

ASMS 2007, Poster# 438: Desalting Phosphopeptides by Solid-Phase Extraction

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ABSTRACT

Some procedures for isolation of phosphopeptides produce samples containing nonvolatile salt that must be removed prior to further analysis. Solid-phase extraction (SPE) is a straightforward method for doing this. Few studies in the literature compare alternative materials for the purpose or assess the effects of salt levels on the effectiveness of the materials involved. This study does so for titania, HyperCarb® (graphitic carbon) and C-18 silica. Samples included a synthetic peptide with 1-4 phosphates, the tryptic digest of beta-casein, and two fractions from a tryptic digest of HeLa cell lysate: a low-salt fraction rich in singly phosphorylated peptides and a high-salt fraction enriched in peptides with more than one phosphate. All SPE materials were packed into TopTips™ or Sep-Pak® cartridges. Filtrates, washes and eluates were analyzed via ERLIC (Electrostatic Repulsion-Hydrophilic Interaction Chromatography), a new mode with high selectivity and resolution for tryptic phosphopeptides. Detection was via absorbance or RPC-MS.

Titania performed reasonably well with all phosphopeptides but retention was less than absolute under high-salt conditions. C-18 silica retained most peptides but failed to retain some phosphopeptides. High levels of salt promoted retention of phosphopeptides by C-18 silica, although some tended to leach out during intermediate water washes prior to elution. HyperCarb was effective at retention of all phosphopeptides from low-salt solvent but retention of phosphopeptides was antagonized by high levels of salt. Accordingly, with the low-salt HeLa cell fraction, HyperCarb retained and released ~ 50% more phosphopeptides than did C-18 silica. With the high-salt HeLa fraction the C-18 material retained and released more phosphopeptides than did HyperCarb. While the objective was to desalt phosphopeptides and not to enrich them, HyperCarb proved to be more selective for phosphopeptides than was C-18 silica under all conditions.

INTRODUCTION

Phosphorylation of proteins is an important cellular regulatory process. There is considerable interest in determining the location and stoichiometry of phosphorylation of proteins as a means to assess its effects. Presently this is approached chiefly by digestion of the protein with trypsin followed by efforts to identify the phosphopeptides. This is frequently done by selective isolation of phosphopeptides via the following methods:

- 1) Immobilized Metal Affinity Chromatography (IMAC).
- 2) Use of a Lewis acid such as titania (phosphate being a strong Lewis base).
- 3) Strong cation-exchange (SCX). At pH 2.7, most tryptic peptides have a net charge of +2. Attachment of a phosphate group lowers the net charge to +1. Thus, the earliest-eluting fractions are enriched in phosphopeptides [1,2].

A fourth method is via a new mode of chromatography: Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) [3]. This is Hydrophilic Interaction Chromatography (HILIC) performed on a column with the same charge as the solutes in the sample. ERLIC of peptides is done with an anion-exchange column at pH ~ 2.0. Most tryptic peptides have a net positive charge at low pH and are electrostatically repelled by anion-exchange materials, eluting prior to the void volume [4]. In ERLIC the mobile phase contains enough organic solvent to confer hydrophilic interaction sufficient for peptides to be retained despite the repulsion. While nonphosphopeptides can be well-retained this way, this study uses conditions where retention of nonphosphopeptides is marginal. Attachment of a phosphate group promotes retention of a peptide in ERLIC through both electrostatic attraction and hydrophilic interaction. This combination suffices to pull most phosphopeptides away from the nonphosphopeptides, permitting their isolation and separation [5]. While isocratic conditions suffice for singly phosphorylated peptides, a salt gradient is required to insure elution of peptides with two or more phosphate groups.

The phosphopeptides obtained through the above methods are frequently in a medium containing nonvolatile salt, sometimes in high concentration. This must be removed prior to mass spectroscopy or some other methods of peptide analysis. Peptides are frequently desalted using solid-phase extraction (SPE) cartridges of a C-18 silica or other reversed-phase chromatography (RPC) material. Such materials are effective at retaining most tryptic peptides but are reported to fail at retention of many phosphopeptides, which tend to be more hydrophilic than other peptides. This caused some concern about the loss of the phosphopeptides in the fractions collected from our ERLIC runs. Accordingly, this study compares the ability of C-18 silica, graphitic carbon media (e.g., HyperCarb®) and titania to retain tryptic phosphopeptides from salty solutions and then release them again following washes with water. HyperCarb [6-8] and titania and other ceramics [9-11] have been reported to retain phosphopeptides that were not retained by C-18 media. The objective did not include assessment of any of these as an affinity material, since the selectivity for phosphopeptides was conferred by the preceding ERLIC step. Some observations were made nevertheless regarding selectivity of binding.

