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Review

Current methods for phosphoprotein isolation and enrichment☆

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Abstract

The phosphorylation of proteins is a central paradigm of signal transduction. The substitution of neutral hydroxyl groups of serine, threonine and tyrosine with a negatively charged phosphate group alters the physicochemical and immunogenic properties of the protein, which then can be used to isolate these isoforms. In the last decades several different techniques were applied, attempting to selectively enrich protein populations with this post-translational modification. This review aims to give an overview on the arsenal of available methods to extract phosphoproteins focusing on chromatographic approaches.

Keywords: Phosphoprotein; Chromatography; IMAC; Chromatofocusing; Immunoprecipitation

Contents

1. Introduction 155
2. Sample pre-treatment 155
3. Separation on the basis of charge differences 156
   3.1. Chromatofocusing 156
      3.1.1. Principle 156
      3.1.2. Examples 156
   3.2. Ion exchange 156
      3.2.1. Principle 156
      3.2.2. Examples 157
4. Separation on the basis of chelated metal ions 158
   4.1. Immobilised metal ion affinity chromatography 158
      4.1.1. Principle 158
      4.1.2. Examples 158
   4.2. Hydroxyapatite 159
      4.2.1. Principle 159
      4.2.2. Examples 159
   4.3. Metal oxides 160
5. Immunoaffinity techniques 160
6. Conclusions and future prospects 161
   Acknowledgements 162
   References 162

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1. Introduction

It is well accepted that protein phosphorylation is one of the most important post-translational modifications of proteins. Proteins can be phosphorylated on specific amino acid residues by the action of protein kinases. Hereby, serine, threonine and tyrosine are the most common residues in eukaryotes whereas in prokaryotes His, Asp and Glu are also found to be phosphorylated [1].

Approximately one third of all proteins in eukaryotes are estimated to be phosphorylated. Protein phosphorylation–dephosphorylation is one of the major signalling mechanisms for modulating the functional properties of proteins involved in gene expression, cell adhesion, cell cycle, cell proliferation, and differentiation [2].

A typical example where phosphorylation is responsible for function and structure is the control of eukaryotic cell cycle by numerous cyclin dependent kinases (CDKs). This process is highly dynamic and therefore the relative abundance of phosphorylated proteins involved is low, combined with a relatively short half-life. Aberrant phosphorylation is one of the underlying mechanisms for cancer or can be a sign for metabolic diseases. Therefore, phosphorylated proteins are important drug discovery targets for therapeutic intervention.

To understand these processes, structural information about the involved phosphorylated isoforms is highly desired. The purification of certain phosphoisoforms in sufficient amounts is very often a major obstacle. Technologies to purify phosphoisoforms of proteins are of great relevance for the production of specific antibodies, or for the separation of an active phosphoprotein from its inactive, non-phosphorylated counterpart [3].

During the last 45 years, several chromatographic techniques have been applied to purify or selectively enrich phosphorylated proteins. The purification of phosphoproteins can been seen under many different aspects. Isolation in a larger scale or for analytical purposes, purification for proteomic studies or extraction of a protein pool from cell extracts are only some examples. Further, many studies are describing the selective isolation of phosphopeptides from complex mixtures. All methods take advantage of several unique features resulting from the addition of a phosphate group to amino acids. In general three different effects can be distinguished and applied as selection principle (Fig. 1.). The first is the alteration of the isoelectric point (p*I*) or the net charge of the protein. This enables the separation by ion exchange chromatography (IEX) or chromatofocusing (CF). Second, the phosphate group can chelate metal ions caged in immobilized metal affinity chromatography (IMAC) resins or Ca^{2+} as part of hydroxyapatite (HTP). Finally, a change of the immunogenic properties of the protein after phosphorylation allows the generation of antibodies, which in turn can be used for immunoprecipitations. Methods that covalently and permanently modify phosphate groups are not covered in this review, although they have been successfully applied to isolate phosphopeptides in several cases [4].

In the following, we are focusing on phosphoproteins, giving an overview on the current repertoire of methods.

2. Sample pre-treatment

Protein phosphorylation is a highly dynamic event controlled by the activity of kinases and phosphatases. Cell lysates contain a number of active phosphatases that can dephosphorylate very efficiently. Therefore, these enzymes must be inhibited to avoid the loss of phosphorylation during sample preparation and cell lysis. A number of chemical compounds have been applied for that task. Vanadate is a highly potent inhibitor of tyrosine phosphatases [5]. Serine and threonine phosphatases can be blocked by ocadaic acid [6]. Additionally, several broad-acting or non-specific reagents can be used to inhibit protein phosphatases. These include sodium fluoride and sodium pyrophosphate or glycerophosphate [7]. Other techniques to suppress protein dephosphorylation in cell lysates and fractions include the denaturation of samples by adding urea [8] or the elution by an electrophoresis sample buffer [9]. In certain cases a specific pre-adsorption of highly abundant proteins can be necessary to be able to enrich the phosphoprotein of interest. It might also

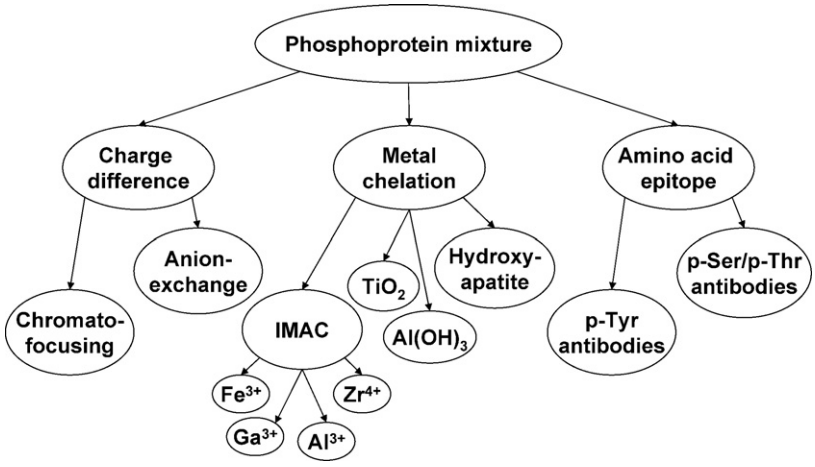

Fig. 1. Overview on different chromatographic isolation strategies for phosphoproteins.

be favourable to increase the accessibility of phosphorylated epitopes by partial or total denaturation if immunoaffinity purification is applied [10–12].

