Review

Strategies for Tag Design and Removal in the Expression and Purification of Recombinant Proteins

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Abstract: Recombinant proteins find extensive applications in the biomedical and industrial fields, and efficient protein purification is often critical for achieving their functional value. Adding specific tags to the target proteins significantly enhances expression and purification efficiency and reduces time and costs. Tags can be classified into interfering and non-interfering tags, based on their effect on protein function during purification. However, interfering tags may need to be removed after purification to prevent interference with the protein's function in downstream applications, presenting challenges for the design and utilization of tagged fusion proteins. In this article, we discuss the recent advancements in solubility tags and controllable aggregation tags, which have emerged as powerful tools to improve purification efficiency and address these challenges. We further outline strategies for optimal tag design and on-demand cleavage, and emphasize emerging trends, technical features, and forthcoming challenges that are shaping the future of tagged fusion protein production.

Keywords: recombinant proteins; protein purification; solubility tags; aggregating tags; tag removal; proteases; self-cleavage tags

1. Introduction

In recent years, the demand for recombinant proteins has been steadily increasing due to their broad applications in food [1], medical [2], and biotechnology research [3]. With the market revenue of recombinant proteins projected to exceed $460 billion in the 2020s [4], there is a growing emphasis on developing methods to achieve high-level production of purified proteins.

However, challenges in producing recombinant proteins include protein insolubility, improper folding, protein instability, and host cell toxicity [5]. Fortunately, several methods have been proposed to address this problem, including the use of various mutated host strains [6], assisting folding techniques [7], the addition of functional tags [8,9], and the optimization of cultivation conditions [10]. Among these approaches, the incorporation of solubility tags stands out as a widely adopted and effective strategy for improving protein solubility and overall yields.

On the other hand, solubility is not a prerequisite for obtaining properly folded and active proteins, as high-activity inclusion bodies (IBs) have been identified as a viable source [11]. In addition, the aggregation of proteins into IBs is a kinetically controlled process. Therefore, an essential shift in perception is that IBs are not unwanted byproducts of proteins, but rather functional materials with increasing applications [12]. In line with this perspective, active aggregation peptides have been employed as fusion protein partners. Studies have shown that they can induce the formation of active inclusion bodies in *E. coli*, which contrasts with the traditional definition of inactive waste products [13]. This approach offers enhanced stability, simplified purification, and convenient immobilization of recombinant proteins [14].

Still, although tag technology offers notable benefits for recombinant proteins, their addition may influence protein production and downstream applications [15]. To address this issue, incorporating protease recognition sites into tags allows for their precise removal, resulting in proteins that more closely resemble their native state [16]. However, this process also increases the complexity and cost of production.

Considering that many outstanding reviews have thoroughly discussed this topic from various perspectives [4,17], this article focuses specifically on solubility-enhancing tags and controllable aggregation tags, examining key factors in their design and evaluating various tag removal strategies. This review serves as a reference for researchers in the design and application of tags, facilitating the optimization of protein purification workflows.

2. Solubility Enhancing Tags

Several widely used and newly developed solubility-enhancing fusion tags are summarized in Tables 1 and 2, providing an overview of current strategies to improve protein solubility. The following sections highlight notable examples of these tags and their applications.

2.1. GST

In 1988, various foreign peptides used novel pGEX vectors that include GST sequences to express soluble proteins [18]. Because GST-fused proteins bind specifically to glutathione resin, this affinity facilitates their efficient purification, making GST one of the most popular and multifunctional tags. Optimizing fermentation conditions–such as lowering temperature, extending induction time, and adjusting the IPTG concentration–can further improve the solubility of GST-tagged proteins [19–21].

In recent years, innovative analytical approaches have expanded the utility of GST fusion proteins, such as real-time characterization via a nanosensor [22] and a plug-and-play chemical proteomic approach [23]. Notably, the novel design of a heterodimeric sjGST, a stable mutant consisting of sjGST (+) and sjGST (−), simplifies the analysis of protein-protein interactions by allowing the fusion of two different target proteins in each variant [24]. Similarly, His-GST and GST-biotin fusion proteins, when expressed in the wheat germ cell-free system, enable the production of well-folded proteins and facilitate protein-protein interaction studies [25]. Another notable GST mutant GST^{4QN}, designed by site-directed mutagenesis, can be expressed in various mammalian cells (e.g., HeLa, 293FT, DU145, and H1299), which promotes gene expression or cancer research [26]. This variant can eliminate the influence of protein modification, thereby improving the reliability of its pull-down assay. Therefore, based on the progress of high-throughput technology and genetic engineering skills, a wide range of proteins effectively maintain or enhance their activity through GST tagging, including peptides (e.g., MzDef [27]) and enzymes (e.g., ASP [10], MET [19]).

2.2. MBP

Maltose-binding protein (MBP), separated from *E. coli*, functions as a receptor and participates in the active transport and maltose chemotaxis [28]. Decades ago, the expressed production of the pMAL vector with an MBP gene capitalized MBP's specific binding to amylose, allowing for efficient purification of MBP-tagged proteins. MBP tags could also assist in the proper folding of proteins that contain disulfide bonds [29]. Furthermore, the solubility enhancement within MBP fusion proteins is due to the formation of folding intermediates [30]. Glycerol helps stabilize fusions' structure and further prevents the advent of aggregations by reducing proteins' hydrophobic interaction [31].

However, MBP has its limitation in effectively binding to amylose resin in some cases [32]. To address this limitation, researchers have developed dual-tag constructs that combine MBP with supplementary affinity tags, such as His6-MBP [33] and HE-MBP [34]. Furthermore, a 14-residue tag, called the mm tag, was designed by identifying the MBP epitope, contributing to broader applications in purification and protein detection [35]. A further improvement involves a modified version of the bacterial maltose-binding protein (mMBP), designed through codon optimization and the introduction of specific mutations, which can efficiently express complex eukaryotic proteins with post-translational modifications at high levels (15 mg/L in HEK293T cells). This modification enhances protein solubility and facilitates fusion crystallization, making it a valuable tool for producing hard-to-express proteins [36].

