Why Is Phosphopeptide Enrichment Important?

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Studying Cancer Cell Signalling Using TiO_2 Mag Sepharose Magnetic Beads

Phosphorylation is a common reversible post-translational modification involved in the regulation of many essential biological processes, for example cell signalling, which is of prime importance for the study of various disease states such as cancer. The phosphoproteins and phosphopeptides resulting from these processes are important to the understanding of tumour progression, however, there are various barriers to their study. Within the cellular environment, phosphopeptides are usually transient and found in very low concentrations. In addition when proteins are phosphorylated they attain a negative charge and, compared to their non-phosphorylated counterparts, they are poorly ionised which further complicates their measurement using mass spectrometry. Therefore, determination of their relative levels as a function of disease progression is an ongoing challenge for researchers. One approach is to use a simple enrichment technique that 'concentrates' the phosphopeptides in samples to aid detection using standard mass spectroscopy approaches. This paper outlines how such a simple enrichment process using TiO_2 Mag Sepharose™ magnetic beads was developed, validated using a model sample, and tested in a complex sample derived from a leukaemia cell line.

About the TiO_2 Mag Sepharose magnetic beads TiO_2 Mag Sepharose magnetic beads use titanium dioxide (TiO_2)-based chromatography to simplify the capture and enrichment of phosphopeptides. TiO_2 has a high affinity for phosphopeptides and provides efficient enrichment of these from complex samples. When the tubes are placed in MagRack, the magnetic beads are attracted to the magnet within a few seconds. This allows fast removal of the supernatant whilst the beads remain in the tubes. The easy visibility of the beads ensures reliable collection of all the targeted peptides.

Table 1. Phosphopeptide enrichment protocol

1. Equilibration with binding buffer (1 M Glycolic acid, 80% Acetonitrile, 5% Trifluoroacetic acid).
2. Sample application and incubation with mixing for 30 min.
3. Washing with 1 × binding buffer and 2 × wash buffer (80% Acetonitrile, 1% Trifluoroacetic acid).
4. Elution of the target peptides (i.e., phosphopeptides) with elution buffer (5% Ammonium hydroxide).

Study 1: Investigation of the Effective Binding of Phosphopeptides to TiO_2 Mag Sepharose Magnetic Beads

To demonstrate the affinity of TiO_2 to phosphopeptides, an initial binding study was carried out in which four pure phosphopeptides with masses ranging from 1126.8 to 2192.4 (AnaSpec, Inc), a Phosphate Colorimetric Assay Kit (BioVision, Inc.) and a Shrimp Alkaline Phosphatase (USB) were used to investigate the amounts of bound phosphopeptides to the TiO_2 Mag Sepharose magnetic beads. The amounts of bound protein were determined using absorbance at 650 nm (SpectraMax Plus 384, Molecular Devices) and the data given in Table 2. On average, 71% of added phosphopeptides were bound to the beads.

Table 2. Describes amount of phosphopeptides with different MW bound to 10 µl TiO_2 magnetic beads.

Peptide	MW	Sequence[1]	Added amount (µg)	Added amount (nmol)	Bound amount (%)
Angiotensin II Substrate	1126.8	DRVyIHPF	2.5	2.2	56
Kinase Domain of Insulin Receptor-3	1702.8	TRDIYETDyYRK	2.5	1.5	87
Bovine β-Casein, Monophosphopeptide	2062.0	FQsEEQQQTEDELQDK	2.5	1.2	77
PKA Regulatory Subunit II Substrate	2192.4	DLDVPIPGRFDRRVsVAAE	2.5	1.1	65

[1] **s** indicates serine and **y** indicates tyrosine phosphorylation.

