Protein Purification by Affinity Chromatography

6

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Abstract

Affinity chromatography involves targeted purification of biological macromolecules from a crude mixture on the basis of highly specific interaction between the macromolecule and a tag protein or peptide. The interaction is typically reversible and purification is implemented by keeping one of the molecules (the affinity ligand or fusion tag) immobilized to the support matrix (containing respective binding resin for interaction with the tag) while its partner (the target protein) is in a mobile phase as part of the crude mixture. In this chapter we will be discussing recombinant protein purification using different affinity tags that are routinely used in a laboratory setup that include polyhistidine, GST (glutathione-S-transferase), maltose-binding protein (MBP) and Strep-tag. As affinity chromatography is a sophisticated purification method that requires significant expertise, the protocol and the problem-solving approaches described in this chapter will act as essential guides to the protein biochemists.

Keywords

Affinity chromatography · Affinity tag · Support matrix · MBP · GST · Strep-tag

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K. Bose (ed.), *Textbook on Cloning, Expression and Purification of Recombinant Proteins*, https://doi.org/10.1007/978-981-16-4987-5_6

6.1 Introduction

As discussed in the earlier chapter, purification of recombinant proteins came into picture when Sumner et al. purified enzymatic proteins from prokaryotic system in the year 1926 [1]. Since its inception, protein purification process has evolved considerably, giving rise to different types of chromatographic techniques that often in combination give proteins of very high purity (>95%). Affinity chromatography is one such technique that was first introduced by Campbell and co-workers in 1951, when they isolated rabbit anti-bovine serum albumin antibodies using bovine serum albumin as the affinity ligand [2]. In subsequent years, the application of this technique went beyond just antigen-antibody separation and researchers slowly adopted its principle for separation of different types of protein molecules [3]. The salient features that give this process an extra edge over other purifying techniques are high-precision specificity, ease of handling and high yield. A classic affinity chromatography process mainly involves following three steps:

- Incubating the given sample (containing mixture of proteins and other molecules) along with the support matrix (containing affinity ligand) that allow the target protein in the sample to bind with the affinity (immobilized) ligand.
- Binding of the target protein and the affinity ligand depends on the buffer conditions. An optimum buffer condition ensures that the target molecule interacts effectively with the ligand and is retained by the affinity medium as all other molecules wash through the column.
- Post-washing, elution (dissociation from the ligand and recovering the protein) of the target protein from the ligand is done by changing the buffer conditions in a way conducive to disruption of the binding interactions between the target and the ligand.

Generally, elution of the desired protein using affinity-based purification depends on reversible interactions between the target protein and the immobilized ligand bound to support or chromatographic matrix [4]. Most proteins have their designated binding sites that are taken into consideration while selecting the suitable affinity ligand. However, it is necessary that the binding interaction between the target protein and the chosen ligand is reversible as well as specific. In case of recombinant proteins purification, a reversible but specific binding with the ligand is achieved through fusion tags. Fusion tags are generally proteins or peptide molecules that are capable of facilitating expression of the target protein in the expression system by providing resistance to proteolytic degradation [4]. A well-characterized fusion tag enables affinity chromatography of the tagged protein (target protein with the fusion tag attached) using immobilized version of the respective affinity ligand. His_6, MBP (maltose-binding protein) and GST (glutathione-S-transferase) are some of the fusion tags that are generally used in affinity purification [5]. Though in recombinant protein purification fusion tags have their own benefit, choosing a suitable tag, using it for affinity-based protein purification and separating it post-purification are a challenging process. Hence, in this chapter, with detailed protocols for various

types of affinity chromatography techniques, we will elucidate its role in purification of recombinant proteins.

6.2 Types of Tags

Selecting suitable fusion tags for the target proteins is an important aspect of recombinant protein purification process. Generally, underlying physiochemical properties of the target protein determine the type of tags to be used for purification. Some of the widely used tags are given below in detail.

6.2.1 Polyhistidine Tag

The polyhistidine affinity tag, *aka* His_6-tag, normally comprises six consecutive histidine (His) residues, but can vary in length from two to ten histidine residues [6]. Protein purification using His_6-tag employs the ability of His to interact with transition metal ions, such as Cu^{2+}, Co^{2+} and Ni^{2+}. His_6-tag is non-toxic, and due to its small size, it induces no effect that would alter the physiochemical properties of the target protein [7]. Usage of His_6-tag is advantageous for protein purification involving prokaryotic expression systems where different vectors such as pET-16b, pET-28a or pET-28b are used [8]. However, in case of eukaryotic systems, where the percentage of His residues is high, protein purification becomes complicated due to non-specific binding of the His residues with the affinity ligands (metal ions). This non-specific background binding is generally circumvented through application of stringent wash conditions [9].

6.2.2 Glutathione-S-Transferase (GST) Tag

GST is a 26 kDa (kilo Dalton) fusion tag belonging to the cytosolic family of eukaryotic and prokaryotic enzymes that catalyse the fusion of the reduced form of glutathione (GSH) to external chemical substrates present within organisms for the purpose of detoxification [7]. It is one of the most extensively used tags for protein purification in prokaryotic expression systems that uses multiple cloning sites of pGEX vectors for generating the fusion tag protein [10]. Due to its ability to enhance tagged-protein solubility, GST affinity tag is also considered as a solubility tag [11]. Since GST-tag has high affinity towards glutathione, glutathione-coated beads are coupled to the chromatographic support matrix during purification of the fused protein of interest [12]. GST-tag is normally attached to the N- or C-terminal region of the target protein, depending on the structural properties of the protein. Owing to its slow binding kinetics with the glutathione ligand, high-scale protein purification using GST-tag sometime becomes time-consuming [13].

6.2.3 Maltose-Binding Protein (MBP) Tag

Similar to GST, MBP also enhance recombinant protein solubility in bacterial expression system [14]. pMAL vector in the *E. coli* expression system is generally used to generate the protein of interest with MBP attached at the N- or C-terminus [15]. One of the advantages of MBP-based purification over polyhistidine tag is the ability to generate high yield of difficult-to-purify proteins [16]. An MBP-fusion protein binds to the cross-linked amylose resin present in the chromatographic matrix [14]. The target protein is eluted by running maltose in the elution buffer solution. MBP-based affinity purification is considerably resistant to denaturing and reducing agents. However, any amylase activity in the crude sample (cell lysate) substantially reduces the efficiency of the amylose resin that is used as the affinity ligand in the support matrix [7].

6.2.4 Calmodulin-Binding Peptide (CBP) Tag

The calmodulin-binding peptide (CBP) is a 26 amino acid fragment that belongs to the C-terminal region of the muscle myosin kinase protein [17]. CBP utilizes its mild binding affinity ($K_d = 10^{-9}$ M) towards calmodulin (affinity ligand) in order to purify the protein of interest from the bacterial system [17]. The mild binding and elution conditions of this purification system enable the fusion protein to maintain its native form post-purification. Expression vectors such as pCAL-c or pCAL-n are used for the expression of CBP-tagged target proteins, where crude cell lysate (containing the fused protein) is passed through the calmodulin affinity resin for its purification and subsequent elution [18, 19]. The 4 kDa size of the CBP tag itself is relatively small and is much less likely to affect the properties of the protein of interest, thus making this an appealing affinity tag in comparison to tags of larger sizes.

6.2.5 Streptavidin-Binding Peptide (SBP) Tag

There are two different versions of SBPs—Strep-tag I and Strep-tag II—that are generally used to immobilize fusion proteins on a streptavidin matrix [20]. In terms of binding specificity, Strep-tag II exhibits stronger interactions with the streptavidin core (StrepTactin Sepharose medium) as compared to Strep-tag I [20]. Strep-tag II is a very small tag of 1 kDa that comprises only eight amino acid residues (WSHPQFEK) [21]. Small size and chemically balanced amino acid composition make it an appropriate tag for purifying functional proteins as it does not affect the protein structure or folding mechanisms. Generally, pASK-IBA or pASG-IBA vector is used to strategically fuse Strep-tag II onto the N- or C-terminus of the target protein for expression in the bacterial system [22]. The affinity core, StrepTactin, is a derivative of streptavidin and exhibits high stability in the presence of various proteases and SDS [23]. This property of streptavidin ensures long-lasting

affinity columns that can be re-used several times. Apart from Strep-tag II's small size, its specific and fast binding kinetics with StrepTactin affinity medium make this purification system user-friendly and straightforward.

