Phosphopeptide Enrichment using Zirconium Dioxide and Titanium Dioxide

Literature Review

Technical Bulletin # 312

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Previously, immobilized metal affinity chromatography (IMAC) was the most widely utilized technique for phosphopeptide enrichment by mass spectroscopy. IMAC methods can vary widely in effectiveness depending on the type of metal ion and loading/elution procedure. The technique also uses valuable research time for the required metal ion loading and washing steps and is difficult to incorporate into an on-line application (6). As non-specific binding of non-phosphorylated peptides further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1, 2). Recently, several papers and posters have been published demonstrating the unique ability of titanium dioxide and zirconium dioxide to selectively retain phosphopeptides contained in complex biological mixtures (1, 2).

Titania Pre-Columns

For example, Pinkse et al. report and innovative approach to automate the method for the enrichment of phosphopeptides using a 2D technique with titanium dioxide particles as the first dimension and a reversed phase silica C18 column as the second dimension (1). The complex proteolytic digests were loaded onto the titanium dioxide pre-column using acidic conditions; retaining the phosphorylated peptides and allowing the rest of the digest to concentrate on the reversed phase column. After the analysis of the digest peptides on the reversed phase column is complete the phosphopeptides are eluted, under alkaline conditions, from the titanium dioxide for analysis. The authors report the method has a recovery above 90% and allows for the identification of previously uncharacterized phosphorylation sites (1). Additionally, they report the titanium dioxide pre-columns could be used for over 200 runs without reduced performance (1). However, using this method, the titanium dioxide does appear to have non-specific binding issues especially of nonphosphorylated peptides with acidic residues.

Larsen et al. took the Pinkse research one step further and dramatically improved the selectivity of the Pinkse method by loading the peptide samples onto the titanium dioxide in 2,5-dihydrobenzoic acid (DHB) (2). In a direct comparison of the titanium dioxide and IMAC methods for semi-complex samples the titanium dioxide pre-columns had a greater yield of phosphorylated peptides and fewer contaminating non-phosphorylated peptides (2). This effect was enhanced as the complexity of the samples increased (2).

Zirconia Microtips

At the recent ASMS 2005 conference Kweon et al. report the successful use of a zirconium dioxide microtip for the enrichment of phosphopeptides (3). Phosphopeptides from proteolytic peptide mixtures were selectively isolated and enriched by binding to zirconia microtips. For this application, the zirconia phosphopeptide enrichment proved superior to titanium dioxide and IMAC methods.

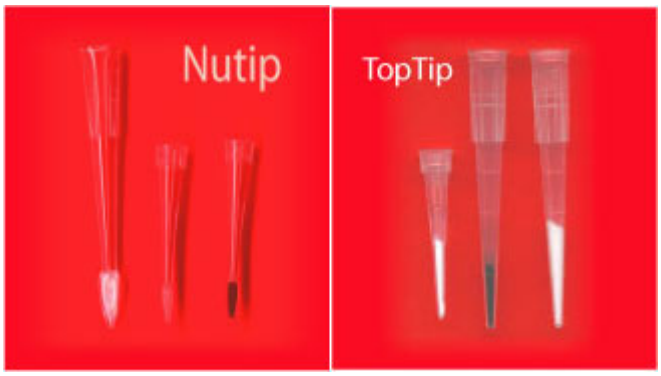

Figure 1. Glygen's Lab-in-a-tip™ SPE pipette tips

Sachtopore-NP (titanium dioxide) and ZirChrom-PHASE (zirconium dioxide) are available as bulk particles or packed analytical, semi-prep or prep sized HPLC columns. In addition, both materials are available as packed or embedded particle SPE pipette tips (Glygen's Lab-in-a-tip™ SPE pipette tips, Figure 1). More information is available on our website at www.zirchrom.com or by contacting a ZirChrom technical specialist by phone at 1-866-STABLE-1 or by e-mail at support@zirchrom.com.

