Amino Acids (2005) 28: 105–109
DOI 10.1007/s00726-004-0130-4

Amino Acids
Printed in Austria

Application of metal-chelate affinity chromatography to the study of the phosphoproteome

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Received August 31, 2004
Accepted September 13, 2004
Published online January 13, 2005;

Summary. With the increasing importance of proteome analysis, studying the phosphoproteome is a priority for functional studies. Therefore, a rational approach to simplifying the proteome is needed. In this work, we examined the use of immobilized metal affinity chromatography (IMAC) using ferric ions-chelated column for enriching crude cell extracts in phosphoproteins. The adsorption of the proteins on Fe^{3+} was obtained at an acidic pH 5.6, and their elution at a more basic pH in Tris buffer. To evaluate the separation, western blots were performed with either anti-phosphotyrosine or anti-phosphoserine/threonine. The analysis of the eluates demonstrated the selectivity of the separation, particularly for proteins phosphorylated on serine or threonine. In conclusion, the advantages and the limits of this approach are discussed.

Keywords: Affinity – Chromatography – Phosphorylation – Proteomics

Introduction

The publication of the human genome has focused attention on complexity and diversity of proteomes. The modifications at the transcriptional, translational, and post-translational levels are what define the complexity of proteins. The most commonly used experimental techniques in proteomics are two-dimensional gel electrophoresis (2-DE) for separating proteins and mass spectrometry (MS) for the identification of separated proteins. However, the separation of co- and post-translationally modified forms of proteins, which reflect the true functional state of the proteome, is essential and 2-DE currently remains the only technique that offers sufficient resolution to address this question at a proteomic level. The capacity of 2-DE to resolve post-translational modifications (PTMs) is considered an especially important strength of this technology.

Affinity chromatography is also a powerful protein separation method. This method is based on the interaction between an immobilized ligand and the target proteins to be separated. Affinity chromatography can also be used as part of a traditional 2-DE/MS workflow. Most application of affinity purification attempt to purify a small group of proteins from complex mixtures. Even the best 2-DE gels can routinely resolve no more than two thousands proteins; therefore, only the most abundant proteins can be visualized if a crude protein mixture is used. For this, affinity-based protein purification prior to electrophoresis is imperative to reduce sample complexity and to permit the analysis of lower-abundance proteins. Enrichment in phosphoproteins can be obtained by specific immunoprecipitation methods, using anti-phosphoaminoacid antibodies. However, even if the efficiency of this approach has been demonstrated for proteins phosphorylated on tyrosine (Imam-Sghiouar et al., 2002), its efficiency is dramatically reduced for phosphoproteins modified on serine and/or threonine. An other method for enriching in phosphorylated proteins is immobilized metal affinity chromatography (IMAC) using metal ions-chelated columns that can selectively bind to the negatively charged phosphate groups (Andersson and Porath, 1986; Muszynska et al., 1986; Cao and Stults, 2000). However, even if this mode of enrichment is currently proposed for the purification of phosphopeptides from trypsin digests (Cao and Stults, 2000; Stensballe et al., 2001; Ficarro et al., 2002), the pertinence of its application to crude protein extracts before proteomic analysis has not been actually evaluated. In this work, we tested this approach in the context of the study of the phosphoproteome, and tried to precise what are the advantages and limits of this method.

Materials and methods

Except when otherwise indicated, chemicals and other reagents were obtained from Sigma (St. Louis, MO, USA), as well as gradient former (2×50 mL). Antiprotease were from Roche Diagnostics (Mannheim, Germany). Sepharose 4B and DTT were from Amersham Biosciences (Uppsala, Sweden). Protean II xi cell, GS-700 densitometer and QuantityOne software were from Bio-Rad (Richmond, CA, USA). Molecular weight calibration kit used for SDS-PAGE from Novex (Invitrogen, Cergy-Pontoise, France). The monoclonal anti-phosphoserine/threonine and anti-phosphotyrosine (mAb4G10) were purchased from Upstate Biotechnology (Lake Placid, USA).