3. Separation on the basis of charge differences

The phosphate esters of serine, threonine and tyrosine have two acidic protons, with pK_a values in H_2O of <2 and about 5.8, respectively. Effects on the p*I* of a protein upon phosphorylation are essential for purification strategies based on ion exchange or chromatofocusing. The measurable p*I* effects upon phosphorylation strongly depend on the primary and secondary structure of the protein. The reduction of the p*I* can vary, from almost no shift at all up to more than one pH unit towards the acidic per introduced phosphate group.

Various algorithms have been developed in the past to calculate a theoretical p*I* of a protein [13,14]. But these calculations are not capable to predict a p*I* shift upon phosphorylation [15,16]. Recently a new algorithm was presented to calculate these effects [17]. Zhu et al. studied the effect of phosphorylation on the p*I* shift and found, on the one hand, very good match with the theoretical model, on the other hand, still considerable differences for certain proteins [16].

Deviations from the theoretical p*I* can be observed in cases where the protein is post-translationally modified. In that context phosphorylation contributes to drastic charge changes when neutral hydroxyl groups of serine, threonine and tyrosine are substituted with a negatively charged phosphate group. Thus, phosphorylation at one site can cause an acidic shift of p*I*, which can be decreased by 1–2 pH units. The effect of multiple phosphorylations can even be higher.

3.1. Chromatofocusing

3.1.1. Principle

The technique of chromatofocusing was invented several decades ago to separate complex protein mixtures, and is still attractive due to its powerful resolution [18,19].

Proteins are separated according to their p*I* on a liquid chromatography column. CF uses the buffering of the charged group on the ion exchanger and the pH gradient is created as the eluting buffer titrates the ion exchanger. A typical amphoteric buffer (polybuffer) contains a variety of charged species to allow an even buffering capacity. CF creates a linear pH gradient, a feature, which cannot be achieved by similar techniques like gel based isoelectric focusing. CF is performed in solution, simplifying downstream applications. Other advantages are the continuous on-line control of the pH and the mild elution conditions at the p*I* of the proteins. No protein is exposed to a higher pH than its own approximate p*I*. Even membrane proteins can be separated in their native conformation by addition of detergents. CF also tolerates the addition of strong denaturants like urea. However, CF is sometimes limited by poor yields, difficulties in scaling up and the need to remove ampholytes form proteins. In some cases a very tight binding of phosphorylated proteins can be observed which requires then an acidic wash to elute those proteins.

3.1.2. Examples

Recently the effect of phosphorylation on CF was assessed more systematically [16]. Whole cell lysate was fractionated under denaturing conditions by CF. It could be observed, that the acidic shift can vary depending on the initial p*I* of the unmodified protein and the amount of phosphorylation. For proteins having a p*I* above 6.4 the change is larger than for those with a p*I* of 5.0 and lower. CF was also applied in a proteomics study to fractionate the mouse liver phosphoproteome by two-dimensional chromatography. A pool of unfractionated phosphoproteins was initially extracted from the lysate by metal affinity chromatography. Then CF in the first dimension was combined with reversed phase chromatography, again demonstrating the huge resolving power of CF [20]. A protein whose phosphorylation has been studied in detail is rhodopsin. Rhodopsin kinase transfers several phosphates to serine or threonine residues in the carboxyl terminus of freshly bleached rhodopsin. All these isoforms can be differentiated by their p*I*. Six different isoforms could be eluted in a pH gradient from 6.4 to 4.0. The observed stoichiometry was 0, 2, 4, 5, 6 or 8 phosphate molecules per molecule rhodopsin. The variants having a higher degree of substitution with phosphate eluted later in the gradient at a lower pH. This example shows that CF is capable to resolve differences of only a single phosphate group [21]. In another experiment with phosphorylated rhodopsin, some isoforms with more phosphate groups bound so tightly to the column matrix, that an elution with a salt gradient became necessary [22]. However, in spite of the high resolution CF does not discriminate against different positions of the phosphate group, it only separates according to the alteration of p*I*. Although CF reliably resolves different rhodopsin isoforms according to their stoichiometry, two monophosphorylated rhodopsin species with phosphorylation at different sites could not be fractioned [23]. CF can also be applied to separate a phosphorylated protein from its native unmodified counterpart. This was done for prolactin. Immunopurified prolactin was loaded to a standard CF column and a typical pH gradient from 7.0 to 4.0 was applied, eluting only unphosphorylated prolactin. The phosphorylated prolactin required much harsher conditions to come off the column. Only an injection of 70% acetic acid (pH 2.7) quantitatively released the monophosphorylated prolactin. That unexpected behaviour could be caused by a conformational change in the phosphoisoform, now exposing substantially different surface charge properties [24]. In another example CF was used to separate the phosphorylated pyruvate kinase from its inactive, non-phoshorylated isoform [25]. Table 1 summarises the examples.

