Simultaneous Identification of Unmodified Tryptic Peptides, Phosphopeptides, Glycopeptides, and Deamidated Peptides by Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) -MS/MS

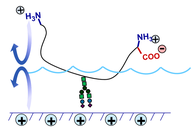
Piliang Hao[1]; Jingru Qian[1]; Wei Meng[1]; Yan Ren[1]; Andrew J. Alpert[2]; Siu Kwan Sze[1]

[1]Nanyang Technological University, Singapore, SINGAPORE; [2]PolyLC Inc, Columbia , MD, USA

ASMS 2011, poster 470

INTRODUCTION

Protein functions are modulated by a wide range of post-translational modifications (PTMs). Characterizing them is time-consuming if possible at all because most methods can only characterize one particular PTM per experimental run. The ERLIC mode of chromatography has recently been introduced, a combination of hydrophilic interaction and electrostatic repulsion. This mixed-mode method is especially powerful for fractionation of complex peptide digests because multiple chromatographic properties can be exploited to enhance selectivity. We present here the fractionation of unmodified peptides and the selective isolation and identification of glycosylated, phosphorylated, and deamidated peptides.



At pH 2.0, most tryptic peptides have a net charge of (+2). When forced by hydrophilic interaction to associate with an anion-exchange column, they migrate in a highly oriented fashion as shown. This enhances the percentage of the retention due to modifications such as glycan side chains. Functional groups that retain negative charge at pH 2.0 are attracted to the anion-exchange material, resulting in selective retention of the peptide. These include phosphate groups (pKa ~ 2.1), sialic acid residues (pKa ~ 2.6), and isoaspartic and isoglutamic acid residues (pKa ~ 3.1). The attraction does not dominate the chromatography. Consequently, the peptides with PTMs can be separated from each other in ERLIC with high resolution, unlike affinity methods involving IMAC or titania.

At 70% ACN, the hydrophilic interaction and electrostatic repulsion are in balance and unmodified peptides elute in or near the void volume while modified peptides are retained. At 90% ACN, the hydrophilic interaction is strong enough for all tryptic peptides to be retained.

MATERIALS AND METHODS

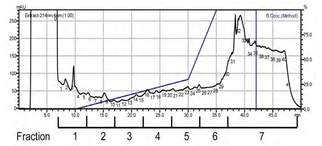
Columns: PolyWAX LP® (for ERLIC) and PolySULFOETHYL A™ (for SCX); both 200x4.6-mm; 5-µm, 300-Å (PolyLC Inc., Columbia, MD, USA)

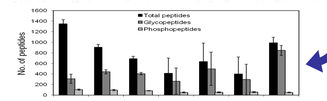
LC-MS/MS: Peptides from ERLIC fractions were separated on a 100x0.2-mm capillary of C18 AQ (5-µm, 100-Å; Michrom BioResources, Auburn, CA, USA). An LTQ-FT was used with an ADVANCE CaptiveSpray Source (Michrom BioResources). Positive ion mode was used with a *m/z* range of 350-1600 in the full MS scan.

Data Analysis: Raw data was converted to dta format and then into MASCOT generic file format. The IPI rat protein database and its reverse complement were combined and used for the searches. Two missed trypsin cleavage sites were allowed. Oxidation (M), phosphorylation (S, T and Y) and deamidation (N and Q) were set as variable modifications.

Theoretical p*I* and GRAVY values of peptides were calculated with an in-house program based on the algorithms from ENBOSS and SWISS-PROT's ProtParam respectively.

B) Isolation of Phosphopeptides and Glycopeptides

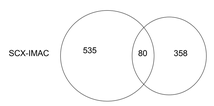




70% ACN:

Hydrophilic interaction is so weak that the electrostatic repulsion causes unmodified peptides to elute in the void volume.

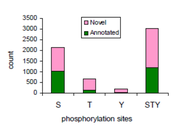
The retained peptides are largely phosphopeptides and glycopeptides [NOTE: This selectivity requires a pH low enough to uncharge –COOH's]



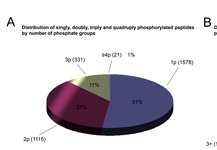
Gastric Cancer Cell Lysate Digest:

SCX-IMAC identifies somewhat more phosphopeptides than does ERLIC, but:
1) It's a lot more work;
2) The sets overlap by only ~ 8-20%

∴ You need *both* methods in parallel to cover the phosphoproteome.

