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Sample preparation & protein enrichment for proteomics and mass spectrometry

Helen Kim
934-3880
helenkim@uab.edu
Dept of Pharmacology and Toxicology
McCallum Building, room 460
http://www.uab.edu/proteomics

Learning objectives for next two lectures:

WEDNESDAY (Jan 8, 2014):

- **Rationales for proteomics versus genomics versus Western blots versus immunoprecipitations.**
- **SMART PROTEOMICS: reduce proteome complexity, increase biological specificity;**
- **Take advantage of properties of proteins and experimental information to reduce proteome complexity**

FRIDAY (Jan 10, 2014):

- **How do we know what we have**
- **How do we enhance what we have**
- **Sample preparation for MS analysis**
- **Quality control in protein purification/enrichment and analysis**

Experimental rationale determines extent and nature of protein purification for proteomic analysis

Different rationales for studying proteins in biological samples:

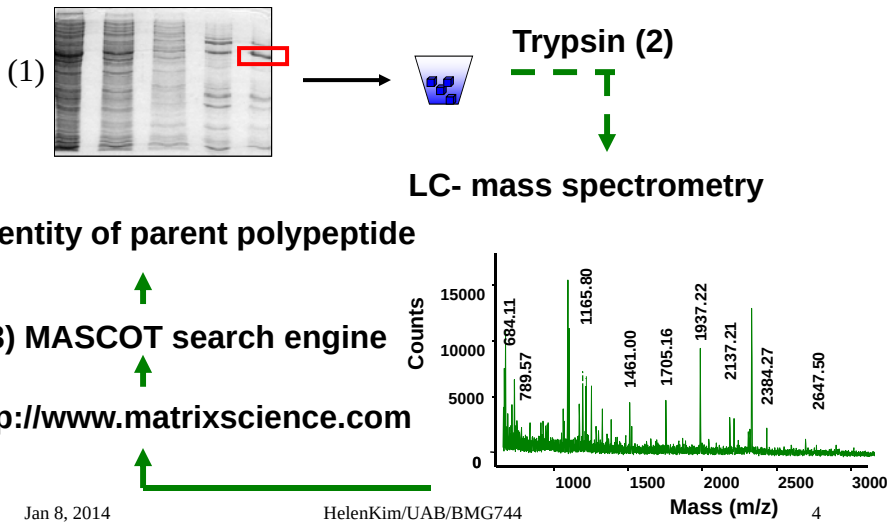
A. Nothing is known, and all proteins are of interest;

B. Nothing is known, but only proteins that are different between disease and healthy or treated and control or novel versus familiar are of interest;

C. PTM of a single protein or a PARTICULAR PTM category

D. Protein *interactions* involving a specific protein are of interest

The basic elements of intact (top-down) protein proteomics: (1) purification/separation, (2) processing for MS analysis, (3) identification and characterization



Parameters that govern the choice of protein separation method

- Purity of protein
- Speed of purification
- Quantity of protein
- What is the question: most important
  - Discovering a new protein/proteome
  - Identifying protein-protein interactions
  - Identifying potential modifications of known proteins

Properties of polypeptides that enable separation from each other

- Intrinsic properties
  - Size—number of amino acids
  - Net Charge
- Biological/functional properties
  - Intracellular location
  - Enzyme activity
  - Undergoes oligomerization
  - Undergoes modification

Some protein separation methods are both preparative and analytical

- **2D electrophoresis**
  - **Resolves polypeptides dissociated from each other**
  - **Good for isoform detection/quantitation, PTM changes detection/quantitation**
- **2D blue native electrophoresis:**
  - **Resolves complexes containing intact proteins**
  - **Can be quantified by MS, or by blotting for specific proteins**

Separating proteins by size

- For purification: Gel filtration chromatography
- Analytic: SDS-PAGE

Separating proteins by size

- Gel filtration chromatography:
- Separate under native or denaturing conditions: when do you want to do which?
- DRAW: separation under denaturing conditions vs native.
- Use of standards allows estimation of globular mw
- SDS-PAGE of fractions allows assessment of complexity in each, extent of separation

