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Abstract	The reversible interaction between an affinity ligand and a complementary receptor has been widely explored in purification systems for several biomolecules. The development of tailored affinity ligands highly specific towards particular target biomolecules is one of the options in affinity purification systems. However, both genetic and chemical modifications on proteins and peptides widen the application of affinity ligand-tag receptor pairs towards universal capture and purification strategies. In particular, this chapter will focus on two case studies highly relevant for biotechnology and biomedical areas, namely, the affinity tags and receptors employed on the production of recombinant fusion proteins and the chemical modification of phosphate groups on proteins and peptides and the subsequent specific capture and enrichment, a mandatory step before further proteomic analysis.	
Keywords (separated by “-”)	Recombinant proteins - Fusion proteins - Affinity tags - Affinity purification - Phosphoproteomics	

Chapter 14

Affinity Tags in Protein Purification and Peptide Enrichment: An Overview

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Abstract

The reversible interaction between an affinity ligand and a complementary receptor has been widely explored in purification systems for several biomolecules. The development of tailored affinity ligands highly specific towards particular target biomolecules is one of the options in affinity purification systems. However, both genetic and chemical modifications on proteins and peptides widen the application of affinity ligand-tag receptor pairs towards universal capture and purification strategies. In particular, this chapter will focus on two case studies highly relevant for biotechnology and biomedical areas, namely, the affinity tags and receptors employed on the production of recombinant fusion proteins and the chemical modification of phosphate groups on proteins and peptides and the subsequent specific capture and enrichment, a mandatory step before further proteomic analysis.

Key words Recombinant proteins, Fusion proteins, Affinity tags, Affinity purification, Phosphoproteomics

1 Introduction

The production of recombinant proteins became possible with the emergence of DNA technology in the 1970s [1], which contributed for the facile manipulation of DNA sequences and consequently for the production of an encoded protein in different hosts (e.g., bacterial, fungal, and eukaryotic host cells) [2]. Bacterial hosts are usually more attractive due to their simplicity, well-established methods for genetic manipulation, high product yields, rapid expression, and cost-effectiveness [3]. However, a major drawback is that protein expression can lead to the formation of insoluble aggregates. These aggregates, termed inclusion bodies (IBs), are formed by unfolded or highly misfolded polypeptides [3]. In order to address these challenges, target proteins can be fused to affinity tags to enhance the fusion partner solubility and proper folding and also overcome problems as protein instability and host cell toxicity [4, 5]. However, the main purpose of introducing affinity

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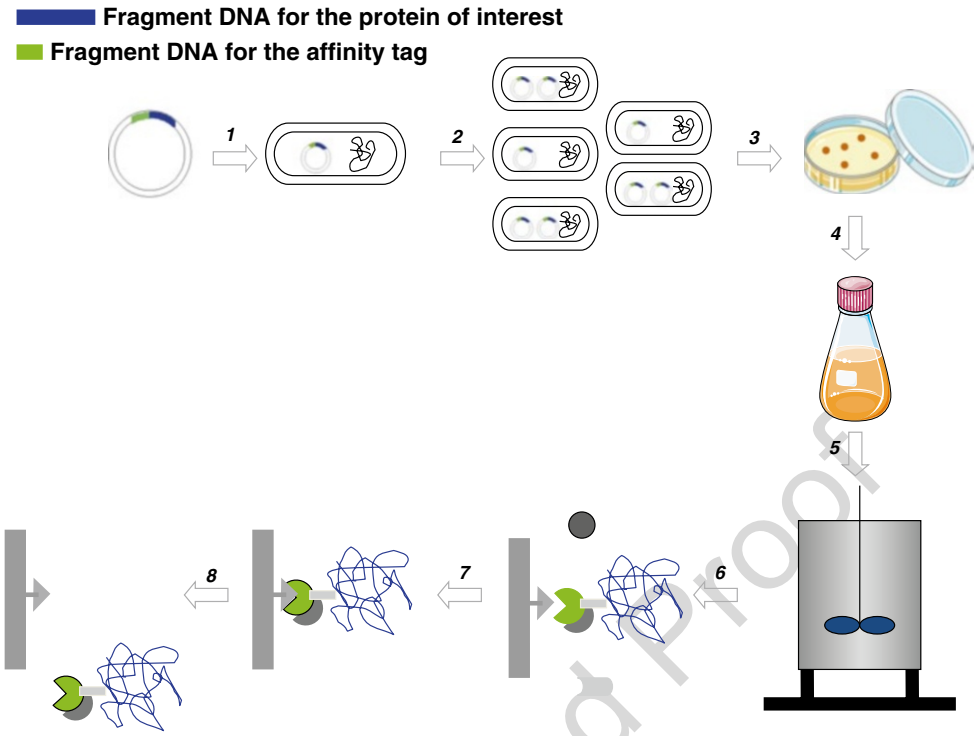

Fig. 1 Production and purification of recombinant protein scheme, involving (1) insertion of recombinant DNA in host cells and transformation process, (2) cloning process, (3) selection of the host cells containing recombinant DNA, (4) growth of the host cells, (5) upscaling, (6) fusion protein purification through affinity chromatography, (7) recognition of fused protein by the affinity ligand through affinity tag, and (8) elution of the purified fusion protein

tags is to facilitate the purification of recombinant proteins (Fig. 1). The tag usually presents high affinity for a specific biological or chemical ligand immobilized onto a chromatographic matrix. Besides their main applications in purification and as solubility enhancers, affinity tags may be used in many other applications, including labeling for imaging and localization studies, protein-protein interactions, and subcellular localization or transduction [4].