6.3 Types of Affinity Chromatography

In the subsequent sections, we will be discussing three major types of affinity-based purification system that utilizes following types of tags:

1. A tandem repeat fusion tag (polyhistidine) that binds to the affinity matrix comprising metal ions
2. A larger-sized solubility tag such as GST or MBP that can act as chaperone during protein expression and purification process
3. A smaller size binding peptide (Strep-tag II) that is faster and does not cause any structural perturbation in the protein of interest

6.3.1 Purification of Polyhistidine Tag Protein

As mentioned in the last chapter, IMAC (immobilized metal-affinity chromatography) is a widely employed method to purify recombinant proteins containing a short affinity tag of consecutive histidine residues. The basic principle behind IMAC is interactions between an immovable transition metal ion (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) bound to the matrix and side chain of a specific amino acid such as Histidine [24]. Histidine residue displays strong affinity towards metal ion matrices, due to the presence of imidazole ring that acts as an electron donor group facilitating coordinate bond formation with the transition metal ions. This interaction is reversed during the elution of the target protein by adjusting the concentration of imidazole in the elution buffer or altering the pH of the buffer.

6.3.1.1 Binding with the Polyhistidine Tag

A consecutive stretch of six polyhistidine residues (His_6-tag) is the most commonly used tag in IMAC [6]. Though tags of six histidine residues are generally long enough to yield high-affinity interactions with the matrix, various studies have exhibited successful implementation of either shorter or longer affinity tags [6]. Usage of longer His_6-tag might increase the purity of the final product in some cases [25, 26]. However, it is advisable to use the smallest number of histidine residues wherever applicable, so that structural and functional perturbation of the protein can be minimized [25, 26]. In general, for purification of a novel recombinant proteins whose structure and functions have not been studied extensively, a six-histidine tag is always the most suitable choice [25]. Placement of the polyhistidine tags is done at either extremities of the N- or the C-terminal region, depending upon the physicochemical nature of the protein. For unknown proteins, it has been observed that changing the location (moving the tag to opposite terminus)

of affinity tag often resolves the issues related to low yield of the target protein in the expression vector [27]. Moreover, the choice of N- or C-terminal tags also depends upon the downstream studies designed with the purified protein. For example, if protein-protein interaction studies through C-terminal domain are envisaged post-purification, the tag is usually attached to the N-terminus of the protein of interest, keeping the other end free [28].

The main advantage of polyhistidine affinity tag is their small size and non-toxic nature. Owing to their tiny size, they are simple to handle and can be incorporated easily into a variety of expression vectors. Normally, His-tags are introduced into the target DNA (Deoxyribonucleic Acid), encoding the protein of interest, by site-directed mutagenesis [29]. In addition, polymerase chain reaction (PCR) methods can also be applied using primers that have tandem histidine codons (CAC or CAT) [30]. For six histidine tags, eighteen bases encoding the histidine residues are inserted either after the start codon or before the stop codon in the DNA fragments encoding the desired protein [29]. Apart from synthetically preparing the His-tag in laboratory conditions, there are commercially available cloning vectors too that are used for the generation and expression of fusion tag recombinant proteins in various prokaryotic expression systems such as *E. coli.*

6.3.1.2 Components of the Chromatographic Matrix

Among the commercially available metal matrices, nickel-nitrilotriacetic acid (Ni^{2+}-NTA) and Co^{2+}–carboxyl methyl aspartate (Co^{2+}-CMA) are the two widely used immobilized ligands for IMAC [31, 32]. Both Ni^{2+} and Co^{2+} ions have six coordination sites, out of which four sites bind with the IMAC matrix resin and the two remaining sites coupled with the polyhistidine affinity tags [33]. Mechanistic models of their interactions are given in Fig. 6.1. IMAC matrices are generally robust in nature and have the ability to withstand broad range of variable conditions, such as physiological pH, stringent washing procedure as well as varieties of protein denaturants [33]. In terms of binding affinity, Ni^{2+}–NTA matrix has higher affinity towards histidine residues when compared to the Co^{2+}–CMA matrix [35]. At pH 8.0, Ni^{2+}–NTA exhibits binding affinity of $K_d = 10^{-13}$ M with an overall binding capacity exceeding 5 mg protein/ml of matrix resin, making it a more efficient IMAC matrix for purification of polyhistidine-tagged proteins [35].

6.3.1.3 Purification Under Different Conditions

Highly purified fraction of the polyhistidine-tagged recombinant proteins can be obtained under two conditions—native and denaturing. Proteins that are highly soluble in the cytoplasm are normally purified in native condition. Purifying the target protein under native condition helps in preserving its biological activity as this condition rarely alters the structural aspect of the protein [33]. Moreover, native purification with high protein yield is even feasible in mild buffer and imidazole concentration [7]. However, purification under native conditions might not be possible if the protein of interest is insoluble in cell cytoplasm, possesses a tertiary structure that disrupts the bound polyhistidine affinity tag or have the tendency to form aggregates [36]. These issues can be circumvented using denaturing agents

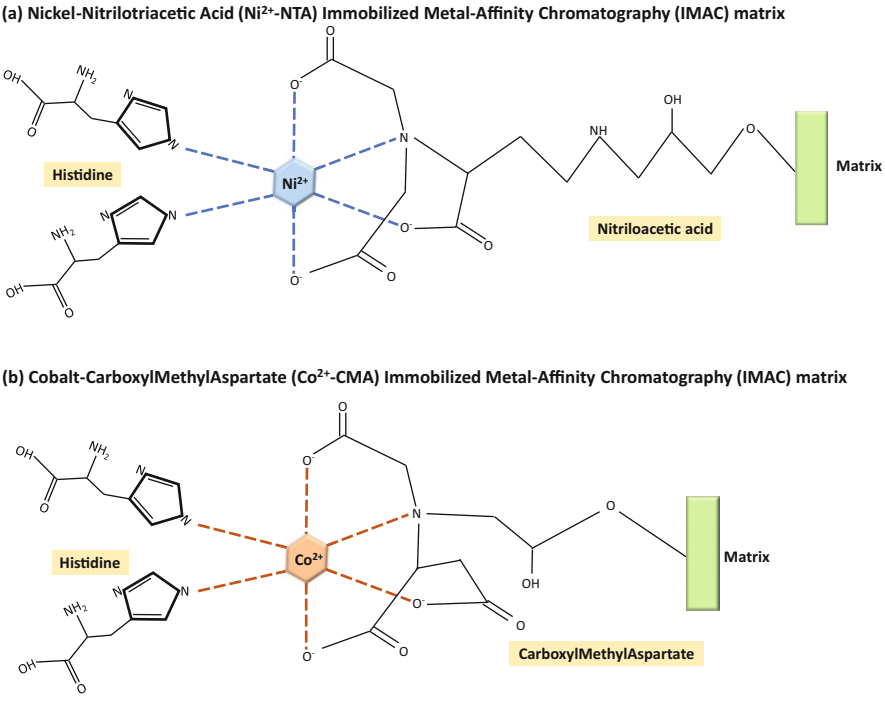

Fig. 6.1 Mechanistic models of interactions between his-tag and IMAC (Immobilized Metal Affinity Chromatography) matrices. (**a**) The nickel–nitrilotriacetic acid matrix (Ni^{2+}-NTA) [34] and (**b**) the cobalt–carboxyl methyl aspartate matrix (Co^{2+}-CMA) [34] are shown here. In both, the metal ion exhibits octahedral coordination by four matrix ligands and two histidine side chains, the latter provided by the polyhistidine affinity tag

including 6 *M* guanidinium hydrochloride or 8 *M* urea during the purification under denaturing conditions [36]. Since polyhistidine tag is an oligopeptide comprising series of tandem histidine repeats, it does not require any specific structural conformation for its proper functioning [37]. Thus, denaturing agents have no adverse effect on the interaction between the matrix resin and the His-tag, which further makes the purification process under denaturing conditions more effective. Between the two agents, denaturation using urea is more preferable for recombinant protein purification as it does not precipitate in the presence of SDS (sodium dodecyl sulphate) unlike guanidinium hydrochloride [37]. Since, SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) analysis is one of the standard steps involved in detecting purity of recombinant proteins, formation of aggregates in contact with the SDS should be avoided. Under denaturing conditions, the obtained purified proteins are generally misfolded with reduced or no activity. Hence, the subsequent refolding of the protein into its biologically active state is achieved by washing away the denaturants stepwise through dialysis [38].

