Chapter 4

Purification Using Affinity Tag Technology

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Abstract

Affinity tag technology is a prerequisite for high and rapid purification of recombinant proteins in structural studies because of specific interactions of tags. Widely used tags are polyhistidine tags specific to metal-chelating ligands and glutathione S-transferase tag for glutathione-immobilized ligands. Furthermore, tags binding to antibodies, such as FLAG, Fc, and HA, are also popular for protein preparation and, in addition, are utilized for biological and biochemical analyses, e.g., western blotting, immunoprecipitation, immunofluorescence assay, and flow cytometry. Some tags improve the solubility of proteins. In this chapter, we introduce the features of these representative tags and show several practical examples.

Keywords Affinity purification, Affinity tags, Solubility enhancement, Biologics

1 Introduction

Recombinant DNA technology is essential for large-scale protein preparation in structural studies. In the early days, purification of recombinant proteins was performed using traditional ion-exchange chromatography and gel filtration. However, the introduction of the hexa-histidine tag used for immobilized metal affinity chromatography (IMAC) dramatically changed the strategy for purification, because this tag confers easy and rapid purification in good yields and with high purity. Nowadays, many tags are commercially available and have additional advantages such as the improvement of the characteristics of the target proteins (Table 1). A schematic image of affinity purification methods are shown in Fig. 1. In this chapter, we describe the basic concepts of representative tags and detail the procedures for purification using some tags.

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Table 1
General tags that are widely used for expression and purification of proteins. In the "sequence" column, the terms "protein" and "compound" indicate a tag that is relatively huge and chemical modification of a specific sequence, respectively

Purification methods	Tag	Residues	Sequence
By tag-ligand affinity	His	6	HHHHHH
	GST	262	Protein
	MBP	375	Protein
	Biotin		Compound
	Strep-Tag	8	WSHPQFEK
By antibody	FLAG	8	DYKDDDDK
	Myc	11	EQKLISEEDL
	Fc	ca. 250	Protein
	1D4	9	TETSQVAPA
	HA	9	YPYDVPDYA
	GFP	238	Protein
None	SUMO	75–100	Protein

2 Polyhistidine Tag

IMAC, introduced by Porath et al., is one of the most powerful methods for protein purification [36]. IMAC is based on the coordination interaction between transition metal ions immobilized on a resin and cationic amino acids in proteins. Transition metal ions, such as Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Fe^{3+}, are immobilized on an agarose, sepharose, or silica gel resin through spacer ligands, such as N,N,N′-tris-(carboxymethyl)-ethylenediamine, nitrilotriacetic acid (NTA), and iminodiacetic acid (IDA). Ni ions and NTA chelator are the most commonly used. The main amino acid interacting with metal ions is histidine. The imidazole ring in histidine serves as an electron donor and can form a coordination bond with a transition metal ion immobilized on the resin. Using genetic engineering, the polyhistidine tag can be added to the target protein to enable strong and specific binding to transition metal ions. The side-chain imidazole ring of the histidine residues interacting with the metal ions can be replaced with imidazole during the elution. The first use of the polyhistidine tag was developed by Hochuli et al. [16]. Terpe investigated the effect of the tag length, ranging from two to ten residues, and demonstrated that the hexahistidine tag