2 Affinity Tags in the Purification of Recombinant Proteins

Affinity tags vary in size, going from a single amino acid to whole proteins [6, 7]. The affinity tag can be placed at both ends of the protein of interest, with the majority being placed at the C-terminal [7]. These tags must exhibit some characteristics as stability, selectivity, and formation of reversible molecular complex with inexpensive, physically and chemically stable ligands/binding partners [7, 8]. Ideally, the dissociation of the tag-receptor system should be performed at mild conditions to facilitate the recovery of the fusion protein [8].

Table 1
Summary of short peptides used as affinity tags

Type of affinity tag	Size	Ligand	References
Enzymes			
β-Galactosidase	116 kDa	Thiogalactosidyl Sepharose	[114]
Glutathione S-transferase	26 kDa	Glutathione Sepharose	[12]
Chloramphenicol acetyl transferase	24 kDa	Chloramphenicol Sepharose	[115]
Thioredoxin	12 kDa	Require a purification tag	[22]
Polypeptide-binding proteins			
Staphylococcal protein A	14–31 kDa	IgG	[33]
ZZ domains	7 kDa	Protein A	[34]
Albumin-binding domain	75–25 kDa	I-Albumin	[116]
Phosphate-binding domain	34 kDa	Hydroxyapatite	[117]
Carbohydrate-binding domains			
Maltose-binding domain	40 kDa	Cross-linked amylose	[19]
Cellulose-binding domain	~100 kDa	Cellulose	[118]
Starch-binding domain	133 aa	Starch	[119]
Exoglucanase CBD	128 aa	Cellulose	[120]
Other protein tags			
N-Utilization substance (NusA)	55 kDa	Require a purification tag	[29]
Small ubiquitin modifier (SUMO)	11 kDa	Require a purification tag	[24]

Currently, there are a wide range of developed affinity tags to be used on the production and purification of recombinant proteins, and it is possible to assemble these affinity tags in two main groups, the protein affinity tags and the peptide affinity tags. The protein affinity tags enclose enzymes (e.g., GST), polypeptide-binding proteins (e.g., SpA), and carbohydrate-binding domains (e.g., MBP, CBD) (Table 1). The use of small peptide tags presents advantages over larger tags in terms of metabolism of the host cell, as less energy is consumed [8]. Also, short tags are less likely to interfere with the structure and function of the target protein; therefore, they may not need to be subsequently removed [8]. The small peptides used as affinity tags fall into two categories: the peptides that bind to small ligands (e.g., poly-arginine and poly-histidine) and the peptide tags that are recognized by proteins (e.g., FLAG) (Table 2). The affinity tags based on small peptides can also be categorized in (a) metal affinity tags, (b) charged peptides, (c) epitope peptides, (d) protein-binding peptides, and (e) streptavidin-binding proteins [4, 6–8].

The presence of the affinity tag may affect characteristics or functions of the target protein, and, depending on its final

Table 2
Summary of protein native domains as affinity tags

Type of affinity tag	Tag sequence	Ligand	References
Metal affinity tags			
Poly-His	HHHHHH	Ni^{2+}NTA, CO^{2+}CMA	[39, 41]
FlAsH tag	CCXXCC	Bis-arsenical fluorescein dye FlAsH	[121]
HAT	KDHLIHVHLEEHAHAHNK	CO^{2+} CMA	[122]
Charged peptides			
Poly-Arg	5–15 aa (R)	Anionic resins	[123]
Poly-Asp	5–16 aa (D)	Cationic resins	[124]
Poly-Cys	4 aa (C)	Thiopropyl Sepharose	[125]
Glu	1 aa (E)	Cationic resins	[126]
Poly-Phe	11aa (E)	IEC	[127]
Epitope peptides			
FLAG™	DYKDDDDK	mAb M1, M2	[50, 128]
c-myc	EQKLISEEDL	mAb 9E10	[51]
T7	MASMTGGQQMG	Anti-T7 9E10	[52, 53]
Protein-binding peptides			
S-tag	KETAAAKFERGHMDS	S-protein	[58]
Calmodulin-binding protein	KRRWKKNFIAVSAANRFKKISSSGAL	Calmodulin	[60, 63, 64]
Streptavidin-binding proteins			
Bio tag	LGIFEAMKMEWR	Streptavidin/avidin	[129]
Strep-tag	SAWRHPQFGG	Streptavidin	[65, 66]
Strep-tag II	WHPQFEK	Strep-Tactin	[70]
Avi tag	GLNDIFEAQKIEWHE	Streptavidin/avidin	[129]
Nanotag	DVEAWLGAR	Streptavidin/avidin	[130]

application, it might be necessary to remove the tag (Table 3) [8]. Specifically, in case of therapeutic proteins, there is a demand for tag cleavage as protein function can be lost and its integrity and biological activity are not achieved [5]. A variety of peptidases and other chemical methods are available for tag cleavage [9]. However, enzymatic methods may lead to unwanted consequences as the incomplete cleavage by the protease or the retention of additional amino acids in the fusion protein sequence from the cleavage site. Also, these enzymatic methods can contribute to increase the costs of the manufacturing process. Nowadays, there are other emerging alternatives such as self-cleaving tags [10, 11] used to overcome some drawbacks of the existent methods.

t3.1 **Table 3**
t3.2 **Summary of enzymatic and chemical methods for tag removal**
t3.3 **(↓ = indicated chemical cleavage site; X = unspecific amino acid)**

t3.4	Cleavage agent	Cleavage specificity	References
t3.5	*Enzymes*		
t3.6	Exopeptidases		
t3.7	Carboxypeptidase A	Poly H—↓—X	[41]
t3.8	Carboxypeptidase B	Poly R—↓—X	[131]
t3.9	Aminopeptidase I	EAE—↓—X	[126]
t3.10	Endopeptidases		
t3.11	Enterokinase	DDDDK—↓—X	[73]
t3.12	Factor Xa	IEGR—↓—X	[75]
t3.13	Thrombin	LVPR—↓—X	[76]
t3.14	TEV protease	EQLYFQ—↓—X	[74]
t3.15	SUMO	SUMO tertiary structure	[25]
t3.16	*Chemical*		
t3.17	Cyanogen bromide	XM—↓—X	[132]
t3.18	Hydroxylamine	XN—↓—G	[133]
t3.19	Acetic acid	XN—↓—P	[134]

