Isolation and Identification of Phosphopeptides by Combining Micropurification, Strong Cationic Exchange and Hydrophilic Interaction Chromatography

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Introduction

Phosphorylation is one of the most common of the reversible posttranslational modifications, but also one of the most challenging to measure. During the last few years, phosphopeptide analysis has benefited from the rapid improvement in biological mass spectrometry, in addition to innovations such as immobilized metal affinity chromatography (IMAC), the development of antibodies specific for phosphorylated amino acids, and most recently, chromatography using TiO_2.

During our analysis of the saliva phosphoproteome, we were concerned that there may be a bias against monophosphorylated peptides when using TiO_2 columns. We therefore decided to explore alternatives that rely on fractionation rather than affinity purification. Using the approach of Roepstorff and colleagues of packing columns in pipette tips [1] to make disposable microcolumns, we compared fractionation using reverse phase (RP), strong cationic exchange (SCX) and hydrophilic interaction chromatography (HILIC) with affinity purification using titanium dioxide [2, personal communication with M. Larsen].

Parotid saliva and its phosphopeptides

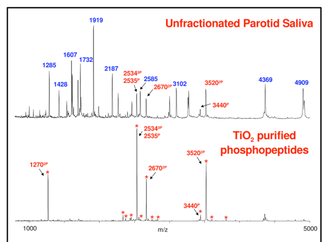


Figure 1: MALDI-TOF spectra (linear mode) of unfractionated peptides from parotid saliva (top), phosphopeptides isolated using TiO_2 (bottom). Phosphopeptides are marked with a red star (*), with some of the masses and phosphorylation states indicated. Non-phosphorylated peptide masses are marked in blue.

Methods

Saliva collected from human parotid glands was precipitated with acetone, washed with 80% acetone, 10% methanol and 0.2% acetic acid, followed by digestion with trypsin in 30% DMSO [3]. After drying down the peptides, about 4 µg were used as starting material for each of the fractionations.

Microcolumns were either packed in Eppendorff GELoader tips (for the isolation of peptides after dephosphorylation) [1] or in P10 tips using plugs of 3M Empore C8 extraction discs (3M Bioanalytical Technologies). The columns were packed to a length of ~4 mm. The stationary phases used were 4 µm Jupiter Proteo (C12) and 5 µm Luna SCX from Phenomenex, 12 µm PolyHYDROXYETHYL Aspartamide™ (PHEA; for HILIC) and 5 µm PolyWAXTM LP (PWAX; for HILIC) from the Nest Group, Inc. and 4 µm TiO2 from GL sciences Inc.

After loading the samples onto the columns (C12 in 5% formic acid; SCX in 20% acetonitrile, 1% formic acid, HILIC in either 85% acetonitrile, 1% formic acid, or 4mM ammonium formate, 20% acetonitrile), the peptides were eluted using step-gradients. C12 was eluted with 3x10µl acetonitrile in 0.1% formic acid and analyzed with MALDI-TOF MS directly. The SCX column was eluted with increasing amounts of ammonia formate (each step 50µl), while the PHEA HILIC column was eluted with decreasing amounts of acetonitrile and increasing amounts ammonium formate (first 10 steps 50µl, then 25ul). In contrast, the peptides were eluted from the PWAX HILIC column by changing the pH, acetonitrile, formic acid and ammonium formate concentrations. Each elution solvent was used for 2-3 fractions of each 50 µl. For the 2-dimensional fractionations, the flow-through from SCX was loaded onto a HILIC column, while the fractions after 10mM ammonia formate from the HILIC fractionation were pooled and loaded onto a C12 column.

For the purification using TiO2, the samples were diluted 5 times with 80% acetonitrile and 3% trifluoroacetic acid. The peptides were loaded onto the column, followed by a 30µl wash with the same solution. The peptides were eluted using 2µl ddH2O, 30µl 0.8% ammonia hydroxide, 2µl 40% acetonitrile and 0.1% formic acid. All samples (but those from C12) were dried in speed-vac and resuspended with 0.5µl formic acid and 10µl ddH2O.

1µl of the phosphopeptides from the TiO2 purification was dephosphorylated in a R2 microcolumn with 20µl 0.05U alkaline phosphatase in 50mM ammonium bicarbonate for 45 minutes at 37°C [4]. After acidification, the peptides were loaded onto the column, washed, and eluted directly onto the MALDI plate using the DHB matrix.