3.2. Ion exchange

3.2.1. Principle

Differences in the net surface charge of molecules are the basis of separation by ion exchange chromatography. These charge properties vary considerably between molecules and therefore they will interact differently with charged chromatography media according to differences in their surface charge distribution, charge density and overall charge. The chemical microenvironment and the structure influences the pK_a values

Table 1
Overview of examples applying chromatofocusing

	Matrix	Chemistry	Buffer (dilution)	pH-range	Proteins	Ref.
1	ProteoSep (Eprogen)	n.a.	Polybuffer 74 (1:10)	pH 7.4–4.0	Epithelial cell lysate	[16]
2	PBE 94	Crosslinked agarose	Polybuffer 74 (1:8)	pH 6.4–4.0	Rhodopsin	[21]
3	Mono P	Polystyrene/divinyl benzene	Polybuffer 74 (1:10)	pH 7.4–4.0 and 0–0.4 M KCl	Rhodopsin	[22]
4	Mono P	Polystyrene/divinyl benzene	Polybuffer 74 (1:12.5)	pH 7.4–4.0 and 0–0.4 M NaCl	Rhodopsin	[23]
5	Mono P	Polystyrene/divinyl benzene	Polybuffer 74 (1:10)	pH 7.0–4.0 and 70% acetic acid	Prolactin	[24]
6	PBE94	Crosslinked agarose	Polybuffer 74 (1:10)	pH 7.4–4.0	Pyruvate kinase	[25]

of charged groups within a molecule and contribute to its net surface charge.

The net charge of a protein is highly pH-dependent because all amino acids with ionisable groups can contribute differently to the overall charge. These ionisable groups can change their charge depending on the pH of the environment. The relationship between net surface charge and pH is unique for a specific protein and this principle is exploited in IEX. Reversible interactions between charged IEX media and oppositely charged molecules are controlled to favour binding or elution of specific molecules and achieve separation in an IEX experiment. At a pH equivalent to its p*I* a protein has no net charge at will not interact with a charged sorbent. However, at a pH below its p*I*, a protein will bind to a cation exchanger and at a pH above its p*I*, a protein will bind to an anion exchanger. Bound proteins are either eluted by a pH shift or by ion displacement, e.g. by adding salt.

The substitution of neutral hydroxyl groups of serine, threonine and tyrosine by a negatively charged phosphate group causes an acidic shift of p*I*. This alteration in p*I* is the underlying principle of separating phosphorylated proteins from unphosphorylated by IEX. It even allows the gradual elution of multiphosphorylated isoforms if the elution is performed in a very long, shallow gradient.

3.2.2. Examples

IEX played a major role in the prefractionation of an 80 kDa bone protein into three isoforms that differed in their p-Ser content. Higher phosphorylated versions of that protein had a longer retention time and eluted later in the gradient [26]. The separation of prolactin was also performed using IEX. As already observed during CF, the phosphorylated isoform bound so tight, that elution with acetic acid was necessary [27]. Acid cystatins from human saliva were separated by IEX into three isoforms having an identical amino acid sequence but differing in their phosphorylation. The non-phosphorylated protein eluted first, followed by the mono- and later the bi-phosphorylated form, eluting last from a strong anion exchanger column [28]. One of the typical examples is the capture of radioactively labelled phosphoproteins from human fibroblasts on a strong anion exchanger column and the elution with a very shallow NaCl gradient from 0 to 0.8 M over 100 column volumes [29]. Anion IEX was also applied to isolate a non-phosphorylated 65 kDa protein from its phosphorylated isoform after stimulation of human peripheral blood monocytes with interleukin 1. As in most other cases the phosphorylated form eluted later at higher salt concentrations [30]. Applying DEAE as IEX matrix could separate three different myosin light chain isoforms. One of them was phosphorylated and eluted last in a relatively short gradient of only seven column volumes [31]. IEX was also used to purify rhodopsin. Here a rather unconventional method was applied. Instead of 1 large column, up to 20 mini columns of ECTEOLA cellulose were arranged in series in form of a column cascade. As expected, highly phosphorylated rhodopsin bound stronger to the matrix. The capacity of the individual mini columns was adjusted so that the first columns, which bound the heavily phosphorylated rhodopsin, were smaller than the last columns. After loading, the columns were separated and eluted in one step of 300 mM NaCl. Although not a complete separation of isoforms could be achieved, at least a quantitative gradual enrichment of up to nine different isoforms was observed [32]. Table 2 summarises the examples.

Table 2
Overview of examples applying ion exchange chromatography

	Matrix	Chemistry	Gradient	Length	Protein	Isoforms	Ref.
1	DE-52	Cellulose	0.1–0.5 M NaCl	18 CV	80 kDa protein	3	[26]
2	DEAE-2025	Methacrylate	0.5 M NaCl 20% acetic acid (step-wise)	n.a.	Prolactin	2	[27]
3	MonoQ	Polystyrene/divinyl benzene	0–0.5 M NaCl	46 CV	Cystatins	3	[28]
4	MonoQ	Polystyrene/divinyl benzene	0–0.8 M NaCl	100 CV	Fibroblast phosphoproteins	Multiple	[29]
5	AX300	Crosslinked polyethyleneimine	0–0.3 M NaCl	46 CV	65 kDa protein	2	[30]
6	DEAE-sepharose	Crosslinked agarose	0.05–0.25 M KCl	7 CV	Myosin light chain	3	[31]
7	ECTEOLA cellulose	Cellulose	0.3M NaCl (step-wise)	n.a.	Rhodopsin	9	[32]

4. Separation on the basis of chelated metal ions

Phosphoproteins exhibit a strong tendency to chelate metal ions. For chromatographic purposes these metal ions are often caged in IMAC resins. Other matrices suitable for the enrichment of phosphoproteins are hydroxyapatite or metal oxide particles. In the following sections an overview on current techniques is given.

4.1. Immobilised metal ion affinity chromatography

4.1.1. Principle

It was originally observed by Andersson and Porath that phosphorylated amino acids and proteins selectively bind to Fe(III)-charged iminodiacetic acid (IDA) agarose [33]. They also showed that other metal ions (Lu(III), Sc(III) and Th(IV)) had similar properties and that phosphorylated proteins could be eluted either by the addition of free phosphate or by a shift in pH. In a later study a wider range of metal ions were screened for their affinity towards phosphoproteins/peptides and it was shown that Ga(III) and Zr(IV) bind phosphoproteins/peptides with good retention and elution properties [34].