3 Proteins as Affinity Tags

One of the classes of affinity tags consists of proteins or large peptides. Protein affinity tags can be divided in solubility-enhancing tags and purification tags. The solubility of the target proteins produced in bacterial hosts is the bottleneck of the production of recombinant proteins. Therefore, a few fusion proteins are already used to enhance protein expression and solubility of the target. Examples of these tags include glutathione S-transferase (GST), maltose-binding protein (MBP), staphylococcal protein A (SpA), thioredoxin A (Trx), small ubiquitin-related modifier (SUMO), and N-utilization substance A (NusA) [5]. GST and MBP tags not only improve the solubility of their fusion partners but also increase the efficiency of protein purification. GST tag is a 26 kDa protein derived from *Schistosoma japonicum* and belongs to a family of enzymes that can modify toxic substances by transferring sulfur from glutathione [12]. The proteins fused to GST tag can be purified from crude extracts by using affinity chromatography through the glutathione immobilized on the solid support [12]. The bound fusion proteins can be eluted under mild conditions through a competitive elution with reduced glutathione [12, 13]. Other main advantages of this tag include the protection and stabilization of the recombinant protein against intracellular protease cleavage in

the expression host, the cost-effectiveness of the affinity resins, and the use of mild conditions on the elution step [14]. Despite being considered a solubility-enhancing tag, when used as a partner of oligomeric proteins, hydrophobic regions enriched proteins, or with proteins larger than 100 kDa, the solubility of the target protein is poor, as the expression of the fusion proteins is a high metabolic burden for the host cell, contributing for an insoluble form expression [15]. GST tag has been successfully used on protein-DNA binding studies and protein-protein interactions [16].

MBP is a 42 kDa periplasmic protein involved on the maltose transport system of *E. coli*, being responsible to transport maltose and maltodextrins across the cytoplasmic membrane [17]. The one-step purification is based on the strong affinity of MBP with cross-linked amylose—a low-cost matrix. Also the bound tag can be removed by using non-denaturing conditions (e.g., competitive elution with maltose) [18, 19]. One of the greatest advantages of using this tag is that MBP is an impressive solubilizing agent due to the evidence that this tag can act as general molecular chaperone preventing the self-aggregation of the fusion partner [20, 21].

The other solubility-enhancing tags already mentioned present a higher impact on the solubility of the fusion partner; however, the use of these tags requires additional affinity tags for use in protein purification. TrxA is a small protein with 11.675 kDa, belongs to a family of oxidoreductases, and presents in its active site a redox couple for a number of biological reactions [22]. This tag allows a high overall gene expression but lacks the formation of inclusion bodies. In particular, the production of wide variety of secreted mammalian cytokines and growth factors fused to the tag C-terminal was possible in a soluble form using *E. coli* as a host [13, 23]. Overall, TrxA presents robust folding properties that contribute for this tag to be a covalently joined molecular chaperone [13]. Also, TrxA is a cytoplasmic protein and presents an inherent thermal stability, and these characteristics become helpful purification tools, facilitating the recovery of the fusion partner of the cell by osmotic shock and enabling heat treatments [23]. However, the purification can be facilitated by using an extra affinity purification tag.

The SUMO protein is involved in posttranslational modifications in eukaryotic cells through the covalent binding to lysine side chains of the target protein, and this presents high relevance on various cellular processes (e.g., nuclear-cytosolic transport, apoptosis, and stability) [24, 25]. Once fused to the N-terminal of the partner, it greatly promotes the target protein correct folding and solubility when compared to untagged version [26]. Although this tag also needs an additional tag for purification, it presents an attractive feature that is the recognition by a SUMO protease (*S. cerevisiae* Ulp1). This SUMO protease recognizes SUMO conformation, more specifically the conserved Gly-Gly motif [25]. Although this technology is an effective tool for prokaryotic hosts, in eukaryotic hosts there is the drawback of the natural occurrence

of SUMO-tag cleavage by the SUMO proteases in vivo [26, 27]. NusA is a 55 kDa transcription elongation and termination factor that modulates transcription by enhancing and pausing at some sites [28, 29]. Recently, it was reported that NusA is also involved in the coordination of cellular responses to DNA damage [30]. NusA is one of the largest proteins being used as carrier protein; however, it presents good solubilizing characteristics and high expression levels [31]. Moreover, this tag increases solubility of proteins (e.g., human interleukin-3) that were being produced as IBs by itself or fused to other tags (TrxA) [31]. This might be related with their biological activity. Once again, this tag is a solubility tag and cannot be purified with a specific affinity matrix, requiring a purification affinity tag [31, 32].

Other protein tags used to increase solubility or to facilitate purification include SpA and its derivatives (Z domain or Z tag) [5]. The SpA protein is present on the surface of the gram-positive bacterium *Staphylococcus aureus* and mainly interacts with the constant region (Fc) of most mammalian class G immunoglobulins (IgG) [33]. This protein tag has been used for the purification of a variety of fusion proteins produced in different hosts such as *E. coli*, yeast, CHO cells, baculovirus-infected insect cells, and plant cells, by using IgG affinity chromatography [34, 35]. The use of this tag presents several advantages, namely, proteolytic stability, the absence of disulfide bonds, and the presence of inherent high solubility [34]. The major drawback is related with the fragility of IgG as a ligand, contributing for ligand leakage and consequently end-product contamination [34]. The Z domain tag emerged as a mutated version of B domain, which is a homologous domain of SpA with high affinity for IgG. This affinity tag has been developed to improve the resistance of undesirable cleavage of the purified fusion protein when using chemical tag removal strategy [36]. Also in this case, the main disadvantages associated with this technology are regarding the immobilized binding partner (e.g., IgG) which presents high costs of production and purification, poor stabilization under sterilization and cleaning-in-place conditions, as well as potential leakage and end-product contamination.

