

Enrichment techniques employed in phosphoproteomics

Jan Fíla · David Honys

Received: 28 January 2011 / Accepted: 26 September 2011
© The Author(s) 2011. This article is published with open access at Springerlink.com

Abstract Rapid changes of protein phosphorylation play a crucial role in the regulation of many cellular processes. Being post-translationally modified, phosphoproteins are often present in quite low abundance and tend to co-exist with their unphosphorylated isoform within the cell. To make their identification more practicable, the use of enrichment protocols is often required. The enrichment strategies can be performed either at the level of phosphoproteins or at the level of phosphopeptides. Both approaches have their advantages and disadvantages. Most enriching strategies are based on chemical modifications, affinity chromatography to capture peptides and proteins containing negatively charged phosphate groups onto a positively charged matrix, or immunoprecipitation by phospho-specific antibodies.

In this article, the most up-to-date enrichment techniques are discussed, taking into account their optimization, and highlighting their advantages and disadvantages. Moreover, these methods are compared to each other, revealing their complementary nature in providing comprehensive coverage of the phosphoproteome.

Keywords Phosphoproteomics · Enrichment · IMAC · MOAC · Titanium dioxide (TiO₂) · Antibodies

Abbreviations

2D-GE	Two-dimensional gel electrophoresis
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DHB	2,5-dihydroxybenzoic acid
DIPEA	<i>N,N'</i> -diisopropylethylamine
DTT	Dithiothreitol
EDC	<i>N,N'</i> -dimethylaminopropyl ethyl carbodiimide
ESI-MS	Electrospray ionization mass spectrometry
IDA	Iminodiacetic acid
HILIC	Hydrophilic interaction liquid chromatography
IMAC	Immobilized metal affinity chromatography
MALDI-MS	Matrix-associated laser desorption/ionization mass spectrometry
MOAC	Metal-oxide affinity chromatography
MS	Mass spectrometry
NTA	Nitrilotriacetic acid
PolyMAC	Polymer-based metal ion affinity capture
pKa	Acid dissociation constant
PPh ₃	Triphenylphosphine
pS	Phosphoserine
pT	Phosphothreonine
pY	Phosphotyrosine
PySSPy	2,20-dithiopyridine
SAX	Strong anionic ion-exchange chromatography
SCX	Strong cationic ion-exchange chromatography
SDS	Sodium dodecyl sulfate
<i>t</i> Boc	<i>t</i> -butyl-dicarbonate
TFA	Trifluoroacetic acid

J. Fíla · D. Honys (✉)
Laboratory of Pollen Biology, Institute of Experimental Botany,
Academy of Sciences of the Czech Republic, Rozvojová 263,
165 02 Prague 6, Czech Republic
e-mail: honys@ueb.cas.cz

J. Fíla
Department of Plant Experimental Biology, Faculty of Science,
Charles University in Prague, Viničná 5, 128 44 Prague 2,
Czech Republic

Introduction

Reversible protein phosphorylation represents one of the most dynamic post-translational modifications. Phosphorylation plays a key role in many cellular processes, including cell cycle regulation, signal transduction, cytoskeletal dynamics regulation, protein targeting, metabolism, transcription and translation regulation (Augustine et al. 2008; Ballesta et al. 1999; Baskaran et al. 1997; Dephore et al. 2008; Fletterick and Sprang 1982; Garnak and Reeves 1979; Mishra et al. 2006; Moll et al. 1991; Rihs et al. 1991). The most abundant amino acids that are phosphorylated are serine (pS), threonine (pT) and tyrosine (pY). On the other hand, under conventional conditions, phosphohistidine remains undetectable.

The importance of phosphoproteins is often not reflected in their abundance, as their non-phosphorylated counterparts are usually present in much greater quantities within the cell. Moreover, phosphorylation is a transient modification, so the protein in question could be present in both native and phosphorylated forms. Phosphoprotein identification is further complicated by technical issues with mass spectrometry (MS). Ion suppression can result in less efficient phosphopeptide ionization in comparison with its non-phosphorylated counterparts, and as a result the phosphorylated species would then barely be detected (Marcantonio et al. 2008).

To increase the number of phosphoproteins that can be identified, it is necessary to remove non-phosphorylated proteins or peptides from samples and enrich for the phosphorylated isoforms prior to MS. This task is carried out by the use of enriching techniques that can be performed at two levels—at the level of intact phosphoproteins, or at the level of peptides (Fig. 1). Typically, hydrophilic interaction liquid chromatography (HILIC), as well as two types of ion-exchange chromatography—strong anionic ion-exchange chromatography (SAX), and strong cationic ion-exchange chromatography (SCX)—represent prefractionation techniques rather than specifically enriching methods. The actual enriching methods are then represented by immunoprecipitation, immobilized metal affinity chromatography (IMAC), metal-oxide affinity chromatography (MOAC), Phos-Tag chromatography, polymer-based metal ion affinity capture (PolyMAC), hydroxyapatite chromatography, enrichment by chemical modification, and phosphopeptide precipitation (Fig. 2). IMAC, MOAC and Phos-Tag share the same principle of using a positively charged chromatography matrix that binds to negatively charged phosphate moieties. A few commercial phosphopeptide enrichment kits are based on variations of IMAC or MOAC and are not reviewed here due to the unknown composition of their buffers.

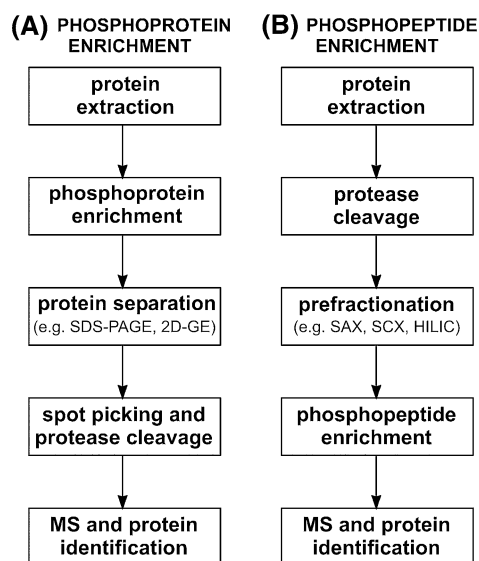


Fig. 1 The schematic workflow of **a** phosphoprotein and **b** phosphopeptide enrichment strategies

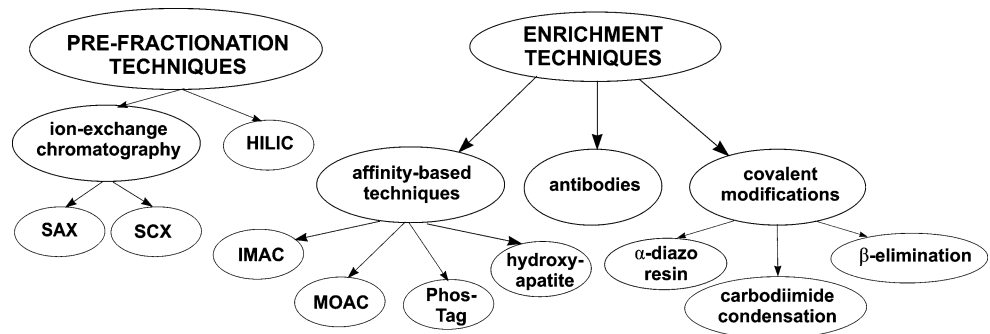
This article offers an overview of currently used enrichment protocols. It will pinpoint their advantages as well as their limitations. Although MS has been a major enabling technology for phosphopeptide identification and mapping, MS techniques and their principles are not covered by this review since they have recently been reviewed extensively elsewhere (Boersema et al. 2009; Yates et al. 2009).

Comparison of phosphoprotein and phosphopeptide enrichment

Protein extraction represents the first step for both phosphoprotein and phosphopeptide enrichment (Fig. 1). The removal of nucleic acids and other interfering contaminants is especially important for plant phosphoproteomic experiments where removal of cell-wall components and various secondary metabolites is essential. The inclusion of protease and phosphatase inhibitors in extraction buffers is often necessary to prevent sample degradation and/or dephosphorylation, especially with “soft” extraction buffers which do not contain protein denaturants such as detergents, chaotropic agents (urea and guanidinium chloride) and organic solvents. Moreover, kinase activity also has to be blocked in order to prevent non-biological phosphorylation of the sample. Such inhibition is necessary since blocked phosphatases could be overwhelmed by kinases, with the result that the explored phosphorylation pattern would be artificial.

Phosphoprotein enrichment is usually performed on the crude protein extract immediately after preparation. The

Fig. 2 A diagram of the most frequently employed methods for the prefractionation and the enrichment of phosphoproteins or phosphopeptides



enriched fraction can be separated by two-dimensional gel electrophoresis (2D-GE) or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 2D-GE electrophoretogram generally contains a number of spots mostly corresponding to single proteins separated in two dimensions on the basis of molecular weight and isoelectric point. The spots or bands to be analyzed are excised from the gel and the proteins are digested by a specific protease—commonly trypsin. The obtained peptides are analyzed by MS. Since this approach could result in partial non-specificity, the non-specific 2D-GE spots could be undistinguishable, especially from the less abundant specific ones. To overcome this limitation, trypsin digest can be directly performed, and the peptides can be separated by chromatography techniques. Moreover, a second round of enrichment can be performed at the phosphopeptide level.

The advantage of phosphoprotein enrichment is that it usually reveals the molecular weight and the isoelectric point of proteins. Such information could be helpful for subsequent protein identification by MS. Another advantage with the phosphoprotein-enriching approach is that intact proteins are separated. The peptide spectrum obtained is mostly derived from one protein, and hence protein identification is more probable since it has been achieved on the basis of several peptides (including the non-phosphorylated ones) and not according to only a single peptide (as is often the case with phosphopeptide enrichment). However, there are also several disadvantages to be considered, such as insufficient re-suspension of some proteins (e.g. hydrophobic ones), the difficulty or impossibility of separating extremely alkaline or acidic proteins, and/or protein losses (of tiny and hydrophobic proteins) during the inevitable precipitation steps. It was shown that, during 2D-GE, as much as 80% of the proteins were lost (Zhou et al. 2005), so less abundant species are also unlikely to be identified. Another disadvantage is that the enrichment of intact phosphoproteins is likely to be less specific than the enrichment of phosphopeptides. This could simply be caused by the higher complexity of protein structure compared with peptides, and by the intactness of protein domains formed by properly folded distal chains in three-dimensional space. These

structures are absent following protein cleavage into short peptides. It remains a possibility that some domain(s) could bind non-specifically even under denaturing conditions, such as calcium-binding domains.

Phosphoprotein enrichment is advantageous when it is the most abundant proteins that are being considered, since less abundant species are usually lost. The process is also feasible for non-model organisms that are often not sequenced since the isoelectric point and the molecular weight could lead to the rejection of less favorable alternative identifications. It can be also used as a first enrichment step followed by phosphopeptide enrichment of the cleaved phosphoproteins from the enriched fraction, as mentioned above.

Phosphopeptide enrichment also starts with protein extraction. Since the total protein extract from a given tissue is usually highly complex, multistep protein extraction leading to more proteome fractions—such as three-fraction protein extraction applied for *Arabidopsis thaliana* pollen—is advantageous (Holmes-Davis et al. 2005). In the first extraction a “soft” Tris-based extraction buffer is applied in order to gain a salt-soluble fraction. The pellet resulting from this step is further re-extracted with a buffer containing urea and CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). The parallel extraction leading to the third fraction relies on a buffer containing sodium dodecyl sulfate (SDS) that extracts the wall proteins. After protein extraction, the protein extract is cleaved by a specific protease, commonly trypsin (Fig. 1b). Due to the sample complexity, it is advantageous to apply some of the prefractionation techniques. The most common prefractionation techniques—namely, SAX, SCX and HILIC—are discussed below. An alternative approach relies on gel separation of intact proteins and subsequent work with the excised band (or bands) or larger gel areas (Carrascal et al. 2008). The peptide mixture that includes modified peptides (e.g. phosphorylated and glycosylated ones) is enriched for phosphopeptides. The enriched fraction is subsequently MS-analyzed.

A key advantage of phosphopeptide enrichment is that peptides represent less complex three-dimensional structures,

as discussed above. Another advantage is that tiny, lipophilic, and extremely acidic or alkaline proteins are not handicapped. In general, chromatography techniques are more easily applied to peptide separation than to protein separation, and are more sensitive than 2D-GE, allowing less abundant phosphopeptides to be identified. Accordingly, more experimental data have been obtained by these methods than by phosphoprotein enrichment.

The fact that the enriched fraction represents a mixture of phosphopeptides originating from multiple proteins can be considered disadvantageous since it represents a limitation for phosphopeptide identification. Moreover, non-phosphorylated peptides can no longer contribute to the protein identification since they were removed during the enrichment step. Protein identification thus usually relies on a single phosphopeptide. Proteins containing more widespread domain(s) with conserved phosphorylation site(s) can be confused with each other, and in these cases protein identification is more challenging. Another disadvantage is the inability to determine the molecular weight and isoelectric point of given proteins. Despite major efforts to overcome this, the apparent non-specificity still represents a significant limitation of most phosphopeptide-enriching methods.

In general, phosphopeptide enrichment is very useful when the phosphoproteome of such isolated organelles as mitochondria is analyzed (Ito et al. 2009), since such samples are less complex than total tissue protein extracts. The approach is also more amenable to automation and high-throughput proteomic experiments than 2D-GE. Moreover, phosphopeptide enrichment and identification is so accurate that the precise phosphorylation site can be identified.

Since many of the enrichment methods described here can be applied to both protein and peptide purification, we will discuss each enrichment method in the context of both phosphoprotein and phosphopeptide enrichment.