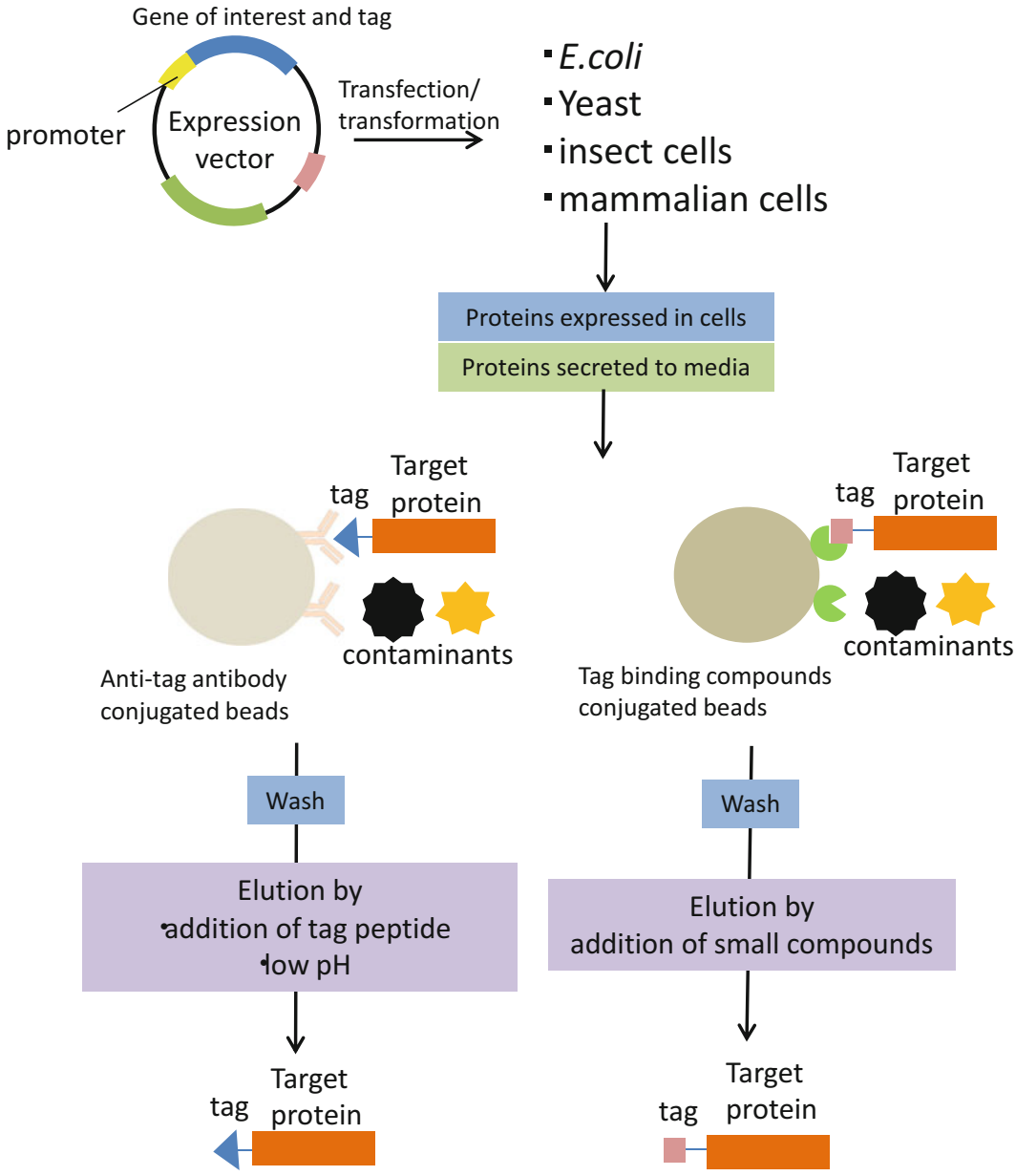

Fig. 1 Schematic image of the process from expression to purification of proteins

containing six consecutive histidines is the most effective tag to purify proteins [43]. While some proteins contain consecutive histidine residues and these proteins can bind with IMAC resins, they can generally be washed out by buffers with a low concentration of imidazole. After the washing procedure, the target protein can be purified in high purity by eluting with a buffer solution containing a high concentration of imidazole (typically 500 mM of imidazole) [43].