The two most commonly applied chelating groups are iminodiacetic acid or nitrilotriacetic acid (NTA), which are immobilized to chromatographic resins, membranes or magnetic beads (Fig. 2a and b). The number of coordination sites available for the metal ion–phosphate interaction varies depending on the chelating group used. The IDA moiety forms a tri-dentate complex with the metal ion leaving three coordination sites unoccupied for phosphate binding. The NTA moiety is a tetradentate ligand having two free coordination sites at the immobilized metal ion.

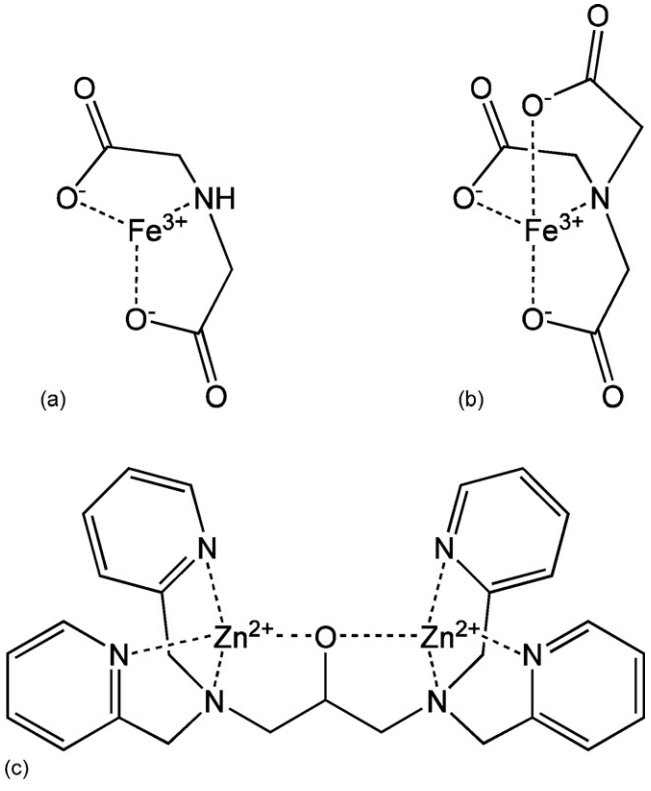

Fig. 2. Chemical structures of metal ion chelating molecules. (a) Iminodiacetic acid (IDA); (b) nitrilotriacetic acid (NTA); (c) phos-tag.

In a recent attempt to develop a more efficient IMAC resin for phosphoprotein separation a new type of chelating group was designed that forms an alkoxide-bridged di-nuclear Zn(II) centre (termed phos-tag) (Fig. 2c) [35]. This was shown to be able to selectively bind phosphorylated proteins at physiological pH as opposed to IMAC resins using Ga(III) or Fe(III), where the optimum pH for binding is reported to be in the range of 2.0–3.5 [34].

The strategy for elution of phosphoprotein/peptides from the IMAC matrix is similar to those in purification of his-tagged proteins, either by the addition of excess amount of free ligand (e.g. by use of a phosphate buffer), or by pH elution. In the latter case an increase in pH promotes the dissociation of the phosphoprotein/peptide from the IMAC matrix [34].

These attractive properties (the selective affinity for phosphorylated proteins/peptides and the reversible nature of the binding) are exploited in several variants of IMAC applications to enrich phosphoproteins and phosphopeptides from complex samples.

Some drawbacks do exist when using IMAC to enrich phosphorylated proteins/peptides. The major drawback is that little or no binding to Fe(III) or Ga(III) charged resins is observed at neutral pH [33]. Therefore, low pH is normally used in the binding buffers [34]. Some proteins will not withstand such conditions but may denature or precipitate on the column. This will either cause the protein not to be recovered in its native form, or not to be recovered at all.

Another drawback is the non-specific binding of acidic proteins and peptides to the IMAC matrix. This can be, at least partly, circumvented by addition of acetate to the sample, or in the case of phosphopeptide isolation, by methyl-esterification of the carboxyl groups in the sample prior to IMAC [36].

4.1.2. Examples

There are reports of IMAC being used not only to enrich phosphoproteins/peptides but also to separate different phosphoisoforms of the same protein. This was first reported by Andersson and Porath, who separated different phosphoisoforms of ovalbumin on a Fe(III)-IDA matrix by gradient elution from pH 5.7–7.2 [33]. Three phosphoisoforms of tomato sucrose synthase could be isolated by applying a very basic gradient from pH 6.3–9.5 on a Fe(III)-IDA matrix [37]. Another example is the separation of different forms of pepsinogen, also performed on Fe(III)-IDA by elution with a pH gradient (pH 5.6–7.0) or increasing concentrations of malic or tartaric acid [38]. IMAC was also applied to extract large numbers of phosphoproteins for proteomic studies. In one case cloned fusion proteins were captured by a Fe(III)-NTA matrix and eluted with a mixture of high pH and competition of phosphate ions [39]. In a similar procedure the phosphoproteome of Jurkat T cells was captured on a Fe(III)-NTA matrix at acidic pH and eluted at pH 8.5. This study also exemplifies some limitation of that method which can result in protein loss, very dilute eluates and contamination with non-phosphorylated proteins [40]. Fe(III)-NTA was also used