Separating proteins by size

- SDS-PAGE = analytical method, but sensitivity of MS instruments enables identification/analysis of “bands” on SDS-PAGE; therefore it is a purification approach as well.
- DRAW: can resolve down full length of gel, or run into resolving gel to get into the gel, but before any mw resolution, keeping all proteins together in one band.
- MW can be estimated by calibration with known standards; DRAW

Separating proteins by charge

- **Purification: Ion exchange chromatography**
- **Analytic: isoelectric focussing**

Separating proteins by charge

- **Ion exchange chromatography**
- **Takes advantage of the charged character of proteins; can greatly concentrate one fraction from the other, by the former binding to the ion exchange resin;**

Separating proteins by charge

- Isoelectric focussing:
- As with SDS-PAGE, was an analytical method;
- With the sensitivity of MS, can be a purification method for resolving multiple proteins
- Particularly effective at resolving proteins that differ by PTMs

Extrinsic properties of proteins that can be basis for purification

- Oligomerization:
  - Each cytoskeletal component undergoes reversible oligomerization from its monomers
- Intracellular location:
  - Change in
  - Increase in same location
- Protein or ligand interactions

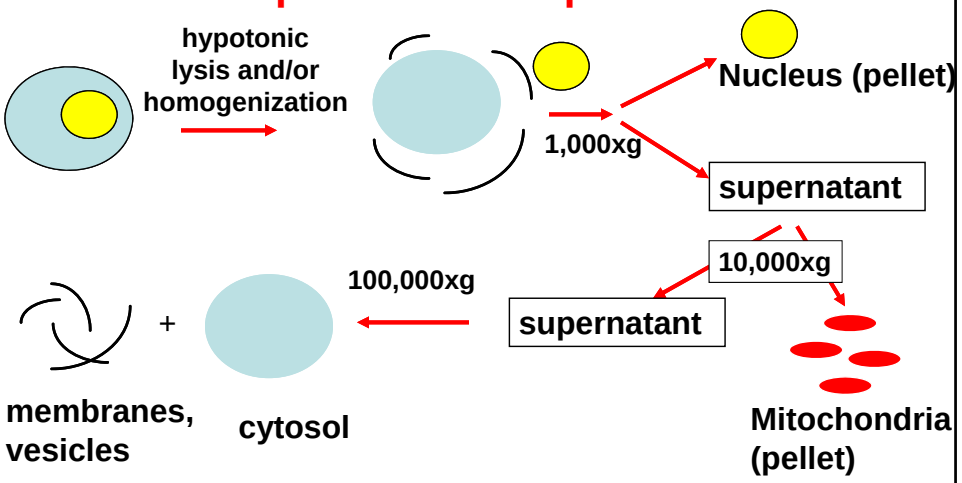
Wide dynamic range for proteins in most cells: rationale for protein enrichment

- A gel that is overloaded with respect to the abundant proteins, may still have only *barely detectable or nondetectable* amounts of a low abundance protein
  - If you can't see it, you don't know it's there;
  - Even if software detects and quantitates it, you can't do MS of it, because there isn't enough protein.
- No one stain will detect 9 orders of magnitude differences in abundance of proteins.
- In MS experiments, bypassing gels, the greater the complexity of the peptide mixture, the lower the chance of detecting very low abundance proteins/peptides.