Immunoprecipitation

In general, antibodies bind various epitopes. Immunoprecipitation used for phosphoprotein or phosphopeptide enrichment employs antibodies raised against phosphorylated amino acids. Phosphoamino acid-selective antibodies have been particularly well suited to identifying tyrosine-phosphorylated proteins (Imam-Sghiour et al. 2002; Lind et al. 2008; Pandey et al. 2000) or peptides (Rush et al. 2005; Villén et al. 2007; Zhang and Neubert 2006; <http://www.springerprotocols.com/cdp/search/searchResultPage?text=phospho+immunoprecipitation>). However, many commercially available phospho-selective antibodies against phosphoserine (pS) and phosphothreonine (pT)

have not given satisfactory results during phosphoprotein enrichment, although they are still useful for Western blotting (Grønberg et al. 2002). Moreover, these antibodies were targeted against the phosphoamino acid within the context of its surrounding residues. Consequently, the antibodies did not bind with the same efficiency to all pS/pT sites, and application of more types of anti-pS/pT antibodies was needed to ensure the detection of all phosphorylation sites. In spite of these difficulties, several antibodies compatible with immunoprecipitation were found, and therefore this technique was also applied to the studies of serine/threonine phosphorylation (Grønberg et al. 2002).

Immunoprecipitation is unsuitable for large-scale studies covering the whole phosphoproteome. Antibodies are phosphoamino acid-specific, so it is necessary to perform several parallel immunoprecipitation reactions (one with anti-pY antibodies, and at least one with anti-pS/pT antibodies). Due to this, they are mostly used when one particular phosphorylated amino acid is being searched for. This advantage becomes especially apparent in the case of tyrosine phosphorylation, since phosphotyrosine is notably less common than phosphoserine and phosphothreonine (Molina et al. 2007). Its pull down by specific antibodies can therefore improve the sensitivity of the enrichment process.

Immobilized metal affinity chromatography (IMAC)

Immobilized metal affinity chromatography employs a matrix composed of resins with associated metal ions (Andersson and Porath 1986; <http://www.springerprotocols.com/cdp/search/searchResultPage?text=IMAC>). These metal ions are positively charged, and hence can catch negatively charged phosphate groups.

For both phosphoprotein and phosphopeptide enrichment, the most widely used resins were iminodiacetic acid (IDA, Fig. 3) and nitrilotriacetic acid (NTA, Fig. 3; Neville et al. 1997). For phosphopeptide enrichment, the metal ions that were used were, for instance, Fe^{3+} (Neville et al. 1997), Ga^{3+} (Posewitz and Tempst 1999), Zr^{4+} (Feng et al. 2007) and Ti^{4+} (Zhou et al. 2008). The results of a few

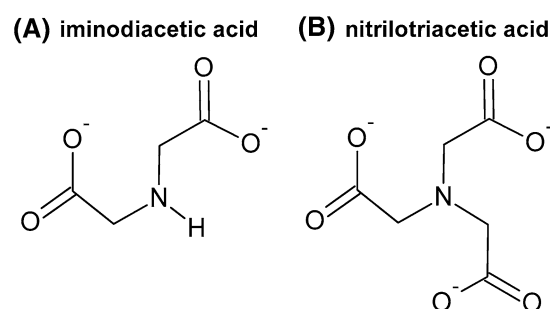


Fig. 3 Chemical formulas of IMAC resins

comparative phosphopeptide-enriching studies were not unequivocal. NTA combined with Fe^{3+} resulted in higher specificity compared with IDA (Neville et al. 1997), but in combination with Ga^{3+} , IDA was superior to NTA (Posewitz and Tempst 1999). Although the results obtained with Ga^{3+} ions were superior to those obtained with Fe^{3+} ions (Posewitz and Tempst 1999), most experimental data were obtained using Fe^{3+} -IMAC. Moreover, IMAC specificity is highly influenced by the purity of the ions used for matrix preparation, as shown for Fe^{3+} ions (Ye et al. 2010). We would suggest using Fe^{3+} ions for phosphopeptide enrichment, mainly due to the fact that they are most widely used, and consequently the protocols have been tested more. It is debatable whether protocols applying Fe^{3+} would work with the same efficiency for other ions (e.g. Ga^{3+}).

However, phosphoprotein-enriching protocols applied the following combinations: Fe^{3+} -NTA, Fe^{3+} -IDA, Ga^{3+} -NTA, Ga^{3+} -IDA (Collins et al. 2005; Dubrovskaya and Souchelnyskiy 2005; Machida et al. 2007) and Ni^{2+} -NTA (Lenman et al. 2008). It was assumed that IDA was more efficient than NTA and that Ga^{3+} ions were superior to Fe^{3+} ions (Collins et al. 2005), and this was subsequently verified (Machida et al. 2007). However, as the number of phosphoprotein-enriching studies is very limited, these conclusions are still questionable.

For sufficient IMAC phosphoprotein/phosphopeptide enrichment to occur, it is crucial to perform protein extraction properly in order to dispose of all traces of nucleic acids (Li et al. 2009b). The contaminating nucleic acids would bind to the IMAC matrix and thus result in phosphopeptide losses. However, the necessity for nucleic acid removal is not confined to proteomics, since it is well established that nucleic acids cannot be present during isoelectric focusing either.

Selection of the protease has a significant impact on phosphopeptide IMAC specificity. Trypsin is the most commonly used specific protease. Tryptic peptides may contain more than just one acidic amino acid since trypsin cleaves behind lysine or arginine (except when either is followed by proline). Protease glu-C cleaves behind glutamic or aspartic acid, giving rise to peptides with just one acidic amino acid (if mis-cleaved peptides are not taken into account). Since acidic peptides are likely to bind non-specifically to the chromatography matrix, the application of glu-C accordingly improved the method specificity (Seeley et al. 2005).

Blocking of acidic carboxyl groups by methylesterification (Ficarro et al. 2002) can also improve the IMAC selectivity. Although the peptide modification was satisfactorily applied in the case of a complex yeast extract, this did not represent an optimal treatment. Esterification was usually not complete, so several acidic peptides remained

unmodified (Trinidad et al. 2006). Moreover, side reactions such as glutamine or asparagine deamination and their subsequent methylation had also occurred, so the sample complexity increased (Larsen et al. 2005). Since a number of additional steps were included in the process, the probability of the detection of less abundant phosphopeptides declined because they could be lost during these procedures. Many studies have avoided this step (Kokubu et al. 2005; Tsai et al. 2008), thus simplifying the sample handling and avoiding the possible reaction incompleteness; we therefore suggest skipping this step and using an alternative IMAC protocol with optimized incubation buffer (see below).

For improved phosphopeptide enrichment, the incubation buffer had to be acidified to pH 2–2.5 with an organic acid—e.g. acetic acid (Posewitz and Tempst 1999) or TFA (Kokubu et al. 2005). The principle of the best IMAC specificity at this pH range was based on the different acid dissociation constant (pKa) between acidic amino acids and the phosphate group. The phosphate residue had a pKa of 2.1, whereas that for glutamic and aspartic acid was 3.65 and 4.25, respectively (Kokubu et al. 2005). These specific values were valid for the sole residues; when part of a peptide was involved, the respective values could shift up or down. If the pH of the loading buffer lay between the pKa values of acidic amino acids (aspartic and glutamic acid) and phosphoric acid (i.e. pH 2–2.5), most acidic amino acids were protonated. Their protonation masked the negative charge of carboxy-groups and therefore they were not likely to bind to the positively charged chromatography matrix. Most phosphate moieties, on the other hand, were deprotonated, so they could exhibit a negative charge that would enable binding to the positive metal ions. It should be noted that complete protonation of all peptides containing acidic amino acids can be achieved only by using highly acidic pH (pH < 1–1.5), whereas only a pH higher than 3 guaranteed complete phosphopeptide deprotonation. Consequently, the highest sensitivity could be achieved at pH > 3, whereas the maximal selectivity would be exhibited at an acidic pH (pH < 1–1.5). Therefore, the pH of the incubation buffer should be considered as a compromise between the specific antagonistic demands (Tsai et al. 2008). However, the higher pH (5.5–8.0) of the incubation buffer was applied for phosphoprotein enrichment since the lower pH (that is used for phosphopeptide enrichment) caused protein precipitation (Machida et al. 2007).

The optimal incubation buffer was determined on the basis not only of an optimal pH but also in terms of optimal ionic strength and composition. The addition of 0.5 M sodium chloride (NaCl) into the incubation buffer for phosphoprotein enrichment improved its specificity. Superoptimal NaCl concentration resulted in excessive ionic strength of the solution and weakened the interactions

between phosphates and gallium ions. On the other hand, suboptimal NaCl concentration was insufficient to reduce the binding non-specificity (Machida et al. 2007).

The presence of NaCl, on the other hand, did not reduce the non-specificity during phosphopeptide enrichment (Lee et al. 2007; Ndassa et al. 2006). Comparing the influence of different acids on the specificity during phosphopeptide enrichment resulted in the following order being determined (listed from the most to the least specific): trifluoroacetic acid (TFA) \geq hydrochloric acid $>$ formic acid $>$ acetic acid. Each particular acid had to be added in the correct concentration. Suboptimal concentration was not able to prevent the non-specific binding of acidic peptides, whereas superoptimal concentration prevented both acidic-peptide and phosphopeptide binding. It was believed that the ability to increase the binding specificity positively correlated with acid strength (Kokubu et al. 2005). Later, an alternative explanation of TFA superiority was addressed (Tsai et al. 2008), wherein it was suggested that the fluorine atoms were responsible for the selectivity rather than the higher strength of TFA compared with acetic acid. Finally, it was stated that stronger acids (such as fluoroacetic acid) could result in phosphopeptide losses, so it was preferable to use acetic acid instead (Tsai et al. 2008). A further test of an alternative incubation buffer with a pH that was higher than usual (above 3.5) resulted in increased sensitivity, whilst the selectivity was not affected if a sufficient concentration of acetic acid (6%) was added (Tsai et al. 2008). Only at a higher pH were the phosphate moieties sufficiently deprotonated; hence, lower amounts of phosphopeptides were lost during the procedure. The deprotonated carboxyl-groups of glutamic and aspartic acids had to cope with acetic acid, the presence of which led to the sufficient exclusion of acidic peptides.

Possible non-specific hydrophobic interactions during phosphopeptide enrichment were repeatedly blocked by acetonitrile (Ficarro et al. 2002; Ndassa et al. 2006; Posewitz and Tempst 1999). It was proven to be superior to sole methanol, ethanol or acetone (Kokubu et al. 2005). Moreover, a higher concentration of acetonitrile improved the phosphate deprotonation and acidic amino acid protonation. It also intensified the measured phosphopeptide spectra and thus enabled the identification of less abundant phosphopeptides (Ye et al. 2010).

The presence of several compounds in the phosphopeptide enrichment incubation buffer interfered with IMAC sensitivity and/or selectivity. Detergents caused greater abundance of multiply phosphorylated peptides in the enriched fraction (Jensen and Larsen 2007). Such changes in the phosphopeptide spectra that were obtained were undesirable since some weakly binding singly phosphorylated peptides were lost. Moreover, IMAC was incompatible with EDTA (Jensen and Larsen 2007). It not

only chelated magnesium and calcium ions but also bound the ions from the IMAC matrix. Unlike their ionized forms, the stripped resins were not able to bind phosphopeptides or phosphoproteins.

Once the phosphopeptides or phosphoproteins are bound to the chromatography matrix, it is of key importance to elute the vast majority of bound phosphopeptides/proteins (ideally all of them) out of the matrix. Moreover, the elution buffer should be compatible with subsequent MS. Phosphoproteins were eluted at various pH levels, ranging from weakly acidic (pH 6.8; Dubrovskaya and Souchelnyskiy 2005) to alkaline (pH 8.0; Machida et al. 2007).

IMAC-bound phosphopeptides, on the other hand, were originally eluted by phosphoric acid, ions of which had to be removed before MS could be carried out (Posewitz and Tempst 1999). Due to this incompatibility between phosphoric acid and MS, elution by ammonium hydroxide (pH 10.5) was carried out instead. This elution was found to be insufficient since several phosphopeptides were retained, and so a new buffer containing 2,5-dihydroxybenzoic acid (DHB) was introduced (Hart et al. 2002). DHB is one alternative that can be used in the form of a matrix-associated laser desorption/ionization mass spectrometry (MALDI-MS) matrix, so the eluted phosphopeptides can be directly loaded onto the steel MALDI target. The multiply phosphorylated peptides were not eluted by this buffer, so a second elution with ammonium dihydrogenphosphate was needed. Next, all phosphopeptides were eluted in one step using a mixture of DHB and phosphoric acid (Stensballe and Jensen 2004). Although DHB is highly suitable for elution before MALDI-MS, it is incompatible with electrospray ionization mass spectrometry (ESI-MS). Therefore, a new elution buffer containing phosphoric acid and acetonitrile was optimized for the enrichment prior to ESI-MS (Imanishi et al. 2007).

Although the flow-through from IMAC one-step phosphopeptide enrichment was discarded in most experiments, it was shown to contain a number of phosphopeptides that had a weak affinity to the chromatography matrix. This problem was dealt with by a second enrichment being applied to the flow-through (Thingholm et al. 2008). The vast majority of multiply phosphorylated peptides were captured during the first IMAC, whilst most singly phosphorylated ones were seized during the second enrichment, achieved with titanium dioxide. These results were in accordance with the increased amount of multiply phosphorylated peptides observed in several studies after one round of enrichment (Ficarro et al. 2002; Nousiainen et al. 2006). The second enrichment was less specific than the first one, and this was probably due to the absence of multiply phosphorylated peptides. The space on the matrix that would be occupied by them was vacant, so that weakly binding peptides (including several non-specific ones)

could also be caught. The different affinity to the chromatography matrix of singly and multiply phosphorylated peptides also enabled the step-wise elution in the SIMAC protocol (Thingholm et al. 2008); details of this will be given in the SIMAC section, below.

The advantages of IMAC phosphopeptide enrichment are mainly that it has a long tradition and a large amount of available data. Several possibilities for protocol improvement have been published, so it is now possible to adjust the protocol to the optimal level for the desired sample. On the other hand, a lower specificity in comparison with MOAC has been observed in several studies (Aryal and Ross 2010; Gates et al. 2010; Kweon and Håkansson 2006; Larsen et al. 2005; Zhou et al. 2007) even though the two methods have also been reported to have similar selectivity (Tsai et al. 2008). Furthermore, the incompatibility with detergents and EDTA is more severe in the case of IMAC compared with MOAC. In spite of these disadvantages, IMAC is still a widely used technique for phosphopeptide enrichment.