Table 3
Overview of examples applying IMAC

	Chelator	Metal	Binding buffer	Elution buffer	Phosphoprotein	Isoforms	Ref.
1	IDA	Fe(III)	0.1 M acetic acid pH 5.0	0.05 M MES/PIPES pH 5.7–7.2	Hen ovalbumin	3	[33]
2	IDA	Fe(III)	0.2 M MES pH 6.3	0.1 M MES-Tris pH 6.3–9.5	Tomato sucrose synthase	3	[37]
3	IDA	Fe(III)	0.1 M sodium acetate pH 5.6 or 0.1 M MES pH 5.6	0.05 M MES pH 5.6–7.0 or 0.001–0.02 M malic or tartaric acid pH 5.6	Human and pig pepsinogen	3	[38]
4	NTA	Fe(III)	Lysate pH 3.0	0.2 M NaH_2PO_4 pH 8.4	Pig testis fusion proteins	n.a.	[39]
5	NTA	Fe(III)	0.05 M acetate pH 5.6	0.05 M Tris pH 8.5	Jurkat T cell proteins	n.a.	[40]
6	NTA	Fe(III)	1% Triton X 100, 0.15 M NaCl, 0.01 M Tirs pH 8.0	0.1 M DTT, 2% SDS, 0.05 M Tris pH 6.8 (95 °C)	Human breast epithelial cell proteins	n.a.	[9]
7	Phos-tag	Zn(II)	0.1M Tris-acetate pH 7.4	0.01– 0.5 M NaH_2PO_4 pH 7.0	Hen ovalbumin, bovine casein	n.a.	[35]
8	IDA or NTA	Ga(III)	6 M urea, 0.05M Tris acetate	6 M urea, 0.05M Tris acetate 0.1 M EDTA, 0.1 M EGTA	Mouse synaptic proteins	n.a.	[8]

as enrichment step for phosphoproteins from human epithelial cells. Phosphoproteins were eluted with electrophoresis sample buffer, thus simplifying the subsequent gel electrophoresis [9]. In an attempt to isolate phosphorylated peptides from synaptic proteins a number of approaches were evaluated to establish a protocol to isolate as many phosphorylated peptides as possible [8]. The most successful approach started with an enrichment of phosphorylated proteins using IMAC under denaturing conditions (6 M urea), followed by tryptic digestion and a second IMAC step to enrich for phosphorylated peptides. The success of this approach is partly because the urea extraction allows for the isolation of peptides from phosphorylated membrane proteins as well as phosphorylated soluble proteins. Another factor is probably the rapid denaturation and inactivation of contaminating phosphatases by the urea buffer, thereby capturing a larger fraction of the phosphorylated proteins/peptides. Table 3 summarises these examples.

4.2. Hydroxyapatite

4.2.1. Principle

Hydroxyapatite is a form of calcium phosphate with the formula $Ca_{10}(PO_4)_6(OH)_2$ which can be used as chromatographic matrix. HTP chromatography is considered to be a "mixed-mode" ion exchange or a "pseudo-affinity" chromatography. It can effectively be applied as purification mechanism in a variety of processes, providing biological molecule selectivity complementary to more traditional ion exchange or hydrophobic interaction techniques. The HTP interactions with biomolecules are complex. Generally, it is thought that amino groups are attracted to negatively charged phosphate sites and repelled by positively charged calcium sites. The opposite is true for negatively charged, phosphorylated residues of proteins. The more phosphates are present, the more dominating is the calcium site binding and thus the tighter is the binding of the protein to the matrix. A competitive elution is achieved by increasing the phosphate concentration in the buffer. HTP was a very popular method between the 1960s to 1980s when few alternative chromatography matrices were available. It has primarily been used to enrich phosphoproteins although a separation of isoforms can be achieved. In the last decades new, more stable formulations of HTP have been prepared which might support a renaissance of this formerly well-established technique.

4.2.2. Examples

One of the earliest descriptions of the resolving power of HTP is from 1960. Here the separation and characterization of two lipovitellin isoforms was performed with a home-made HTP matrix [41]. Almost 30 years ago, phosphofructokinase from rabbit skeletal muscle was fractionated into two differently phosphorylated species by HTP chromatography [42]. Obviously HTP was applied, as most other classical methods, in purification procedures for rhodopsin derivatives. The most highly phosphorylated rhodopsin was most strongly adsorbed to the HTP matrix and eluted last [43]. The same effect could be observed during the purification of the glucocorticoid receptor. Here the higher phosphorylated isoform also required a higher phosphate concentration of 0.4 M KH_2PO_4 and the addition of up to 0.9 M NaCl for elution [44]. HTP has been frequently applied to separate identical proteins that only differ in their post-translational modifications, e.g. phosphorylation. One of those examples is a 64 kDa protein that could be isolated as two isoforms from stimulated, human leukocytes. Again the phosphorylated protein eluted slightly later than its unmodified counterpart from a HTP column in a phosphate gradient [45]. Many studies have been performed on bone proteins that naturally have a high affinity to bone structure and thus to HTP as well. One of those, an 80 kDa protein from calf bone was separated into four isoforms by HTP chromatography. Two of those isoforms differed strongly in their content of p-Ser and could be eluted with increasing KH_2PO_4 concentrations [26]. Recently a combination of HTP chromatography and 2D SDS-PAGE was used to characterize low abundant phosphoproteins involved in cellular responses to a growth factor, demonstrating the specificity of that matrix [46]. However, HTP is not always applicable, as it was shown in the purification of phosvitins. Here the phosphoproteins adhered too tightly and could only be eluted with concentrations above 1.5 M phosphate buffer [47]. Table 4 summarises the examples.

Table 4
Overview of examples applying HTP

	Matrix	Gradient	Phosphoprotein	Isoforms	Ref.
1	Hydroxyapatite	50–150 mM KH_2PO_4	α,β-Lipovitellin	2	[41]
2	Bio-Gel HTP	50–250 mM KH_2PO_4	Phosphofructokinase	2	[42]
3	Hydroxyapatite	5–300 mM KH_2PO_4	Rhodopsin	2	[43]
4	Hydroxyapatite	10–400 mM KH_2PO_4	Glucocorticoid receptor	2	[44]
5	Hydroxyapatite MP	5–500 mM NaH_2PO_4	64 kDa protein	2	[45]
6	Hydroxyapatite	0–200 mM KH_2PO_4	80 kDa protein	4	[26]
7	CHT-II	10–400 mM NaH_2PO_4	Cell extract	n.a.	[46]