The chromatographic equipment was composed of a LKB 2245 gradient pump with a loop of 200 μL, a LKB 2510 Uvicord detector, and a Gilson FC 205 fraction collector. IMAC HPLC experiments were carried out at room temperature using a PEEK column (30 mm $\times$ 4 mm I.D.) filled with 0.4 mL of chelating gel.

Protein extraction

Jurkat T cells were washed in ice-cold PBS 4×10 min, suspended in PBS, and then counted. The number of cells was adjusted to 3.10^8 cells/mL in extraction buffer (50 mM acetate buffer, pH 5.6, 10 mM CHAPS, 0.5 mM orthovanadate, 30 mM PMSF and 1 tablet of proteases inhibitor for 10 mL). Mechanical lysis was done on ice 5×5 min with a Dounce glass grinder, followed by an ultracentrifugation at 105,000 g for 25 min at 4°C. The supernatant was collected and samples were frozen and stored at −20°C until used.

Preparation of the affinity gel

Preparation of the chelating gel was essentially carried out according to (Porath et al., 1975) as schematically described in Fig. 1. Briefly, Sepharose 4B gel (suction-dried) was washed several times with Milli-Q water and again suction-dried on a sintered glass funnel. The gel was resus-

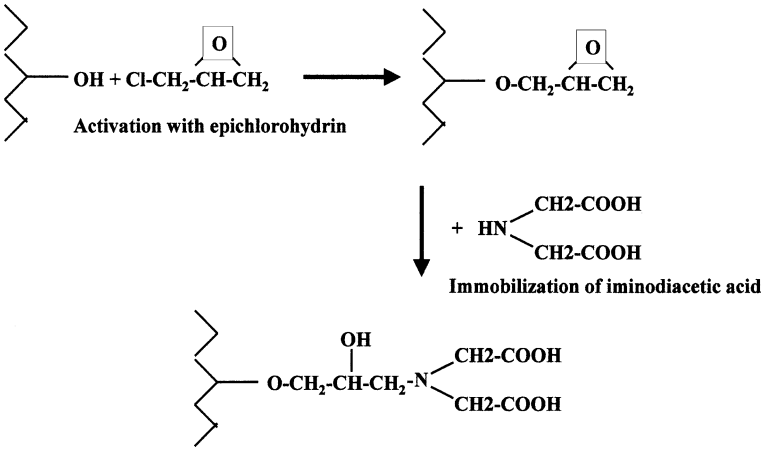

Fig. 1. Brief synthetic scheme and structure of the IMAC support

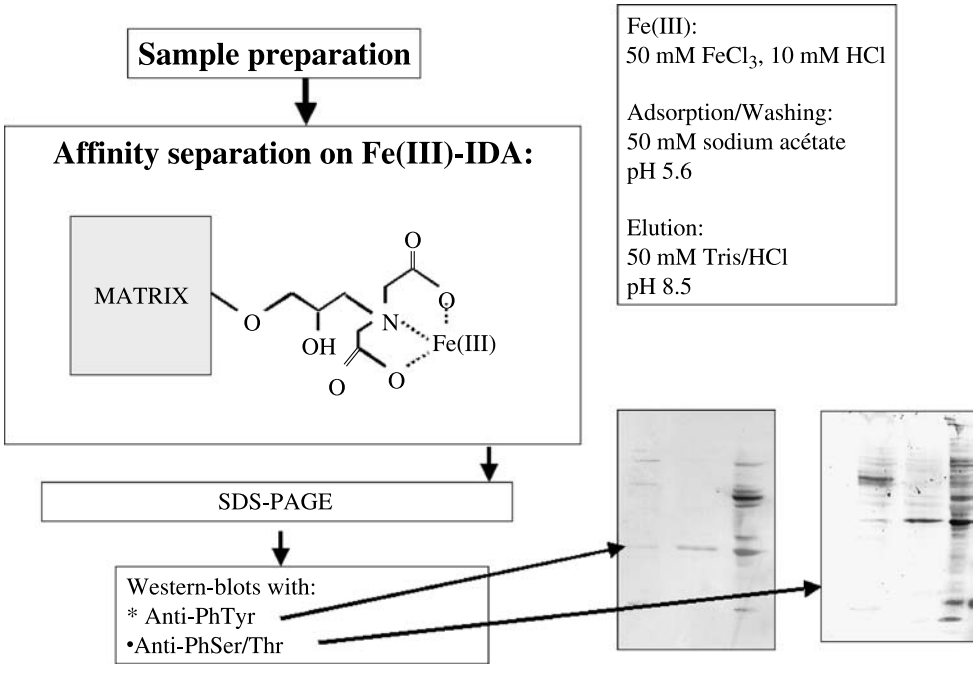

Fig. 2. Process followed to enrich cell extracts in phosphoproteins on IMAC column and to test the eluates. Schematic representation showing the main steps in the procedure (see Experimental for details)

pended in 0.6 M NaOH (20 mL/10 g of gel) and epichlorhydrine added (2.5 mL). The reaction was allowed to proceed 2 h at 40°C with periodic manual agitation. The excess of reagents was removed by washing the gel with a large volume Milli-Q water (>500 mL). For the coupling of the chelating ligand, 10 mL of 1 M iminodiacetic acid were added to 5 g of activated support. The coupling was allowed to proceed overnight at room temperature with gentle agitation.

The chelating gel was suspended in Milli-Q water degassed and packed into the column using the HPLC gradient pump. After extensive washings with Milli-Q water, 0.1 M EDTA solution, and again Milli-Q water, the column was charged with Fe^{3+} ions by passing 50 mM $FeCl_3$ solution in 10 mM HCl through the column until saturation. Non specifically bound ions were removed by washing the column with the adsorption and elution buffers used for the chromatographic runs.

