Lecture 1
Fundamentals of Protein Chemistry and Mass Spectrometry

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Why Proteomics?

Same Genome

Different Proteome

DNA – what could be

RNA – what is trying to be

Protein – what is

Black Swallowtail – larvae and butterfly
Proteomics is a fundamentally interdisciplinary activity. It exists at the intersection of three major domains.
Amino Acids are the Basic Structural Units of Proteins

General Structure of an Amino Acid

L-Isomer

D-Isomer

The Ionization State of Amino Acids Changes with pH
Formation of Peptide Bond

Note the loss of water in forming the peptide bond.

This loss must be recognized when calculating the molecular weight of peptides from a list of molecular weights of amino acids.

http://en.wikipedia.org/wiki/Amino_acid
The charge state and hydrophobicity are key physicochemical attributes that clearly impact not only protein structure and function, but also impact how we process and analyze proteins by mass spectrometry.
Acid-Base Chemistry in Protein Characterization

The net charge of a peptide or protein at any pH depends on the combined pK values for its amino acids and terminal groups.

\[ \text{pH} = \text{pK} + \log \left[ \frac{[A^-]}{[HA]} \right] \]

- pK of alpha-COOH groups: 1.8 - 2.4
- pK of alpha-NH2 groups: 9.0 - 10.8
- pK of ionizable side chains: 3.9 - 12.5

The isoelectric point is the pH at which there is no net charge.

It is important to remember how protein and peptide pK values affect chemistry and separations:

- Chemical Modification (e.g. Reduction / Alkylation)
- Proteolysis (e.g. specificity of Glu-C)
- Chromatography (e.g. Ion Exchange, Reversed Phase)
- 2D Gel Separations (Isoelectric Focusing)
- Ionization for Mass Spectrometry
## Properties of The Twenty Amino Acids

<table>
<thead>
<tr>
<th>Name</th>
<th>Side Chain</th>
<th>pKa</th>
<th>pI</th>
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<tbody>
<tr>
<td>Alanine</td>
<td>Ala A</td>
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<td>Arginine</td>
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<td>Aspartic Acid</td>
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<td>Cysteine</td>
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<td>Ile I</td>
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<td>Leu L</td>
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<tr>
<td>Valine</td>
<td>Val V</td>
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<td>6.00</td>
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Average Molecular Weight, Monoisotopic Molecular Weight, and Peptide Molecular Weight

• “The atomic weight of an element is the weighted average of the isotope masses. For example, 98.89% of all carbon atoms are in the form of carbon-12 and almost all of the remainder are carbon-13. The atomic weight of carbon is then calculated to be $0.9889 \times 12.00000 + 0.0111 \times 13.003354 = 12.01$.”
  – See http://www.i-mass.com/guide/aweight.html for listing of atomic weights

• Question – will you ever analyze a carbon atom with an atomic weight of 12.01?
  – NO!
  – If you measure the weight of a peptide with 100 carbon atoms, 98.89% of the carbons will have an atomic weight of 12, and 1.11% will have an atomic weight of 13.003354
  – The average atomic weight of carbon is 12.0111
  – The monoisotopic molecular weight is 12.0000

• Mass spectrometers measure “real” isotopes, not “average” isotopes
  – In the 2000 US Census the average family had 1.86 kids
  – In the 2000 US Census no family had 1.86 kids

• Pro-Glu-Pro-Thr-Ile-Asp-Glu (PEPTIDE)
  – Average MW = 799.8228
  – Monoisotopic MW = 799.3599
Amino Acids are Linked by Amide Bonds to Form Peptide Chains

General Structure of a Peptide

Note loss of water
Note that these are the forms & weights of the amino acids as they would exist in a peptide chain

Each residue is “missing H2O”
- N-terminal hydrogen and C-terminal hydroxyl groups

The peptide DEVIL would weigh
- \( H_2O = 2(1.00783)+15.9949 = 18.01056 \)

http://www.i-mass.com/guide/aamass.html
Really Useful App
Molecular Weight Calculator Tool