4.3. Metal oxides

One of these techniques is based on the ability of TiO_2 to absorb water-soluble phosphate [48]. Ikeguchi used this method already in 1990 for the selective binding of phospholipids [49]. Titanium dioxide in form of HPLC microspheres was described by Jiang and Zuo [50]. The characteristics of the material are described as an amphoteric ionexchanger with a basic surface. The exact mechanism of the phosphate binding is not yet understood. In contrast to the IMAC principle, no additional metal ions are required. Sano and Nakamura applied this concept on phosphopeptides successfully [51,52]. Phosphopeptides are adsorbed under slightly acidic conditions (0.015% TFA) and eluted with a 50 mM borate buffer, pH 8.0 containing 100 mM LiCl [52]. Pinkse et al. could identify eight phosphorylation sites in cGMP-dependent kinase by using a TiO_2 microcolumn for the isolation of the phosphopeptides [53]. Recent work describes improved chromatographic material with higher loading capacity [54] and TiO_2 material with a magnetic core [55]. The binding of phosphopeptides to TiO_2 and ZrO_2 was compared in pipette tips [56]. ZrO_2 showed a higher selectivity for singly phosphorylated peptides and TiO_2 showed preference for multiply phosphorylated compounds. A proof of this concept for the purification of phosphoproteins remains to be done.

Another technique called metal oxide affinity chromatography, MOAC, utilizes $Al(OH)_3$ to selectively adsorb phosphorylated compounds. Wolschin et al. studied in a systematic way the adsorption of several standard phosphoproteins, as well as phosphorylated proteins and peptides of *Arabidopsis thaliana* leaf protein extracts [57]. Proteins can be adsorbed in several physiological buffer systems whereas a phosphate or pyrophosphate buffer was used for the elution. An early study on the interaction between different phosphate esters and phosphonates and the $Al(OH)_3$ tried to enlighten the absorption process, but the exact binding mechanism remains unclear [58].

5. Immunoaffinity techniques

Immunoaffinity isolation of phosphoproteins with antibodies directed against p-Ser, p-Thr and p-Tyr has been performed since many years. The first use of antibodies to purify p-Tyr containing proteins was published by Ross et al. in 1981 [59]. Since then, several polyclonal and monoclonal antibodies have been developed, both specifically against primary structure elements of phosphorylated protein isoforms and to p-Ser, p-Thr and p-Tyr as such. To reach the necessary immunogenicity the phosphoamino acids, or phosphopeptides, have been coupled to keyhole limpet hemocyanin (p-Ser/p-Thr) or bovine serum albumin (p-Tyr) [60,61]. The cross reactivity among the different phosphoamino acids was hereby found to be low or absent. Phosphotyrosine antibodies are far more frequently used for immunoprecipitation than anti p-Ser and anti p-Thr antibodies, mainly due to the higher efficiency. Antibodies generated against p-Ser and p-Thr are very often dependent on a consensus sequence flanking the phosphorylated residue. This might be a consequence of the lower immunogenicity of p-Ser/p-Thr compared to p-Tyr. So far, only anti p-Tyr antibodies are used in a generic way.

Although antibodies appear to be an ideal tool for purifying phosphorylated proteins, today, no application can be found describing a protein purification process in a multi milligram scale. One reason is certainly the relatively high costs of appropriate affinity media. On the other side, many studies are published describing the enrichment of phosphoproteins out of cell extracts in small scale, mostly for analytical purposes.

Many authors apply an anti p-Tyr antibody for the selective enrichment of pools of p-Tyr containing proteins [10,11,62,63]. The isolated phosphoproteins have all been subjected to further analysis under denaturing conditions. In 2002, Palomo-Jimenez et al. isolated active phosphocalmodulin, free of the unphosphorylated variant, by using anti p-Tyr antibody in a 1–2 μg scale [64]. Anti p-Tyr antibodies show variations in binding efficiency between different suppliers and clones. Some are tolerant against 0.1% SDS; others fail to bind p-Tyr under this condition [62]. Another difference is the influence of sterical factors on binding. The recovery of an immunoaffinity purification can be increased by partial or total denaturation of the protein structure [10–12]. Alternatively a mix of several antibodies from several clones or suppliers can be advantageous to improve the results. Gronborg et al. have investigated the use of different anti p-Ser, anti p-Thr and anti p-Thr-Pro (threonine flanked by a proline) antibodies for immunoprecipitation in cell lysates of HeLa and 293T cells [65]. The lysates were incubated with the respective antibody overnight. Immunoprecipitated proteins were further analyzed by Western blotting and mass spectrometry. This study showed that p-Ser and p-Thr antibodies initially designed for Western blotting were also suitable and efficient for selective enrichment of phosphoproteins. Many different antibodies are commercially available today. Even generic antibodies against p-Ser/p-Thr and p-Tyr exist in a high number comprising polyclonal, monoclonal,

Table 5
Overview of immunoaffinity applications

	Protein sample	Antibody	Antigen	Application	Year	Ref.
1	RSV-transformed CEF and 3Y1 cells	Two different rabbit polyclonal custom made	pTyr	Immunoprecipitation,	1988	[12]
2	6–10 p-proteins from rat-1 and primary chicken embryo cells expressing src oncogene	Rabbit polyclonal	pTyr	Immunoprecipitation	1989	[11]
3	p-Thr coupled to KLH or BSA as antigen	Rabbit polyclonal	pThr	Antibody generation	1989	[60]
4	pTyr conjugates with keyhole limpet hemocyanin as antigen	1G2 mouse monoclonal	pTyr	Antibody generation	1991	[61]
5	Activitated Bal17 murine B lymphocytes	PY20 monoclonal	pTyr	Immunoprecipitation affinity chromatography on AB-agarose beads	1994	[10]
6	Active phosphocalmodulin	PT66 mouse monoclonal	pTyr	Purification antibody coupled to agarose beads	1999	[64]
7	EGF receptor signal transduction pathway	4G10 monoclonal	pTyr	Immunoprecipitation, antibody coupled to agarose beads	2002	[63]
9	Cell lysates of HeLa and 293T cells	pSer 7F12, 1C8 mouse monoclonal pThr 1E11 mouse monoclonal	pSer/pThr	Immunoprecipitation, antibody coupled to agarose beads	2002	[65]

