Mass Spectrometric Integration of Robust Phosphoprotein Profiling Strategies

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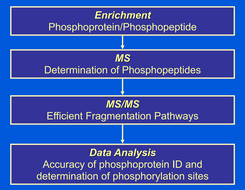
Overview / Introduction

Protein phosphorylation plays an important role in intracellular signal transduction and is involved in regulating cell cycle progression, differentiation, transformation, development, peptide hormone response, and adaptation. This important post-translational modification often occurs at very low stoichiometry. As a result, it is important that studies directed at identifying phosphoproteins and characterizing phosphorylation sites incorporate a phosphoprotein or phosphopeptide enrichment step.

In this poster we demonstrate steps taken towards an integrated and robust enrichment and mass spectral analysis methods for the identification of phosphoproteins obtained from complex cellular environments.

Methods

Crucial steps and challenges frequently encountered in Phosphoprotein/Phosphopeptide Profiling



Enrichment: It's been shown that strong cation exchange (SCX) chromatography can enrich for tryptic phosphopeptides based on their solution charge states ([1]Beausoleil et al.). We have adapted similar approaches. Results are shown with simple test systems and with complex protein mixtures obtained from mouse brain synaptoneurosomes.

MS platforms: Three mass spectrometric platforms with different MS and MS/MS approaches are utilized, Only one with online RP LC-MS/MS on a QSTAR XL mass spectrometer (ABI, Foster City, CA) will be discussed here.

Data Analysis: Enrichment results are compared from mouse synaptoneurosomes using both MASCOT and SEQUEST protein database search algorithms after statistical validation by PeptideProphet™ and ProteinProphet™ (Institute for Systems Biology, Seattle, WA)

Mouse Brain Synaptoneurosomes:

1.5 mg Synaptoneurosomes from 7 days old mouse pups stimulated with KCl to increase kinase activity. Phosphatase inhibitor Coctail I and II (Sigma) are added at 1% concentration in all buffers used throughout the preperation. Synaptoneurosome preparations containing 1.5 mg of total protein were lysed by the addition of urea (10 M) to a final concentration of 6 M urea. Gentle sonication was used to increase the efficiency of the lysis. Samples were then diluted to a concentration of 1 M urea with Milli-Q water, since trypsin is sensitive to urea at concentrations >1 M. Trypsin Gold (Promega) was reconstituted in 50 mM acetic acid, to 1 microgram trypsin per microliter. Proteins were digested by trypsin in a ratio of 1 part trypsin to 20 parts total proteins (by mass) at 37 degrees C for 8 h.

References

[1]Beausoleil, S.A., Jedrychowski, M., Schwartz, D., Elias, J.E., Villen, J., Li, J., Cohn, M.A., Cantley, L.C., and Gygi, S.P. (2004). Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. U S A* 101:12130-12135

[2]Pinkse, M.W., Uitto, P.M., Hilhorst, M.J., Ooms, B., and Heck, A.J. (2004). Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal. Chem.* 76:3935-3943

[3]Collins MO, Yu L, Coba MP, Husi H, Campuzano I, Blackstock WP, Choudhary JS, and Grant SG. (2005). Proteomic analysis of in vivo phosphorylated synaptic proteins. *J Biol Chem.* 280(7):5972-82

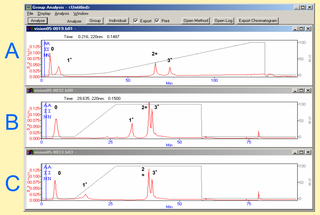
Results

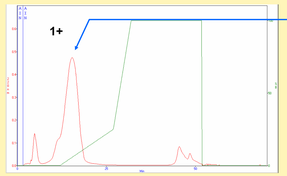
SCX chromatography optimization of the 4 model peptides under three different experimental conditions

(A) Buffer A=10mM KH2PO4+30% ACN; Buffer B= same as Buffer A+ 1M KCl; pH= 3.0
(B) Buffer A=5mM KH2PO4+30% ACN; Buffer B= same as Buffer A+ 1M KCl; pH=2.7
(C) Buffer A=5mM KH2PO4+30% ACN; Buffer B= same as Buffer A+ 1MKCl; pH= 3.0