- This program can calculate the molecular weight and percent composition of chemical formulas and amino acids
- Provides a graphical user interface to edit the formulas and parsing options.
- It recognizes user-definable abbreviations, custom elemental isotopes, and can display up to 20 compounds simultaneously.
- It also includes a
  - Mole/Mass Converter
  - Formula Finder
  - Capillary Flow Modeler
  - Amino Acid Notation Converter
  - Peptide Sequence Fragmentation Modeler
  - Isotopic Distribution Calculator
  - and last but not least
    - built-in calculator.
Protein Post-Translational Modification

Protein modifications performed by “extra-translational” processes.

Cannot be definitively predicted from DNA sequence

Can involve very complex systems of enzymes

In some cases, “consensus” sites of modification can be identified

Ubiquitous in eukaryotes

Frequently critical for:
- initiation or modulation of biological activity,
- transport,
- and/or secretion
Protein Post-Translational Modification

- **Proteolytic Cleavage**  N-term Met of all proteins removed by aminopeptidases
- **N-terminal Acylation**  formyl, acetyl, myristyl, etc. by acyltransferases
- **Glycosylation**  Asn, Ser, and Thr
- **Sulfation**  Tyr
- **Phosphorylation**  Ser, Thr, and Tyr
- **Carboxyl Terminal Amidation**
- **Hydroxylation**  Pro, Lys, Asp
- **N-Methylation**  Lys, Arg, His, Gln
- **Carboxylation**  Glu, Asp
- **Modifications introduced by us**  Met [O], Cys-acrylamide

http://www.abrf.org/index.cfm/dm.home
UNIMOD Protein Modifications for Mass Spectrometry

http://www.unimod.org/  (login as Guest)

<table>
<thead>
<tr>
<th>Accession #</th>
<th>PSI-MS Name</th>
<th>Interim Name</th>
<th>Description</th>
<th>Monoisotopic mass</th>
<th>Average mass</th>
<th>Composition</th>
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<tbody>
<tr>
<td>View 766</td>
<td>Met-loss</td>
<td>Met-loss+Acetyl</td>
<td>Removal of initiator methionine from protein N-terminus, then acetylation of the new N-terminus</td>
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<td>-89.1594</td>
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<td>Acetyl</td>
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UNIMOD Protein Modifications for Mass Spectrometry

http://www.unimod.org/  (login as Guest)

![UNIMOD web page](image.png)
Knowledge of PTMs is Essential to Understanding Function

P00734 Prothrombin  http://www.uniprot.org/uniprot/P00734

<table>
<thead>
<tr>
<th>Molecule processing</th>
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<table>
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<th>Amino acid modifications</th>
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<td>Disulfide bond 391 – 467</td>
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<tr>
<td>Disulfide bond 536 – 660</td>
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<td>Disulfide bond 564 – 594</td>
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Classical Analytical Methods for Protein Characterization

Polyacrylamide Gel Electrophoresis (PAGE)
- Simple, inexpensive and fast
- A crude measure of molecular weight and purity
  - Analytical or preparative separations
  - Coupled with Blotting- sensitive & selective detection

Isoelectric Focusing (IEF)
- Analytical or preparative separations
- Used for mapping disease markers (e.g. CGDs)
- Variety of pH gradients
- Automated, high throughput instruments

Two Dimensional IEF – PAGE (2D Gels)
- Orthogonal separations- large separation space
- Detection of small changes in complex samples
- Separation of post-translationally modified proteins
- Dynamic Range problems due to sample loading capacity
Isoelectric Focusing (IEF)

In a pH gradient, under an electric field, a protein will move to the position in the gradient where its net charge is zero.

An immobilized pH gradient is created in a polyacrylamide gel strip by incorporating a gradient of acidic and basic buffering groups when the gel is cast.

Proteins are denatured, reduced, and alkylated, and loaded in a visible dye. The sample is soaked into the gel along its entire length before the field is applied.

Resolution is determined by the slope of the pH gradient and the field strength.
Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gels may be have many different constant or gradient compositions.