The Z domain was also engineered to create the Z tag (basic or acidic), a highly charged domain to be used on the purification of recombinant protein through ion-exchange chromatography [37, 38]. The Z_{basic} tag has been employed on matrix-assisted refolding strategies of proteins that were solubilized with chaotropic agents after being produced as inclusion bodies [37].

4 Peptides as Affinity Tags

4.1 Metal Affinity Peptides

Immobilized metal affinity chromatography (IMAC) was introduced in 1975 by Porath and co-workers, being this type of chromatography based on the affinity between proteins and heavy metal

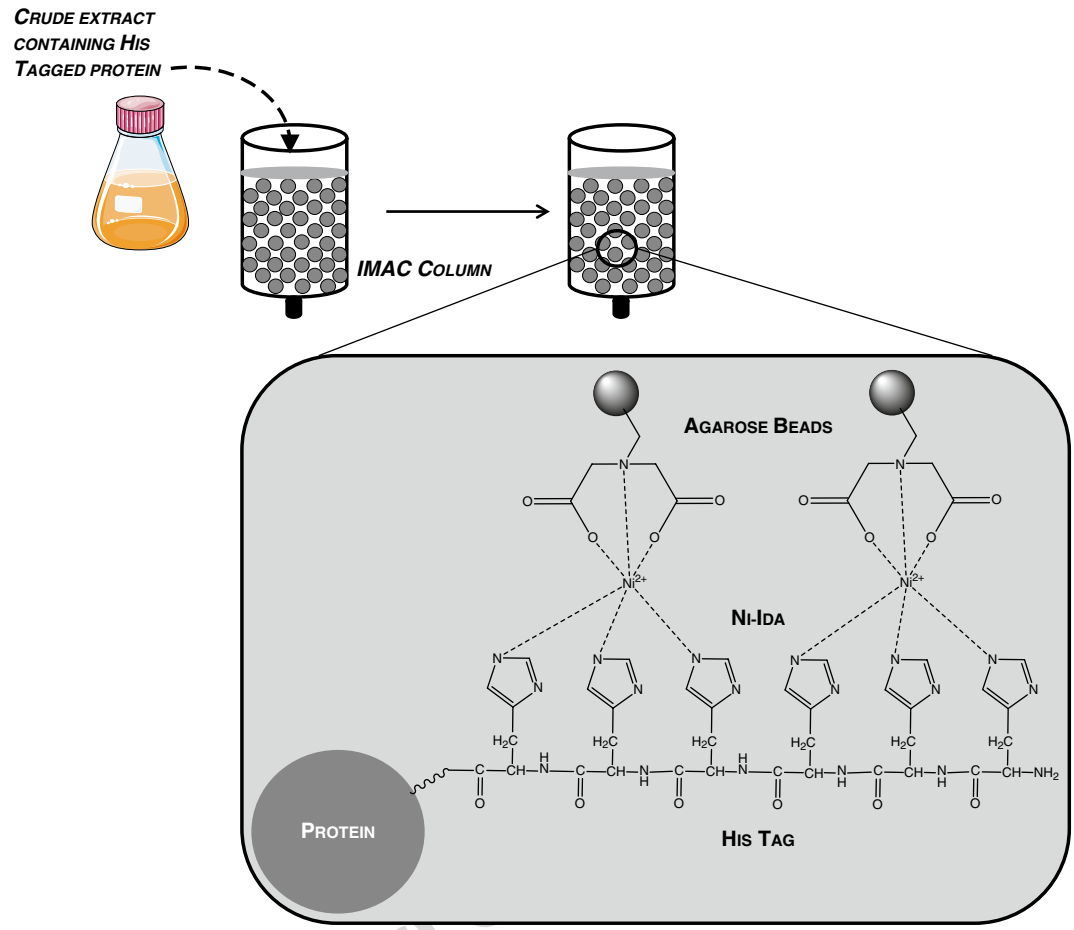

Fig. 2 Principle of protein purification through histidine affinity tag

ions (Zn^{2+}, Cu^{2+}, Ni^{2+}) [39]. This type of chromatography exploited the formation of stable complexes in aqueous solution between histidine (His) and cysteine (Cys) residues and zinc and copper [39]. After this, Hochuli et al. developed a new metal chelate affinity resin that once charged with nickel ions (Ni^{2+}-NTA) presents selectivity for neighboring His of proteins or peptides [40].

Subsequently, Hochuli et al. were also the pioneers on using a poly-histidine peptide (His-tag) genetically fused to the mouse dihydrofolate reductase protein and then produced in *E. coli* and purified on Ni^{2+}-NTA adsorbent with a subsequent His-tag removal by carboxypeptidase A [41]. Nowadays, the purification by using His-tag is one of the most used methodologies for protein purification and has been extensively described (Fig. 2) [42–44]. The main advantages of IMAC technology are related with high protein loading capacity, ligand stability, and lower costs. Also, this technology can be easily scaled up with reproducibility and affordable costs [42]. Other advantage of using IMAC for the purification of recombinant proteins is the compatibility with denaturant agents

for the solubilization and refolding of insoluble protein aggregates produced in *E. coli* host. IMAC technology has been described as a successful strategy for the one-step refolding of His-tagged proteins [45]. The major drawback of using a metal affinity tag is the metal ion leakage that leads to metal contamination of the end product. These metal ions are toxic, and therefore additional steps of purification are required, especially for therapeutic proteins. Moreover, the metal resin disposal constitutes an environmental problem [42].