To evaluate the fractionation, 0.5 µl of each sample (~5% of total) were analyzed with MALDI-TOF MS (Voyager DE-STR, Applied Biosystems) using the dried droplet method (10mg DHB/ml in 25% acetonitrile, 15% isopropanol, 0.5% phosphoric acid on a hydrophobic surface of lanolin). Representative spectra were acquired with 300 shots and optimized for spectrum quality with varying laser intensity. PSD spectra with 200-500 shots were acquired for the region containing peaks resulting from neutral loss of the phosphate groups. The peptides from the HILIC fractionation were identified by MSMS using nanoLC Q-STAR (Applied Biosystems) and nanoLC LTQ-Orbitrap MS (Thermo Fischer Scientific).

Microcolums

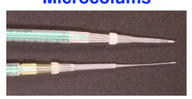


Figure 2: Microcolumn in GELoader tip (bottom) vs. P10 tip (top).

Results

The saliva phosphoproteome is dominated by a few related phosphopeptides

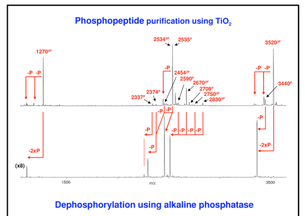


Figure 3: MALDI-TOF MS spectra (reflector mode) comparing phosphopetide isolated using TiO_2, before and after dephosphorylation using alkaline phosphatase. The losses of phosphate due to dephosphorylation and PSD are indicated by arrows. For the identity of the peptides, see table 1.

Overloading TiO_2 columns lead to loss of monophosphorylated peptides

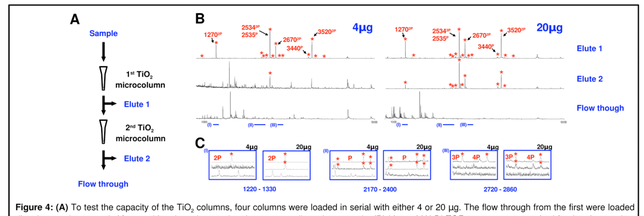
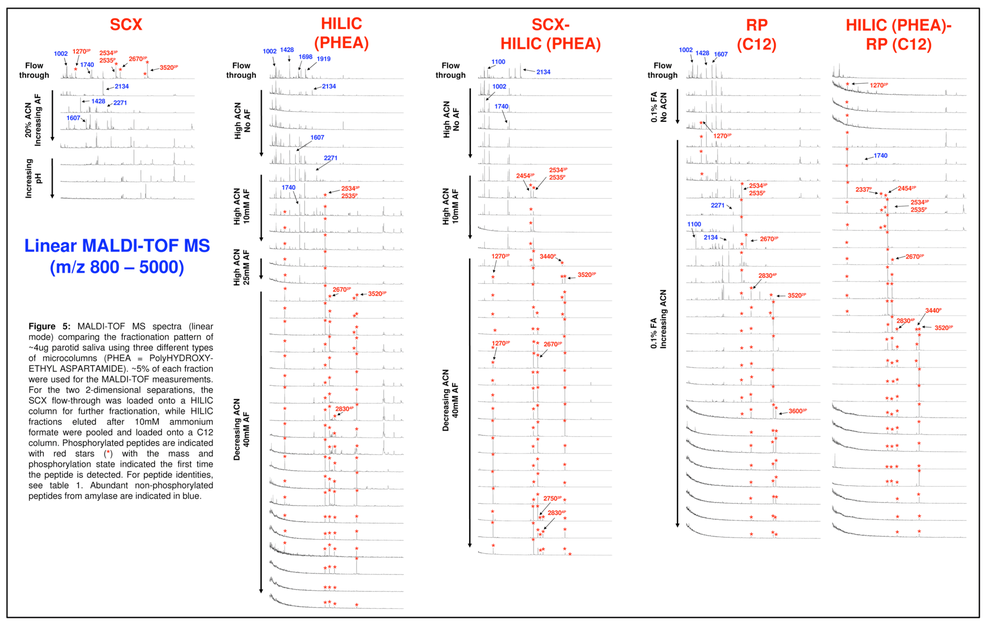