Many if not most recent studies of SPE of phosphopeptides have used mass spectrometry to identify the peptides obtained. Mass spectrometry detection is not entirely quantitative for singly phosphorylated peptides and is appreciably less so for more heavily phosphorylated peptides. Accordingly, recovery from the SPE materials was assessed chiefly through absorbance detection.

MATERIALS AND METHODS

Samples and reagents

SPE was performed with TopTip™ cartridges (PolyLC Inc., Columbia, MD) in the size for samples 10-200 µl. The part numbers for C-18 silica, HyperCarb, and tania TopTips were TT200C18, TT200CAR, and TT200TIO, respectively. C-18 material was also used in the form of Sep-Pak® SPE cartridges from Waters Corp. (Milford, MA). Reagents were HPLC grade if available and the purest grade obtainable if not.

A set of synthetic phosphopeptides with a tryptic sequence was a gift of Karl Mechtler and colleagues (IMP/ Vienna, Austria). The sequence was WWGSGPSGSGGGGK, with 0-4 phosphate groups on the serine residues. The tryptic digest of β-casein was prepared as described [5]. The tryptic digest of HeLa cell lysate was prepared as described for mouse liver protein [11] except that 10 mg. of total protein from HeLa cells was used.

The β-casein test sample was prepared as follows: 1 mg. of lyophilized digest was dissolved in 100 µl of hexafluoro-2-propanol (HFIP). Water was added to a total volume of 800 µl. This was divided into 80-µl aliquots (0.1 mg. peptide each). Each aliquot was brought to 100 µl with additional reagents, resulting in the compositions listed in the figure legends. A variety of agents has been used to release adsorbed phosphopeptides in SPE. One reference used piperidine [12], which has a boiling point of 106 °C and a pKa of 11.1. Pyrrolidine is both more volatile (b.p. 88°C) and more basic (pKa = 11.3) and so was used here instead.

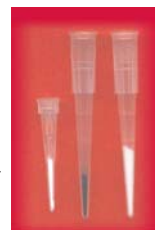
Analysis of Samples via ERLIC

HPLC analysis of SPE filtrates, washes, and eluates was performed with a PolyWAX LP™ column (PolyLC item# 104WX0503; 100x4.6-mm, 5-µm, 300-Å) used in the ERLIC mode.
Running conditions:

- 1) Mobile Phase A: 20 mM sodium methylphosphonate, pH 2.0, with 70% acetonitrile [ACN].
- 2) Mobile Phase B: 200 mM triethylamine phosphate, pH 2.0, with 60% ACN.

Flow rate: 1.0 ml/min. Absorbance detection: A₂₂₀.

Gradient: 0-5': 0% B; 5-25': 0-100% B; 25-35': 100% B. Between runs the column was reequilibrated with 0% B for 35' at 2 ml/min.



TopTips →

SPE with TopTips

Before use, microcentrifuge tubes were soaked overnight in 10 mM formic acid, rinsed with water and 2-propanol and allowed to dry. This procedure was developed to minimize the contamination of samples by components leached from the plastic.

Each TopTip was conditioned with a full gradient cycle, as follows: 3 washes with 50 µl (3 x 50 µl) of binding solvent, then 3 x 100 µl of eluting solvent, then 3 x 50 µl of water. They were then equilibrated for use with 6 x 50 µl of binding solvent. Samples were added in two 50-µl portions. The TopTip was attached to a pipettor and air displacement was used to force out the filtrate. This was collected in a microcentrifuge tube for analysis. The TopTips were then washed with 2 x 50 µl of the appropriate washing solvent. In the case of binding from low-salt media, this was the binding solvent. In the case of binding from high-salt media, this was water. The washings were combined for analysis. Retained peptides were released with 4 x 25 µl of eluting solvent, with the released portions being combined. All collected portions were dried in a SpeedVac® (Thermo Savant). To insure removal of volatile components, the residue was resolubilized with 30 µl of a 1:1 blend of water:methanol (MeOH) and dried a second time; this was repeated a third time. The residue was then dissolved for analysis by wetting the walls of the microcentrifuge tube with 4 µl water + 2 µl HFIP, then adding 19 µl of Mobile Phase A.