References

(1) Pinkse, M.W.H.; Uitto, P.M.; Hilhorst, M.J.; Ooms, B.; Heck, A.J.R., Analytical Chemistry 2004, 76, 3935-3943.

(2) Larsen, M.R.; Thingholm, T. E.; Jensen, O.N.; Roepstorff, P.; Jorgensen, T.J.D., Mol Cell Proteomics, 2005, 4, 873-886.

(3) Kweon, H.K; Hakansson, K.; ASMS 2005 Poster "Characterization of Phosphopeptides by EDD in FT-ICR Mass Spectrometry"

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Phosphopeptide Enrichment using Sachtopore®-NP Titanium Dioxide

ZirChrom Separations, Inc.

Technical Bulletin # 322

We report a rapid highly selective enrichment procedure utilizing 2,5-dihydroxybenzoic acid (DHB) to enhance the selective enrichment of phosphorylated peptides on a titanium dioxide micro-column. This unique technique dramatically increases the selectivity, and thus sensitivity, of enrichment purification of phosphorylated peptides from complex mixtures of non-phosphorylated and phosphorylated peptides.

Introduction

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Previously, immobilized metal affinity chromatography (IMAC) was the most widely utilized technique for phosphopeptide enrichment by mass spectroscopy. However, IMAC methods can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into on-line applications (1). As non-specific binding of non-phosphorylated peptides further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1, 2).

The following rapid (less than 5 min/sample) highly selective enrichment procedure, developed by the Department of Biochemistry and Molecular Biology, University of Southern Denmark (Odense, Denmark), dramatically increases the selectivity of enrichment in comparison to traditional IMAC methods.

Experimental

A complex mixture of phosphorylated and non-phosphorylated peptides, diluted 1:5 in loading buffer, was enriched using titanium dioxide bulk particles packed into a 3mm long micro-column. The enrichment procedure was as follows:

Column:	Sachtopore®-NP titanium dioxide micro-column [Part# TI02-0310-5(100)]
Loading Buffer:	10uL-300 mg/mL DHB in 80/20 ACN/1% TFA, pH 1.9
Wash Buffer:	1. 10uL-300 mg/ml DHB in 80/20 ACN/0.1% TFA pH 1.9 2. 20uL-80/20 ACN/0.1% TFA, pH 1.9
Elution Buffer:	20uL - NH4OH, pH 10.5

Figure 1 demonstrates performance of the material with a relatively simple mixture (1:1 ratio) of non-phosphorylated and phosphorylated peptides. At this level of complexity the titania based method compares favorably with traditional techniques, enabling detection of equal numbers of phosphopeptides and reducing the number of non- phosphorylated peptides retained. As sample complexity increases so does the selectivity of the binding for the phosphorylated versus the non-phosphorylated peptides (see reference 2 for additional data).

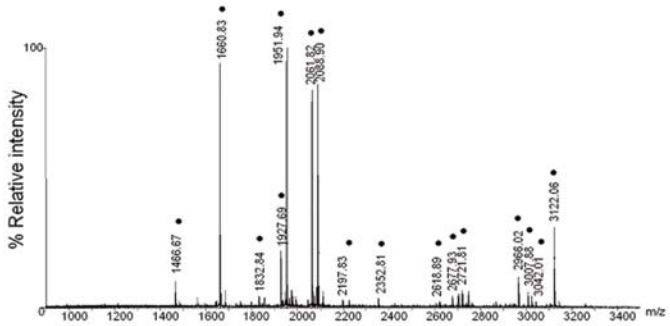

Figure 1: This MALDI mass spectra demonstrates the performance of TiO2 micro-columns for the selective enrichment of phosphorylated peptides (marked with dots) from a complex mixture (a tryptic digestion of 0.5 pmol of the phosphorylated proteins [β-casein, α-casein and ovalbumin] and 0.5 pmol of the non-phosphorylated peptides [serum albumin, β-lactoglobulin and carbonic anhydrase]).

This method can be tailored to your specific application needs. ZirChrom technical support can help to optimize and transfer this method to your site. Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for details about Sachtopore®-NP bulk or Sachtopore®-NP guard inserts.

