7.02 Fall 2001 PBC DAY 2 Recitation

Version 1.02

1) Today in lab
   a) Obtain and resuspend AS pellet.
   b) Desalt entire sample on PD-10 column.
   c) Test fractions using qualitative assay, determine fractions for DEAE/AF load.
   d) Load half of DEAE/AF load on DEAE anion exchange column.
   e) Assay resulting fractions quantitatively.
2) Chromatography 101
   a) Separation of molecules based on differential interaction with an immobilized matrix.
   b) We do column chromatography; immobile matrix is packed in a column.
   c) What types of column chromatography will we be using? What methods of separation do they use?
      i) Gel filtration—size
      ii) Ion exchange—charge
      iii) Affinity—affinity for a substrate molecule
   d) General considerations for using when running columns:
      i) Don t let column run dry watch the meniscus.
      ii) Don t disturb the column bed add liquid along side of column tube.
      iii) Always cetrifuge tubes prior to loading insoluble material gunks up the column.
      iv) Use cold column buffer and place fractions on ice as soon as they are collected keep in mind ABC or raw meat principal.
      v) Chase protein sample with a small amount of column buffer.
3) Gel Filtration
   a) A.k.a. Size exclusion, desalting column
   b) Matrix is porous agarose beads pores can fit things us to 5 kDa in size.
   c) Small molecules can get stuck in pores and are impeded by the column. Larger molecules are not imeped by the column as much. (Complex PLINKO game).
   d) Therefore, as buffer flows through the column, how will small molecules and large molecules elute?
      i) Small—impeded—elute last
      ii) Large—not impeded—elute first
   e) What are the types of molecules present in the AS-P sample? How will each behave in the column?
      i) Protein molecules—not impeded—elute first.
      ii) *Ammonium and sulfate ions—impeded by matrix—elute last.*
   f) Given enough time and buffer pushed through the column, salt molecules will elute.
   g) How does this help in our purification?
      i) Doesn't significantly separate β-gal from other proteins, but
      ii) Does effectively separate β-gal from ammonium and sulfate ions.
      iii) Protein elutes with the buffer salts already present on column—allows one to switch buffer.
   h) **Is there any other method one could use for changing the buffer that a protein is in?** *Answer: Dialysis.* Allows exchange of buffer salt ions across selectively permeable membrane, but requires large amounts of buffer and takes a long time. PD-10 column much more time efficient.

4) DEAE chromatograpy
   a) After PD-10 column, assay fractions qualitatively. Pool one or 2 fractions. Load half of this onto DEAE column (after brief spin), save the other half.
   b) DEAE anion exchange column.
   c) (Draw DEAE bead) Matrix is positivlely charged negative things stick to it.
   d) **How could we then elute something that is bound to the column?**
      i) *Elute with something that has a lot of negative charges—will compete for positive charges and knock off what ever is bound*.
   e) What properties of proteins allows us to use this as a purification technique?
      i) Proteins have surface charges.
      ii) Some proteins have a more negative charge than others.
      iii) β-gal is one such protein.
   f) Isoelectric point
      i) At low pH, equilibrium pushes H+ ions to protonate negative surface charges. How does this effect the surface charge of the protein.
      ii) At high pH, equilibrium favors H+ ions deprotonating.
      iii) At some point, protein is neutral in surface charge**. This is called the protein's Isoelectric Point (IEP)**. The pH at which this occurs is called the pI
   g) What happens when pH<pI? When pH>pI?
      i) pH<pI, proteins positive
      ii) *pH>pI, proteins negative.*
   h) pI for β-gal is 5.3. **The pH of the column buffer is what?** *Answer: 7.5*. **So, therefore, what is the charge of β-gal?** *Answer: Negative.* **Cool, ain't?**
   i) **Once we stick β-gal to the column, how will we get it off?** *Answer, apply large amounts of negative ions, i.e. Cl-*.
   j) Phases of DEAE column
      i) Load protein. **Salt concentration?** 0.1 M. This salt concentration is near physiological levels, some salt must be present to stabilize protein. Collect fraction while loading the protein. This phase is called binding .
      ii) Add a volume of 0.1 M NaCl. **Will this elute protein?** *Answer: no*. Collect this wash fraction.
      iii) Add volumes of 0.4 M NaCl. **What will this higher salt concentration do?** *Answer: knock off β-gal (hopefully)*. This step is known as elution .
      iv) Add volume of 1.0 M NaCl. **What will this salt concentration do?** *Answer: knock off anything else bound to the column.* This step is known as flushing the column.
      v) Thought question: If this method of using three NaCl concentrations didn't give us sufficiently pure protein, how could we improve upon the protocol?
         (1) Use "step elution" of smaller increments.
         (2) *Use "gradient elution". Both of these require the collection of "many more fractions.*