Fractionation of HeLa cell lysate digest by ERLIC and Analysis via Mass Spectrometry

1.5 mg. of the digest was dissolved in Mobile Phase A and run via ERLIC [above]. Fractions were collected between 6-7' (low-salt fraction in Mobile Phase A) and 24.5-27.5' (high-salt fraction in Mobile Phase B). The ACN was removed by drying the fractions for 1 hour in a SpeedVac. The remainder of each fraction was divided into two halves. One half was applied directly to a HyperCarb TopTip for desalting as above. The other was desalted with a C-18 Sep-Pak®, 1-cc size (Waters Corp. item# WAT036820). After lyophilization overnight, the residue was dissolved in 0.1% TFA and loaded onto the Sep-Pak. It was flushed with 0.1% TFA to eliminate salts. Peptides were then eluted with 0.1% formic acid in 40% ACN. The eluates were analyzed via nano-scale RP-LC-MS/MS by an FT10 method on an LTQ-FT™ (Thermo Fisher Scientific) as described [13]. MS/MS spectra were searched using the SEQUEST algorithm against a nonredundant human database (PI Human). A composite database was constructed consisting of sequences in the forward (target) orientation and then reversed (decoy). This allowed for an estimation of the false positive rate for the final phosphopeptide data set [14], which was 1%. Searches allowed for phosphorylation as a dynamic modification on serine, threonine, and tyrosine residues and filtering of data was performed using Xcorr, dCorr, and mass deviation [15].

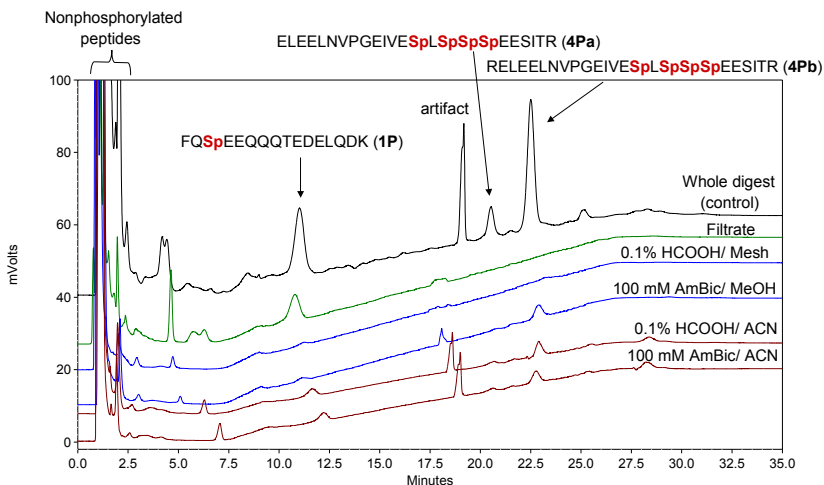


Fig. 1. β -Casein Tryptic Digest on C-18 (low salt) - ERLIC analysis.

100 μ g. of digest was loaded on the TopTip in 100 μ l of 20 mM ammonium formate, pH 3.0. Washings, elution and ERLIC analysis were performed as in Methods. Four samples were processed in parallel this way, each eluted with the solvent indicated (with 60% MeOH or ACN). The tetraphosphopeptide labelled 4Pa is also present in the commercially available standard (Sigma item# P9615-1SET), in the same ratio with peptide 4Pb as seen here [5].

Most of the singly phosphorylated peptide was not retained by C-18, while the tetraphosphopeptides were hard to elute. AmBic was a stronger eluting additive than formic acid, leading to elution of some of the tetraphosphopeptide with MeOH as the solvent. ACN was a stronger eluting solvent than MeOH, leading to elution of all these peptides even with formic acid as the additive.