Proteins may be run directly, or are denatured, reduced, and alkylated. Samples, with a visible dye added, are loaded in wells cut into the top of the gel.

Loading capacity depends on gel size and thickness.

In 2D IEF/PAGE, the gel strip from IEF is loaded into a single large well.

Molecular weight standards are often run to calibrate the gel.

After separation, the gel is removed from the rig and stained, or bands are blotted onto a membrane.
Chemical Methods for Protein Characterization: Some Reagents

**Denaturation**- dissociates and unfolds proteins

Chaotropes: Urea, Guanidine

**Disulfide Bond Cleavage**

Two Common Reducing Agents:
Dithiothreitol, beta-mercaptoethanol

Note pH dependence

**Cysteine Alkylation**- prevents reoxidation to form disulfides

Two common Alkylation Agents:
Iodoacetamide, Vinylpyridine

Note reactivity with reducing agents
Methods for Protein Characterization: Proteolysis
An essential step for mass spec analysis

Chemical Methods:
Acid Hydrolysis, various [H+], time, temp
Cyanogen Bromide cleavage, C-term to Methionine gives a homoserine lactone at Met

Enzymes:
- Trypsin: C-term to Lys, Arg pH 8.5
- Chymotrypsin: C-term to Y, F, W, H, L pH 8.5
- S. aureus V8 protease: C-term to Glu pH 8
- S. aureus V8 protease: C-term to Glu, Asp pH 5
- Achromobacter protease (Lys-C): C-term to Lys pH 8
- Arg-C: C-term to Arg pH 8
- Asp-N: N-term to Asp pH 8
- Thermolysin: N-term to L, I, M, F, W pH 8.5

The specificities of proteases is a very useful tool to bear in mind.
Specificity of these methods is variable: some excellent, some almost none
Peptide Cutter
one of many useful tools at ExPASy Bioinformatics Resource Portal
http://ca.expasy.org/proteomics
also see Peptide Mass http://web.expasy.org/peptide_mass/

http://www.uniprot.org/uniprot/P04626
Receptor tyrosine-protein kinase erbB-2
EC=2.7.10.1
Alternative name(s):
Metastatic lymph node gene 19 protein
Short name=MLN 19
Proto-oncogene Neu
Proto-oncogene c-ErbB-2
Tyrosine kinase-type cell surface receptor HER2
Using MS-Digest to perform in-silico Digests
- particularly useful for insuring coverage of a specific peptide sequence, such as in phosphorylation mapping

http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest
1. **TCA Precipitation:** (if [protein] is >0.05 mg/mL)
   - Chill protein in a microcentrifuge tube to 0°C
   - Add 1/9 volume of cold 100% w/v trichloroacetic Acid (TCA)
   - Vortex, Incubate at 0°C for 30-60 min
   - Spin down in microcentrifuge. Remove supernatant
   - Wash pellet 3x w/ 200 µL cold acetone (do not vortex)
   - Air dry pellet >30 min

2. **Redissolving the sample in a chaotrope:**
   - Redissolve protein in 50 µL of fresh 8 M urea/0.4 M Amm. Bicarb.
   - For efficient digestion, [protein] of >0.025 µg/mL is required.
   - (Final volume = 4x the volume of urea added)-adjust as necessary

3. **Reduction and alkylation of cysteines:**
   - Add 5 µL (or 1/10 volume) of 45 mM dithiothreitol (DTT)
   - Incubate at 50°C for 15 min. Cool to room temperature
   - Add 5 µL of 100 mM iodoacetamide
   - Incubate in the dark at room temperature for 15 min
4. Trypsin digestion:
Add 140 µL of ddH₂O, vortex. Check that pH is between 7.5 and 8.5. Trypsin added should be a 1:25 weight:weight ratio of protease to sample. Concentration of trypsin should be such that 1 to 5 µL is added to sample. Incubate at 37°C for 24 h. Stop digest by freezing.

