Phosphorylation of proteins

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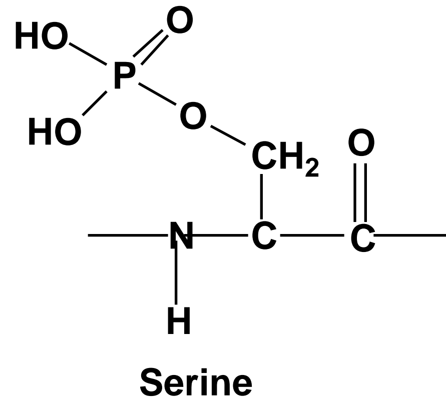
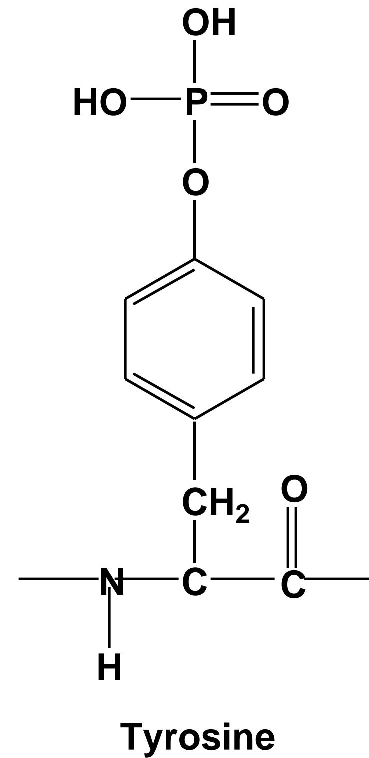
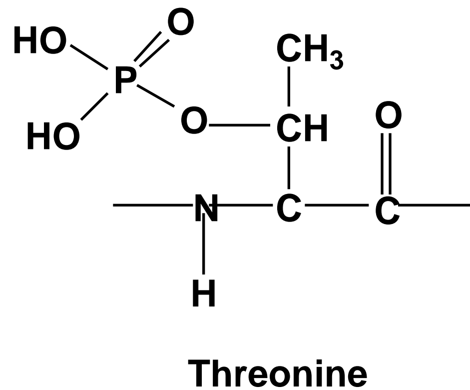
- in some cases, proteins are found in a stable, hyperphosphorylated state, e.g., casein
- more interestingly, in most other cases, it is a transient event that causes 10-100 fold increase in enzyme activity. This is the way signals are propogated through a signal transduction pathway. However, the molar abundance of phosphorylation at an individual site may only be 1-2%

Chemistry of phosphorylation



Serine





Threonine

Tyrosine

Mass spectrometry of proteins

- Adds H_3PO_4 (+98)
- Eliminates water (-18)
- Net change +80
- if the phosphate group is ionized (i.e., in negative ion spectra), net change is +79, otherwise it is +80 in positive ion spectra

Mass spectrometry of proteins

Mass spectrometry has several advantages over other techniques

- it is very accurate
- it eliminates ambiguity by defining the site of phosphorylation
- it is very fast
- it does not require ^{32}P labeling

Limitations of mass spectrometry

- **Although it can deliver sensitivity in the low fmol to high attomole range (similar to immunomethods), because it is a universal detection method, finding the needle in the "haystack" of all the other peptides is a challenge**

- **Recovering the phosphopeptides from the matrix of the sample is more important than the mass spectrometry measurement**

Finding a phosphate group

Three main methods are in current use for detection of phosphopeptides

- use of parent ion scanning
- affinity chromatography for enrichment of phosphopeptides
- phosphatase sensitivity

Recovery and enhancement of phosphopeptides

The biggest problem in the detection of phospho-peptides is how to convert the initial sample matrix into a form suitable for mass spectrometry analysis.

- **how to handle minute samples with minimal losses**
- **how to recover and detect all the phosphopeptides**
- **how to recover and detect the non-phosphorylated proteins to determine the extent of phosphorylation at individual sites**

Antibodies and phosphopeptides

- **In this approach, both the phosphorylated and non-phosphorylated forms of a protein may be recovered from the sample matrix**
- **This can most easily achieved by immuno-precipitation of the protein with an antibody that recognizes epitope(s) that is(are) in common with both forms**

NOTE ***that as with all immunoprecitiation methods, the best results will be achieved if the antibody is coupled to agarose beads. This allows selective immuno-absorption of the antigen, washing free of contaminating proteins, AND following elution, minimization of the amount of antibody that is in the eluate and therefore would be analyzed by mass spectrometry***

Detection of phosphopeptides based on their sensitivity to phosphatase

- **An alternative source of potentially phosphorylated proteins are individual spots on 2D-IEF/SDS gels. The protein preparation so isolated is either hydrolyzed by trypsin in solution (or in the gel piece) or using solid-phase trypsin**

- **One portion of the resulting tryptic peptides (in 50% acetonitrile:water) is analyzed by MALDI-TOF-MS. A second portion is diluted into 50 mM NH_4HCO_3 buffer and reacted with 0.5 U calf intestinal alkaline phosphatase at 37°C for 30 min. Sample is dried with a SpeedVac, redissolved in 50% acetonitrile:water, and re-analyzed by MALDI-TOF-MS**

- **Phosphopeptides shift down by *m/z* 80 (or units of 80 in the case of multiply phosphorylated peptides)**

The peaks identified as phosphopeptides can then be analyzed in a nanoelectrospray experiment where collision-induced dissociation is used to determine the identity of the peptide and the phosphorylation site in the sequence