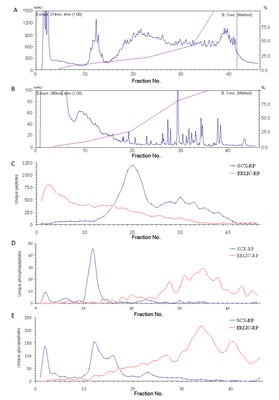


SCX-IMAC tends to isolate the basic tryptic phosphopeptides; ERLIC isolates the acidic ones. Those include most peptides with phosphotyrosine; **ERLIC id's ~ 3x more pY peptides than do other methods**. Here, most of the pT's and nearly all the pY's are novel.



ERLIC is also effective at isolating multiphosphorylated peptides that tend to get lost with other protocols.

80% ACN: A compromise affording *both* fractionation of unmodified peptides and isolation of modified peptides



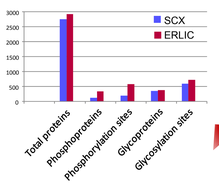
SCX of rat kidney protein digest

ERLIC of same digest. Conditions:
MP A: 80% ACN with 0.1% formic acid
MP B: 10% ACN with 2% formic acid
- unlike with 70% ACN, unmodified peptides are retained well enough to be distributed into a number of fractions while still eluting earlier than most modified peptides

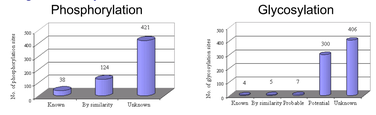
Distribution of total peptides
- still more uniform with ERLIC than with SCX

Distribution of phosphopeptides
- better separated both from unmodified peptides and from each other with ERLIC than with SCX

Distribution of glycopeptides
- same advantages with ERLIC as for phosphopeptides



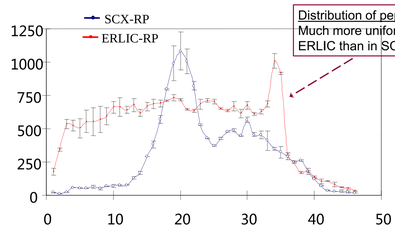
Annotation with SWISS-PROT of sites identified via ERLIC
- again, mostly novel



∴ ERLIC id's more total proteins and more modified proteins and peptides than does SCX alone, with a lot less effort; the solvents are volatile and modified and unmodified peptides are identified at the same time. More phosphopeptides are identified with SCX-IMAC, but again this is more work and the phosphopeptide sets show little overlap.

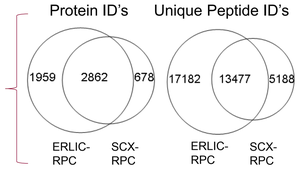
A) Fractionation of Unmodified Peptides with Volatile Solvents (90% ACN)

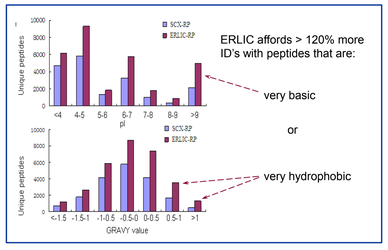
Sample: Rat kidney lysate digested with trypsin, desalted (SPE-C18) and dried; 2 mg. run
Flow Rate: 0.5 ml/min
Mobile Phases: A) 90% ACN, 0.1% Acetic Acid; B) 30%ACN, 0.1%Formic Acid
Gradient: 10', 0% B; 20', 0-8% B; 30', 8-27% B; 10', 27-45% B; 20', 45-81% B; 20', 81-100% B; 30', 100% B
46 fractions were collected, dried and redissolved in 0.1% formic acid for LC-MS/MS.



