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Review

The FLAG™ peptide, a versatile fusion tag for the purification of recombinant proteins

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Abstract

A fusion tag, called FLAG™ and consisting of eight amino acids (AspTyrLysAspAspAspAspLys) including an enterokinase-cleavage site, was specifically designed for immunoaffinity chromatography. It allows elution under non-denaturing conditions [Bio/Technology, 6 (1988) 1204]. Several antibodies against this peptide have been developed. One antibody, denoted as M1, binds the peptide in the presence of bivalent metal cations, preferably Ca^{+}. Elution is effected by chelating agents. Another strategy is competitive elution with excess of free FLAG™ peptide. Antibodies M2 and M5 are applied in this procedure. Examples demonstrating the versatility, practicability and limitations of this technology are given.

Keywords: Chromatography; FLAG™; Recombinant proteins

1. Introduction

Over the years, fusion protein technology has become an important tool for solving numerous problems linked to recombinant protein production. A number of gene fusion systems have been developed to benefit from the molecular interactions arising from gene fusion technology. These systems include various extensions of the target protein, leading to an increase in solubility and yield of the protein to be expressed. Furthermore, the properties of the additional tag facilitate identification and provide a one-step purification procedure of the fusion protein by passing cell extracts or supernatants through columns of an appropriate (e.g. affinity) matrix. A variety of expression vectors with different tag sequences has been designed for fusion to almost any target protein that can be cloned and expressed in a microbial host (in one or more reading frames).

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The size of these tags can range from only one or a few amino acids to complete proteins, which in some cases, consist of several subunits and can be attached to either the *N*- or *C*-terminus of the desired protein, or in some applications, to both termini. To ensure good translational initiation, the fusion partner gene is typically positioned at 5′ of the gene of interest. Positioning at the 3′-end of the desired gene is also possible, although in this configuration, expression yields may be more variable. Optimal positioning must be elicited as needed. There is also the possibility to express short peptide tags as internal gene fusions, such as in the active-site loop of *Escherichia coli* thioredoxin TrxA, where short peptide sequences of up to 22 residues can be inserted. These "loop peptides" can be of additional use, e.g. serving as antigens, as library members in binding screens. Each of these tags has intrinsic features, making them

Table 1
Common fusion tags for recombinant protein production

Type of fusion tag	Size	*C*- or *N*-terminal fusion	References
(A) Enzymes			
β-Galactosidase	116 kDa	*N,C*	[41]
Glutathione-S-transferase	26 kDa	*N*	[2]
Chloramphenicol acetyl transferase	24 kDa	*N*	[42]
TrpE	27 kDa	*N*	[43]
(B) Polypeptide-binding proteins			
IgG-binding domain of Staphylococcal protein A	14–31 kDa	*N*	[44]
IgG/albumin-binding domain of Streptococcal protein G	28 kDa	*C*	[6]
(C) Carbohydrate-binding domains			
Maltose-binding protein	40 kDa	*N*	[9,45,46]
Starch-binding domain	119 aa	*C*	[47]
Cellulose-binding domain	111 aa	*N*	[7,8]
(D) Biotin-binding domain	8 kDa	*N*	[10]
(E) Antigenic epitopes			
recA	144 aa	*C*	[48]
FLAG™	8 aa	*N*	[1,11,12]
(F) Charged amino acids			
Poly (Arg)	5–15 aa	*C*	[13,49]
Poly (Asp)	5–16 aa	*C*	[50]
Glutamate	1 aa	*N*	[51]
(G) Poly (His) tails	1–9 aa	*N,C*	[4,14]
(H) Other poly (amino acid) tails			
Poly (Phe)	11 aa	*N*	[15]
Poly (Cys)	4 aa	*N*	[15]

suitable also for certain purification procedures. Subsequently, specific cleavage sites engineered between the affinity tag and the protein of choice enable the removal of these tags.

