

January 9, 2013

Sample preparation & protein enrichment for proteomics and mass spectrometry

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1

Learning objectives for next two lectures:

WEDNESDAY (Jan 9, 2013):

- 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 11, 2013):

- 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

S Barnes BMG 744 1/07/13

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

Different rationales for studying proteins in biological samples:

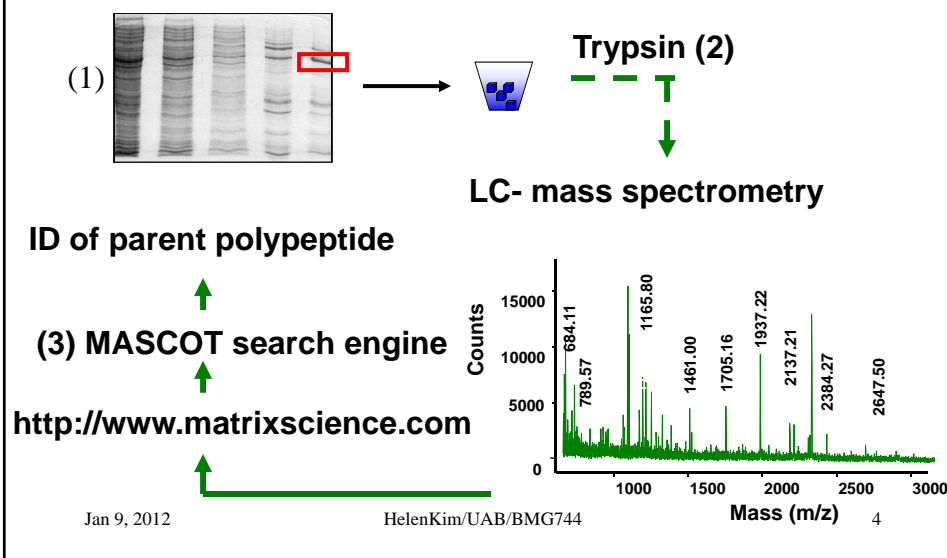
- A. Nothing is known, and all proteins are of interest;
- B. Nothing is known, but only proteins that are different between disease and healthy or treated and control or novel versus familiar are of interest;
- C. PTM of a single protein or a PARTICULAR PTM category
- D. Protein *interactions* involving a specific protein are of interest

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3

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



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4

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**

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5

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**

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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

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7

Separating proteins by size

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

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8

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**

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9

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**

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10

Separating proteins by charge

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

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11

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;**

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12

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**

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13

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**

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14

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.

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15

Tissue disruption/cell lysis

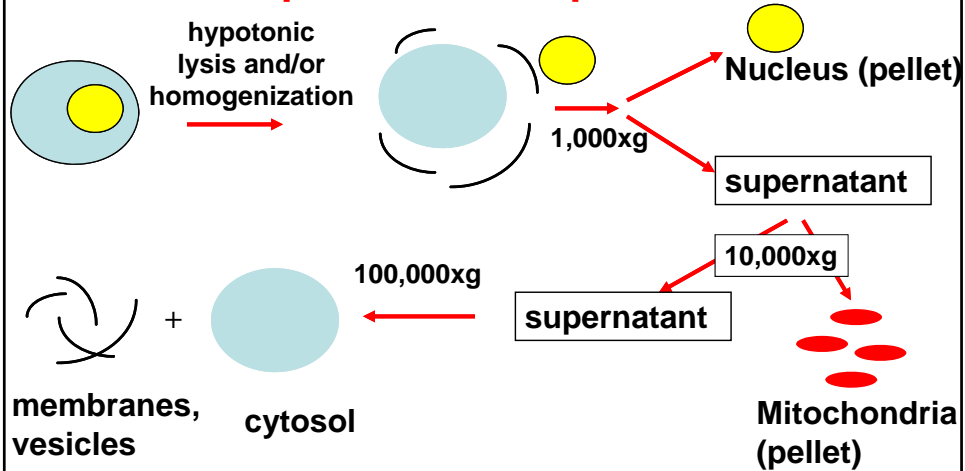
- **Manual and mechanical homogenization**
 - Mortar and pestle, Dounce and Potter-Elvehjem homogenizers, Waring blender
- **Grinding with beads, sonication and freeze-thaw**
- **Osmotic shock**
- **Bugbuster™ for bacteria**
- **Detergents: CHAPS, Triton X-100, cholate and deoxycholate**
- **Protease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