Acknowledgements

Martin R. Larsen, Tine E. Thingholm, Ole N. Jensen, Peter Roepstorff and Thomas J.D. Jørgensen
Department of Biochemistry and Molecular Biology, University of Southern Denmark (Odense, Denmark)

References

(1) Pinkse, M.W.H.; Uitto, P.M.; Hilhorst, M.J.; Ooms, B.; Heck, A.J.R., *Analytical Chemistry*, **76**, 3935-3943 (2004).

(2) Larsen, M.R.; et al., Mol Cell Proteomics, **4**, 873-886 (2005).

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Phosphopeptide Enrichment Using Titanium Dioxide & Zirconium Dioxide SPE Tips

Dr. Ashok Shukla, Glygen Corporation
Kelly S. Johnson, ZirChrom Separations, Inc.

Technical Bulletin # 323

We report a rapid, highly selective enrichment procedure for phosphopeptides utilizing titanium dioxide (TiO_2) & zirconium dioxide (ZrO_2) SPE tips. β-casein digest samples purified via NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), display exceptional signal to noise ratios for phosphopeptide analysis and eliminate many difficulties present in traditional IMAC methods.

Introduction

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Previously, immobilized metal affinity chromatography (IMAC) was the most widely utilized technique for phosphopeptide enrichment by mass spectroscopy. However, IMAC methods can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into on-line applications (1). As non-specific binding of non-phosphorylated peptides further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1).

The following rapid enrichment procedure, developed by Glygen Corporation and New Objective, Inc. (Woburn, MA), maintains high enrichment selectivity without the complications and irreproducibility inherent in traditional IMAC methods (2).

Experimental

An overnight trypic β-casein digest was performed and the sample was then diluted with a 0.1% formic acid solution to generate a 1 pmol/µL solution. The enrichment procedure was as follows:

Product:	Titanium Dioxide & Zirconium Dioxide NuTip™ (part # NT1TIO & NT1ZRO)
Conditioning:	Tips conditioned with 5 aspiration/expulsion (A/E) cycles of HPLC grade water
Loading:	10 µL of sample loaded in 10 A/E cycles
Wash:	10 µL of HPLC grade water for 10 A/E cycles
Elution:	2 µL of 50/50 50mM NH_4HCO_3/50mM TEA in 5 A/E cycles
Post Elution:	Addition of 2 µL of a 50mM TEA in methanol solution followed by immediate mixing and centrifugation.
Detection:	All samples were analyzed via ESI-MS in negative-ion mode.

Figure 1 demonstrates performance of TiO_2 and ZrO_2 Trap'nTip™ (Trap'nTip™ is a miniaturized form of NuTip™, manufactured exclusively by Glygen Corporation for New Objective, Inc.). A phosphopeptide control set was used for tuning purposes and to confirm the identity of peaks in Figure 1. The results obtained on both TiO_2 and ZrO_2 compare favorably with traditional techniques, successfully enriching the phosphopeptides and thus greatly improving the signal-to-noise ratio for phosphopeptide analysis (2).

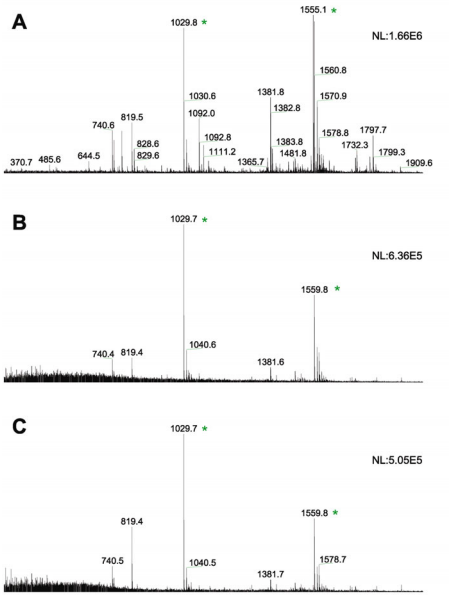

Figure 1: A) Spectrum of β-casein without enrichment B) Spectrum of β-casein after purification by TiO_2 Trap'nTip™ C) Spectrum of β-casein after purification by ZrO_2 Trap'nTip™ (2)

ZirChrom technical support can help to optimize and transfer this method to your site. Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for details about using TiO_2 and ZrO_2 SPE tips for phosphopeptide enrichment.

