Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 27:

Other Forms of Chromatography

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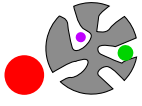
©David P. Goldenberg
University of Utah
goldenberg@biology.utah.edu

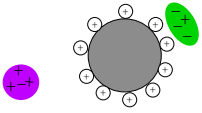
Chromatography Methods Commonly Used for Biomolecules

- Gel filtration chromatography - based on molecular size

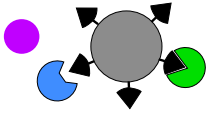


Form that we are using in lab.

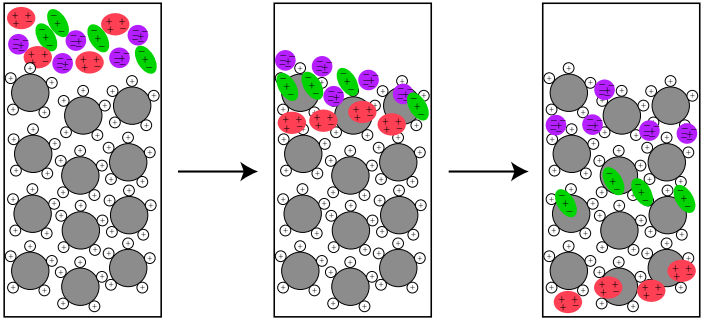
- Ion exchange chromatography - based on electric charge



- Affinity chromatography - based on specific biochemical interactions

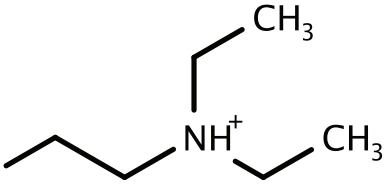


Ion Exchange Chromatography

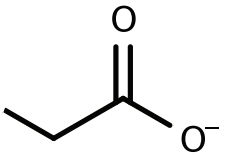


- Beads are chemically modified to introduce positively or negatively charged functional groups. Pores are kept small to minimize effect of molecular size.
- What determines strength of interaction between proteins and beads?
  - Net charge of proteins and, secondarily, distribution of charges.
    Choose column material based on net charge of proteins of interest.
  - Solution pH
  - Ionic strength (salt concentration)

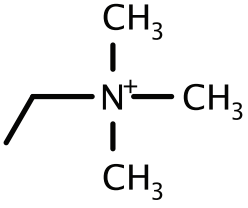
Charged Functional Groups Commonly Used for Ion Exchange Chromatography



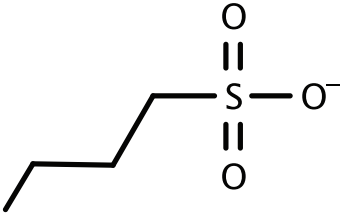
Diethylaminoethyl (DEAE)



Carboxymethyl (CM)



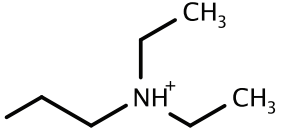
Quaternary amine (QA)



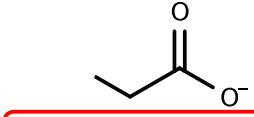
Sulfopropyl (SP)

Clicker Question #1

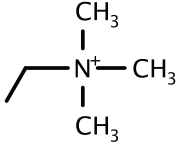
Suppose that we want to purify a protein with an isoelectric point of 8.3 using an ion-exchange column at pH 7.5. Which type of media would should we use?



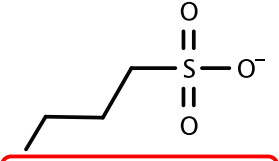
A) Diethylaminoethyl (DEAE)



C) Carboxymethyl (CM)

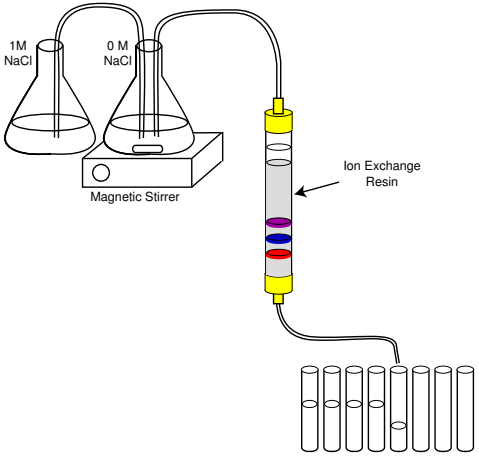


B) Quaternary amine (QA)



D) Sulfopropyl (SP)

A “Salt Gradient” is Usually Used to Elute Proteins from an Ion Exchange Column



- Column is first equilibrated with a buffer with low ionic strength.
- Sample is applied to top of the column.
- Proteins with no net charge or charge with same sign as the beads do not interact with the beads and pass through quickly.
- Ionic strength of buffer is gradually increased by mixing solutions with low and high salt concentrations.
- Individual proteins elute at characteristic salt concentrations.
- Very effective method for purifying proteins. Proteins differing by a single charge can often be separated.
- There is fancier equipment available!