MALDI-TOF analysis of phosphopeptides

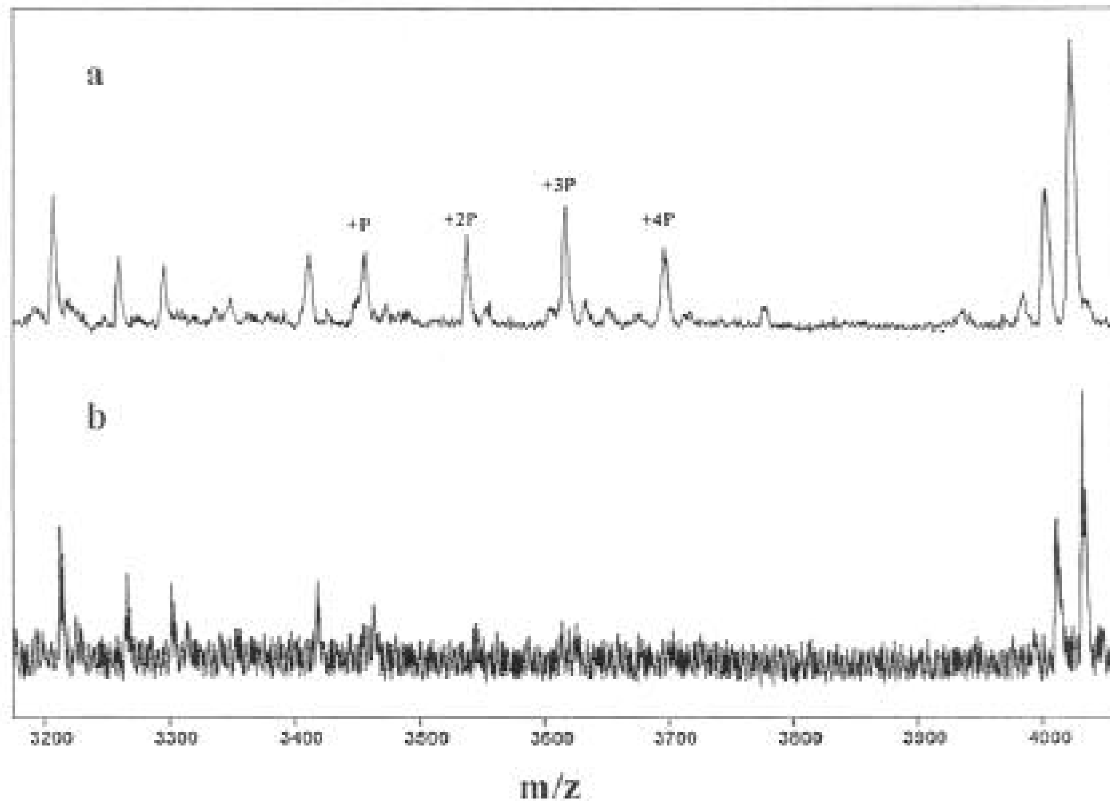


Fig. 3. The short linear MALDI/TOF mass spectrometer offers better sensitivity for the detection of multiply-phosphorylated peptides than the big reflector instrument. MALDI/TOF spectra of the in-gel trypsin digestion of a phosphoprotein taken with (a) the standing alone linear instrument and (b) the reflector instrument. The phosphopeptides were labeled with +P, +2P, +3P and +4P.

Use of alkaline phosphatase

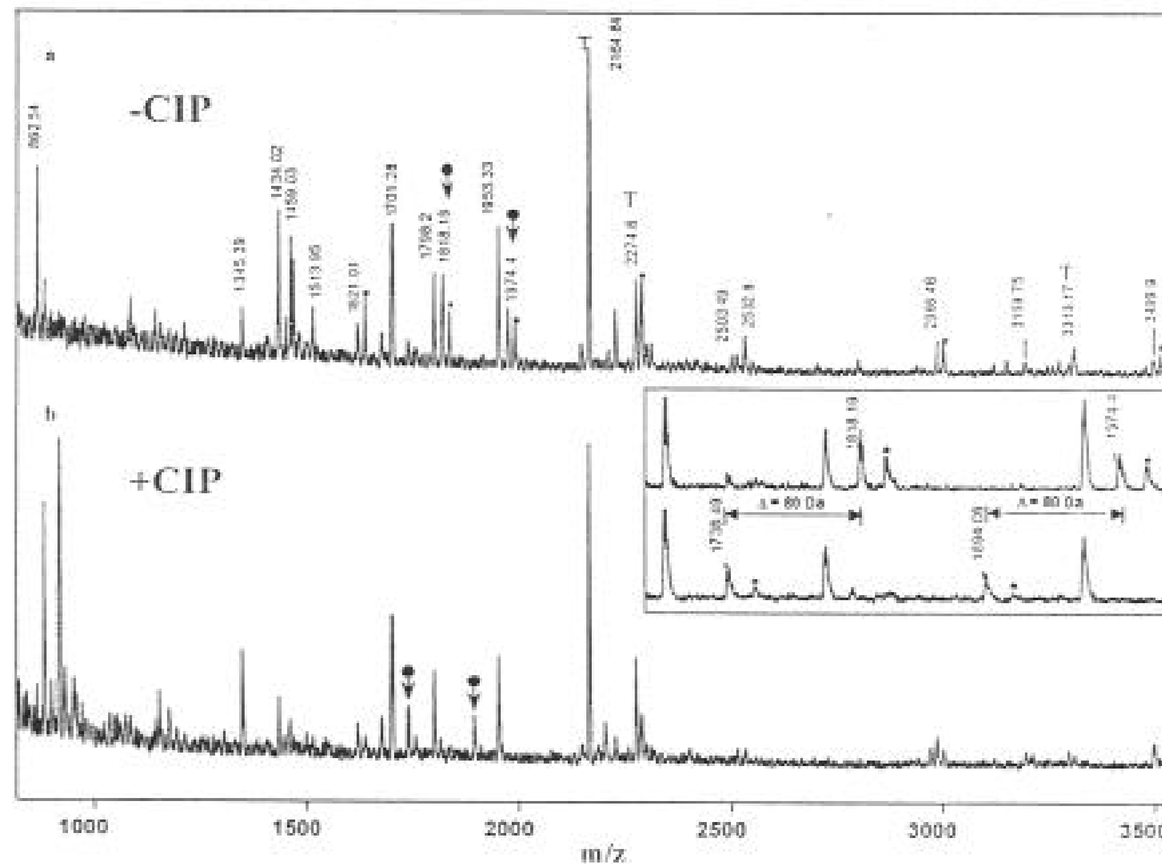


*Fig. 1. Identification of the phosphopeptides in the in-gel tryptic digest of 20 ng of the catalytic domain of MIHCK by MALDI/TOF spectrometry. (a) Before CIP treatment. Arrows indicate peptides that are modified. (b) After CIP treatment. Arrows indicate new peaks arising from dephosphorylation of the phosphopeptides. The insert shows detailed spectra of the region of phosphopeptides before (top) and after (bottom) CIP treatment. T: trypsin autolysis peaks; *: methionine-oxidized peptides of mass 16 Da higher.*