6.3.1.4 Elution

In general, elution of the purified polyhistidine-tagged proteins involves two popular methods—altering the pH level of the elution buffer and inducing imidazole at different concentrations [33]. In case of Ni^{2+}–NTA, reduction of pH to a range between 5.3 and 4.5 results in protonation of the nitrogen atom of the imidazole ring in histidine residue (pK_a 6.0) [34]. This subsequently impedes the coordination bond between the histidine and the transition metal. On the other hand, imidazole that acts as a histidine analogue can be used to competitively elute the bound polyhistidine residues by increasing the imidazole concentrations to 100 m*M* or higher [33]. If the tagged protein forms oligomers, more stringent conditions such as lower pH or higher concentrations of imidazole might be required to elute the protein. While both of these elution methods are effective, the use of imidazole is often preferable as exposure to low pH may damage the protein of interest [7]. The overall mechanism behind binding and elution of His-tagged protein is illustrated in Fig. 6.2.

A detailed protocol of IMAC involving Ni^{2+}–NTA as the support matrix is given below [34]:

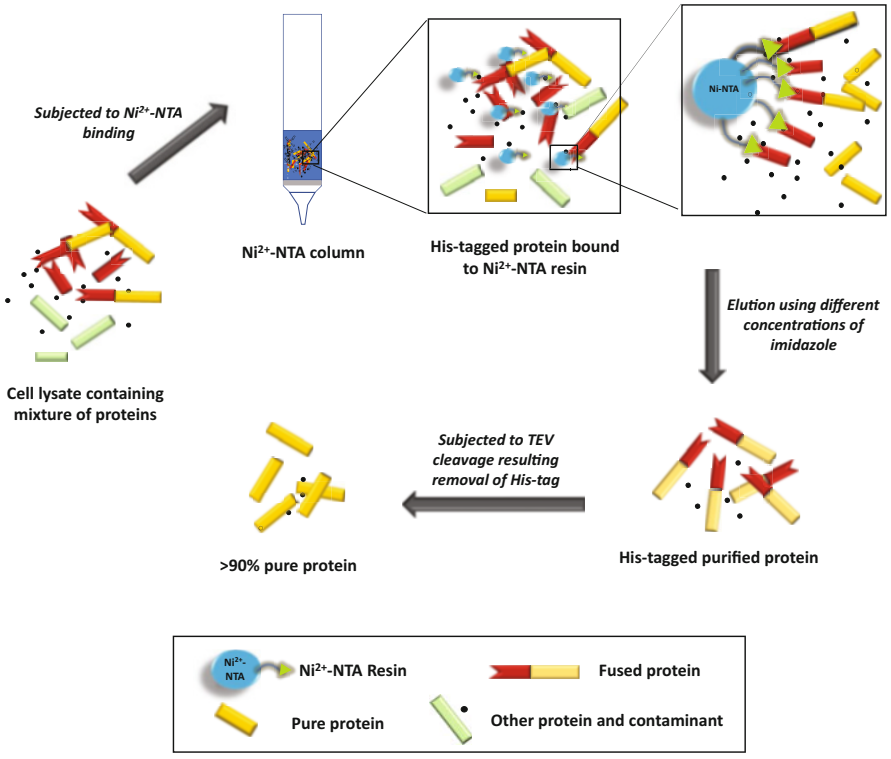

Fig. 6.2 An overall mechanism showing His-tag based affinity purification using Ni^{2+}–NTA. Cell lysate containing mixture of proteins along with the one with His-tag is passed through Ni^{+2}–NTA IMAC column. The fused protein binds to the column and gets eluted subsequently. His-tag removal is carried out using TEV protease, resulting in generation of the pure protein

- Lyse the *E. coli* cells expressing His-tagged recombinant protein suspended in the lysis/loading buffer by sonication on ice. Approximately 3–5 ml of loading buffer should be used per gram (wet weight) of cell pellet. It is important to keep the lysate under cold conditions to avoid any possible proteolysis.
- Centrifuge the cell lysate at 30,000 × *g* for 30 min at 4 °C.
- Add the clear lysate supernatant in the Ni^{2+}–NTA resin pre-equilibrated with an ice-cold loading buffer. Generally, 5–10 mg of protein binds per ml of the resin. Incubate the sample at 4 °C for 1 h on a rocker.
- Load the resin onto a column, followed by washing of the resin with 20 column volumes of a loading buffer containing 10 mM imidazole at 4 °C.
- Elute with 10 column volume gradient of 10–250 mM imidazole prepared in the loading buffer, pH 8.0 and collect the fractions.
- The purified protein is treated with site-specific proteases, such as TEV (Tobacco Etch Virus protease), to cleave the His-tag, followed by overnight dialysis (optional). Usually, most of the proteins are purified with the tag since it is too small and most of the time non-interfering.
- To obtain pure homogenous recombinant protein, the dialysed protein is concentrated using concentrators like Centricon [39], and subjected to SDS-PAGE to check for the purity and expected molecular weight of the recombinant protein.
- If the purification is not >95%, a second round of purification using gel filtration chromatography (discussed in detail in Chap. 8) is performed.

6.3.1.5 Troubleshooting

Problems	Possible reasons	Potential solutions
Purified fraction containing very low yield of the target protein.	Less stringent elution condition might result His-tag to still be bound with the protein.	Buffer condition can be made more stringent by increasing imidazole concentration or decreasing pH.
	Non-specific binding, especially hydrophobic interactions.	Add a non-ionic detergent to the elution buffer (e.g. 0.2% Tween-20).
	Undue protein precipitation in the column or wells.	Reducing protein concentration by eluting with slowly rising imidazole concentration gradient; avoiding steep rise and also decrease the amount of sample in the first place.
Impurity in the eluted protein fraction (SDS-PAGE analysis showing multiple bands).	Contaminants have affinity towards the tagged protein.	During protein expression in the bacterial system, adding detergent and/or reducing agents before sonicating cells might reduce contaminants. Moreover, increasing detergent levels (e.g. up to 2% Tween 20),

(continued)

Problems	Possible reasons	Potential solutions
		or adding glycerol (up to 20%) to the wash might further help in preventing non-specific interactions.
	Insufficient removal of the unbound materials during the washing step.	After application of the sample, repeat the wash step once or twice till highly pure target protein is obtained.
His-tag protein is getting eluted prematurely during loading or washing.	The tag is not adequately exposed.	Usage of denaturing agents such as guanidinium hydrochloride (Gdn-HCL) or urea during purification can be done to verify whether the tag is sufficiently exposed. Alternatively, application of longer polyhistidine tag or addition of linker between the tag and the target protein can significantly increase tag exposure.
	Incubation period is insufficient.	Increasing the incubation time of the crude lysates in the well or decreasing the speed of the centrifugation. Flow rate also must be regulated for efficient his-tag binding to the matrix.
	Imidazole concentration in the loading and/ or binding buffer is above limit.	Imidazole concentration must be lowered. Alternatively, usage of sophisticated affinity matrices such as Ni-Sepharose excel or TALON Superflow might be useful as these matrices do not require imidazole in the loading or binding buffer [40].

6.3.2 Purification of GST-Tagged Protein

GST tag system is a robust mode of affinity purification that involves protein expression and purification at high level in prokaryotic system. GST is a naturally occurring eukaryotic protein of 211 amino acid residues, which exhibits strong affinity towards glutathione [13]. Salient components of GST-tag-based purification are a pGEX plasmid vector, a GST moiety attached at the N- or C-terminus of the protein of interest, and an affinity ligand comprising series of immobilized glutathione beads bound to the chromatographic matrix [12]. One of the striking characteristics that gives GST-based purification an edge over IMAC is the ability of GST to act as a chaperone [41]. Moreover, application of GST enhances protein solubility and avoids its expression in the inclusion bodies [13]. The fusion protein is

then captured by immobilized glutathione and impurities are washed away. Elution of the protein is performed under mild, non-denaturing conditions using reduced glutathione. If desired, the removal of the GST affinity tag is accomplished by using a site-specific protease recognition sequence located between the GST moiety and the target protein [7].

6.3.2.1 pGEX Vectors and Their Gene Fusion Construct

GST-tagged proteins are generally constructed by inserting a gene or gene fragment into the multiple cloning sites of pGEX vectors [10]. There are 13 types of commercially available pGEX vectors each containing a tac promoter that is capable of high-level protein expression, if induced by IPTG (Isopropyl ß-D-1-thiogalactopyranoside) [11]. An internal $laqI^q$ gene facilitates tight control over expression of the gene insert by binding to the tac promoter until IPTG is applied. All these vectors possess cleavage site so that GST can be separated from the protein of interest after purification, using any of the three enzymes, namely, Thrombin, PreScission Protease and Factor Xa [11, 42]. Generally, the pGEX-T series, pGEX-X series and pGEX-P series contain protease cleavage sites for Thrombin, Factor Xa and PreScission protease, respectively, as represented in Fig. 6.3 [11, 42]. A suitable vector is determined on the basis of the future application of the target protein. While selecting the protease cleavage site, it is to be noted that the target protein must not possess an internal recognition sequence for this protease. Out of the three proteases, thrombin is the most cost-effective, as relatively small amounts of thrombin and short incubation times at 37 °C are sufficient to cleave the protein with high efficiency [43]. On the other hand, Factor Xa has very high specificity, but is expensive and generally requires high enzyme-to-substrate ratios for efficient cleavage [11, 43]. PreScission protease, in particular, has several advantageous characteristics, namely, it is effective at low temperature (5 °C) [11].