Notes:
Trypsin used should be treated with TLCK to inhibit residual chymotrypsin. Trypsin is made up at 1 or 5 mg/mL in 1 mM HCl. Aliquots can be stored frozen for up to 3 mos (use once and discard).

This protocol can be used for chymotrypsin or Achromobacter protease (Lys-C)
Why In-Gel Digestion Works:

1. The gel piece behaves like a sponge. It shrinks and swells in response to addition of aqueous or organic solvent. A gel piece shrunk with organic solvent will suck in an aqueous buffer containing reagents, thus bringing them “inside” the matrix to access the protein.

2. The intact protein is trapped in the gel, so many chemical steps can be performed without significant loss.

3. Many of the peptides resulting from proteolysis within the gel freely diffuse out of the matrix.
1. **Destaining the Gel Band:**

Mince gel spot or band with razor blade (cut into 1 mm cubes).

Wash gel pieces with 50% acetonitrile in 50 mM Ammonium bicarbonate pH 8.0

0.5 – 1.0 ml depending on the volume of gel pieces; wash 20 min. on rotator.

If volume of pieces is large (greater than 500 ul) do 2 washes at 20 min each.

Shrink pieces with neat acetonitrile.

Dry in speed vac.
In-Gel Digestion Protocol

• Directions for in-gel tryptic digestions of coomassie-stained 1D Bands and 2D Spots.
  – NOTE: Although nearly any SDS-PAGE system can be utilized upstream of an LC-MS analysis, the DPCF recommends Invitrogen’s NuPAGE Bis-Tris mini-gel system.
  – A good general purpose gel covering a large MW range (6-200 kDa) is the 4-12% gradient gel (Cat. No NP0321) using MES running buffer. These pre-cast gels provide excellent resolution, fantastic staining sensitivity (10 ng BSA using colloidal blue staining kit, Cat. No. LC6025), and are highly compatible with downstream LC-MS analysis. Additional product information can be found at: http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Protein-Gel-Electrophoresis/1D-Electrophoresis/NuPAGE.html.

• A couple of very important things to avoid keratin contamination:
  – Any gel manipulation prior to trypsin digestion should be done in a BSC or laminar flow hood.
  – Wear nitrile (not latex) gloves.
  – Wear a lab coat and make sure there is no gap between your coat sleeve and the gloves (lab tape works well).
  – Never, ever stand over you gel and look down at the bands
    • shedding of skin flakes will ruin the gel
    • LC/MS/MS will give great results for keratin
In-Gel Digestion Protocol


- After aspirating off liquid that sample is stored in, cut into equal size pieces using either a scalpel (cutting plug on the side of the Epi tube) or pinch it between the tongs of small tweezers to break it apart.
- Wash gel pieces with 200-500 μL (depending on the volume of the gel pieces) of a mix of 40% acetonitrile and 60% 50 mM ammonium bicarbonate (“AmBic”), pH 8, for 15-30 minutes on rotator. Repeat with a second or third wash if necessary to remove stain.
- Discard washes and shrink pieces with neat acetonitrile (pieces will turn white and stick together). Discard acetonitrile and dry pieces with tubes open at 50°C.
- Reduction and Alkylation: Add 100 μL of 10 mM dithiothreitol stock (15.4 mg DTT in 10 mL 50 mM AmBic). Incubate 30 min at 80°C. Add 100 μL of 55 mM iodoacetamide stock (102 mg IAA in 10 mL 50 mM AmBic). Incubate 20 min at room temperature in the dark. Use Sigma DTT (D5545) and IAA (I1149).
- Remove excess reduction and alkylation mix; wash gel pieces twice with 500 μL aliquots of 50 mM AmBic. Remove excess.
- Shrink gel pieces with 200 μL neat acetonitrile (gel pieces will again shrink, turn white, and stick together). Remove acetonitrile. Swell in 200 μL 50 mM AmBic. Remove AmBic. Repeat shrink and swell step.
- Shrink gel pieces with 200 μL neat acetonitrile, and remove acetonitrile. Dry gel pieces with tubes open at 50°C briefly.
- Swell gel pieces in 50 mM AmBic containing 10 ng/μL trypsin. Use Promega sequencing grade modified trypsin (porcine). Prepare stock from 20 μg vial at 0.1 μg/μL by diluting with 50 mM AmBic. This can be stored at -20°C for 6 months. Dilute this stock 1:10 with 50 mM AmBic pH 8.0 to give 10 ng/μL. Gel pieces should be just covered, but not in a large excess of volume (for a single 2D gel spot, use 25-30 μL of 10 ng/μL trypsin).
- Digest overnight for 16-18 hours at 37°C.
- Following digestion, centrifuge condensate to bottom of vial. Add extraction solution of 1% formic acid, 2% acetonitrile in water. The amount needed will vary with the gel volume, but typically you can use the same amount as the volume of trypsin solution you added (25 to 30 μL for a 2D gel spot). Vortex pieces in extraction solution occasionally over 30 minutes (optional: after adding extraction solution, sonicate the gel for 5 minutes. Be careful of contamination).
- Remove supernatant containing peptides and pipette into LC vial. Depending on amount of protein expected, more peptides can sometimes be recovered by shrinking gel pieces in ~50 μL of neat acetonitrile, then adding this to the LC vial. Speed vac to dryness and resuspend in LC buffer (0.2% formic acid, 2% acetonitrile in water) if last acetonitrile shrinking step is used.
- In-gel digest protocol is based on the procedure from M. Mann’s lab: “Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry.” M. Wilm, A. Shevchenko,
Ion Sources and Mass Analyzers in Protein Characterization