Chromatography

The procedure followed to fractionate the cell protein extract by IMAC is schematically represented in Fig. 2. The column saturated with Fe^{3+} ions was washed with 50 mM acetate buffer, pH 5.6 (adsorption buffer), 25 mM Tris/HCl, pH 8.5 (elution buffer), and then equilibrated with the adsorption buffer. The protein extract was applied onto the column at a flow rate of 0.4 mL/min. The column was washed with adsorption buffer, until no further proteins were detected. Adsorbed proteins were subsequently eluted in elution buffer, and the metal ions were finally eluted in 100 mM EDTA.

Electrophoresis and Western blotting

For SDS-PAGE, 1 mm thick homemade 15% gels were used. Electrophoresis were carried out at 200 V (15 mA/gel, 25 W), for 2 h 30. The gels were stained with silver nitrate. Stained gels were digitized using the GS-700 densitometer, and the images were exported to QuantityOne Software (Bio-Rad).

For Western blotting, proteins were separated by SDS-PAGE in the same conditions and transferred to an Immobilon-P membrane (Millipore). Bots were incubated with anti-phosphoserine/threonine (1:500), or anti-phosphotyrosine (1:1000) for 2 h at room temperature. The blots were visualized by incubation with goat anti-mouse HRP-conjugated Ig diluted 1:10,000. Peroxidase activity was visualized with the Amplified Opti-4CN kit (Bio-Rad). The image of the membranes was acquired from GS-700 densitometer, and analysed with QuantityOne Software.

Results

Immobilized metal (ion) affinity chromatography was introduced in the mid 1970s by Porath et al. (1975). Iminodiacetic acid (IDA) is the standard, most commonly used metal chelating ligand for the immobilization of metal ions in IMAC supports (Porath and Olin, 1983). In the mid 1980s, the use of ferric ions immobilized on IDA-agarose to fractionate phosphoproteins was proposed (Andersson and Porath, 1986; Muszynska et al., 1986). However, in the last twenty years, most of the applications of IMAC concern the purification of proteins with a high content in histidine, and more recently the use of histidine tags for separation of recombinant proteins (Sulkowski, 1989; Porath, 1992; Boden et al., 1998; Gaberc-Porekar and Menart, 2001). Compared to the capture of histidine-tagged proteins, the application of IMAC for the fractionation of phosphoproteins remained limited to some model studies (Andersson and Porath, 1986; Muszynska et al., 1986; Porath, 1992).