Parent ion scanning to detect phosphopeptides

- The procedure depends on the detection of the *m/z* 79 ion fragment (PO_3^-) during collision-induced dissociation in a triple quadrupole instrument operating in the negative ion mode

- Parent ion scanning is a reversal of the more familiar daughter ion MS-MS where the parent ion is selected (in Q1) and a mass spectrum of the daughter ion fragments is obtained by scanning in Q3

- In parent ion scanning, the daughter ion fragment (in this case *m/z* 79) is held constant in Q3 and a mass spectrum of parent ions that give rise to the daughter ion obtained by scanning in Q1.

- having identified the phosphopeptides, the sample can be re-analyzed to obtain daughter ion MS-MS spectra on selected ions in the positive ion mode

Parent ion scanning to detect phosphopeptides - Q-tof

- Since all daughter ions are collected and sampled in this type of instrument, a parent ion scan cannot be simply obtained
- However, by preparing a selected ion chromatogram from all the data sets collected, individual peaks containing a phosphate fragment ion can be identified
- Since the MS-MS spectra are associated with this peak, spectra for the phosphorylated peptide made available
- This approach is enhanced if the sample is analyzed during a chromatographically effective LC-MSMS run

MS-MS of a phosphopeptide

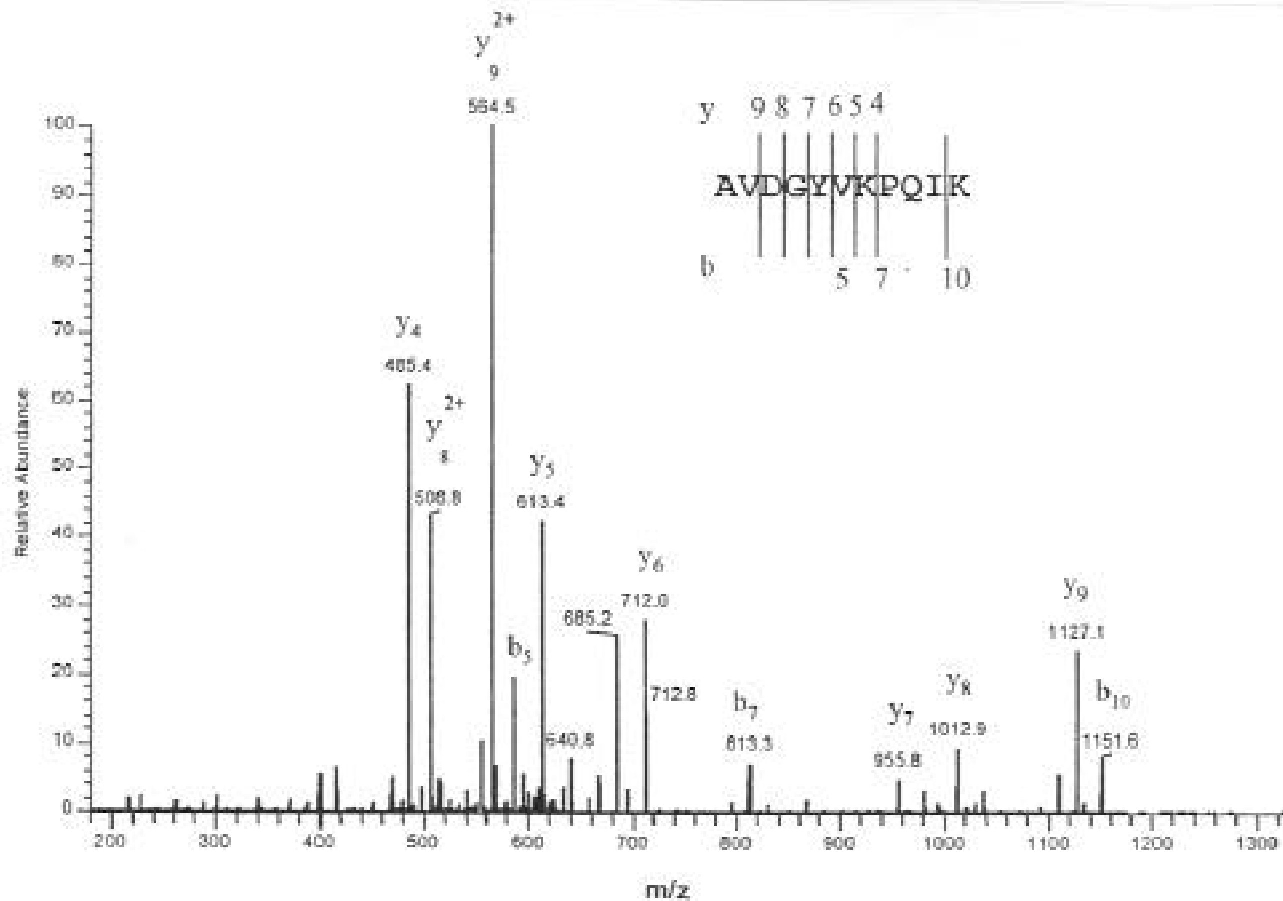


Fig. 7. The LC/MS/MS spectrum of the doubly charged small phosphopeptide as identified in Fig. 6. Y is the residue that is identified as phosphorylated.