HPLC: High Performance (or Pressure) Liquid Chromatography

(A relatively primitive version, circa 1990)

Photograph from https://en.wikipedia.org/wiki/High-performance_liquid_chromatography

- Smaller beads allow higher resolution.
- But, smaller beads reduce flow rate.
- High pressure allows small beads *and* high flow rates.
- Typical pressures: 1,000–3,000 psi.
- Requires high-pressure pumps and plumbing, usually made of stainless steel.
- Solvent gradients can be controlled precisely by computer control of the pumps.
- Newer versions aren't so different, but the parts are hidden inside!

HPLC: High Performance (or Pressure) Liquid Chromatography

Common separation modes:

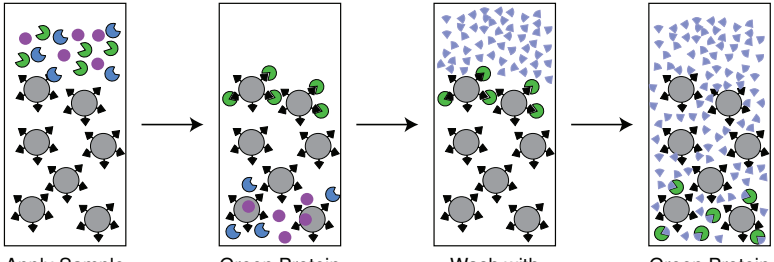
- Size exclusion.
  - Rigid porous beads (not gels), typically made of silica.
- Ion exchange.
- “Reversed phase”
  - Stationary-phase beads coated with a non-polar surface, *e.g.,* alkyl chains 8 to 20 carbons long.
  - Aqueous mobile phase with increasing concentration of a water-miscible non-polar compound, such as acetonitrile.
  - Molecules are separated on the basis of their polarity: More polar compounds elute earlier in the gradient.
  - Very widely used for organic compounds.
  - Works very well for smaller peptides (up to about 50 amino-acid residues), but not so well for larger polypeptides or proteins.

FPLC: Fast Protein Liquid Chromatography

Photograph from
https://en.wikipedia.org/wiki/Fast_protein_liquid_chromatography

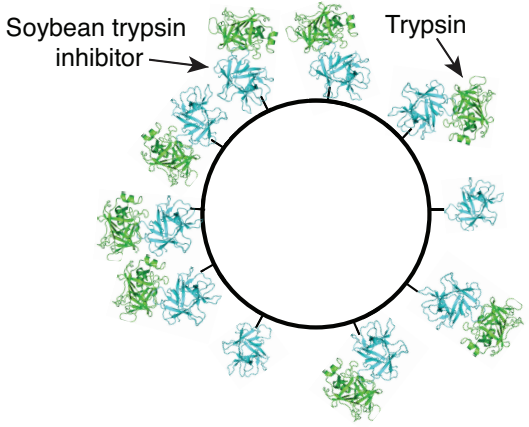
- First marketed by Pharmacia (now Cytiva) as an alternative to HPLC, better suited for proteins. Similar systems sold by Bio-Rad and others.
- Typical pressures are about 50 psi, versus 2000 psi.
- Allows the use of more protein-friendly materials, such as glass columns and plastic tubing.
- Media are similar to traditional media for biochemical chromatography, based on gels, but somewhat more rigid and with smaller beads.
- Typically faster separations and better resolution than with lower pressures, but somewhat limited in column and sample sizes.
- Very widely used now.

Affinity Chromatography



- Bound proteins can also be eluted by changing solution conditions:
  - pH
  - Salt concentration
  - Denaturants, such as urea or GuHCl
- Protein has to survive the treatment!