2.1 Materials and Methods

2.1.1 Buffers

Lysis buffer	50 mM Tris·HCl, pH 8.0, 150 mM NaCl (0.02 % Triton-×100, 5–10 % glycerol); not below pH 4.0
Wash buffer	50 mM Tris·HCl, pH 8.0, 100 mM NaCl, 10 mM imidazole (~500 mM NaCl)
Elution buffer	50 mM Tris pH 8.0, 100 mM NaCl, 250–500 mM imidazole

2.1.2 Methods

When the protein of interest is in an insoluble form in the lysis solution, a denaturant such as guanidinium chloride or urea can be utilized to solubilize the proteins from the pellet. Adding the IMAC resin to the lysis solution, the mixture is incubated with gentle shaking at 4 °C for 1 h. The supernatant and the resin precipitate are separated by centrifugation or filtration. The IMAC resin is washed a few times using the wash buffer containing the low concentration of imidazole (e.g., 10 mM), which is useful for the removal of nonspecifically bound proteins (but it is also important to note that in some cases, His-tagged proteins are removed even at a low concentration of imidazole). Adding the elution buffer containing 250 mM imidazole or more, IMAC resin is incubated with gentle shaking at 4 °C for 1 h. The target protein is eluted in the supernatant.

3 Glutathione S-Transferase Tag

Glutathione S-transferase (GST) is a major member of detoxification enzymes [4]. GSTs are composed of three superfamilies: cytosolic, mitochondrial, and microsomal GST proteins. These GSTs are also classified in terms of cytosolic and membrane-bound isoenzymes. There are five types of cytosolic enzymes, including alpha, mu, pi, sigma, and theta isoforms. The microsomal GST isoforms, delta, kappa, omega, and zeta isoenzymes, and the mitochondrial superfamily are membrane bound. In the cellular signaling pathways, these GST proteins inhibit some kinases such as those of the MAPK cascade, which regulates cell proliferation and death [2, 24]. Furthermore, GSTs can interact with glutathione (GSH) strongly and regulate the oxidation/reduction environment in the cell. GSH comprises three amino acids, Glu, Cys, and Gly. This peptide plays an important role to protect cells from reactive oxygen species such as peroxides and free radicals. The interaction between GST and GSH is strong with the dissociation constant (K_d) at a nanomolar level. Thus, using this high recognition ability, the GST tag can be applied to GSH-based affinity chromatography. The GST tag can be fused with the protein of interest either at the N- or C-terminal. The GST tag sometimes exhibits the

increment of stability and solubility of target proteins. Fusion with GST can facilitate the proper folding of the target proteins because GST is rapidly folded right after translation. After the lysis of the host cells expressing the GST fusion proteins, the lysate is added to the GSH resin, which is generally composed of agarose or sepharose. GST-tagged proteins are immobilized on resins through the interaction of the GST tag with GSH. Notably, the GST tag can bind GSH at above pH 7, and thus, the buffer for the GST purification is normally prepared at pH 8 or above. After washing the resin to remove nonspecific proteins, the GST-tagged protein can be purely eluted from the GSH resin by adding an excess of GSH.

3.1 Materials and Methods

3.1.1 Buffers

Lysis buffer	50 mM Tris pH 7.5, 150 mM NaCl, 0.02 % Triton X-100
Wash buffer	50 mM Tris pH 8.0, 100 mM NaCl (~500 mM NaCl)
Elution buffer	50 mM Tris pH 8.0, 100 mM NaCl, 20 mM *reduced* glutathione (*make fresh every time*)

3.1.2 Methods

1. Lysis of *E. coli* cells by the ultrasonic sonicator or a French press with the lysis buffer. Centrifuge the lysate at 20,000 × *g* at 4 °C for 10 min.
2. Filtrate the supernatant using a 0.22-μM pore size filter unit.
3. Wash the GSH resin with the wash buffer. Add 10 resin bed volumes of the wash buffer and centrifuge the mixture until the resin collects at the bottom. Remove and discard the supernatant. Wash the resin at least two more times with the wash buffer.
4. Transfer the lysate to the tube that contains the GSH resin. Incubate at 4 °C for 30–60 min with agitation.
5. Centrifuge and wash the resin three times with the wash buffer.
6. Add the elution buffer to the GSH resin. Incubate at 4 °C for 30 min and collect the supernatant. If desired, the elution can be repeated multiple times.