Selective enhancement of phospho-peptides in tryptic digests

- Immobilized metal affinity chromatography (IMAC). Similar to Ni-affinity resins used in the purification of 6xHis-tagged proteins. The affinity phase can be charged with different metal ions (as their chlorides)

- Fe(III) and Ga(III), and to a lesser extent Zr(IV), were the most effective for the recovery of two synthetic phosphopeptides

- A tryptic digest containing both phosphorylated and non-phosphorylated peptides is passed over the IMAC column at acid pH (pH 2.5-3)

- The column is washed with 0.1 M acetic acid to remove unbound peptides

Metal ion affinity chromatgraphy

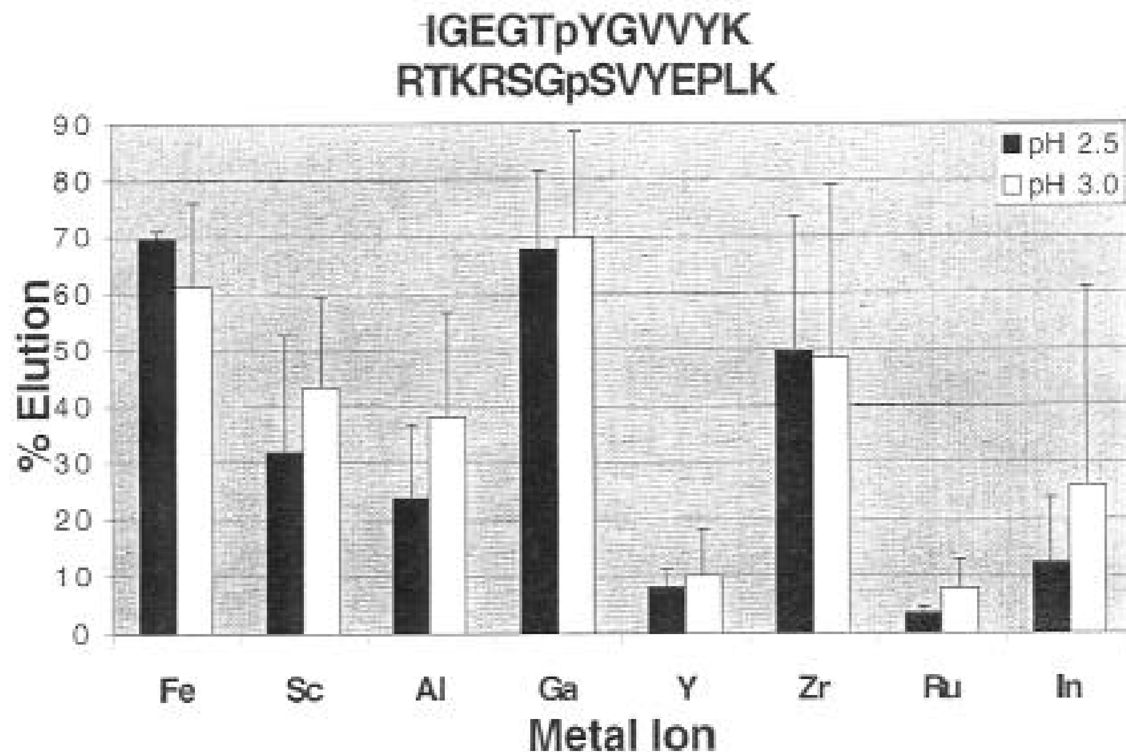
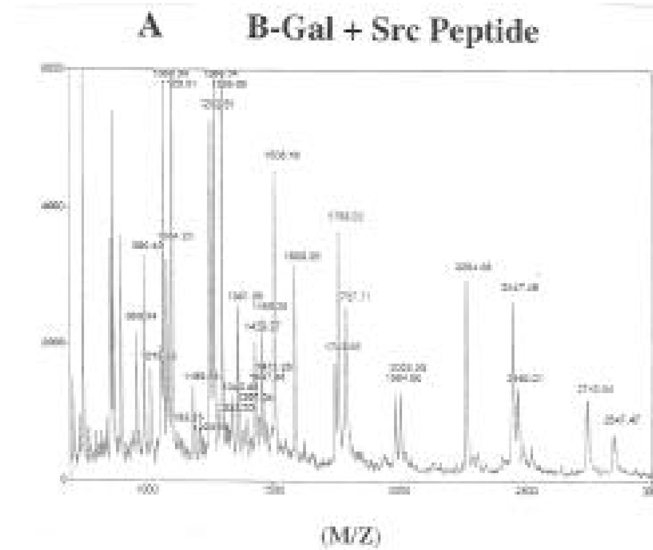
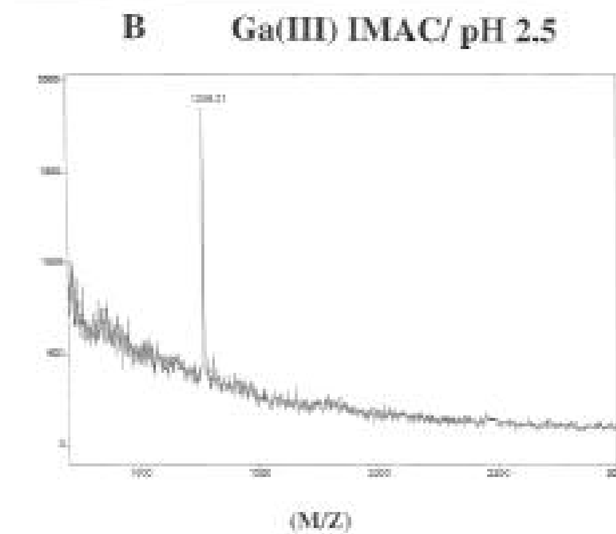


Fig. 5. Recovery of phosphopeptides from IMAC micro-columns: effects of metal ion. Two [^{32}P]-labeled phosphopeptides were separately loaded (at 2 different pHs, as indicated) onto POROS MC micro-columns to which different metal ions had been immobilized, extensively washed, eluted with 0.2M phosphate, and recoveries monitored by Cerenkov counting. Results are presented as average for the two peptides.

