Beyond Trypsin: *Strategies to Improve Mass Spec Sequence Coverage and PTM Analysis*

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Presentation Outline

I. Introduction to Bottom-Up Proteomics

II. The Role of Trypsin

III. Trypsin Enhancers

IV. When Trypsin is not enough…

1) Lys-C – a tool for proteolysis under denaturing conditions
2) Other proteases to increase coverage
3) Membrane protein analysis
4) Post-Translational Modification (PTM) analysis

V. Glycosidases

Applications of Mass Spec in Biology

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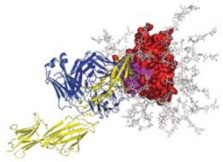
Mass spec can answer multiple questions in biology

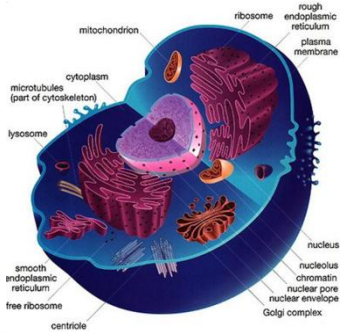
Protein Structure (HDX Mass Spec)



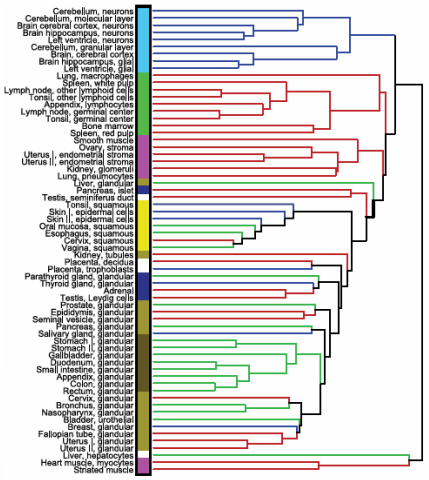
Protein Interactions

Biomarker Discovery

Subcellular Localization

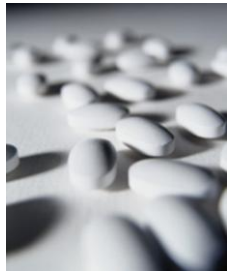


Protein Expression



Drug Discovery

Drug Binding Studies (Chemical Proteomics)



Biologics

Why is Mass Spec a Powerful Tool for Biology?

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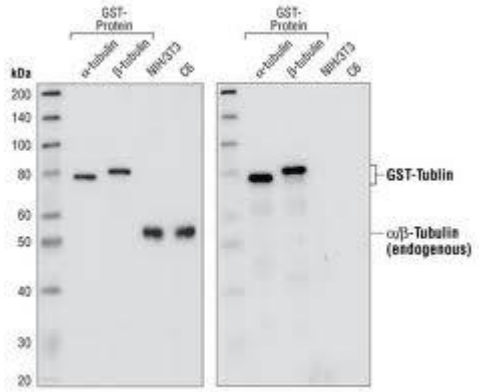
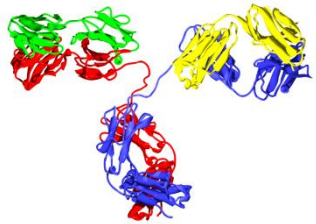
Western Blot: Traditional Approach

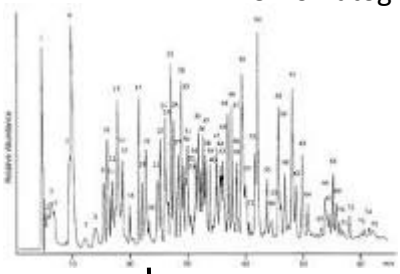




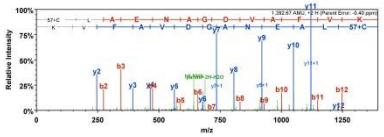
Antibody Required

Mass Spec: Unbiased Analysis

Chromatogram



MS/MSpectra



Protein ID based on matches to Spectra

Mass Spec Advantages:

- No pre-existing knowledge required - unbiased
- Ability to identify 1000's of proteins in single run
- Quantitative
- Highly reproducible
- High dynamic range
- Automatable
- Eliminates need for Antibody

Bottom-Up Proteomics: Unbiased Profiling of Complex Protein Extracts

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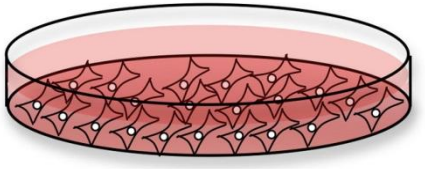
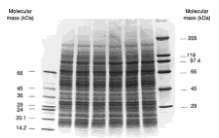
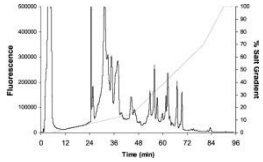
Proteases convert protein to peptides to be analysed by the Mass Spec

Step #1: Sample Preparation (Pure Protein, Protein Complex, IP, Cells, Tissue, Serum etc.)

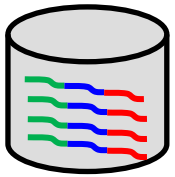


Step #2: Reduction of Sample Complexity (SDS-PAGE, SCX/HILIC, affinity enrichment, subcellular fractionation etc.)

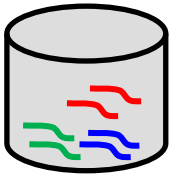




Step #3: Protein Isolation (excise gel band, collect fractions etc.)

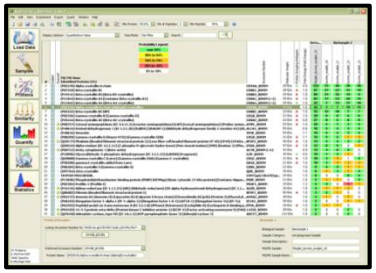


Step #4: Protein digestion (trypsin or other proteases)

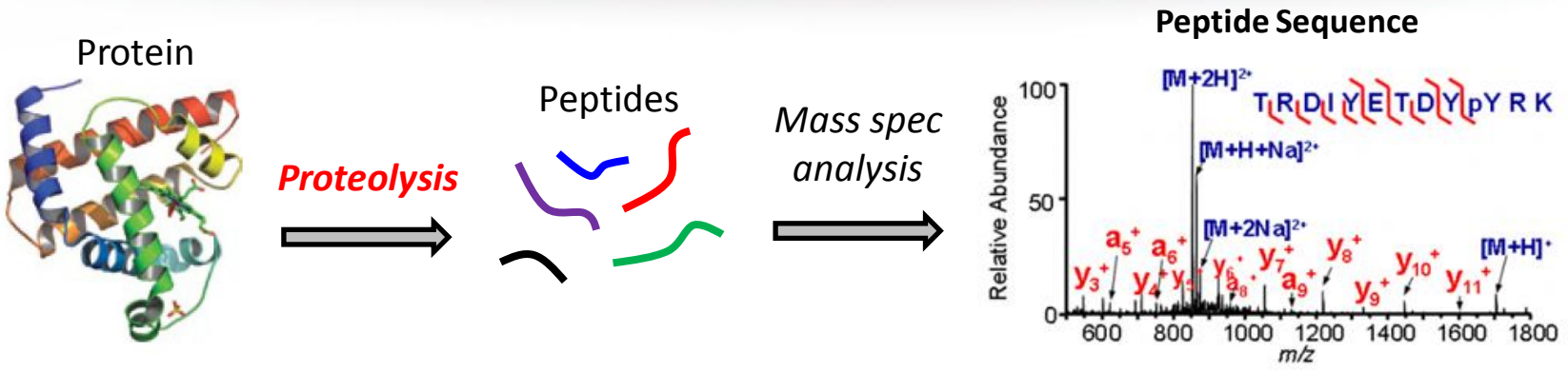


Step #5: LC-MS/MS Analysis

Step #6: Software assisted protein identification



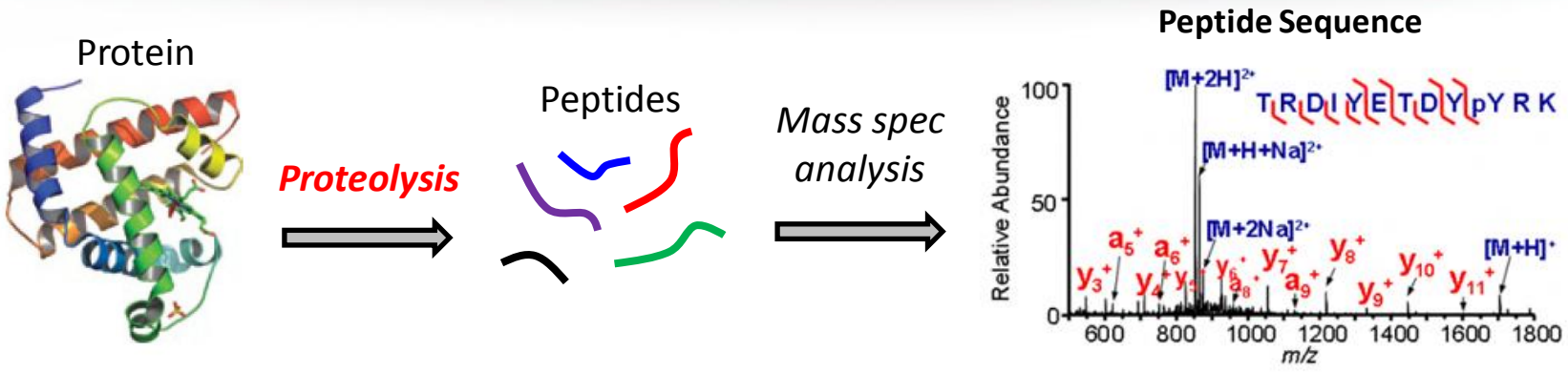
Why is Proteolysis Required?



The **range of masses** is limited in the typical mass spec (250-4000 daltons). This is not a problem for small molecule measurements, but large molecules are challenging…

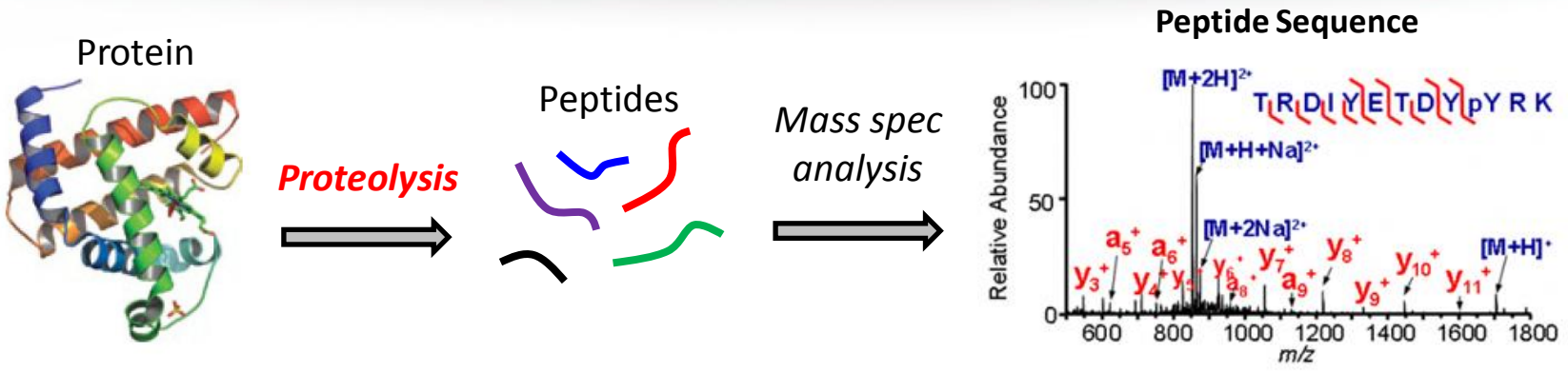
The problem is solved by using site-specific endoproteinases (proteases).