There are some challenges including some proteins still cannot be expressed with high solubility and the variability in solubility cannot be explained [29,33]. Therefore, it is strongly recommended to perform biological assays at an early stage to ensure that the target proteins retain their native conformation, rather than relying solely on solubility measurements.

2.3. SUMO

Small ubiquitin-related modifier(SUMO) tags, which are similar to GST and MBP tags, can overcome the barriers during protein production, including solubility, activity, degradation, stability, and toxicity to the host

[37,38]. A unique advantage over other tags is its cleavage specificity, enabling precise removal of the tag and recovery of near-native proteins [39]. However, the difference is that the SUMO tag typically binds to affinity tags (e.g., StrepII-tag [40]) for purification.

Attributing to appropriate protein engineering SUMO is now commonly applied to produce antimicrobial peptides (AMPs) that may affect the viability of the host cell [37,41]. Linker sequences between the SUMO tag and target proteins enable complete cleavage by Ulp1 at a fast speed due to steric hindrance elimination. However, whether the linker remains on the AMPs is still a concern. In addition to the *E. coli* expression system, an R64T R71E double mutant SUMO, termed SUMOstar, was developed to address the challenge of pre-cleavage induced by endogenous desumoylases in eukaryotic systems (e.g., mammalian cells [42] and baculovirus-insect cells [43]), thereby enabling higher expression levels. However, the expression level of recombinant proteins failed to increase as expected by the codon optimization strategy, likely due to the reduced stability of the transcribed mRNA and inefficient formation of the translational initiation complex.

Several researchers have emphasized developing genetic engineering, whereas the freeze-thawing method contributes to the reconstitution of soluble fusion proteins [44]. Additionally, further research analyzed and optimized media conditions such as pH and concentration of $Mgcl_2$ by response surface methodology [45].

2.4. NusA

N utilization substance protein A (NusA) is applicable for different regions and can produce various soluble proteins when overexpressed, such as IsPETase [46], astaxanthin [47], and human interleukin-3 (HIL-3) [48].

However, the effectiveness of NusA, like other tags, depends on the target protein's inherent properties. Compared to Trx fusion proteins, NusA avoids the formation of inclusion bodies in interferon production and simplifies the expression process, while the protein activity is somewhat lower [49]. However, when expressing recombinant lysyl oxidase, although the solubility of NusA fusions is lower than that of Trx-tagged proteins, NusA exhibits higher enzymatic activity, allowing for higher expression levels of active enzymes [50]. Furthermore, the NusA tag is one of the best choices for expressing HIL-3 [48], while the SUMO tag is more suitable for expressing HIL-7 [51]. These examples highlight the importance of selecting tags based on the specific requirements of each target protein.

2.5. Trx

As a small-sized solubility tag, Thioredoxin (Trx) can directly enhance expression levels, protect proteins from misfolding, and produce soluble proteins without affecting their function [52]. However, for larger proteins, Trx may not sufficiently prevent inclusion body formation, prompting the consideration of larger fusion partners such as MBP or NusA [53].

Notably, the thermal stability of Trx allows for increasing Trx fusions' purity. Trx fusion proteins retain their secondary structure integrity while many contaminants are denatured and precipitated within a heat treatment procedure ranging from 50 °C to 90 °C. This approach, therefore, allows for more convenient purification, especially for various membrane proteins [54].

2.6. GB1

The immunoglobulin binding domain B1 of Streptococcal protein G, known as GB1, contains only 56 amino acid residues [55,56]. Compared to GST and MBP, the GB1 tag circumvents the size limitation that obstructs the study of fusion proteins in NMR [56]. As a well-folded and structurally stable model system, the GB1 tag is also applicable for determining protein oligomeric state, structure, and dynamics due to its well-folded and intrinsically stable structure [7,57,58]. Notably, three structural elements of the GB1 β-hairpin, including the turn region, the hydrophobic core, and the tails, significantly affect the balance between folded and unfolded states [59].

Additionally, it has been demonstrated that GB1 can act as an epitope tag [60] and a molecular imaging probe [55] due to its affinity to the Fc domain. Moreover, it has been used in genetic algorithms to optimize the recognition specificity of mimetic antibodies, illustrating GB1's adaptability for diverse biotechnological applications [61].

2.7. TrpLE

It is challenging to obtain soluble proteins fused with a solubility tag, for example, Trx fusions accompanied by optimized expression conditions simply significantly increased the production of IBs rather than solubility [53].

Under such circumstances, an alternative strategy involves intentionally expressing TrpLE-tagged proteins as IBs, then solubilizing them using denaturants and refolding to obtain functional proteins [62].

TrpLE, as a fusion partner, is recognized to assist the expression of membrane proteins, such as G-protein coupled cannabinoid receptors [63] and viral membrane proteins from SARS-CoV-2 [64]. The cleavage of fusion proteins can be accomplished by CNBr [7], TEV [63], and nickel (Ni^{2+}) [64], Factor Xa [65], each exhibiting specific advantages. However, the harsh conditions required for chemical cleavage may lead to undesirable modifications and functional changes in the target protein, complicating downstream applications [62]. Notably, the cleavage catalyzed by Ni^{2+} circumvents the usage of toxic reagents, thereby providing another efficient way for the general characterization of small transmembrane proteins [64].

Table 1. A set of common solubility tags and their characteristic properties.