Selective isolation of phosphopeptides





Different IMAC columns and phosphopeptides

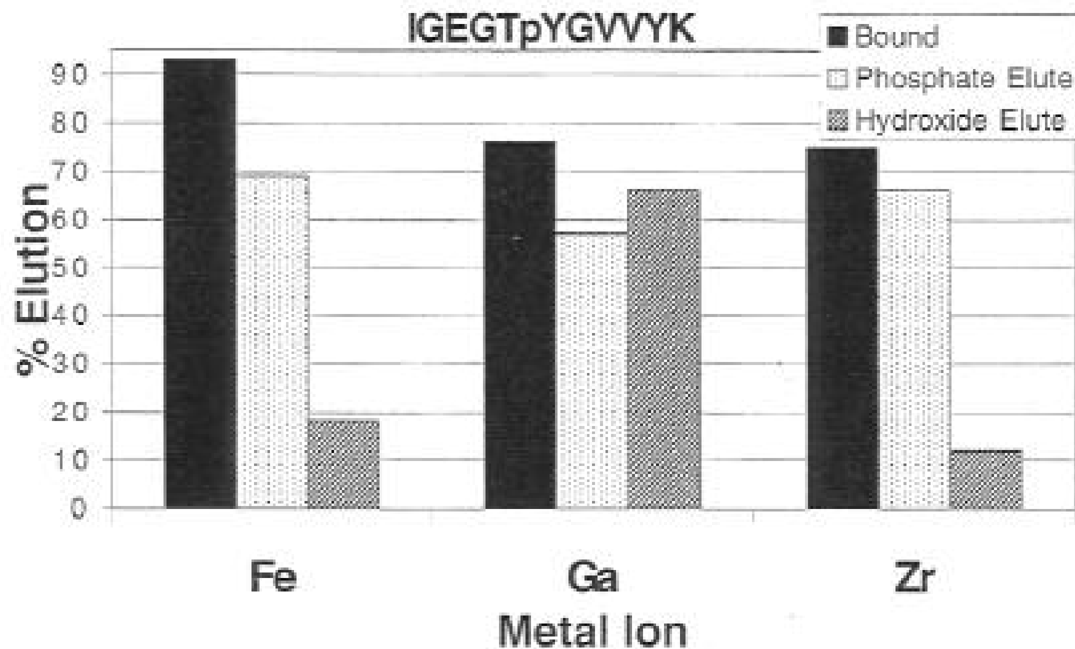


Fig. 8. Phosphate versus base elution of phosphopeptides from IMAC micro-columns. $[^{32}P]$-labeled phosphopeptide was loaded (at pH 2.5) onto a POROS MC (containing immobilized metal ions, as shown) micro-column, extensively washed, eluted with either 0.2M phosphate or 0.075% ammonium hydroxide pH 10.5, and recoveries monitored by Cerenkov counting. % bound was calculated (100-x %) by counting of the flow-through and wash solutions (combined x%).

Selective enhancement of phospho-peptides in tryptic digests

- An alternative elution method is to use ammonium hydroxide (0.075%) which is volatile and the salt it forms will be with acetic acid - this does not interfere with the electrospray ionization process

  - however, elution from Fe(III) and Zr(IV) matrices was poor - in this application Ga(III)-IMAC is a far superior phase