Although IMAC has been used mainly for protein purification, different applications have been explored such as protein refolding and solubilization [45], protein microarrays [46–48], and phage display [49]. In this last application, phage display-derived peptide sequences were developed to bind to a novel class of chelating ligands complexed with Ni^{2+} [49]. These chelating agents are based on 1,4,7-triazacyclononane (TACN) structure and have been chosen to overcome ligand leaching in IMAC purification.

4.2 Epitope Peptides

Epitope peptides are used as tags but usually not for purification purposes because the affinity matrices are antibody based, which contributes for higher purification costs [4]. The most frequently antigenic peptides used are Flag-tag [50], c-myc [51], T7 epitope tag [52, 53], and Softags [54].

The Flag-tag is an eight-amino acid peptide with a hydrophobic sequence consisting of DYKDDDDK [50]. The Flag technology allows a rapid purification of fusion proteins in a mild, highly specific, and calcium-dependent affinity chromatography procedure with an Anti-Flag M1 monoclonal antibody immobilized on the affinity support [50]. One of the features of this tag is the recognition of the five C-terminal amino acids of the peptide sequence by the protease enterokinase, facilitating tag removal [50]. Main drawbacks of this system are related with ligand leakage and stability due to their own natural character and low scalability. Also, this system cannot be used for the purification of fusion proteins produced as IBs because denaturant agents are required. Although this tag presents a highly specific sequence for enterokinase recognition, unwanted cleavage may occur in the presence of contaminant proteases [50].

Softags are epitope tags used for immunoaffinity chromatography which present high affinity for "polyol-responsive" monoclonal antibodies (mAbs) [54]. These mAbs present a particular feature regarding the elution conditions, being possible to use mild conditions supplemented with a low molecular weight polyol (e.g., ethylene glycol) and a non-chaotropic salt [5, 54]. Softag 1 is a 13-amino acid sequence near the C-terminal of the β′ subunit of *E. coli* RNA polymerase [55]; Softag 2 is a repeat heptapeptide found on C-terminal of RNA polymerase I [56]; and Softag3 is an epitope near the N-terminal of human transcription factor [57].

4.3 Protein-Binding Peptides

S-peptide tag is a 15-amino acid sequence polypeptide resultant fragment from the cleavage of ribonuclease A by the protease subtilisin. The other remaining product is S-protein [58]. S-peptide binds to S-protein with high affinity, and this interaction allows the efficient affinity purification of recombinant proteins [58, 59].

Calmodulin-binding protein (CBP) is a calcium-binding protein that plays a key role as a regulator on a wide range of calcium-dependent intracellular processes [60, 61]. Calmodulin-binding unit is a 26-amino acid peptide derived from the carboxyl-terminal of rabbit skeletal muscle myosin light chain kinase [62, 63]. This peptide binds to calmodulin with a nanomolar affinity and is also calcium dependent [60–63]. The elution can be carried out under milder conditions and requires a calcium-chelating agent such as ethylene glycol tetraacetic acid (EGTA) [60, 63]. This tag was found out to be a versatile tag for antibody fragments [64]. However, its use in eukaryotic cells is hampered by its interference in calcium signalling pathways [5].

4.4 Streptavidin-Binding Peptides

The Strep-tag is a nine-peptide sequence (AWRHPQFGG) and was originally developed by selection from a genetic peptide library for its capability to bind to streptavidin protein in a highly specific and reversible manner [65, 66]. Strep-tag recognizes the same pocket of streptavidin as biotin, the natural ligand, allowing one-step purification on immobilized streptavidin columns. However, the original Strep-tag is needed to be fused only to the C-terminal of the recombinant protein [67, 68]. A new improved version—Strep-tag II, an eight-residue-peptide sequence (WSHPQFEK)—was developed and optimized to overcome this constrain and also presents affinity for streptavidin [67, 69]. Simultaneously, progresses have been made to optimize the respective chromatographic matrices, and an engineered streptavidin support with improved binding capacity (Strep-Tactin) has been developed [70]. The main advantages of these systems are the resistance to host cell proteases, the fact that the binding is not dependent on metal ions, the elution that can be carried out at mild conditions, and the biological inertness of this tag [70].

A new streptavidin-binding protein (SBP) was also developed for the purification of recombinant proteins [71, 72]. This SBP tag presents a sequence of 38 amino acids long with a nanomolar affinity for streptavidin. The main applications of this tag are in high-throughput protein expression and purification procedures, existing already in several streptavidin-derivatized materials (plates, beads, enzymes, fluorophores, etc.) commercially available [71].

5 Tag Removal

The removal of the affinity tag can be carried out by harsh chemical treatments (e.g., cyanogen bromide or hydroxylamine) or by enzymatic cleavage, with the latter being preferred since it can be

performed under physiological conditions [6]. The chemical treatment presents significant drawbacks such as protein denaturation and side chain modification of amino acids in the target protein [6]. Several endoproteases have been utilized for tag removal [9], such as enterokinase [73], tobacco etch virus (TEV) [74] Factor Xa [75], thrombin [75, 76], and SUMO protease [25]. Enterokinase (EK) is a serine proteinase constituted by a high chain and a light chain linked by a disulfide bond. This enzyme presents high specificity for the $(Asp)_4$-Lys sequence, which contributes for a useful tool for fusion protein cleavage [73, 77]. Factor Xa and thrombin are trypsin-like serine proteases, and both recognize specific amino acid sequences (Table 3) [75]. TEV is a 49 kDa proteinase of tobacco etch virus (TEV) that cleaves the polyprotein derived from the TEV genomic RNA at five locations [74]. Most of these enzymes are able to cleave without requiring a specific sequence at the C-terminal, allowing for the complete removal of the tag [6, 9]. The major drawback associated with these enzymes is related with the high enzyme/protein ratios and the long incubation times required. Moreover, for an efficient tag removal, it is also necessary to take into account the absence of cryptic sites recognized by endoproteases in the native protein sequence [9].

