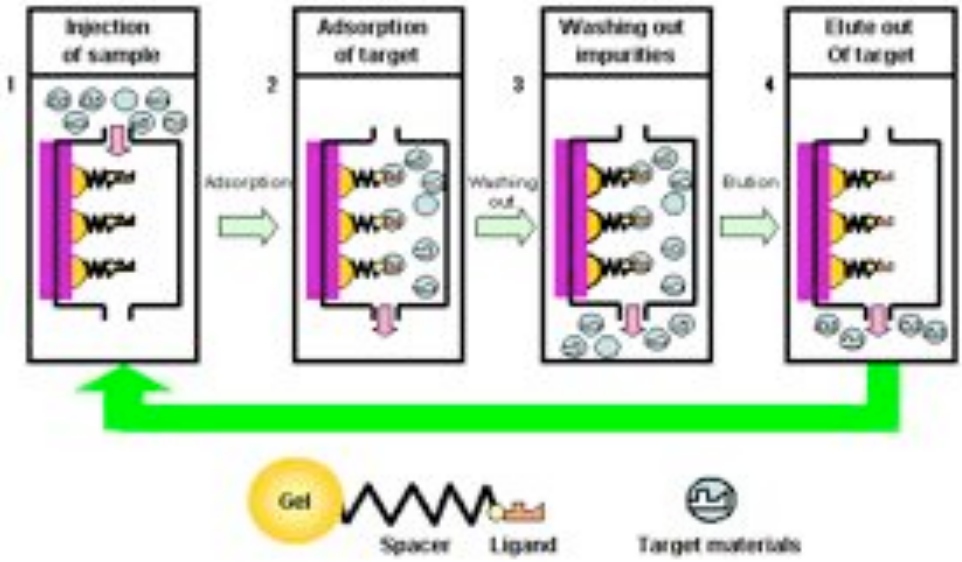
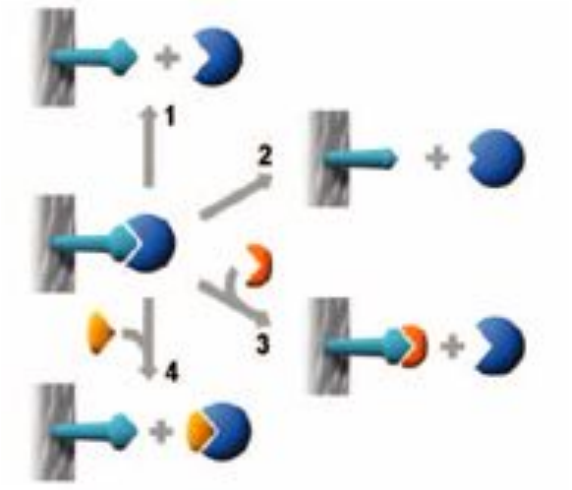
Cromatografia di affinità (AC)

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix. The technique is ideal for a capture or intermediate step in a purification protocol and can be used whenever a suitable ligand is available for the protein(s) of interest.



Elution

There is no generally applicable elution scheme for all affinity media. Reference to manufacturer's instructions, the scientific literature and a few simple rules should result in an effective elution method that elutes the target protein in a concentrated form. Elution methods may be either selective or non-selective, as shown in Figure 5.



Method 1
The simplest case. A change of buffer composition elutes the bound substance without harming either it or the ligand.

Method 2
Extremes of pH or high concentrations of chaotropic agents are required for elution, but these may cause permanent or temporary damage.

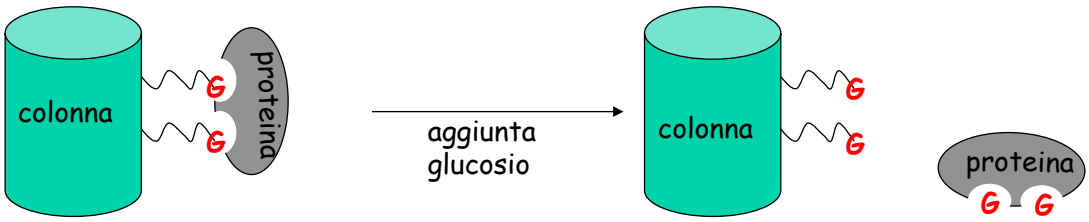
Methods 3 and 4
Specific elution by addition of a substance that competes for binding. These methods can enhance the specificity of media that use group-specific ligands.