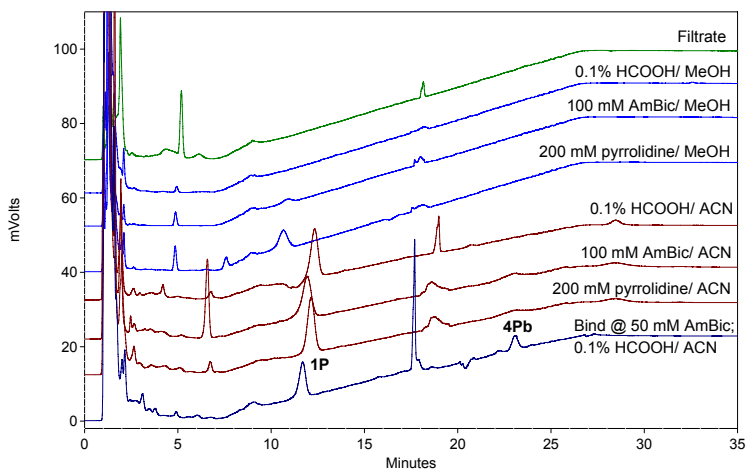


Fig. 2. β -Casein Tryptic Digest on HyperCarb (low salt).

Binding and procedures were as in Fig. 1 except for the bottom sample. HyperCarb bound all the phosphopeptides effectively. Retention was stronger than with C-18; AmBic + 60% MeOH released almost none. Pyrrolidine + MeOH only eluted some of the singly phosphorylated peptide. With ACN as the solvent, any additive eluted the singly phosphorylated peptide. No combination eluted any of the tetraphosphopeptide unless the sample was bound in a solvent containing a moderately high level of salt (50 mM AmBic).

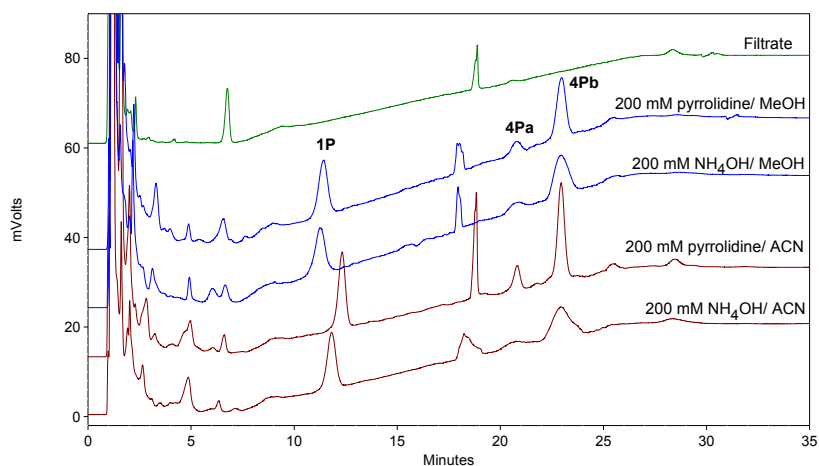


Fig. 3. β -Casein Tryptic Digest on Titania (low salt).

The binding solvent and procedures were as in Fig. 1 except that the binding solvent contained 10% ACN and the release solvents contained 10% MeOH or ACN. Titania bound all phosphopeptides effectively. Either NH_4OH or pyrrolidine were effective as releasing additives. Chromatography peaks for the phosphopeptides were consistently sharper when pyrrolidine was used for release. No difference was noted between MeOH and ACN as a releasing solvent component.

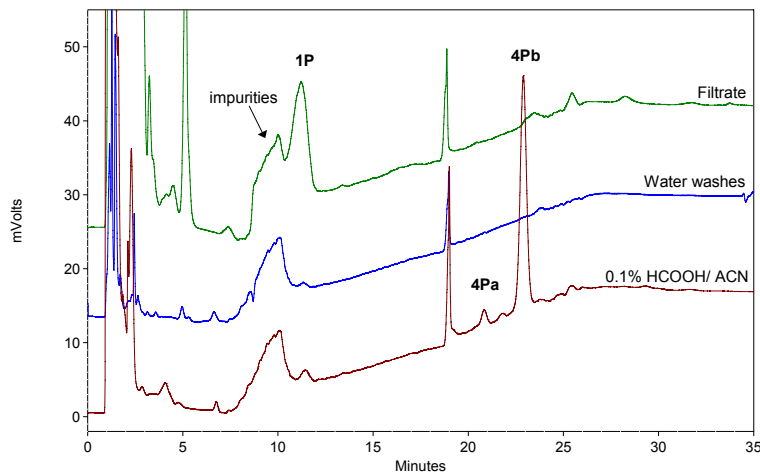


Fig. 4. β -Casein Tryptic Digest on C-18 (high salt).