Table 6
Comparison of different enrichment technologies

	Method	Scale	Elution	Resolution	Drawbacks	Sample
1	Chromatofocusing	Limited scale	pH gradient	Isoform separation	Removal of ampholytes	Low conductivity
2	Anion exchange	Fully scalable	Salt gradient	Isoform separation	Very flat and long gradient	Low conductivity
3	Immobilised metal affinity	Fully scalable	pH or phosphate gradient	Isoform separation	Low pH for binding, dilute eluate	No phosphate buffer
4	Hydroxyapatite	Fully scalable	Phosphate gradient	Isoform separation	Mechanical instability, sometimes too tight binding	No phosphate buffer
5	Immunoaffinity	Limited scale	Denaturation	Phosphoamino acids and small epitopes	High costs for larger scale, loss of structure and activity	Low volume

different host, different clones, formulations and conjugations. Newly developed antibodies appear on the market continuously. Search engines on the internet that are specialized on commercially available antibodies are helpful tools for the researcher to follow this dynamic field [66–68]. Table 5 summarises these examples.

6. Conclusions and future prospects

All the above-cited methods offer specific advantages or suffer from certain drawbacks. A comparison of these technologies is given in Table 6. Some of them require sample of low ionic strength or the absence of phosphate. Most of the techniques are not totally selective for phosphoproteins and co-purification of non-phosphorylated proteins may occur. Additionally, not all scales are recommended for all techniques.

In the context of phosphoprotein purification not much progress was made regarding classical techniques like ion exchange and chromatofocusing during the last decades. Although the physical stability of hydroxyapatite matrices has improved, they have only rarely been used. Interestingly the greatest potential for improvements is covered by affinity chromatography. The most dynamic development can be observed for the variations of immobilised metal ion affinity chromatography, where novel chelating groups or metals have been applied recently. Similarly the number of available phosphospecific antibodies has increased drastically.

Both affinity techniques share the advantage to be able to selectively enrich phosphoproteins from very complex, crude samples. This is very attractive for proteomics studies. However, the separation on the basis of charge difference offers a very high resolution of isoforms, distinguishing between the numbers of attached phosphate groups. Therefore, a combination of different methods in the right order is advisable. Starting with IMAC as first enrichment step, followed by CF or IEX to separate isoforms and applying immunoaffinity as final selective step to distinguish between the phosphorylated amino acid epitopes could be the most favourable logical sequence of methods to achieve very pure, well defined single isoforms of phosphoproteins. Generally, the above-cited approaches should enable the researcher to efficiently and specifically enrich or isolate phosphoproteins even from complex mixtures. For the analysis of phosphorylation status and stoichiometry of certain protein isoforms, a successful enrichment or isolation is an essential prerequisite. Since protein phosphorylation is a highly dynamic process and almost never reach complete stoichiometry, there is an additional challenge to enrich and analyze phosphopeptides for suitable analysis methods. These strategies follow in general

the same guidelines and principles as for proteins, but are very likely condemned to fail without successful enrichment work at the protein level.