4 Maltose-Binding Protein

Maltose-binding protein (MBP) is another popular protein tag, similar to the abovementioned GST tag and polyhistidine tag. MBP is composed of 388 amino acids (42 kDa) and works in the *E. coli* maltose/maltodextrin system, which regulates the uptake and catabolism of maltodextrin. This protein tag binds with the "maltose," as its name suggests, and, furthermore, can bind a few similar sugar groups, such as trehalose and amylose [7, 33]. Almost

all of the marketed resins are conjugated with amylose. Maltose is a reduced disaccharide that consists of two α-glucose monomers joined by the α-1,4 glycosidic bond, and amylose is its polymer. In other words, MBP recognizes the maltose part of amylose. Maltose has a higher affinity toward MBP than amylose; therefore, maltose can be used as an elution compound by the competition of the interaction between the amylose and MBP. Similar to the GST tag, the MBP tag can induce the increment of the solubility and stability in some cases. As a common vector system, the pMAL vector can be purchased from New England Biolabs. In this system, the MBP tag is located at the N-terminal side of the target protein via a specific proteinase cleavage site.

5 Avidin/Biotin System

Avidin is a glycoprotein, approximately 70 kDa, first found in the white of a chicken egg. The physiological functions of the avidin protein in the egg have not been clarified. Biotin is one of the B-group vitamins and is also known as vitamin H. In 1976, the avidin–biotin interaction was first reported as a powerful tool in biological science [14, 18]. The interaction is extremely strong among noncovalent bonds, $K_d = 10^{-15}$ M; this affinity exhibits a much higher value than antigen–antibody reactions [12]. Because avidin is a tetrameric protein and its monomer can bind one biotin, avidin can bind up to four biotins. Other biotin-binding proteins such as streptavidin and neutravidin also have the ability to interact with four biotin molecules. Streptavidin is derived from the bacteria *Streptomyces* and thus does not require the sugar modification. Neutravidin does not have any sugar chains, either. These proteins do not interact with sugar-binding proteins such as lectins. Combined with their neutral isoelectric point (pI), these proteins show less nonspecific interactions compared to the avidin protein and are suitable as an experimental tool. The interaction between avidin and biotin is very rapid and nearly irreversible, so it can be used for enzyme-linked immunosorbent assay (ELISA), immunocytochemistry, pull-down assays, and protein immobilization. Recently, this interaction was modified and used extensively as a purification system. It has become possible to use this interaction reversibly by using desthiobiotin [15] and Strep-tag® composed of octapeptides [40, 41]. Because these molecules or peptides have weaker affinities against the avidin, biotin can be used as an additive for elution from these avidin-related proteins.

6 FLAG Tag

The FLAG tag consists of eight hydrophilic amino acids (DYKDDDDK, from the N-terminus to the C-terminus) [9]. The developmental history of FLAG tag is rather unique. Some other tags (e.g., myc and HA discussed below) are part of native proteins and a monoclonal antibody was first isolated against the proteins, then the epitope was characterized. In contrast, the FLAG epitope was artificially designed first, and then monoclonal antibodies were prepared. So far, monoclonal anti-FLAG antibodies, such as M1, M2, and M5, have been developed and are commercially available (i.e., Sigma-Aldrich). It is known that the aromatic amino acid (tyrosine) of the FLAG tag is the major factor in tag–antibody interactions [19], but each commercially available antibody has a different epitope and affinity to the FLAG tag (Table 2). For instance, if the α-amino group of the first amino acid of FLAG tag is freely accessible, the M1 antibody binds with three to four orders of magnitude higher affinity [37]. Combining the use of the

Table 2
The affinities of monoclonal antibodies (M1, M2, and M5) with different fusion positions of the FLAG tag to the protein are shown

