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Review

Affinity chromatography: A useful tool in proteomics studies☆

Mohamed Azarkan[a], Joëlle Huet[b], Danielle Baeyens-Volant[a], Yvan Looze[b], Guy Vandenbussche[c,*]

[a] *Laboratoire de Chimie Générale (CP: 609), Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, 808, route de Lennik, B-1070 Bruxelles, Belgium*
[b] *Laboratoire de Chimie Générale (CP: 206/4), Institut de Pharmacie, Université Libre de, Bruxelles, Campus de la Plaine, Boulevard du Triomphe, B-1050 Bruxelles, Belgium*
[c] *Laboratoire de Structure et Fonction des Membranes Biologiques (CP: 206/2), Faculté des Sciences, Université Libre de Bruxelles, Campus de la Plaine, Boulevard du Triomphe, B-1050 Bruxelles, Belgium*

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Abstract

Separation or fractionation of a biological sample in order to reduce its complexity is often a prerequisite to qualitative or quantitative proteomic approaches. Affinity chromatography is an efficient protein separation method based on the interaction between target proteins and specific immobilized ligands. The large range of available ligands allows to separate a complex biological extract in different protein classes or to isolate the low abundance species such as post-translationally modified proteins. This method plays an essential role in the isolation of protein complexes and in the identification of protein–protein interaction networks. Affinity chromatography is also required for quantification of protein expression by using isotope-coded affinity tags.

Keywords: Proteomics; Affinity chromatography; Mass spectrometry; Phosphoproteome; Glycoproteome; Disulfide proteome; Isotope-coded affinity tags

Contents

1. Introduction

During the past decades, important technological progresses realized in the genomic field, and particularly the automation of analytical methods, have led to sequencing completion of an increasing number of organism genomes [1]. At the present time, 387 complete genomes are published and more than 1600 are ongoing (http://www.genomesonline.org/). The sequencing of complete genomes provides an opportunity to analyze the different functions governed by the genes. Nevertheless, despite the important data gained from these genomes, this information remains "static" and cannot by itself describe the modifications occurring during the cell cycle or after environmental stim-

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* Corresponding author.
E-mail address: vbussche@ulb.ac.be (G. Vandenbussche).

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uli. The description at the molecular level of cellular functions also requires the complete analysis of the gene product expression. Proteomics is one of the most important of the so-called post-genomic approaches to understanding gene function. The purpose of this methodology is to deliver a complete qualitative and quantitative description of the proteome of a biological system under given conditions. However, while proteins may yield the most important clues to cellular function, they are also the most difficult of the cells components to detect on a large scale due to the large diversity of their properties (size, dynamic range, hydrophilicity/hydrophobicity, post-translational modifications, etc., . . .). Due to the complexity of a biological system, capturing this dynamic state represents a technological challenge that requires efficient tools. Most of the proteomic approaches are generally based on the same scheme: separation of the proteins present in a biological sample followed by their identification. Major advances in mass spectrometry allowed this technique to become the method of choice for protein identification (for review see [2]). The limiting step in proteomic approach is the protein separation rather than identification. Two-dimensional (2D) electrophoresis remains the most widely used separation tool for analyzing complex mixtures of proteins. This technique developed independently by Klose [3] and O'Farrell [4] allows the orthogonal separation of the proteins according to the net charges by isoelectric focusing (IEF) in one direction and on the basis of their apparent molecular masses by electrophoresis in the presence of sodium dodecyl sulfate in the other. The major advantage of the 2D electrophoresis methodology is linked to its capability for the simultaneous separation, visualization and quantification of thousands of proteins at different modification states [5,6]. Using the 2D electrophoresis method with large gels, it has been demonstrated that protein patterns obtained from mouse tissues reveal more than 10,000 polypeptide spots [7]. No other method can achieve this at the present time. The 2D electrophoresis gel delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications (PTMs). However, this technique presents several limitations. The dynamic range of 2D electrophoresis is at best 10^4, a value that is largely inadequate to cover the dynamic range of 10^6–10^9 found in certain biological samples [8]. The analysis of the low copy number proteins requires an enrichment or prefractionation step. Some classes of proteins, particularly hydrophobic membrane-bound proteins that are favorite targets for drug development, will not run on 2D electrophoresis gels. Generally, these kinds of proteins precipitate during the isoelectric focusing step. Proteins with extreme p*I*s or molecular weights are not well resolved by 2D electrophoresis. Finally, the automation of the 2D electrophoresis is difficult or even impossible for certain steps. The limitations of this technology have hindered the analysis of complete proteomes. To overcome some of these limitations, "gel-free" approaches were developed. The most impressive one, regarding the number of proteins identified by the method, is the multidimensional protein identification technology (MUDPIT) [9,10]. This method is based on the separation of a total protein digest by multidimensional chromatography (i.e., a strong cation-exchange followed by a reverse phase column) interfaced on-line with a MS/MS spectrometer (for review of different chromatographic approaches, see [11,12]). The information gained from the 2D electrophoresis or the MUDPIT approaches is roughly a list of identified proteins and their level of expression when suited. Analysis of protein functions requires additional methodology. In this context, affinity chromatography is a powerful proteomic tool. This separation method is based on the specific interaction between immobilized ligands and their target proteins. As this method is versatile, it can be adapted to different needs. In this review, we will describe the usefulness of affinity chromatography in the study of different post-translational modifications, protein complexes and protein quantification.