Why Trypsin?



Why is Trypsin viewed as the “Gold Standard” for bottom up proteomics?

Why Trypsin….Multiple Reasons!



….

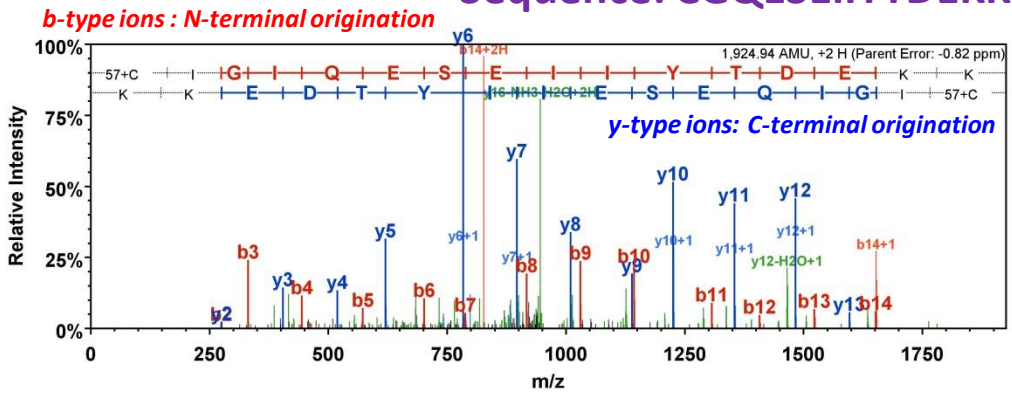
- ✓ Average size of peptides is between 700-1500 daltons (ideal for MS analysis)
- ✓ All peptides have a C-terminal charge (due to K/R)
- ✓ Highly active
- ✓ Highly specific
- ✓ Autolysis can be controled by lysine/arginine modification

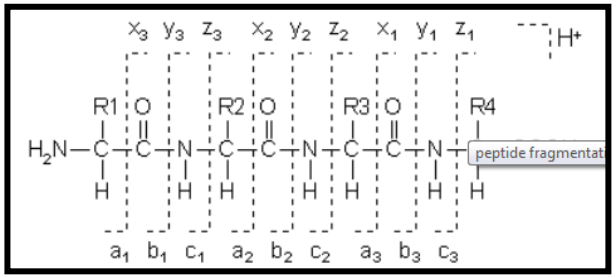
How Does Mass Spec "Sequence" Proteins?

Steps in the Process:

1. Protein sequences are stored in a database (i.e. UNIPROT)
2. Sequences are **digested *in silico*** (based on the appropriate protease)
3. Peptide masses are measured and MS/MS spectra recorded
4. Peptide sequence candidates which match the mass of the peptides measured (within a certain tolerance) are selected and a theoretical MS/MS spectrum is generated (i.e. a bar-code)
5. Bar-code is then matched to the MS/MS spectrum.

Sequence: CGQESEIIYTDEKK





The spacing between the ions correspond to the masses of the amino acids

What are the Challenges With Proteolysis?

Challenges:

- *Protein folded too tightly – Protease can't access*
- *Protein is insoluble and require additives*

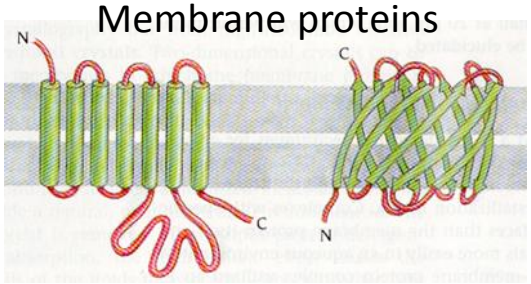
Solutions:

- *Urea, Guanidine HCl and organic solvents (e.g., acetonitrile) –denaturing agents*
- *Detergents such as Triton X-100 and SDS*

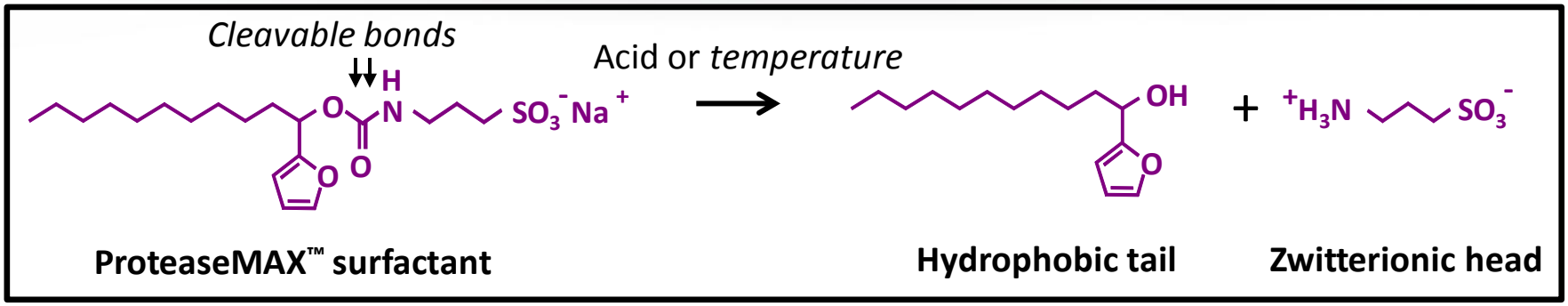
Limitations:

- *Protease Inhibition*
- *Unwanted side effects (i.e., protein modification).*
- *Detergents hurt the hardware (LC and Mass Spec)!*

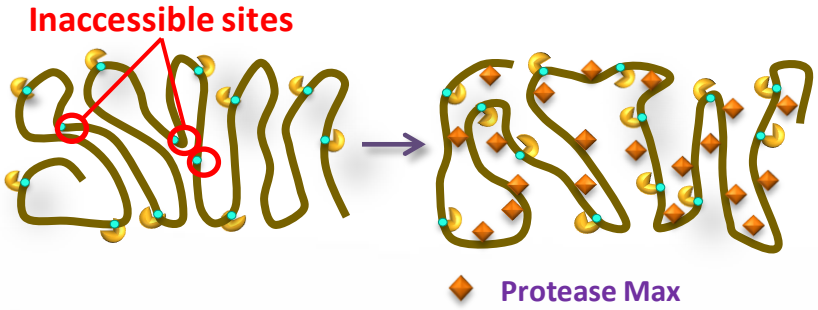
Membrane proteins



ProteaseMAX™ Surfactant

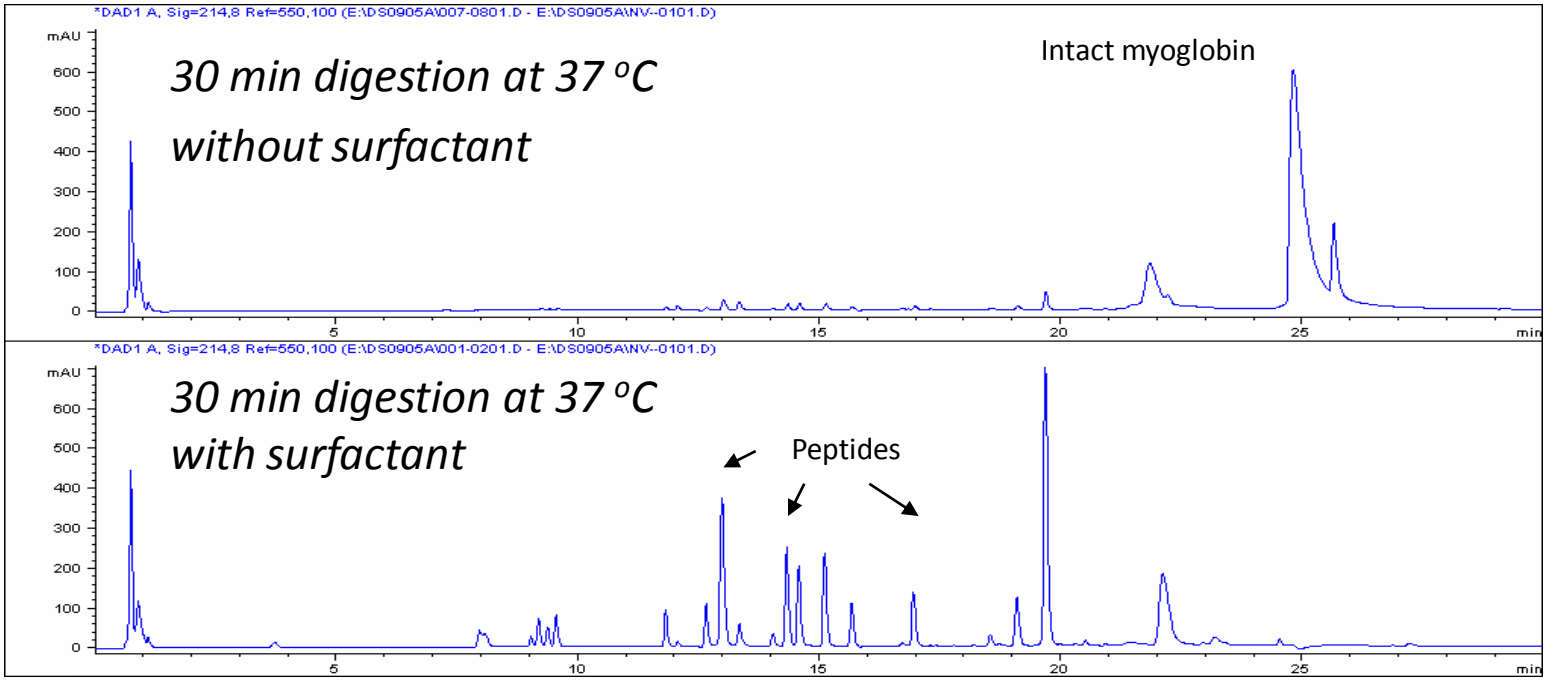


- Solubilizing and denaturing properties of surfactant – no effect on LC-MS/MS
- Decomposes within 8 hours at Room Temperature (in solution)
- Improves digestion efficiency for compact/tightly folded proteins
- Solubilizes Membrane Proteins



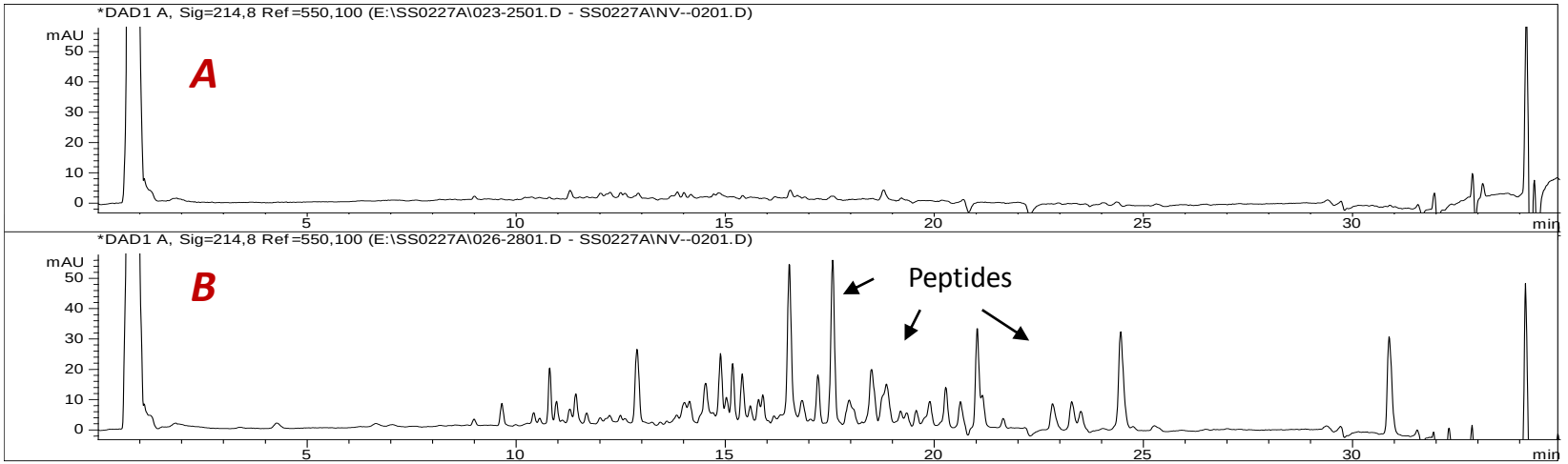
ProteaseMAX™ Surfactant: Rapid Digestion

