Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) for Specific Enrichment and Identification of Phosphopeptides

Goran Mitulovic[1)]; Andrew Alpert[2)]; Karl Mechtler[1,3)]

1) IMBA Institute of Molecular Biotechnology, Vienna, AUSTRIA; 2) PolyLC Inc., Columbia, MD, USA;

3) IMP Research Institute of Molecular Pathology, Vienna, AUSTRIA

Overview

Aim: Development of efficient and simple chromatographic separation method for phosphorylated peptides.
Methods: ERLIC chromatographic fractionation in the first dimension combined with reversed phase HPLC MS/MS detection.
Results: A total of 7130 peptides was identified. Phosphopeptides contributed with 77% or 5496 peptides. The amount of unphosphorylated peptides is 23% or 1634 peptides.

Introduction

- At a pH low enough to uncharge Asp- and Glu- residues (so as to distinguish them from phosphate groups), the electrostatic repulsion of the positively-charged termini causes the elution of tryptic fragments prior to the void volume [1], and attachment of a single phosphate group does not suffice to overcome this repulsion. Such phosphopeptides are not well-resolved from nonphosphopeptides in AEX (Anion Exchange Chromatography) [2]. When the column is operated in the HILIC (Hydrophilic Interaction Chromatography)[3] mode, though, then the considerable hydrophilicity of the phosphate group plus its electrostatic attraction accomplishes this separation despite the repulsion from the termini. This combination is called ERLIC (Electrostatic Repulsion- Hydrophilic Interaction Chromatography) [4]. ERLIC is sensitive to aspects of peptide composition besides the phosphate group. This makes it suitable as a high-resolution mode for samples containing thousands of phosphopeptides.

This poster explores the use of ERLIC for isolation of phosphopeptides via gradient elution of a ERLIC column.

Materials, Instrumentation and Methods

- **Sample**: Tryptic digest of 3.6 mg HeLa proteins. Sixty fractions (500 µl each) were collected using Probot µ-fraction collector (Dionex, The Netherlands) and 3% of the fraction was injected onto the RP HPLC MS/MS for separation and dentification.
- **Instrumentation**: The ERLIC separation of digested HeLa proteins was performed on a 4.6 mm ID x 10 cm, 5 µm, 300Å PolyWAX LP ™ (PolyLC, Columbia, MD, USA) mounted in a BioLC HPLC system, Dionex (Sunnyvale, CA, USA).
- Reversed phase HPLC was performed on 75 µm ID x 15 cm length Acclaim PepMap C18, 100Å, 3µm nano separation column mounted in an UltiMate 3000 Dual Gradient nano HPLC system (Dionex, The Netherlands). Detection was performed with both UV @ 214 nm and with MS (OrbiTrap, Thermo Fisher, Bremen, Germany) operated in positive nano ESI mode and applying multistage activation for enhanced phosphopeptide detection [5].
- **HPLC mobile phases:**

For the ERLIC separation a 2-solvent gradient was used: mobile phase A was 20 mM Na-MePO4, pH 2.0, containing 70% acetonitrile (ACN) (v/v) and mobile phase B was 200 mM TEAP (triethylammonium phosphate), pH 2.0, containing 60% ACN (v/v).
For reversed phase HPLC mobile phase A was 95% of 0.1% aqueous formic acid (FA) containing 5% ACN (v/v).

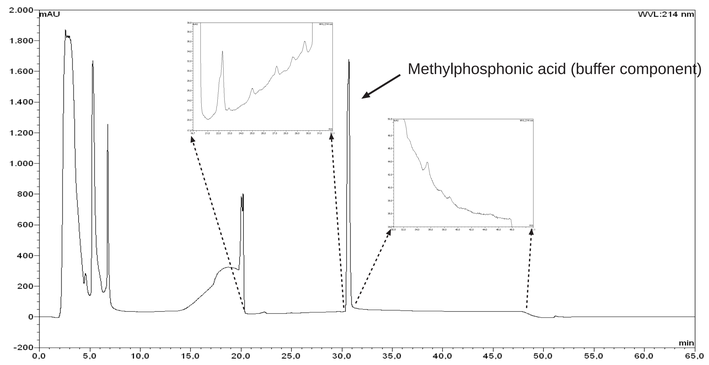


Fig. 1 Unphosphorylated peptides are the major part in early fractions (1-6) during the isocratic part of the separation and are efficiently separated from phosphopeptides, which elutes in the, later, gradient part.
The inserts show the blown-up part of the chromatogram where phosphopeptides represent the majority of detected peptides. Large peak @ 31 minutes is an artifact originating from methylphosphonic acid.