Fig. 5. Elution methods.

esempio: Concanavalina A alta affinità per il glucosio

colonna con glucosio ⇒ la proteina si attacca
il resto scorre

aggiunta di glucosio ⇒ liberazione della proteina



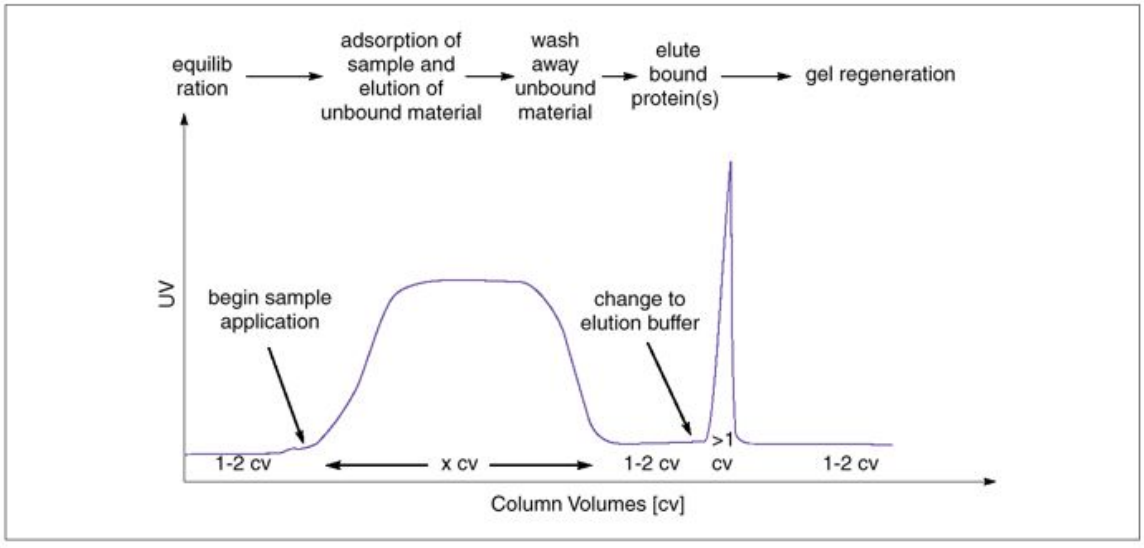
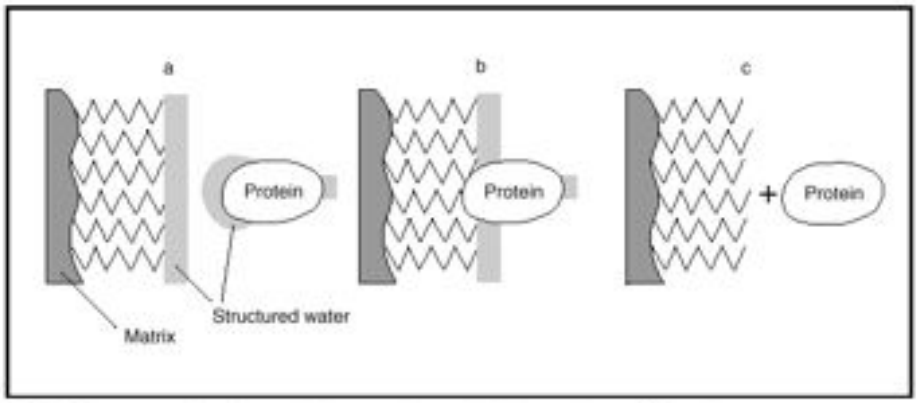


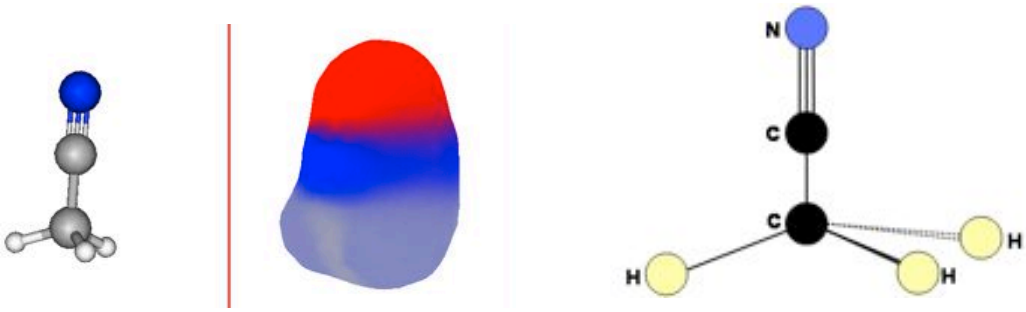
Fig. 40. Typical affinity separation.