Myoglobin (Proteolytically resistant protein) is rapidly digested in presence of ProteaseMAX™



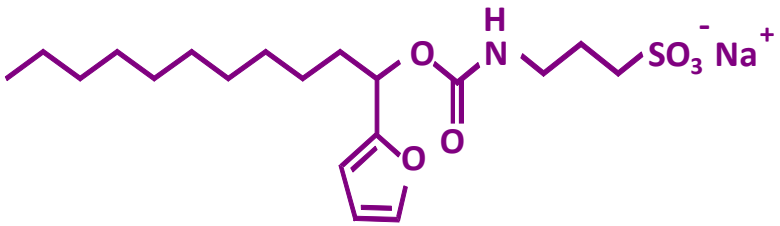
ProteaseMAX™ Surfactant: Solubilization

ProteaseMAX™ readily solubilizes the membrane protein bacteriorhodopsin



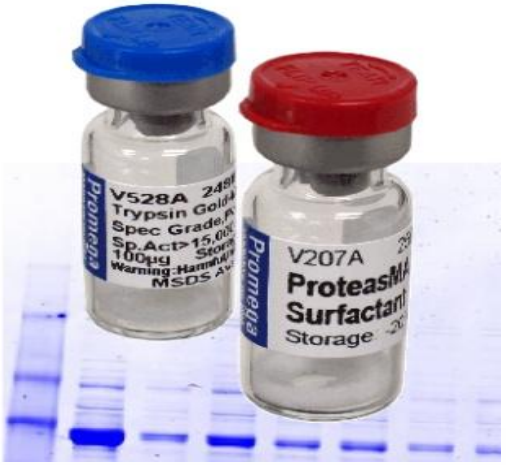
- Bacteriorhodopsin is insoluble in aqueous solutions and resistant to proteolysis (panel A)
- With the surfactant, this protein solubilizes within 1-2 minutes at room temperature and easily digested (panel B)

ProteaseMAX™ Surfactant: Conclusions



ProteaseMAX™ surfactant:

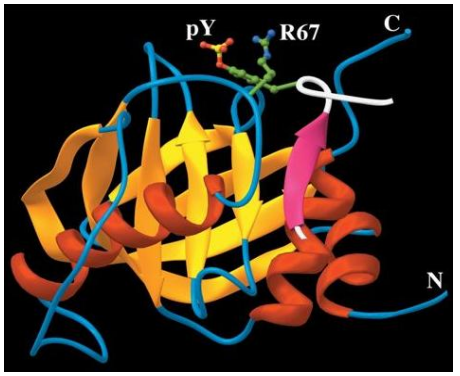
- *Improved digestion of Stably folded proteins*
- *Improved solubility of membrane proteins*
- *Faster overall digestion kinetics*
- *Higher throughput*
- *No cleanup required*



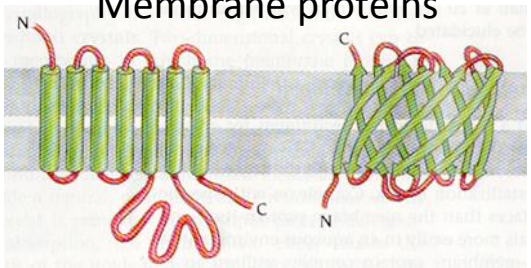
But, When is Trypsin Not Enough?

- *Substantial number of tryptic peptides are too long or too short for Mass Spec analysis*
- *Tryptic cleavage sites might not be accessible due to PTM's (i.e phosphorylation, glycosylation, histone methylation/acetylation)*
- *Certain proteins are not efficiently digested by trypsin (i.e. membrane proteins and proteins in tight conformation)*
- *Additional proteases allow for more sequence coverage and protein ID's (especially in complex samples)*

pY containing protein



Membrane proteins



Alternative Proteases Overcome the Limitations of Trypsin

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Examples to be discussed:

- Using other proteases to increase protein identifications
- improve digestion efficiency of compact proteins with Lys-C
- “Sequence” membrane proteins with Elastase, Pepsin, and Thermolysin
- Complementation of trypsin in Histone analysis with Arg-C
- Identification of phosphorylation sites on MAPK requires Trypsin, Chymotrypsin, and Elastase

Alternative Proteases For Mass Spec

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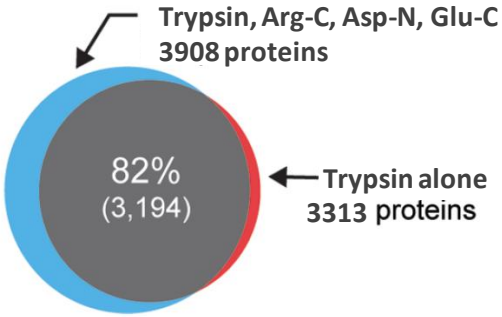
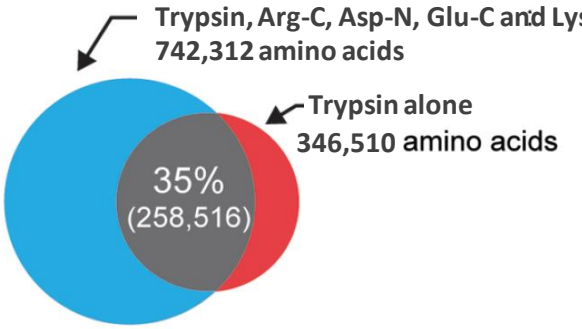
Examples of the Most Commonly Used Proteases (in addition to trypsin)

Protease	Cleavage site	Example of use
Lys-C	NNNN**K**▼NNN (**K** is lysine)	• Active under denaturing conditions • Produces larger peptides that trypsin • Useful for ETD applications
Glu-C	NNNN**E**▼NNN (**E** is glutamate) Glu-C can also cleave at aspartate residue also depending on the pH	• Alternative to trypsin when trypsin produces peptides outside of required mass window (too small or larger) • E cleavage in Phosphate buffer (pH = 7.8) • E and D in ammonium buffers.
Asp-N	NNNN▼**D**NNN (**D** is aspartate)	Alternative to trypsin when trypsin produces peptides outside of required mass window (too small or larger)
Chymotrypsin	NNNN**(F/Y/W)**▼NNN (**F**, **Y** and **W** are aromatic residues phenylalanine, tyrosine, and tryptophan)	Digests hydrophobic proteins (i.e . membrane proteins)
Arg-C	NNNN**R**▼NNN (**R** is arginine) (can also cleave c-terminal side of K)	Analysis of histone posttranslational modifications
Pepsin	Nonspecific protease (advantage – digestion at low pH)	Hydrogen-deuterium Exchange Mass Spec
Thermolysin	Nonspecific protease (advantage – digestion at high temperature)	• Digestion of proteolytically difficult proteins • Structural Studies
Elastase	Nonspecific protease	• Used to increase protein coverage • Protein structural studies

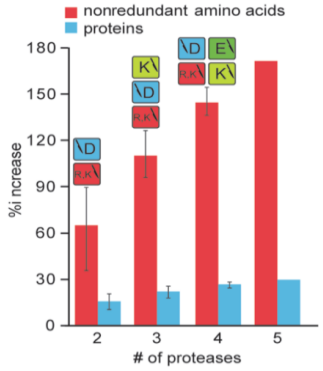
Alternative Proteases Increase Protein Identifications by 20 %



Identified proteins increased from 3313 to 3908 (20%), upon applying alternative proteases



Sequence coverage increased by 172%



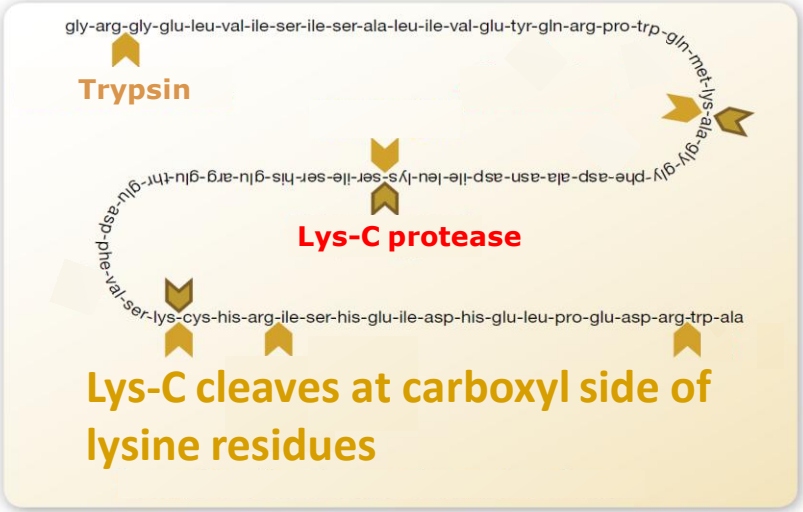
Number of identified protein and sequence coverage gradually increased upon adding additional alternative proteases