The three of the many optimization conditions are shown. All SCX runs were acquired with the Vision Chromatography System (ABI, Forest City, CA) using the Poly2-Sulfoethyl Aspartamide) 5µm, 200 Å pore , 2.1 x 150mm column (PolyLC Inc., Columbia, MD).
Approximately 3.6 nmole or 5 micrograms of each peptide was injected.
Flow Rate = 200ml/min; UV detection = 220nm.
SCX model peptides have solution charge states of 0, 1, 2, and 3 (marked on peaks). The positive (H)N-; Arg, Lys, His) and negative (HPO_3) charge contributing residues at low pH are highlighted below).

0 H_2N-xxxxxxpSxxxxxx
1+ H_2N-xxxxxxpSxxxxKx
2+ H_2N-xxxxxRpSxxKxxx
3+ H_2N-xxxxxRSxxKxxx

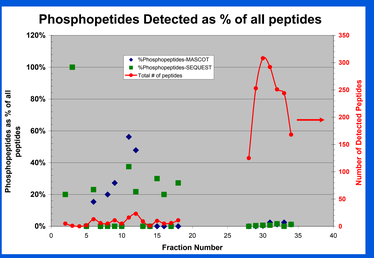




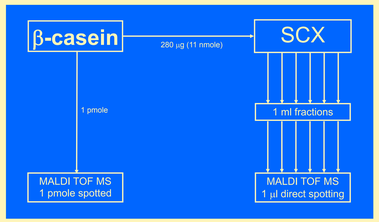
SCX chromatogram of a test mix of 1000 phosphopeptides with 1$^+$ solution charge state

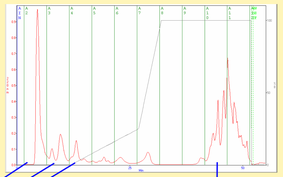
Peptides were synthesized at the Keck Lab Small Scale Peptide Synthesis core facility. Sequences were adapted from reference (1) as GAPXPXpSXFEAR where for each X a random mix of A, D, E, F, G, L, S, T, V, and Y amino acids were inserted making a mixture of 1000 different phosphopeptide species each having a solution charge state of 1+ at low pH. As preferred from the 4 model peptide SCX runs, the elution profile of the phosphopeptides were observed near the early part of the gradient (max ~ 14 min).

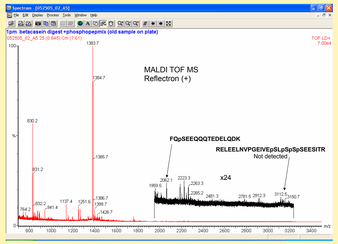
SCX conditions are the same as above (C), except with slight modification of the gradient.
UV detection = 220nm.
Sample loading: 280 µg (approximately 0.2nmole of each peptide) was injected.

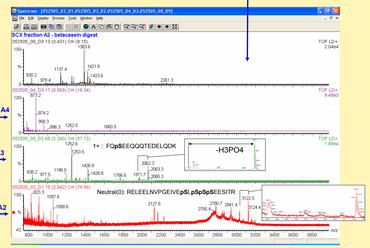


The figure above shows that early SCX fractions produced proportionately larger number of phosphopeptides when compared to the overall number of peptides identified from that fraction.







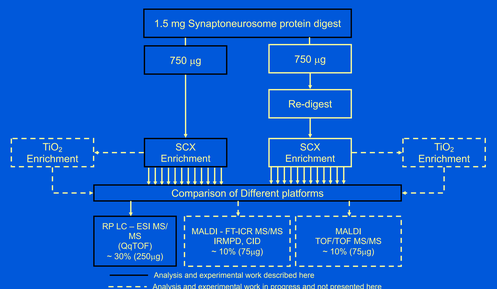


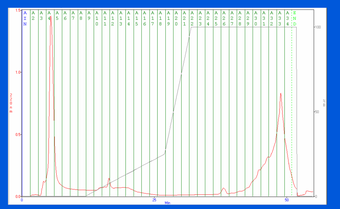
SCX Enrichment for the two known phosphopeptides that are mass spectrometrically detected from a tryptic digest of bovine β-casein:

Solution Charge state = 1$^+$ FQpSEEQQQTEDELQDK (MH$^+$ = 2061.0Da)
Solution charge state = 0 RELEELNVPGEIVEpSLpSpSpSEESITR (MH$^+$ = 3121.9 Da).