During the last few years, with the entry in the post-genome era and the increasing importance of proteomics, mining the phosphoproteins (the so-called phosphoproteome) appeared as a priority for functional studies. This is particularly true for the studies of the proteome during signal transduction, where protein phosphorylation is clearly the more important event that occurs in cells. This leads to a revival of the use of IMAC, applied to the capture of phosphopeptides before their analysis by MALDI-TOF (Cao and Stults, 2000; Stensballe et al., 2001; Haydon et al., 2003). In the present work, we tested the pertinence of using the same type of separation for crude protein extracts before proteomic analysis. The separation was performed on a microcolumn packed with an affinity support with ferric ions immobilized on IDA-agarose (Fig. 2). The adsorption of the proteins on the metal ions was obtained at an acidic pH 5.6, and their elution at a more basic pH in Tris buffer. In order to evaluate the capacity of the column, preliminary experiments were performed by increasing the amount of protein solution injected on the affinity support. In our experimental conditions, the support was able to adsorb approximately 2 mg protein/1 mL of packed support. When several injections of Jurkat T cells proteins (extracted in acetate buffer) were performed in the same conditions, the amount of protein that was retained and eluted by the Tris buffer was of approximately of 590 μg with a protein concentration of about 110 μg/ml (Table 1). These elutions represented 37% of the total amount of injected proteins, but the volume of elution was about ten fold the volume of injection. The different chromatography fractions were then tested by Western blots with either anti-phosphotyrosine or anti-phosphoserine/threonine, following SDS-PAGE. Even if the accuracy of the detection was necessarily limited by the heterogeneity of the eluted material, using the QuantityOne Software allowed analyzing the images of stained SDS-PAGE and of Western blots of numerous proteins present in the cell extract. This analysis clearly showed that phosphoproteins with both types of phosphorylation were retained by the affinity support. Very generally, the same phosphoproteins were detected in the crude extract before chromatography, in the eluate, but not at all in the flow-through. Some minor exceptions were found where the same band was detected in the crude extract and in the flow through (one detected with the anti-tyrosine and two by the

Table 1. Elutions obtained in three independent experiments

	Elution volume (ml)	Eluate concentration (μg/ml)	Eluted proteins (μg)	Enrichment factor
Chromatography 1	5.5	113	560	2.9
Chromatography 2	5.0	104	520	3.1
Chromatography 3	5.7	123	701	2.3

Each experiment corresponds to the injection of 1.6 mg protein of Jurkat cells extract