2. Affinity chromatography in phosphoproteomics

Phosphorylation is one of the most prevalent post-translational protein modifications occurring in eukaryotic cells [13]. This reversible modification plays a key role in the regulation of different cellular processes including signal transduction, activation/inactivation of enzyme activity, complex formation, and protein degradation. Phosphorylation is a dynamic process reversibly controlled by the concerted actions of protein kinases and protein phosphatases. Abnormal activation of the kinases leads to perturbation in the signal transduction pathways resulting in severe disorders including several types of cancers [14–16]. Complete description of the phosphorylation events is therefore required to understand the regulation of these pathways.

It has been estimated that about one-third of the eukaryotic proteins are phosphorylated at any given time [17]. Four different phosphorylation types have been described [18], but the most common in eukaryotic cells is the *O*-phosphorylation of hydroxyamino acids such as serine, threonine or tyrosine [19]. Phosphoproteomic approaches have to face technical problems: phosphoproteins may be of low abundance especially for the signaling pathways where 1–2% of the total protein amount is phosphorylated; the same protein may be phosphorylated in different ways and at different sites; the proteins may only be transiently phosphorylated. The enrichment of phosphoproteins or phosphopeptides is thus a prerequisite to any analysis.

Different strategies were developed to enrich phosphorylated proteins or peptides. Classical enrichment protocols involve immunoaffinity purification with phosphospecific antibodies. Antibodies specific for phosphorylated tyrosine residues were used for the selection of phosphorylated proteins [20–22]. Due to a lower specificity, antibodies that bind to phosphorylated serine or threonine residues were less successfully used to purify phosphoproteins [23–25].

Another purification approach takes advantage of the affinity of the phosphate group for different stationary phases. Immobilized metal ion affinity chromatography (IMAC), firstly introduced by Hellferich [26] and Porath et al. [27] for purification of proteins, is the most widely used method for enriching phosphopeptides [28–31]. In this technique, the phosphate group interacts through nonbonding ion pair electron coordination with metal ions, usually Fe^{3+} or Ga^{3+} cations, which have been chelated to a multidentate ligand immobilized onto a sup-

port material. The commonly used solid supports for IMAC include porous [32,33] and nonporous [34] silica, agarose [35], sepharose [36,37] or cross-linked poly(styrene-divinylbenzene) [38,39]. IMAC has been used in combination with electrospray ionization (ESI) tandem MS [40] or matrix-assisted laser desorption/ionization (MALDI) MS after alkaline phosphatase treatment in order to localize phosphorylation sites [29]. IMAC beads containing immobilized phosphopeptides have also been directly applied onto a MALDI target plate for phosphorylation analysis [35]. The major drawbacks of the IMAC method are a preferential selection of multi phosphorylated peptides and contamination with very acidic peptides [29]. The latter can be overcome by the esterification of the side chains of glutamate and aspartate residues with HCl-saturated methanol prior to purification on the IMAC column [38]. Recently, a new method using a TiO_2 microcolumn was demonstrated to be more selective for binding phosphorylated peptides than IMAC [41].

The last category of enrichment methods is based on the chemical substitution of the phosphate moiety for an affinity tag that allows subsequent purification. Oda et al. [42] designed a strategy where the phosphate group on serine and threonine was replaced with ethanedithiol by a beta-elimination and Michael addition reaction followed by introduction of a biotin-containing tag. Biotinylated peptides could be selectively captured using immobilized streptavidin [42]. Zhou et al. [43] have proposed an alternative chemistry where the phosphopeptides were modified by attachment of cysteamine (1-amino-2-thioethane) to the phosphate group using a carbodiimide condensation reaction. The resulting peptides were purified by covalent binding to iodoacetyl resin and released by acidification.

3. Affinity chromatography in glycoproteomics

Glycosylation is widely recognized as one of the most important factors in determining protein activity. It has been estimated that more than half of all the proteins in the nature should be glycoproteins [44]. Modulation of glycosylation alters biological functions and impacts on cellular processes [45]. Recognition between carbohydrates moieties and proteins is crucial in a variety of processes, including protein trafficking [46], protein folding [47], cell–cell interaction [48] and tagging and recognition of proteins for proteolytic degradation [49]. The glycoproteome is also one of the major subproteomes of human plasma, as many proteins are secreted from the tissues, such as the liver, in a glycosylated form [50,51]. About 50% of all plasma proteins are glycosylated [59]. The plasma glycoproteome has important clinical value, as many biomarkers are glycosylated [52,53]. Zhang et al. [54] have developed a method to specifically enrich glycoproteins from human serum by capturing N-linked glycoproteins using hydrazide chemistry. After immobilization on a solid support, the nonglycosylated proteins were washed off and the glycoproteins were proteolyzed on the solid support. The immobilized glycopeptides were then isotopically labeled and released following peptide-*N*-glycosidase F before to be analyzed and identified using microcapillary high-performance liquid chromatography electrospray ionization MS/MS. By applying this glycoproteins capture approach, the authors identified 145 unique peptides mapping 57 unique serum proteins [54].