The sample was dissolved in ERLIC Mobile Phases A + B in a 1:2 ratio without the ACN: 6.7 mM sodium methylphosphonate + 133 mM triethylamine phosphate, pH 2.0. This mimics a solution one might get from collection of an ERLIC fraction and evaporation of the ACN. Because of the importance of the water washes here for elimination of nonvolatile salt, they were pooled and analyzed.

The prominence of the impurities peak reflects a switch to methylphosphonic acid reagent from Sigma instead of Fluka

Again, most of the singly phosphorylated peptide eluted in the filtrate. A trace is evident in the washes. Elution releases the remaining singly phosphorylated peptide and, for the first time for C-18, apparently all of the tetraphosphopeptide. As with Fig. 2 (bottom trace), this suggests that binding from high salt facilitates subsequent elution of highly phosphorylated peptides.

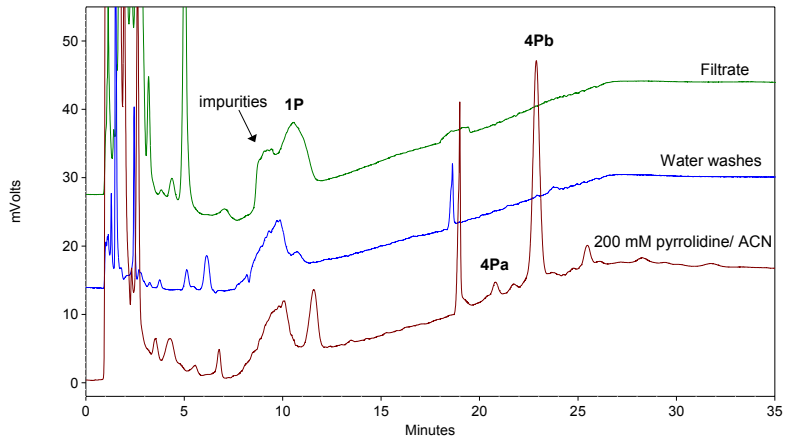


Fig. 5. β -Casein Tryptic Digest on HyperCarb (high salt).

As before, the high salt seems to interfere with binding of phosphopeptides; much of the monophosphopeptide eluted in the filtrate. A small amount eluted in the water washes as well. As with C-18, the releasing solution eluted the tetraphosphopeptides as well as the remaining singly phosphorylated peptide.

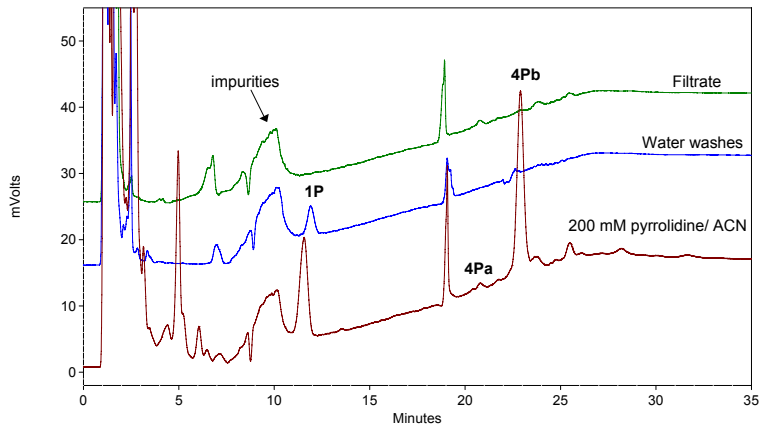


Fig. 6. β -Casein Tryptic Digest on Titania (high salt).

The binding solvent (per Fig. 4) is far from optimal for titania. One might expect the phosphate and methylphosphonate, being strong Lewis bases themselves, to compete with the phosphopeptides for binding. Evidently the TopTip had sufficient capacity for that not to happen. No phosphopeptides are apparent in the filtrate and only a modest amount (~20%?) of the monophosphopeptide leaks out in the water washes. Elution (with 10% ACN) releases both the tetraphosphopeptides and the remaining monophosphopeptide.