Flag-tag fusion proteins	Affinity with each monoclonal antibody		
	M1	M2	M5
Unprocessed N-terminus tagged proteins Signal peptide — FLAG — protein	ND	+	ND*
Met-N-terminus tagged proteins Methionine — FLAG — protein	–	+	++
N-terminus tagged proteins FLAG — protein	+	+	weak
Taginserted proteins protein — FLAG — protein	–	+	ND*
C-terminus proteins protein — FLAG	–	+	weak
Calcium dependent binding	+	–	–

ND indicates not detected. "++" "+" "weak" "–" indicate the binding affinity from strong to weak, and none

Table 3
Enzymes generally used for tag removal

Protease names and digestion site	Representative available company	Protease capture
Thrombin	GE, Merck Millipore,	Benzamidine–agarose
LVPR▼GS	SIGMA, Roche	
Factor Xa	GE, New England Biolabs,	Benzamidine–agarose
I(D/E)GR▼	Roche	
Enterokinase	New England Biolabs,	Trypsin inhibitor–agarose
DDDDK▼	Merck Millipore, Roche	
TEV protease	Promega, Nacalai, SIGMA	Ni-NTA (6 His recombinant TEV)
ENLYFQ▼G		
PreScission	GE	GSTrap for GST fusion enzyme
LDVLFQ▼GP		
HRV 3C Protease	Takara, Merck Millipore, Pierce	Ni-NTA (6 His recombinant enzyme)
LEVLFQ▼GP		
SUMO Protease	Life Technologies, LifeSensors	Ni-NTA (6 His recombinant enzyme)
Recognize the tertiary structure of SUMO		

FLAG tag with other tags leads to more efficient purification methods [25].

Elution of FLAG-tagged proteins from anti-FLAG antibody can be performed by two different methods. One is a low-pH elution method similar to other antibody-based purification systems. The other is elution by addition of 2–5 mM EDTA, which is a mild elution procedure for many proteins. Furthermore, another advantage is that the tag itself is cleaved by an enterokinase without any insertion of additional amino acids because the sequence of FLAG is recognized by the enzyme (Table 3). A weak point of the system is that the monoclonal antibody matrix for purification is not so stable as others, e.g., Ni^{2+}–NTA or streptavidin beads [43].

7 Myc Tag

The myc tag, EQKLISEEDL sequence, is a short tag derived from the c-myc gene. Myc (c-Myc) is one of the transcription factors, which regulates the cell cycle. The myc gene has been extensively studied as an oncogene because the mutations in myc are found in

many cancer cells. A monoclonal antibody, 9E10, which was raised against the myc peptide in mice, is available from the noncommercial Developmental Studies Hybridoma Bank [10, 20]. The agarose gels or beads covalently linked with anti-myc tag antibody are also commercially available from suppliers. The expression of myc fusion proteins in several expression hosts, such as bacteria, yeasts, insect cells, and mammalian cells, has also been successful. Purified c-myc-tagged proteins have been crystallized [32]. The myc tag can be fused to either the C-terminus or the N-terminus of a target protein. It should be noted to avoid fusing the tag directly behind the signal peptide of a secretory protein because it interferes with correct intercellular trafficking.

8 Fc Tag

Fc fusion is also highly used for the expression and purification of proteins. The immunoglobulin Fc domain is a 25 kDa protein with a sugar modification for structural stability. Sugar modification does not generally occur in prokaryotes: therefore, the expression of Fc fusion proteins is normally performed in eukaryotic cells. However, recent biotechnological developments have allowed us to express Fc fusion proteins in *E. coli* by introducing *Campylobacter jejuni* glycosylation machinery into *E. coli* and subsequent enzymatic transglycosylation [27, 42]. Although the Fc regions are originally located at the C-terminus of immunoglobulin, the Fc tag can link to either the N-terminus or the C-terminus of target proteins. Fc-tagged proteins can be utilized for pharmacological purposes (Table 4) [6, 38]. The most important feature of the Fc tag fusion is its ability to increase the protein half-life in the plasma, extending the efficacy of drugs. This phenomenon is mainly thought to be because of the following reasons: (1) Fc-tagged proteins interact with the salvage neonatal Fc receptor (FcRn) [39], and (2) larger molecules have slower renal clearance [23]. The attached Fc domain also enables the fused protein to interact with Fc receptors (FcRs) expressed in immune cells, which is particularly important for their antibody-dependent cellular cytotoxicity (ADCC) in oncological therapies and in the application for vaccines [28, 34]. In addition, in regard to their biophysical features, the Fc domain folds independently and can improve the solubility and stability of the fused proteins both in vitro and in vivo. Furthermore, the Fc region provides easy and cost-effective purification by using protein G/A affinity chromatography [5]. Protein G and protein A are cell-surface proteins from *Streptococcus* and *Staphylococcus* species, respectively. They have different binding