Tag cleavage by using enzymatic or chemical methods always requires additional purification steps that contribute for the higher costs. In this way, other emerging technologies have been developed, namely, self-cleaving tags. There are different types of self-cleaving tags, such as inteins, sortase A, N-terminal protease, and FrpC module [10, 11]. In this particular case, these tags present inducible proteolytic activity under certain conditions as pH and/or temperature shift and addition of specific reagents (e.g., dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), Ca^{2+}) [11]. Although these self-cleaving tags seem to be attractive from the economic point of view, there are still a few drawbacks associated with premature cleaving and consequently target-protein losses and minor product compatibility with cleaving conditions [10].

6 Affinity Tags for the Enrichment of Phosphorylated Proteins and Peptides

Posttranslational modifications (PTMs) are involved in the regulation of several cellular processes, such as gene expression, signal transduction, metabolism, homeostasis, cell division, and apoptosis, by modulating protein folding and function [78, 79]. Over 300 types of PTMs are known, but only a few play determinant roles in biological processes [80, 81]. Protein phosphorylation is one of the most common PTMs and exhibits a transient and reversible character, being regulated by the dynamic action of kinases and phosphatases. There are more than hundred thousand potential phosphorylation sites in the human proteome, being estimated that 30–50 % of all proteins are phosphorylated at some point

during their lifetime. In eukaryotic systems, phosphorylation occurs essentially at serine and threonine residues, followed by tyrosine, with a ratio of 1,800:200:1 [79, 82, 83]. Phosphorylation events have been associated to a variety of diseases, such as cancer [84], type II diabetes [85], cystic fibrosis [86, 87], neurological diseases such as Parkinson's [88] and Alzheimer's [89], and neuropsychiatric disorders (e.g., schizophrenia) [90]. The degree of phosphorylation and the localization of specific phosphosites provide meaningful insights to better understand disease-associated signalling pathways, contributing for the development of novel biomarkers and drug targets.

Currently, the characterization of phosphoproteins and correspondent chemical or proteolytic digests is generally performed using mass spectrometry (MS) techniques. However, this analysis is not always straightforward since phosphopeptides present lower ionization efficiency than their non-phosphorylated counterparts, which results in lower signal intensities in positive ion mode. Moreover, phosphorylated species are usually present at substoichiometric levels and are easily adsorbed by plastics and metals during sample handling [80, 91]. These problems can be partially overcome by using materials with low protein-binding properties and efficient enrichment methods before MS analysis.

Enrichment methods are generally based in the affinity capture of the phosphate groups, either by charge interactions (e.g., chromatofocusing, ion-exchange chromatography), chelation (e.g., IMAC, metal oxide affinity chromatography (MOAC), hydroxyapatite, phosphate-binding ligands), or molecular recognition (e.g., immunoaffinity chromatography, affinity chromatography based in phosphoprotein-binding domains) (Fig. 3). For further details on this subject, the reader should consult recent reviews [79, 92, 93]. However, strategies consisting on the chemical modification or replacement of the phosphate moieties by affinity tags are also viable alternatives (Fig. 4). Table 4 summarizes the chemical tags used for phosphoprotein and phosphopeptide enrichment.

Both phosphoserine (p-Ser) and phosphothreonine (p-Thr) residues undergo β-elimination of phosphoric acid under strong alkaline conditions, yielding dehydroalanine and β-methyldehydroalanine, respectively [94]. These analogues are susceptible to Michael addition by several nucleophiles, such as amine, alcohol, and thiol groups [95]. Oda et al. replaced phosphate moieties of p-Ser and p-Thr by a biotin affinity tag via a maleimide group, using ethanedithiol (EDT) as a Michael donor and cross-linker. These biotin-labeled peptides were then enriched using avidin chromatography [96]. A similar approach using a phosphoprotein isotope-coded affinity tag (PhIAT) allows the determination and comparative quantification of the phosphorylation sites of proteins, by using either EDT or its deuterated version and a biotinylation reagent—(+)-biotinyliodoacetamidyl-3,6-dioxaoctanediamine. One of the advantages of the latter method is that it does not use maleimide group, which undergoes partial