Due to several factors, such as the possibility of an adverse effect of the fusion on protein function, definitive removal of the tag and the applicability to the host system of choice, the selection of an appropriate fusion tag requires thorough consideration. Several fusion tags of different biospecific and biochemical interactions have been utilized as the basis of fusion protein recovery systems (Table 1). They include enzymes with affinity for substrates or inhibitors [2,3], peptide-binding proteins [4–6], carbohydrate-binding proteins or domains [7–9], biotin-binding domains [10], antigenic epitopes with affinity to specific immobilized monoclonal antibodies [1,11,12], charged amino acids for use in charge-based recovery methods [13], poly(His) residues for binding to metal chelates and recovery by immobilized metal-affinity chromatography [14], and other poly(amino acid)s with various binding specificities [15].

In this review, we will focus on the FLAG™ tag, a hydrophilic and immunogenic purification tag, which was specifically designed for antibody-mediated identification and purification of recombinant proteins [1]. We will introduce the major features of this purification tag and illustrate some practical applications as well. Subsequently, we will briefly mention some limitations of this purification system.

2. The FLAG™ tag

To create an efficient detection and purification system based upon fusion polypeptides, several requirements must be met. One major demand on the added marker segment is that it should not interfere with the native folding of proteins to which it is attached. Secondly, the marker peptide sequence should be water-soluble and should retain a high degree of exposure on the surface of the protein, so that it can readily interact with its ligand. It should also be suitable for a mild and inexpensive affinity purification procedure. Finally, an easy removal of the marker peptide leading to a native product is also advantageous.

In choosing the specific sequence encoding for the FLAG™ marker peptide, several factors were considered. The sequence was limited to eight amino acids (AspTyrLysAspAspAspAspLys). Due to this small size, the marker peptide can be encoded by a single synthetic oligonucleotide. It is known that aromatic amino acids are the major factors in antigen–antibody interactions [16]. Therefore, Tyr was placed in position 2, flanked by charged amino acids, since the likelihood of aromatic amino acids being involved in antigenic sites is much higher than in less polar environments [17]. The negatively charged Asp at the *N*-terminus of the peptide was chosen to aid the antigenic properties of Tyr. Lys at position 3 in the marker sequence leads to a hexapeptide sequence LysAspAspAspAspLys, which ensures a maximum value on the hydrophilicity scale according to Hopp and Woods [18]. Such hydrophilic sequences have been shown to express strong antigenicity and are thus likely to adopt a highly exposed conformation in the three-dimensional folding of proteins [17]. Another virtue of FLAG™ is that the longest trypsinogen prosequences are of this length. This enables the removal of the tag

and the production of an authentic *N*-terminus of the fusion protein partner by enterokinase treatment. The protease recognizes the five *C*-terminal amino acids of the marker peptide (AspAspAspAspLys) as minimal specificity site. The FLAG™ peptide can be fused to either the *N*- or *C*-terminus of a given fusion protein. Nevertheless, the *N*-terminal fusion has several advantages. Inhibition ELISA experiments showed that the anti-Flag antibody M1 binds three to four orders of magnitude better under conditions where the α-amino group of the first amino acid is freely accessible [19]. Expression experiments of recombinant antibody fragments in *E. coli* with antibody M1 demonstrated that the *N*-terminal fusion of the FLAG™ peptide leads to a correctly processed product. This is important for the development and comparison of expression strategies for various antibody fragments or fusion proteins. It was also shown that the FLAG™ sequence at the *N*-terminus of a fusion protein is stable and is not removed by *E. coli* proteases [20]. Additionally, the *C*-terminus is frequently used for fusion of extra modules or different affinity handles and consequently should not be blocked by a detection tag in a flexible vector system. The FLAG™ peptide exerts similar properties in yeast as observed for *E. coli* [21]. The FLAG™ tag is usually not degraded and it seems that it stabilizes heterologous proteins. Furthermore, it seems that the FLAG™ peptide enhances the expression level in yeast, although comprehensive studies on this subject have not been published.