References

(1) Pinkse, M.W.H et al, *Analytical Chemistry*, **76**, 3935-3943 (2004).

(2) Toher, C.J., Perala, A.W., Shukla, A.K., Valaskovic, G.A., Shukla, M.M., Poster # TP13-215, ASMS 2006.

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Effect of Loading Buffer on Phosphopeptide Enrichment using Zirconium Dioxide SPE Tips

ZirChrom Separations, Inc.

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Technical Bulletin # 324

The following text investigates the effect of sample loading buffer on a rapid, highly selective enrichment procedure for phosphopeptides utilizing zirconium dioxide (ZrO_2) SPE tips. For α-casein digest samples enriched using ZrO_2 NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), a low pH formic acid loading buffer enabled the most effective and specific enrichment of phosphopeptides.

Introduction

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Immobilized metal affinity chromatography (IMAC) techniques, the most widely utilized technique for phosphopeptide enrichment, can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into on-line applications (1). As non-specific binding further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1).

This rapid enrichment procedure, developed by Kweon and Hakansson at the University of Michigan (Ann Arbor, MI), optimizes sample loading conditions for the enrichment of phosphopeptides using zirconium dioxide SPE tips (2).

Experimental

An overnight tryptic α-casein digest was performed and the sample was then diluted with loading solution (see figure 1) to generate a 100 pmol solution. The enrichment procedure was as follows:

Product:	50 µg Zirconium Dioxide NuTip™ (part # NT1ZRO)
Conditioning:	Tips conditioned with 10 µL loading solution (see figure 1) for 3 aspiration/expulsion (A/E) cycles.
Loading:	10 µL of sample loaded in 10-20 A/E cycles
Wash:	10 µL of HPLC grade water for 2 A/E cycles
Elution:	10 µL of 0.5% piperidine solution for 2 A/E cycles
Post Elution:	Eluted samples were dried and reconstituted in 2-propanol/ACN/water (1:1:2) with 0.25% piperidine.
Detection:	All samples were analyzed via ESI FT-ICR in negative-ion mode.

Figure one compares the spectra of five different sample loading buffer conditions. The superior loading buffer is the 2.4% formic acid buffer (pH 2.0). Even raising the pH to 3.0 makes a large difference in the number of contaminating non-phosphopeptides (2). To achieve maximum recovery of the bound analytes the washing and elution solutions were also optimized. The highest phosphopeptide recovery was achieved with water washing solution and a 0.5% piperidine (pH 11.5) elution solution (2).