Adding additional proteases improves proteomic analysis

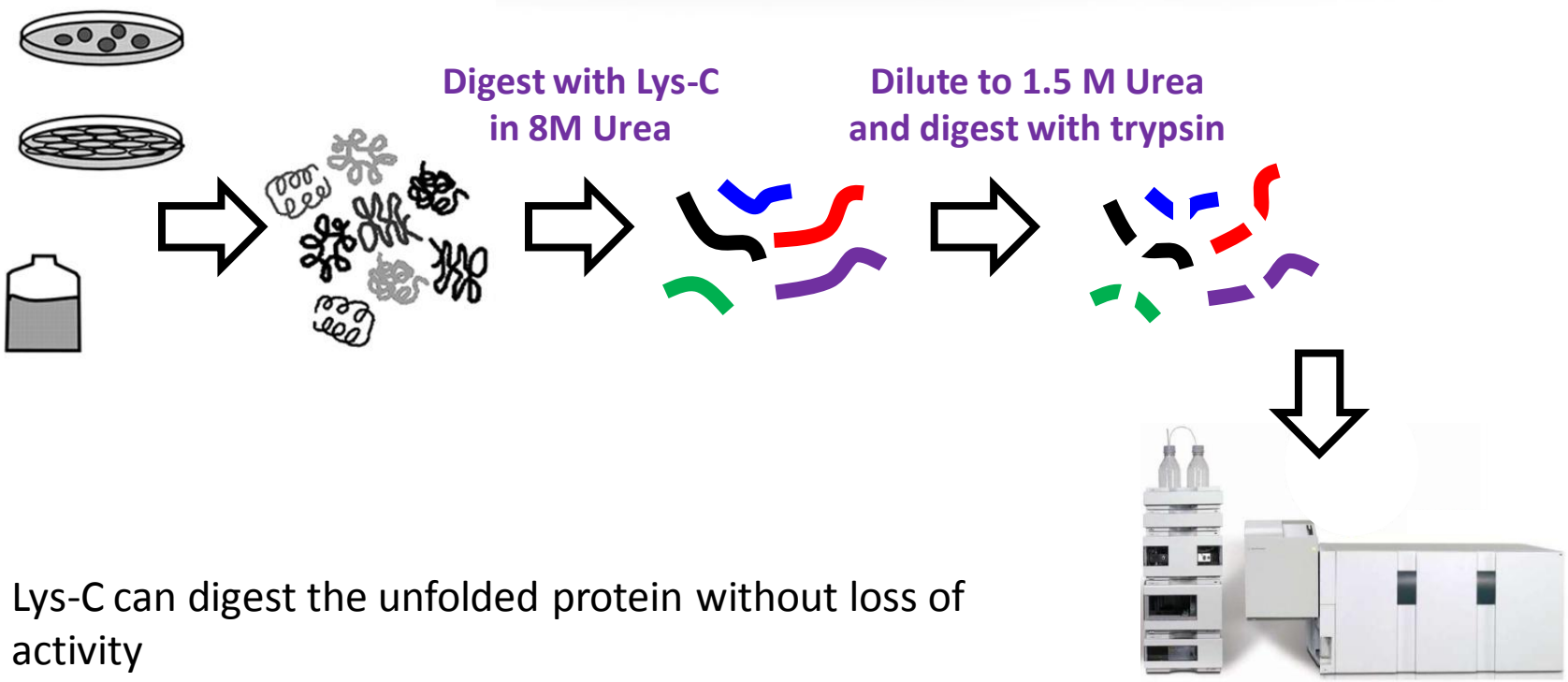
Swaney et al. (2010) J. of Proteome Res. 9:1323-1329

Lys-C: A Valuable Tool for Proteomics Studies

- As ***highly active and specific*** as trypsin
- ***Unlike trypsin, lys-C remains active under highly denaturing conditions*** (8M Urea) allowing for digestion of unfolded proteins (which don't digest when folded)
- ***Generates larger peptides*** than trypsin (useful for ETD studies)



An Alternative Workflow: Sequential Digestion with Lys-C and Trypsin

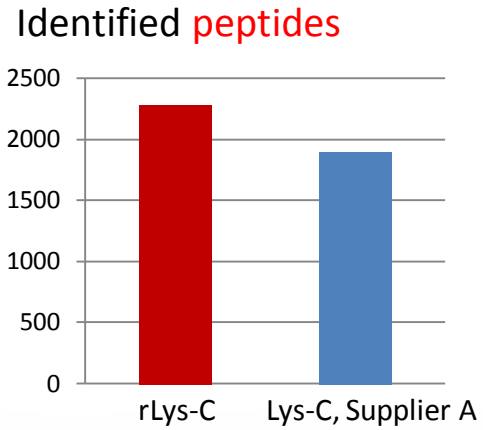


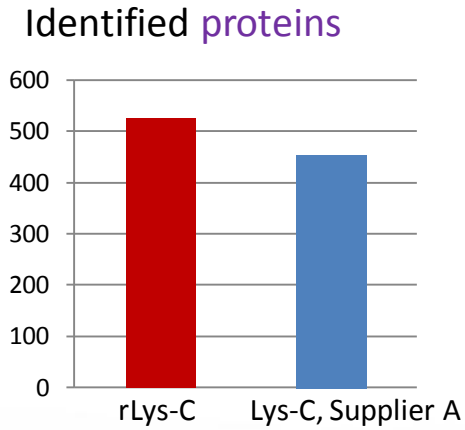
- Lys-C can digest the unfolded protein without loss of activity
- Sequential digestion with Lys-C followed by trypsin is often used to ensure high digestion efficiency
- *Many proteins require denaturation prior to digestion*

A Recombinant Lys-C: A Low-Cost Alternative to the Native Enzyme

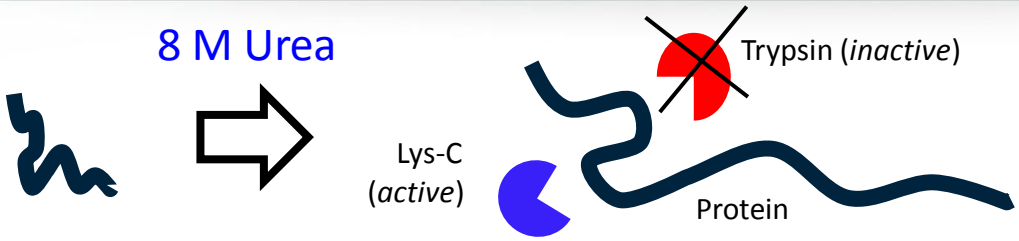
- Promega has developed a novel recombinant Lys-C ($8/µg versus $30/µg)
- rLys-C is as active and robust as the native enzyme with performance comparable to other suppliers
- rLys-C is also active under denaturing conditions

Total Protein Coverage (Yeast Extract)



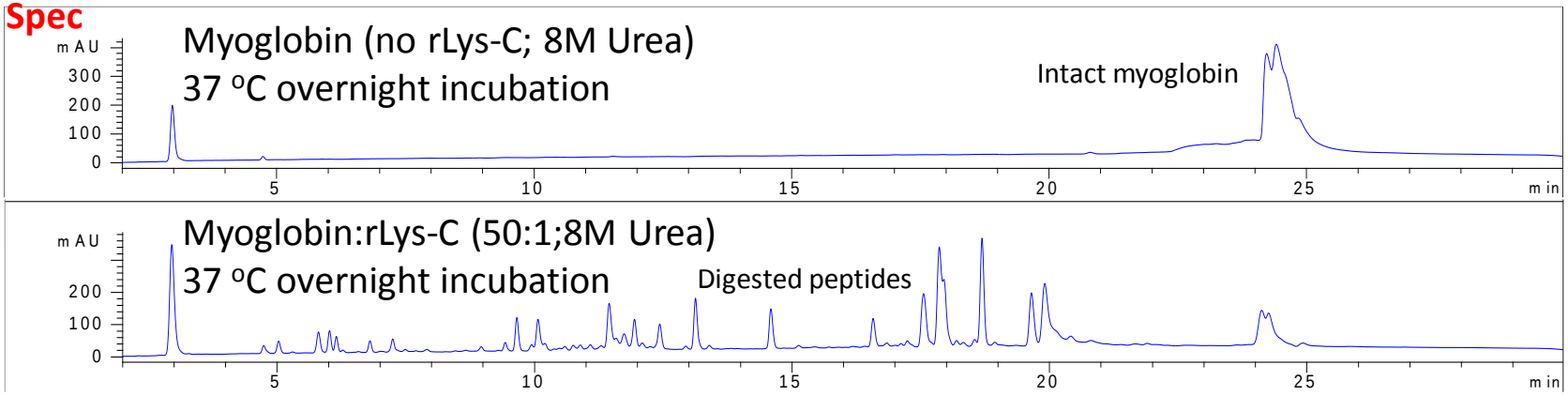


rLys-C Can Tolerate Denaturation



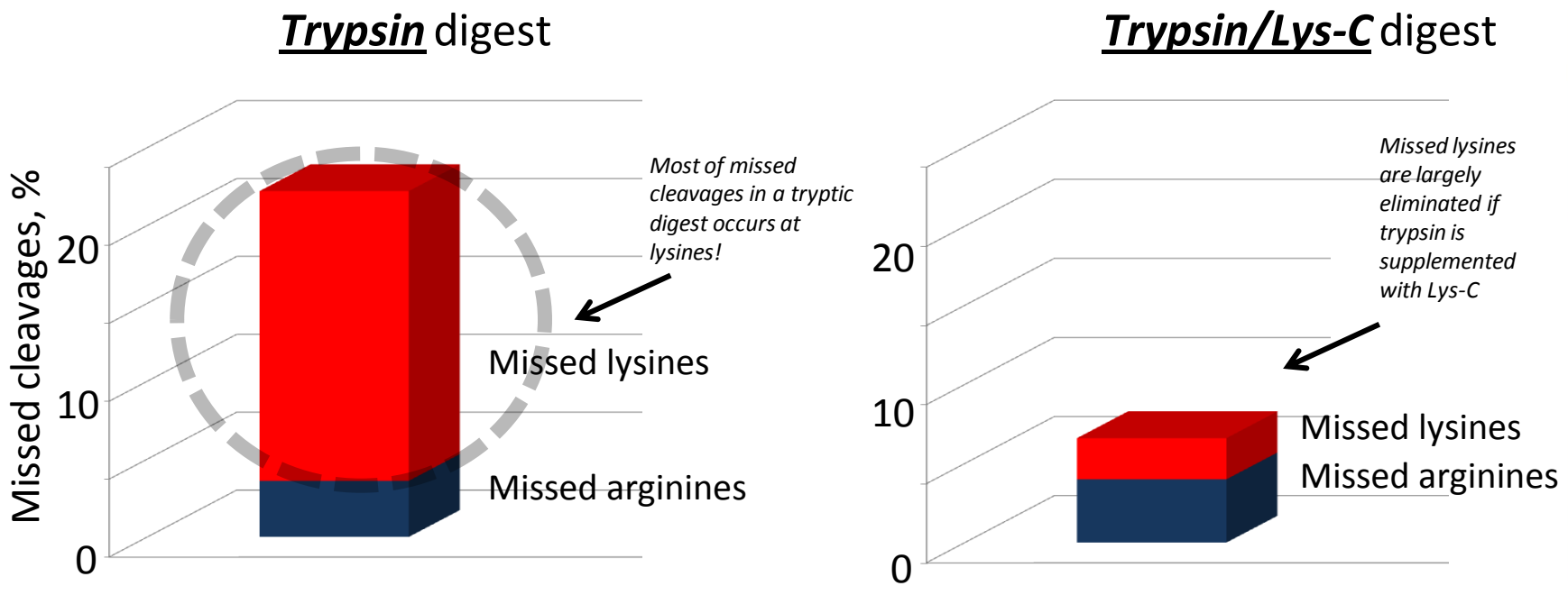
Strong denaturing conditions

RP HPLC chromatograMass Spec



Recombinant Lys-C retains activity under strongly denaturing conditions (8M Urea) digesting a proteolytically resistant protein

A Trypsin/Lys-C Mixture: Improved Proteolytic Efficiency



Supplementing trypsin with Lys-C dramatically improves digestion efficiency

Product available later this year: e-mail mike.rosenblatt@promega.com *or gary.kobs@promega .com for details*