Study 2: Model Sample Study: Phosphopeptide Enrichment From 50 pmol -Casein, -Casein, and BSA

The aim of this study was to show enrichment of a model sample system using known quantities of phosphorylated proteins (α-casein and β-casein) in a background of nonphosphorylated bovine serum albumin. A mixture containing 50 pmol of the two forms of casein and 50 pmol non-phosphorylated bovine serum albumin (BSA), was trypsin digested and applied to the TiO_2 Mag Sepharose magnetic beads following the same protocol. The eluates with enriched phosphopeptides were then lyophilised, dissolved in 20% Acetonitrile with 0.1% Trifluoroacetic acid and analysed with MALDI-ToF MS (Autoflex III Smartbeam, Bruker Daltonics, Germany). The resultant MS spectra are given in *Figure 1*, in which the enriched phosphopeptides found in B and C are marked with*, also metastable phosphopeptide is also seen, marked with a dot. Enrichment of phosphopeptides was achieved with both α-casein and β-casein phosphopeptides found including the tetraphosphopeptide from β-casein. Even when the eluate was diluted a 100 times the two phosphopeptides could still be detected.

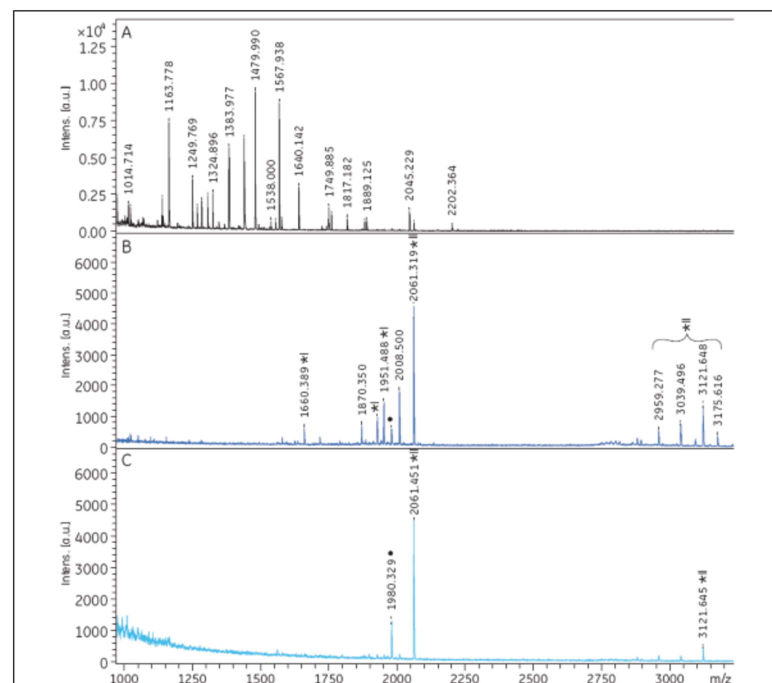

*Figure 1. MALDi-ToF spectra of tart materials with α-casein, β-casein and BSA (A), the corresponding eluate (B) and the eluate diluted 100 times (C). The enriched phosphopeptides found in B and C are marked with *and also metastable phosphopeptide is seen, marked with a dot.*

Study 3: Complex Sample Study to Investigate Enrichment of Phosphorylated Peptides From a Human Leukaemia Cell Line

In a complex sample study carried out by a multidisciplinary group* at Uppsala University, the aim was to map the phosphorylation pattern, of digested proteins from a human leukaemia cell line proteins expressing the BCR-ABL oncogene.

In this study two separate batches of trypsin-digested lysate from human cells were prepared for MS analysis. The first batch used the complete protocol outlined in *Figure 2* to prepare an enriched sample, whilst the second batch was processed without the enrichment step as a control sample. The levels of phosphopeptides were then measured and compared. Phosphopeptides were only detected in the material that had undergone enrichment. The MS analysis identified 15 phosphopeptides and 14 phosphorylation sites were found, which are listed in *Table 2*. A total of 16% of proteins of the leukaemia cell line were phosphorylated in the enriched sample.

Table 3. Lists the phosphopeptides detected by MS in the enriched peptide mixture from the cell division protein kinase 3.