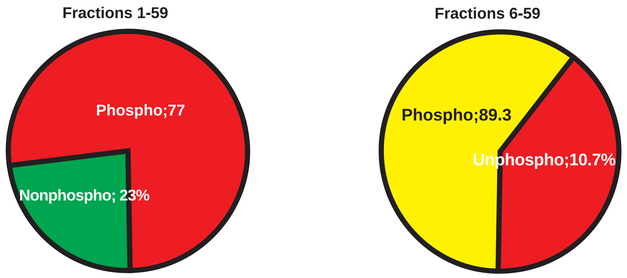


Fig. 2 A total of 7130 peptides was identified in 3.6 mg of HeLa proteins. A significant amount of identified peptides were phosphorylated, 5496 or 77%. Unphosphorylated peptides contribted with 23 %[A].The ratio of phosphopeptides/unphosphorylated peptides detected in laterERLIC fractions [B] is very high and underscores the ability of the ERLIC approach to efficiently separates the two classes of peptides.

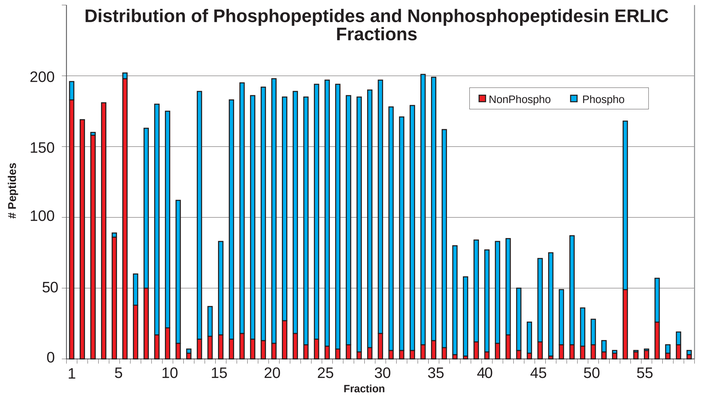


Fig. 3 When operated in HILIC mode, the anion exchange column is able to bind and separate phosphopeptides and nonphosphopeptides due to the considerable hydrophilicity of the phosphate group. As predicted, the nonphosphorylated peptides will mainly elute in early fractions while phosphorylated peptides are retained on the column and eluted with increasing AEX charcter of the mobile phase-increasing concnetration of TEAP (triethyl amino phosphate)

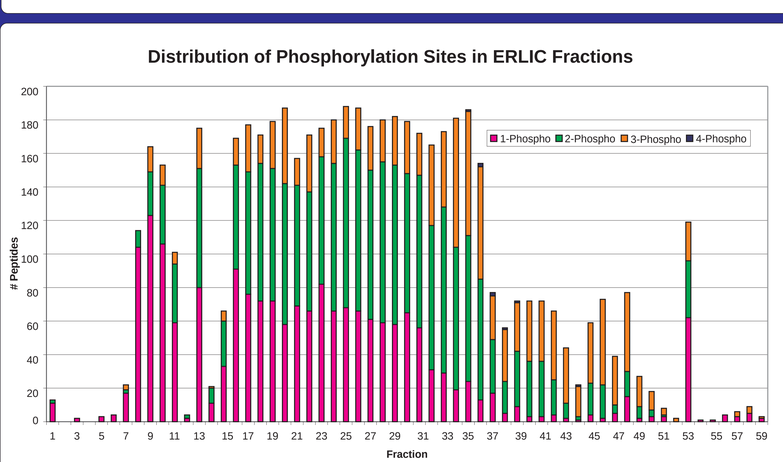


Fig. 4 In ERLIC, separation of phosphopeptides with different phosphorylation state is possible. The majority of singly phosphorylated peptides was eluted in early fractions (1-11). For the remaining fractions, the majority of identified peptides was multiple phosphorylated.

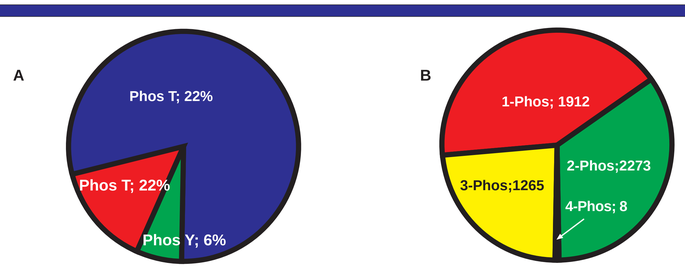
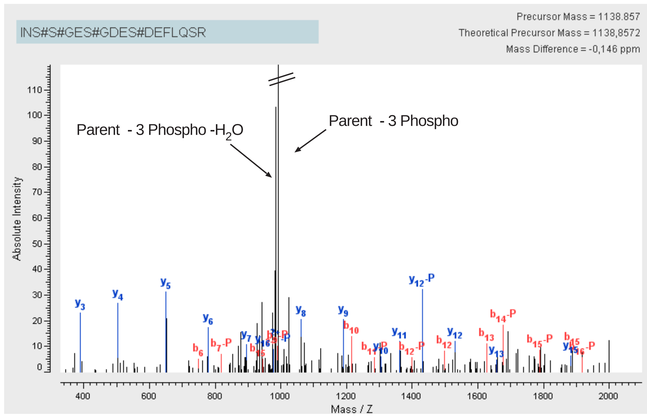