Robust and Consistent Proteolysis of Complex Protein Mixtures with Trypsin/Lys-C

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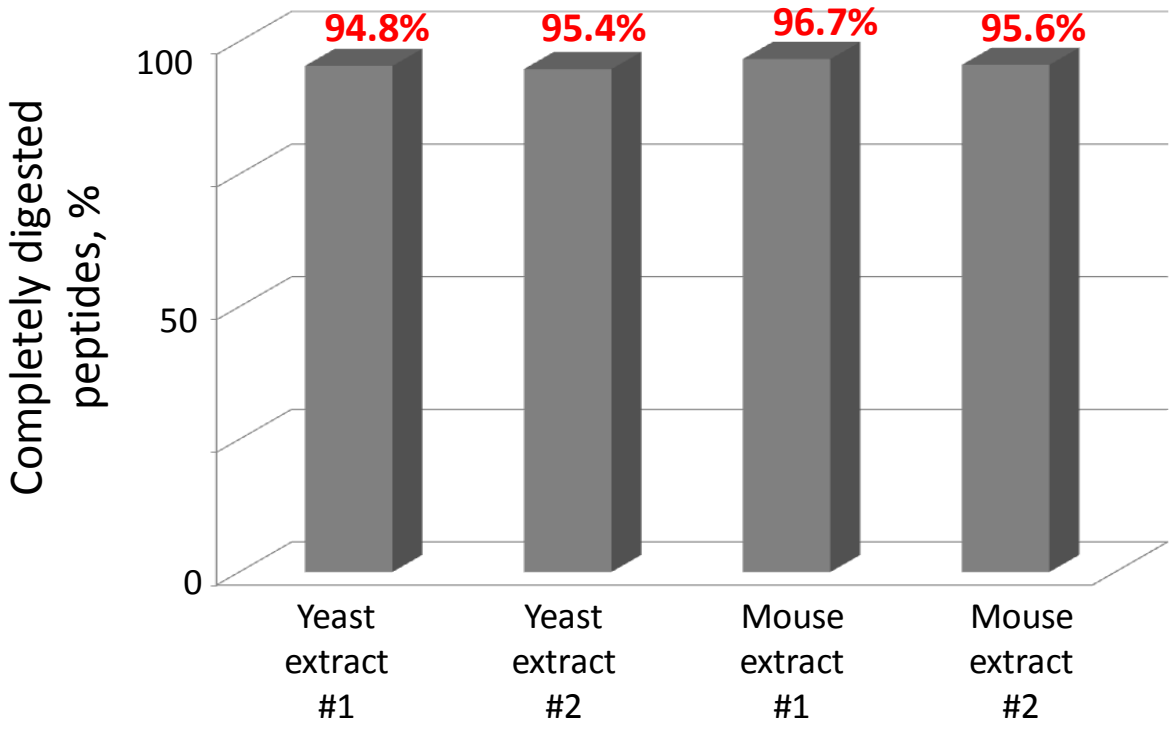
Digestion of yeast and mouse protein extracts with Trypsin/rLys-C mix



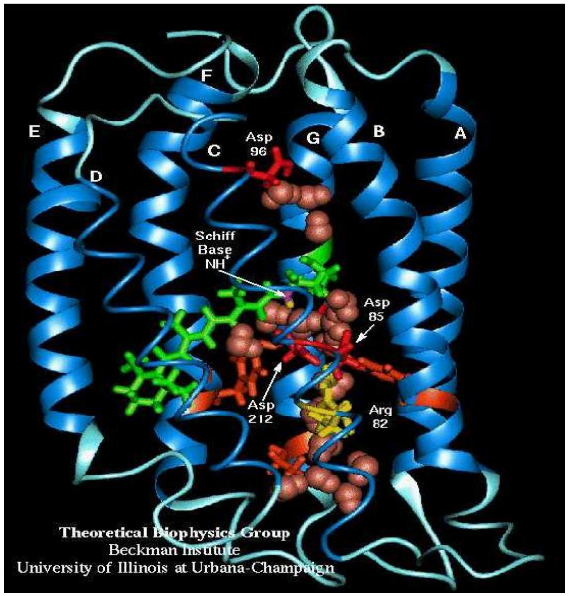
The remaining missed cleavages represent a few percent of total peptide population and predominantly occur at (R/K)/(D/E) sites or at peptide N-termini

Membrane Proteins: A Proteolytic Challenge

- Membrane Proteins contain a large number of hydrophobic residues and therefore are challenging for trypsin to digest
- They also require solubilizing agents for proteolysis, another problem for trypsin
- Additional proteases, like Pepsin, elastase, and thermolysin, which will cut around hydrophobic sequences are beneficial

Bacteriorhodopsin:

- Prototypical membrane protein (7-TM GPCR)
- Trypsin does not cleave any of the 7 helices



Multiple Proteases Improves Coverage of Membrane Proteins

Bacteriorhodopsin:

MLELLPTAVEGVSQAQITGRPEWIWLALGTALMGLGTLYFLVKGMGVSDPDAKKFYAITTL
VPAIAFTMYLSMLLGYGLTMVPFGGEQNPIYWARYADWLFTTPLLLLDLALLVDADQGTIL
ALVGADGIMIGTGLVGALTKVYSYRFVWWAISTAAMLYILYVLFFGFTSKAESMRPEVASTF
KVLRNVTVVLWSAYPVVWLIGSEGAGIVPLNIETLLFMVLDVSAKVGFGLILLRSRAIFGEA
EAPEPSAGDGAAATSD

- Red and Blue are alternating tryptic peptides
- Underlined sequences correspond to embedded TM regions

Trypsin 27% Coverage

tr|B0R5N9|B0R5N9_HALS3 (100%), 28,257.6 Da
Bacteriorhodopsin OS=Halobacterium salinarum (strain ATCC 29341 / DSM 671 / R1) GN=bop PE=4 SV=1
14 unique peptides, 15 unique spectra, 22 total spectra, 72/262 amino acids (27% coverage)

MLELLPTAVE GVSQAQITGR PEWIWLALGT ALMGLGTLYF LVKGMGVSDP
DAKKFYAITT LVPAIAFTMY LSMLLGYGLT MVPFGGEQNP IYWARYADWL
FTTPLLLLDL ALLVDADQGT ILALVGADGI MIGTGLVGAL TKVYSYRFVW
WAISTAAMLY ILYVLFFGFT SKAESMRPEV ASTFKVLRNV TVVLWSAYPV
VWLIGSEGAG IVPLNIETLL FMVLDVSAKV GFGLILLRSR AIFGEAEAPE
PSAGDGAAAT SD

Combination of coverage from Elastase, Thermolsyin and Pepsin 90% Coverage

tr|B0R5N9|B0R5N9_HALS3 (100%), 28,257.6 Da
Bacteriorhodopsin OS=Halobacterium salinarum (strain ATCC 29341 / DSM 671 / R1) GN=bop PE=4 SV=1
222 unique peptides, 236 unique spectra, 579 total spectra, 236/262 amino acids (90% coverage)

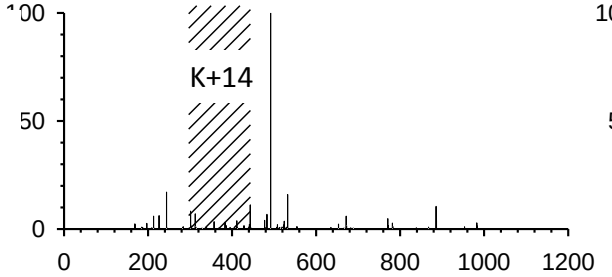
MLELLPTAVE GVSQAQITGR PEWIWLALGT ALMGLGTLYF LVKGMGVSDP DAKKFYAITT
LVPAIAFTMY LSMLLGYGLT MVPFGGEQNP IYWARYADWL FTTPLLLLDL ALLVDADQGT
ILALVGADGI MIGTGLVGAL TKVYSYRFVW WAISTAAMLY ILYVLFFGFT SKAESMRPEV
ASTFKVLRNV TVVLWSAYPV VWLIGSEGAG IVPLNIETLL FMVLDVSAKV GFGLILLRSR
AIFGEAEAPE PSAGDGAAAT SD

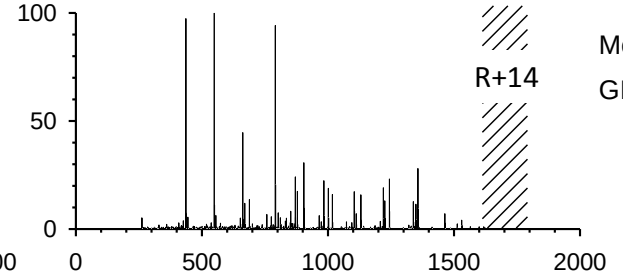
The combination of 3 additional enzymes increased overage by over 60 % and identified 7/8 trans-membrane domains

The Principle of PTM analysis by MS/MS

The spacing between the fragment ions will have a specific mass shift that is PTM specific