6.3.2.2 Expression of the Fused Protein

Generally, it is very difficult to predict whether the protein of interest will remain soluble in the cytoplasm or will accumulate in inclusion bodies. With increase in the protein size, structural complexity increases resulting requirement of intricate series of protein folding to avoid accumulation in the inclusion bodies. Though GST's chaperone-like behaviour ensures proper folding of the GST fusion protein, high-scale protein expression also requires maintaining of optimum culture conditions such as appropriate growth temperature and proper aeration [44]. Yields of GST fusion proteins using prokaryotic expression system like *E. coli* are highly variable, often ranging from 10 to 50 mg/l, but could potentially be much lower in cases where the fusion protein is toxic to the cells or unstable [11, 44]. Since expression levels are typically high, adequate amounts of protein usually can be obtained from several litres or less amount of the bacterial culture. The expressed protein can further be analysed using SDS-PAGE analysis. A detailed protocol explaining the cloning and expression of the fused protein is given below [11, 44, 45]:

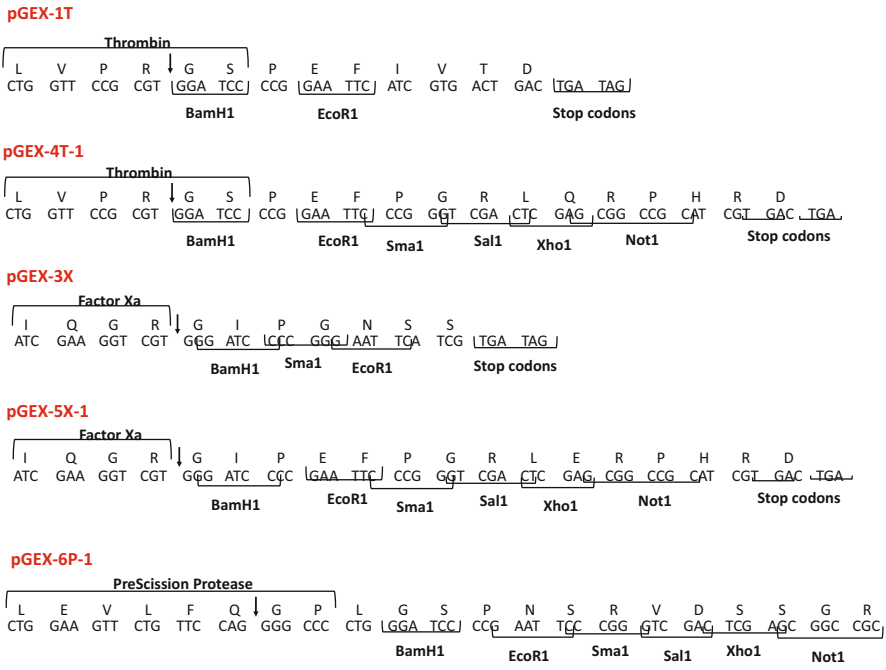

Fig. 6.3 An illustration showing various forms of pGEX vectors along with their proteolytic cleavage sites. T, X and P series of pGEX vectors are shown, which contain enzymatic cleavage sites for proteases such as Thrombin, Factor Xa and PreScission Protease, respectively. The vectors also contain different restriction sites represented by restriction endonucleases including BamH1 (from *Bacillus amyloliquefaciens*), EcoR1 (from *Escherichia coli*), Sma1 (from *Serratia marcescens*), Xho1 (from *Xanthomonas holcicola*), Not1 (from *Nocardia otitidis-caviarum*) and Sal1 (from *Streptomyces albus*)

- PCR amplification of the target gene sequence (DNA sequence of the target protein) containing the restriction sites at the ends of the target gene fragment that are in-frame and complementary to the vector are chosen.
- Digestion of the PCR product using relevant restriction enzymes followed by cleaning using gel electrophoresis.
- Transformation into the *E. coli* system, and growing the transformants on LB agar plates at 37 °C overnight.
- Screening the colonies for verifying that the gene insert is oriented properly and the reading frame is in correct order. Colonies are then transferred to separate tubes consisting PCR beads with 10 picomole each of pGEX primer being added.
- The colonies showing positive PCR are selected and an individual mini-culture of 5 ml is grown for each transformant for further screening of the expression of the protein of interest.
- Glycerol stocks can further be prepared using the exponential phase of the bacterial culture and 70% glycerol for storage purposes. Integrity of the target sequence is analysed using DNA sequencing.

- For expression procedure, some of the glycerol culture containing the isolated colonies are transferred to 100 ml LB (Luria-Bertani broth) with 100 μg/ml ampicillin (antibiotic) added as supplement.
- The inoculated culture is incubated overnight at 37 °C, inside an incubator shaker with 250–300 rpm, that is followed by extraction of 1 ml aliquot of the semi-opaque culture (based on the optical density, OD_{600}) the next morning.
- The obtained culture is termed as the starting culture, which is further diluted to 1:20 ratio using 600 ml fresh LB supplemented with 100 μg/ml ampicillin.
- Incubate the culture at 37 °C at 250–300 rpm until the optical density measured at 600 nm wavelength (OD_{600}) is within the range of 0.5 to 0.7 (log phase) [45].
- Prior to IPTG induction, 1 ml aliquot of the uninduced culture is kept for SDS-page analysis.
- Post-induction, the culture is incubated at 37 °C at 250–300 rpm for an additional 3 h, while monitoring the growth at OD_{600}. At saturation, they will stop dividing.
- Harvest cells by centrifugation at 4000 × *g* for 20 min at 4 °C.
- Carefully decant the supernatant, leaving ~15–50 ml in the centrifugation bottle.
- Resuspend the cells and transfer to a 50 ml centrifuge tube.
- Centrifuge for 20 min at 4000 × *g*, 4 °C.
- Decant the supernatant.
- Analyse un-induced and induced samples by SDS-PAGE to check protein expression levels.

6.3.2.3 Affinity-Based Purification of the GST-Fused Protein

After attaining the desired expression level, the GST fusion protein is subjected to purification from the bacterial cell lysate using immobilized glutathione coupled to a Sepharose column. There are different types of glutathione chromatography resin commercially available for the purification process. One of them is Sepharose 4B resin that is poured into the chromatographic column with an attached peristaltic pump that can control flow rates of the cell lysates [5, 44]. A detailed protocol describing the affinity purification using glutathione Sepharose 4B column is given below [11, 44, 45]:

- Prior to addition of the cell lysate, the glutathione Sepharose 4B column is washed thoroughly with 5–10 bed volumes of phosphate-buffered saline (PBS) for removal of any contaminants such as ethanol solution.
- Pelleted *E. coli* cells are lysed by sonication on ice for 10 s with 1 min interval between the bursts. This must be done at least 10 times with a time-gap between every burst to avoid sample heating. 50 μl of the resultant cell lysate is kept for SDS-PAGE analysis and the rest is subjected to centrifugation at 48,000 × *g* for 20 min at 4 °C.
- Supernatant is decanted into a sterile 50 ml centrifuge tube and kept for further gel analysis.
- 5–10 μl each of both the cell lysate and the decanted supernatant are run on SDS-PAGE gel to assess whether the fusion protein is in the decanted

supernatant. Upon verifying the presence of the fused protein in the supernatant, the fraction is subjected to affinity ligand binding.

- Keeping a flow rate of 0.1 ml/min (flow rate is kept low due to the slow binding kinetics between glutathione and GST), the supernatant fraction is loaded onto the Sepharose 4B column for binding with the glutathione resin. To verify whether the target protein with the GST-tag has bound with the glutathione, SDS-PAGE gel analysis is carried out.
- At this point, the obtained protein should be more than 90% pure. For the removal of the GST affinity tag, the purified fraction is further subjected to enzymatic cleavage through digestion by the serine proteases.