The main disadvantage of IMAC phosphoprotein enrichment, however, is its partial non-specificity, which results in its rare use at the level of phosphoproteins. This partial non-specificity was shown by phosphoprotein-specific ProQ Diamond staining and by the presence of several non-specific proteins that were identified in the enriched fraction (Collins et al. 2005). It showed significantly higher intensity while staining eluates compared with the flow-through, but in the eluate there were also proteins that were undetectable by ProQ Diamond and visible only after SyproRuby staining. It should be mentioned that the non-specificity that was concluded is not completely definite since it was assumed on the basis of only one study, and further data are lacking. One can speculate whether the combinations of buffers and chromatography matrices used were really optimal since still details of only a limited number of IMAC phosphoprotein-enrichment applications were published. However, further optimization of incubation and elution buffers can increase IMAC performance. A complex study comparing a wider range of protocols for phosphoprotein enrichment, ideally on the same model in the same laboratory, is needed.

Phos-Tag

Phos-Tag enrichment can be considered as an IMAC variation, and it was applied in the cases of both phosphoprotein and phosphopeptide enrichment. Its matrix is composed of Phos-Tag, chemically named 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olato dizinc(II) complex, that is anchored to agarose (Kinoshita et al. 2005; [\[Page?text=phos-tag\]\(#\)\). The Phos-Tag complex carries two zinc atoms that can accept two electrons each, whilst the phosphate moiety can donate two oxygen-bound electrons. Phosphoproteins or phosphopeptides can be captured by Phos-Tag since electrons can be shared by these compounds. Similarly, zinc ions were shown to be responsible for the phosphopeptide binding. EDTA treatment of Phos-Tag \(the removal of zinc atoms\) rendered Phos-Tag itself unable to bind phosphopeptides \(Kinoshita et al. 2005\).](http://www.springerprotocols.com/cdp/search/searchResult</p>
</div>
<div data-bbox=)

The incubation buffer for phosphoprotein enrichment had a nearly physiological pH value of 7.5, containing 0.1 M Tris-acetic acid and 1 M sodium acetate (Kinoshita-Kikuta et al. 2006). Sodium acetate probably increased binding specificity by reducing the non-specific interactions. NaCl at the same concentration did not work, unlike Ga^{3+} -IMAC, where even 0.5 M NaCl reduced the non-specificity (Machida et al. 2007). Again, the optimal elution buffer was required to release the vast majority of bound proteins out of the chromatography matrix. The elution buffers had the same pH value as the incubation buffer, and differed in only a few components. Although sodium acetate was proven to be efficient in reducing the non-specificity reaction, its ability to elute bound phosphoproteins was suboptimal, as some proteins were retained in the matrix after the wash. The yield of eluted phosphoproteins was higher when NaCl was present in the elution buffer.

On the other hand, the phosphopeptide Phos-Tag enrichment occurred in the incubation buffer that was free of both NaCl and sodium acetate (0.1 M Tris-acetic acid, pH 7.4; Kinoshita et al. 2005). This buffer was further improved by the addition of acetonitrile that, at a concentration of >40%, reduced the non-specific hydrophobic interactions and thus reduced the non-specificity (Nabetani et al. 2009). This was consistent with the role of acetonitrile in IMAC (see above). Phosphopeptides were released by various elution buffers and it is hard to conclude which of them resulted in optimal performance since they worked with similar efficiency and, as far as we know, were tested in only one study (Kinoshita et al. 2005).

A neutral pH value of the Phos-Tag incubation buffer is advantageous since it can help the complete phosphopeptide/phosphoprotein deprotonation and thus improve the sensitivity of the method. Moreover, proteins enriched under these conditions could be more easily further used for experiments in which biological activity of proteins is of key importance. Unfortunately, acidic amino acids are also deprotonated, resulting in their non-specific binding. The risk of higher non-specificity seemed to be reduced with a higher concentration of acetonitrile. It is debatable whether the detergent-free incubation of proteins at room temperature would also be compatible with other systems (e.g. plant samples). However, this technique was used in

only a limited number of studies, making it premature to draw any solid conclusions.

Metal oxide affinity chromatography (MOAC)

Unlike IMAC, the MOAC matrix is itself composed of metal oxides or hydroxides, and so the resin anchoring is not needed. The most commonly used MOAC phosphopeptide enrichment strategy employed titanium dioxide (TiO_2 ; <http://www.springerprotocols.com/cdp/search/searchResultPage?text=TiO2>), whereas phosphoprotein enrichment relied mostly on aluminium hydroxide ($\text{Al}(\text{OH})_3$). The alternative metal oxides—for instance, zirconium dioxide (ZrO_2), gallium oxide (Ga_2O_3), ferric oxide (Fe_3O_4), niobium oxide (Nb_2O_5), stannic oxide (SnO_2), hafnium dioxide (HfO_2) and tantalum oxide (Ta_2O_5)—were used only rarely, and will not be discussed in this text (Leitner 2010). These compounds are solid, insoluble in liquid buffers, and positively charged at an acidic pH (Tombácz 2009).

Titanium dioxide (TiO_2)

The TiO_2 affinity to organic phosphates has been known for quite a long time (Ikeguchi and Nakamura 1997; Matsuda et al. 1990). After pilot experiments with various organic compounds, TiO_2 was used to retain phosphopeptides during high-performance liquid chromatography (HPLC; Sano and Nakamura 2004) and to enrich phosphopeptides (Pinkse et al. 2004).

First of all, the physical properties of the TiO_2 particles themselves have to be taken into account. Different manufacturers (Cantin et al. 2007) and/or different crystallinity (Imami et al. 2008) of these particles resulted in different degrees of specificity. Such differences could lead to contradictory results being obtained in different laboratories, and thus to different interpretations. It was advantageous to use mesoporous particles instead of smooth ones since they had a higher surface-to-volume ratio and thus greater binding capacity (Tang et al. 2010).

Once the correct type of particles was chosen, it was important to apply the optimal ratio for the titanium-dioxide particles and the peptide amount (Li et al. 2009a). Up to a certain concentration, the number of identified phosphopeptides increased in steps. Within a particular range of concentrations, the yield of identified phosphopeptides was high and constant. Once this concentration was exceeded, the yield declined rapidly.

As with IMAC, the non-specific peptide binding was reduced by methyl-esterification of carboxyl groups prior to the enrichment (Pinkse et al. 2004). The methyl-esterification had its limitations, as discussed in the IMAC section, and so a novel protocol developed by Larsen et al.

(2005) optimizing the incubation buffer was used, eliminating the need for methylesterification.

Again, the pH value of the loading buffer for phosphopeptide enrichment was between 2.7 and 2.9 to enable protonation of acidic amino acids and deprotonation of phosphate groups (see above in IMAC section). The buffer contained 60% acetonitrile acidified by acetic acid (0.1–0.25 M; Pinkse et al. 2004). The method was more selective if the buffer was acidified by TFA instead (Jensen and Larsen 2007; Larsen et al. 2005), and the ability to block non-specific binding again decreased in the following order: TFA > fluoroacetic acid > acetic acid (Aryal and Ross 2010; Jensen and Larsen 2007).

Buffer acidification itself was not sufficient to reduce the binding non-specificity during phosphopeptide enrichment. Therefore DHB alone (Hsieh et al. 2007; Larsen et al. 2005) or in combination with octanesulphonic acid (Mazanek et al. 2007; Mazanek et al. 2010) was added to the incubation buffer. The amount of non-specifically binding peptides that were detected negatively correlated with DHB concentration. The fact that even higher concentrations of DHB did not compete with phosphopeptide binding was probably due to the different geometry of phosphopeptide binding compared with non-specific peptide binding (Fig. 4). This hypothesis was further supported by the inability of phosphoric acid (mimicking a phosphopeptide) to block non-specific binding and by its ability to block phosphopeptide binding. Various acids were tested, and their suitability for acidic-peptide exclusion decreased as follows: DHB = salicylic acid = phthalic acid > benzoic acid = cyklohexanecarboxylic acid > phosphoric acid > TFA > acetic acid (Larsen et al. 2005).

Although DHB can improve the MOAC specificity, its co-elution with several phosphopeptides during liquid chromatography could decrease the number of identified phosphopeptides (Sugiyama et al. 2007). To avoid the chromatography complications caused by traces of DHB, another washing step should be included (Mazanek et al. 2010). DHB did not block the non-specificity in several other studies (Aryal and Ross 2010; Simon et al. 2008) where use of an optimized loading buffer without any additives seemed to be sufficient (Ahn et al. 2007; Aryal and Ross 2010). In general, it seems wise to include some additives to the buffer and not to rely on sole buffer

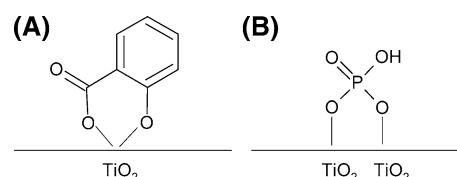


Fig. 4 The geometry of phthalic acid binding and phosphate binding to titanium dioxide

components to set up the correct ionic strength and pH level.

Due to DHB side effects as well as unequivocal results, other compounds thought to be useful for the improvement of selectivity were also tested. Aliphatic hydroxy acids were more hydrophilic and thus did not make any troubles during liquid chromatography. They decreased the amount of bound acidic peptides (Sugiyama et al. 2007), and lactic acid was demonstrated to be the best alternative (Sugiyama et al. 2007; Wu et al. 2007). It is noteworthy that imidazole, glutamate and aspartate in combination (used in a standard phosphoprotein or phosphopeptide $\text{Al}(\text{OH})_3$ -MOAC protocol [see below]) did not lead to a significant increase in specificity (Sugiyama et al. 2007). On the other hand, the efficiency of sole glutamic acid has also been reported (Wu et al. 2007). Moreover, an increasing concentration of glycolic acid was shown to positively correlate with the binding non-specificity (Aryal and Ross 2010). Such contradictory results make it difficult to choose the optimal conditions, and we suggest testing more contenders for non-specific binding in order to discover the optimal conditions for particular TiO_2 particles and sample.

Since various detergents were known to improve protein resuspension, their influence on phosphopeptide enrichment was tested. Some (e.g. SDS) were not only compatible with enrichment protocols but actually beneficial, since their addition blocked any binding of the phosphopeptide to plastic tubes and/or tips and thus reduced peptide losses (Jensen and Larsen 2007). Phosphatase inhibitors were applied in order to protect the phosphorylated proteins and/or peptides before dephosphorylation by phosphatases present in the sample took place. Although phosphatase inhibitors were beneficial for phosphate protection, they also decreased the enrichment specificity (Aryal and Ross 2010).

Up to now, phosphopeptide enrichment has been discussed. However, in discussing phosphoprotein enrichment, two buffers will now be compared—namely, denaturing and native supplied with phosphatase inhibitors (Lenman et al. 2008). Every standard phosphoprotein was captured exclusively under different conditions (i.e. either native or denaturing). The application of each condition showed different phosphoproteomic spectra, in complex samples as well. Although Lenman et al. (2008) considered the difference between native and denaturing conditions, we would also point to the different composition of these two buffers. Buffer content could influence what protein fraction would be extracted (Sheoran et al. 2009). Moreover, phosphatase inhibitors (present in the native buffer) could decrease the method specificity as it did during phosphopeptide enrichment (Aryal and Ross 2010).

The release of the bound peptides during phosphopeptide enrichment was carried out by application of 250 mM

ammonium bicarbonate (pH 9.0; Pinkse et al. 2004), but several phosphopeptides (mainly multiply phosphorylated) remained entrapped in the matrix (Larsen et al. 2005). Ammonium hydroxide (pH 10.5) improved the efficiency of phosphopeptide elution (Larsen et al. 2005; Sugiyama et al. 2007). Since the elution buffer still had reserves, the influence of various amines and salts was tested (Kyono et al. 2008). Pyrrolidine turned out to be the most efficient. It was shown that the pH itself was not responsible for the improved elution efficiency, but, rather, the properties of the eluting compounds had a role to play. Different eluents gave different phosphopeptide spectra, showing the usefulness of carrying out sequential elution with various elution buffers rather than relying on just one (Kyono et al. 2008). The different affinity to the matrix of singly and multiply phosphorylated peptides enabled their separation by stepwise elution (Simon et al. 2008). The first elution was done by 100 mM triethylammonium bicarbonate (pH 8.5), whilst the second one was achieved by 3% ammonium hydroxide (pH 11.5). A higher proportion of non-specific peptides was found in the second eluate, showing that several acidic peptides could bind more tightly to the chromatography matrix in comparison with monophosphorylated peptides. The possibility of eluting the phosphopeptides stepwise has recently been shown to a greater extent (Park and Maudsley 2011). Such stepwise elution is beneficial for reducing sample complexity and thus for increasing the probability of phosphopeptide identification.

TiO_2 -MOAC is frequently used for phosphopeptide enrichment, and a number of protocols are available. On several occasions TiO_2 -MOAC has been shown to be superior to IMAC in either selectivity (Aryal and Ross 2010; Kweon and Håkansson 2006; Larsen et al. 2005) or sensitivity (Hsieh et al. 2007). Although MOAC seems to be more promising than IMAC, it still faces problems with the specificity issue.

On the other hand, a TiO_2 protocol for phosphoprotein enrichment was not verified on other models, and it is therefore arguable whether the optimal protocol was used. Another issue is the enrichment specificity: as with IMAC phosphoprotein enrichment, it was still not clear whether the method was specific enough.