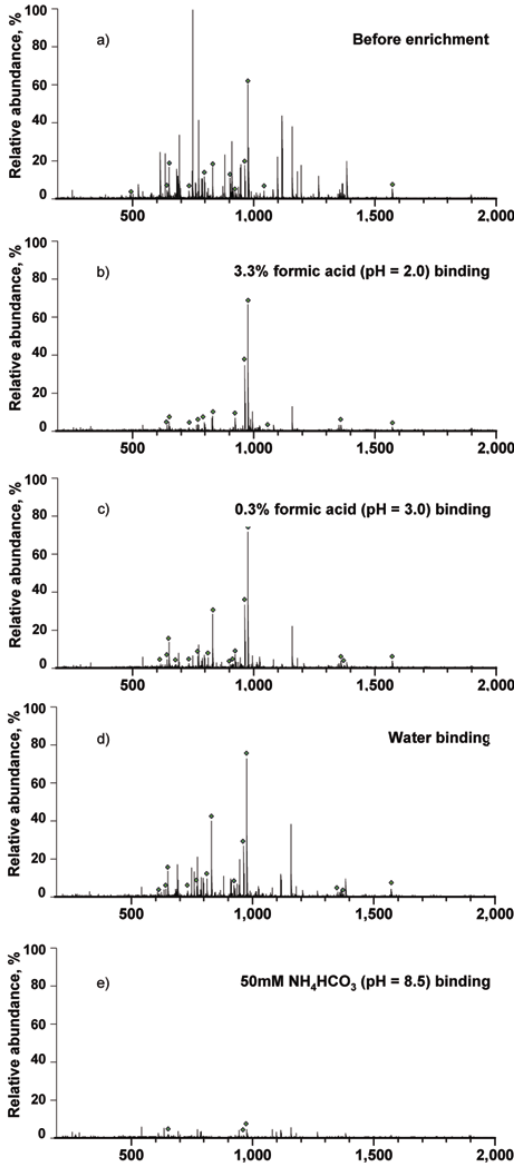

Figure 1: Negative mode ESI FT-ICR mass spectra (8 scans) of a tryptic digest of α-casein obtained prior to enrichment (a) and following enrichment using various loading solutions (b-e). Phosphopeptides indicated by green diamonds.

This method can be tailored to your specific application needs. Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for details.

References

(1) Pinkse, M.W.H et al, *Analytical Chemistry*, **76**, 3935-3943 (2004).

(2) Kweon, H.K; Hakansson, K.; *Analytical Chemistry*, **78**, 1743-1749 (2006).

Comparison of Titanium Dioxide & Zirconium Dioxide SPE Tips for Phosphopeptide Enrichment

ZirChrom Separations, Inc.

Technical Bulletin # 325

The following compares and contrasts zirconium dioxide (ZrO_2) and titanium dioxide (TiO_2) SPE tips for rapid enrichment of phosphopeptides. Although, for the α-casein digest samples tested, either technique proves more effective than traditional methods, interestingly, the ZrO_2 tips enriched singly phosphorylated peptides in greater abundance.

Introduction

Historically, researchers have been unable to fully realize the benefits of mass spectrometry as an analysis method for phosphopeptides because isolation of the molecules from non-phosphorylated peptides is frequently required before examination of the complex samples can proceed (1). Immobilized metal affinity chromatography (IMAC) techniques, the most widely utilized technique for phosphopeptide enrichment, can vary widely in effectiveness, use valuable research time for the required metal ion loading/washing steps and are difficult to incorporate into on-line applications (1). As non-specific binding further hampers the technique, researchers using mass spectroscopy needed a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1).

The following rapid enrichment procedure, developed by Kweon and Hakansson at the University of Michigan (Ann Arbor, MI), was applied to both the ZrO_2 and the TiO_2 NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), to compare and contrast the enrichment of phosphopeptides from a trypic α-casein digest (2). The ZrO_2 and TiO_2 materials used in this study were manufactured by ZirChrom Separations and Sachtleben Chemie GmbH, (Duisburg, Germany), respectively.

Experimental

An overnight tryptic α-casein digest was performed and the sample was then diluted with 3.3% formic acid (pH 2) to generate a 100 pmol solution. The enrichment procedure was as follows:

Product:	50 µg Zirconium Dioxide NuTip™ (part # NT1ZRO) & 50 µg Titanium Dioxide NuTip™ (part # NT1TIO)
Conditioning:	Tips conditioned with 10 µL 3.3% formic acid (pH 2) for 3 aspiration/expulsion (A/E) cycles.
Loading:	10 µL of sample loaded in 10-20 A/E cycles
Wash:	10 µL of HPLC grade water for 2 A/E cycles
Elution:	10 µL of 0.5% piperidine (pH 11.5) for 2 A/E cycles
Post Elution:	Eluted samples were dried and reconstituted in 2-propanol/ACN/water (1:1:2) with 0.25% piperdine.
Detection:	All samples were analyzed via ESI FT-ICR in negative-ion mode.

Figure 1 compares three mass spectra; (a) before enrichment , (b) after enrichment using ZrO_2 SPE tips, and (c) after enrichment using TiO_2 SPE tips . Phosphopeptides are numbered and non-phophorylated peptides are labeled with their corresponding amino acid residue numbers.