6.3.2.4 Elution and Removal of the GST Tag

On the basis of the vector chosen during cloning, different proteases such as thrombin, factor Xa or PreScission are used for cleavage of the GST affinity tag. These proteases are added in the glutathione buffer for inducing cleavage; however, it is to be ensured that the buffer must not contain any protease inhibitors. Post-cleavage, the target protein is separated from the GST moiety through re-chromatography using the glutathione Sepharose column. The detailed protocol of the elution process is given below [11, 44, 45]:

- Sufficient amount of thrombin or factor Xa or PreScission is added to the purified GST-tagged protein and kept for incubation at appropriate temperature (37 °C for thrombin, 25 °C for factor Xa and up to 5 °C for PreScission proteases).
- Enzymatic cleavage is carried out for a suitable length of time before being subjected to a non-specific protease inhibitor, called Phenylmethylsulphonyl Fluoride (PMSF). 0.3 mM of PMSF is added to terminate the cleavage process.
- The resultant fraction is given for dialysis, at least twice using 2 l of PBS or EDTA (Ethylenediamine Tetra-acetic Acid) for a minimum of 4 h per dialysis.
- The dialysed sample is subjected to centrifugation for 20 min at 4000 × *g*. This step ensures removal of unwanted precipitated materials that might have been developed during dialysis or pre-dialysis stages.
- Re-chromatography of the dialysed sample is done by loading the sample onto the Sepharose column at a flow rate of 0.1 ml/min. This fraction is collected for further gel analysis.
- The column is further washed using 2–3 bed volumes of PBS or EDTA at 1.5 ml/min.
- Finally, the bound GST and un-cleaved fusion protein are eluted using a reduced glutathione buffer at 0.3 ml/min for 5 bed volumes. All these fractions are analysed using SDS-PAGE, and are subsequently pooled to obtain the protein of interest.
- It is to be noted that the sample might contain some residual GST that did not rebind to the Sepharose column during re-chromatography. Moreover, there can be other contaminants too, such as proteolytic fragments, precipitates and aggregates. Hence, for further polishing, ion-exchange chromatography or gel

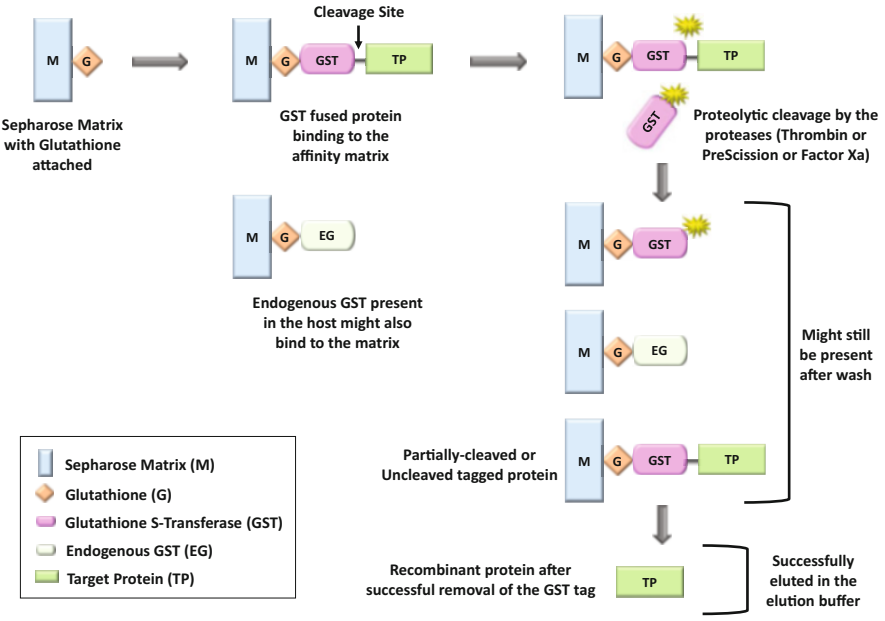

Fig. 6.4 Schematic representation of purification of GST-tagged protein. The target protein fused with GST gets attached to the Glutathione matrix, which is followed by the sequential purification steps as described in the text. The GST tag is subsequently removed by the proteolytic actions of proteases like Thrombin, Factor Xa or Precision Protease

filtration chromatography can be used. These procedures are discussed in detail in the subsequent chapters.

An illustration elucidating the affinity purification of GST-tagged protein is given in Fig. 6.4.

6.3.2.5 Troubleshooting

Problems	Possible reasons	Potential solutions
GST-tag fusion protein poorly binds to the affinity matrix column.	The flow rate used during sample loading is too high.	The binding kinetics between GST and glutathione is very slow. Thus, it is important to keep the flow rate low during sample loading onto the column. This ensures proper binding and increases the binding capacity of the column.
	The tagged protein has already aggregated into the sample during expression.	Prior to sonication of the cells, addition of reducing agents like DTT (Dithiothreitol) may

(continued)

Problems	Possible reasons	Potential solutions
		significantly reduce precipitation of the fusion protein and increase the yield.
	Low concentration of the tagged protein.	The binding kinetics between GST and glutathione Sepharose medium is directly proportional to sample concentration. A highly expressed protein binds more efficiently than the one with low expression. Hence, increasing the concentration of the tagged protein might improve binding.
	The Sepharose column has not been equilibrated properly prior to the fusion protein loading.	Before adding the cell lysates to the Sepharose medium, the column must be equilibrated with 5–10 bed volumes of buffers such as PBS (ensure that the buffer pH is between 6.5 and 8.0).
	The glutathione medium has been used too many times.	Time to time change of the Sepharose medium and use of freshly prepared medium considerably increases the binding efficiency of the affinity matrix column.
GST-tagged protein is not eluted efficiently.	The pH and ionic strength of the elution buffer is low.	Sometimes, increasing the basicity of the elution buffer or adding NaCl salt (0.1–0.3 M) might improve the elution yield of the target protein
	Concentration of glutathione is low.	Normally, the presence of 10 mM glutathione in the elution buffer is sufficient. However, for some tricky proteins, increasing the concentration of glutathione to a range of 20–40 mM might improve elution process.
	Non-specific interactions.	Solubility of the fusion proteins might get affected by non-specific hydrophobic interactions with the affinity medium, resulting aggregation. Addition of detergent molecules such as 0.1% Tween-20 or 2% n-octylglucoside into the elution buffer might prevent non-specific binding
Multiple bands are seen in SDS-PAGE analysis.	Partial degradation of the tagged protein during enzymatic cleavage using thrombin, factor Xa or PreScission.	Adding protease inhibitors like PMSF to the cell lysate solutions might improve elution. AEBSF or 4-(2 aminoethyl)-

(continued)

Problems	Possible reasons	Potential solutions
		benzenesulphonyl fluoride hydrochloride is another alternative to PMSF [46].
	Cell disruption during cell lysis process.	Decreasing lysis duration as over-lysis generally leads to the co-purification of the host cell (*E. coli*) proteins along with the GST-tagged one. Moreover, addition of lysozyme solution before the commencement of mechanical lysis prevents frothing and denaturation of the tagged protein
	Antibodies that react with various *E. coli* proteins may be present in the tagged protein sample.	Sometimes, the commercially available anti-GST might contain antibodies that can interact with *E. coli* proteins, resulting in non-specific background binding. This can be avoided using anti-GST, which has already been cross-adsorbed against all kinds of *E. coli* proteins.
Attached GST-tag is not cleaved properly.	Insufficient enzyme concentration and/or less incubation time might result in partial or no detachment of the GST-tag from the target protein.	Normally increasing the incubation time to 24 h or more facilitates efficient cleavage of the tag. However, higher reaction time might result in target protein degradation, which can be alternatively circumvented by increasing the enzyme concentration of the particular protease (PreScission Protease, thrombin, or Factor Xa) [11, 44, 45].
	During cloning of the fused protein, specific sites for protease cleavage might get altered.	Nucleotide sequence of the generated construct must be checked thoroughly to verify the presence of cleavage sites. Comparative alignment of the resultant sequence with a known sequence might help in identifying the altered site.
	Presence of endogenous protease inhibitors might interfere with the enzymatic action of the proteases.	In case of PreScission Protease, the inhibitors can be removed by dialysing the fused protein against 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT at pH 7.5 [11, 44, 45]. For factor Xa, fused protein can be subjected to buffer exchange on a desalting column, or dialysis

(continued)

Problems	Possible reasons	Potential solutions
		against 50 mM Tris–HCl, 150 mM NaCl, 1 mM $CaCl_2$ at pH 7.5 [11, 44, 45].