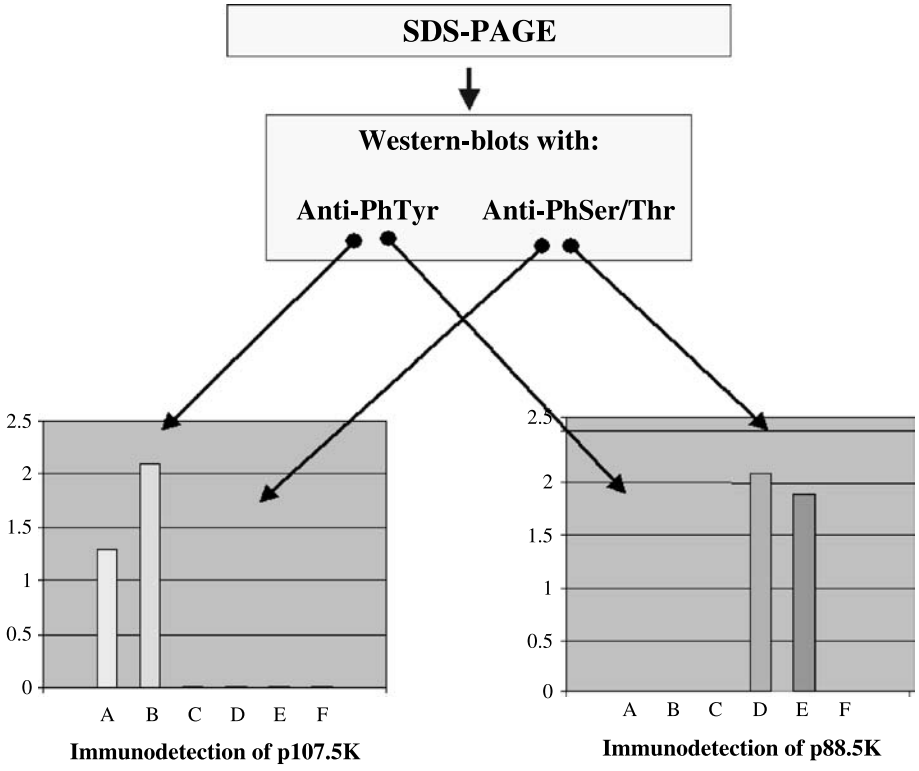

Fig. 3. Relative amounts (intensity) of proteins in different chromatography fractions, analyzed using the QuantityOne software. The figure shows the examples of two proteins of approximately 107 kDa and 88 kDa, respectively, and of their detection by the two specific anti-phosphoaminoacids antibodies. Detection with anti-phosphotyrosine: (*A*) crude extract, (*B*) eluate, (*C*) flow-through; detection with anti-phosphoserine/threonine, (*D*) crude extract, (*E*) eluate, (*F*) flow-through

anti-phosphoserine/threonine antibodies, respectively). The results that were obtained are illustrated in Fig. 3, showing the analysis performed for two proteins differentially phosphorylated. The protein of 107 kDa was recognized by anti-phosphotyrosine antibodies in the crude extract and in the eluate. No detection was obtained in any fraction with the antibody directed against phosphorylated serine/threonine. On the opposite, the protein of 88 kDa that was also retained by the affinity support was detected only by anti-phosphoserine/threonine.

Discussion

The use of IMAC-based pre-fractionation as a step in the study of the phosphoproteome has to be considered. Its major advantage is certainly that it can allow an efficient separation not only for proteins containing phosphotyrosines but also for proteins phosphorylated on serine or threonine. Therefore, it can be complementary to affinity captures on anti-phosphotyrosine antibodies. During this study, we have used this method in the context of the fractionation of a crude protein extract typical of what

is currently studied in proteomics, and not on model systems. It was important to enlighten some limits, or at least problems of this approach. Particularly, one may mention that:

- An acidic pH is certainly far from being optimal for protein solubilisation. Comparing the extracts obtained either in these conditions or in an extraction buffer at pH 8.5, currently used in proteomics, we observed that many proteins were lost during the extraction at pH 5.6 (not shown).
- The eluates were clearly too diluted to be directly separated in a current proteomic experimental flow. One consequence is that the advantage of eluting the proteins in a buffer that could be directly compatible for instance with 2-DE is lost. Further experimentation would necessitate to add stages of concentration and desalting.
- The amounts of protein eluted from the column suggest that the eluates did not contained only phosphoproteins. As the same phenomenon has previously been observed using an other method of pre-fractionation of the phosphoproteome (immuno-affinity) (Imam-Sghiouar et al., 2002), it may be attributed to the nature of the proteins retained on the column rather than to the IMAC method. Most of the phosphoproteins participate to supra-molecular networks, and during their adsorption on an affinity support some members of these networks are certainly captured and retained by their phosphorylated partners.

In conclusion, considering that in the next future new developments in proteomics will need the study of protein extracts enriched in a given sub-proteome, the combination of affinity separation and of current proteomic approaches will certainly be more and more employed. However, one may keep in mind that affinity methods have currently been developed in model systems (mixtures of pure proteins or plasma) and that their use in complex systems will need further optimization and adaptation.

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