Comparative IMAC

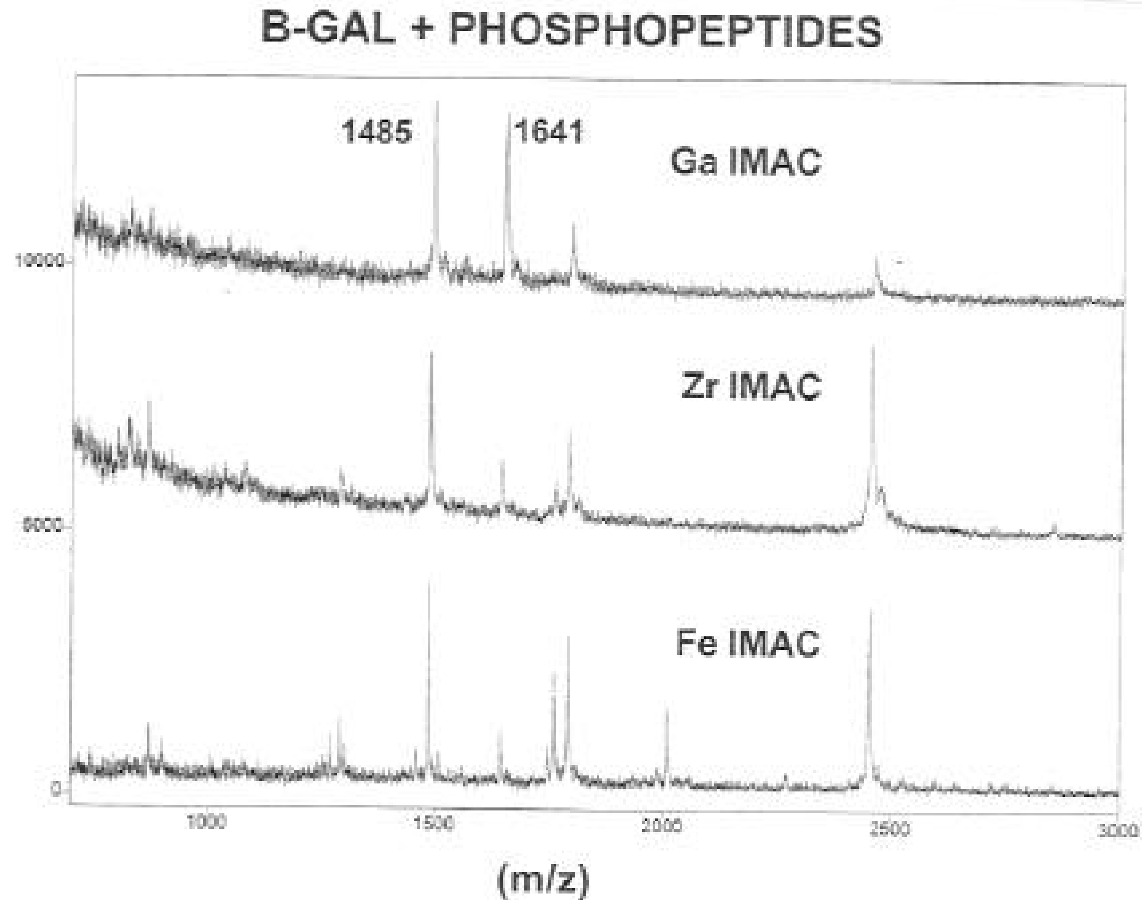


Fig. 7. Comparative IMAC micro-column isolation of phosphopeptides. Two phosphopeptides (RRLIEDAEpYAARG-amide; RLIEDAEpYAARG-amide) were added to a tryptic digest mixture, which was then divided into three equal parts and processed in parallel over IMAC micro-columns to which three different metal ions (as indicated) had been immobilized, desalted over an RP micro-column and analyzed by MALDI-TOF MS; all as described under Fig. 6.