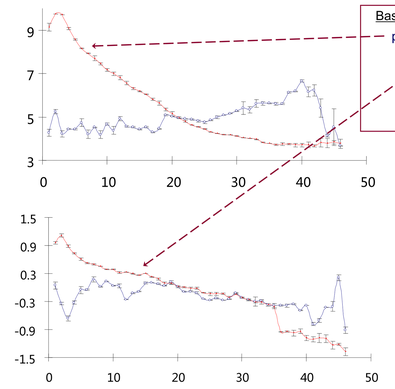
Distribution of peptides:
Much more uniform in ERLIC than in SCX

	Unique Protein ID's	Unique Peptide ID's
SCX:	3540	18,665
ERLIC:	4821 (32% more)	30,659 (64% more)





ERLIC affords > 120% more ID's with peptides that are:
very basic
or
very hydrophobic



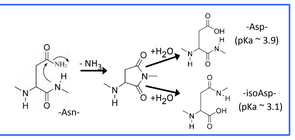
Basis of separation in ERLIC:
pI of peptide (high to low) and polarity (low to high)
- both variables are complementary to selectivity in RPC -

Advantages of ERLIC for tryptic digests:

1) Salt-free!
2) Fast and simple
3) Uniform distribution of peptides in the collected fractions
4) More ID's and more rugged ID's than with SCX
5) Separation of peptides by isoelectric points - *without ampholines*!

C) Glycopeptide or artifact? Isolation of deamidated peptides

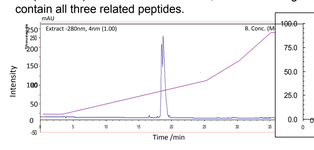
Deamidation of Asn produces Asp and isoAsp residues, with isoAsp predominating. isoAsp is a stronger acid than Asp. Asn residues susceptible to this reaction frequently have an unhindered residue such as Gly on the C-terminal side. This is also a common motif in N-linked glycopeptides. The reaction is catalyzed by high pH.



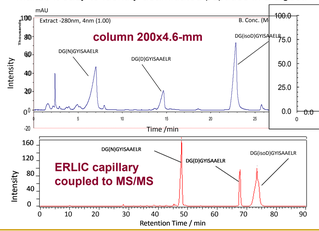
N-linked glycopeptides are frequently identified by treating digests with PNGase F and then identifying peptides with Asp- where Asn- should be. However, the high pH of both tryptic and PNGase F digestions may cause artifactual deamidation of susceptible Asn- residues. How prevalent are such artifacts?

A. Locate deamidation: RPC-ERLIC

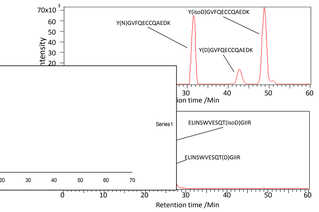
1) In most cases the triad of N/D/isoD peptides is poorly resolved by reversed-phase chromatography (RPC), per this example with DG(N/D/isoD)GYISAAELR. Therefore, RPC fractions generally contain all three related peptides.



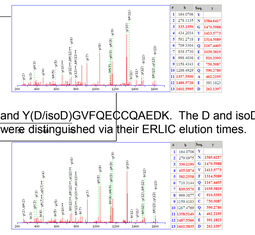
2) ERLIC of the RPC fractions: The three peptides of each triad are readily separated. The more acidic isoD form elutes last. This pattern of three peaks in *nonadjacent fractions* makes it easy to identify deamidated peptides in a digest.



3) Extracted ion chromatograms (XIC) of more deamidation triads.



4) MS/MS identification of Y(N)GVFQECCQAEDK



and Y(D/isoD)GVFQECCQAEDK. The D and isoD peptides were distinguished via their ERLIC elution times.

B. How much N-deamidation is artifactual or *in vivo*? Here, 14%

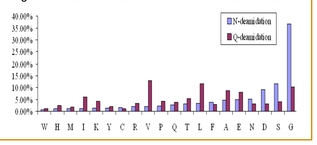
Sample: Rat liver homogenate tryptic digest, analyzed via RPC-ERLIC (80-10% ACN gradient)

Digestion pH: 8.0 (both trypsin and PNGase F; 25 mM NH_4HCO_3)

Both digests were treated identically except that the enzyme was left out in the (-PNGase F) sample.

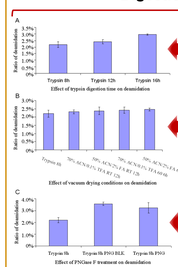
	ERLIC-RPC (-PNGase F)	ERLIC-RPC (+ PNGase F)
Unique peptides	10396	11939
Unique glycopeptides	107	781
Unique N-deamidated peptides	821	1020
Ratio of N-deamidation	7.90 %	8.54 %
Unique Q-deamidated peptides	307	418
Ratio of Q-deamidation	2.95 %	3.50 %

In addition to identifying Asn deamidation sites, ERLIC also identified so many Gln deamidation sites that it was possible to compile motifs for this. The C-terminal side neighbors differ from those for Asn: V > L > G > A > E > I.