Using agarose-linked α-D-mannose column, Andon et al. [55] isolated 136 distinct mannose-binding proteins from different rice tissue extracts. After separation by SDS-PAGE and in-gel trypsin digestion, the proteins were identified, on the basis of exact peptide matching to sequences in the rice genomic database, by reverse phase LC–MS/MS. Nearly 15% of the identified proteins do not have a known function, indicating the potential of this combined chromatographic approach to assign a preliminary function to novel proteins in a high-throughput fashion. In fact, the affinity chromatographic support not only enriched the desired protein population, as is common with any affinity technique, but also provided information about the functional role of the captured proteins in their natural environment, in this case acting as lectins [55].

To characterize the human brain lysosomal proteome with a focus on the proteins containing mannose-6-phosphate, Sleat et al. [56] used an affinity support with immobilized mannose 6-phosphate receptor. The fractions enriched in Man6-P glycoproteins were separated by 2D electrophoresis, and proteins in each spot were identified using a combination of MALDI-TOF MS and MALDI-TOF MS/MS analysis of the tryptic peptides or by N-terminal sequencing by Edman degradation. In total, 61 different proteins were identified, of which 11 had not previously been reported to contain mannose 6-phosphate. The authors underlined the usefulness of this affinity approach to study such highly complex source.

Lectin affinity chromatography technology, also named "glyco-catch" [57], is another method designed for the enrichment of glycoproteins from complex samples. Bukenborg et al. [58] developed a strategy for mapping *N*-glycosylation sites in complex mixtures by reducing sample complexity and enriching glycoproteins with the aid of lectin affinity chromatography on immobilized concanavalin A and wheat germ agglutinin. Glycosylated proteins were selectively captured with an initial lectin chromatography step and digested with endoproteinase Lys-C. The digest mixture containing the glycosylated peptides was then subjected to a second lectin chromatography step. After removal of glycan components with *N*-glycosidase F, the peptides were digested by trypsin and analyzed by on-line reverse phase LC–MS. Using this approach, 86 *N*-glycosylation sites in 77 proteins were identified in human serum [58]. Aiming at investigating the human serum proteome, Yang and Hancock [59] used a multi-lectin affinity column. After having evaluated the ability of five commonly used immobilized lectins to capture glycoproteins, the authors developed a multi-lectin affinity support containing concanavalin A, wheat germ agglutinin and jacalin lectin. The selection of these lectins was also based on the known N-linked and O-linked glycan structures that are considered representative of the serum proteome. By using this multi-lectin affinity column, 10% (w/w) of human serum proteins were found to be glycosylated. Furthermore, analysis of a serum sample depleted from the six most abundant proteins (albumin, IgG, IgA, antitrypsin, transferin and haptoglobin) after chromatography on the multi-lectin affinity support revealed that 50% (w/w) of the remaining serum proteins are glycosylated.

The multi-lectin affinity approach was found to successfully remove a large portion of the serum albumin fraction (estimated greater than 80% of the total protein fraction), resulting in more reproducible protein identifications (more peptides characterized per protein and consistency between serum proteomic analyses [59]. Indeed, the presence of high abundance proteins, such as albumin, can effect the identification of low abundance proteins and it has been shown that depletion of highly abundant proteins can improve the dynamic range of protein identification [60].

Kaji et al. [61] have developed a strategy for large-scale identification of *N*-glycosylated proteins from a complex biological sample. The approach, termed isotope-coded glycosylation-site specific tagging (IGOT), is based on the lectin column-mediated affinity capture of a set of glycopeptides generated by tryptic digestion of protein mixtures, followed by peptide-*N*-glycosidase-mediated incorporation of a stable isotope tag, ^{18}O, specifically into the *N*-glycosylation site. The ^{18}O-tagged peptides were then identified by LC–MS based technology. The application of this approach to the characterization of N-linked high mannose and/or hybrid-type oligosaccharide chains glycoproteins from a bacterial extract allowed the identification of 250 glycoproteins, including 83 putative transmembrane proteins, with the simultaneous determination of 400 unique *N*-glycosylation sites. The lectin affinity capture was found effective to remove major nonglycosylated protein components, allowing detection of low abundance N-linked glycoproteins [61].

4. Affinity chromatography in thiol/disulfide proteomics

Oxidation–reduction of cysteine residues is also increasingly recognized as an important dynamic post-translational modification and is described as a significant modulator of protein function. Cysteine residues present in proteins can undergo oxidation to form a disulfide bond (–SSR; protein–protein disulfide or protein–glutathione disulfide), sulfenic acid (–SOH), sulfinic acid ($–SO_2H$) or sulfonic acid ($–SO_3H$). Sulfinic and sulfonic acids are irreversibly oxidized forms of cysteine and would likely be associated with a loss of biological activity. In contrast, disulfide bonds and protein sulfenic acid moities can be readily recycled into a reduced form by cellular redox systems. This redox cycling of cysteine residues has been demonstrated, in some cases, to play a key role in the regulation of protein activity and signal transduction [62,63]. Thus, oxidation of critical cysteine residues can either activate or inactivate protein functions in various physiologically important reactions.