Cromatografia per fase inversa (RPC)
(interazione idrofobica)

RPC separates proteins and peptides with differing hydrophobicity (dal gr. *hydrophòbos*: paura dell'acqua) based on their reversible interaction with the hydrophobic surface of a chromatographic medium. The binding interaction is the result of a favourable entropy effect. Due to the nature of the reversed phase matrices, the binding is usually very strong and requires the use of organic solvents for elution.



The initial mobile phase binding conditions used in reversed phase chromatography are primarily aqueous which indicates a high degree of organised water structure surrounding both the solute molecule and the immobilised ligand. As solute binds to the immobilised hydrophobic ligand, the hydrophobic area exposed to the solvent is minimised. Therefore, the degree of organised water structure is diminished with a corresponding favourable increase in system entropy. In this way, it is advantageous from an energy point of view for the hydrophobic moieties, i.e. solute and ligand, to associate



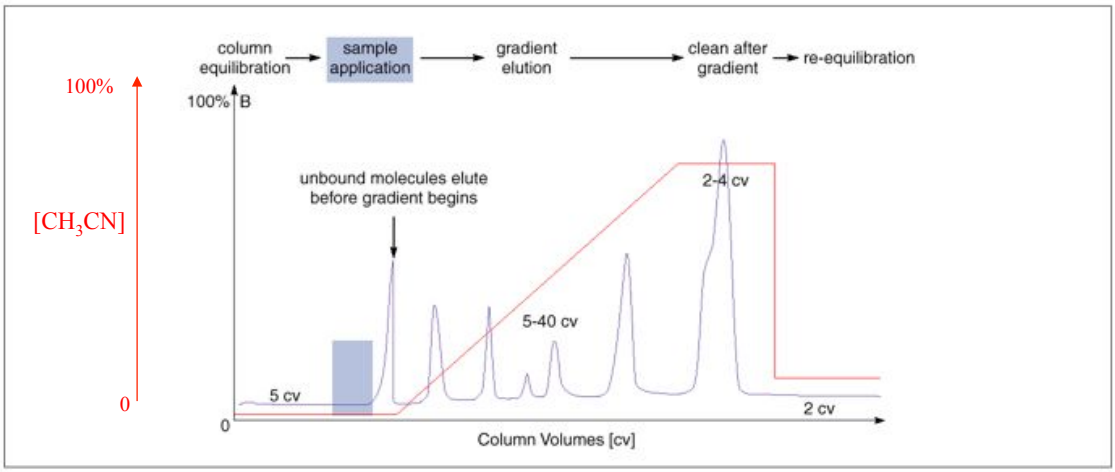
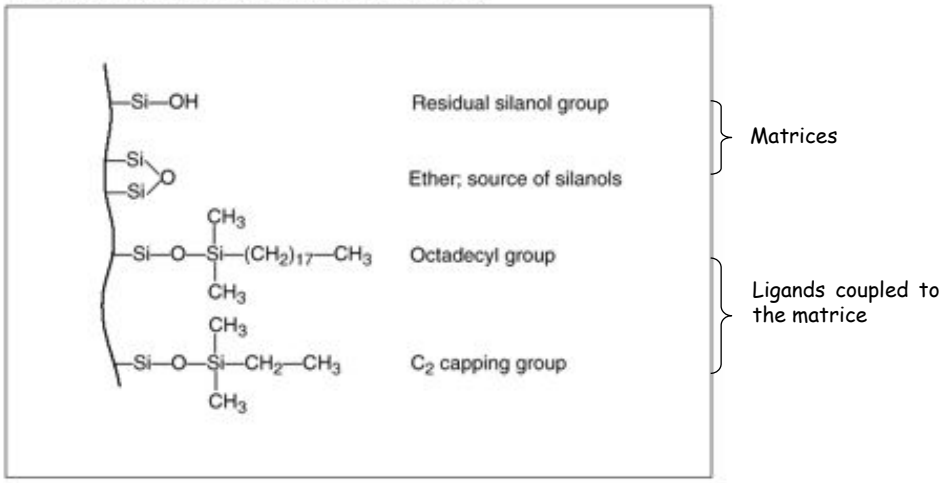


Fig. 43. Typical RPC gradient elution.