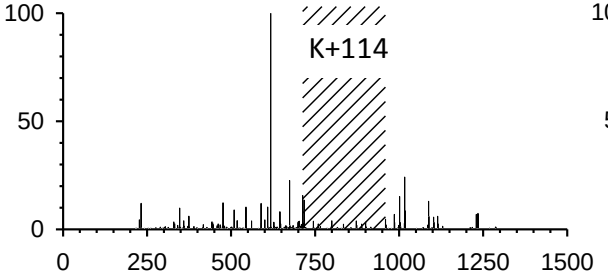
Methylation (K)
VPDVDIkGPK

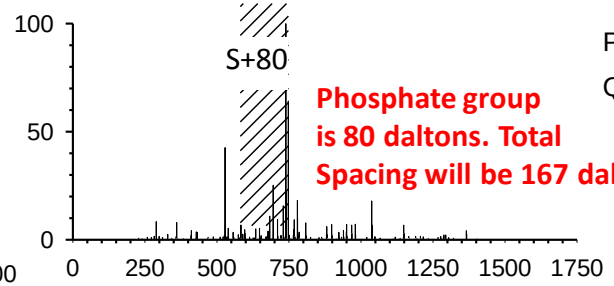




Methylation (R)
GLGTDEDSLIELLcSr

Ubiquitination (K)
LIFAGkQLEDGR

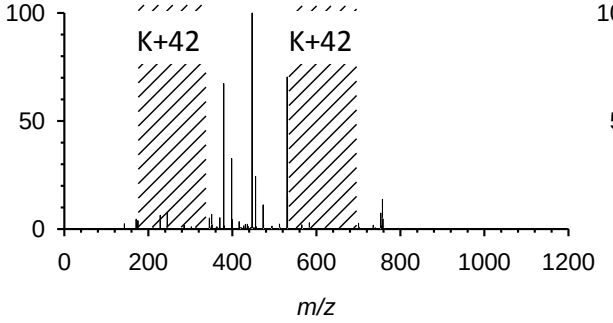


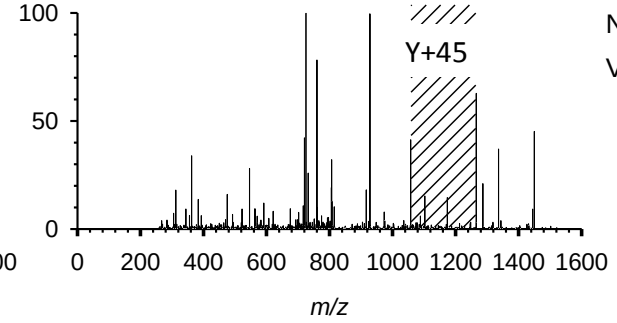


Phosphorylation (S/T/Y)
QVAEQGGDLsPAANR

Phosphate group is 80 daltons. Total Spacing will be 167 daltons

Acetylation (K)
GLGkGGAkR



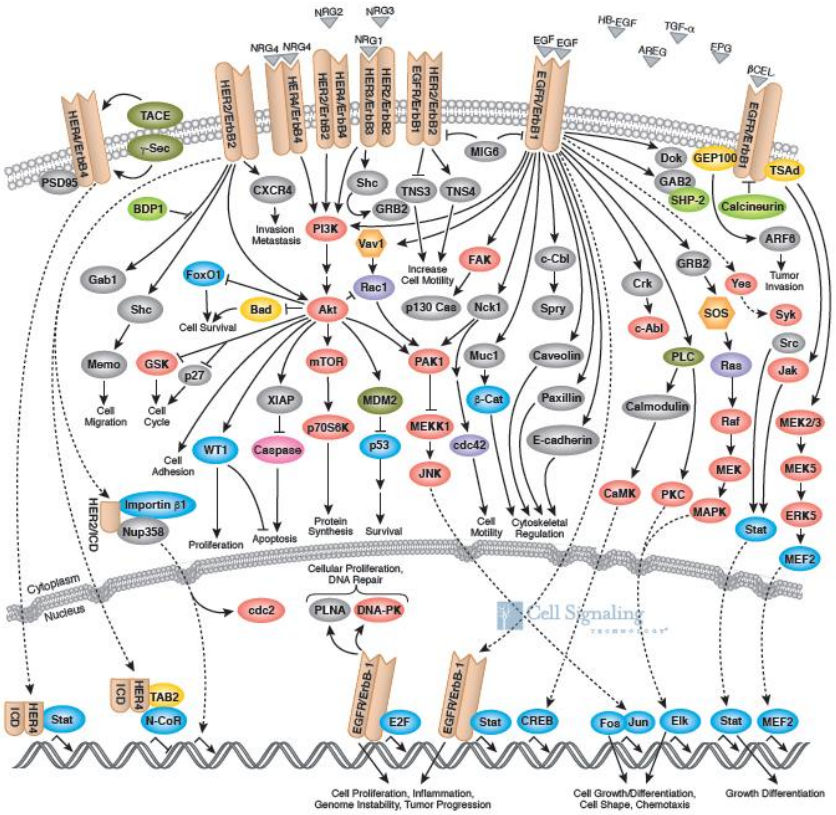


Nitration (Y)
VVLAyEPVWAIGTGK

Modified residues are in blue

Data courtesy of MS Bioworks LLC

Cell Signaling: A Post-Translational Modification (PTM) Driven Process



- Signal Transduction is central to cell growth and plays a central role in multiple diseases including cancer and diabetes
- Much of the signalling is governed by PTM's like phosphorylation, acetylation, ubquitination, and O-GlcNac to name a few
- Mass Spec is a powerful tool for both site-specific Identification of not only PTM modified proteins but the specific site of modification

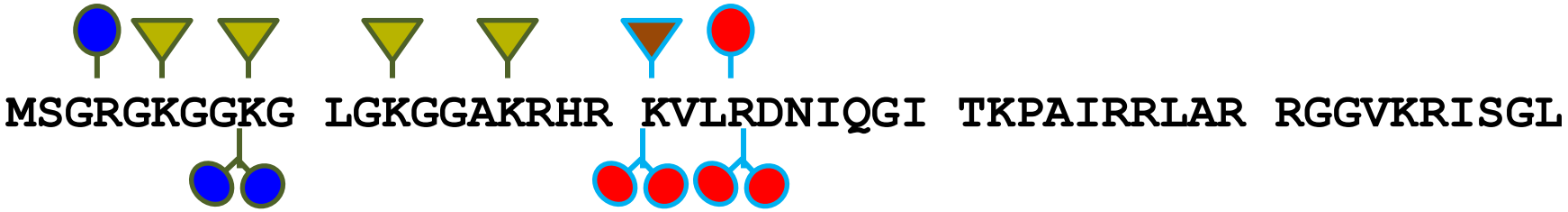
Some Major PTM's

PTM	Affected Residue
Acetylation	Lysine
Methylation	Lysine (up to 2 methyl groups) /Arginine (up to 3 methyl groups)
Ubiquitination	Lysine
Phosphorylation	Serine /Threonine /Tyrosine
O-GlcNac	Serine /Threonine /Tyrosine
O-glycans	Serine /Threonine
N-glycans	Asparagine
Nitration	Cysteine/tyrosine

Arg-C Complements Trypsin for Analysis of Histone Modifications

Promega

N-terminus of Histone H4:



Methylated site identified with trypsin

Di-methylated site identified with trypsin

Acetylated sites identified with trypsin

Additional methylated site identified with Arg-C

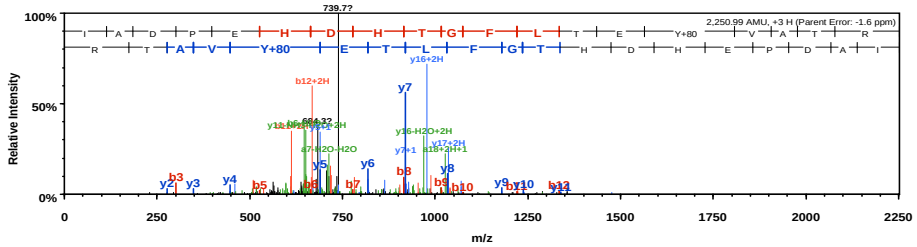
Additional di-methylated sites identified with Arg-C

Additional acetylated site identified with Arg-C

Protease Combinations Increase Identification of Phospho Sites

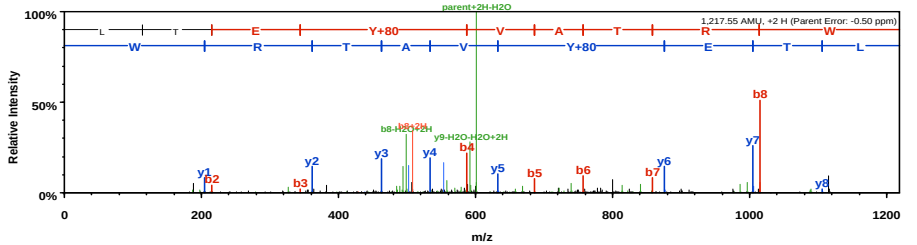
Identification of Tyrosine Phosphorylation in Erk1/2

Trypsin – IADPEHDHTGFLTE(pY)VATR



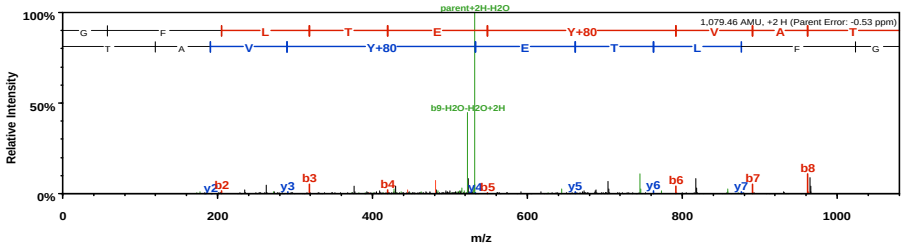
MS/MS spectrum is of poor quality

Chymotrypsin – LTE(pY)VATRW



Note complimentary y- and b-ion pairs around pY

Elastase – GFLTE(pY)VAT



Note complimentary y- and b-ion pairs around pY

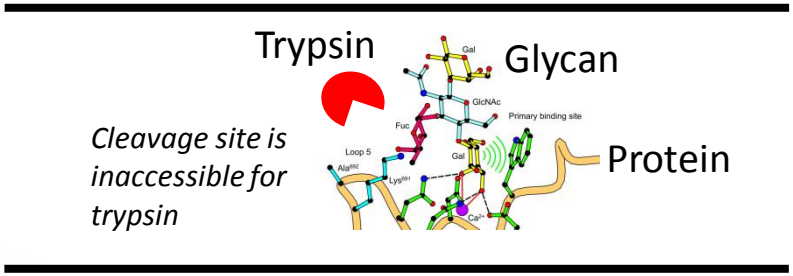
Data courtesy of MS Bioworks LLC

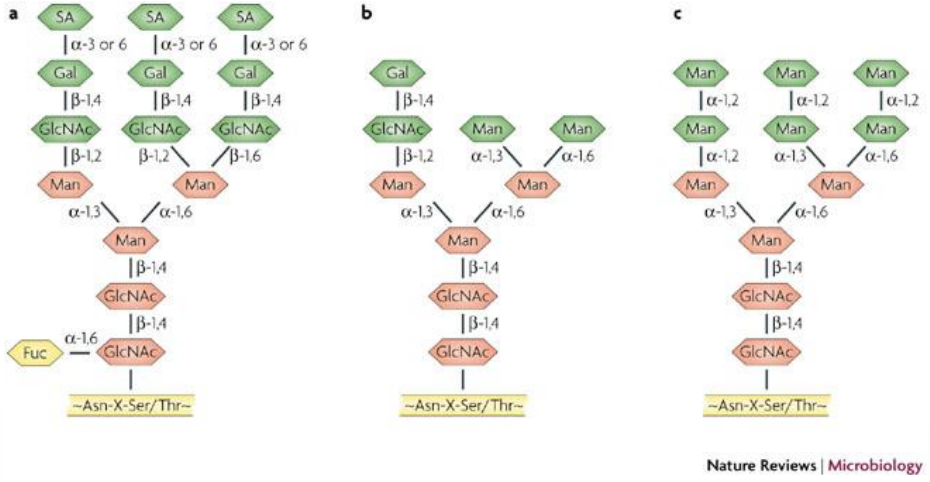
Glycosidases Have Multiple Roles in Proteomics

Promega

Glycosidases are useful for :

- Glycomics
- Glycoproteomics
- Improving protease coverage by unmasking protease sites