The systematic study of redox regulation requires the isolation and characterization of proteins containing redox-regulated cysteine residues. In this context, the term “disulfide proteome” was introduced by Yano et al. [64]. New methodologies for the isolation of redox-regulated proteins based on thioredoxin-Sepharose affinity column [65,66] or covalent chromatography using thiol disulfide interchange [67], were reported. Lee et al. [68] have developed a simple and powerful method for identifying proteins with disulfide bonds *in vivo*. In this method, free thiol functions in proteins were first fully blocked by alkylation after denaturation. The disulfide bridges that are not modified during this first step were thereafter converted to sulfhydryl groups by reduction. Proteins with free thiol functions generated during the reduction step were selectively captured and enriched by thiol affinity chromatography, and were identified by MALDI-TOF MS and nanoelectrospray MS/MS after separation by SDS-PAGE and in-gel digestion with trypsin. Using this approach, soluble as well as secreted and membrane disulfide containing proteins were successfully isolated in the presence of detergent from *Arabidopsis thaliana*. A total of 65 putative disulfide-containing proteins were identified, including 20 that had not previously been demonstrated to be regulated by redox state. The protein fraction isolated using this approach did not exceed 1% of the total protein content, underlying the potential of this affinity approach to identify low abundance proteins [68].

Chromatographic approaches to selectively isolate cysteinyl peptides were also developed to reduce the complexity of a protein extract. Spahr et al. [69] used an affinity selection to specifically capture cysteinyl peptides after reversible cysteine biotinylation of the digested mixture. The biotinylated digest was applied to an immobilized avidin column. The avidin-bound fraction, eluted with dithiotreitol, and the flow-through fraction were then analyzed by LC–MS/MS after being fully alkylated. The approach was applied to the analysis of a mixture of purified standard proteins as well as to a protein mixture of unknown complexity represented by proteins released from isolated mitochondria following atractyloside treatment. Such a treatment induced mitochondria membrane permeabilization, a situation also observed during apoptosis process [70,71]. The authors aimed at defining more broadly the nature of protein components released and their results demonstrated that cysteine affinity labeling is a selective procedure. Selectivity is crucial taking into account the low abundance of cysteine residues among the other amino acids. In fact, this residue in proteins constitutes, as a mean, 1.7% of all amino acids [72], making their capture from a complex mixture challenging. Using this affinity approach, 43 proteins were identified in the avidin-bound fraction. Furthermore, analysis of the avidin flow-through fraction revealed the absence of cysteinyl peptides. Even though this fraction is far more complex than the specifically bound fraction, reduction of its complexity (through removal of cysteinyl peptides) resulted in additional peptide matches and hence additional protein identifications [69].

Covalent chromatography represents another powerful tool to selectively capture cysteine-containing proteins through a reversible thiol-disulfide interchange process [73]. However, this approach has only recently been exploited in the context of proteomics studies as a strategy to enrich cysteine-containing peptides. Tryptic digests are generally prepared after reduction and S-alkylation of the protein before that proteolysis proceeds. This should be avoided because such a practice precludes disulfide interchange. Wang and Regnier [74] and Wang et al. [67] described an elegant procedure in which thiol containing proteins from a *Escherichia coli* lysate were allowed to react with 2,2′-dipyridyl disulfide at first, a reversible and specific thiol-blocking reagent. The derivatized proteins were then digested with trypsin and, thereafter, acylated with succinic anhydride.

Cysteine containing peptides were then selected from the acylated digest by disulfide interchange with sulfhydryl groups on a thiopropyl Sepharose gel. Captured cysteine containing peptides were released from the gel with dithiothreitol and alkylated with iodoacetic acid, subsequently fractionated by reverse-phase liquid chromatography and analyzed by MS. This chromatographic approach offers the advantage of following the binding process of cysteine-containing peptides because the thiol-disulfide interchange is accompanied by the release of 2-thiopyridone (λ_{max} = 343 nm) that is easily monitored [67,74].

Another strategy designed to specifically and reversibly capture thiol-containing proteins and peptides was developed in our laboratory. This method can be used as a preliminary step before other purification approaches, like affinity chromatography, to reduce the complexity of a protein sample. The method consists in the synthesis of monomethoxy(polyethylene glycol) (mPEG) derivatives selected to react specifically and instantaneously with free thiol functions by forming mixed disulfide bonds (Fig. 1). The developed mPEG derivatives are themselves a mixed disulfide between a PEG derivative containing a free thiol and 2-thiopyridone. They offer the advantage, when reacting with a thiol-containing polypeptide, to liberate 2-thiopyridone. The usefulness of the reversible modification by these mPEG derivatives, a process called "thiol pegylation," was demonstrated with a monothiol proteinase, ananain, from crude stem bromelain [75] as well as monothiol proteases and a dithiol protease isolated from the latex of the tropical tree *Carica papaya*. After mPEG derivatization, proteins carrying one or several mPEG chains acquire quite different chromatographic properties when compared to their non-derivatized counterparts. These new properties are exploited for their separation on classical chromatographic supports, such as cationic-exchangers [76–80].