SCX conditions are the same as above (C), except with slight modification of the gradient shown in gray line.
Sample loading: 10 nmole; 280 µg;
12 collected fractions are shown on the SCX Chromatogram taken at 5 minute intervals (1ml). Representative MALDI TOF mass spectra from 1µl aliquots are shown from early phosphopeptide enriched fractions (A2, A3). Also shown are two of the later fractions mostly containing the unphosphorylated peptides (i.e. A4, A10). For comparison, spectrum from 1 pmole bovine β-casein digest mixture is shown above left.

Mouse Brain Synaptoneurosomes:



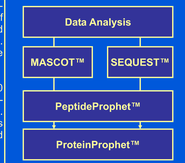


SCX enrichment for the synaptoneurosome phosphopeptides.

SCX conditions are the same as above (C), except with slight modification of the gradient.
Sample loading: 250 µg;
34 SCX fractions are collected at 1.5 minute intervals (300 µl). Only the first 18 and the last 5 peptide rich fractions were examined.
Each fraction was subsequently dried down, and reconstituted with 15 µl of 0.1 % TFA. 5µl of the sample was injected and desalted on a reversed phase C18 trap column (Waters Symmetry, Nanoease 180µm x 23.5 mm, 5µm) and was separated on a C18 analytical column (Waters, Atlantis, Nanoease 0.1mm x 150mm, 3µm, 100Å) using Dionex Ultimate chromatography system. On-line MS analysis was performed on the ABI QSTAR XL system. MS data was surveyed for 0.25 s, and the highest peptide peak was chosen for MS/MS analysis at low collision energy (0.25 s) and at optimum collision energy (1.5 s). Low collision energy step was added to confirm neutral loss on putative S and T phosphopeptides.

Protein Prophet results from SEQUEST searches with 0.5 and higher probability produced 527 protein identifications (226 from 2 or more peptides) of which 33 were phosphoproteins. These phosphoproteins were represented by 38 phosphopeptides that had 0.5 or more peptide prophet probability score. 23 peptides were phosphorylated on the Ser, 9 peptides on Thr, and 2 on the Tyr residues. 4 more had both Ser and Thr phosphorylation sites.

Individual MASCOT search results revealed 40 phosphopeptides from 20 phosphoproteins of which only five overlapped with the SEQUEST results requiring further inquiry into the differences between the two search algorithms. Close examination of the Mascot search results also revealed 14 peptides from Microtubule associated proteins in agreement with previously published reports[3] confirming the highly phosphorylated nature of these proteins.



Conclusions / Future Improvements

- It's demonstrated that the SCX chromatography offers a simple and rugged means to enrich for phosphopeptides from complex protein samples.
- The phosphopeptide enrichment from synaptoneurosomes and MS/MS results show similar qualitative observations of phosphopeptides and phosphoproteins published by Collins et. al.[3] However, much lower number of phosphopeptides and phosphoproteins were detected here reflecting the much lower starting protein amounts (12.5 mg vs. 0.25mg).
- Larger loading amounts of proteins on to SCX column (milligrams) at semi-preparative or preparative levels may be necessary for the identification of larger number of phosphopeptide and proteins in the future.
- Implementation of TiO_2 on line with the SCX column, especially in earlier fractions, is expected to result in better enrichment of phosphopeptides.
- Future development of improved protein database search and validation algorithms on MS/MS spectra of phosphopeptides are essential.
- Improvements on mass spectral detection and prioritization of surveyed phosphopeptides for MS/MS are needed
  - Better MS and MS/MS platform (MALDI FTMS with IRMPD or ECD or TOF/TOF with upfront RP LC)
  - Development of real time algorithms for high probabilistic determination of phosphopeptides in survey mode

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