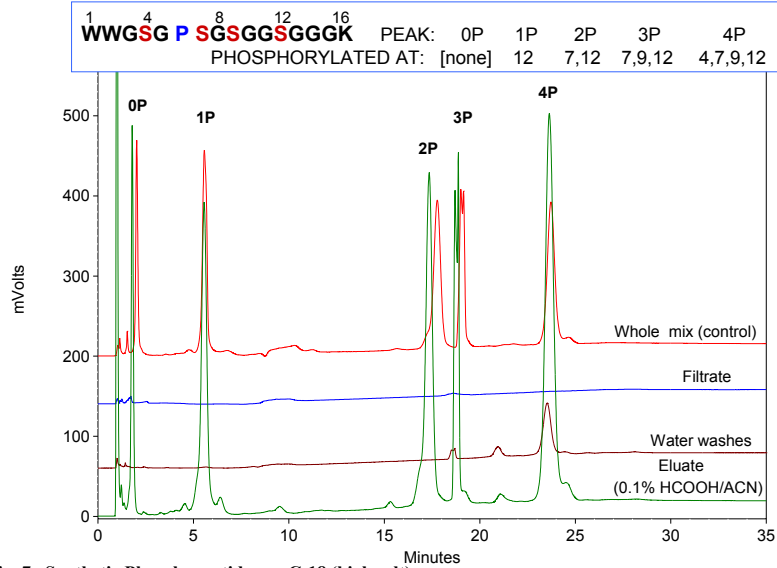


Fig. 7. Synthetic Phosphopeptides on C-18 (high salt).

The binding solution was as in Fig. 4. All peptides were retained by C-18, including the nonphosphorylated standard; none appeared in the filtrate. Retention of the more hydrophilic, highly phosphorylated peptides was shaky; a significant amount of the tetraphosphopeptide eluted in the water washes. Everything else eluted with formic acid + 60% ACN.

The control sample contained 63% ACN to match the mobile phase, essential in either ERLIC or HILIC. The doublet peaks seen with the later-eluting standards probably represent isomers with different conformation around the central proline residue.

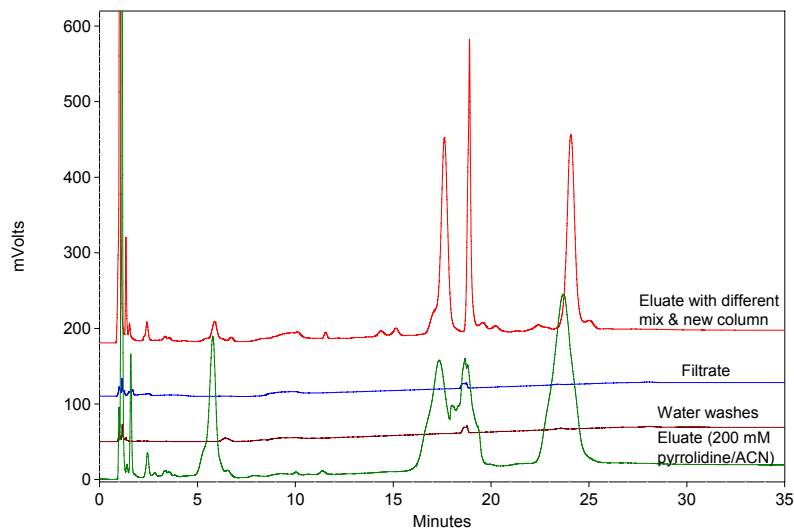


Fig. 8. Synthetic Phosphopeptides on HyperCarb (high salt).

All peptides were bound. None leaked out during the water washes, in contrast with C-18 silica (Fig. 7). All were eluted by pyrrolidine + 60% ACN. The poor shape of the resulting peaks reflected the imminent failure of the PolyWAX LP column after some months of use. A new column (demonstrated with a mixture of the standards in a different ratio) afforded much better shaped peaks for the HyperCarb eluate [top].