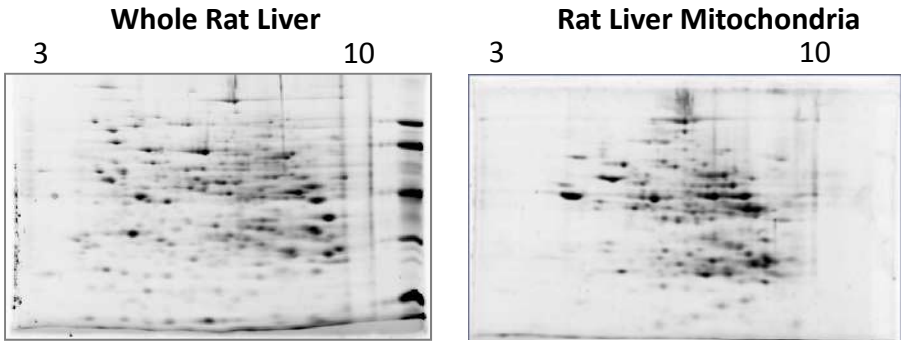
Tissue disruption/cell lysis

- Manual and mechanical homogenization
  - Mortar and pestle, Dounce and Potter-Elvehjem homogenizers, Waring blender
- Grinding with beads, sonication and freeze-thaw
- Osmotic shock
- Bugbuster™ for bacteria
- Detergents: CHAPS, Triton X-100, cholate and deoxycholate
- Protease inhibitors if necessary

Use “old” information and “conventional” approaches like differential centrifugation to enhance proteomics experiments.



Analysis of mitochondrial proteins enhanced by purifying that subproteome



2D gel analysis using the Invitrogen ZOOM system

(Courtesy of Shannon Bailey Lab – Whitney Theis and Kelly Andringa)

The good news: Several subcellular proteomes have been "catalogued."

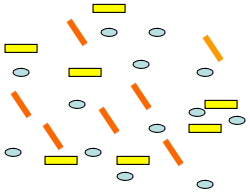
Compartment	# polypeptides in SWISSPROT as of 2000
Mitochondria (1000/cell)	269------5% of total
Lysosome (400/cell)	50------1% of total
Peroxisome	35------0.6%
ER and Golgi apparatus	157------3%
Nuclei (5% cell volume)	964-----17%
Others (cytosol, membrane)	4228----75%
	total: 5703

(Jung et al. [2000] Electrophoresis)
Note date: this is old!!! But the principle is the same; homework for class, find current numbers, please.

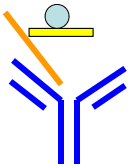
Affinity purification: Antibodies are available for many protein posttranslational modifications

- Phosphorylation
- Glycosylation
- Oxidative modifications:
  - Protein carbonyls
  - Reactive aldehyde adduct formation: 4HNE
- Keep in mind that these modifications each involve mass changes, thus can be detected directly by MS.

Antibodies can reduce the complexity of the proteome, as well as enhance biological specificity, by 10,000-fold



A cell lysate: 6,739 polypeptides



An immune complex of 1-10 polypeptides (why might there be more than one polypeptide?)

Which sample would you rather deal with on a 1D or 2D gel?

Affinity purification re-visited: mass spectrometry and immunoprecipitation, with some new insights

LESSONS: No matter how much you "clarify" a homogenate, some proteins aggregate over the timeframe of incubation with antibody, and co-precipitate with the antibody… these are "nonspecific."

BUT THIS CAN BE DEALT WITH, simply by doing a second "clarification" spin after the antibody incubation, before incubation with the secondary antibody- or Protein A- beads.

The "immune complex" has an S value of ~ 10, will not spin down in the same timeframe or centrifugation conditions as Protein A-agarose beads.
(taken from O'Malley's paper------GET)

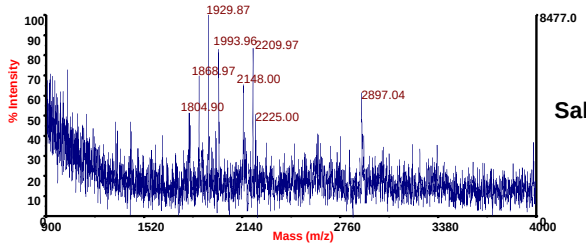
Preparing proteins for peptide mass fingerprinting (1)

- **The mass spectrometry procedure has to be preceded by high resolution protein chromatography steps……the LC part of LC-MS.**
- **Or, it can be a sample with a few intense bands of interest - e.g., a recombinantly expressed protein in bacteria lysate.**
- **SDS-PAGE may be the best thing you can do for your protein preparation…it gets rid of lots of potential contaminants, and at the same time concentrates the proteins into "bands."**