5. Affinity chromatography for the purification of ubiquitinated proteins

Ubiquitination is a common post-translational modification consisting in the covalent attachment of isopeptide-linked chains of ubiquitin to target eukaryotic proteins that marks them for degradation by the proteasome system [81]. In addition to its role in protein turnover, ubiquitination contributes directly to the regulation of cellular functions such as the repair of DNA damage [82] and trafficking, endocytosis, and sorting of transmembrane proteins [83,84]. Aberrations in ubiquitination and deubiquitination underlie, directly or indirectly, the pathogenesis of many diseases including several types of cancer [85]. Comprehensive analysis of proteins that are ubiquitinated during biological processes is thus crucial.

The peculiarity of this post-translational modification is to be large (∼8 kDa), in comparison to other PTMs, which makes its identification more complicated. Mass spectrometry approaches were developed to identify critical cellular targets of ubiquitin and to map ubiquitination sites on proteins [86,87]. A second characteristic is the low level of the protein–ubiquitin conjugated present in a cell as its turnover is very rapid. Robust purification

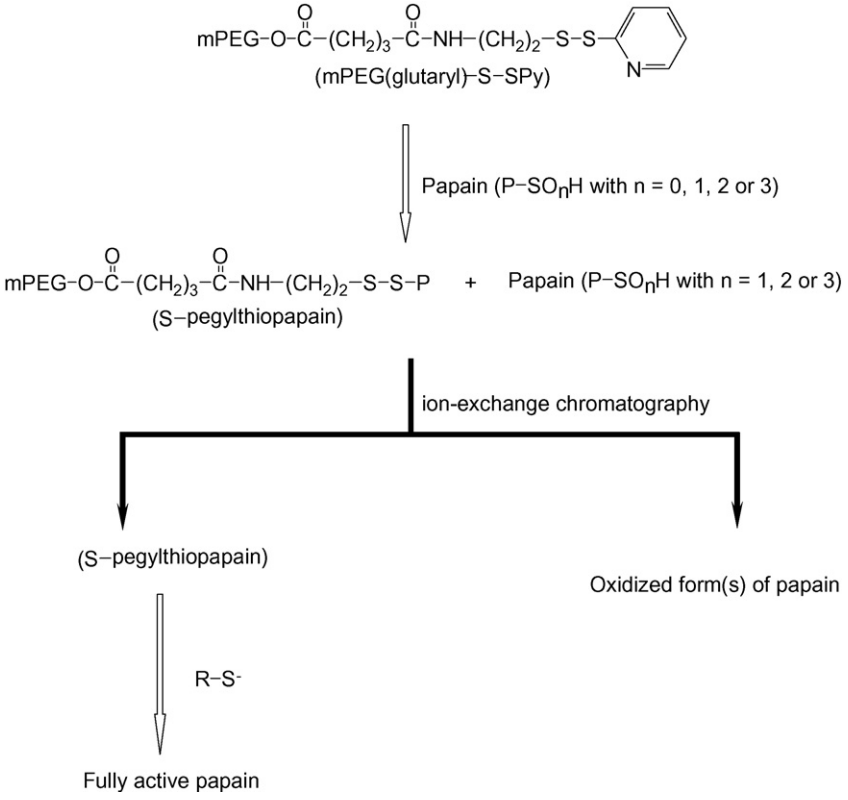

Fig. 1. Schematic representation of the covalent grafting of an mPEG derivative to free thiol groups. Papain, a cysteine endopeptidase, was taken as an example ([78–80]).

procedures of large quantities of ubiquitinated protein conjugates are then required. Different successful approaches were based on tag affinity purification. Layfield et al. [88] used an immobilized glutathione-S-transferase (GST)-S5a fusion protein to purify poly-ubiquitinated proteins from mammalian tissues. The S5a subunit of the 26S proteasome was originally identified as a protein capable of binding poly-ubiquitin chains. Using this affinity chromatographic strategy, a complex mixture of poly-ubiquitinated proteins was successfully purified from normal pig brain extract, following induction of *in vitro* ubiquitination. Peng et al. [87] provided a general tool for large-scale analysis and characterization of protein ubiquitination. Ubiquitin conjugates from a *Saccharomyces cerevisiae* strain expressing 6xHis-tagged ubiquitin were selectively captured using nickel-affinity chromatography, proteolyzed with trypsin and analyzed by multidimensional liquid chromatography coupled with tandem MS. A total of 1075 proteins have been identified and 110 precise ubiquitination sites were found in 72 ubiquitin–protein conjugates [87]. A similar approach based on IMAC purification of protein-His(6x)-ubiquitin-GFP conjugates from human embryonic kidney cells, digestion in solution of the purified ubiquitinated proteins with trypsin, and separation and microsequencing of the complex mixtures of peptides by nano LC–MS/MS, has led to the identification of 21 proteins [89]. A large-scale analysis of the human ubiquitin-related proteome was conducted by immuno-affinity chromatography using immobilized ubiquitin antibody [90]. By a combination of affinity chromatography, trypsin digestion of the purified fractions and 2D LC–MS/MS analysis of the resulting peptides, the authors developed a comprehensive characterization of ubiquitin-conjugated and ubiquitin-associated proteins in human cells treated with a proteasome inhibitor. The inhibition treatment stabilized such labile protein complexes and allows further analysis. The 670 proteins identified in this study were separated in two populations depending on the conditions of purification: (i) proteins identified under denaturing conditions that included ubiquitin-conjugated proteins, and (ii) those identified only under the native condition that comprised the proteins associated to ubiquitinated proteins [90].