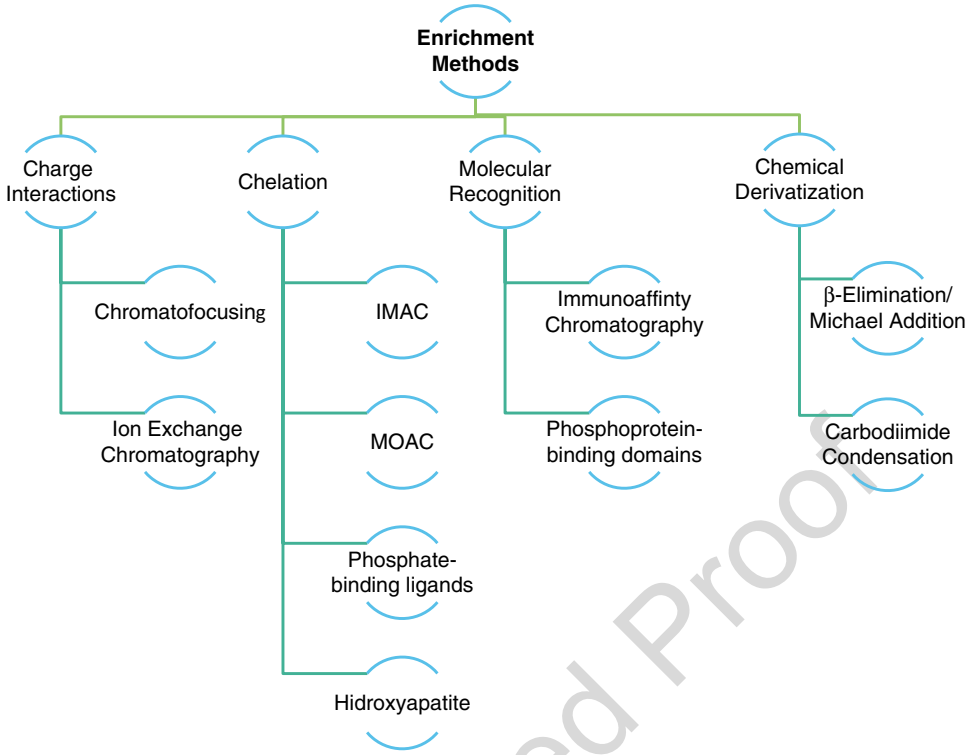

Fig. 3 Phosphoprotein and phosphopeptide enrichment methods. The most common are based in charge interactions, chelation, molecular recognition, and chemical derivatization

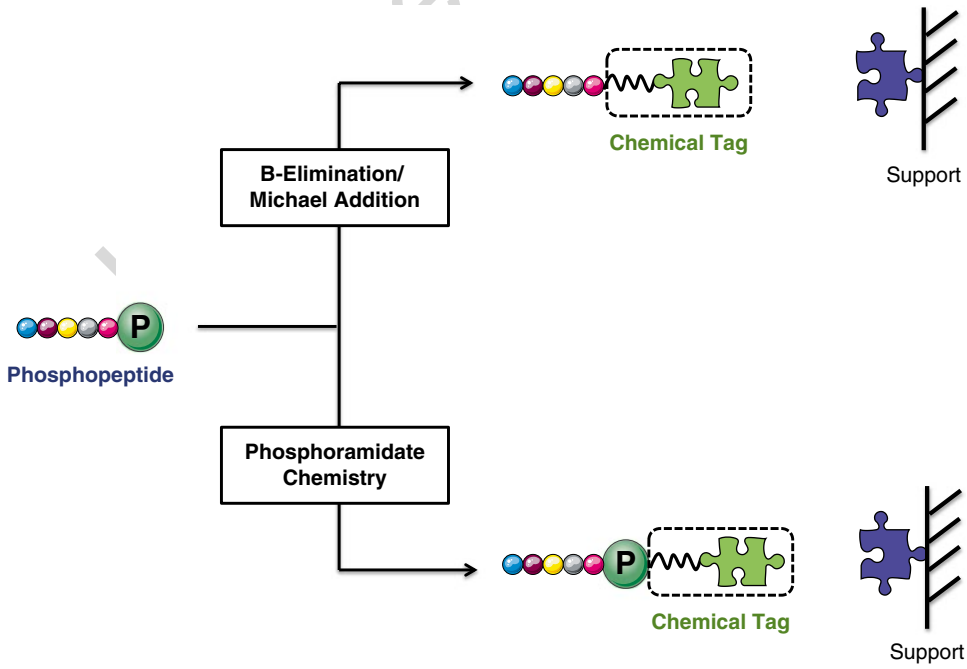

Fig. 4 Phosphopeptide enrichment using chemical tags. B-elimination/Michael addition and phosphoramidate chemistry are the most common chemical derivatization strategies

Table 4
List of chemical tags used in phosphoproteomics and their correspondent solid supports

Chemistry	Chemical tag	Solid support	Elution	References
B-Elimination/ Michael addition	Ethanedithiol (EDT) coupled to biotin	Avidin resin	Trifluoroacetic acid (TFA)	[96, 97, 100]
			Dithiothreitol (DTT)	[99]
	Engineered biotin tag		Triethylamine (TEA)	[109]
	EDT	Thiol-activated resins	DTT	[101]
	Propanedithiol			[102]
	EDT	PhIST[a]	UV light	[103]
	Cysteamine	PEG-PS resin[b]	TFA	[104]
	Guanidinoethanethiol (GET)	–	–	[105, 106]
	Fluorescent affinity tag (FAT)	Anti-rhodamine antibodies	TFA	[107]
	Engineered His-tag	Ni^{2+}-IMAC	Factor Xa	[108]
Carbodiimide condensation	Cystamine	Glass beads with iodoacetyl groups	TFA	[111]
	Cystamine	Glass beads with maleimide groups		[112]
	Dendrimer	–		[113]

[a]PhIST—aminopropyl beads with a photosensitive linker, a stable isotope-coded leucine moiety, and a thiolate-reactive group
[b]PEG-PS resin—polyethyleneglycol-polystyrene copolymer base resin with cystamine as the benzyl carbamate

hydrolysis [97]. Biotin-avidin chromatography presents some drawbacks associated with the nonspecific binding of samples containing endogenous biotin and biotin-binding proteins and the harsh conditions used during elution, which might denature target proteins. The utilization of monomeric avidin, which has lower affinity towards biotin, allows the employment of milder elution conditions. Yet, a weaker biotin-avidin interaction may lead to an inefficient capture of biotinylated molecules in the presence of strong detergents, which are often used to solubilize hydrophobic molecules, such as membrane proteins [98]. Adamczyk et al. used a pyridyldithiol-activated biotinylation reagent—biotin-HPDP (*N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide)—which conjugates via a cleavable disulfide bond, allowing the reversible biotinylation of the phosphopeptides [99]. However, this type of reagents may be unstable in some biological conditions. Van der Veken et al. developed an alternative approach by introducing an acid-labile linker within a biotin-based tag, allowing full recovering of affinity-purified material and elimination of affinity tag prior to MS analysis [100].