Affinity Purification of Trypsin



Actual dimensions:

Beads: $\approx 200\,\mu$m

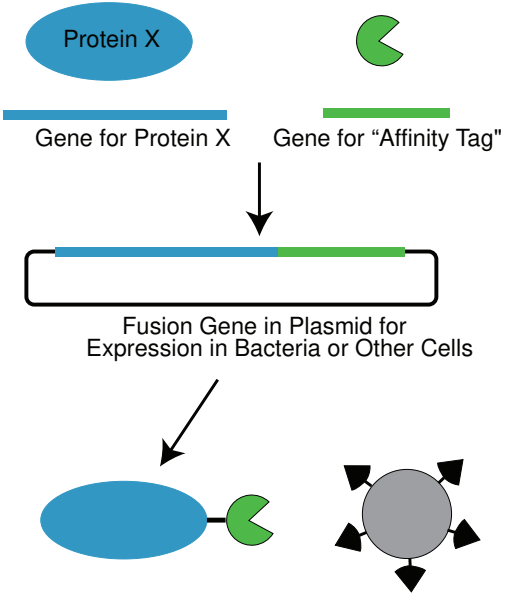
Protein complex: ≈ 5 nm

- Trypsin inhibitor from soybeans covalently attached to agarose beads. Soybean inhibitor is more selective for trypsin than is BPTI.
- Trypsin solution is applied to column and other proteins, and trypsin fragments, pass through.
- Trypsin is eluted with a solution that is 0.01 M HCl and 0.2 M KCl.
  Low pH disrupts the trypsin-inhibitor complex.
  Low pH also reversibly inactivates the trypsin and minimizes autolysis.
- Purified trypsin has higher activity and is more reproducible!

Affinity Targets for Chromatography

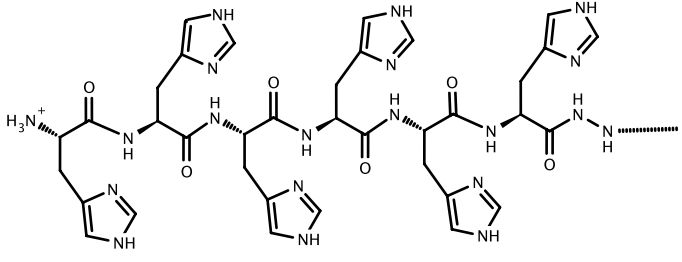
- Antibodies
  - Can be prepared to recognize almost any protein.
  - But, tend to be fragile, making it hard to dissociate complex without killing the antibody.
- Enzyme inhibitors; proteins or small molecules
- Nucleotide analogs
- DNA
  - Introduced by Bruce Alberts to purify proteins involved in DNA replication.
- Immobilized metal ions
- Dyes

Engineering Proteins for Affinity Purification



- Allows the same affinity beads and protocol to be used for many different proteins.
- Affinity purification doesn’t depend (much) on properties of a particular protein.
- But, the purified protein has an extra bit!
  - Can, in principle, remove the tag with a protease.
  - Need a protease that won’t cut other sites in the protein.

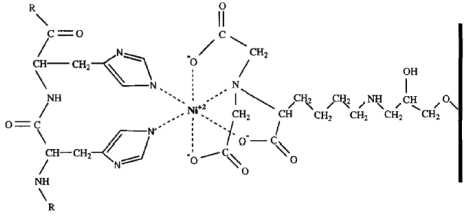
Polyhistidine: A Widely Used Engineered Affinity Tag



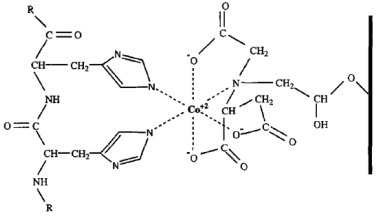
- Nitrogen atoms of imidazole act as ligands to transition-metal ions, *e.g.,* Ni^{+2}, Cu^{+2}, Co^{+2} or Zi^{+2}.
- Ions can be immobilized to beads by other ligands.
- Polyhistidine is easily added to N- or C-termini of proteins by genetic engineering.
- Short polyhistidine segments often have minimal effects on structure and function of proteins.

Immobilized Ions for Affinity Purification

a. Nickel-nitriloacetic acid (Ni^{+2}-NTA)



b. Cobalt-carboxylmethylaspartate (Co^{+2}-CMA)



- Liganding compound is covalently attached to beads.
- Column is “charged” with metal ions.
- Sample with tagged protein is applied to column.
- Column is washed with buffer to remove untagged proteins.
- Tagged protein is eluted by lowering pH or adding excess imidazole.

Illustration from Bornhorst, J. A. & Falke, J. J. (2000). Purification of proteins using polyhistidine affinity tags. *Methods Enzymol*, 326, 245–254. https://doi.org/10.1016/S0076-6879(00)26058-8