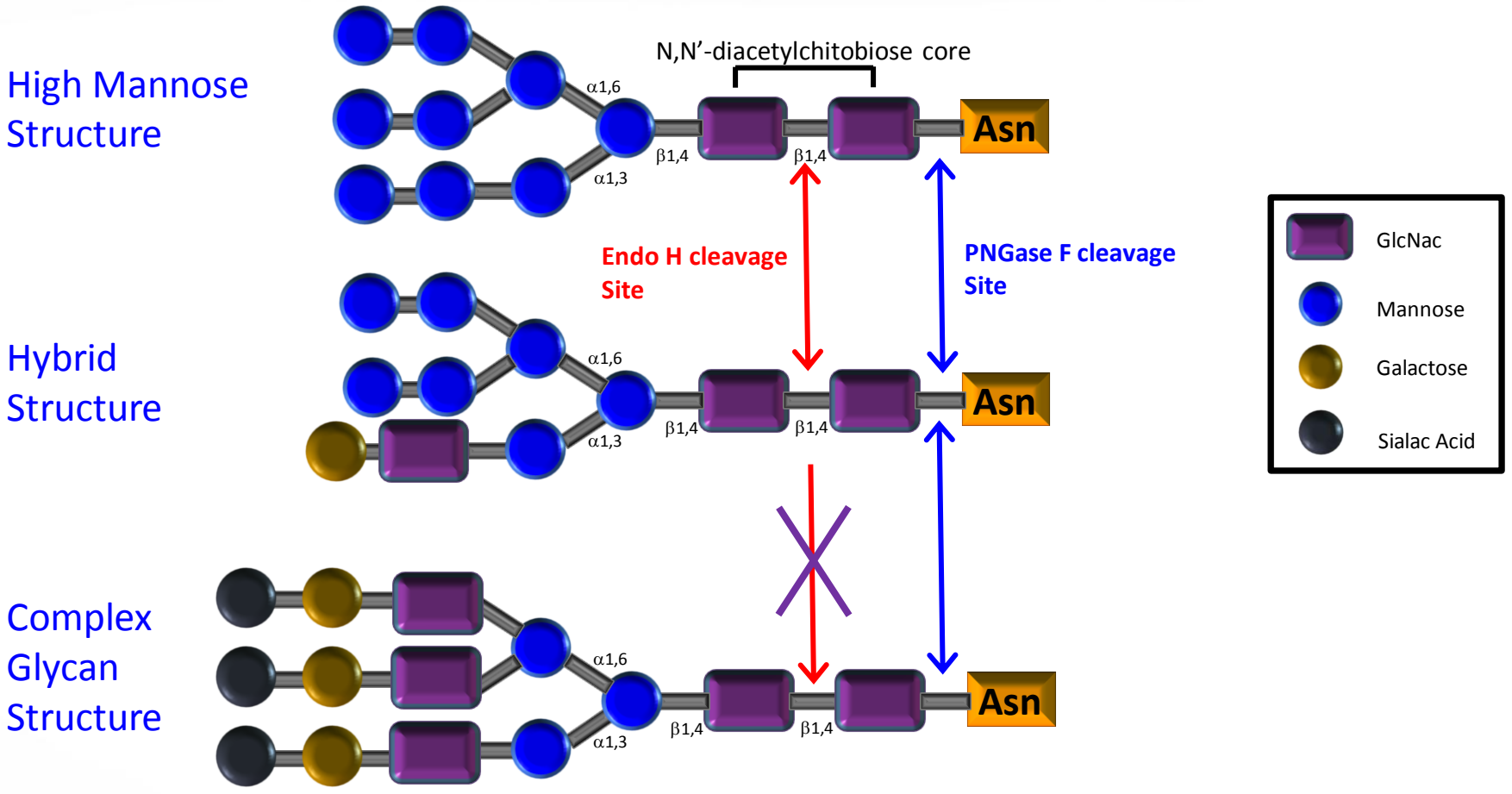
Some Common Glycosidases

Promega

Glycosidase	Substrate	Product	Change Detected
PNGase F	XX**N**X(S/T)XX N = Asn (glycan containing)	XX**D**X(S/T)XX D = Asp	1 dalton Mass Shift – N to D conversion
Endo-H	XX**N**X(S/T)XX N = Asn (glycan containing)	XX**N(GlcNac)**X(S/T)XX N(GlcNac) = Asparagine bonded to a single GlcNac	203 daltons – Mass of one GlcNac
Protein Deglycosylation Mixture (**contains both N and O-glycosidases**)	All glycoproteins	XX**D**X(S/T)XX D = Asp (O-linked amino acids are unchanged)	• 1 dalton for N-linked glycans • No change for O-linked, but no glycan attached, so peptide is unmodified.

Glycosidases will launch in Fall 2012

PNGase F /Endo H Cleavage Specificity

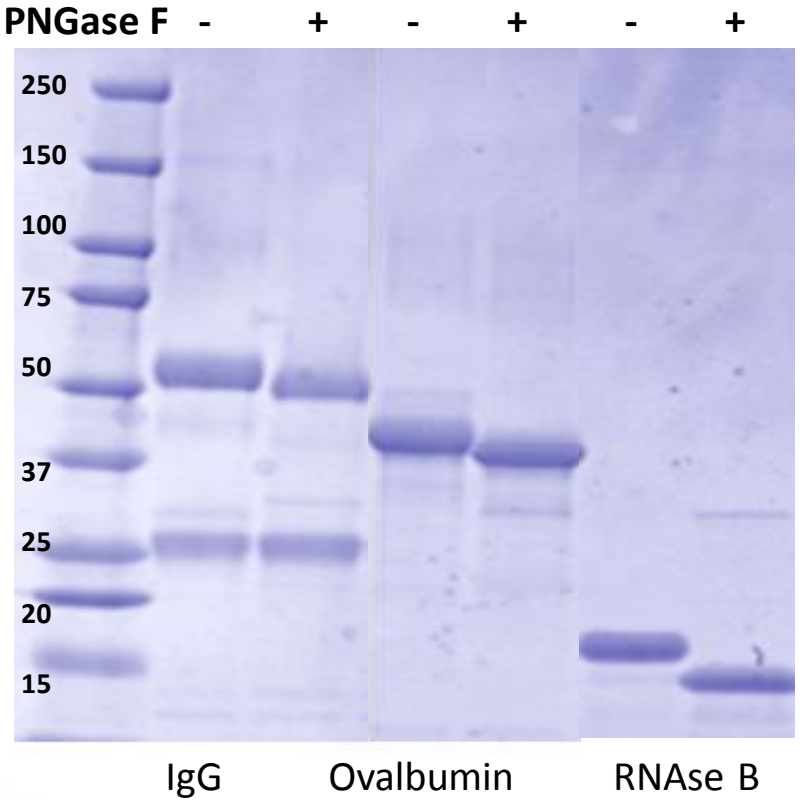


Endo H is unable to cleave N-linked complex-type glycans

A Recombinant PNGase F

- Protein is based on the 34 kDa protein secreted by *flavobacterium meningosepticum*
- Total Activity is comparable to the endogenous enzyme
- Active under native and mildly denaturing conditions and therefore very effective for proteomic workflows
- *Note: After removal of the glycan, the Asparagine (N) residue is converted to Aspartic Acid (D)*

Characterization of PNGase F Treated Proteins



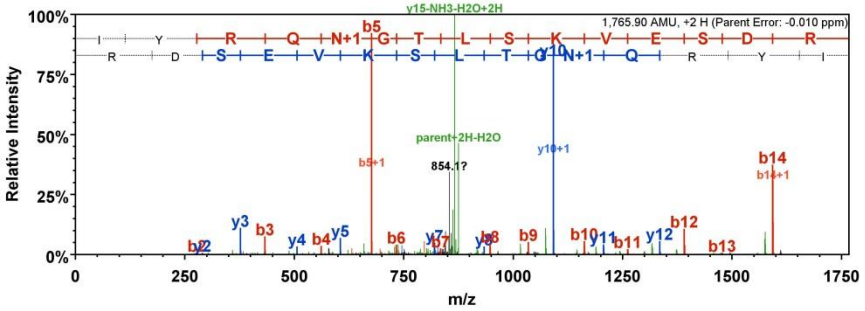
Protein Sequence:

Alpha-1-acid glycoprotein OS=Bos taurus GN=ORM1 PE=2 SV=1

16 unique peptides, 22 unique spectra, 32 total spectra, 132/202 amino acids (65% coverage)

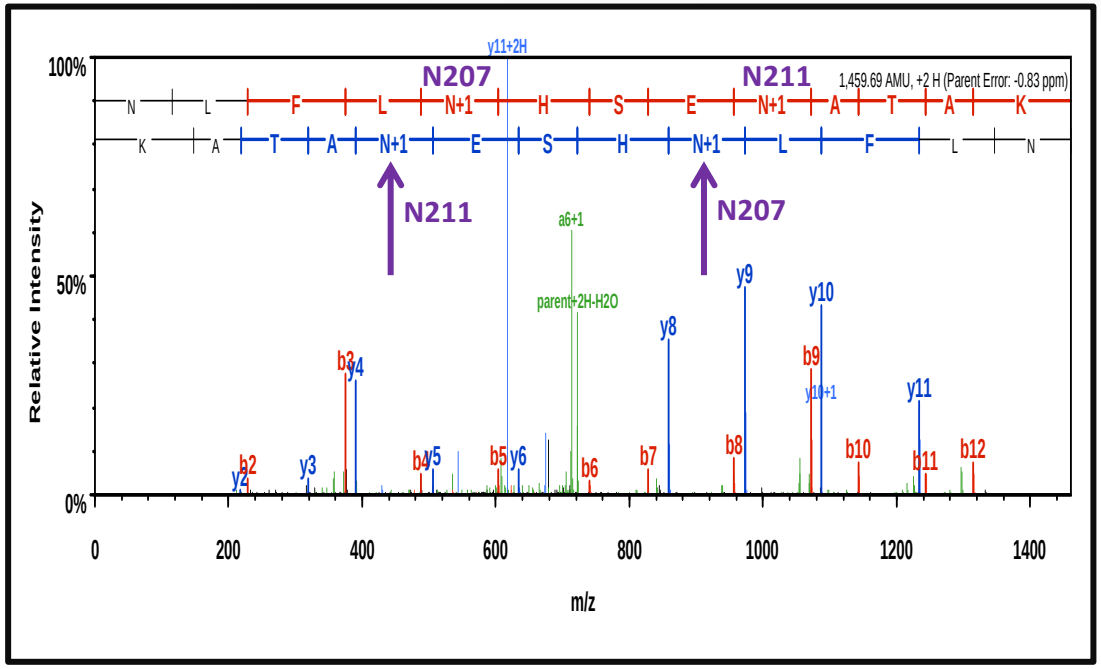
MALLWALAVL SHLPLLDAQS PECANLMTVA PITNATMDLL SGKWFYIGSA
FRNPEYNKSA RAIQAAFFYL EPRHAEDKLI TREYQTIEDK CVYNCSFIKI
YRQNGTLSKV ESDREHFVDL LLSKHFRTFM LAASWNGTKN VGVSFYADKP
EVTQEQKKEF LDVIKCIGIQ ESEIIYTDEK KDACGPLEKQ HEEERKKETE
AS

Identification of Glycosylation Sites:

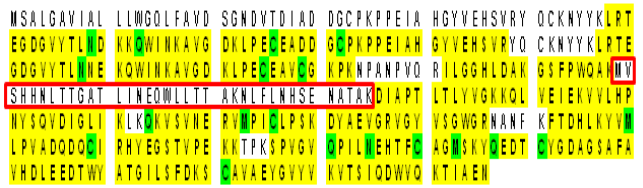


Band shifted to lower MW indicates removal of N-glycan chains

PNGase F Treatment Improves Sequence Coverage



Haptoglobin (Untreated)



Haptoglobin (PNGase F treated)

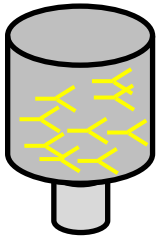
- Majority of sequence covered without PNGase F treatment
- However, highlighted glycan was not observed due to attached glycan
- Treatment with PNGase F gave sites of attachment ((N 207 and N211) were identified)
- Site of attachement confirmed by MS/MS analysis (see above spectrum)

An Example of a Typical Serum Glycopeptide identification Workflow

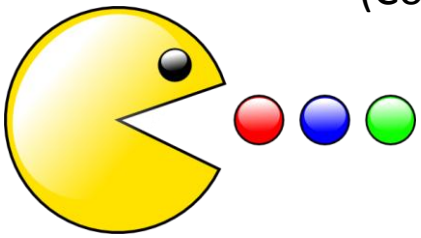
Promega

Step #1: Obtain Serum Sample

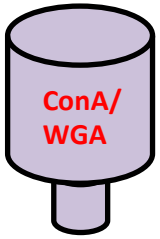
Step #2: Deplete Serum of IgG/ Albumin



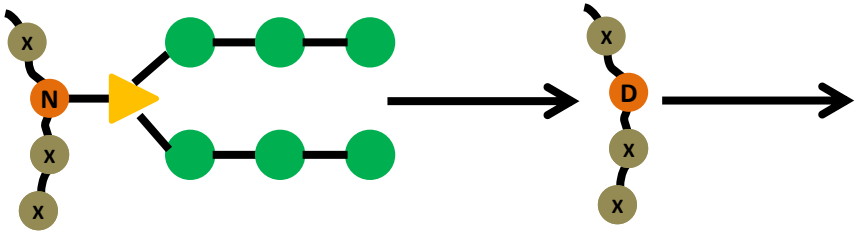
Step #3: Trypsin digestion



Optional Step #4: Enrich Glycopeptides with Lectins (ConA/WGA)