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16

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

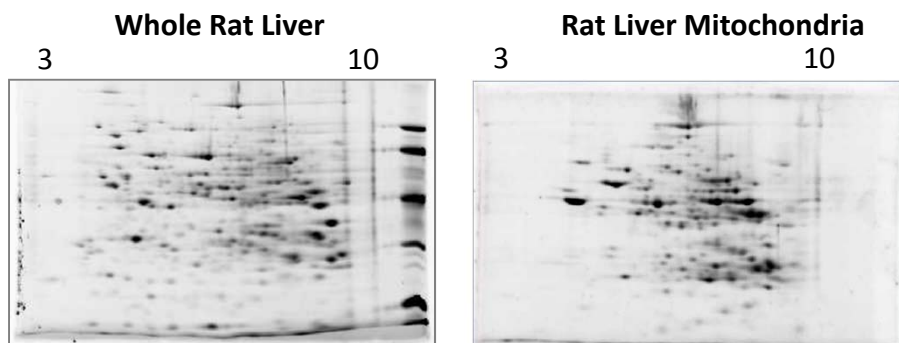


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17

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)

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18

**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.

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19

**Affinity purification: Antibodies are
available for many protein
posttranslational modifications**

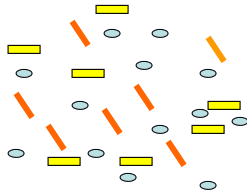
- **Phosphorylation**
- **Glycosylation**
- **Oxidative modifications:**
 - Protein carbonyls
 - Reactive aldehyde adduct formation: 4HNE
- **Keep in mind that these modifications
each involve mass changes, thus can
be detected directly by MS.**

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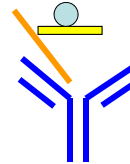
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20

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?

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21

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 time frame or centrifugation conditions as Protein A-agarose beads.

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22

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.”

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23

Preparing proteins for peptide mass fingerprinting (2)

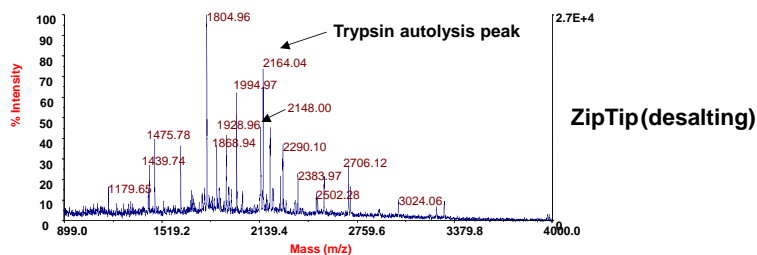
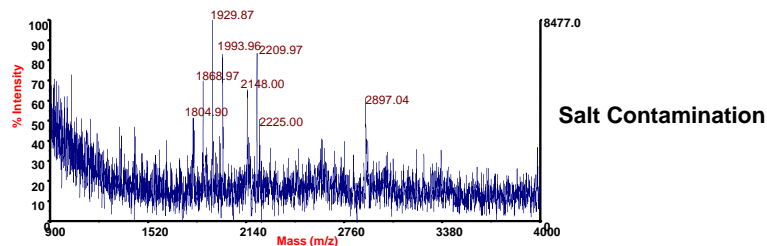
- Once the protein is precipitated in the SDS gel matrix, electrolytes/salts and the SDS are largely removed by washing the gel pieces with 50% aqueous acetonitrile containing 25 mM NH_4HCO_3 buffer, pH 8
- The gel is dehydrated and rehydrated in 25 mM NH_4HCO_3 buffer, pH 8 to which trypsin added for 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.

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24

Benefit of removing salt from tryptic digest



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25

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 N₂)**
 - 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.

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26

Quality control issues in handling proteins and peptides, part II

- Reduce variance by thinking of what actions causes 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.

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27

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 want to purify extensively if very little information
 - What technologies you can access readily

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28

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.

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29

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.”

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30