3. FLAG™ antibodies

To date, three monoclonal antibodies (M1, M2 and M5) with different requirements for epitope binding are available. Initially, the FLAG™ tag was engineered onto the *N*-terminus of lymphokine interleukin 2 by means of synthetic oligonucleotides. The fusion protein was expressed in yeast, and the product was purified by conventional methods. Further on, the purified peptide was used as an immunogen to produce a monoclonal antibody M1 (also called anti-Flag or 4E11) specific for the marker sequence [1]. The binding of the anti-Flag M1 monoclonal antibody to the FLAG™ epitope is calcium-dependent and has been used frequently for the identification and purification of numerous recombinant proteins which bear the FLAG™ tag as an *N*-terminal fusion [12,21–27]. ELISA experiments have shown that among divalent metals, binding affinity decreases with an increasing radius above or decreasing radius below that of calcium. For several smaller metals, such as nickel, an inhibitory effect upon the binding reaction could be observed. Substantial binding was seen for heavy metals, such as cadmium, lanthanum, and samarium [28]. An amino acid scan established that the antibody binds primarily to the first four amino acids and requires a free *N*-terminal amino group [12]. Studies with combinatorial peptide libraries further clarified the importance of the *N*-terminal region and established that Asp at position 7 is also required for complex formation [29]. A limitation of the anti-Flag M1 monoclonal antibody is that it is specific for the *N*-terminus of the FLAG™ fusion protein [12]. Therefore, the anti-Flag M1 monoclonal antibody cannot be used for affinity purification of FLAG™ fusion proteins in which the FLAG™ marker peptide is preceded by a methionine at the *N*-terminus of the protein (Met-FLAG fusion proteins) or the FLAG™

marker peptide is fused to the *C*-terminus of the protein. In order to extend the application area of the FLAG™ tag, another anti-Flag monoclonal antibody (anti-Flag M2) for use in affinity purification of FLAG™ fusion proteins was raised. The binding of the anti-Flag M2 antibody is not calcium-dependent, therefore, bound antigens cannot be eluted from the affinity column by chelating agents, such as EDTA. Furthermore, the FLAG™ sequence can be preceded by additional amino acids [30]. Applying this antibody, elution can be carried out at either low pH [31], or a competitive elution with synthetic peptide can be performed [22]. A major advantage of this antibody is its applicability to both *N*-terminal Met-FLAG and *C*-terminal FLAG™ fusion proteins. A third anti-Flag monoclonal antibody, anti-Flag M5, was raised against the sequence *N*-MetAspTyrLysAsp$_4$Lys-*C*. Its binding does not require calcium, but exhibits a higher affinity for *N*-terminal FLAG™ fusion proteins in which the FLAG™ epitope is preceded by a methionine. The anti-Flag M5 monoclonal antibody is therefore the preferred anti-Flag antibody for the detection of cytoplasmically expressed FLAG™ fusion proteins. In contrast, the anti-Flag M2 monoclonal antibody will bind to both of these types of amino-terminal FLAG™ fusion proteins and, as a result, is more generally applicable [32].

4. FLAG™ technology

The FLAG™ marker peptide fusion system comprises a unique and widely useful technique for protein identification and purification (Table 2). Elution of the fusion protein can be accomplished either by antibody-mediated affinity chromatography in a calcium-dependent manner, by lowering the pH, or by competitive elution with synthetic peptides.

When covalently attached to a solid support, the anti-Flag M1 antibody can be used for the rapid purification of FLAG™ fusion proteins in a mild, calcium-dependent affinity chromatography procedure [1,12,27]. FLAG™ fusion proteins are typically purified to homogeneity in a single step, starting from a crude cell homogenate or supernatant, without ever exposing the protein to conditions other than physiological saline at pH 7.2 (with calcium or EDTA). The entire purification process can be carried out within several hours. This process has been applied to several important classes of molecules, including gene-regulatory proteins [22], members of the seven-transmembrane helix receptor family [26], immunoglobulins [24] and cytokines [1,12]. Different types of supports were applied to antibody immobilization. Agarose affinity gels are available for the purification of *N*-terminal free FLAG™ fusion proteins [1,24]. Schuster et al. [33], report on a short-cut protein purification procedure, where mechanical cell disintegration and affinity purification by immobilization of the anti-Flag M1 antibody on magnetic glass beads (from Bioprocessing, Consett, UK) are combined. The modified glass beads were added to yeast cells and mixed on a laboratory vortex mixer. The magnetic glass beads capture the target protein, while the yeast cells are disrupted. By making contact with a stationary magnet, the magnetic glass beads can very efficiently be separated from yeast cell debris. Chelating agents, such as EDTA, were capable of eluting the FLAG™ fusion proteins from the magnetic glass beads. An example of the