Fig. 3. Some typical structures on the surface of a silica-based reversed phase medium. The hydrophobic octadecyl group is one of the most common ligands.



RPC is often used in the final polishing of oligonucleotides and peptides and is ideal for analytical separations, such as peptide mapping.

RPC is not recommended for protein purification if recovery of activity and return to a correct tertiary structure are required, since many proteins are denatured in the presence of organic solvents.

ESEMPIO 2

Proteina espressa con un tag di 6 istidine

Protein information:

Tag N-His	MW (+tag) : 48942.5	PI (+tag) : 8.41
ε 280 nm : 1.3	Cysteines : 6	Methionine : 10

Expression :

Expression Conditions

Date	E. coli Strain	Medium	Volume	Température	Lysis buffer
20-07-05	Rosetta DE3pROS	SB*	3L	17°C	Tris50,NaCl300, imid25, pH8 + Lysozyme0.25mg/mL, antiprotéases

Cultures

DO Before/After induction	
Induction	0.5mM IPTG
Induction time	O.N
Lysis buffer volume	
Solubility	yes

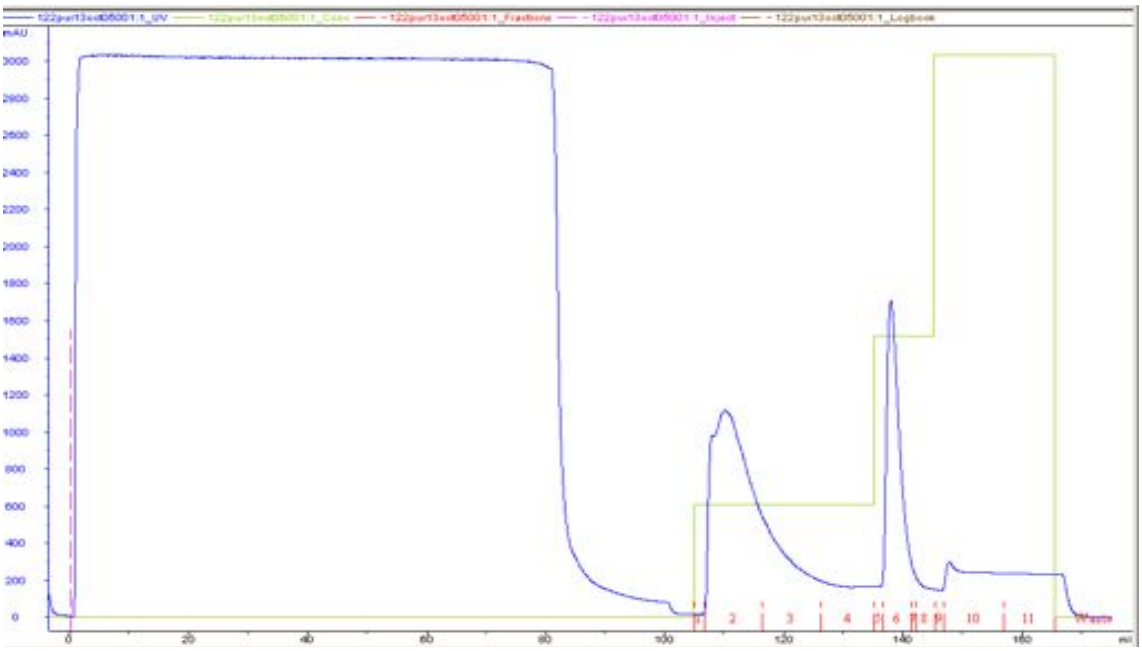
Purification : AKTA XPRESS

Purification on Hi Trap Ni 1 ml (Amersham)

Sample 80 ml of total lysate

Buffer A: Tris 50mM, NaCl 300 mM, imidazole **10mM**
Buffer B: Tris 50mM, NaCl 300 mM, imidazole **500mM**