Several other tags were developed as alternatives to the biotin-based ones but still using β-elimination/Michael addition protocols. Biotin-HPDP can be substituted by different pyridyldithiol-activated resins, presenting similar reaction mechanisms. Thiol-activated peptides displace the thiopyridyl group by disulfide exchange, which

rearranges to form a thione [101, 102]. Thaler et al. used propanedithiol as a Michael donor, possessing six hydrogen atoms instead of the four of EDT, which when replaced by deuteriums provide a higher difference in mass [102]. Using a similar protocol, McLachlin and Chait observed a problematic side reaction in which 1–2 % of the thiol tag was incorporated into non-modified serine residues, since some of these residues undergo β-elimination of water to form dehydroalanine. This will lead to sample enrichment in both phosphorylated and non-phosphorylated species [101].

A phosphoprotein isotope-coded solid-phase tag (PhIST) was introduced as an improvement of the PhIAT. The biotin tag was replaced by a photosensitive linker covalently bound to aminopropyl glass beads, a leucine isotope-coded linker containing six ^{12}C and one ^{14}N (light) or six ^{13}C and one ^{15}N (heavy), and a thiolate-reactive group. B-elimination, Michael addition, tryptic digestion, and solid-phase labeling may be all performed in the same vial. Moreover, the reaction is not affected by the presence of denaturants or detergents, and the beads can be thoroughly washed without the risk of sample losses, leading to high reaction yields. The bound peptides are simply released by UV photocleavage of the photosensitive linker [103].

Knight et al. converted p-Ser and p-Thr residues into lysine analogues, aminoethylcysteine and β-methylaminoethylcysteine, respectively. As aminoethylcysteine and lysine are isosteres, the modified peptides are then easily cleaved using a LysC endoprotease. They successfully enriched the samples in p-Ser by using a polyethyleneglycol-polystyrene (PEG-PS) resin functionalized with a methoxybenzylcarbamate spacer and cystamine. The methoxybenzylcarbamate linkage is stable under the alkaline conditions used during β-elimination reaction but is highly acid-labile, allowing peptide release at acidic pH. This methodology allows direct enzymatic cleavage of the peptides at the site of phosphorylation, which facilitates phosphorylation site mapping [104].

In a different work, p-Ser residues were converted into guanidinoethylcysteine (Gec), by adding a guanidinoethanethiol (GET) tag to β-eliminated peptides. Gec is recognized as a trypsin cleavage site, providing selective enzymatic digestion and thus facilitating the assignment of phosphorylation sites. Also, the basic guanidine moiety of the tag possesses superior proton affinity, increasing peak intensities in MS [105, 106].

In a one-step reaction, a fluorescent affinity tag (FAT) consisting of rhodamine conjugated to a cysteamine moiety selectively modifies p-Ser and p-Thr through a β-elimination/Michael addition strategy. FAT-labeled peptides may then be enriched simply by using commercially available anti-rhodamine affinity columns [107].

His-tag may also be used to chemically derivatize p-Ser- and p-Thr-containing peptides. An engineered His-tag possessing six histidines, a specific recognition site of protease Factor Xa (IEGR),

a glycine spacer, and a sulfhydryl-containing cysteine residue was used to enrich phosphopeptides by Ni^{2+}-IMAC. The thiol group of the side chain of cysteine functioned as a nucleophile in Michael addition reaction. His-tag peptides were then cleaved at the C-terminal side of arginine of the recognition sequence [108]. A chemically engineered biotinylated tag, consisting of a biotin group, a base-labile 4-carboxy fluorenylmethoxycarbonyl group, and a sulfhydryl moiety, was developed as a refinement of the previously described His-tag. This engineered biotin tag is smaller and easier to couple and requires mild alkaline conditions instead of the expensive Factor Xa upon release of the peptides [109].

Although β-elimination/Michael addition reactions are very well established and straightforward procedures, there are some drawbacks related to their application in phosphoproteomics. First, tyrosine residues are not able to undergo β-elimination. Second, cysteine residues need to be protected by oxidation or alkylation to prevent side reactions. Third, O-glycosylated residues also undergo β-elimination to form dehydroanalanyl residues, and therefore enzymatic deglycosylation is recommended to reduce nonspecific labeling. Fourth, deamidation of asparagine may occur, especially under strong alkaline conditions. Finally, Michael addition might occur at both Cα and Cβ, leading to the formation of epimers [80, 96].

Combining both IMAC and β-elimination methods minimizes their individual limitations. Phosphopeptides can be captured using an IMAC resin, which discriminates them from O-glycosylated residues, and then directly eluted by β-elimination. Several chemical tags can be reacted with the β-eliminated peptides, making them easily distinguishable from non-modified peptides that were also bound to the resin [110].

Phosphoramidate chemistry is a standard alternative to β-elimination/Michael addition procedures. Zhou et al. used a series of six chemical reactions, involving two carbodiimide-catalyzed condensations. Phosphate groups of the peptides were derivatized with sulfhydryl groups and then captured using iodoacetyl groups immobilized on glass beads. This method is highly selective and allows the identification of p-Ser, p-Thr, and p-Tyr residues. However, it presents a low recovery yield of approximately 20 % [111].

Using a different approach, phosphate groups can be activated using carbodiimide and imidazole and reacted with cystamine to form phosphoramidate bonds in a single step, eliminating the need to protect amine groups on the peptides. After the generation of free thiol groups by reduction, the peptides can be captured using maleimide groups immobilized on glass beads [112]. Using the same chemistry and as an alternative to the solid-phase strategies, phosphorylated peptides can be coupled to a soluble synthetic polyamine (dendrimer), allowing for homogenous reaction [113].

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Author Queries

Chapter No.: 14 0002077658

Queries	Details Required	Author's Response
AU1	Please check the term "Thiogalactosidyl" for correctness.	
AU2	Please check if edit to sentence starting "The main applications…" is okay.	
AU3	Please check if the term "dehydroanalanyl" should be changed to "dehydroalanine".	
AU4	Please provide publisher's location in references [1, 4, 5, 13, 16, 18, 26, 60, 61, and 66].	