6.3.3 Purification of MBP-Tag Recombinant Proteins

MBP, encoded by malE gene, is a part of the maltose/maltodextrin system of *E. coli* that can be expressed in both secreted and non-secreted forms in the prokaryotic system [47]. Usage of MBP enhances solubility of the target protein by facilitating proper folding that further results in high yield [14, 48]. Fostering protein-folding ability of MBP lies in its ability to function as a chaperone magnet that enable it to recruit chaperones at the vicinity of the fused protein [49]. Although MBP exerts a high-metabolic burden on the expression system due to its large size (42 kDa), its exquisite ability to circumvent problems associated with heterologous protein expression overshadows its limitations and makes it a popular affinity tag [50]. It also enables segregation of the fused protein from its heterogeneous forms (resulting due to posttranslational modifications), and expedites its expression in the host system [14, 50]. Generally, in MBP-based affinity purification, a series of pMAL vectors can be used for aiding fusion of the MBP-tag with the target protein. Followed by successful attachment of the MBP-tag, the fused protein is subjected to binding with the amylose resin (affinity chromatographic matrix). Similar to GST, MBP is also a highly efficient fusion system due to its smooth purification methods, mild elution criteria and high compatibility with almost all downstream applications. A detailed methodology depicting the purification of high-quality MBP-fused protein is discussed below.

6.3.3.1 Expression of MBP-Tag Protein Using pMAL Vector

E. coli competent cells are subjected to transformation using pMAL vectors for expression of the MBP-tag protein. These vectors enable the expression of the secreted or cytosolic form of MBP, fused to the target protein, under the regulation of an IPTG-induced *tac* promoter. The use of this promoter allows pMAL vectors to be used in a wide variety of bacterial hosts, since the *tac* promoter utilizes the bacterial RNA polymerase for transcription. A detailed protocol describing the expression process is given below [14, 48]:

- *E. coli* competent cells are transformed with 10 ng of pMAL vector and plated on LB agar plates containing 100 mg/ml ampicillin and incubated for 16 h at 37 °C. Generally, 1 l of the LB medium is prepared by 10 g bacto-tryptone [51], 5 g bacto-yeast [51] extract, 10 g NaCl, 166 mL NaOH (10 N) and 10 ml $MgSO_4$ (1 M).

- A single colony is used to inoculate a tube containing 10 ml LB with 100 mg/ml ampicillin.
- The cells are grown in a shaker incubator for 16 h, before being transferred into 1 L of LB medium where the inoculum to medium ratio is kept at 1:100. The cells are placed in an incubator shaker having temperature 37 °C.
- When the OD_{600} reaches around 0.6, IPTG is added to a final concentration of 0.3 mM.
- The cells are harvested after 6 h of incubation at 30 °C and the pellets are stored at −80 °C if not immediately used for purification.

6.3.3.2 Binding to the Amylose Affinity Column and Purification

The main component of the chromatographic matrix for purification of the MBP-fused protein is amylose-agarose resin. For generation of the matrix, 25 ml of Sepharose 6B is washed with water in a Sinter glass and with 1 M sodium carbonate kept at pH 11 [47]. It is then allowed to react with 5 ml vinyl sulphonic acid for 70 min at room temperature. After washing with 500 ml of water, the resin is resuspended in a 25 ml solution of 2.6 g amylose in 1 M sodium carbonate at pH 11, with continuous stirring overnight. The resultant column is rigorously washed and kept in a solution of 20% ethanol and 80% water at 4 °C for proper maintenance. When the fused protein is passed through the column, MBP binds to amylose primarily via hydrogen bonds [52]. Addition of high ionic salts such as NaCl (1 M) can be used in order to reduce non-specific adsorption of proteins onto the resin [52]. The overall procedure of the purification method is discussed here:

- The frozen cell pellet from a 1 l culture is thawed on ice and resuspended in 70 ml of buffer. The cells are then mechanically lysed using a Micro-fluidizer, where the pressure is kept at 21,000 psi (pounds/inch^2).
- Post-lysis, the lysate is subjected to centrifugation at 4 °C for 20 min (15,000 × *g*), followed by filtration for removal of insoluble cell debris.
- Pre-equilibrated amylose-agarose column is loaded with filtered lysate at 1.7 ml/min and washed with buffer A at 2.5 ml/min up to low optical density (~5 CV, column volume).
- Protein is eluted with elution buffer (Binding buffer comprising 20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA at pH of 7.4 and 10 mM maltose) at 1.5 ml/min, collecting fractions of 9 ml during 4 CV.
- Samples from each fraction are analysed for protein content by SDS–PAGE.

6.3.3.3 Removal of the MBP-Tag Through Proteolytic Cleavage

Presence of maltose in the elution buffer allows the binding of the MBP-fused protein with the maltose and subsequent elution. However, the generated eluate still contains MBP tag fused with the protein of interest, which is required to be removed to obtain pure protein. Post-purification, removal of MBP-tag is done by the application of proteases such as Factor Xa, Thrombin, Enterokinase or TEV proteases [14, 53]. The MBP-containing vectors consist of designated proteolytic cleavage site for these proteases at the junction between the MBP and the target

protein. Several studies have shown that usage of proteases like Factor Xa and Thrombin might result in non-specific digestion of the target protein; however, other proteases such as Enterokinase, Rhinovirus 3C protease and TEV are more specific [53–55]. Among them, TEV protease is the most predominantly used protease for MBP-based purification of the target protein [53]. TEV remains active even at 4 °C, whereas the other proteases usually require higher temperatures and a long period of incubation, which further increase the risk of aggregation and inactivation of the cleaved protein [53]. Another advantage of using this protease is its resistance to detergents that are often essential in the preparation of membrane proteins and other hydrophobic proteins [56]. After successful removal of the tag, the purified protein (70–90%) is further polished using ion-exchange chromatography or gel filtration chromatography that are discussed in detail in the subsequent chapters. The purification process is culminated by the regeneration of the affinity column, where the amylose–agarose resins are regenerated with 0.1% SDS at room temperature, followed by water wash, and maintained in 20% ethanol at 4 °C.

6.3.3.4 Troubleshooting

Problems	Possible reasons	Potential solutions
Inadequate binding of MBP-tagged proteins to the amylose resin.	Presence of endogenous amylases in the bacterial system might competitively inhibit binding to the amylose column.	This can be prevented by the application of 0.2% glucose in the growth medium that would substantially reduce the expression of endogenous amylase [52].
	The presence of non-ionic detergents.	Occasionally, the presence of detergents such as triton X-100 and Tween-20 can interfere with amylose and MBP binding. Concentration of the detergents can be reduced to 0.05% or less in order to solubilize the extract [52, 53]. Moreover, binding can be improved by screening alternative detergents.
	Oligomeric property of the target protein also can affect its binding to the amylose column.	Due to oligomerization, soluble aggregates are formed, which can be detected by gel filtration. The formation of oligomers can be reduced by changing the expression conditions or the purification procedure and by screening different buffers and additives [52].
Protein is not eluted efficiently from the affinity column.	Elution kinetics is very slow.	In case of slow elution kinetics, following changes might improve the elution process [57]:

(continued)

Problems	Possible reasons	Potential solutions
		(a) Decreasing the elution flow rate. (b) Overnight incubation in the elution buffer, when performing batch purification. (c) Increasing the concentration of maltose in the elution buffer using varied concentration of maltose (20–100 mM).
	SDS-PAGE analysis showing multiple bands post-elution.	Non-specific proteolysis can be identified by Western blot analysis. Undue proteolysis or degradation of the target protein can be prevented by conducting all purification steps at 4 °C. Moreover, use of protease inhibitors during the cell lysis process, can also help in reducing the chance of proteolysis [14, 57].
	Contaminants are non-covalently linked to the recombinant protein.	Increase ionic strength in all buffers for cell lysis and purification (up to 1 M NaCl) or add mild detergents, 0.1% Tween, 0.1% CHAPS) [14].
Column has clogged.	Top filter is clogged.	Top filter can be changed in case of clogging. Moreover, amylose column can be replaced if repetitive column regeneration has exhausted the column capacity.
	Cell debris in the lysate may have clogged the column.	Centrifuge and/or filter the sample through a 0.22 μm or a 0.45 μm filter or otherwise optimize sample pre-treatment before loading the next sample [52, 57].

6.3.4 Purification of Strep-Tag II Recombinant Proteins

The Strep-tag II is a short peptide that is capable of high selective binding with StrepTactin [21, 58]. StrepTactin is an engineered streptavidin, which in comparison to streptavidin, possesses 100-fold higher binding affinity ($K_d = 1$ μM) with Strep-tag II [58]. One of the striking advantages of this purification system is that it facilitates one-step purification of a wide range of recombinant proteins under natural physiological conditions, thus preserving its bioactivity [22]. Moreover, unlike GST or MBP tags, the Strep-tag II does not disturb the structural aspect of the protein of interest due to its small size. The Strep-tag II purification system can be implemented in various expression systems, such as bacterial, mammalian and

insect; however, in this section we will mainly focus on its application using the bacterial expression system.