Tag	Protein	Size (aa [a])	MW [b] (KD_a)	Cleavage	Advantage	Disadvantage	Ref.
GST	Glutathione-S-transferase	211	27	TEV, HRV 3C	Specific binding to the matrix, Wide applicability	Fluctuations in solubility enhancement, Production loss after tag cleavage, Potential interference with protein activity	[19,25]
MBP	Maltose-binding protein	396	40	TEV	Stabilize proteins, Wide applicability	Fluctuations in solubility enhancement, Limited binding effectiveness to amylose resin	[66]
SUMO	Small ubiquitin-related modifier	100	11	Ulp1	Cleavage specificity, High expression levels	Induces steric hindrance, incomplete protection of AMPs production	[41,37]
NusA	N utilization substance protein A	495	55	EK	Minimal effect on protein structure	Fluctuations in solubility and yield enhancement, Loss production after tag cleavage	[67]
Trx	Thioredoxin	109	12	EK, Thr	Thermostability, Minimal effect on protein structure, Increased production purity	Fluctuations in solubility and yield, Unsuitable for large proteins, Production loss after tag cleavage	[54,68]
GB1	immunoglobulin binding domain B1 of protein G	56	8.4	TEV, Thr	Minimal effect on protein structure, Intrinsically stable	NA [c]	[69,70]
TrpLE	Tryptophan Leader Sequence	106	14	CNBr, Ni^{2+}, TEV, Factor Xa	Suitable for difficult-to-express proteins	Lacking high-throughput compatibility	[62]

[a] aa: amino acids; [b] MW: molecular weight; [c] NA: not available.

2.8. Novel Solubility Tags

In the following section, we introduce several novel tags that have been employed to achieve high solubility, including 6k, MsyB, NEXT, NT*, NT11, PCDS, SEP, SPY, and XAA. Each of these tags provides structural features to improve protein solubility and facilitate successful expression.

Mysb is a small, acidic protein derived from *E. coli* that improves the solubility of fusion proteins. Its hyper-acidic nature promotes correct protein folding, providing a distinct advantage over less acidic solubility tags [71,72]. Similarly to its size, the 11-peptide sequence (NT11) derived from the N-terminal region of carbonic anhydrase [73] and the Protocatechuate 3,4-Dioxygenase Solubility tag (PCDS tag) with 15-residue peptides [74] –show promising abilities to enhance both solubility and expression levels.

Another innovative option, the NEXT tag, is an N-terminal extension sequence of α-type CA. This intrinsically disordered peptide enhances protein solubility without altering the passenger protein's inherent

properties [75,76], it has little effect on the inherent properties of passenger proteins. Notably, a linker sequence with a shorter length between protein and tag leads to lower production.

Based on the high solubility of silk proteins, a mutant of the spidroin N-terminal domain (NT*) can significantly enhance protein solubility [77], and help express TEV proteases with stability and solubility even under harsh conditions [78]. Similarly, lysine- and arginine-based SEP tags have been shown to prevent aggregation and support the expression of various difficult targets, including enzymes [79], antibody fragments [80], and transmembrane proteins [81], in solution. Notably, increasing the charge on spy surface allows for higher anti-aggregating activity [82]. Furthermore, the Spy fusion partner (Spy tag) and its Tandem fusion (Spy-Spy) have also been developed to increase the solubility of both small and large proteins [83]. Moreover, the Spy Tag/Spy Catcher system is also noteworthy for its ability to bind proteins, improving specificity in whole-cell biosensors [84], and offering an effective tool for designing modular vaccines [85].

Additionally, a retro-protein (XAA) [86], as a novel solubility fusion tag, is superior to other solubility tags commonly employed, such as GST, Trx, and NusA, due to their suitable size. This advantage arises from its balanced size and the electrostatic interactions that resemble the mechanism of the 6k tag, where strategic charge interactions reduce aggregation [87].

Table 2. A list of some novel tags and their featured characteristics.

Tag	Protein	Size (aa [a])	MW [b] (KD_a)	Cleavage	Advantage	Disadvantage	Ref.
6k	hexa-lysine tag	6	0.79	NA [c]	Enhance solubility, activity, and protein yield	NA [c]	[88]
MsyB	an acidic protein from *E. coli*	124	14	TEV, Ulp1	Assist in proper folding, high solubility, and increased yield	Limited widespread applicability	[71,72]
NEXT	N-terminal extension sequence of α-type CA	53	5.5	No required	Minimal effect on protein structure, Wide applicability	NA	[76,89]
NT11	N-terminal 11 residues of a carbonic anhydrase	11	1.38	No required	Thermostability, Minimal effect on protein structure	Limited widespread applicability	[73]
NT*	spidroin N-terminal domain	130	17	TEV, HRV 3C	Stabilize proteins, Insensitivity to PH	Instability of solubility after tag removal	[77,78]
PCDS	Protocatechuate 3,4-Dioxygenase Solubility tag	15	1.44	No required	Minimal effect on protein structure	Limited compatibility with alkaline proteins	[74]
SEP	solubility enhancing peptide	12	1.60	No required	Thermostability, Minimal effect on protein structure, Wide applicability	NA	[79]
SPY	Spheroplast Protein Y	138	17	TEV	Minimal effect on protein structure, Retention of solubility after tag cleavage	Limited effectiveness of solubility enhancement in certain cases	[83]
XAA	The reversed sequence of antifreeze protein	192	25	TEV	Thermostability, Retention of solubility after tag cleavage	Impurity contamination during high temperature expression in certain cases	[86]

[a] aa: amino acids; [b] MW: molecular weight; [c] NA: not available.

3. Controllable Aggregation Tags

As represented in Table 3, there are some controllable tags exploit different environmental triggers, such as thermo-responsive (ELPs), pH-responsive like CspB or TIP60, salt-inducible peptides (CpA), and magnetoreceptor protein (MagR).

3.1. ELPs

Elastin-like proteins (ELPs) are a class of genetically encoded biopolymers characterized by a repeating pentapeptide sequence Val-Pro-Gly-Xaa-Gly (VPGXG), where the guest residue Xaa represents any naturally occurring amino acid other than proline [90]. In tobacco plants, high-level accumulation of ELP fusion proteins is achieved following stable transformation, while preserving the activity of antimicrobial peptides [91]. ELPs exhibit reversible, thermo-responsive behavior, which enables their purification under mild, biocompatible conditions, including at near body temperature (37 °C) [92].