Aluminium hydroxide ($\text{Al}(\text{OH})_3$)

Aluminium hydroxide is a prevailing matrix that has been used for MOAC phosphoprotein enrichment, and its feasibility for phosphopeptide enrichment has also been shown (Wolschin et al. 2005). The original protocol for phosphoprotein enrichment was set up according to the results achieved with a standard protein mixture composed of both phosphorylated and non-phosphorylated proteins (Wolschin et al. 2005). A high concentration of urea

together with CHAPS in the incubation buffer led to protein denaturation that resulted in the reduction of the non-specificity. Particular protein domains in their native conformations could be responsible for non-specific binding, so their denaturation partly prevented this undesirable effect from occurring. On the other hand, it is possible that native conditions might lead to the identification of additional phosphoproteins, since denaturing and native conditions differed significantly during TiO₂ phosphoprotein enrichment (Lenman et al. 2008). Imidazole in the incubation buffer mimicked histidine (having an imidazole ring in its structure), and so reduced the non-specific binding of higher-affinity histidine-containing peptides. Acidic amino acids showed a strong affinity to aluminium hydroxide, probably due to their negative charge. Their non-specific binding was greatly reduced by the addition of their sodium and potassium salts into the buffer. The optimal concentration of these salts was set up as 0.2 M each (Wolschin et al. 2005), and this concentration was satisfactorily applied for complex protein samples like *Arabidopsis* leaf and seed proteins, as well as *Chlamydomonas reinhardtii* (Wolschin and Weckwerth 2005; Wolschin et al. 2005). However, the 0.2 M concentration of acidic amino acid salts led to very high specificity on the one hand, but caused losses of weakly bound proteins on the other, in the case of *Craterostigma plantagineum* leaves (Röhrig et al. 2008).

In this study, several concentrations of acidic amino acid salts were tested. The leaf protein extract was enriched by MOAC using an aluminium hydroxide matrix, and the original extract, the flow-through and the phosphoprotein-enriched eluate were separated by SDS-PAGE. The gel was then stained with phosphoprotein-specific ProQ Diamond stain and by Coomassie that displayed the total proteins. The 0.2 M concentration resulted in quite a low concentration of proteins being present in the eluate (Fig. 5). Since the flow-through contained quite a high concentration of proteins, even though most of them were non-phosphorylated, there remained a possibility that less abundant and/or low-affinity phosphoproteins were lost in this fraction. In order to improve the method sensitivity, 0.05 and 0.01 M concentrations of acidic amino acid salts were tested. These concentrations resulted in a very great abundance of protein in the eluate, but almost no proteins were present in the flow-through. This showed that this protocol was not selective enough, even though its sensitivity was very high. Such lower specificity was probably caused by a suboptimal concentration of acidic amino acid salts that were thus incapable of competing with non-specifically bound proteins. The original extract had very similar spectra in all three cases, ruling out the possibility of differences originating from a different starting sample. The compromise between high specificity and sensitivity was established at a 0.1 M concentration of acidic amino

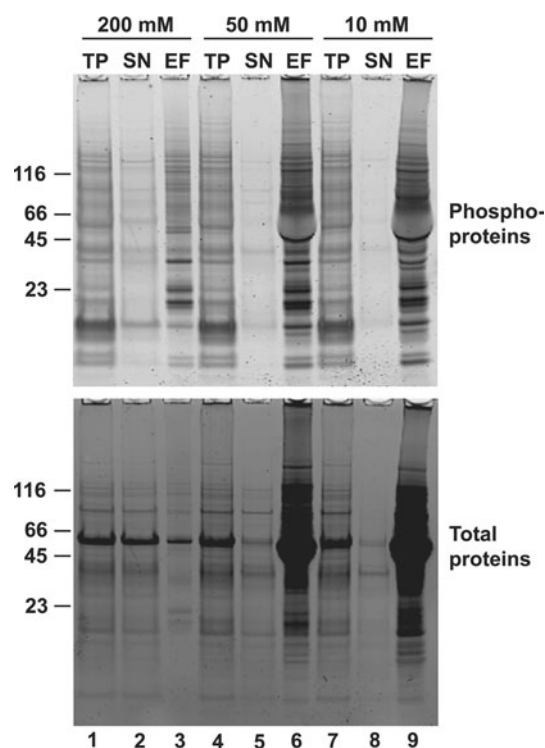


Fig. 5 The dependence of the selectivity and sensitivity of Al(OH)₃-MOAC on the concentration of acidic amino acid salts in incubation and washing buffers. The concentrations tested were 200, 50 and 10 mM for each acidic amino acid (glutamic acid and aspartic acid). Proteins (5 mg) in incubation buffer before (TP), and after incubation with the aluminium hydroxide matrix (SN) together with TCA precipitated candidate phosphoproteins from the eluted fraction (EF, corresponding to 0.5 mg of total proteins each lane) were analyzed by NuPAGE 4–12% Bis–Tris gels. Gels were stained with Pro-Q Diamond to detect phosphoproteins, and subsequently with SYPRO Ruby to visualize total proteins. The original 200 mM concentration showed high selectivity but quite low sensitivity since a number of phosphoproteins ended up in the flow-through. On the other hand, 50 and 10 mM concentrations showed high sensitivity since no phosphoproteins remained in the flow-through, but the selectivity was too low because almost all the proteins (not only the phosphoproteins) appeared in the eluate. (Röhrig et al. 2008. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)

acid salts in the incubation buffer and at a 0.15 M concentration in the washing buffer.

However, the phosphopeptide enrichment by aluminium hydroxide was performed in an almost identical incubation buffer to that of the phosphoprotein enrichment, only without urea (Wolschin et al. 2005). Although glutamate, aspartate and imidazole improved the specificity of the original protocol applied for casein tryptic peptides, they were not able to compete with non-specific peptides derived from standard proteins, and so a number of non-phosphorylated peptides were also present in the eluate (Sugiyama et al. 2007).

The elution buffer for the phosphoprotein enrichment was composed of 100 mM potassium pyrophosphate

(pH 9.0) and 8 M urea. The pyrophosphate elution buffers were more efficient than the phosphate ones. The high elution efficiency was necessary, especially for phosphoproteins carrying more phosphates, such as α -casein, which had eight phosphates (Wolschin et al. 2005). A 100 mM potassium pyrophosphate concentration was originally set up as optimal (Wolschin et al. 2005), but to avoid protein retention in the matrix, it was increased to 200 mM (Röhrig et al. 2008). On the other hand, the phosphopeptide elution was carried out in two steps, with an increasing concentration of sodium pyrophosphate.

MOAC specificity for phosphoprotein enrichment was tested in several ways. The non-phosphorylated standard proteins were excluded from the eluate, whereas the phosphorylated ones were captured by the matrix (Wolschin et al. 2005). Eight standard proteins could not cover the whole complexity of the biological samples, so it remained a possibility that some proteins could bind non-specifically. Another sign of MOAC specificity occurred in μ LC-ICP-MS (Krüger et al. 2007). The phosphate-to-sulfur ratio was higher in the eluate compared with the crude extract, and this was in agreement with the expected presence of phosphoproteins in the eluate. Last but not least, another proof of MOAC specificity was given by alkaline phosphatase dephosphorylation of the enriched fraction that showed a lower signal on ProQ Diamond staining after the dephosphorylation than it did before (Röhrig et al. 2008). Since ProQ Diamond is very sensitive and also able to faintly stain non-phosphorylated proteins (Steinberg et al. 2003), it is possible that the observed signal after dephosphorylation was caused by the presence of non-phosphorylated proteins. On the other hand, it is possible that alkaline phosphatase did not remove all the phosphates due to its substrate specificity (Morton 1955).

Collectively, phosphoprotein-enriching $\text{Al}(\text{OH})_3$ -MOAC did not seem to be less specific than other phosphoprotein-enriching affinity methods (IMAC and TiO_2 -MOAC). An advantage of its having been tested on several models is that many phosphoproteins were identified. However, its possible non-specificity must be regarded as a disadvantage.

On the other hand, the phosphopeptide enrichment with the aluminium hydroxide matrix was not widely used, and this prevents a broader discussion here about the method efficiency. Moreover, it is debatable whether the tested conditions would also be optimal for complex samples.

Sequential elution from IMAC (SIMAC)

SIMAC is a phosphopeptide-enriching method combining both MOAC and IMAC (Thingholm et al. 2008). It results in the separation of multiply and singly phosphorylated

peptides. IMAC enrichment was first performed according to Kokubu et al. (2005); singly phosphorylated peptides were eluted by an acidic buffer (1% TFA, 20% acetonitrile, pH 1.0), whereas multiply phosphorylated peptides were released by an alkaline solution (ammonium hydroxide, pH 11.3). Singly phosphorylated peptides as well as flow-through peptides were loaded onto TiO_2 -MOAC (Jensen and Larsen 2007; Larsen et al. 2005). The second enrichment was performed because of the presence of non-phosphorylated peptides in the first eluate, and due to the presence of phosphorylated peptides in the flow-through. A variation of SIMAC combining both IMAC and MOAC on the flow-through was repeatedly satisfactorily applied to various samples (Carrascal et al. 2008; Rampitsch et al. 2010). Again, it was worthwhile to re-enrich the flow-through.

The combination of MOAC and IMAC strengthened binding selectivity as well as sensitivity. SIMAC led to the identification of a greater amount of phosphopeptides than MOAC itself and was more efficient, especially for multiply phosphorylated peptide enrichment (Thingholm et al. 2008). An advantage is that the combination of MOAC and IMAC broadened the phosphopeptide spectrum. The sequential elution is another advantage, since having a greater amount of less complex phosphopeptide fractions heightened the probability of their ionization and identification by MS. Taken together, the protocols combining more methods and re-enriching the flow-through can be considered promising.

Polymer-based metal ion affinity capture (PolyMAC)

Recently, an alternative affinity technique, PolyMAC, was introduced for phosphopeptide enrichment (Iliuk et al. 2010). The phosphopeptide-capturing compound was composed of a soluble dendrimer, to which two types of side group were bound (Fig. 6). The majority of side chains contained titanium atom, enabling the phosphopeptide binding that occurs in the soluble phase. Subsequently, the PolyMAC reagent and the bound phosphopeptide(s) were captured via the side chains of the second type of side

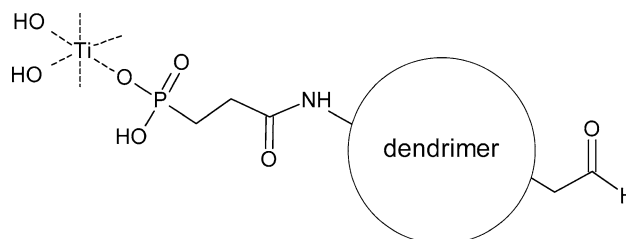


Fig. 6 The two types of side chains emerging from the PolyMAC dendrimer. Note that in reality many of the side chains are present with prevailing titanium-containing chains. The chain containing a titanium atom binds phosphopeptides, whereas the aldehyde group enables the conjugation with the solid-phase beads

group (in the original study, the aldehyde group) onto solid-phase beads containing hydrazide groups. The non-phosphorylated peptides were washed out and the phosphopeptides subsequently eluted.

This method seemed very promising, since the phosphopeptide binding in the soluble phase was more efficient than the binding to solid-phase TiO₂ or to IMAC resins, so the phosphopeptide recovery was higher. Moreover, the selectivity and reproducibility of this method were greater in comparison with IMAC and MOAC. Another advantage was that the dendrimer could be synthesized with a number of alternative side groups, and accordingly the fishing on the solid phase could be performed by several alternative reactions. Also, the phosphopeptide-catching titanium atom could be substituted with iron. However, the PolyMAC method was only published recently and thus has not yet been widely tested, so its presumed superiority to MOAC and IMAC cannot yet be said to be based on solid evidence.

Hydroxyapatite chromatography

Another chromatography approach for phosphopeptide enrichment used hydroxyapatite as a matrix (Mamone et al. 2010). Hydroxyapatite affinity to phosphate moieties has been known for years, and it was used, for instance, in the separation of differentially phosphorylated forms of casein (Addeo et al. 1977). Hydroxyapatite is a crystalline compound with the summary formula Ca₁₀(PO₄)₆(OH)₂. For phosphopeptide enrichment, the loading buffer contained 20 mM Tris–Cl (pH 7.4). On the other hand, 1 M K₂HPO₄ (pH 7.4) decreased the ability of singly phosphorylated peptides to bind, and so was not further used. This was probably caused by the competitive binding of hydrogen-phosphate ions to hydroxyapatite. When washing steps were included, the non-specific binding was reduced (Mamone et al. 2010). Phosphopeptides can be separated according to their phosphorylation level by sequential elution with an increasing concentration of potassium hydrogen phosphate in the elution buffer (Mamone et al. 2010). Alternatively, the bound phosphopeptides can be directly subjected to MS measurements. By omitting the elution steps, the sample handling time is shortened and sample losses are less likely to occur (Pinto et al. 2010). The method worked for phosphoprotein enrichment, as was shown for casein and egg shell proteins (Pinto et al. 2010).

Although the method seemed very efficient when applied to both phosphoproteins and phosphopeptides (especially to multiply phosphorylated peptides), it was not verified on a wider spectrum of models. It is questionable, too, whether it would exhibit similar efficiency with more complex samples.

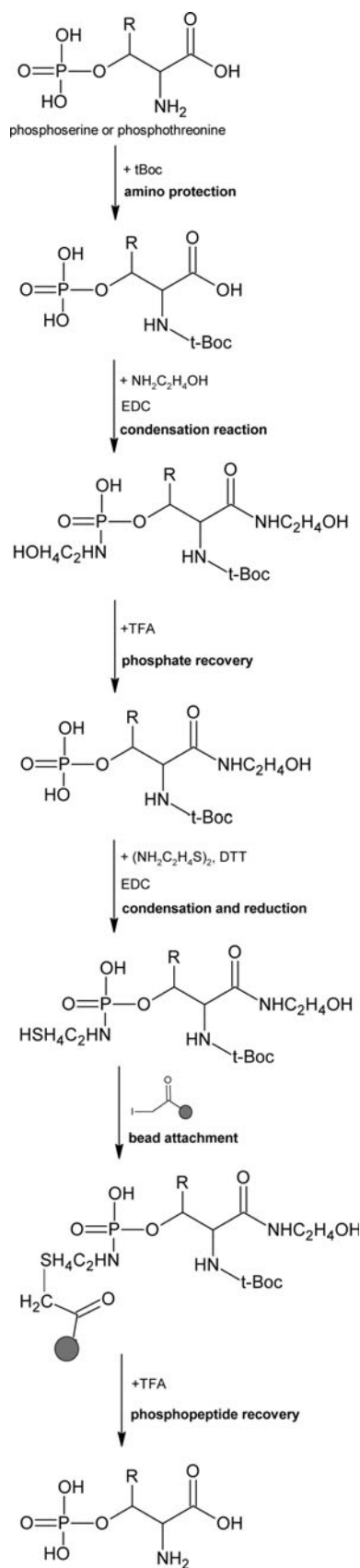
Enrichment by phosphopeptide chemical modification

Several methods were introduced for phosphopeptide enrichment by chemical modification—for example: carbodiimide condensation coupled with bead fishing (Zhou et al. 2001); β -elimination coupled with a Michael addition (Oda et al. 2001); oxidation–reduction condensation (Warthaka et al. 2006); α -diazo substituted resin (Lansdell and Tepe 2004); and carbodiimide condensation using a dendrimer (Tao et al. 2005). In general, these methods provide greater specificity than the affinity-based techniques (IMAC, MOAC and their variations). On the other hand, the reactions are often not fully completed, and several phosphopeptides can therefore remain undetected. Moreover, the reaction is mostly performed in several steps, so throughout the procedure part of the sample is necessarily lost during handling.