6.3.4.1 Expression of the Strep-Tag II Fused Protein

There are two types of vector systems used for expressing the Strep-tag II fused protein in the bacterial cells such as *E. coli*. One of them is pASK-IBA/pASG-IBA vector system that carries an inducible tetracycline promoter/operator (*tet*-promoter) responsible for fusion of the Strep-tag II at the N- or C-terminus of the recombinant protein [59]. pPR-IBA/pPSG-IBA vector system, on the other hand, utilizes T7 promoter and T7 RNA polymerase for high-level expression of the target protein in BL21 strain of *E. coli* [59]. Generally, *tet* system is preferred to T7 system, as it is independent of *E. coli* strain unlike T7 system, which needs a definite source of the T7 RNA polymerase (present in BL21 strain of *E. coli*) recombinant protein expression [60]. Hence, the following protocol describes the generation of cell lysates for the expression of Strep-tag II fusion proteins using *tet* system [20, 61, 62]:

- LB medium is prepared using 10 g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl. 100 μg/ml of Ampicillin is added to the LB medium. Upon addition, 100 ml of the resultant medium is inoculated with a fresh bacterial colony containing the pASK-IBA expression plasmid and shake overnight (200 rpm) at 37 °C.
- Optical density of the sample is measured at 600 nm (OD_{600}) wavelength. When OD_{600} reaches between 0.5 and 0.6 range, 1 ml of the sample is taken out to use as uninduced control and then subjected to induction. The sample is induced by adding 10 μl stock solution of tetracycline antibiotic derivative such as anhydrotetracycline (prepared by 2 mg/ml of anhydrotetracycline in Dimethylformamid or DMF).
- The induced cell sample is subjected to shaking for 3 h at 200 rpm. However, for some proteins overnight induction might increase its yield (standardization with small amount is preferred prior to large scale purification). Culture is harvested and pellets are prepared by centrifugation at 4500 × *g* for 12–15 min.
- Washing buffer is prepared by 100 mM Tris/HCl (pH 8.0), 150 mM NaCl and 1 mM of EDTA. The pellet generated from the culture is resuspended in 1 ml of chilled washing buffer (at 4 °C).
- 10 μl of the resultant buffer content is given for SDS-PAGE analysis and rest of the residual suspension is subjected to sonication.
- The suspension is then centrifuged at 13000 rpm (microfuge) for 15 min at 4 °C resulting in sedimentation of the insoluble cell components. The clear supernatant is carefully separated from the sediments and collected in a tube.

6.3.4.2 Purification and Elution of the Fused Protein Using StrepTactin Affinity Column

Purification of Strep-tag II fused protein predominantly depends on the efficiency of binding between Strep-tag II and StrepTactin. The StrepTactin Sepharose column is made up of miniscule StrepTactin beads (average 30 μm), having high-resolution separating capacity, which is capable of generating target proteins in highly pure

form [62]. Once the Strep-tag II bound protein binds with the StrepTactin resin, the unbound proteins in the cell lysates are washed away by the wash buffer (100 mM Tris/HCl at pH 8.0, 150 mM NaCl and 1 mM EDTA), prepared to maintain physiological condition [60]. The StrepTactin bound Strep-tag II protein is then eluted by addition of the elution buffer (wash buffer supplemented with 2.5 mM desthiobiotin) [20, 61, 62]. The desthiobiotin is a competitive inhibitor of Strep-tag II, which results in competitive binding at the biotin binding pocket and leads to recovery of the target protein [20, 22]. A complete protocol discussing the purification and elution procedure is given below [20, 61, 62]:

- Using 2 column bed volumes (CVs) of wash buffer, the StrepTactin column is equilibrated. During equilibration, addition of the wash buffer is done by removing the top cap of the column first, followed by the outlet cap. This order is maintained to avoid the column from becoming dry.
- If the storage solution of the column is 20% ethanol, it is recommended that the column is washed with at least 5 CVs of the wash buffer or distilled water. The flow rate during equilibration is set between 50 and 100 cm/h.
- After equilibration, clear supernatant of the cell lysates (cell or protein extract) generated during protein expression is added onto the column. The volume of the cell extract can be in the range of 0.5 to 10 CVs. It is to be noted that higher the concentration of the extract, more is the yield. Generally, the concentration within the range of 50–100 nanomolar is recommended for generating good yield.
- Once the cell extract has completely entered the column, wash the column 5 times with 1 CV of wash buffer. These washings will remove all the unbounded host proteins. Eluate is collected in fractions of 1 CV and each fraction is subjected to SDS-PAGE analysis.
- Elution buffer, containing desthiobiotin [63], is added for 6 times in 0.5 CV. Eluate is collected in fractions of 0.5 CV. 20 µl of each fraction is then given for SDS-PAGE analysis to verify whether the target protein has been successfully recovered. It is to be noted that addition of desthiobiotin results in its binding with StrepTactin followed by release of the target protein.
- Post-elution, the column is needed to be regenerated through removal of the desthiobiotin. The column is cleaned with 3 CVs of distilled water and 3 CVs of 0.5 M NaOH, followed by another wash by 3 CVs of distilled water.
- Subsequently, the column is re-equilibrated using 5 CVs of wash buffer prior to next purification. Alternatively, column re-equilibration can also be done using 15 CVs (large amount) of 1 mM HABA (2-[4′-hydroxy-benzeneazo] benzoic acid) in the wash buffer. When HABA is added in excess, it binds to the biotin by displacing desthiobiotin from the binding pocket. This binding results in colour-change of the medium from yellow to red, indicating that the column regeneration has been successfully accomplished.

An illustration elucidating the affinity purification of recombinant proteins using Strep-tag II is given in Fig. 6.5.

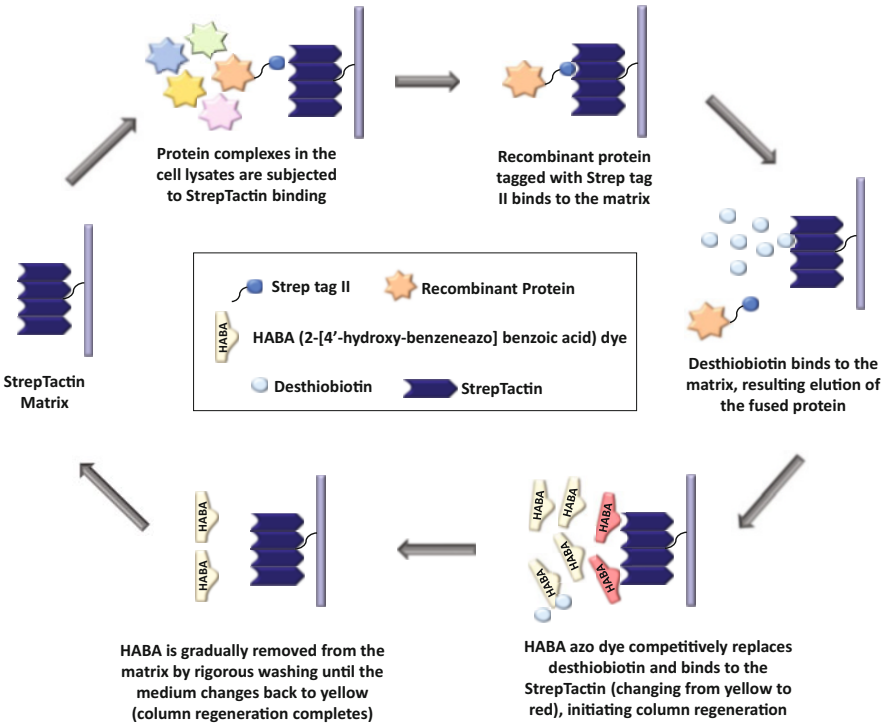

Fig. 6.5 A model delineating the mechanism behind purification of Strep-tag II-fused protein. Cell lysates containing the cocktail of proteins is subjected to binding to StrepTactin column. Target protein containing the Strep-tag II gets attached to the affinity column. Addition of desthiobiotin leads to its competitive binding with the affinity matrix resulting releasing of the fused target protein. The StrepTactin column is regenerated by implementation of HABA dye in excess amount, which displaces desthiobiotin, followed by rigorous washing

6.3.4.3 Troubleshooting

Problems	Possible reasons	Potential solutions
Increased column pressure in the opposite direction.	Unclarified cell lysates and highly viscous solution.	Dilution of the cell extract prior to sonication might reduce the concentration of host nucleic acid. Sometimes, bringing the cell extract at room temperature (from 4 °C) can reduce viscosity.
	Incomplete sonication.	Increasing the sonication time can ensure efficient cell disruption. Moreover, sonication must be conducted in ice as overheating might result in froth formation and denaturation of the target protein.