Specifically, below their transition temperature (Tt), ELPs remain soluble in water, whereas polypeptide aggregation occurs when the temperature rises above Tt [93]. Accordingly, a non-chromatographic purification method called the inverse transition cycle (ITC) is employed to isolate proteins, which is inexpensive and efficient [94]. However, higher purities achieved through more cycles come at the cost of lower product yields [95]. Consequently, a polar organic solvent extraction and precipitation strategy was put forward for producing highly pure material, as a rapid purification workflow [96].

3.2. CspB

Cell surface protein B (CspB), also known as PS2, has been identified as a major secreted protein in several *Corynebacterium glutamicum* strains, where it forms the S-layer [97]. Like TIP60, CspB is pH-responsive, reversibly precipitating and dissolving under changing pH conditions [98,99]. CspB offers a significant advantage over TIP60 in terms of convenience. Under acidic conditions (e.g., pH < 5), impurities and proteases aggregate, while target proteins remain soluble. This aggregation of impurities simplifies and enhances the process of non-chromatographic purification.

Repeated acid precipitation and redissolution in arginine solutions overcome the challenge of co-precipitating impurities, which hinder the use of purified proteins in spectroscopic methods due to their strong absorption and fluorescence emission [100]. By adjusting the concentrations of the salts, including NaCl and Na_2So_4, CspB can be further employed for purifying acid-sensitive proteins [101]. However, this process may take time and effort.

3.3. CpA

CpA is a short peptide composed of two identical GCN4 sequence elements separated by a two-residue alanine insertion [102]. CpA remains soluble or aggregates depending on the salt concentration. Leveraging these properties, a novel protein purification scheme utilizes low salt concentrations to keep the fusion protein soluble, while eliminating insoluble cellular components by centrifugation. High salt concentrations then trigger CpA-mediated self-aggregation of the fusion proteins, effectively removing soluble background host cell proteins [103].

This salt-inducible cSAT (iSAT) scheme is a simple and effective aggregating strategy that eliminates the need for tag cleavage, offering advantages such as cost-effectiveness, high expression yield, and high purity. Though this salt-inducible cSAT (iSAT) scheme simplified the purification process, its efficiency is much lower compared to the SAT approach, due to different pI values.

3.4. MagR

Magnetoreceptor (MagR), a magnetic protein, can serve as a fusion partner to immobilize proteins on magnetic surfaces with efficiency, little impact on target proteins, and strong environmental adaptability [104]. Furthermore, it is easy to manipulate and environmentally friendly. However, it is noteworthy that the adsorption capacity is relatively low, limiting MagR's ability to immobilize larger proteins effectively [105].

Table 3. A set of novel adjustable aggregation tags and their characteristic properties.

Tag	Protein	Induced Factor	Cleavage	Characteristic	Ref.
ELP	Elastin-like protein	temperature	EK	Wide applicability; Time-consuming	[95]
CspB/PS2	Cell surface protein B	pH	TEV	Wide applicability, Acute responsivity; Not suitable acidic unstable proteins	[106]
TIP60	artificial protein nanocage	pH	NA [a]	Easy to purify, Cost-effective	[99]
CpA	short peptide sequence	salt concentrations	Self-cleavage	Easy to purify, Cost-effective	[103]
MagR	Magnetoreceptor	magnetism	Thr	Environmentally friendly, Minimal impact on protein structure; Not suitable for large proteins	[105]

[a] NA: not available.

4. Classification and Design of Tags

4.1. Interfering and Non-Interfering Tags

Based on their influence on downstream applications, tags can be classified as interfering and non-interfering sequences [4]. This classification plays a critical role in determining which tags should be removed to ensure optimal performance in subsequent analyses. Interfering tags, such as GST [19], MBP [107], SUMO [108], and His-tag [16], must be removed because they may have a series of negative influences on proteins of interest [16,109,110] and their therapeutic applications [111].

Non-interfering tags, such as poly-Arg-Tag, FLAG-Tag, poly-His-Tag, c-Myc-Tag, S-Tag, Strep II-Tag, CBP-Tag, and thioredoxin, do not need to be removed in certain cases [4,112,113]. Additionally, several tandem affinity purification tags (e.g., FLAG–Strep, FLAG–2xHA, His–FLAG) also fall under this category, further expanding the toolbox of non-interfering solutions.

However, the classification is not absolute due to the versatility of applications. For example, although the MBP fusion protein cannot be used in the medical field due to the immunogenicity of the MBP tag, this tag does not hinder the biological activity of mLIF, which is applicable for fundamental research regarding stem cells [114]. His-tags, owing to their relatively small size, are not considered to significantly interfere with target proteins' activities and their protease cleavage may be ineffective [115,116]. However, it should be noted that though the functional activity and dynamics of the His-tag protein remain unchanged after phosphorylation, these modifications can lead to the misinterpretation of the data regarding physiological protein phosphorylation [117]. And misfolded proteins carrying a FLAG tag may become sulfated, resulting in reduced detection [118]. Thus, several considerations must be taken into account when analyzing post-translational modifications using mass spectrometry.

4.2. Influential Factors in Tag Design

The appropriate tag strategy for a particular application is crucial to the success of any tag-based protein expression or purification approach [4]. In this context, we outline the key factors that should be considered during tag design.

Attention must be given to several design aspects of these tags, including the choice of tag composition, N- or C-terminal fusions, and optimized techniques employed (e.g., individually or in tandem) that facilitate the purification of the target protein [17,119]. Other important considerations include the characteristics of the target protein itself (such as stability and hydrophobicity), the expression system, and the application or development stage of the purified protein [120]. Cost and scalability issues related to chromatographic supports also influence tag selection [121]. Moreover, attention must be given to the selection of tag removal strategies because of the loss of solubility after fusion digestion [122]. Taken together, balancing these factors ensures that the chosen tagging approach optimally supports both the protein's intended function and the downstream processing requirements.

5. Tag Removal Methods and Techniques

Given the detrimental impacts of interfering tags, their removal is necessary. Tag removal methods belong to overall strategies for tag cleavage, including chemical methods, enzymatic cleavage methods, and self-cleavage tags. Additionally, cleavage techniques provide detailed operational steps to effectively execute these methods, including immobilized protease techniques and co-expression approaches. Table 4 summarizes the key characteristics of various cleavage methods, enabling a comparative assessment that can inform the choice of the most suitable approach.