The most abundant species following enrichment with the ZrO_2 material is the singly phosphorylated peptide 7. Whereas the most abundant signal following TiO_2 enrichment is the doubly phosphorylated peptide 6. This phenomenon is not hypothesized to be due to irreversible binding of the multiply phosphorylated peptides to ZrO_2 as these peptides were present in the solution left-over after enrichment.

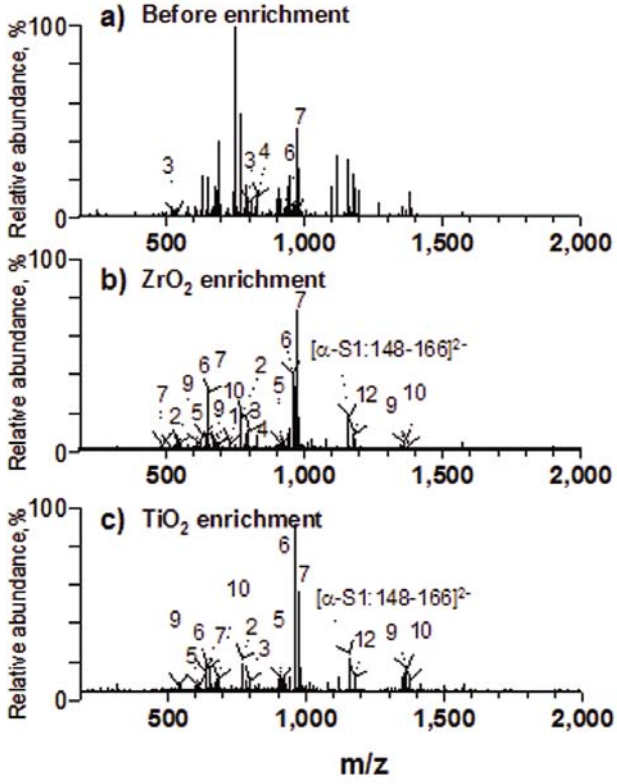

Figure 1: Negative mode ESI FT-ICR mass spectra (8 scans) of a tryptic digest of α-casein obtained; (a) prior to enrichment, (b) after ZrO_2 enrichment, and (c) after TiO_2 enrichment.

ZirChrom technical support can help to optimize and transfer this method to your site. Please contact ZirChrom technical support at 1-866-STABLE-1 or support@zirchrom.com for details.

References

(1) Pinkse, M.W.H et al, *Analytical Chemistry*, **76**, 3935-3943 (2004).
(2) Kweon, H.K; Hakansson, K.; *Analytical Chemistry*, **78**, 1743-1749 (2006).

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Selectivity of Titanium Dioxide & Zirconium Dioxide SPE Tips for Phosphopeptide Enrichment

ZirChrom Separations, Inc.

Technical Bulletin # 326

The following compares and contrasts the selectivity of zirconium dioxide (ZrO_2) and titanium dioxide (TiO_2) SPE tips with varying concentrations of α-casein for rapid enrichment of phosphopeptides. The results demonstrate that phosphopeptide selectivity for α-casein was not compromised when sample amount was decreased to 25 pmol. Below 25 pmol, a smaller (25 μg) SPE tip is required to obtain acceptable signal to noise ratios.

Introduction

Immobilized metal affinity chromatography (IMAC), the most widely utilized technique for phosphopeptide enrichment, can vary widely in effectiveness, uses valuable research time for the required metal ion loading/washing steps and is difficult to incorporate into on-line applications (1). As non-specific binding further hampers the technique, researchers need a more specific on-line technique for isolating the phosphopeptides in order to fully realize the time saving benefits of mass spectroscopy (1).