Figure 4: (A) To test the capacity of the TiO_2 columns, four columns were loaded in serial with either 4 or 20 µg. The flow through from the first were loaded directly onto the second. After washing the columns, the elutes were collected separately. **(B)** Linear MALDI-TOF spectra were acquired for the six samples. Phosphopeptides are indicated with red stars (*) with the major peaks labeled with masses and number of phosphorylated amino acids. When comparing the spectra, there are a few obvious features: the first elutes give very similar spectra with slight differences ratios between mono- and diphosphorylated peptides (e.g. 2535/2670 and 3440/3520). The second elutes are dramatically different with a significant amount of phosphopeptides in the elute from 20 µg. **(C)** Three regions of the spectra are highlighted (indicated with blue bars). The six panels show that multiply phosphorylated peptides are retained efficiently even though high amounts are present, but monophosphorylated peptides appear in the second elute already when loading 4 µg.. Finally, low abundant multiply phosphorylated peptides are competed off the columns and appear in the second elute at higher loading.

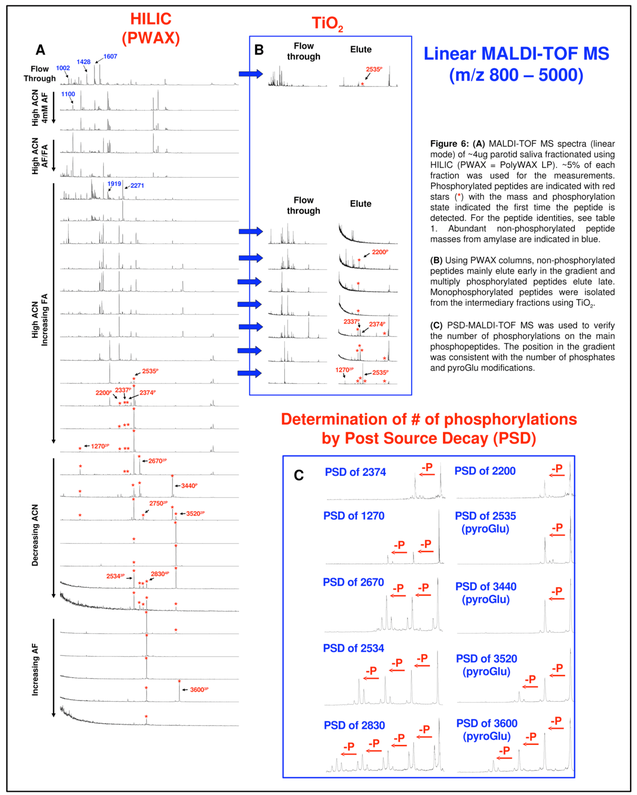
Fractionation of saliva using microcolumns and step gradients



Linear MALDI-TOF MS (m/z 800 – 5000)

Figure 5: MALDI-TOF MS spectra (linear mode) comparing the fractionation pattern of ~4ug parotid saliva using three different types of microcolumns (PHEA = PolyHYDROXY-ETHYL ASPARTAMIDE). ~5% of each fraction were used for the MALDI-TOF measurements. For the two 2-dimensional separations, the SCX flow-through was loaded onto a HILIC column for further fractionation, while HILIC fractions eluted after 10mM ammonium formate were pooled and loaded onto a C12 column. Phosphorylated peptides are indicated with red stars (*) with the mass and phosphorylation state indicated the first time the peptide is detected. For peptide identities, see table 1. Abundant non-phosphorylated peptides from amylase are indicated in blue.

Fractionation using HILIC (PWAX) shows good resolution and can in combination with TiO_2 isolate both monophosphorylated and multiply phosphorylated peptides



Linear MALDI-TOF MS (m/z 800 – 5000)

Figure 6: (A) MALDI-TOF MS spectra (linear mode) of ~4ug parotid saliva fractionated using HILIC (PWAX = PolyWAX LP). ~5% of each fraction was used for the measurements. Phosphorylated peptides are indicated with red stars (*) with the mass and phosphorylation state indicated the first time the peptide is detected. For the peptide identities, see table 1. Abundant non-phosphorylated peptide masses from amylase are indicated in blue.

(B) Using PWAX columns, non-phosphorylated peptides mainly elute early in the gradient and multiply phosphorylated peptides elute late. Monophosphorylated peptides were isolated from the intermediary fractions using TiO_2.