Selective biotinylation of phospho-groups

Ba(OH)$_2$

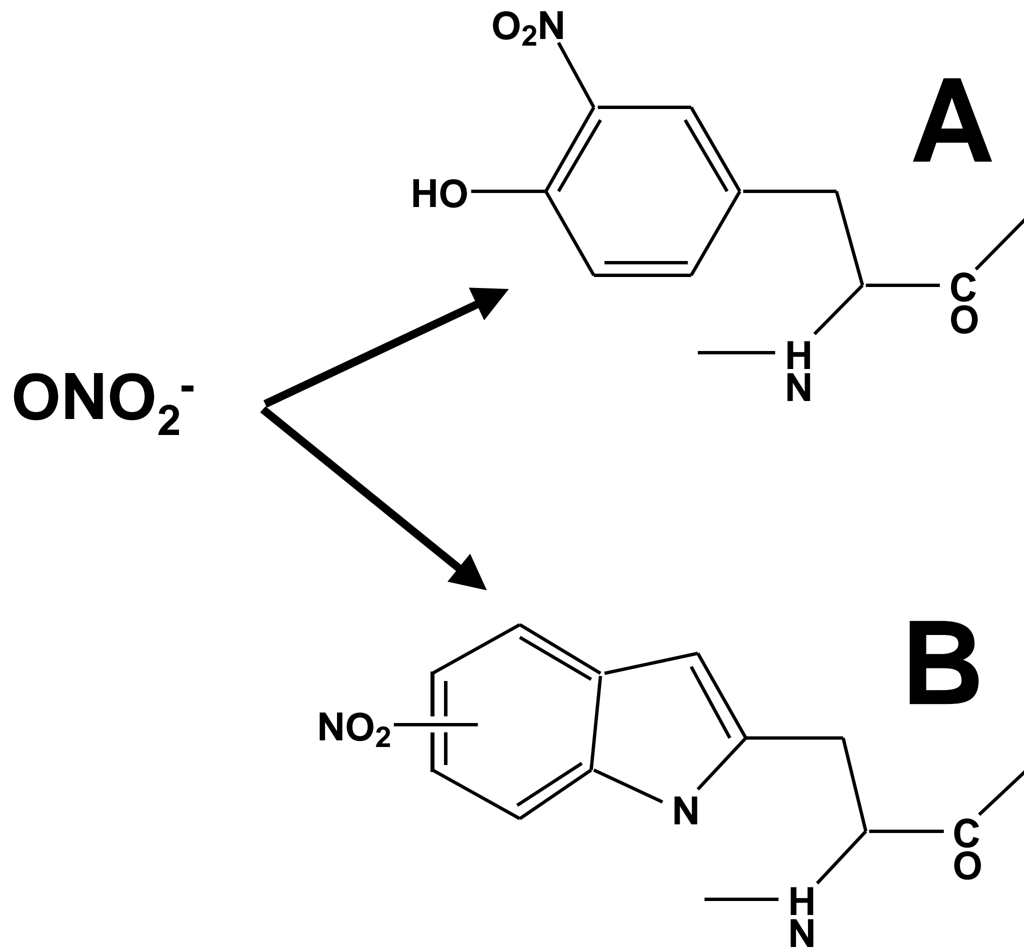
EDT

$C(=O)$, H, N–H, CH_2–O–PO_3H → $C(=O)$, N–H, $=CH_2$ → $C(=O)$, N–H, CH_2–SCH_2CH_2SH

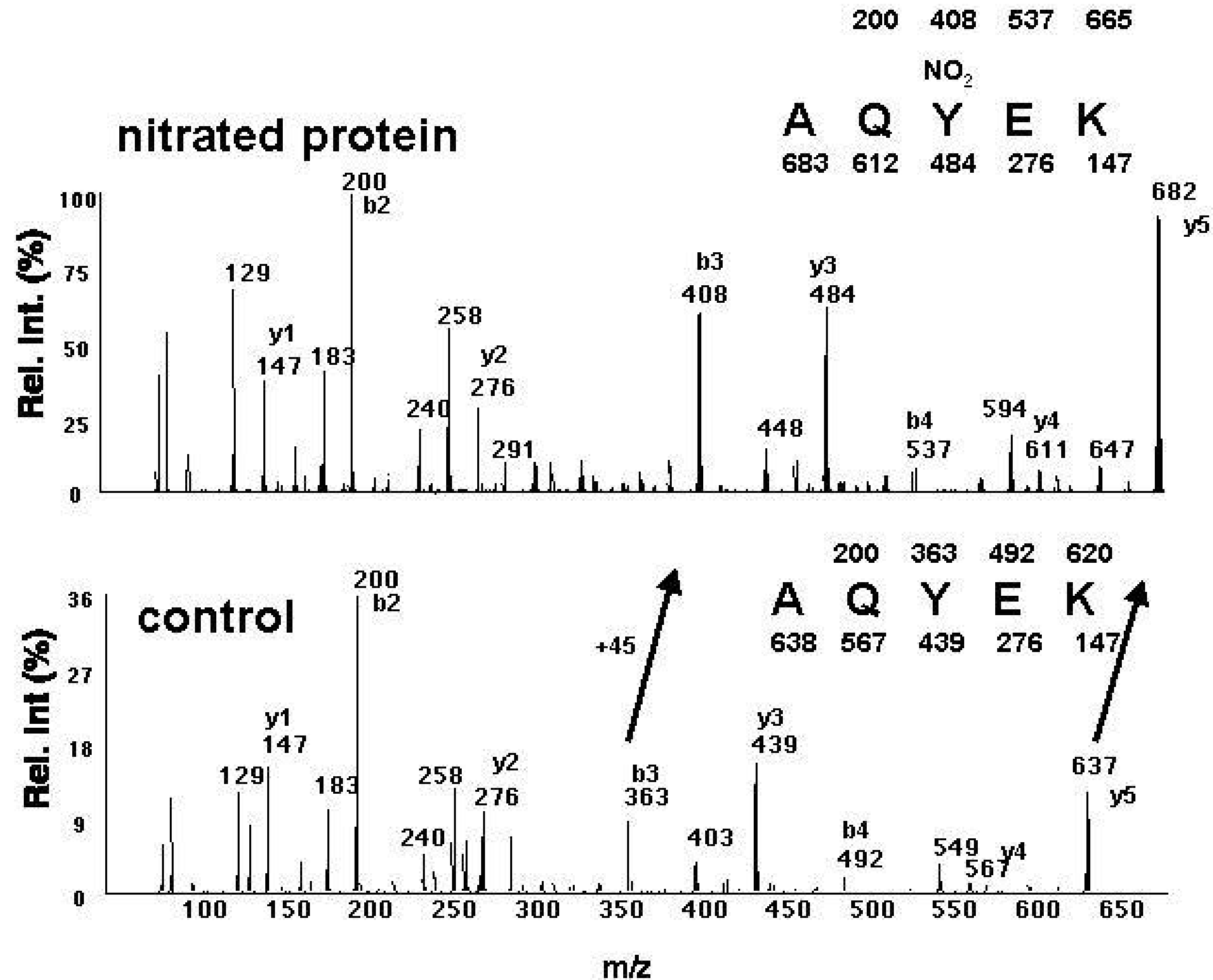
→ $C(=O)$, N–H, CH_2–$SCH_2CH_2SCH_2CONHCH_2CH_2OCH_2CH_2NHCOCH_2CH_2CH_2$-biotin

Goshe et al., Anal Chem 73: 2578 (2001)
Adamcyk et al., Rapid Commun Mass Spec 15; 1481 (2001)