5.1. Chemical Cleavage Methods

Reagents such as CNBr [7], Formic Acid [123], Hydroxylamine [124], and BNPS-Skatole [125] are employed for removing affinity tags, particularly in the preparation of small proteins [126], and membrane proteins [127]. In some cases, compared to enzymatic methods, chemical cleavage offers advantages, including cost-efficiency and high cleavage efficiency [125]. However, drastic reaction conditions may lead to alterations in protein properties due to modifications of the side chain [128]. In addition, the toxicity of these reagents demands careful handling and safety measures [64]. Balancing these advantages and drawbacks is essential when selecting chemical cleavage for sensitive or large-scale applications.

5.2. Enzymatic Cleavage Methods

Traditional serine proteases, such as factor Xa [125], thrombin [105], and enterokinase [91], have long been used to cleave fusion proteins. However, proteases may lose their activity of cleavage due to their sensitivity to low concentrations of detergents [111,125]. Furthermore, unwanted cleavage of the protein at nonspecific sites (e.g., factor Xa) can result in protein degradation and reduced yields.

To overcome these limitations, more stable, specific, and easily produced proteases have been adopted, including Tobacco Etch Virus (TEV), Human Rhinovirus 3C Protease (HRV 3C) [129], and SUMO protease (Ulp1) [39] The drawback of endoprotease-mediated tag cleavage, however, is the increased cost due to the high requirement for enzymes, extended incubation time to achieve complete removal, and additional steps to isolate enzymes [130,131].

TEV protease remains stable and active over a range of pH, ionic strengths, and temperatures [132]. With high specificity, TEV protease has solved the problem of nonspecific cleavage, whereas processing efficiency varies unpredictably with different fusion proteins [133]. Recent advancements, such as the development of TEV protease variants with improved catalytic efficiency, solubility, stability, and specificity [134,135]. Furthermore, the YESS 2.0 platform provides methods to produce TEV Protease Variants by optimizing gene transcription and improving reaction efficiency [136]. With the increasing number of TEV mutants, a fluorescence dequenching assay and protein graph convolutional network have been developed to identify or estimate their function [137,138].

Ulp1, which is responsible for cleaving SUMO fusions, has been extensively studied [39,139]. It can avoid the presence of residual amino acids after cleavage because it recognizes the tertiary structure of SUMO specifically. Compared to AcTEV protease, Ulp1 often exhibits higher catalytic efficiency, and it supports various purification formats, including on-column techniques [140] and an alternative novel strategy as follows [141]. Fusion proteins and Ulp1 are separately displayed on the surface of Escherichia coli cells, which requires only centrifugation to obtain purified proteins.

The human rhinovirus (HRV 3C) protease is highly active and specific even at 4 °C, making it the preferred protease for processing temperature-sensitive recombinant proteins [129,142]. The specificity of tag removal is attributed to the precise interaction between the P1 (P1′) position and the S1 (S1′) pocket of proteases [143]. Engineered HRV 3C variants with reconstructed S1′ pockets exhibit enhanced specificity [144]. Furthermore, kosmotropic anions (such as sulfate) contribute to catalytic activity enhancement because of hydrogen bond formation [145].

Similarly, the enzyme T7AC-cpCasp2 was generated through the circular permutation of caspase-2, incorporating both a solubility tag and an affinity tag [146]. Notably, its variants, designed via PROFICS and CASPON techniques, are beneficial for advancing industrial applications applicable to different proteins. They feature high selectivity, rapid kinetics, and high stability [147,148]. Most importantly, a simple strategy concerning computational modeling and simulation has been developed to estimate the cleavage ability of a variety of target proteins [149].

Table 4. Cleavage strategies and their characteristic properties.

Cleavage Tool	Name	Source	Cleavage Site [a]	Characteristic	Ref.
Chemical regent	CNBr	Synthesis	Met	High cleavage efficiency; Impact structure, Toxic	[7]
	Formic acid	Synthesis	Asp-Pro		[123]
	Hydroxylamine	Synthesis	Asn-Gly		[124]
	BNPS-Skatole	Synthesis	Trp		[125]
Traditional enzymes	EK	Enterokinase	DDDDK *	Nonspecific cleavage, Sensitivity to reagents	[68]
	Factor Xa	NA	IGGA *		[150]
	Thr	Thrombin	LVPR * GS		[151]
Popular enzymes	TEV	Tobacco Etch Virus	ENLYFQ * G/S	High specificity; Variable cleavage Efficiency	[152]
	HRV 3C	Human Rhinovirus	LEVLFQ * GP/M	Wide applicability, High activity	[129,143]
	Ulp1	Ubiquitin-like protease 1	Gly-Gly *	High specificity	[37]
	cpCasp-2	circular permutation Caspase-2	VDVAD *	High activity, High specificity	[148]

*: Indicates the specific amino acid sequence where cleavage occurs. [a] Cleavage site: Refers to an individual amino acid or a specific amino acid sequence recognized by different cleavage tools.

5.3. Self-Cleavage Methods

Inteins have long been recognized as parasitic elements, with self-splicing induced by the homing endonuclease domain [153]. In nature, they widely exist within functional proteins, and get involved in genetic and metabolic processes [154]. More recently, the discovery of highly efficient splicing inteins and accurate adaptation of splicing by environmental alteration have propelled their basic research and innovative biotechnological applications [155–157].

A variety of environmental cues can regulate cleavage and is mainly categorized into several classes, involving pH-induced or/and thiols-induced inteins chemical reagent-induced inteins (e.g., *Sce* VMA intein [158] and *Mxe* GyrA intein [159]), salt-induced inteins (e.g., MCM2 intein and Npu DnaE [160]), and pH-induced or/and temperature-induced inteins (e.g., Δ*I-CM* mini-intein [161], and Δ*I-CM* intein mutant [162]), and pH-induced and temperature-induced inteins (e.g.,Ssp DnaB intein [131]). The overall experimental procedure with a self-cleaving tag is visualized in Figure 1.