Carbodiimide condensation coupled with bead fishing

The first method that was used for phosphopeptide enrichment by chemical modification was carbodiimide condensation coupled with bead fishing (Zhou et al. 2001). This is a technique consisting of many reaction steps (Fig. 7). First of all, the peptide amino groups are protected by *t*-butyl-dicarbonate (tBoc) in order to prevent side condensation reactions. After this initial step, the ethanolamine reacts with phosphate and carboxylic groups with the help of carbodiimide (*N,N*-dimethylaminopropyl ethyl carbodiimide, EDC) catalyzation. Phosphate groups are then regenerated by cleavage in the acidic environment of TFA. The regenerated phosphate group is subjected to cystamine attachment. The cystamine reduction by dithiothreitol (DTT) results in terminal thiol (–SH) groups being reduced and prepared for subsequent reaction. Thiol groups of modified phosphopeptides react with iodoacetyl groups attached to solid beads. The unbound peptides can be washed out. Finally, the captured phosphopeptides are regenerated by TFA that cleaves the phosphoramidate bonds as well as the protecting tBoc group.

This method has a very poor recovery rate for phosphopeptides, and this can cause losses of less abundant phosphopeptides, which can be considered a disadvantage of this method. Such losses are likely due to many steps being performed. Not every step is completed with one-hundred-percent efficiency, so the increase in sample complexity is also a potential risk of this method. We would not use this method, since there are better and simpler chemical modification methods. On the other hand, this method works with the same efficiency for phosphoserine, phosphothreonine and phosphotyrosine, and this should be regarded as an advantage.



◀ **Fig. 7** Schematic workflow of carbodiimide condensation coupled with bead fishing. The R-group is a hydrogen atom (–H) in a serine and methyl group (–CH₃) in a threonine. *t*Boc—*t*-butyl-dicarbonate, EDC—*N,N'*-dimethylaminopropyl ethyl carbodiimide, TFA—trifluoroacetic acid

β -elimination coupled with a Michael addition

This phosphopeptide-enriching method relies on β -elimination coupled with a Michael addition (Oda et al. 2001; Thaler et al. 2003). To avoid side reactions on thiol groups, cysteine was oxidized with peroxyformic acid. After cysteine side chains were blocked, β -elimination of phosphate groups was achieved by application of barium hydroxide (Fig. 8). Phosphoserine gave rise to dehydroalanine, whereas phosphothreonine changed into dehydro amino-2-butyric acid. The new double bond on the acid side chain enabled a Michael addition of propanedithiol. Thus a side chain with a terminal thiol group was attached to the treated amino acids. Through this group, the peptide was bound to dithiopyridine resin (Thaler et al. 2003). This resin can only bind modified phosphopeptides since cysteine was blocked during the initial step. The bound peptides were subsequently unbound by 2-mercaptoethanol or DTT, causing the thiol-group reduction. As an alternative to dithiopyridine resin, biotiny tag was attached to the peptide (Oda et al. 2001). The modified peptides were then captured by avidine through its strong affinity to biotin.

Unfortunately, β -elimination is unable to modify phosphotyrosine, so it remains undetectable by this method. Moreover, β -elimination causes modifications not only of phosphorylated serine and threonine but also of their *O*-glycosylated forms (Rusnak et al. 2004). In addition, some non-modified serine and threonine residues could also undergo this modification (Li et al. 2003). Due to this fact, it is unclear whether the captured peptide was formerly phosphorylated or glycosylated, and whether it was modified at all is not even completely certain. Another disadvantage of this method concerns significant sample losses as well as the reaction incompleteness that further increases the sample complexity.

Oxidation–reduction condensation

This protocol was used for both phosphopeptide and phosphoprotein enrichment (Warthaka et al. 2006). The procedure began with blocking of the hydroxyl (–OH) part of the carboxyl groups of both the C-terminus and acidic amino acids by methylesterification, since they would be also susceptible to oxidation–reduction (Fig. 9). Afterwards, the phosphogroups were condensed with glycine-conjugated Wang resin. The glycine amino group enabled the reaction with the phosphate group under the catalysis of

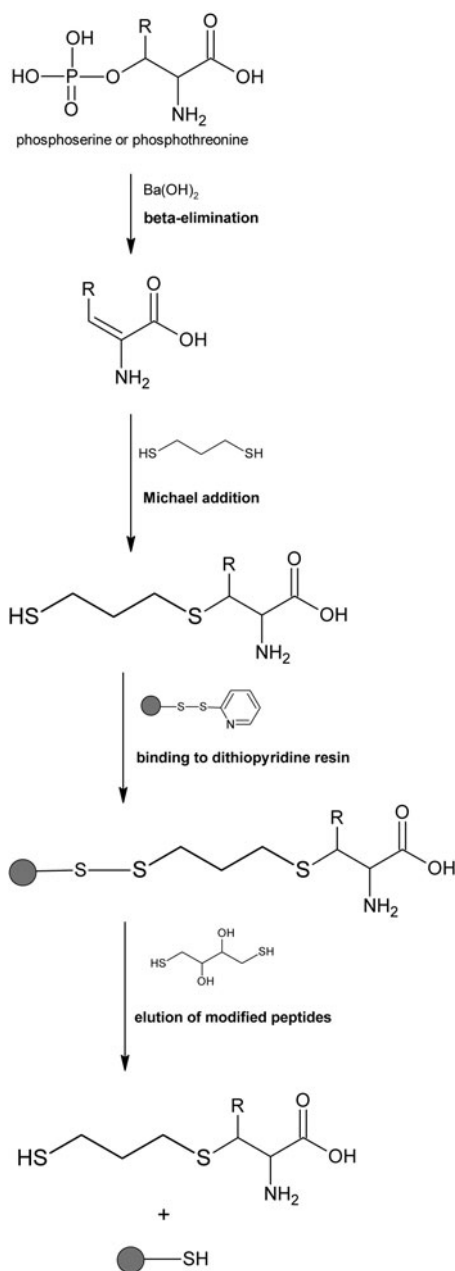


Fig. 8 Schematic workflow for phosphopeptide enrichment by β -elimination coupled with a Michael addition. The procedure for fishing of modified phosphopeptides by the dithiopyridine resin is depicted. The R-group is a hydrogen atom (–H) in a serine and methyl group (–CH₃) in a threonine

triphenylphosphine (PPh_3), 2,20-dithiopyridine (PySSPy), and *N,N'*-diisopropylethylamine (DIPEA). The resin–peptide complex represented a solid phase, so non-modified peptides were washed away. Subsequently, bound phosphopeptides were eluted under acidic conditions.

An advantage of this method is that it contains fewer steps than β -elimination and carbodiimide condensation coupled with bead fishing, so the peptide losses are less dramatic. Moreover, phosphoserine, phosphothreonine and

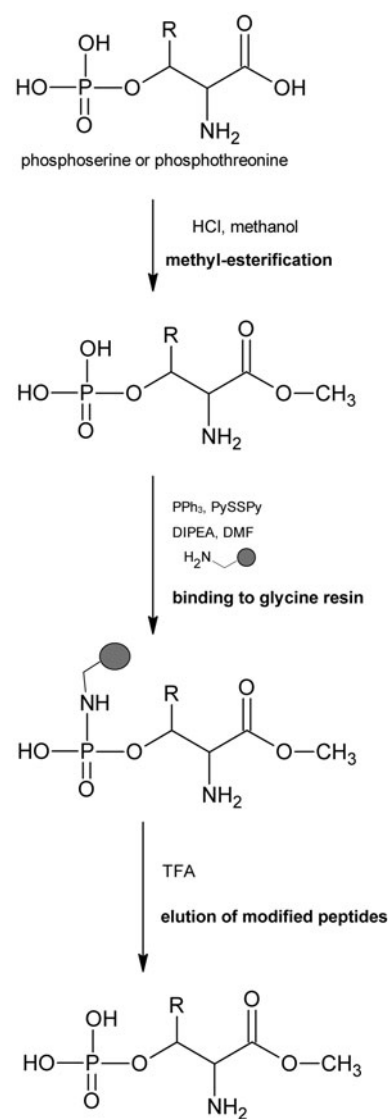


Fig. 9 Schematic workflow for phosphopeptide enrichment by oxidation–reduction condensation. The R-group is a hydrogen atom (–H) in a serine and methyl group (–CH₃) in a threonine. PPh_3 —triphenylphosphine, PySSPy—2,20-dithiopyridine, DIPEA—*N,N'*-diisopropylethylamine, DMF—dimethylformamide

phosphotyrosine could be enriched, but phosphotyrosine is modified to a lesser extent than the former phosphorylated amino acids (Warthaka et al. 2006). The disadvantage is that not every step is completed, that results in peptide losses, since only those that were completely converted could be detected.

α -Diazo resin

Another protocol for the phosphopeptide enrichment of all three phosphorylated amino acids employed

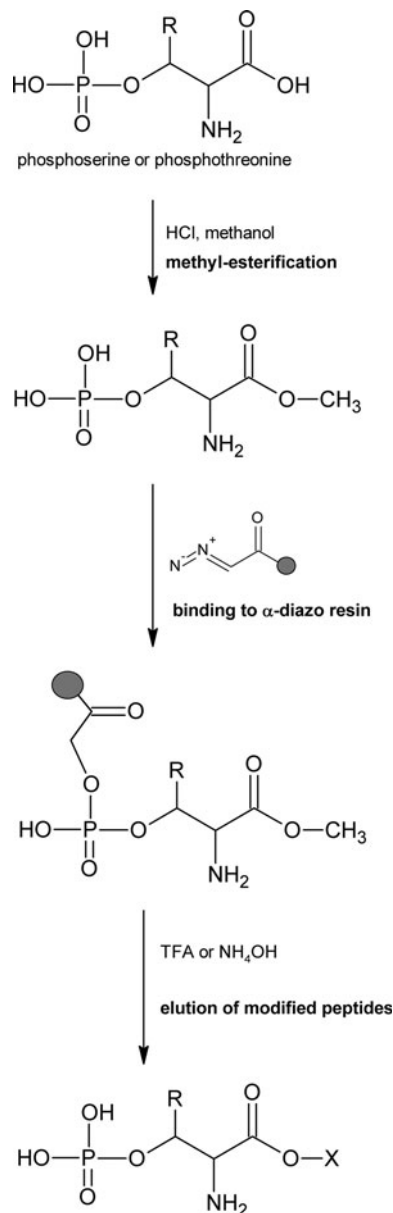


Fig. 10 Schematic workflow for α -diazo resin phosphopeptide enrichment. The R-group is a hydrogen atom ($-H$) in a serine and methyl group ($-CH_3$) in a threonine. TFA—trifluoroacetic acid. The X is the $-CH_3$ group if the peptide was eluted by trifluoroacetic acid, and the hydrogen atom if it was eluted by ammonium hydroxide

α -diazocarbonyl resins (Lansdell and Tepe 2004). As with the above-mentioned oxidation–reduction condensation, the first step was methylesterification of carboxyl groups to protect them before the subsequent reaction steps were taken (Fig. 10). Then the peptide reacted with the α -diazo resin, leading to its attachment. After the washing step, bound peptides were eluted either in TFA, that resulted in esterified-peptide elution, or in ammonium hydroxide, that led to the regeneration of the carboxyl group.

Again, advantages are that all amino acids can be enriched by this method and that it consists of fewer steps than

β -elimination coupled with a Michael addition or carbodiimide coupled with bead fishing. As with all of the above techniques, it is likely to result in peptide losses, since not all the steps will be completed with 100% efficiency.

Carbodiimide condensation using a dendrimer

The last modifying protocol for phosphopeptide enrichment to be discussed here is carbodiimide condensation (Tao et al. 2005). Again, the carboxyl group has to be blocked by methylesterification (Fig. 11). The esterified peptides are then subjected to a reaction with the dendrimer with a terminal amino ($-NH_2$) group on each chain. To enable the reaction to occur, carbodiimide (EDC) and imidazole activate the phosphopeptide that in turn reacts with the amino groups on the dendrimer. The bound proteins are then separated from the unbound non-phosphopeptides by a membrane-based filter device. The phosphopeptides are again eluted in the acidic pH. The advantages and disadvantages are the same as for α -diazo resin, except that the reaction is more likely to be completed since the dendrimer contains an excess of amino groups that favor the reaction taking place.

Enrichment by phosphopeptide precipitation

This approach is based on calcium phosphate precipitation from a solution (Gomori and Benditt 1953). First, the sample was resuspended in $NaHPO_4$ (Zhang et al. 2007). After the addition of calcium chloride, phosphopeptides together with calcium phosphate were precipitated. Subsequently, it was possible to pellet phosphorylated peptides by centrifugation. After the washing step, the pellet was resuspended in an appropriate buffer. In the original study, the phosphopeptide concentration was very low and some phosphopeptides could not be identified. When Fe^{3+} -IMAC was coupled with phosphopeptide precipitation, more phosphopeptides were detected.

The advantages were that the combination of the precipitation method with IMAC led to a higher specificity than with just IMAC, and that this method was also applied to complex samples (Xia et al. 2008; Zhang et al. 2007). However, its specificity remains uncertain, since several acidic peptides were thought to be present in the enriched fraction as well (Zhang et al. 2007).