Table 4
Fc fusion proteins used as drugs

Drug name	Description	Indication	Expression system	Approved year	Company
Belatacept	Modified CTLA-4 fused to the Fc of human IgG1	Organ rejection	Mammalian and COS cells	2011	Bristol-Myers Squibb
Aflibercept	Second Ig domain of VEGFR1 and third domain of VEGFR2 fused to the Fc of human IgG1	Age-related macular degeneration	CHO cells	2011	Regeneron Pharmaceuticals
Rilonacept	IL-1R fused to the Fc of human IgG1	Cryopyrin-associated periodic syndromes	CHO cells	2008	Regeneron Pharmaceuticals
Romiplostim	Thrombopoietin-binding peptides fused to the Fc of human IgG1	Thrombocytopenia in chronic immune thrombocytopenic purpura patients	*E. coli*	2008	Amgen/Pfizer
Abatacept	Mutated CTLA-4 fused to the Fc of human IgG1	Rheumatoid arthritis	Mammalian cells	2005	Bristol-Myers Squibb
Alefacept	LFA-3 fused to the Fc of human IgG1	Psoriasis and transplant rejection	CHO cells	2003	Astellas Pharma
Etanercept	Human p75 TNF receptor fused to the Fc of human IgG1	Rheumatoid arthritis	CHO cells	1998	Amgen/Pfizer

affinities depending on the kind of immunoglobulins (Table 5). Cleavage of the Fc domain from the Fc-tagged protein is performed by papain. This enzyme specifically cleaves the hinge region between the target protein and the Fc domain. Recently, for better stability of the cleaved protein, a 3C protease cleavage site was introduced into the hinge region because this enzyme has high specificity and a low optimal reaction temperature [3].

Table 5
The affinities of various kinds of immunoglobulins from several species with protein G or A

Immunoglobulins	Affinity for protein A	Affinity for protein G
Human IgG_1	++++	++++
Human IgG_2	++++	++++
Human IgG_3	−	++++
Human IgG_4	++++	++++
Human IgM	±	−
Human IgA_1	−	−
Human IgA_2	+	−
Human IgD	−	−
Human IgE	±	−
Mouse IgG1	+	++++
Mouse IgG_{2a}	++++	++++
Mouse IgG_{2b}	+++	+++
Mouse Ig_{G3}	++	+++
Mouse IgM	±	−
Mouse IgA	−	−
Mouse IgE	−	−
Rat IgG_1	±	+
Rat IgG_{2a}	−	++++
Rat IgG_{2b}	−	++
Rat IgG_{2c}	+	++
Bovine IgG_1	±	+++
Bovine IgG_2	+++	+++
Bovine IgA	−	−

The number of "+" reflects the affinity strength. "±" indicates slight affinity
"−" indicates no binding affinity

9 SUMO Tag

Small ubiquitin-like modifier (SUMO) tag is a recently developed tag that accelerates the solubility of the target protein. In *Saccharomyces cerevisiae*, the posttranslational modification of SUMO, Smt3, provides proteins with wide biological function, such as nuclear–cytosolic transport, transcriptional regulation, and apoptosis [13]. In contrast to ubiquitin, which is a "tag" for degradation, the SUMO tag often extends the lifetime of the proteins. When the