Nitration of Tyr and Trp residues

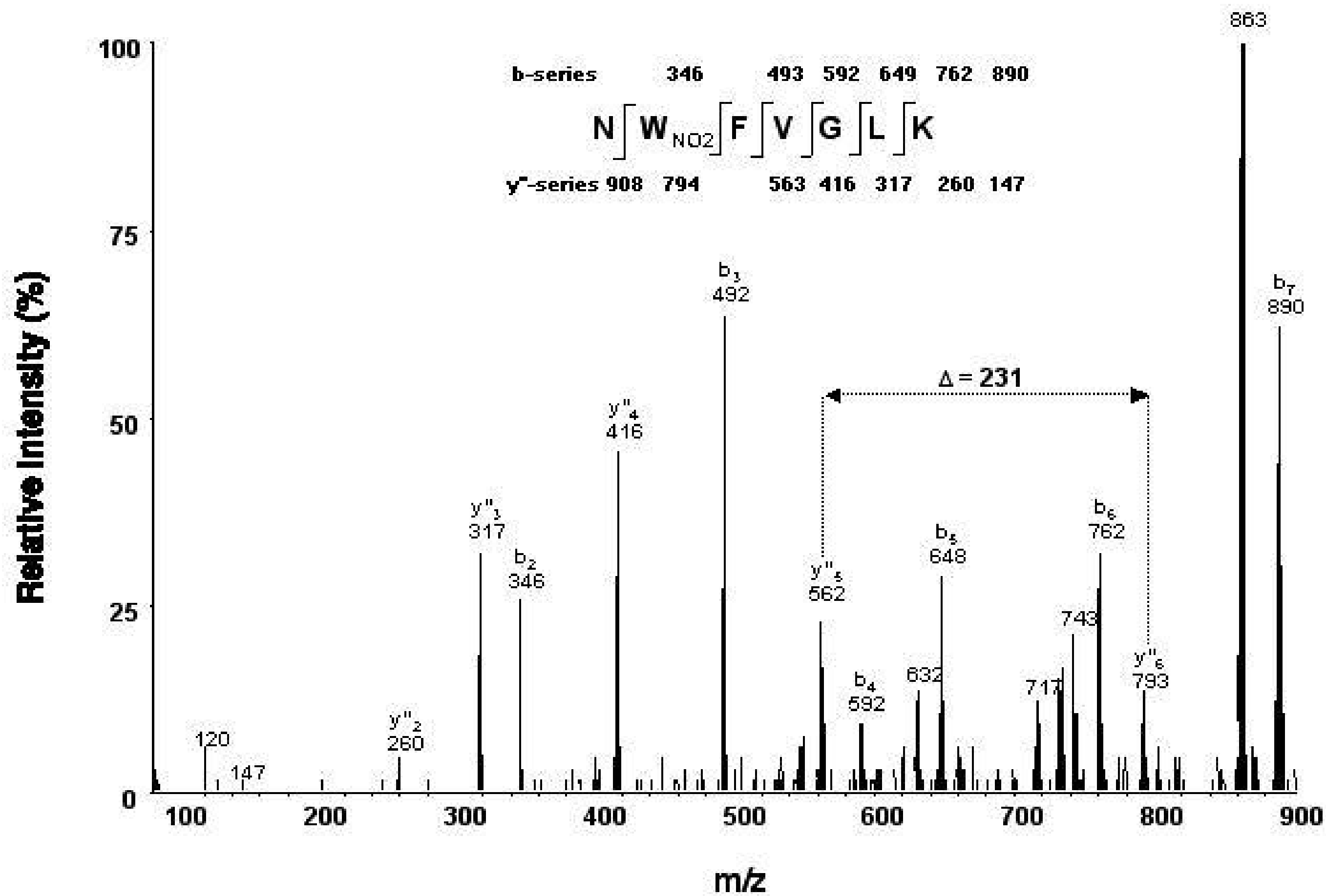


Nitration of NF-L protein in motor neurons from ALS patients

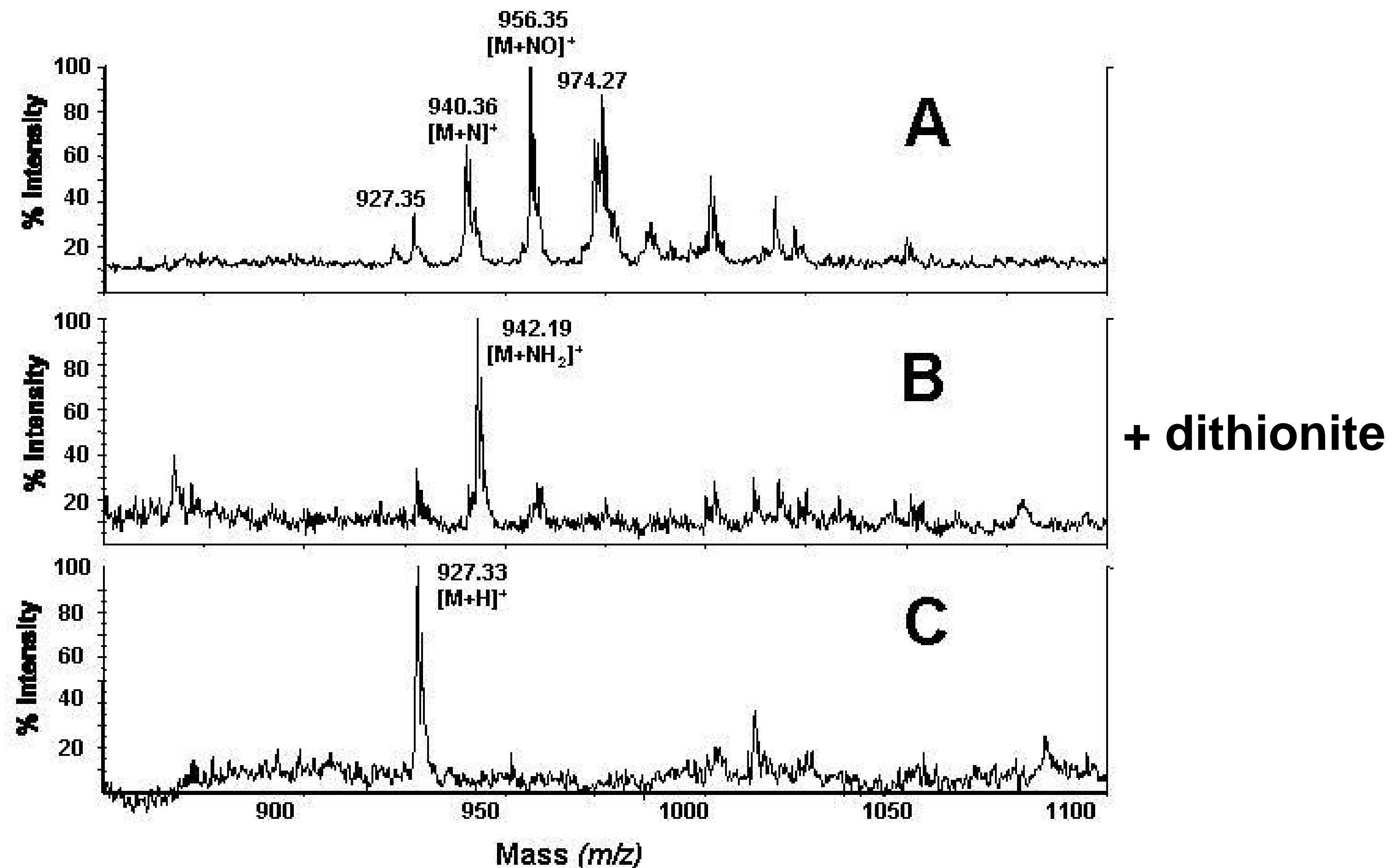


Crow et al., 1997

Trp nitration in FGF peptide



Laser-induced decomposition of nitrated peptides using MALDI-TOF



+ dithionite

How to identify modified peptides by searching databases

- **The databases you have already seen have some ability to predict the expected masses for a limited number of posttranslational modifications**

  - MASCOT (http://www.matrixscience.com)
  - PROTEIN PROSPECTOR (http://prospector.ucsf.edu/)
  - PROFOUND(http://www.proteometrics.com/prowl.cgi/ProFound.exe)

How to identify posttranslational modifications at a new site

FindMod at

http://www.expasy.ch/tools/findmod/

It examines mass fingerprinting data for mass differences between empirical and theoretical peptides. If the mass difference corresponds to a known modification, it also makes intelligent guesses as to the site of modification.

FindMod

Modifications considered are:

acetylation	amidation	biotinylation
C-mannosylation	deamidation	flavinylation
farnesylation	formylation	geranyl-geranylation
g-carboxyglutamic acid	hydroxylation	lipoylation
methylation	myristoylation	N-acyl diglyceride
O-GlcNac	palmitoylation	phosphorylation
pyridoxal phosphate	phospho-pantetheine	pyrrolidone-carboxylic acid
sulfation		

NOTE that none of the common chemical modifications (alkylation of sulfhydryl groups with iodoacetic acid, iodoacetamide, 4-vinylpyridine, and acrylamide) were included.

The list also omits nitration and the recently discovered halogenation of peptides.

See the article by Wilkins et al. (1999) in J. Mol Biol. for details on FindMod