(continued)

Problems	Possible reasons	Potential solutions
Poor binding with the column matrix.	Strep-tag II is missing or inaccessible.	Proteases that are capable of disrupting the 8-amino acid tag might be present in the *E. coli* strain used for cell lysates. Implementation of proper protease inhibitor can avoid this degradation of the Strep-tag II.
	Biotinylated proteins present in the cell extract might competitively block the ligand.	The host cell proteins might contain biotin in significant concentration that interferes with the StrepTactin binding of Strep-tag II. Addition of avidin (a biotin blocking buffer) facilitates Strep-tag II binding [62].
	Protein has already formed inclusion bodies in the column.	Very high protein concentration leads to precipitation. Decreasing the flow rate and amount of sample load can block protein.
Protein contaminants	Contaminants are generally referred to as the shorter fragments of the fused protein generated during elution.	Uncontrolled proteolysis can be prevented either by using protease-deficient *E. coli* strains or by inducing protease inhibitors post cell lysis [64]. Sometimes, reversing the fusion terminus of Strep-tag II and addition of chelating agents like EDTA can improve protein purity by inhibiting protease activity.
	Formation of covalent and electrostatic interactions between the contaminants and the tagged protein.	Covalent interactions such as disulphide bonds can be disrupted by adding reducing agents such as DTT during cell lysis step. Electrostatic bond formation can be prevented by either increasing the ionic strength up to 1 M NaCl of the wash buffer or by adding weak detergents like 0.1% Tween and 0.1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate).

6.4 Role of Affinity Tags in Identifying Protein-Protein Interactions

Apart from column-based protein purification, affinity tags also play a crucial role in identifying protein-protein interactions [28, 65, 66]. Generally, for validating interactions between two proteins, pull-down assay is one of the most common methods, which involves segregation of a protein complex with the help of

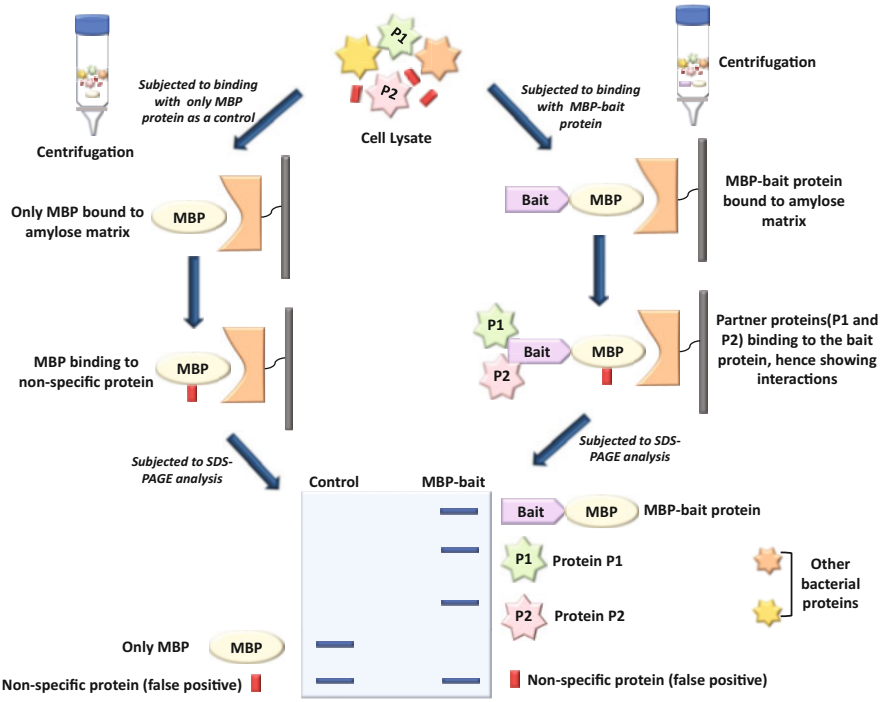

Fig. 6.6 Outline of an MBP-based pull-down assay. The procedure on the left shows the control experiment. In this example, two proteins (P1 and P2) are identified by SDS-PAGE as interacting partners with the bait protein (right lane) bound to the MBP tag. One additional non-specific protein (red) was pulled down as a false positive (MBP binder) by the control (left lane)

immobilized beads [67]. The beads are made of specific ligand resins that bind to the affinity tags such as histidine, GST or MBP attached to the either extremities of the protein complexes [67, 68]. Besides, identifying the interaction between two binding partners, affinity pull-down assays are also used for isolation of low amount (in μg) of complexes, mainly to recognize discrete domains or subunits [52, 67].

One such affinity pull-down assay is MBP pull-down assay that involves affinity purification of one or several unknown proteins from a biological sample using an MBP-tagged bait protein. The MBP-tagged bait protein is attached to the immobilized amylose matrix, which acts as the affinity ligand. The basic principle is that the MBP-tagged bait protein binds to its interacting partner/s forming a complex that gets captured by the affinity matrix. A control is also included to identify non-specific false positive bindings where only MBP is kept without the bait protein. The control can either be lysate from separately transformed cells that express MBP (not the bait fusion protein) or lysate from non-transformed cells to which MBP is added. An illustration explaining the overall process of MBP-based affinity pull-down assay is given in Fig. 6.6. Similar principle can also be used for pull-down using other tags like GST, histidine or Strep-tag II.

6.5 Conclusion

In this chapter, we have discussed about affinity tags where their reversible interactions with the target protein are utilized in the purification process. Though affinity-based chromatography is one of the most sophisticated chromatographic techniques, it has its own limitations. As it relies on the specific interactions of the affinity ligand with the protein of interest, preparation of these ligands sometimes becomes too expensive. The shelf-life of these ligands and the support matrix further adds to the problem, as they require frequent regeneration of the column and regular monitoring. Moreover, choice of tags, media selection, maintaining optimum flow rate and preparation of precise washing and elution buffer require extensive analytical skills of the users for successful execution. Troubleshooting is a key aspect of the affinity chromatographic technique and this chapter has vividly explained how in different scenarios these problems can be dealt with.

Nevertheless, it is a powerful technique that can be utilized at its highest potential with prior knowledge about the structure and function of the target protein. Although affinity chromatography alone is sufficient for purification of a wide variety of recombinant proteins, sometime it might require combination of two or more techniques for purification of few specific proteins to attain highest level of purity for subsequent biophysical or structural studies. In the forthcoming chapters, we will further discuss other chromatographic techniques and purification of proteins which are difficult to purify using so-called conventional methods.

Acknowledgements The authors thank Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) for providing necessary infrastructure and resources for successful completion of the chapter. The authors acknowledge Ms. Chanda Baisane, Bose Lab, ACTREC for formatting the manuscript.

Problems

Multiple choice questions

1. The specific biological interaction that is not used in affinity chromatography purification is:
   (a) Receptor-ligand
   (b) Antigen-antibody
   (c) Cations-anions
   (d) Enzyme-substrate
2. The first step of affinity chromatography purification process is:
   (a) Addition of affinity ligand into the matrix
   (b) Precipitation
   (c) Elution
   (d) Binding of the ligand with the tag

(continued)

3. The property of an ideal affinity chromatography matrix is:
   (a) The matrix materials should be polymeric and organic
   (b) The matrix should be based on inorganic compounds
   (c) The matrix should be mechanically stable and exhibit good flow property
   (d) The matrix should form reversible but specific interaction with the affinity tag

Subjective questions

1. A 50 kDa $His_{6\times}$-tagged protein was adequately expressed in *E. coli* BL21 (DE3) host strain and subsequently purified using Ni-NTA column. Buffer conditions were as follows:
   Binding buffer: 50 mM NaH_2PO_4, 150 mM NaCl and 10 mM Imidazole, pH 7.4
   Elution buffer: 50 mM NaH_2PO_4, 150 mM NaCl and 100 mM imidazole, pH 7.4
   On purification, the protein co-elutes with chaperones and non-specific bands. In addition, white precipitates were observed in the eluted fractions. List a few strategies that can be employed to obtain better yield and purity of target protein.
2. To assess the interactions between protein A and protein B, an MBP pull-down assay was performed. Protein A (22 kDa) was tagged with MBP and considered the bait protein, whereas protein B (48 kDa) was kept untagged. The proteins were incubated in following buffer conditions:
   Binding buffer: 20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4
   Elution buffer: 10 mM Maltose, 20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4
   Post-elution, the pull-down eluates were subjected to SDS-PAGE analysis keeping only MBP as the control. The resultant gel analysis showed an unexpected band at 70 kDa which neither corresponds to protein A nor B. State a reason that can explain the band and also provide a strategy that can be implemented to avoid such bands.

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