Prefractionation methods

The prefractionation methods usually result not specifically in purifying phosphopeptide fractions, but rather in the separation of complex peptide samples into fractions with a higher representation of phosphopeptides. It is important

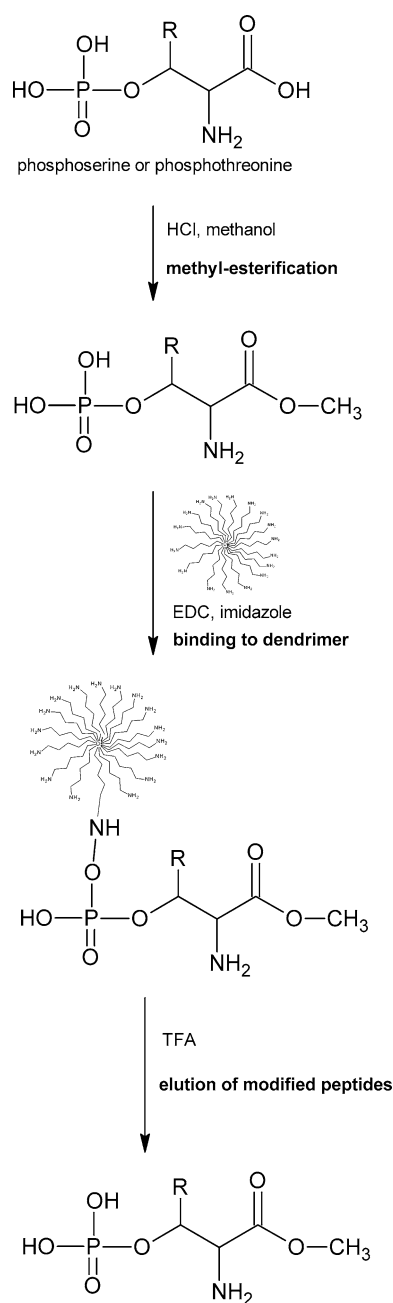


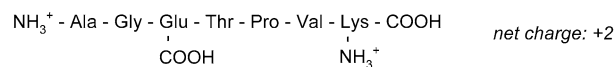
Fig. 11 Schematic workflow for phosphopeptide enrichment by carbodiimide condensation using a dendrimer. The R-group is a hydrogen atom (–H) in a serine and methyl group (–CH₃) in a threonine. EDC—*N,N'*-dimethylaminopropyl ethyl carbodiimide, TFA—trifluoroacetic acid

that these methods are applied prior to the phosphopeptide enrichment in order to reduce the sample complexity and increase the efficiency of the enrichment.

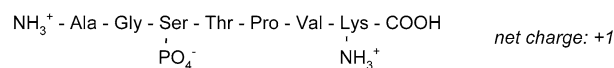
Ion-exchange chromatography

Both strong cationic ion-exchange chromatography (SCX) and strong anionic ion-exchange chromatography (SAX)

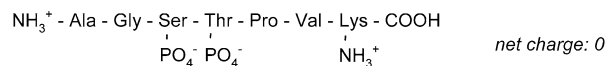
(A) typical non-phosphorylated peptide



(B) typical phosphorylated peptide



(C) multiply-phosphorylated peptide



(D) C-terminal non-phosphorylated peptide

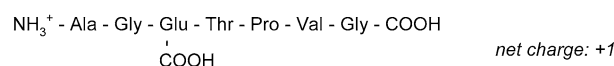


Fig. 12 Charges of illustrative peptides in the acidic buffer (pH 2.7) used for SCX prefractionation

fractionate peptides according to their charge. Although the principle of both methods has been known for over 60 years (Partridge 1949), it is only recently that they have been adopted for phosphopeptide prefractionation (Beausoleil et al. 2004; Nühse et al. 2004).

SCX chromatography involves a negatively charged chromatography matrix (also called catex) selectively binding to positively charged peptides. SCX chromatography is carried out with acidic buffers (pH 2.7; Beausoleil et al. 2004) in order to protonate the N-terminal amino group and the side chains of lysine and arginine residues. With trypsin digestion, most peptides contain a C-terminal arginine or lysine residue since trypsin cleaves the polypeptide chain that lies behind these amino acids. Consequently, most trypsin-digested peptides possess on average at least one basic amino acid, so that most non-phosphorylated peptides have a net charge of +2 (Fig. 12a). In contrast, phosphorylated peptides carry an additional negative charge on their phosphate group(s), so that a peptide containing a single-phosphate moiety has a net charge +1 instead of +2 (Fig. 12b). This method cannot distinguish phosphopeptides from other peptides with the same net charge. For instance, *N*-acetylated peptides and peptides derived from the carboxyl terminus of proteins can also have a +1 charge (Fig. 12d; Gruhler et al. 2005). Peptides containing two or three phosphate groups have on average a net charge of 0 (Fig. 12c) or +1, respectively, and are not captured by the SCX column matrix. Instead, these multiply phosphorylated peptides are present in the flow-through fraction and are probably lost, since only rarely is the flow-through further analyzed (Dai et al. 2007). On the other hand, a minority of phosphopeptides can also appear in the later elution fractions—that is, phosphopeptides with a

missed cleavage with a net charge of +2 (3× positive charge due to 2 basic amino acid side chains and an N-terminal amino group and 1× negative charge of the phosphate). Such phosphopeptides would thus be eluted with non-phosphorylated peptides. The bound peptides are separated by a step-wise elution with increasing ionic strength and/or increasing pH, so that phosphopeptides elute predominantly in the early elution fractions together with some non-phosphorylated peptides (Gruhler et al. 2005). A second purification step utilizing different selection matrices, such as Fe³⁺-IMAC (Gruhler et al. 2005; Neville et al. 1997) or TiO₂-MOAC (Olsen et al. 2006; Pinkse et al. 2004), can be used to remove the remaining non-phosphorylated peptides prior to MS analysis.

In contrast, SAX chromatography relies on a positively charged chromatography matrix (also called anion exchange) selectively binding negatively charged peptides. SAX chromatography is typically carried out in neutral-to-alkaline buffer conditions (e.g. 7.0; Nie et al. 2010), so that the C-terminal carboxyl group and the side chains of glutamate and aspartate amino acids are deprotonated. Under such buffer conditions, peptides display a net negative charge as the side groups of lysine and arginine residues tend to be uncharged, whereas the C-terminal carboxyl group and the acidic side chains of aspartate and glutamate each carry a single negative charge. Phosphopeptides are amongst the most acidic peptides due to their negatively charged phosphate group(s). Hence, non-phosphorylated peptides predominate in early elution fractions, whereas phosphopeptides are eluted later. Unlike SCX, this method retains multiple phosphorylated peptides in the elution fractions (Han et al. 2008). However, the loss of alkaline phosphopeptides is more likely (Dai et al. 2007), since they tend to appear in the early elution fractions and/or in the flow-through fraction. As with SCX chromatography, the enriched phosphopeptides are still contaminated with unphosphorylated peptides, so a further enrichment step is often required using an alternative selective chromatography criterion.

It is noteworthy that the parallel application of both SAX and SCX was shown to increase the number of identified phosphopeptides since the results obtained by SCX and SAX were complementary rather than overlapping (Dai et al. 2007; Nie et al. 2010). SCX led to the identification of more alkaline phosphopeptides with theoretical pI > 4, whereas SAX resulted in the identification of more acidic phosphopeptides with pI < 4 (Dai et al. 2007). Multiply phosphorylated peptides were more likely to be detected by SAX than by SCX coupled with TiO₂-MOAC (Nie et al. 2010).

Hydrophilic interaction liquid chromatography

The last prefractionation technique discussed here is hydrophilic interaction liquid chromatography (HILIC;

Alpert 1990). Unlike SCX and SAX, HILIC fractionates according to the peptide hydrophilicity. Recently, it has been adopted for phosphopeptide pre-fractionation (McNulty and Annan 2008). The more hydrophilic a peptide, the longer it takes to travel through the chromatography column. The opposite principle is used in reverse phase liquid chromatography, where the most hydrophobic peptides are retained on the column for the longest time (Rosenbaum 1974). With phosphorylation, peptides become more hydrophilic and are eluted in later fractions, often co-eluting with longer and/or acidic/basic peptides. The HILIC fractions can be further enriched by other protocols, such as Fe³⁺-IMAC (McNulty and Annan 2008). On the other hand, reversing the order of the separation methods (Fe³⁺-IMAC followed by HILIC) results in significantly lower phosphopeptide enrichment, with more non-phosphorylated peptides being present (McNulty and Annan 2008). This is probably caused by lower IMAC specificity when dealing with more complex samples. When HILIC is used as the first enrichment method, many peptides binding non-specifically to the Fe³⁺-IMAC resin are discarded in the HILIC flow-through and so do not bind to the IMAC matrix. HILIC prefractionation can also partially separate multiply phosphorylated peptides from singly phosphorylated ones. This conveniently prevents multiply phosphorylated peptides from competing with mono-phosphorylated peptides for binding to the secondary IMAC column and thereby influencing the profile of phosphopeptides subsequently identified by MS.

HILIC should be considered complementary to SCX rather than an alternative strategy, since the two techniques can lead to the identification of different phosphopeptides from those in the sample (Chen et al. 2011).

Conclusion

In this review, we have presented and discussed various enrichment techniques employed in phosphoproteomics and showed their advantages and disadvantages.

The phosphoprotein-enriching strategies are generally used less frequently than the phosphopeptide-enriching ones. The spectrum of available methods for the former is narrower and there are not many experimental data available. These phosphoprotein-enriching strategies' main problem is the issue of non-specific binding that is apparently more obvious at the protein level than at the peptide level. This is probably due to the greater complexity of protein structures compared with peptides. Moreover, the buffers used have to be optimized not only for the phosphoprotein enrichment but also for sufficient protein resuspension. It remains a possibility that the theoretically most optimal conditions are unusable due to protein

resuspension limitations under such conditions. In spite of these challenging issues, phosphoprotein enrichment offers a few attractive advantages, such as the determination of protein molecular weight and isoelectric point, as well as phosphoprotein identification according to more than just one peptide. Although they are partly non-specific, these methods could have roles to play, since they would be able to serve as prefractionation methods that would be followed by phosphopeptide enrichment strategies. However, it remains a challenge to improve the existing protocols and/or to develop and introduce completely novel one(s). The most promising methods for phosphoproteomic studies seem to be IMAC, TiO₂-MOAC and Al(OH)₃-MOAC. The disadvantages of IMAC and TiO₂-MOAC are that these methods have not been widely used and that the composition of buffers therefore might be suboptimal. IMAC suffers from being at least partly non-specific; the evidence of this is that several proteins present in the eluate were not stained by phosphoprotein-specific dye ProQ Diamond and were not shown to be phosphorylated by mass spectrometry (Collins et al. 2005). In any case, a single experiment cannot lead to unequivocal conclusions being drawn about method specificity. Phos-Tag remains a promising affinity protocol, but it has not been broadly tested. Its use is advantageous due to the physiological conditions under which the enrichment is carried out. TiO₂-phosphoprotein enrichment has to our knowledge only been used once, and thus the shortage of experimental data prevents further discussion. TiO₂-MOAC and Ni-NTA IMAC differed in the phosphoproteomic spectra that were obtained. Each of the two matrices probably bound phosphoproteins in a different way. This hypothesis was supported by the fact that different compounds inhibited phosphoprotein binding in the respective cases. Binding to TiO₂ was blocked by application of a phosphate, whereas Ni-IMAC binding was inhibited by imidazole (Lenman et al. 2008). The findings that were presented showed that these protocols might be valuable for covering a broader fraction of the phosphoproteomic spectrum. However, it remains unclear whether the sole denaturing conditions are sufficient for entire phosphoproteomic coverage. During titanium-dioxide enrichment, every standard phosphoprotein was detectable only under native or denaturing conditions (Lenman et al. 2008). Each of these conditions also resulted in different phosphoproteomic spectra in the case of enriching a complex sample. Al(OH)₃ exclusively employed denaturing conditions since they were more specific than native ones (Wolschin et al. 2005). Now we can only speculate whether additional phosphoproteins can be identified under less specific native conditions. We would consider Al(OH)₃-MOAC the most promising phosphoprotein-enriching protocol. Its specificity seemed to be higher than that of TiO₂-MOAC and IMAC. Accordingly, it has been

employed in the vast majority of relevant studies. Phosphoprotein immunoprecipitation is suboptimal for complex phosphoproteomic studies, but is optimal for selective capturing of tyrosine-phosphorylated proteins. The use of immunoprecipitation for pS/pT phosphorylation is limited by a low availability of functional antibodies.

Phosphopeptide-enriching protocols represented the prevailing set of strategies. During phosphopeptide enrichment, sample prefractionation is of key importance in order to improve enrichment techniques' specificity and to enable the identification of more phosphopeptides. There is not one single optimal pre-fractionation protocol, since any of them are biased towards a different class of phosphopeptides. All the above-mentioned prefractionation methods are well suited, and a combination of more strategies can result in complementary phosphopeptide spectra being discovered, and thus can broaden the number of identified phosphopeptides (Chen et al. 2011; Dai et al. 2007; Nie et al. 2010).

TiO₂-MOAC was the most promising phosphopeptide-enrichment protocol. The parallel application of zirconium dioxide could be helpful, since both metal ions ended up with different phosphopeptides being identified (Mazanek et al. 2010). Thus, together they covered a greater portion of phosphoproteome than IMAC itself (Kweon and Håkansson 2006; Zhou et al. 2007). The tendency was observed for TiO₂ to preferentially capture singly phosphorylated peptides, whilst ZrO₂ preferred multiply phosphorylated species (Aryal and Ross 2010; Kweon and Håkansson 2006). TiO₂-MOAC itself was found to be more robust than IMAC. Moreover, MOAC was also more tolerant to EDTA as well as to various detergents, and in general it was found to be more selective and sensitive (Aryal and Ross 2010; Gates et al. 2010; Kweon and Håkansson 2006; Larsen et al. 2005; Zhou et al. 2007). MOAC was of at least comparable efficiency to IMAC; it was definitely not inferior (Tsai et al. 2008). However, in a few cases, the application of MOAC led to inconclusive results being obtained with the same competitor for non-specific binding. Therefore it remains unclear which published protocol, if any, is optimal.