Enriched phosphopeptides from cancer cells

Accession number[1]	Gene name	Protein name	Phosphopeptide sequence[2]	Site	SWISS-PROT[3]
IPI00012442	G3BP1	Ras GTPase-activating protein-binding protein 1	sssPAPADIAQTVQEDLR	S230/ S231/ S232	Yes, for all 3
IPI00009032	SSB	Lupus La protein	FAsDDEHDEHDENGATGPVKR	S366	Yes
IPI00009032	SSB	Lupus La protein	TKFAsDDEHDEHDENGATGPVKR	S366	Yes
IPI00025512	HSPB1	Heat-shock protein β-1	QLsSGVSEIR	S82	Yes
IPI00185526	SAMSN1	SAM-domain protein SAMSN-1	SSsFGNFDR	S11	No
IPI00017297	MATR3	Matrin-3	RDsFDDRGPSLNPVLDYDHGSR	S188	Yes
IPI00184330	MCM2	DNA replication licensing factor MCM2	GLLyDSDEEDEERPAR	Y137 (or S139)	No, S139 known
IPI00017659	RCSD1	Protein kinase substrate CapZIP	SQsDCGELGDFR	S179	Yes
IPI00023503	CDK3	Cell division protein kinase 3	IGEGTyGVVYK	Y15	Yes
IPI00013721	PRPF4B	Serine/threonine-protein kinase PRP4 homolog	LCDFGSASHVADNDITPyLVSR	Y849	Yes
IPI00337465	KLC1	Isoform P of Kinesin light chain 1	AssLNVLNVGGK	S546/ S547	No[4]
IPI00163505	RMB39	Isoform 1 of RNA-binding protein 39	DKsPVREPIDNLTPEER	S136	Yes
IPI00014177	SEP2	Septin-2	IYHLPDAEsDEDEDFKEQTR	S218	Yes
IPI00299254	EIF5B	Eukaryotic translation initiation factor 5B	NKPGPNIEsGNEDDDASFK	S214	Yes
IPI00178667	TOP2A	183 kDa protein/DNA topoisomerase 2	yLEESDEDDLF	Y1601	No

[1] International Protein Index
[2] Sequences of identified phosphopeptides; s indicates serine and y indicates tyrosine phosphorylation
[3] Phosphorylation is reported in SWISS-PROT protein database
[4] Sequence is specific for isoform P.

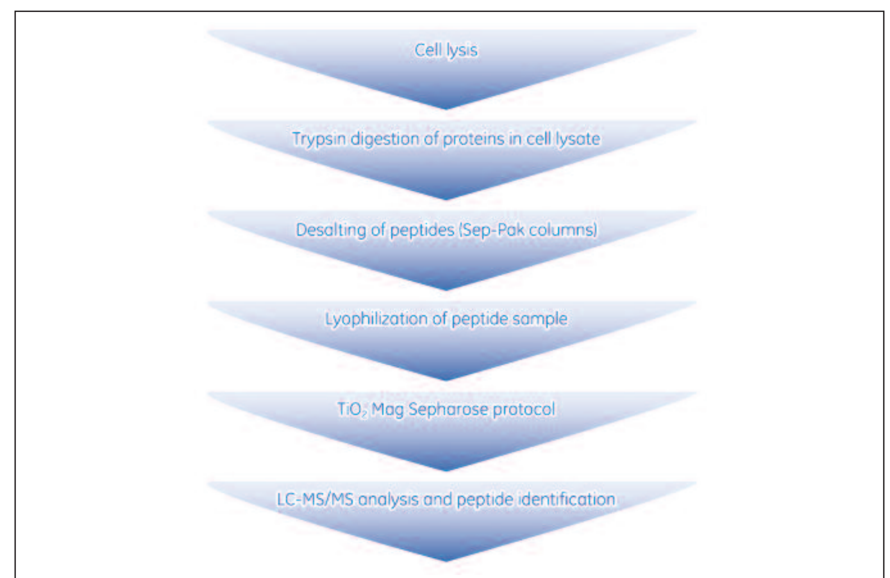

Figure 2

Summary

Phosphorylation is pivotal in cellular regulation and hence one of the mechanisms responsible for disease progression for example in cancer. The cellular signalling proteins and pathways involved need to be identified and understood however there are many challenges to phosphopeptide measurement. Phosphoproteins are generally present in unsuitable forms and in such low concentrations that methods for their enrichment are required to enable routine detection by MS analysis. These studies clearly demonstrate that TiO_2 Mag Sepharose magnetic beads are ideal for selectively enriching phosphopeptide concentrations in both simple and complex cell samples. Their strong affinity for phosphoproteins and physical ease-of-use make them a convenient tool for studying phosphorylation patterns.

*Acknowledgement

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