6. Affinity chromatography for protein complexes characterization

Proteins control and execute the large majority of cellular functions. However, proteins do not act alone and often interact with other biomolecules to form larger entities in a time- and space-dependent manner. Within these protein complexes, each partner has a specialized function that may modulate the activity of its neighbors. Mapping these interactions leading to the formation of stable or transient complexes is required to unravel the mechanisms of cellular processes.

Due to the tremendous amount of information continuously coming from the "genomic pipeline," deciphering of entire protein interaction networks requires a robust and high throughput method. In this context, a yeast two-hybrid screening [91] has been performed on a large-scale in *S. cerevisiae*, predicting new potential protein interaction [92,93]. Although the yeast two-hybrid system has a real potential in the cataloging phase of protein interactome analysis, it presents limitations: it cannot detect interactions involving more than two proteins and those depending on post-translational modifications, it is not suitable for the detection of interactions involving membrane proteins, and there is no guarantee that the inferred interactions are of physiological relevance as the technique suffers from false positive and negative signals [94].

An alternative to the yeast two-hybrid assay is proposed by different affinity chromatography approaches [95]. Among these techniques including immunoaffinity purification, epitope tagging, GST pulldown, etc., . . ., the tandem affinity purification (TAP) method, developed by Rigaut et al. [96], represents a promising new tool for functional proteomic exploration. This approach is based on the fusion of a high-affinity tag to the target protein and the introduction of this construct in a host cell or organism. The TAP tag is composed of two affinity components separated by a short amino acid sequence containing a protease cleavage site. In the original TAP-tagging system, a calmodulin binding peptide (CBP) fused to the target protein is linked to two IgG-binding domains of protein A via a specific tobacco etch virus (TEV) protease recognition sequence [96,97] (Fig. 2). After expression in a relevant type of cell, the

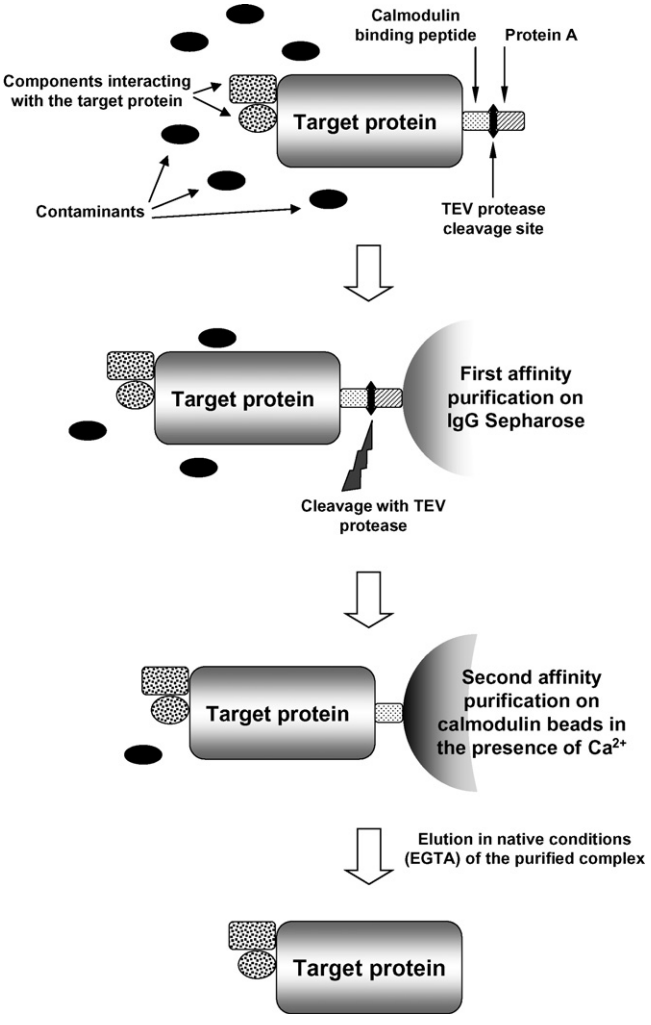

Fig. 2. Schematic representation of the TAP purification steps.

fusion protein and its associated partners are recovered from the cell extract by affinity purification on an IgG matrix. The protein complex specifically released from the matrix after TEV protease cleavage undergoes a second affinity purification step by incubation with calmodulin-coated beads in the presence of calcium. Finally, the target protein and its partners are released with EGTA. The complex components can then be separated by SDS-PAGE and after enzymatic in-gel digestion, the proteins can be subsequently identified after microsequencing by MS/MS [98]. As the protein complex is isolated in a native form, it can be alternatively used for functional or structural studies [99,100]. In comparison to single step affinity purification, the level of contaminating proteins is very low with the TAP method. Contrary to the yeast two-hybrid screening, the TAP methodology is not limited to protein–protein interaction but can also reveal the presence of ligands associated to the target protein. Prior knowledge of complex composition or function is not required and all directly and indirectly interacting components are identified in a single step. Two comprehensive analyses of protein complexes using the TAP method were performed in the yeast *S. cerevisiae* [101,102]. These studies allowed to identify 232 [101] and 547 [102] distinct protein complexes, respectively.