(C) PSD-MALDI-TOF MS was used to verify the number of phosphorylations on the main phosphopeptides. The position in the gradient was consistent with the number of phosphates and pyroGlu modifications.

Determination of # of phosphorylations by Post Source Decay (PSD)

Phosphorylated peptides and proteins in parotid saliva

A Abundant salivary phosphopeptides

Protein	Acc nr	M+H	M+H +HPO3	M+H +2HPO3	M+H +3HPO3	M+H +4HPO3	Peptide sequence
Basic salivary proline-rich protein 4 allele S	P10163	2120.05 2294.12 2510.19	2200.02 2374.08 2590.15	2454.05 2670.12	2534.02 2750.09	2830.06	EDVSQEESLFLISGKPEGR SSEDVSQEESLFLISGKPEGR ESSEDVSQEESLFLISGKPEGR
Basic salivary proline-rich protein 3	Q04118	2257.10 2455.16	2337.06 2535.13				LNEDVSQEESPSVISGKPEGR QSLNEDVSQEESPSVISGKPEGR
Salivary acidic proline-rich phosphopeptide 1/2	P02810	3360.49	3440.46	3520.42	3600.38		QDLNEDVSQEDVPLVISDGGDSEQFDEER
Statherin	P02808	1110.54	1190.51	1270.48			SEEKFLR

B Phosphorylated proteins identified in parotid saliva (so far…)

Salivary α-β-amylase
Salivary acidic proline-rich phosphopeptide 1/2
Basic salivary proline-rich protein 3
Basic salivary proline-rich protein 4 allele S
Statherin

Carbonic anhydrase VI
Fibrinogen α chain
Hemoglobin β chain
α-1-antitrypsin

Immunoglobulins
Keratine

Table 1: (A) The dominating phosphopeptides are listed with the different phosphorylated forms in red and the non-phosphorylated masses in blue. Two of the sequences start with pyroGlu modification indicated with **Q**. **(B)** Proteins where phosphorylated sites been identified, some which have not been reported before (e.g. amylase).

Main difficulty with phosphopeptide purification in parotid saliva is the dynamic range

Advantages of using the microcolumns and step gradients with volatile salts:
- Relatively good resolution was achieved
- Highly enriched phosphopeptide fractions were collected
- Desalting was not necessary

The results of the study indicate that prefractionation using microcolumns is a fast, cheap and efficient way to increase the coverage of the proteome.

Conclusions

Under the conditions employed, SCX columns retain most peptides; only very acidic and/or phosphorylated peptides are unbound. Therefore SCX can quickly segregate phosphopeptides, removing the majority of non-phosphorylated one.

Micropurification using TiO_2 is fast and generates clean phosphopeptide fractions. However, when overloaded, TiO_2 media preferentially lose monophosphorylated peptides in the flow through.

HILIC phosphopeptide separations are an alternative to TiO_2.

Fractionation using HILIC chromatography shows good retention of phosphopeptides, but also of some acidic non-phosphorylated peptides.

In HILIC mode, PHEA binds phosphopeptides stronger than PWAX, making it difficult to elute highly phosphorylated peptides with volatile salts. Separation using PWAX, on the other hand, is very responsive to both changes in pH and salt concentration, making it possible to elute these highly phosphorylated species.

Combination of columns containing SCX/HILIC (PHEA) or HILIC (PHEA)/C12 or a single column of HILIC (PWAX) can resolve the phosphopeptides into fractions with a minimum of non-phosphorylated peptides.

By combining HILIC (PWAX) with TiO_2 monophosphorylated peptides can be isolated from co-eluting non-phosphorylated peptides.

The results indicate that the fractionation using HILIC microcolumns and TiO_2 are complementary.

When comparing the separation between non-phosphorylated and phosphorylated peptides, and the resolution of the phosphopeptides in the gradients, microcolumns using HILIC PolyWAX LP (PWAX) material gave the best results.

References

[1] Gobom et al., *J Mass Spectrom.*, 1999, 34:105-16
[2] Larsen et al., *Mol Cell Proteomics*, 2005, 4:873-86
[3] Ytterberg et al., *Plant Physiol.*, 2006, 140:984-97
[4] Larsen et al., *Proteomics*, 2001, 1:223-38

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