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References

[1] P.J. Kennelly, M. Potts, J. Bacteriol. 178 (1996) 4759.
[2] T. Hunter, Cell 100 (2000) 113.
[3] T. Miliotis, P.O. Ericsson, G. Marko-Varga, R. Svensson, J. Nilsson, T. Laurell, R. Bischoff, J. Chromatogr. B Biomed. Sci. Appl. 752 (2001) 323.
[4] M. Adamczyk, J.C. Gebler, J. Wu, Rapid Commun. Mass Spectrom. 15 (2001) 1481.
[5] J.A. Gordon, Methods Enzymol. 201 (1991) 477.
[6] A.H. Schonthal, Methods Mol. Biol. 93 (1998) 35.
[7] M. Jaumot, J.F. Hancock, Oncogene 20 (2001) 3949.
[8] M.O. Collins, L. Yu, H. Husi, W.P. Blackstock, J.S. Choudhary, S.G. Grant, Sci. STKE 2005 (2005) l6.
[9] A. Dubrovska, S. Souchelnytskyi, Proteomics 5 (2005) 4678.
[10] M.R. Gold, T. Yungwirth, C.L. Sutherland, R.J. Ingham, D. Vianzon, R. Chiu, I. van Oostveen, H.D. Morrison, R. Aebersold, Electrophoresis 15 (1994) 441.
[11] S.B. Kanner, A.B. Reynolds, J.T. Parsons, J. Immunol. Methods 120 (1989) 115.
[12] M. Hamaguchi, C. Grandori, H. Hanafusa, Mol. Cell. Biol. 8 (1988) 3035.
[13] B. Bjellqvist, G.J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J.C. Sanchez, S. Frutiger, D. Hochstrasser, Electrophoresis 14 (1993) 1023.
[14] B. Bjellqvist, B. Basse, E. Olsen, J.E. Celis, Electrophoresis 15 (1994) 529.
[15] H. Steen, J.A. Jebanathirajah, J. Rush, N. Morrice, M.W. Kirschner, Mol. Cell. Proteomics 5 (2006) 172.
[16] K. Zhu, J. Zhao, D.M. Lubman, F.R. Miller, T.J. Barder, Anal. Chem. 77 (2005) 2745.
[17] Y. Kumar, A. Khachane, M. Belwal, S. Das, K. Somsundaram, U. Tatu, Proteomics 4 (2004) 1672.
[18] L.A.A.E. Sluyterman, O. Elgersma, J. Chromatogr. 150 (1978) 17.
[19] L.A.A.E. Sluyterman, J. Wijdenes, J. Chromatogr. 150 (1978) 31.
[20] Y.M. Li, T.X. Chen, L.P. Yang, Y.W. Liu, Y. Jiang, Di Yi Jun Yi Da Xue Xue Bao 25 (2005) 1152.
[21] B.R. Aton, B.J. Litman, M.L. Jackson, Biochemistry 23 (1984) 1737.
[22] G. Adamus, A. Arendt, P.A. Hargrave, T. Heyduk, K. Palczewski, Arch. Biochem. Biophys. 304 (1993) 443.
[23] J.H. McDowell, J.P. Nawrocki, P.A. Hargrave, Biochemistry 32 (1993) 4968.
[24] B.G. Kim, C.L. Brooks, Biochem. J. 296 (Part 1) (1993) 41.
[25] K.N. Ekdahl, P. Ekman, J. Biochem. (Tokyo) 95 (1984) 917.
[26] H.Y. Zhou, E. Salih, M.J. Glimcher, Biochem. J. 330 (Part 3) (1998) 1423.
[27] C.L. Brooks, L.A. Isaacs, J.R. Wicks, Mol. Cell. Endocrinol. 99 (1994) 301.
[28] N. Ramasubbu, M.S. Reddy, E.J. Bergey, G.G. Haraszthy, S.D. Soni, M.J. Levine, Biochem. J. 280 (Part 2) (1991) 341.
[29] P. Leoni, P. Heyworth, A.W. Segal, J. Chromatogr. 527 (1990) 152.
[30] K. Matsushima, M. Shiroo, H.F. Kung, T.D. Copeland, Biochemistry 27 (1988) 3765.
[31] A. Sobieszek, Anal. Biochem. 172 (1988) 43.
[32] U. Wilden, H. Kuhn, Biochemistry 21 (1982) 3014.
[33] L. Andersson, J. Porath, Anal. Biochem. 154 (1986) 250.
[34] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (1999) 2883.
[35] E. Kinoshita, A. Yamada, H. Takeda, E. Kinoshita-Kikuta, T. Koike, J. Sep. Sci. 28 (2005) 155.
[36] S.B. Ficarro, M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, F.M. White, Nat. Biotechnol. 20 (2002) 301.
[37] R. Anguenot, S. Yelle, B. Nguyen-Quoc, Arch. Biochem. Biophys. 365 (1999) 163.
[38] Z. Kucerova, P. Majercakova, J. Biochem. Biophys. Methods 49 (2001) 523.
[39] X. Shi, R.J. Belton Jr., H.R. Burkin, A.P. Vieira, D.J. Miller, Anal. Biochem. 329 (2004) 289.
[40] N. Imam-Sghiouar, R. Joubert-Caron, M. Caron, Amino Acids 28 (2005) 105.
[41] G. Bernardi, W.H. Cook, Biochim. Biophys. Acta 44 (1960) 96.
[42] C.R. Hussey, P.F. Liddle, D. Ardron, G.L. Kellett, Eur. J. Biochem. 80 (1977) 497.
[43] G.J. Sale, P. Towner, M. Akhtar, Biochem. J. 175 (1978) 421.
[44] Y. Tashima, M. Terui, H. Itoh, H. Mizunuma, R. Kobayashi, F. Marumo, J. Biochem. (Tokyo) 108 (1990) 271.
[45] M. Shibata, Y. Yamakawa, T. Ohoka, S. Mizuno, K. Suzuki, J. Leukoc. Biol. 54 (1993) 10.
[46] X.F. Csar, N.J. Wilson, P. Strike, L. Sparrow, K.A. McMahon, A.C. Ward, J.A. Hamilton, Proteomics 1 (2001) 435.
[47] R.A. Wallace, O. Carnevali, T.G. Hollinger, J. Chromatogr. 519 (1990) 75.
[48] H. Matsuda, H. Nakamura, T. Nakajima, Anal. Sci. 6 (1990) 911.
[49] Y. Ikeguchi, H. Nakamura, Anal. Sci. 16 (2000) 541.
[50] Z.T. Jiang, Y.M. Zuo, Anal. Chem. 73 (2001) 686.
[51] A. Sano, H. Nakamura, Anal. Sci. 20 (2004) 861.
[52] A. Sano, H. Nakamura, Anal. Sci. 20 (2004) 565.
[53] M.W. Pinkse, P.M. Uitto, M.J. Hilhorst, B. Ooms, A.J. Heck, Anal. Chem. 76 (2004) 3935.
[54] S.S. Liang, H. Makamba, S.Y. Huang, S.H. Chen, J. Chromatogr. A. 1116 (2006) 38.
[55] C.T. Chen, Y.C. Chen, Anal. Chem. 77 (2005) 5912.
[56] H.K. Kweon, K. Hakansson, Anal. Chem. 78 (2006) 1743.
[57] F. Wolschin, S. Wienkoop, W. Weckwerth, Proteomics 5 (2005) 4389.
[58] M.A. Coletti-Previero, M. Pugniere, H. Mattras, J.C. Nicolas, A. Previero, Biosci. Rep. 6 (1986) 477.
[59] A.H. Ross, D. Baltimore, H.N. Eisen, Nature 294 (1981) 654.
[60] D. Heffetz, M. Fridkin, Y. Zick, Eur. J. Biochem. 182 (1989) 343.
[61] A.R. Frackelton Jr., M. Posner, B. Kannan, F. Mermelstein, Methods Enzymol. 201 (1991) 79.
[62] P.P. Di Fiore, F. Fazioli, Methods Mol. Biol. 124 (2001) 221.
[63] H. Steen, B. Kuster, M. Fernandez, A. Pandey, M. Mann, J. Biol. Chem. 277 (2002) 1031.
[64] P.I. Palomo-Jimenez, S. Hernandez-Hernando, R.M. Garcia-Nieto, A. Villalobo, Protein Expr. Purif. 16 (1999) 388.
[65] M. Gronborg, T.Z. Kristiansen, A. Stensballe, J.S. Andersen, O. Ohara, M. Mann, O.N. Jensen, A. Pandey, Mol. Cell. Proteomics 1 (2002) 517.
[66] The Antibody Resource Page, 2006. Available at http://www.antibodyresource.com/findantibody.html.
[67] Biocompare Inc., The buyer's guide for life scientists [Web Page], 2006. Available at http://www.biocompare.com/jump/2045/Antibodies.html.
[68] Biomol GmbH, Biochemistry Molecular Biology Cellular Communication (BIOMOL) [Web Page], 2006. Available at http://www.biomol.de.