Comparison of the information gained from the different large-scale complex analyses reported in the literature for the yeast *S. cerevisiae* shows a poor overlap of the data. As each method monitors different properties of the proteins (stable versus transient complexes), these data should be considered as complementary and their integration should lead to a more comprehensive description of the protein interactive networks [103–105].

The transfer of this approach for use in higher eukaryotes has been lagging behind for the following reasons: (i) yield of fusion proteins; (ii) protein competition by the endogenous protein and (iii) obtaining a sufficient cell mass to perform analysis. The use of yeast cells easily obviated the problem of a sufficient cell mass. On the other hand, some of the problems dealing with the competition with endogenous protein can be overcome using *Drosophila melanogaster* S2 cells [106] in conjunction with RNAi technology. In mammalian cells, however, this modification is not easily adapted. Although there have been reports of successful use of the TAP tag techniques in mammalian cells [101,107], the protein yield is too low most of the times even for MS analysis. In this context, a modified TAP tag technique for the purification of protein complexes in mammalian cells has been recently reported [108]. The method takes advantage of the high affinity streptavidin–biotin interaction to allow more efficient fusion protein-capture leading to an increased yield of complex proteins. Instead of using CBP as the second affinity tag, these authors have inserted a biotinylation tag at the N-terminus of the target protein to take advantage of the higher biotin–avidin-binding affinity [108]. In another study, Junttila et al. [109] used a streptavidin tag affinity chromatography method, which enabled fast and simple one-step purification of multiprotein complexes from mammalian cells. After separation by SDS-PAGE and in-gel digestion with trypsin, the proteins were subsequently identified by mass LC–MS/MS. Using this affinity approach, the authors successfully purified a functional protein phosphorylase A2 holoenzyme protein complex from a cultured mammalian cancer cell line. They also identified that the complex contained both, known and novel interacting proteins for the protein phosphorylase A2 [109].

7. Affinity chromatography in quantitative proteomics

Important informations to understand the regulation of a biological process can be gained from the evaluation of quantitative changes in protein expression. In the high-resolution 2D electrophoresis approach, quantification is obtained by image analysis of the protein spots detected by staining, radioactivity, immunodetection, etc., ... [110]. In addition to the drawbacks described previously, a limitation comes from variations between gel runs with identical samples. Difference gel electrophoresis (DIGE) is an improvement in the method [111]. In the DIGE method, distinct CyDye fluorophores are used to covalently modify the lysine ε-amino group on proteins via an amide linkage. Before electrophoresis, the samples and a control are separately labeled using different dyes (e.g., Cy2, Cy3 or Cy5). The samples are combined and run in a single 2D electrophoresis gel to minimize gel-to-gel variations. The detection and quantification are then realized at the different excitation/emission wavelengths of each CyDye fluorophore.

In the gel-free approaches, the separation of the complex peptide mixture obtained after sample digestion is based on (multidimensional) liquid chromatography on-line with MS/MS analysis. In mass spectrometry, the ionization efficiency is peptide dependent. Therefore, the only valuable standard that can be used for quantification of a peptide is the same peptide labeled with stable isotopes. The isotope-coded affinity tags were developed in this context. The ICAT reagents consist of three functional components: a thiol reactive group selective for reduced cysteines; a linker group that exist in an isotopically normal and deuterated form, and a biotin group [112]. The reduced cysteine residues of the proteins in the two samples to be compared are labeled with the isotopically heavy or normal reagent, respectively. The two samples are combined, digested with trypsin, and the tagged peptides selected by avidin affinity chromatography and analyzed by mass spectrometry. The relative abundance of each peptide, and therefore of the corresponding protein, is determined by the ratio of signal intensities of the isotopically normal and heavy peptide forms Fig. 3. New cleavable ICAT (cICAT) reagents that employ ^{13}C isotopes and an acid-cleavable biotin group were recently designed to alleviate certain problems encountered with the original ones [113,114].

Comparisons of the 2-DE and ICAT methods show that they both yield quantitative results with reasonable accuracy [115,116]. The types of information obtained with these methods are complementary: ICAT-LC/MS is superior for high molecular weight proteins and membrane proteins and 2D electrophoresis/MS complemented ICAT-LC/MS for low molecular weight, cysteine-free proteins and post-translational modifications.