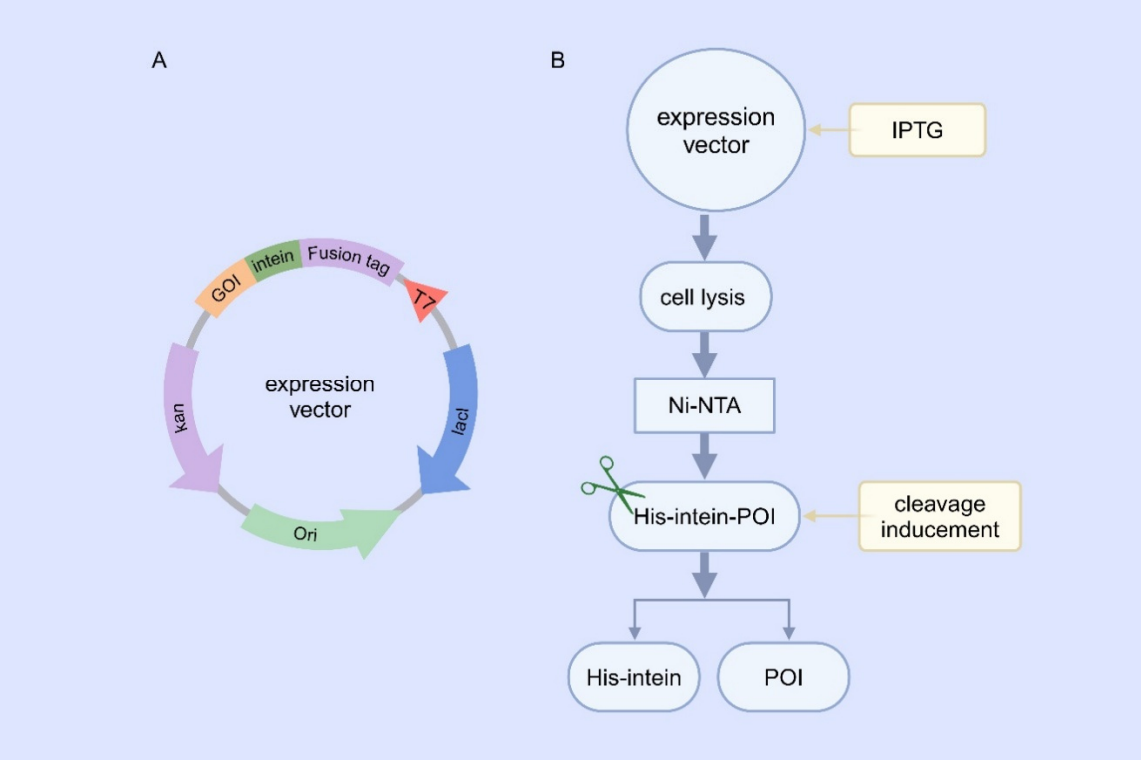

Figure 1. Schematic diagram of self-cleavage tagged POI production. (**A**) Schematic representation of the expression vector. The target gene and intein sequence are cloned into the vector. (**B**) Overview of the workflow for POI expression and purification induced by intein. The process involves intein-mediated self-cleavage, enabling efficient separation of the POI. Abbreviations: GOI, gene of interest; His, His-tag sequence; IPTG, isopropyl β-D-1-thiogalactopyranoside; lacI, lactose operon repressor gene; kan, kanamycin resistance gene; Ori, replication origin; POI, protein of interest; S, cleavage recognition site; T7, T7 promoter.

Beyond controlling intein activity through environmental conditions, selecting an optimal expression system can further improve yields. The Baculovirus Expression System (BES) is another suitable choice for intein-based strategies, as its culture conditions closely match those required for the Ssp DnaB mini-intein [131]. Though the production of fusion proteins under similar conditions to mini-intein cleavage, the enhanced yield of the fusion protein is sufficient to offset any decreases in yield associated with cleavage.

Since classical purification methods using various column chromatographic techniques are often tedious and expensive [163,164], scientists developed a cleavable self-aggregating tag (cSAT) scheme, including ELP-intein [165], CipA-intein [166], and ELK16-intein [167]. The experimental setup is illustrated in Figure 2, which outlines the key steps used in the procedure. It eliminates costly equipment or proteases and only necessitates centrifugation and self-cleavage steps. Thus, it represents an efficient and economical approach to purification. Unfortunately, inteins usually experience some degree of pre-cleavage, significantly reducing final yields. However, the fusion cleavage induced by split-inteins can overcome this problem since each segment is capable of cleavage upon its assembly [168,169].