Principles of MS and MS/MS

Matrix Assisted Laser Desorption Ionization (MALDI)
Electrospray Ionization (ESI), Nano-ESI

Time of Flight
Quadrupole Mass Filter
Quadrupole Ion Trap
Fourier Transform Ion Cyclotron Resonance
Orbitrap FTMS
Mass Spectrometry

Lenses and prisms focus and refract light.

Analogous systems can focus and deflect ions in a vacuum.

1. Get molecules into the gas phase & ionize them.
2. Give the ions a defined energy or velocity.
3. Separate or sort the ions on the basis of that defined property.
4. Detect the ions & assign their masses.
Larger Peptides = More Complex Isotope Patterns

As ions grow larger, the "\(^{12}\text{C}\)" peak is not necessarily most abundant.

The mass resolution of analyzers may not always be adequate to distinguish individual peaks.

It is important to be aware of the capabilities of the mass analyzer one is using.

These Isotopic Distributions are calculated at a m/z resolution of 10,000.
Typical Tryptic Peptide – EGVNDNEEGFFSAR

- what is the minimum resolution needed to accurately analyze +3 charge state?
- what is the ideal resolution to analyze +3 charge state?

A resolution of 10,000 is required to eliminate crosstalk from individual isotopologues from this peptide.

Running at higher resolution will reduce sensitivity, so why would you consider using higher resolutions?

You must reduce cross-talk from other peptides in the sample.
In-silico Distributions of Peptides and Proteins in Proteomes

- 60% of all proteins ≤ 60 kDa, top ten AA’s ~70% of all AA’s
- 70% of the peptides MW’s are ≤2000, with 50% ≤1500

**Species of interest**
- Homo sapiens (Human)
- Mus musculus (Mouse)
- Rattus norvegicus (Rat)
- Oryctolagus cuniculus (Rabbit)

**Amino Acids**
- 70% of all AA

*The use of proteome similarity for the qualitative and quantitative profiling of reperfused myocardium*  
In-silico Peptide Distributions as a Function of Gradient Elution Liquid Chromatography

50% of all ions
1/5th gradient elution

- Human db (Uniprot Dec 2009) contained 20319 proteins
- 842,000 tryptic peptides
- In silico analysis using SSRCalc

\[ \sim 28,000 \text{ peptides/min at distribution Apex} \]
(average 8,000 peptides/min for a 100 min gradient)
Precursor Ion Density zoomed between m/z 540 and 580