C. Minimizing artifactual N-deamidation

Digestion at pH 8



Deamidation observed after 8 hrs. at pH 8 was present *in vivo* or involves particularly susceptible residues. It then continues at a slower rate.

When digests are dried at low pH, deamidation is minimal even at 60°C.

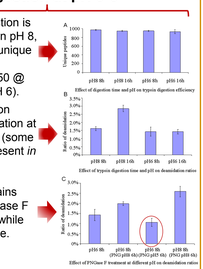
The high pH of PNGase F digestion leads to appreciable deamidation whether or not the enzyme is present.

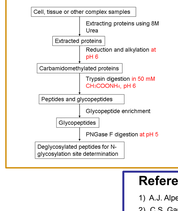
Digestion at pH 5-6

While tryptic digestion is slower at pH 6 than pH 8, it's fast enough; #unique peptide id's were comparable (10,350 @ pH 8, 10135 @ pH 6).

Prolonged digestion increases deamidation at pH 8 but not pH 6 (some deamidation is present *in vivo*).

Deamidation remains minimal with PNGase F digestion at pH 5 while #id's is comparable.





∴ In N-linked glycopeptides analysis, artifactual deamidation can be minimized by digestion with trypsin at pH 6 and with PNGase F at pH 5.

References about ERLIC:

1) A.J. Alpert, *Anal. Chem. 80* (2008) 62 [introduces ERLIC, especially for phosphopeptide isolation]
2) C.S. Gan *et al., J. Proteome Res. 7* (2008) 4869 [compares ERLIC with SCX-IMAC for isolation of phosphopeptides. SCX-IMAC is moderately more effective, at the cost of a lot more work. However, the sets of phosphopeptides identified by the two methods only overlap by about 20%. SCX-IMAC tends to identify basic phosphopeptides while ERLIC tends to identify acidic ones. This includes peptides with pTyr residues, 75% of which are on tryptic fragments with more than one phosphate group. Consequently, one identifies 3x more pTyr-containing peptides with ERLIC than with other methods]
3) U. Lewandrowski *et al., Clin. Proteom. 4* (2008) 25 [isolation of glycopeptides using ERLIC]
4) H. Zhang *et al., Mol. Cell. Prot. 9* (2010) 635 [glycopeptides and phosphopeptides elute in the same fractions in ERLIC; it can be used to enrich for them both]
5) M.V. Bennetzen *et al., Mol. Cell. Prot. 9* (2010) 1314 [isolation of phosphopeptides using volatile solvents in ERLIC]
6) B. Razani *et al., Sci. Signaling 3* (123), ra41 (2010) [more isolation of phosphopeptides using volatile solvents in ERLIC]
7) P. Hao *et al., J. Proteome Res. 9* (2010) 3520 [use of ERLIC to fractionate all peptides in a tryptic digest. Results are superior to those from SCX and it's much less work. Also, peptides elute in order of decreasing pI without ampholines, in a totally volatile solvent]
8) A.J. Alpert *et al., Anal. Chem. 82* (2010) 5253 [explains positional selectivity in ERLIC and ion-exchange chromatography in terms of well-defined peptide orientation on the stationary phase surface]
9) X. Chen *et al., J. Chromatogr. B, 879* (2011) 25 [compares SCX, HILIC, and two versions of ERLIC for isolation of phosphopeptides]
10) P. Hao *et al., PLoS One 6* (2011) e16884 [modifies the method of ref. 7 [above] to get simultaneous isolation of phosphopeptides and glycopeptides along with some fractionation of the unmodified peptides]
11) T. Guo *et al., Cell. Mol. Life Sci. 68* (2011) 1983 [uses SCX and ERLIC in parallel to isolation phosphopeptides from five gastric cancer cell lines. The overlap between the two sets was only about 8%; you need both of them in order to identify as many phosphopeptides as possible]

Acknowledgement: This work is supported by grants from the Nanyang Technological University (Tier 1: RG51/10) and the Agency for Science, Technology and Research of Singapore (BMRC: 08/1/22/19/575).