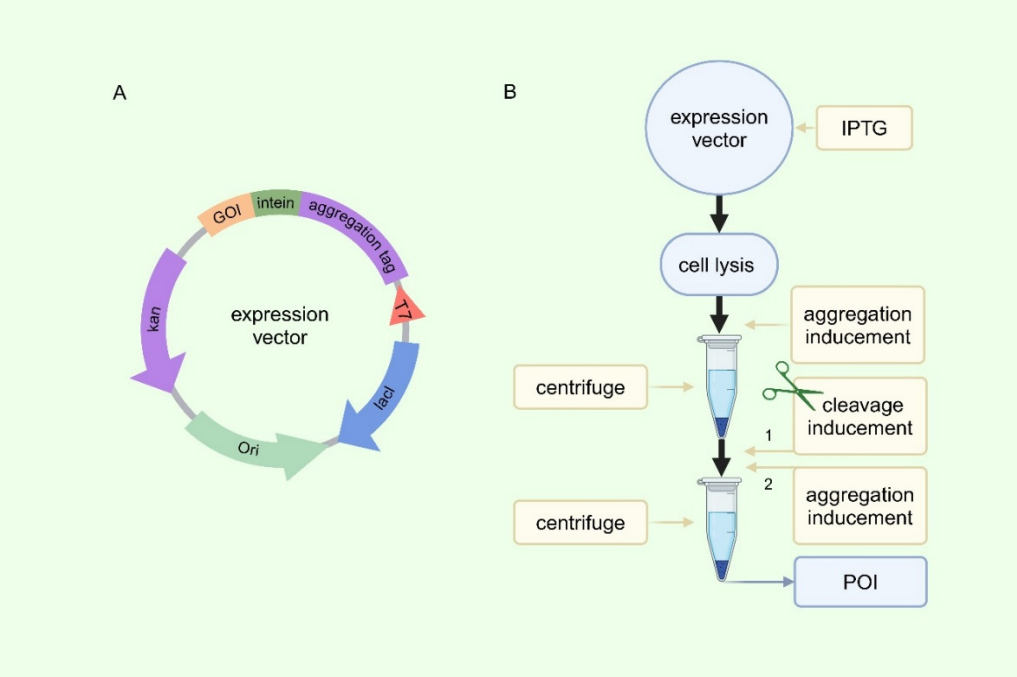

Figure 2. Schematic illustration of the expression and purification process using cSAT. (**A**) Schematic representation of the expression vector. GOI is fused with a cleavable self-aggregating tag (cSAT) and cloned into the expression vector. (**B**) Workflow overview for the expression and purification of the POI using cSAT. Protein expression is induced by IPTG. Aggregation conditions promote the self-aggregation of the cSAT-fused protein, which may undergo several cycles of aggregation. The fusion protein is cleaved at the intein cleavage site, enabling the isolation of purified POI via aggregation and subsequent centrifugation.

5.4. Co-expression of Target Proteins with Proteases

In vitro tag removal increases complexity and costs [170]. To address these challenges, researchers developed a controlled intracellular processing (CIP) strategy more than two decades ago, enabling the co-expression of a specific protease with the fusion protein and ultimately producing the unfused target protein in vivo [171]. The entire experimental setup is illustrated in Figure 3.

Proteases such as TEV [172] and HRV3C [173] are co-expressed, thus it eliminates the need for costly proteases for fusion protein cleavage and streamlines cloning efforts across various expression vectors [173,174]. Furthermore, the expression of the "duet" vector, designed to encode the protein of interest fused to SUMO and a SUMO-Ulp1 fusion protein, allows for the in vivo cleavage of SUMO fusion proteins by Ulp1 [175]. Further study has explored optimized bioprocess conditions, thereby circumventing the challenge of leaky expression and high metabolic burden [176].

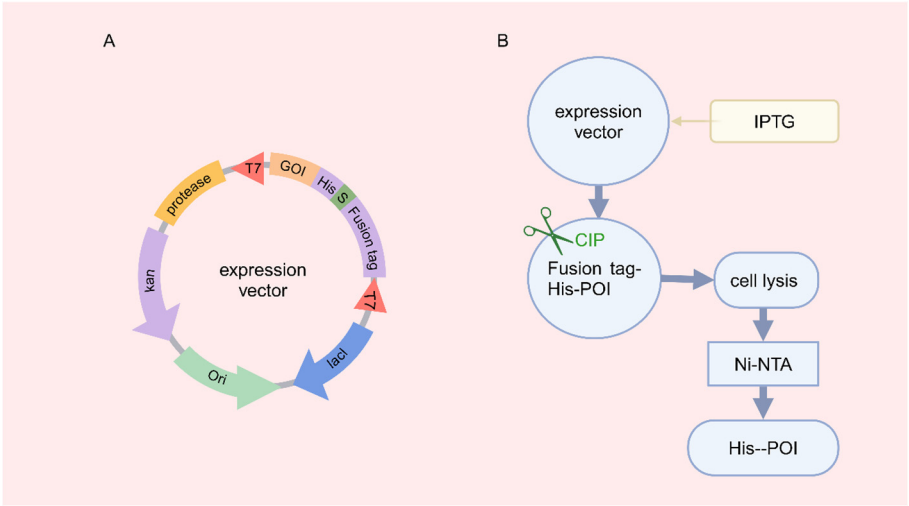

Figure 3. Schematic illustration of a co-expression and purification method. (**A**) Schematic diagram of the expression vector. The target gene and protease sequence are cloned into the same vector, along with regulatory

elements. (**B**) Overview of the co-expression and purification workflow. Protein expression is induced by the addition of IPTG. During the controlled intracellular process, the protease recognizes the cleavage site and cleaves the fusion protein. The His-tagged POI is subsequently isolated using Ni-NTA chromatography.

5.5. Immobilized Proteases Techniques

Figure 4 illustrates several forms of protease cleavage. Tag removal can be accomplished by traditional approaches of using free enzymes [134], as shown in Figure 4A. However, this cleavage is complex and time-wasting, since it requires at least two purification steps to obtain aimed proteins and the high consumption of proteases is expensive. Therefore, it is only common for small-batch purification.