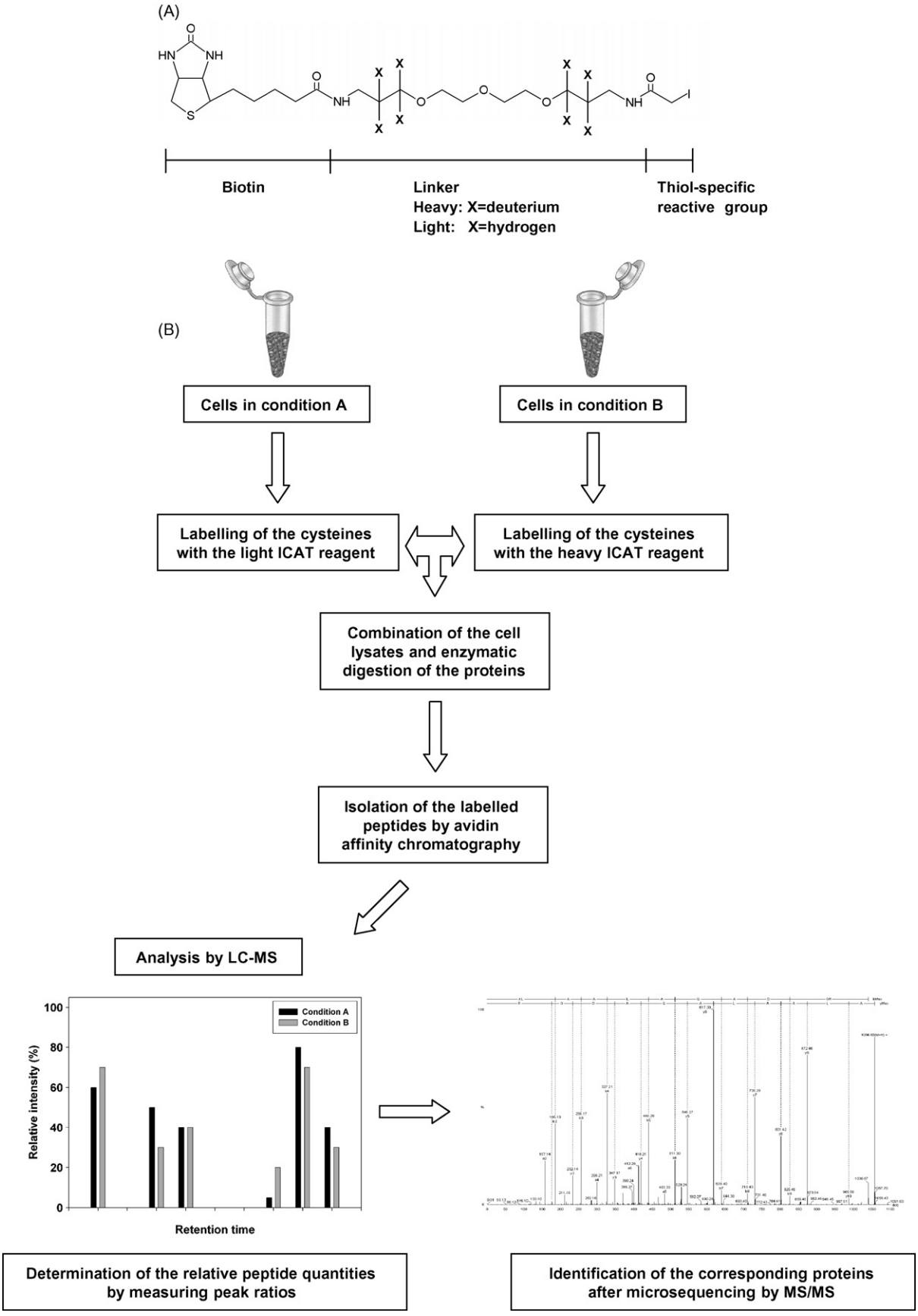

Fig. 3. (A) Chemical structure of the ICAT reagents. (B) Strategy used for the quantitative determination of protein expression levels in cell lysates obtained for two different environmental conditions.

8. Conclusion and perspectives

Chromatographic methods remain the most widely used tools in protein analysis. There is a special interest for affinity chromatography in proteomic approaches. Due to its high specificity, this method allows to decrease the complexity of a protein mixture for subsequent analysis by 2D electrophoresis or gel-free based approaches like LC–MS/MS. Affinity chromatography can be used in two different ways: enrichment of a specific class of proteins or depletion of certain types of proteins, e.g., highly abundant proteins in serum. Both approaches are helpful to increase the probability to identify low copy number proteins. The large variety of antibodies or other ligands immobilized on solid supports has allowed in solving several problems like the selection of phosphorylated, glycosylated proteins, etc. To answer to the high diversity of proteins and of protein modifications on earth, however, scientists will have to resort to the rational design of synthetic *de novo* affinity ligands. The biomimetic ligands that have already been produced circumvent most of the problems associated with biological ligands [117].

An additional advantage of the affinity separation procedure in comparison to 2D electrophoresis or LC–MS/MS, is that the isolated protein or group of proteins can be further characterized in terms of function and structure. This will probably be the challenge for the years to come in the case of protein complexes isolated with the TAP method. A detailed structural description of the protein complex will enable a better understanding of the functions of protein networks.

The high diversity of physico-chemical properties of proteins requires the creation of a large battery of specific ligands adapted to the three-dimensional (3D) structure of the polypeptidic chains. In most cases, each protein affinity chromatography step involves the use of a specific ligand. The situation is simplified when the protein is digested in a sum of peptides. Starting from one protein, the large panel of peptide properties allows to perform multiple assays. Peptidomics, a new area of the proteomics field, will generate the development of new methodologies dedicated to the high-throughput identification of proteins [118].

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