The following rapid enrichment procedure, developed by Kweon and Hakansson at the University of Michigan (Ann Arbor, MI), was applied to various sized ZrO_2 and the TiO_2 NuTip™ SPE tips, manufactured by Glygen Corporation (Columbia, MD), to analyze the sensitivity of the enrichment of phosphopeptides from several concentrations of trypic α-casein digest (2). The ZrO_2 and TiO_2 materials used in this study were manufactured by ZirChrom Separations and Sachtleben Chemie GmbH, (Duisburg, Germany), respectively.

Experimental

An overnight trypic α-casein digest was performed and the sample was then diluted with 3.3% formic acid (pH 2) to generate a 100 pmol solution. Samples of decreasing concentration were created from this solution, and the sensitivity of the enrichment protocol was tested. The enrichment procedure was as follows:

Product:	50 & 25 μg ZrO_2NuTip™ (part # NT1ZRO) 50 & 25 μg TiO_2 NuTip™ (part # NT1TIO)
Conditioning:	Tips conditioned with 10 μL 3.3% formic acid (pH 2) for 3 aspiration/expulsion (A/E) cycles.
Loading:	10 μL of sample loaded in 10-20 A/E cycles
Wash:	10 μL of HPLC grade water for 2 A/E cycles
Elution:	10 μL of 0.5% piperidine (pH 11.5) for 2 A/E cycles
Post Elution:	Eluted samples were dried and reconstituted in 2-propanol/ACN/water (1:1:2) with 0.25% piperdine.
Detection:	All samples were analyzed via ESI FT-ICR in negative-ion mode.

Table 1 demonstrates clearly that phosphopeptide selectivity is not compromised when sample amount is decreased from 100 pmol to 50 and then finally to 25 pmol. As the MS analysis system was not optimized for high sensitivity, poor signal to noise values were obtained with samples lower than 25 pmol in concentration. Halving the size of the SPE tips (refer to Figure 1) dramatically improves the signal to noise ratios for a 1 pmol sample however selectivity is still compromised when compared to higher concentration samples.

Table 1. Selectivity[a] (%) of 50-ug ZrO_2 and TiO_2 Microtips for Phosphopeptide Enrichment of a Tryptic Digest of a-casein as a Function of Sample Amount

	100 pmol	50 pmol	25 pmol
without enrichment	27	29	29
ZrO_2 Enrichment	67	85	83
TiO_2 Enrichment	62	77	74

[a] *Defined as relative phosphopeptide signal*

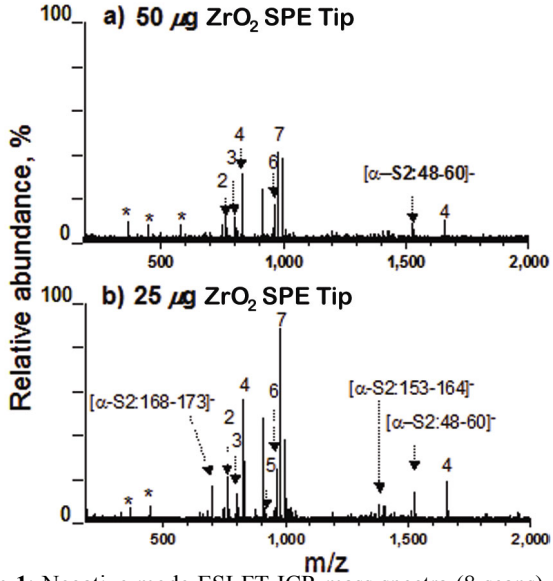

Figure 1: Negative mode ESI FT-ICR mass spectra (8 scans) from 1pmol of a trypsin digest of α-casein obtained following phosphopeptide enrichment with a 50-ug ZrO_2 (a) and a 25-ug ZrO_2 NuTip™ SPE tip (b). Identified nonphosphorylated peptides are labeled with their corresponding amino acid residue numbers and α-casein isoform.

NuTip™ is a trademark of Glygen Corporation.

References

(1) Pinkse, M.W.H et al, *Analytical Chemistry*, **76**, 3935-3943 (2004).
(2) Kweon, H.K; Hakansson, K.; *Analytical Chemistry*, **78**, 1743-1749 (2006).

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