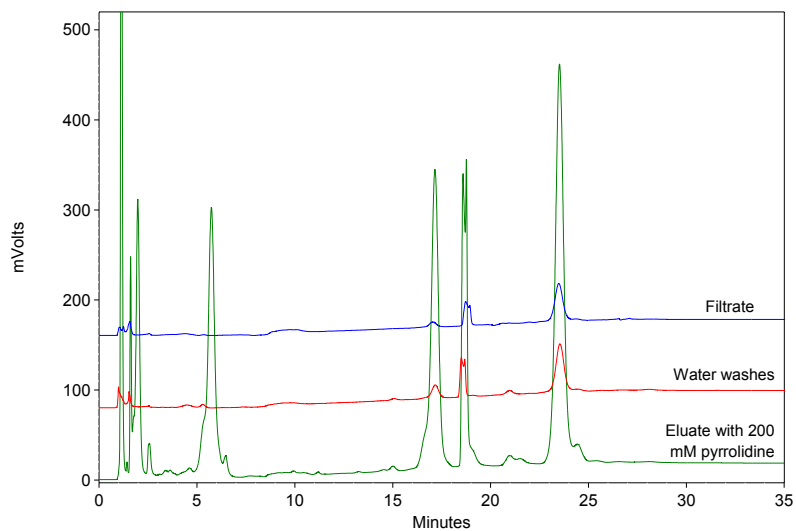


Fig. 9. Synthetic Phosphopeptides on Titania (high salt).

As with the beta-casein digest (Fig. 6), titania proved to be leaky under these conditions; it failed to bind a modest amount of the tetraphosphopeptide and lesser amounts of the tri- and diphosphopeptides. Leakage of the same standards was observed in the water washes as well, in the same ratio. All remaining bound peptide was released by pyrrolidine with 10% ACN.

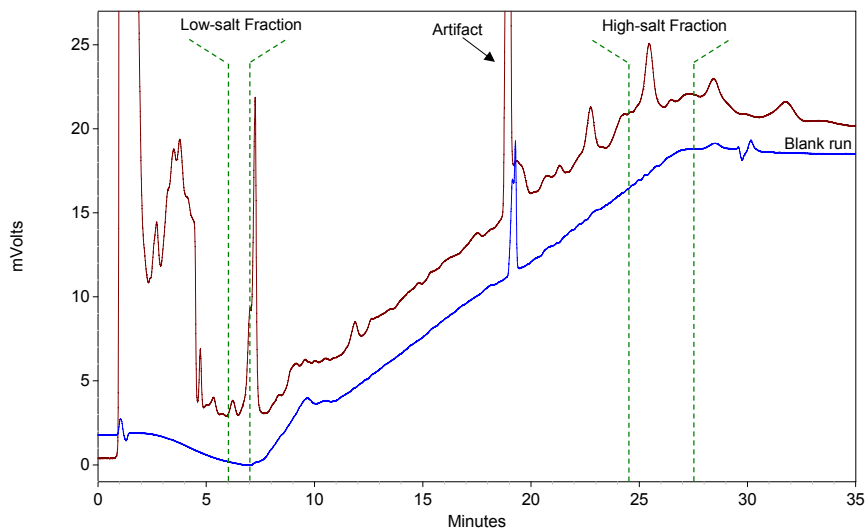


Fig. 10. HeLa Cell Lysate Tryptic Digest: Effect of Salt Level on Recovery of Phosphopeptides in SPE.

The digest was run via ERLIC. The indicated fractions were collected and divided. Half was desalted via C-18 and half via HyperCarb (see Methods). Results are in Table 1.

**Table 1. HeLa cell lysate digest:
C-18 vs. HyperCarb for recovery of peptides from low-salt and high-salt fractions.**

The numbers in the table are net figures after duplicate sequences were deleted from the lists.

FRACTION	# Phospho-peptides		# Nonphospho-peptides		% Phospho-peptides	Distribution of mono/di/tri phosphopeptides
Low salt – C18	102	54 in common	613	123 in common	14%	99/2/1 (97/2/1 %)
Low salt – HyperCarb	182		189		49%	172/10/0 (94/6/0 %)
High salt – C18	133	22 in common	439	18 in common	23%	24/79/30 (18/59/23 %)
High salt - HyperCarb	52		22		70%	4/27/21 (8/52/40 %)

Low-Salt: 78% more phosphopeptides were identified from the HyperCarb-desalted sample than from the C-18 Sep-Pak. At the same time, far fewer nonphosphopeptides were retained by HyperCarb than by C-18. Result: HyperCarb was 3.5x more selective for binding phosphopeptides than was the C-18 material, to the point that half the peptides identified from the HyperCarb eluate were phosphopeptides. This compares favorably with a regular affinity material such as titania or IMAC and reflects the selectivity of the ERLIC separation that preceded the desalting on HyperCarb.