Table 2
Selected samples of fusion proteins expressed with the FLAG system

Fusion protein	Host organism	*C*/*N*-terminal fusion	Applied antibody/matrix	References
Alkaline phosphatase	*E. coli*	*N*	M2/sepharose 4B	[11]
Human serum albumin	yeast	*N*	M1/anti-Flag M1 PROSEP-5CHO	[33]
Alkaline phosphatase	COS-1	*N*	M1/anti-Flag M1 affinity gel	[32]
Yeast transcription factors *SUP1, TUP1*	SF21	*N*	M2/anti-Flag M2 affinity gel	[52]
Murine interleukin-18	*E. coli*	*N*	M2/anti-Flag M2 affinity gel	[53]
Interleukin-3/-4	yeast	*N*	M1/anti-Flag M1 Affigel-10	[12]
Ectodomain of influenza subunit HA_2	*E. coli*	*N*	M2/anti-Flag M2 affinity gel	[54]
Antibody McPC603 (fragments)	*E. coli*	*N*	M1/anti-FLAG M1 affinity gel	[24]
Lymphokine interleukin-2	yeast	*N*	M1/anti-Flag M1 affinity gel	[1]
Humanised γ1 Fab′	*E. coli*	*C*	M2	[55]
Leukemia inhibitory factor	yeast	*N*	M1/anti-Flag M1 affinity gel	[21]
Homo sapiens fau	yeast	*N*	M1/anti-Flag M1 affinity gel	[21]
Human UbA-52	yeast	*N*	M1/anti-Flag M1 affinity gel	[21]
Nuclear protein P68	yeast	*N*	M1/anti-Flag M1 affinity gel	[21]

efficiency of this system is shown in Fig. 1, representing a SDS-PAGE of the affinity purification of a recombinant FLAG–HSA fusion, expressed intracellularly in *Saccharomyces cerevisiae*, with the use of an anti-Flag M1 agarose column.

Competitive elution with synthetic peptides represents a second possibility for affinity purification of FLAG-tagged proteins. In this procedure, described by Brizzard et al. [30], anti-Flag antibody M2 was immobilized on a suitable chromatographic support, such as a Hydrazide AvidGel™ Ax (Bioprobe International, Tustin, CA, USA). Cell lysates were applied to the column by gravity flow. Elution of the fusion protein was accomplished by the addition of synthetic FLAG™ peptide [30]. However, purification of FLAG™ fusion proteins cannot be performed in the presence of denaturants without affecting the stability of the antibody or fusion. For this reason, expression of FLAG™ fusion proteins in inclusion bodies may not be the best choice.

Investigations regarding the metal specificity of anti-Flag M1 antibody in ELISA assays showed an unusual performance of the whole antibody in comparison to its Fab fragments. The M1 antibody lacks a detectable calcium dependency on complex formation of antigen and antibody. This is in contrast to the known behavior of this antibody in affinity chromatography, where calcium is needed for sufficient antigen

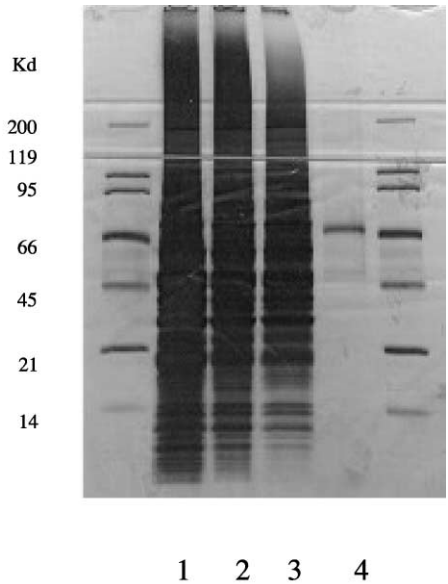

Fig. 1. SDS-PAGE affinity purification of recombinant HSA on an anti FLAG M1 agarose column according to Schuster et al. [33]. Lanes: (1) negative control, expression vector without insert; (2) yeast extract; (3) desalted yeast extract; (4) eluate with EDTA.