In spite of the disadvantages of IMAC, it represents a method that could complement MOAC phosphopeptide spectra, and it has satisfactorily been used in numerous phosphoproteomic studies. The combination of several methods, and especially SIMAC (a combination of MOAC and IMAC), seemed to be very promising. SIMAC offered higher sensitivity and selectivity than sole IMAC or MOAC.

Although Phos-Tag enrichment has led to a greater number of identified proteins than TiO₂-MOAC (Nabetani et al. 2009), it also has to be verified in more studies. Another promising alternative for MOAC and IMAC could

be the recently published PolyMAC, that was shown to be more specific, sensitive and more reproducible than both IMAC and MOAC. However, its superiority has to be further verified in another laboratory.

The importance of testing the actual protocols on more models was shown for IMAC (Barnouin et al. 2005). The casein-optimized conditions could be suboptimal for other proteins from complex biological samples. Casein was cleaved into acidic peptides that were very likely to bind to the matrix by both phosphate moieties and acidic amino acids. On the other hand, strongly alkaline phosphopeptides derived from histone H1 were partly lost under these conditions. The chosen protocol also strongly depended on the subsequent MS ionization technique (MALDI or ESI), as shown, for instance, in the case of IMAC, where DHB elution was compatible with MALDI-MS (Hart et al. 2002) but incompatible with ESI-MS (Imanishi et al. 2007). Another general problem can appear—namely, the fact that the improvements achieved for one protocol may not necessarily be helpful for the others. For instance, the application of glu-C protease instead of trypsin improved IMAC specificity, whereas the non-specificity of ZrO₂-MOAC remained untouched (Kweon and Håkansson 2006). The difference might have been caused by the different geometry of phosphopeptide bonds in the case of Ga³⁺-IMAC and ZrO₂-MOAC.

The enrichment methods by chemical modification could be more selective than the affinity-based techniques, but they handle the samples in more steps and thus are prone to lose parts of the sample. Moreover, several of these techniques include reactions that do not have to be completed, and so the sample complexity increases. On the other hand, these techniques can be considered as complementary protocols to affinity-based techniques, and can broaden the phosphoproteomic coverage (Bodenmiller et al. 2007). For the modification methods, carbodiimide condensation with bead fishing and β -elimination coupled with a Michael addition cannot be recommended. Both these methods comprise a high number of steps. Moreover, the latter is even incompatible with phosphotyrosine, and could also affect *O*-glycosylated serine and/or threonine. The remaining less complicated methods that are equally compatible with all amino acids are α -diazo resin and carbodiimide condensation using a dendrimer, and we would consider these to be more promising. Finally, although the oxidation–reduction technique is comprised of fewer steps, it converts phosphotyrosine with significantly lower efficiency.

For obvious reasons, we cannot generally recommend the sole use of novel strategies that lack verification on a broader spectrum of models (i.e. hydroxyapatite enrichment and enrichment by precipitation). We would try them only in combination with other techniques and/or test them on more models in advance.

Collectively, it should be stressed that complete phosphoproteomic coverage could be only reached by using a combination of prefractionation (Chen et al. 2011; Dai et al. 2007; Nie et al. 2010) and enrichment techniques (Bodenmiller et al. 2007; Ito et al. 2009), and hence, the less specific and less frequently used methods could also play roles in revealing the complete phosphoproteome. We are far from having developed one optimal protocol. Most protocols still face the problems of specificity despite a major effort being made to solve this issue. Another problem is presented by the controversial results frequently obtained from two different models and/or using two parallel techniques. Sometimes a wide range of testing methods and comparison of a number of different protocols have been lacking. For the future, we consider affinity methods to be the most promising, especially a combination of several of them—for example, SIMAC or a combination of SCX and SAX prefractionation coupled with any of the affinity-based techniques.

Acknowledgments The authors thank Věra Čapková, František Novák, Amanda Woods, Jonathan Combe and Said Hafidh for their critical reading of the manuscript and their valuable comments including language corrections. This work was supported by the Czech Science Foundation (P501/11/1462; 522/09/0858; 525/09/0994) and the Ministry of Education, Youth and Sports of the Czech Republic (OC08011).

Conflict of interest The authors declare that they have no conflict of interest.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

- Addeo F, Chobert JM, Ribadeaudumas B (1977) Fractionation of whole casein on hydroxyapatite—application to a study of buffalo kappa-casein. *J Dairy Res* 44:63–68
- Ahn YH, Ji ES, Lee JY, Cho K, Yoo JS (2007) Coupling of TiO₂-mediated enrichment and on-bead guanidinoethanethiol labeling for effective phosphopeptide analysis by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom* 21:3987–3994
- Alpert AJ (1990) Hydrophilic interaction chromatography for the separation of peptides, nucleic acids and other compounds. *J Chromatogr* 499:177–196
- Andersson L, Porath J (1986) Isolation of phosphoproteins by immobilized metal (Fe³⁺) affinity chromatography. *Anal Biochem* 154:250–254
- Aryal UK, Ross ARS (2010) Enrichment and analysis of phosphopeptides under different experimental conditions using titanium dioxide affinity chromatography and mass spectrometry. *Rapid Commun Mass Spectrom* 24:219–231
- Augustine RC, Vidali L, Kleinman KP, Bezanilla M (2008) Actin depolymerizing factor is essential for viability in plants, and its

- phosphoregulation is important for tip growth. *Plant J* 54:863–875
- Ballesta JPG, Rodriguez-Gabriel MA, Bou G, Briones E, Zambrano R, Remacha M (1999) Phosphorylation of the yeast ribosomal stalk. Functional effects and enzymes involved in the process. *Fems Microbiol Rev* 23:537–550
- Barnouin KN, Hart SR, Thompson AJ, Okuyama M, Waterfield M, Cramer R (2005) Enhanced phosphopeptide isolation by Fe(III)-IMAC using 1, 1, 1, 3, 3, 3-hexafluoroisopropanol. *Proteomics* 5:4376–4388
- Baskaran R, Chiang GG, Mysliwiec T, Kruh GD, Wang JYJ (1997) Tyrosine phosphorylation of RNA polymerase II carboxyl-terminal domain by the Abl-related gene product. *J Biol Chem* 272:18905–18909
- Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villén J, Li JX, Cohn MA, Cantley LC, Gygi SP (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc Natl Acad Sci USA* 101:12130–12135
- Bodenmiller B, Mueller LN, Mueller M, Domon B, Aebersold R (2007) Reproducible isolation of distinct, overlapping segments of the phosphoproteome. *Nat Methods* 4:231–237
- Boersema PJ, Mohammed S, Heck AJR (2009) Phosphopeptide fragmentation and analysis by mass spectrometry. *J Mass Spectrom* 44:861–878
- Cantin GT, Shock TR, Park SK, Madhani HD, Yates JR (2007) Optimizing TiO₂-based phosphopeptide enrichment for automated multidimensional liquid chromatography coupled to tandem mass spectrometry. *Anal Chem* 79:4666–4673
- Carrascal M, Ovefletro D, Casas V, Gay M, Ablan J (2008) Phosphorylation analysis of primary human T lymphocytes using sequential IMAC and titanium oxide enrichment. *J Proteome Res* 7:5167–5176
- Chen X, Wu D, Zhao Y, Wong BHC, Guo L (2011) Increasing phosphoproteome coverage and identification of phosphorylation motifs through combination of different HPLC fractionation methods. *J Chromatogr B Anal Technol Biomed Life Sci* 879:25–34
- Collins MO, Yu L, Coba MP, Husi H, Campuzano L, Blackstock WP, Choudhary JS, Grant SGN (2005) Proteomic analysis of in vivo phosphorylated synaptic proteins. *J Biol Chem* 280:5972–5982
- Dai J, Jin WH, Sheng QH, Shieh CH, Wu JR, Zeng R (2007) Protein phosphorylation and expression profiling by Yin-Yang multidimensional liquid chromatography (Yin-Yang MDLC) mass spectrometry. *J Proteome Res* 6:250–262
- Dephoure N, Zhou C, Villén J, Beausoleil SA, Bakalarski CE, Elledge SJ, Gygi SP (2008) A quantitative atlas of mitotic phosphorylation. *Proc Natl Acad Sci USA* 105:10762–10767
- Dubrovskaya A, Souchelnytskyi S (2005) Efficient enrichment of intact phosphorylated proteins by modified immobilized metal-affinity chromatography. *Proteomics* 5:4678–4683
- Feng S, Ye ML, Zhou HJ, Jiang XG, Jiang XN, Zou HF, Gong BL (2007) Immobilized zirconium ion affinity chromatography for specific enrichment of phosphopeptides in phosphoproteome analysis. *Mol Cell Proteomics* 6:1656–1665
- Ficarro SB, McClelland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* 20:301–305
- Fletterick RJ, Sprang SR (1982) Glycogen phosphorylase structures and function. *Acc Chem Res* 15:361–369
- Garnak M, Reeves HC (1979) Phosphorylation of isocitrate dehydrogenase of *Escherichia coli*. *Science* 203:1111–1112
- Gates MB, Tomer KB, Deterding LJ (2010) Comparison of metal and metal oxide media for phosphopeptide enrichment prior to mass spectrometric analyses. *J Am Soc Mass Spectrom* 21:1649–1659
- Gomori G, Benditt EP (1953) Precipitation of calcium phosphate in the histochemical method for phosphatase. *J Histochem Cytochem* 1:114–122
- Grønborg M, Kristiansen TZ, Stensballe A, Andersen JS, Ohara O, Mann M, Jensen ON, Pandey A (2002) A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies—Identification of a novel protein, Frigg, as a protein kinase A substrate. *Mol Cell Proteomics* 1:517–527
- Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen ON (2005) Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Mol Cell Proteomics* 4:310–327
- Han GH, Ye ML, Zhou HJ, Jiang XN, Feng S, Jiang XG, Tian RJ, Wan DF, Zou HF, Gu JR (2008) Large-scale phosphoproteome analysis of human liver tissue by enrichment and fractionation of phosphopeptides with strong anion exchange chromatography. *Proteomics* 8:1346–1361
- Hart SR, Waterfield MD, Burlingame AL, Cramer R (2002) Factors governing the solubilization of phosphopeptides retained on ferric NTA IMAC beads and their analysis by MALDI TOFMS. *J Am Soc Mass Spectrom* 13:1042–1051
- Holmes-Davis R, Tanaka CK, Vensel WH, Hurkman WJ, McCormick S (2005) Proteome mapping of mature pollen of *Arabidopsis thaliana*. *Proteomics* 5:4864–4884
- Hsieh HC, Sheu C, Shi FK, Li DT (2007) Development of a titanium dioxide nanoparticle pipette-tip for the selective enrichment of phosphorylated peptides. *J Chromatogr A* 1165:128–135
- Ikeguchi Y, Nakamura H (1997) Determination of organic phosphates by column-switching high performance anion-exchange chromatography using on-line preconcentration on titania. *Anal Sci* 13:479–483
- Iliuk AB, Martin VA, Alicie BM, Geahlen RL, Tao WA (2010) In-depth analyses of kinase-dependent tyrosine phosphoproteomes based on metal ion-functionalized soluble nanopolymers. *Mol Cell Proteomics* 9:2162–2172
- Imami K, Sugiyama N, Kyono Y, Tomita M, Ishirama Y (2008) Automated phosphoproteome analysis for cultured cancer cells by two-dimensional nano LC–MS using a calcined titania/C18 biphasic column. *Anal Sci* 24:161–166
- Imam-Sghiour N, Laude-Lemaire I, Labas V, Pflieger D, Le Caer JP, Caron M, Nabias DK, Joubert-Caron R (2002) Subproteomics analysis of phosphorylated proteins: application to the study of B-lymphoblasts from a patient with Scott syndrome. *Proteomics* 2:828–838
- Imanishi SY, Kochin V, Eriksson JE (2007) Optimization of phosphopeptide elution conditions in immobilized Fe(III) affinity chromatography. *Proteomics* 7:174–176
- Ito J, Taylor NL, Castleden I, Weckwerth W, Millar AH, Heazlewood JL (2009) A survey of the *Arabidopsis thaliana* mitochondrial phosphoproteome. *Proteomics* 9:4229–4240
- Jensen SS, Larsen MR (2007) Evaluation of the impact of some experimental procedures on different phosphopeptide enrichment techniques. *Rapid Commun Mass Spectrom* 21:3635–3645
- Kinoshita E, Yamada A, Takeda H, Kinoshita-Kikuta E, Koike T (2005) Novel immobilized zinc(II) affinity chromatography for phosphopeptides and phosphorylated proteins. *J Separation Sci* 28:155–162
- Kinoshita-Kikuta E, Kinoshita E, Yamada A, Endo M, Koike T (2006) Enrichment of phosphorylated proteins from cell lysate using a novel phosphate-affinity chromatography at physiological pH. *Proteomics* 6:5088–5095
- Kokubu M, Ishihama Y, Sato T, Nagasu T, Oda Y (2005) Specificity of immobilized metal affinity-based IMAC/C18 tip enrichment of phosphopeptides for protein phosphorylation analysis. *Anal Chem* 77:5144–5154