High-Salt: This late-eluting fraction is enriched in peptides with more than one phosphate group. Again, HyperCarb was quite selective; 70% of the peptides identified from its eluate were phosphopeptides, vs. 23% with C-18. However, the C-18 Sep-Pak bound and released many more peptides of all kinds, resulting in 2.5x more total phosphopeptides being identified from the C-18 eluate. The high-salt conditions prevented efficient retention of monophosphopeptides in particular by HyperCarb, as per Fig. 5. HyperCarb fared better with the more highly phosphorylated peptides, nearly matching the Sep-Pak in the number of triphosphopeptides in its eluate.

DISCUSSION

The following tendencies are evident from this data:

- 1) HyperCarb retains phosphopeptides of all kinds from media low in salt, being considerably more effective than C-18 silica in this regard. It also binds fewer nonphosphopeptides than does C-18 silica.
- 2) Methanol is a weaker eluting solvent for phosphopeptides than is ACN. Thus, 0.1% formic acid elutes the β -casein tetraphosphopeptides from C-18 silica in 60% ACN but not in 60% MeOH (Fig. 1). In the case of HyperCarb, any additive with 60% ACN releases the singly phosphorylated peptide from β -casein, but only 200 mM pyrrolidine does so with 60% MeOH (Fig. 2).
- 3) A high salt level in the solvent antagonizes binding of phosphopeptides by HyperCarb, the effect resembling ion-exchange chromatography. Thus, singly phosphorylated peptides tend not to be retained (Figs. 5 and 10). The good retention of the singly phosphorylated peptide in Fig. 8 probably is due to the two tryptophan residues, which would promote binding by a mechanism independent of that involving phosphate groups. Peptides with more than one phosphate group can be retained under these conditions and are eluted by conditions that fail to elute them when the same peptides are bound from low salt media (Fig. 2 vs. Fig. 5). This suggests that multiphosphorylated peptides bind from high salt media in a more weakly held manner.
- 4) A high salt level seems to promote retention of phosphopeptides by C-18 silica. Thus, the synthetic tetraphosphopeptide was effectively captured from a high-salt solution. With a drop in the salt level during the subsequent water washes, some of it leached out (Fig. 7). This behavior is characteristic of phase partitioning, the salt increasing the polarity of the aqueous mobile phase and promoting partitioning into the hydrophobic stationary phase coating. It may also supply a reason why C-18 silica was more effective than HyperCarb in capturing a variety of HeLa cell lysate phosphopeptides from high-salt media (Table 1).
- 5) Titania captures phosphopeptides effectively from low-salt media (Fig. 3). Its use is more straightforward than with either C-18 silica (which tends not to capture some phosphopeptides) or HyperCarb (which tends not to release more highly phosphorylated peptides unless adsorbed under conditions that antagonize binding of monophosphopeptides). Results were the same with either 10% MeOH or 10% ACN in the releasing solvent. Binding from high salt is weaker, with some phosphopeptide eluting during water washes (Figs. 6 and 9) or not binding at all (Fig. 9). Presumably the phosphate and methylphosphonate in the medium competed with the phosphopeptides for binding.

This study is far from comprehensive. Only a limited selection of salts and concentrations were examined. That said, current literature on phosphopeptide analysis typically involves uncritical use of C-18 silica for desalting prior to MS analysis. The present data serve to alert the field that alternatives exist and should be considered for good reasons. For the time being, preliminary suggestions for desalting phosphopeptides are as follows:

Low-salt conditions: Either titania or HyperCarb is effective. It would be difficult to release some multiphosphorylated peptides from HyperCarb. In ERLIC, at least, such peptides are unlikely to be present in the low-salt fractions.

Intermediate-salt conditions (~ 60-150 mM): Titania is probably best, since retention on C-18 silica may be unreliable in this range.

High-salt conditions (> 150 mM): C-18 silica is best; the salt level promotes binding of peptides to it while antagonizing binding to HyperCarb and titania.

For offline desalting of phosphopeptides, there is no reason for confining desalting to a single material. Each of the materials in Table 1 bound and released a significant number of phosphopeptides that were not obtained with the other material. A more complete set of phosphopeptides would be identified by processing the sample with both materials in parallel and then combining the eluates.

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