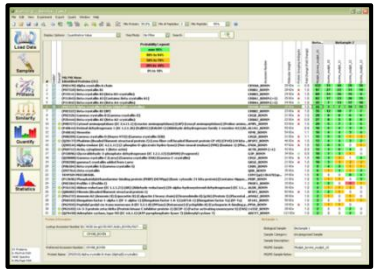


Step #5: Glycosidase (PNGase F) Treatment

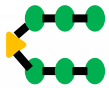


Step #5: LC-MS/MS Analysis

Step #6: Software assisted Protein Identification



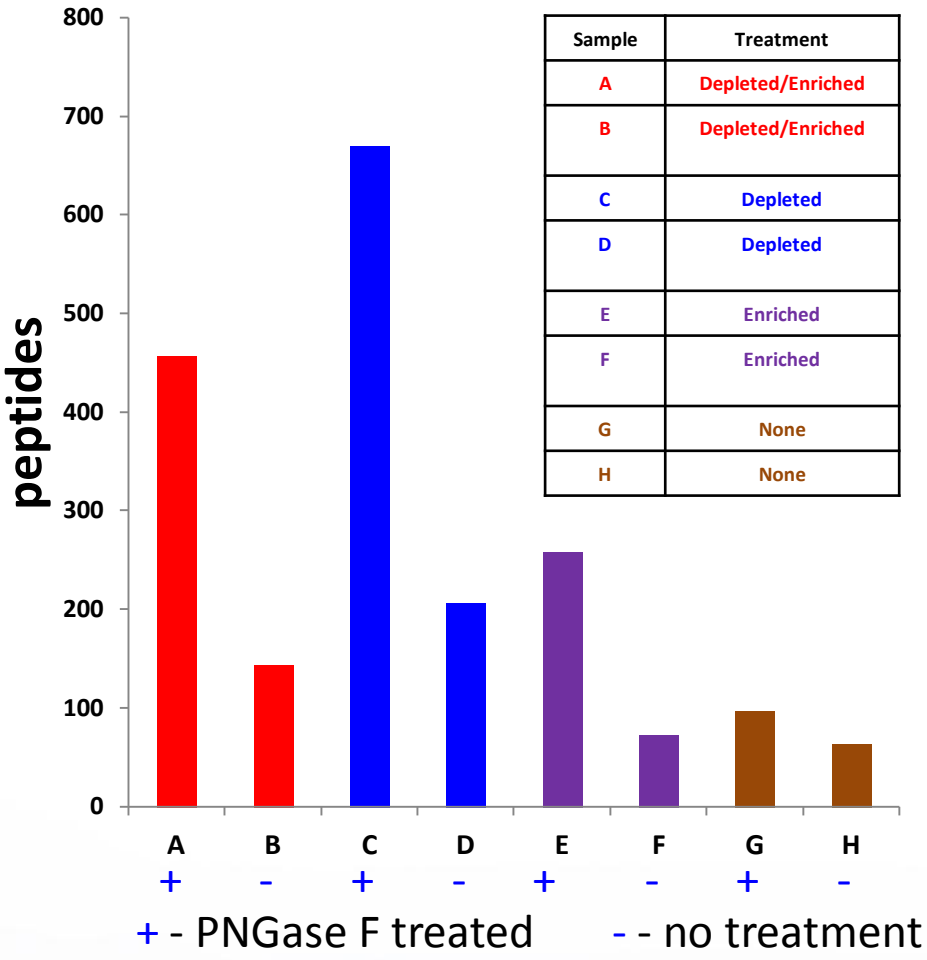
Glycan



PNGase F Increases Identification of Glycopeptides

Promega

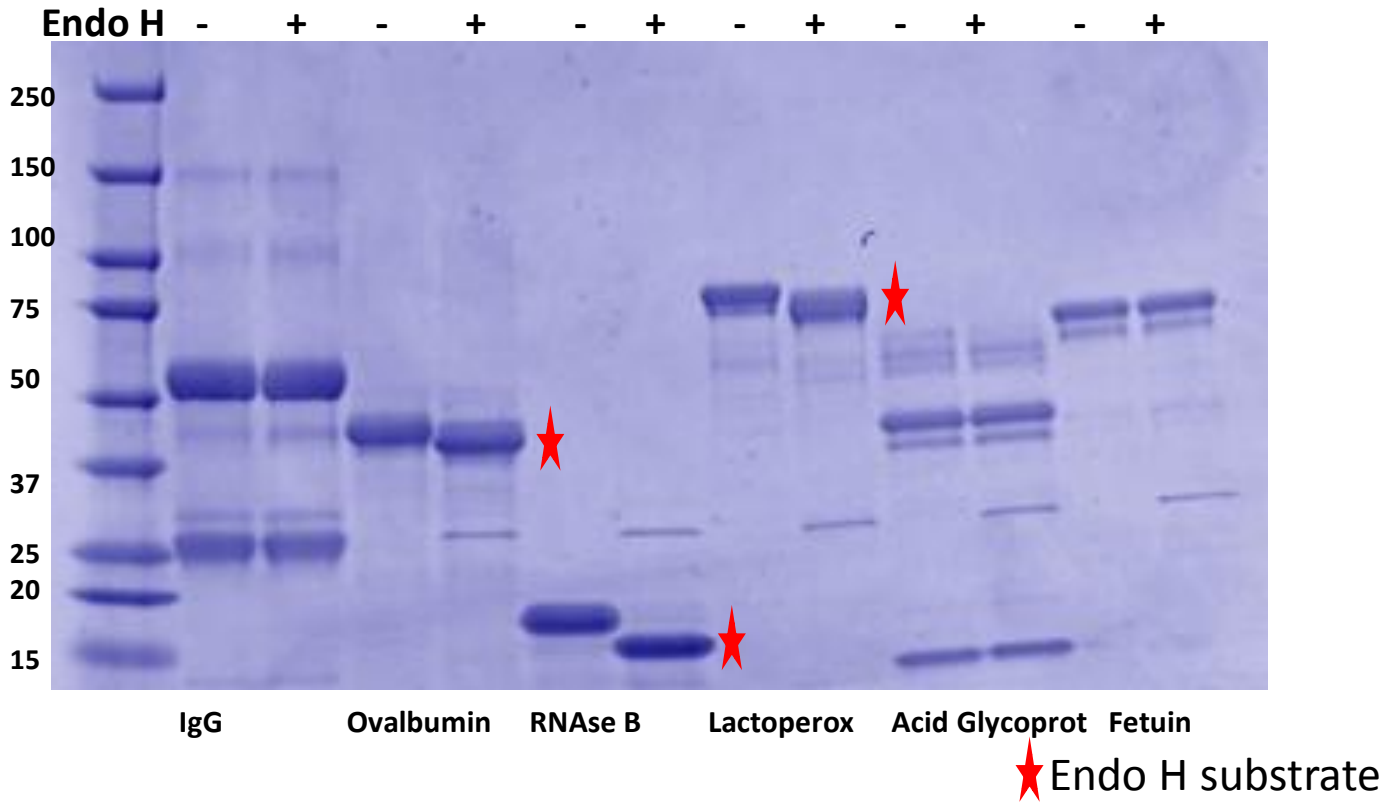
Total Deamidated SpC – Serum Sample



Sample	Treatment
A	Depleted/Enriched
B	Depleted/Enriched
C	Depleted
D	Depleted
E	Enriched
F	Enriched
G	None
H	None

- Albumin/IgG was removed from Serum
- WGA and ConA lectins were used to enrich samples
- Depletion alone appears to yield the greatest number of glyco-peptides identified

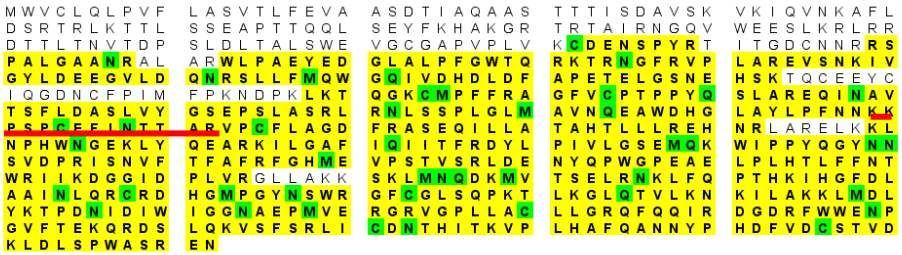
Endo H Treatment is Effective in Deglycosylating Glycoproteins



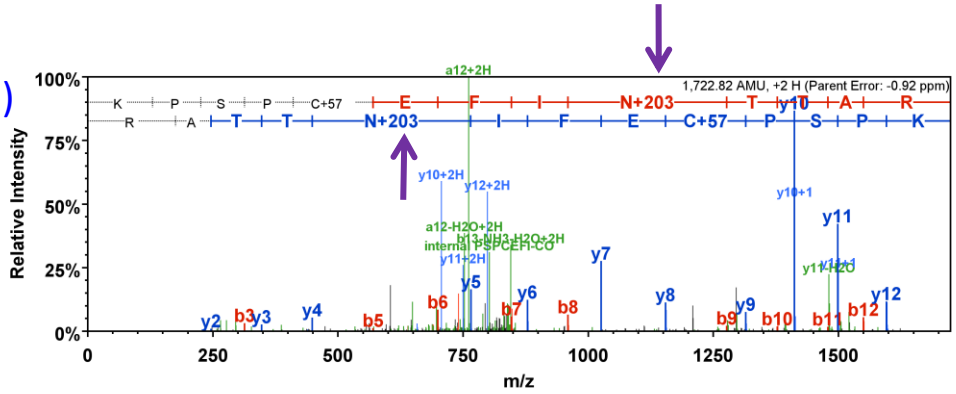
Endo H is reactive toward some, but not all, glycoprotein substrates.
Thus, a powerful biochemical tool to determine glycan compositions

Endo H Treatment of Lactoperoxidase Identifies Glycosylation Sites

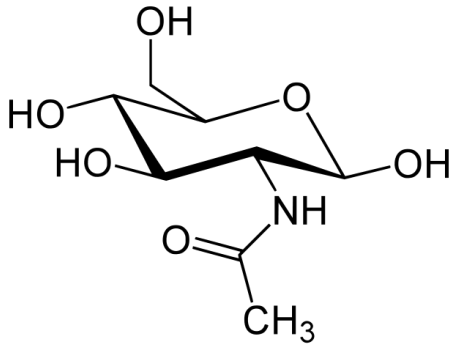
(A)



(B)



GlcNac Modification
Mod. Mass = 203 daltons



Using a combination of Trypsin and Endo H, followed by Mass Spec analysis, the site of glycan attachment (N203) could be determined, precisely.

Conclusions

- Trypsin is the best protease to start with when preparing Mass Spec samples.
- The use of a Mass Spec compatible solubilizing agent (i.e. ProteaseMax™) can dramatically improve the efficiency of proteolysis.
- Using alternative proteases will increase the number of protein ID's.
- Alternative proteases may also increase your identification and confidence in assignment of PTM's.
- For identification of glycosylation sites, the use of either PNGase F or Endo H may be required.

Promega's Protease Portfolio

Product	Catalog #	Quantity
Trypsin Gold	V5280	100 µg
Trypsin, Sequencing Grade	V5111, V5113 (Frozen)	100 µg
Immobilized Trypsin	V9012, V9013	2, 4 mL
Lys-c	V1071	5 µg
Recombinant Lys-c	V1671	15 µg
Arg-C	V1881	10 µg
Asp-N	V1621	2 µg
Glu-C	V1651	50 µg
Chymotrypsin	V1061, V1062	25 µg, 100 µg
Thermolysin	V4001	25 mg
Pepsin	V1959	250 mg
Elastase	V1891	5 mg
Protease Max	V2071 , V2072	1 mg, 5 mg

New Glycosidases from Promega

Product	Catalog #	Quantity
PNGase F	V4831	500 mIU
Endo H	V4871, V4875	10,000 and 50,000 units
Deglycosylation Mixture	V4931	100 µL
Fetuin	V4961	500 µg

These products should be available some time in Q4, 2012