Method: Step1: 100mM: Step 2: 250mM; Step 3: 500mM imidazole



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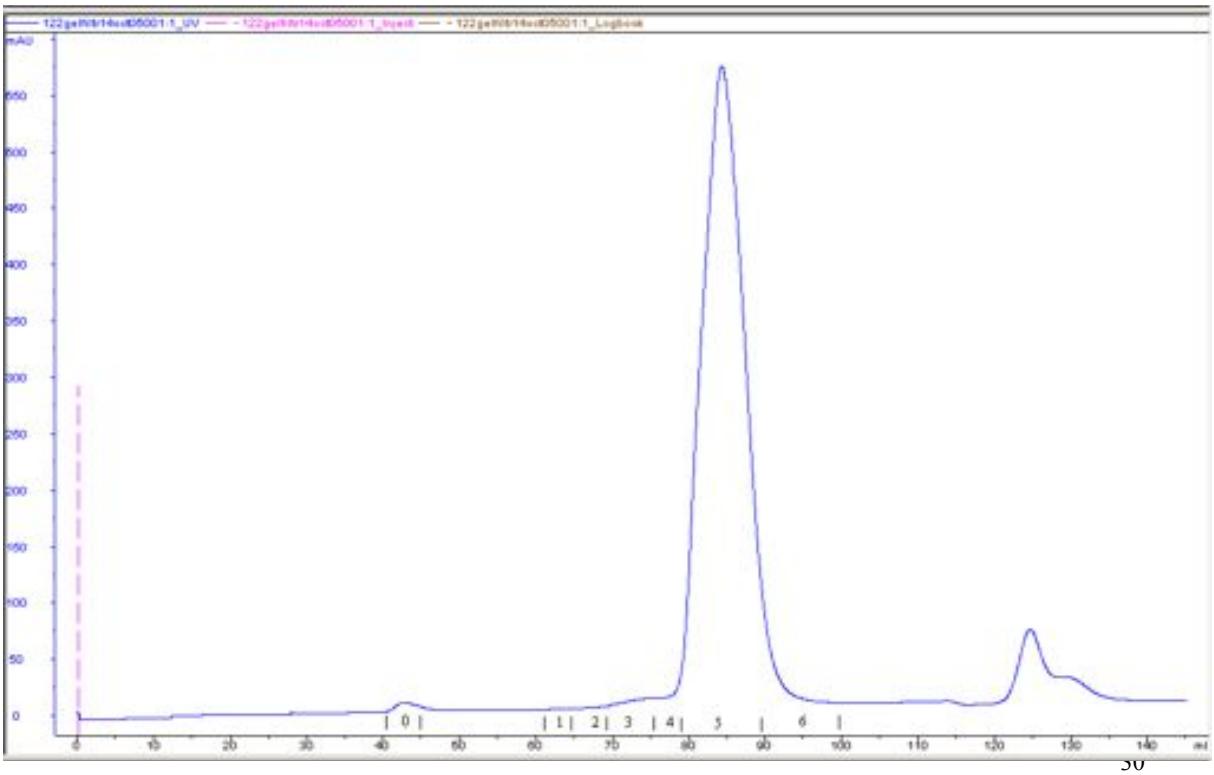
Purification on Sephadex 200 16/60 (Amersham)

Sample fr 6 of Ni-purification
Buffer: BICINE 10mM, NaCl 100 mM



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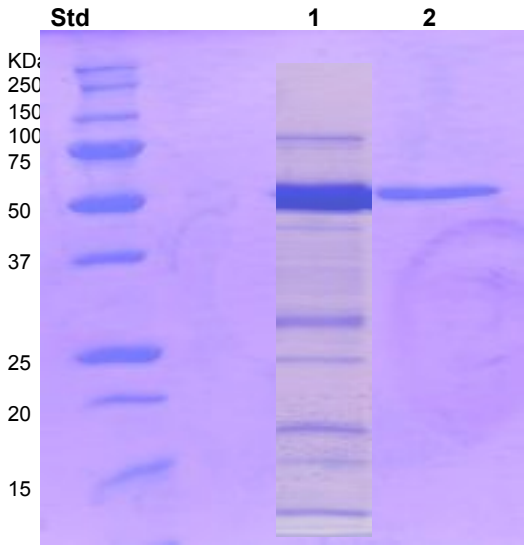
SDS-PAGE



1 = fr 6 da Ni-column
2 = fr 5 da gel-filtration