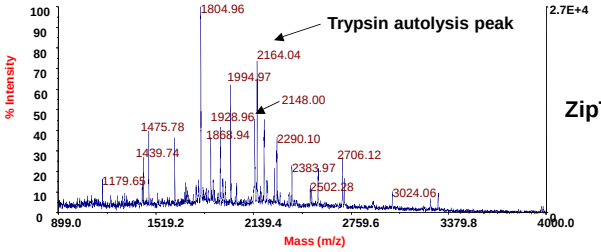
Preparing proteins for peptide mass fingerprinting (2)

- **Once the protein is precipitated in the SDS gel matrix, electrolytes/salts and the SDS are largely removed by washing the gel pieces with 50% aqueous acetonitrile containing 25 mM NH_4HCO_3 buffer, pH 8**
- **The gel is dehydrated and rehydrated in 25 mM NH_4HCO_3 buffer, pH 8 to which trypsin added for overnight digestion.**
- **The resulting peptides are extracted with 50% aqueous acetonitrile containing 25 mM NH_4HCO_3 buffer, pH 8 - the extract is evaporated, and brought up in MS-compatible buffer/solvent.**

Benefit of removing salt from tryptic digest



Salt Contamination



ZipTip (desalting)

Quality control issues in handling proteins and peptides

- **Whatever you do, do it the same (minimize variance);**
  - **Collecting and storing the sample:**
    - **same type of storage device such as centrifuge tube, even the type of plastic**
    - **If one batch at -80, all at -80**
- **Some samples are sensitive to freezing**
  - **Mitochondria and other organelle fractions should be prepared using fresh tissue**
  - **Samples especially fluids and organelle preparations should be placed in same volume aliquots and only thawed one time to avoid the effects of multiple freeze-thaw cycles**
- **Freeze fast (into liquid N_2)**
  - **Buffers such as sodium phosphate can selectively precipitate while ice and water are in equilibrium (down to -20°C) - this can lead to a substantial change in pH**
  - **Similarly, thaw fast rather than slowly, then keep on ice. (thaw by rolling between your hands, vs letting it sit on bench or worse on ice while you eat lunch.**

Quality control issues in handling proteins and peptides, part II

- **Reduce variance by thinking of what actions cause it:**
  - **Help a colleague by processing samples through all of one step in a multi step process, not by processing part of the samples through the same step side by side with him---- no one pipets exactly the same;**
  - **Use the same pipettor for the same volume where feasible,and use the largest volumes where practical; i.e. use 10 ul of a 1/100 dilution, vs 1 ul of a 1/10.**
  - **Use the same vendor of a chemical for the same set of samples; SDS is not SDS is not SDS.**
- **Eliminate variance where possible;**
  - **If you do an overnight freezing at -80 between steps, do it for every sample;**
  - **If you Western blot overnight, always Western blot overnight.**

Take home messages regarding protein purification

- **Goal is to lower the complexity of the subproteome**
  - **by conventional protein separation approaches (size exclusion, ion exchange, reverse phase).**
  - **Let biology work for you**
    - **subcellular compartment**
    - **ligand or protein interaction**
    - **Oligomerization**
- **Choice/extent of purification governed by**
  - **Abundance of sample**
  - **Abundance (if known) of protein in question**
  - **Question being asked…you may not <u>want</u> to purify extensively if very little information**
  - **What technologies you can access readily**

Take home points, part II

- What proteomics technology gives back is like any other analytical approach: the quality is as good as what you put in;
- Be mindful of distinguishing between low abundance proteins versus low level contaminants;
- "Conventional" approaches like immunoprecipitation can be powerful when combined with MS;
- The "end result" in proteomics is just a beginning:
  I. Some changes are causal to the disease/phenotype;
  II. Some are "real" but not causal;
  III. Some could be response of the cells/tissues TO DEAL WITH the disease, not causing the disease.

Suggested readings

- Cañas et al., 2007. "*Trends in sample preparation for classical and second generation proteomics*" Journal of Chromatography A, vol. 1153, pp 235-258.
- Weerapana, Speers, Cravatt, Nature Protocols, 2007

HOMEWORK by Friday:

- find a publication (full citation) within the last 2 years that identifies the proteins in a subproteome (mitochondria, peroxisomes, plasma membrane ie) by mass spectrometry
- OR: read the Weerapana et al paper above, and tell us what is meant by "click chemistry."