~900 ions per each unit mass bin or ~2700 ions per 3 Da mass bin are distributed across the entire chromatographic range
Ion Sources

Matrix Assisted Laser Desorption Ionization

Electrospray Ionization

Mass Analyzers

Quadrupole Mass Filters

Quadrupole Ion Traps

Fourier Transform Ion Cyclotron Resonance

Time of Flight
Ion Sources: MALDI
Matrix Assisted Laser Desorption Ionization

Analyte: 10 – 1000 fmol
1 – 500 kDa
Some Characteristics of MALDI

Ions are easy to generate

Buffers, salts, some detergents easily tolerated

Excellent sensitivity (< 20 fmol for digests)

Protein or peptide mixtures can show suppression effects

Different matrices yield different results

Not quantitative w/o use of “iTRAQ” tags (more later)
Ion Sources: Electrospray

Very gentle and efficient way of getting gas phase ions from solutions.

A fine spray of charged droplets is generated in an electric field.

Droplets evaporate - analyte molecules are left carrying charges.

Multiply Charged Ions are the rule – Lys, Arg, His, and N-terminal amine.

Concentration dependent – High sensitivity at very low flow rates (<< 1 ul/min)

Inherently quantitatively reproducible
Electrospray is a concentration-dependent technique. Lower flow rates are favored significantly.

**FIGURE 1.** Normal flow rate electrospray (top) vs a lower flow rate electrospray (bottom) that produces smaller droplets. By allowing closer proximity to the MS inlet, the lower flow rate electrospray affords more efficient ion introduction. See Table 1.

<table>
<thead>
<tr>
<th>Table 1. Comparison of Electrospray Characteristics at Conventional and Nano-ESI Flow Rates</th>
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<tbody>
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<td>flow rate</td>
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<tr>
<td>droplet diameter$^a$</td>
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<tr>
<td>electrospray current$^d$</td>
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<tr>
<td>droplet generation rate</td>
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<td>molecules/droplet (1 μM analyte concentration)</td>
</tr>
<tr>
<td>charges/droplet</td>
</tr>
<tr>
<td>charges/analyte (1 μM concentration)</td>
</tr>
</tbody>
</table>


**FIGURE 2.** Simplified illustration showing concentration and flow rate effects on the ESI process. For larger flow rates, which produce larger droplets (Table 1), analyte surface activity, concentration, and competition from other species can affect overall ionization efficiency, the extent of ionization “suppression”, and quantitation. At sufficiently low flow rates and analyte concentrations, each droplet contains on average less than one analyte molecule, ionization efficiency is 100%, and suppression/matrix effects are eliminated.
Quasimolecular ions, \([M+nH]\), from myoglobin, Mr = 16,951.5 Da.

Using adjacent pairs of ions, the molecular mass of the myoglobin can be calculated very accurately.

\[
m_1 = \frac{(M+n)}{n} \\
m_2 = \frac{(M+n+1)}{(n+1)}
\]
Tandem Mass Spectrometry (MS/MS) is the Method of Choice for Sequence Analysis of Peptides

Speed
Sensitivity
Tolerance for Amino-terminal Blocking Groups
High Specificity for Protein Identification
Tandem Mass Spectrometry (MS/MS)

**Mass Spectrometer**

Tandem MS permits selection and isolation of specific ions for subsequent analysis.

**Tandem Mass Spectrometer**

Tandem instruments have multiple mass analyzers.
Tandem Mass Spectrometry: Product Ion Scan

1. “Parent” Ions are selected and isolated
2. Collision-Induced-Dissociation Results in fragmentation
3. “Daughter” Ions are characterized with the second mass analyzer
Common Peptide Product Ion Types

- fortunately, peptide fragment in predictable locations along the peptide backbone
- use of trypsin insures that the C-terminal amino acid will be charged, generating a series of y-ions

Low Energy CID (Q-Tof) of Doubly Charged Glu-Fibrinopeptide: y-series ions

**Remember,** y-ions read C->N direction

**Last y ion (y14) = peptide M+H⁺