binding. This effect is explained by the divalent nature of the antibody. Because dissociation of the first of the two Fab arms of the antibody leaves the second arm still attached, there exists the possibility of a re-association of the first arm before the second arm is released. Since calcium is present in all wash steps and subsequent reactions, any complexes formed will contain calcium and will therefore resist further dissociation. In contrast to the whole antibody, the Fab diffuses away, once it is dissociated [28]. These results were confirmed in real-time BIACORE experiments, where binding constants of the anti-Flag M1 monoclonal antibody in the presence and absence of calcium were determined [34].

5. FLAG™ removal

The question of whether to remove the purification tail or not essentially depends on the end-use of the target protein. For the laboratory-scale characterization of a protein that is difficult to obtain in sufficient quantities, it may be possible to leave the tail on, after initial demonstration that the tail does not interfere with the biological function of the target protein. In order to obtain reliable results, the removal of the tail may be necessary when interference is encountered. For antibody production, it may be desirable to leave the fusion tail on as a carrier to promote antigenicity. For pharmaceutical applications, precise removal of the fusion tail is usually desired, due to the need for an absolute product authenticity. Cleavage sites for proteases, such as Factor Xa, RTEV

(recombinant protease from the tobacco Etch virus with 6 × His tag), genenase I, thrombin, and 3C (3C human rhinovirus Type 14 protease) were introduced between the FLAG™ peptide and the target protein [35–38]. The use of such enzymes is usually associated with high costs, as well as variable effectiveness due to steric hindrances of the domain surrounding the target site, which limits the accessibility of the enzyme to the cleavage site. Their use also requires a further, efficient purification step to remove the proteolytic enzyme from the sample.

The five *C*-terminal amino acids of the FLAG™ marker peptide sequence represent the minimal enterokinase specificity site AspAspAspAspLys. A cDNA, encoding the catalytic domain of bovine enterokinase, has been cloned and expressed in mammalian cells and in *E. coli* [39,40]. The enzyme possesses most of the characteristics of a universal cleavage reagent. It is highly active, works over broad ranges of pH, denaturant levels, and detergent concentrations, exhibits high specificity for its pentapeptide recognition sequence and cleaves at the carboxy-terminal side of its recognition site, leaving no additional downstream residues on the cleaved product. Subsequently, even shorter variants, consisting of only four amino acids at the *N*-terminus of the FLAG™ peptide, have been constructed with an even greater sensitivity in immunological detection and purification procedures [24].

6. Conclusion

The FLAG™ tag shows all the advantages and disadvantages of immunoaffinity purification. Although highly selective, the binding capacities are low, making scale-up a costly undertaking. In addition to cost and low capacity, large-scale immunoaffinity chromatography, applied to the production of therapeutic proteins has several disadvantages: ligand leakage, instability, and need for validation of antibody production. The stability of the affinity chromatography column depends on the nature and source of the crude extracts. Furthermore, the FLAG™ tag, designed to be immunogenic, must be removed from therapeutic proteins. In most cases, this can be accomplished with enterokinase. However, contaminating proteases may also produce undesired cleavages. Despite these drawbacks, the FLAG™ fusion is useful in research and development: FLAG™ proteins can be readily purified and assayed by ELISA or any other immunochemical detection method, thus expediting the raising of antisera against a desired protein and characterization studies. The benefits of a simple and rapid quantitative assay for any fusion protein should not be underestimated, and the FLAG™ fusion certainly provides them. The performance of the antibody M1 in affinity chromatography cannot be explained by binding studies carried out with ELISA and Biosensor assays. Further investigations are necessary to clarify the mode of action of this affinity system.

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