- Krüger R, Wolschin F, Weckwerth W, Bettmer J, Lehmann WD (2007) Plant protein phosphorylation monitored by capillary liquid chromatography-element mass spectrometry. *Biochem Biophys Res Commun* 355:89–96
- Kweon HK, Håkansson K (2006) Selective zirconium dioxide-based enrichment of phosphorylated peptides for mass spectrometric analysis. *Anal Chem* 78:1743–1749
- Kyono Y, Sugiyama N, Imami K, Tomita M, Ishihama Y (2008) Successive and selective release of phosphorylated peptides captured by hydroxy acid-modified metal oxide chromatography. *J Proteome Res* 7:4585–4593
- Lansdell TA, Tepe JJ (2004) Isolation of phosphopeptides using solid phase enrichment. *Tetrahedron Lett* 45:91–93
- Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJD (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol Cell Proteomics* 4:873–886
- Lee J, Xu YD, Chen Y, Sprung R, Kim SC, Xie SH, Zhao YM (2007) Mitochondrial phosphoproteome revealed by an improved IMAC method and MS/MS/MS. *Mol Cell Proteomics* 6:669–676
- Leitner A (2010) Phosphopeptide enrichment using metal oxide affinity chromatography. *Trends Anal Chem* 29:177–185
- Lenman M, Sorensson C, Andreasson E (2008) Enrichment of phosphoproteins and phosphopeptide derivatization identify universal stress proteins in elicitor-treated *Arabidopsis*. *Mol Plant Microbe Interact* 21:1275–1284
- Li W, Backlund PS, Boykins RA, Wang GY, Chen HC (2003) Susceptibility of the hydroxyl groups in serine and threonine to beta-elimination/Michael addition under commonly used moderately high-temperature conditions. *Anal Biochem* 323:94–102
- Li QR, Ning ZB, Tang JS, Nie S, Zeng R (2009a) Effect of peptide-to-TiO₂ beads ratio on phosphopeptide enrichment selectivity. *J Proteome Res* 8:5375–5381
- Li YJ, Luo YM, Wu SZ, Gao YH, Liu YX, Zheng DX (2009b) Nucleic acids in protein samples interfere with phosphopeptide identification by immobilized-metal-ion affinity chromatography and mass spectrometry. *Mol Biotechnol* 43:59–66
- Lind SB, Molin M, Savitski MM, Emilsson L, Åström J, Hedberg L, Adams C, Nielsen ML, Engström A, Elfneh L, Andersson E, Zubarev RA, Pettersson U (2008) Immunoaffinity enrichments followed by mass spectrometric detection for studying global protein tyrosine phosphorylation. *J Proteome Res* 7:2897–2910
- Machida M, Kosako H, Shirakabe K, Kobayashi M, Ushiyama M, Inagawa J, Hirano J, Nakano T, Bando Y, Nishida E, Hattori S (2007) Purification of phosphoproteins by immobilized metal affinity chromatography and its application to phosphoproteome analysis. *Febs J* 274:1576–1587
- Mamone G, Picariello G, Ferranti P, Addeo F (2010) Hydroxyapatite affinity chromatography for the highly selective enrichment of mono- and multi-phosphorylated peptides in phosphoproteome analysis. *Proteomics* 10:380–393
- Marcantonio M, Trost M, Courcelles M, Desjardins M, Thibault P (2008) Combined enzymatic and data mining approaches for comprehensive phosphoproteome analyses. *Mol Cell Proteomics* 7:645–660
- Matsuda H, Nakamura H, Nakajima T (1990) New ceramic titania—selective adsorbent for organic phosphates. *Anal Sci* 6:911–912
- Mazanek M, Mitulovic G, Herzog F, Stingl C, Hutchins JRA, Peters JM, Mechtler K (2007) Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis. *Nature Prot* 2:U1059–U1069
- Mazanek M, Roitinger E, Hudecz O, Hutchins JRA, Hegemann B, Mitulovic G, Taus T, Stingl C, Peters JM, Mechtler K (2010) A new acid mix enhances phosphopeptide enrichment on titanium- and zirconium dioxide for mapping of phosphorylation sites on protein complexes. *J Chromatogr B Anal Technol Biomed Life Sci* 878:515–524
- McNulty DE, Annan RS (2008) Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. *Mol Cell Proteomics* 7:971–980
- Mishra NS, Tuteja R, Tuteja N (2006) Signaling through MAP kinase networks in plants. *Arch Biochem Biophys* 452:55–68
- Molina H, Horn DM, Tang N, Mathivanan S, Pandey A (2007) Global proteomic profiling of phosphopeptides using electron transfer dissociation tandem mass spectrometry. *Proc Natl Acad Sci USA* 104:2199–2204
- Moll T, Tebb G, Surana U, Robitsch H, Nasmyth K (1991) The role of phosphorylation and the CDC28 protein-kinase in cell-cycle regulated nuclear import of the *Saccharomyces cerevisiae* transcription factor SWI15. *Cell* 66:743–758
- Morton RK (1955) Substrate specificity and inhibition of alkaline phosphatases of cows milk and calf intestinal mucosa. *Biochem J* 61:232–240
- Nabetani T, Kim YJ, Watanabe M, Ohashi Y, Kamiguchi H, Hirabayashi Y (2009) Improved method of phosphopeptides enrichment using biphasic phosphate-binding tag/C18 tip for versatile analysis of phosphorylation dynamics. *Proteomics* 9:5525–5533
- Ndassa YM, Orsi C, Marto JA, Chen S, Ross MM (2006) Improved immobilized metal affinity chromatography for large-scale phosphoproteomics applications. *J Proteome Res* 5:2789–2799
- Neville DCA, Rozanas CR, Price EM, Gruis DB, Verkman AS, Townsend RR (1997) Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry. *Protein Sci* 6:2436–2445
- Nie S, Dai J, Ning ZB, Cao XJ, Sheng QH, Zeng R (2010) Comprehensive profiling of phosphopeptides based on anion exchange followed by flow-through enrichment with titanium dioxide (AFET). *J Proteome Res* 9:4585–4594
- Nousiainen M, Sillje HHW, Sauer G, Nigg EA, Korner R (2006) Phosphoproteome analysis of the human mitotic spindle. *Proc Natl Acad Sci USA* 103:5391–5396
- Nühse TS, Stensballe A, Jensen ON, Peck SC (2004) Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. *Plant Cell* 16:2394–2405
- Oda Y, Nagasu T, Chait BT (2001) Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat Biotechnol* 19:379–382
- Olsen JV, Blagoev B, Gnäd F, Macek B, Kumar C, Mortensen P, Mann M (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* 127:635–648
- Pandey A, Podtelejnikov AV, Blagoev B, Bustelo XR, Mann M, Lodish HF (2000) Analysis of receptor signaling pathways by mass spectrometry: Identification of Vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. *Proc Natl Acad Sci USA* 97:179–184
- Park SS, Maudsley S (2011) Discontinuous pH gradient-mediated separation of TiO₂-enriched phosphopeptides. *Anal Biochem* 409:81–88
- Partridge SM (1949) Separation of bases and amino acids by displacement chromatography on ion exchange columns. *Discuss Faraday Soc* 7:296–305
- Pinkse MWH, Uitto PM, Hilhorst MJ, Ooms B, Heck AJR (2004) Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-nanoLC-ESI-MS/MS and titanium oxide precolumns. *Anal Chem* 76:3935–3943
- Pinto G, Caira S, Cuollo M, Lilla S, Fierro O, Addeo F (2010) Hydroxyapatite as a concentrating probe for phosphoproteomic

- analyses. *J Chromatogr B Anal Technol Biomed Life Sci* 878:2669–2678
- Posewitz MC, Tempst P (1999) Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal Chem* 71:2883–2892
- Rampitsch C, Subramaniam R, Djuric-Ciganovic S, Bykova NV (2010) The phosphoproteome of *Fusarium graminearum* at the onset of nitrogen starvation. *Proteomics* 10:124–140
- Rihs HP, Jans DA, Fan H, Peters R (1991) The rate of nuclear cytoplasmic protein-transport is determined by the casein kinase-II site flanking the nuclear-localization sequence of the SV40 T-antigen. *EMBO J* 10:633–639
- Röhrig H, Colby T, Schmidt J, Harzen A, Facchinelli F, Bartels D (2008) Analysis of desiccation-induced candidate phosphoproteins from *Craterostigma plantagineum* isolated with a modified metal oxide affinity chromatography procedure. *Proteomics* 8:3548–3560
- Rosenbau D (1974) Quantitative analysis of a multicomponent analgesic product containing butalbital, using high speed reverse phase liquid chromatography. *Anal Chem* 46:2226–2228
- Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat Biotechnol* 23:94–101
- Rusnak F, Zhou J, Hathaway GM (2004) Reaction of phosphorylated and O-glycosylated peptides by chemically targeted identification at ambient temperature. *J Biomol Tech* 15:296–304
- Sano A, Nakamura H (2004) Titania as a chemo-affinity support for the column-switching HPLC analysis of phosphopeptides: application to the characterization of phosphorylation sites in proteins by combination with protease digestion and electrospray ionization mass spectrometry. *Anal Sci* 20:861–864
- Seeley EH, Riggs LD, Regnier FE (2005) Reduction of non-specific binding in Ga(III) immobilized metal affinity chromatography for phosphopeptides by using endoproteinase glu-C as the digestive enzyme. *J Chromatogr B Anal Technol Biomed Life Sci* 817:81–88
- Sheoran IS, Ross ARS, Olson DJH, Sawhney VK (2009) Compatibility of plant protein extraction methods with mass spectrometry for proteome analysis. *Plant Sci* 176:99–104
- Simon ES, Young M, Chan A, Bao ZQ, Andrews PC (2008) Improved enrichment strategies for phosphorylated peptides on titanium dioxide using methyl esterification and pH gradient elution. *Anal Biochem* 377:234–242
- Steinberg TH, Agnew BJ, Gee KR, Leung WY, Goodman T, Schulenberg B, Hendrickson J, Beechem JM, Haugland RP, Patton WF (2003) Global quantitative phosphoprotein analysis using multiplexed proteomics technology. *Proteomics* 3:1128–1144
- Stensballe A, Jensen ON (2004) Phosphoric acid enhances the performance of Fe(III) affinity chromatography and matrix-assisted laser desorption/ionization tandem mass spectrometry for recovery, detection and sequencing of phosphopeptides. *Rapid Commun in Mass Spectrom* 18:1721–1730
- Sugiyama N, Masuda T, Shinoda K, Nakamura A, Tomita M, Ishihama Y (2007) Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. *Mol Cell Proteomics* 6:1103–1109
- Tang J, Yin P, Lu XH, Qi DW, Mao Y, Deng CH, Yang PY, Zhang XM (2010) Development of mesoporous TiO₂ microspheres with high specific surface area for selective enrichment of phosphopeptides by mass spectrometric analysis. *J Chromatography A* 1217:2197–2205
- Tao WA, Wollscheid B, O'Brien R, Eng JK, Li XJ, Bodenmiller B, Watts JD, Hood L, Aebersold R (2005) Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. *Nat Methods* 2:591–598
- Thaler F, Valsasina B, Baldi R, Jin X, Stewart A, Isacchi A, Kalisz HM, Rusconi L (2003) A new approach to phosphoserine and phosphothreonine analysis in peptides and proteins: chemical modification, enrichment via solid-phase reversible binding, and analysis by mass spectrometry. *Anal Bioanal Chem* 376:366–373
- Thingholm TE, Jensen ON, Robinson PJ, Larsen MR (2008) SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Mol Cell Proteomics* 7:661–671
- Tombácz E (2009) pH-dependent surface charging of metal oxides. *Periodica Polytechnica Chem Eng* 53:77–86
- Trinidad JC, Specht CG, Thalhammer A, Schoepfer R, Burlingame AL (2006) Comprehensive identification of phosphorylation sites in postsynaptic density preparations. *Mol Cell Proteomics* 5:914–922
- Tsai CF, Wang YT, Chen YR, Lai CY, Lin PY, Pan KT, Chen JY, Khoo KH, Chen YJ (2008) Immobilized metal affinity chromatography revisited: pH/acid control toward high selectivity in phosphoproteomics. *J Proteome Res* 7:4058–4069
- Villén J, Beausoleil SA, Gerber SA, Gygi SP (2007) Large-scale phosphorylation analysis of mouse liver. *Proc Natl Acad Sci USA* 104:1488–1493
- Warthaka M, Karwowska-Desaulniers P, Pflum MKH (2006) Phosphopeptide modification and enrichment by oxidation-reduction condensation. *ACS Chem Biol* 1:697–701
- Wolschin F, Weckwerth W (2005) Combining metal oxide affinity chromatography (MOAC) and selective mass spectrometry for robust identification of in vivo protein phosphorylation sites. *Plant Methods* 1. doi:10.1186/1746-4811-1-9
- Wolschin F, Wienkoop S, Weckwerth W (2005) Enrichment of phosphorylated proteins and peptides from complex mixtures using metal oxide/hydroxide affinity chromatography (MOAC). *Proteomics* 5:4389–4397
- Wu J, Shakey Q, Liu W, Schuller A, Follettie MT (2007) Global profiling of phosphopeptides by titania affinity enrichment. *J Proteome Res* 6:4684–4689
- Xia QW, Cheng DM, Duong DM, Gearing M, Lah JJ, Levey AI, Peng JM (2008) Phosphoproteomic analysis of human brain by calcium phosphate precipitation and mass spectrometry. *J Proteome Res* 7:2845–2851
- Yates JR, Ruse CI, Nakorchevsky A (2009) Proteomics by mass spectrometry: approaches, advances, and applications. *Ann Rev Biomed Eng* 11:49–79
- Ye JY, Zhang XM, Young C, Zhao XL, Hao Q, Cheng L, Jensen ON (2010) Optimized IMAC–IMAC protocol for phosphopeptide recovery from complex biological samples. *J Proteome Res* 9:3561–3573
- Zhang GA, Neubert TA (2006) Use of detergents to increase selectivity of immunoprecipitation of tyrosine phosphorylated peptides prior to identification by MALDI quadrupole-TOF MS. *Proteomics* 6:571–578
- Zhang X, Ye JY, Jensen ON, Roepstorff P (2007) Highly efficient phosphopeptide enrichment by calcium phosphate precipitation combined with subsequent IMAC enrichment. *Mol Cell Proteomics* 6:2032–2042
- Zhou HL, Watts JD, Aebersold R (2001) A systematic approach to the analysis of protein phosphorylation. *Nat Biotechnol* 19:375–378
- Zhou SB, Bailey MJ, Dunn MJ, Preedy VR, Emery PW (2005) A quantitative investigation into the losses of proteins at different stages of a two-dimensional gel electrophoresis procedure. *Proteomics* 5:2739–2747
- Zhou HJ, Tian RJ, Ye ML, Xu SY, Feng S, Pan CS, Jiang XG, Li X, Zou HF (2007) Highly specific enrichment of phosphopeptides

by zirconium dioxide nanoparticles for phosphoproteome analysis. *Electrophoresis* 28:2201–2215

Zhou HJ, Ye ML, Dong J, Han GH, Jiang XN, Wu RN, Zou HF (2008) Specific phosphopeptide enrichment with immobilized

titanium ion affinity chromatography adsorbent for phosphoproteome analysis. *J Proteome Res* 7:3957–3967