Fig. 5 An intersting observation was made concerning the ratio of phosphorylation sites on Ser, Thr, and Tyr. With 6% of phosphorylated Tyr, the ration issignificantly higher compaire with other studies where 2 - 2.5% phosphorylation on Tyr was reported [A].In ERLIC,phosphopeptides can also be resolved according to the number of phosphorylation sites and this lead to identification of significant number of multiple phosphorylated peptides. The problem with identifying phosphopeptides with >3 phosphorylation sites is rather related to the mass spectrometric detection and the number of quadruply phosphorylated peptides was low with only four identified peptides.



#	b	AA	y
1	114,091340	I	-
2	228,134267	N	2163,622992
3	395,132626	(pS)	2049,580065
4	562,130985	(pS)	1882,581706
5	619,152449	G	1715,583347
6	748,195042	E	1658,561883
7	915,193401	(pS)	1529,519290
8	972,214865	G	1362,520931
9	1087,241808	D	1305,499467
10	1216,284401	E	1190,472524
11	1383,282760	(pS)	1061,429931
12	1499,309703	D	894,431572
13	1627,352296	E	779,404629
14	1774,420710	F	650,362036
15	1887,504774	L	503,293622
16	2015,563352	Q	390,209558
17	2102,595380	S	262,150980
18	-	R	175,118952

Fig. 6 The quadruply phosphorylated peptide INS#S#GES#GDES#DEFLQSR was detected in fraction 37 with Mascot ionscore of 32. Although the ionization and fragmentation of multiple phopshorylated peptides is difficult it was possible to identify and assign all phosphorylation sites as well as the neutral loss signals. A fairly good number of y- and b- ions is assigned to the signals in MS/MS spectrum. The peptide sequence is reach with Glu and Asp residues thus confirming the theory that peptides rich with these amino acids will generally elute later in ERLIC chromatography.

DISCUSSION

Using ERLIC, separation of phosphopeptides from nonphosphopeptides is easy. In addition, ERLIC can also separate phosphopeptides from each other with high resolution, an important consideration in analysis of complex mixtures via mass spectrometry. The number of phosphopeptides detected and identified from the HeLa digest in this experiment compares favorably with the best results reported using titania or IMAC, with appreciably less effort expended. Perhaps the chief advantage of ERLIC over IMAC or metal oxide materials is, paradoxically, the weakness of its selective affinity for phosphopeptides. A single phosphate group typically suffices to increase retention merely by several column volumes; it does not completely dominate the chromatography. Consequently, retention is significantly influenced by the other residues, and the phosphopeptides can be separated from each other with high resolution. As predicted by theory, phosphopeptides with single phosphorylation eluted in early fractions and their amount declined in later fractions. Amount of multiply phosphorylated peptides, however, was rising in later fractions. The chromatographic separation of phosphopeptides will improve the detection by mass spectrometry and enable identification of more phosphopeptides compared to previous methods that did discriminate only the phosphopeptides and unphosphorylated peptides.

Observations of note:

1) ERLIC can handle peptides with > 2 phosphate groups with no difficulty. The fractions eluted with TEAP are likely to contain more peptides with > 3 phosphates than the eight that were identified. The limiting factor in their identification at present is not the chromatography but the capabilities of mass spectrometry. However, the use of Multi Stage Activation technique for peptide fragmentation in MS can enhance both the general number of detected phosphopetides and the number of detected multiple phosphorylated peptides.

2) The ratio of phosphorylated Y residues (6%) in the HeLa lysate fractions is higher than has commonly been reported in the literature (2-2.5%). That may reflect one of the following two explanations:
   a) ERLIC handles multiphosphorylated peptides better than other enrichment methods.
   b) Peptides with pY residues tend to be of low abundance, and the ERLIC method identified many such peptides.

After database search and data analysis of the MS/MS spectra obtained it was found that 1.89% of the protein hits were false positive results. These hits have been removed from the list. The final result of 5496 detected and confirmed phosphopeptides is encouraging for using the ELRIC separation in further analysis.
Establishing the use of voaltile buffer for ERLIC separationcan add to the selectivity of the method and enable easier handling of the samples for the reversed phase MS/MS analysis.

References

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