SUMO tag is used as an N-terminal fusion protein in prokaryotic expression, SUMO promotes folding and structural stability, which leads to enhanced functional production compared to untagged protein [30, 31]. Furthermore, the SUMO tag itself has a unique advantage that a SUMO-specific protease (*S. cerevisiae* UlpI) can digest a Gly–Gly motif of the tag. Thus, the SUMO tag is widely available in both prokaryotic and eukaryotic expression systems. Recently, an engineered SUMO-based tag, SUMOstar, has been established to enhance protein expression in eukaryotic cells because the SUMOstar sequence could not be recognized by the endogenous SUMO protease [22, 26, 35]. Instead of the conventional SUMO protease, this SUMOstar tag could be cleaved by engineered SUMOstar protease. Because of the recent usefulness of the SUMO tag for protein crystals, this tag will become more important and common in the future [1, 21, 29].

10 Other Tags

Hemagglutinin is well known as a surface protein in the human influenza virus and is involved in the adhesion to host cells. The HA tag consists of 9 amino acids, YPYDVPDYA, from the N-terminus to C-terminus, corresponding to the 98–106 amino acid residues in HA. HA monoclonal antibodies (and HA-antibody conjugated agarose) for purification are commercially available.

The GFP tag is widely used to investigate the subcellular localization of a target protein by fluorescence microscopy and the expression of exogenous proteins by FACS or Western blotting. The expression and purification of GFP-tagged recombinant proteins are not common owing to the problem of cost and the amount of protein expression. Recently, it has been reported that the GFP tag has been used for optimization of the expression and purification of a eukaryotic membrane protein [8].

The 1D4 epitope is nine amino acids (TETSQVAPA) derived from the intracellular C-terminus domain of bovine rhodopsin [17, 44]. Combining this epitope and the high-affinity 1D4 monoclonal antibody has established useful tools in antibody-based purification, localization studies, and Western blot analysis of 1D4-tagged proteins [11, 45]. Additionally, the 1D4 enrichment strategy offers a highly specific, non-denaturing method for purifying membrane proteins with yields and purities sufficient enough to use for structural characterization and functional proteomics applications [45].

11 Tag Digestion

Because tags described above have various characteristics, these might affect the physicochemical properties of the target protein fused with these tags. For example, the polyhistidine tag is

composed of several consecutive histidine residues and thus confers a high positive charge to the fusion proteins. Conversely, huge tags such as GST and MBP might inhibit the enzyme activities of fusion proteins through their steric hindrance. In these cases, tags can be a useful tool for purification by avoiding undesired enzymatic function, but it is better to remove the tags for functional assays. Generally, there is a linker composed of some amino acids between a tag and a target protein, wherein the digestion sequences of some proteases are inserted into the linker site. The linker can be used for higher purification by removing tagged proteins from the resin by specific proteases. Typical proteases are 3C protease, thrombin, Factor Xa, and enterokinase (Table 3).

11.1 Removal of Tags

An example of the removal of a tag with an enterokinase site is shown below.

1. Dilute enterokinase in the storage buffer to prepare 0.01, 0.04, 0.1, 0.4, and 1 U/μl enterokinase solution.
2. Mix the following materials in a tube. The total volume should be adjusted to 50 μl by the addition of an appropriate amount of water.

10× reaction buffer	5 μl
Target protein	20 μg
Diluted enterokinase	5 μl
H_2O	X μl
Total volume	50 μl

3. Incubate the tube at room temperature (e.g., 25 °C).
4. Take a 10 μl aliquot after 2, 9, and 24 h of incubation. Each aliquot is mixed with 10 μl 2× SDS-PAGE sample/loading buffer for SDS-PAGE analysis.
5. Check the result of the tag cleavage by SDS-PAGE and decide suitable reaction conditions (time and the protease concentration).
6. Apply suitable reaction condition to a large amount of the tagged protein.
7. Purify the cleaved protein by chromatography, such as gel filtration chromatography.

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