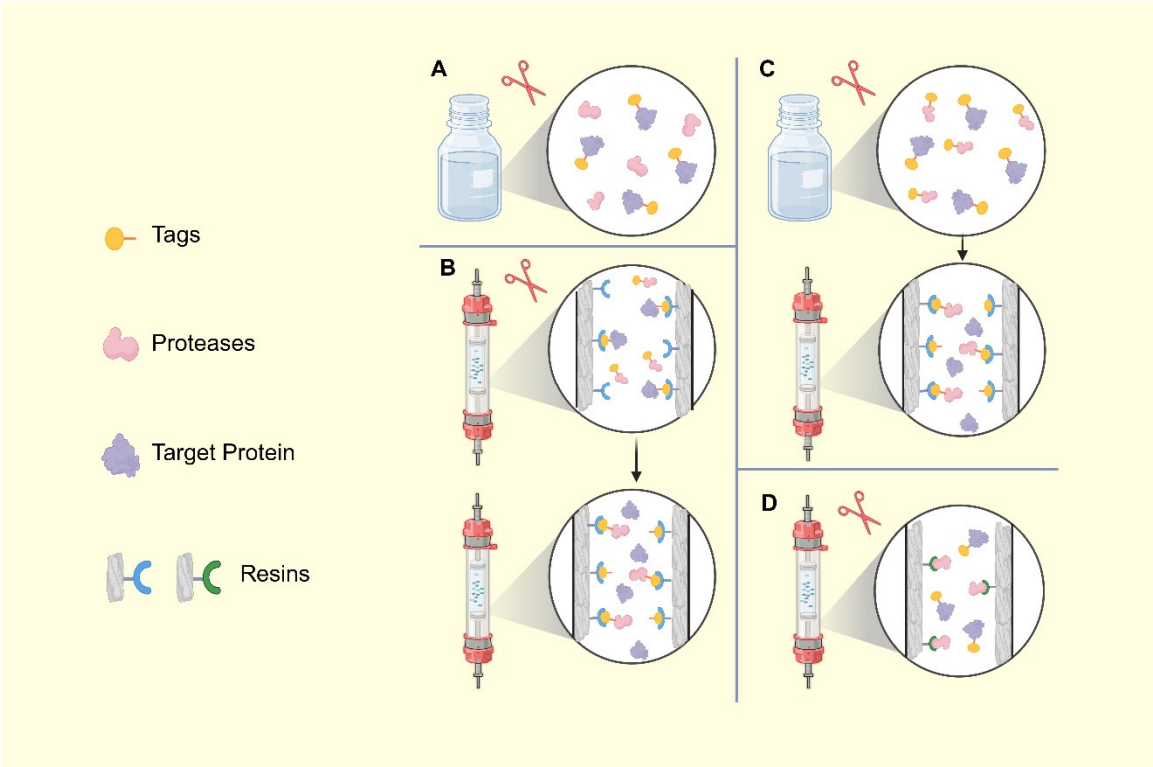

Figure 4. Category of different cleavage conditions. (**A**) Free Enzymes. Following the expression of recombinant proteins, cleavage occurs in solution. (**B**) On-Column Cleavage. Both the POI and proteases are tagged with identical labels, allowing for the purification of the protein through a single-step process. (**C**) Novel On-Column Cleavage. Protease cleavage is performed in solution, after which the mixture is transferred to a chromatographic column, facilitating efficient and complete cleavage. (**D**) Immobilized Protease Cleavage. Proteases are pre-immobilized on the column, enabling protease digestion as the mixture flows through. This method supports large-scale cleavage through multiple cycles.

In Figure 4B, it can be observed that both target proteins and enzymes are encoded by the same tag [19,177]. Though it allows for one-step purification, the cleavage is inefficient since enzymes attach to resin as well, thereby the tagged proteases cannot easily access the target site. However, an alternative method overcomes this challenge when the digestion of recombinant proteases occurs in solution as illustrated in Figure 4C [178].

As depicted in Figure 4D, the immobilization of enzymes facilitates the convenient separation of enzymes from contaminants because of the formation of the covalent bonds between enzymes and the matrix [15]. This form of immobilization also improves the properties of proteases, including cleavage specificity, catalytic activity, and stability [179]. For example, the immobilization of Ulp1 and TEV maintains its catalytic activity, thus allowing for operational reusability and large-scale application [140,179].

6. Discussion

Protein expression and purification play a crucial role in biomedical applications. An increasing number of techniques aim to circumvent solubility problems and promote the development of this field. This review outlines novel solubility tags and emerging aggregation tags. We also discuss the classification, design, and tag cleavage strategies.

Interfering tags may have negative influences without cleavage, while non-interfering tags are not necessary to remove. Recently, novel solubility tags have been developed as non-interfering sequences that leave protein structure and activity intact, facilitating their integration with high-throughput technologies.

With the widespread use of popular affinity tags (e.g., GST, MBP, SUMO, ELP, and intein), researchers have significantly advanced the field of protein expression in eukaryotic systems, including plants, insects, yeast, mammalian cells, and cell-free system. These tags enhance various aspects of protein production, including improving protein folding, increasing solubility, enabling high-level expression, and facilitating the production of complex proteins with post-translational modifications, such as glycosylation. However, efforts are needed to expand various protein employments of the novel solubility tags, because the expression efficiency in different host cells or cell-free systems is still unknown.

Traditional chemical methods are generally suitable for small proteins and membrane proteins, but protease-based approaches have now become widely adopted for a broader range of applications. Among protease-based tag removal methods, options include free enzyme cleavage, on-column cleavage, and immobilized protease cleavage. Immobilized proteases, in particular, can be reused multiple times, making them well-suited for large-scale production, while on-column cleavage or free enzyme treatments offer greater flexibility for smaller-scale or more experimental workflows.

Previously, though inclusion bodies and inteins were considered ineffective additives, subsequent research and validations have offered new approaches to address the “solubility issue” or to provide effective tag removal strategies. Consequently, more and more researchers have made efforts to develop self-cleaving aggregation tags, which significantly simplify the purification process and even eliminate the need for columns.

Controllable aggregation tags are designed to simplify their isolation process by inducing proteins to aggregate or become soluble under different conditions. Furthermore, inteins can achieve efficient cleavage by adjusting environmental factors such as pH, temperature, salt, and magnetic force. However, complexity arises when combining controllable aggregation tags and environment-responsive inteins, as multiple induced conditions may interact, potentially reducing purification efficiency. Finding the right combination of conditions requires careful optimization, adding time and effort to the purification process. Thus, exploring a suitable combination of conditions takes more time, since the induced conditions may interact with each reaction. Even though only a few experiments have so far investigated the co-expression of proteases and target proteins within the same vector, this strategy holds promise for large-scale production. Further research, however, should focus on the exploration of suitable tag removal conditions to prevent pre-cleavage.

7. Conclusions

The utilization of tags has been the standard for solving IBs problems. As a result, several innovative forms of tags have been further developed, including solubility tags with small sizes and controllable aggregating tags. The design of interfering tags combined with reasonable cleavage methods plays a crucial role in the subsequent characterization and applications. This review thus offers a reference for the reasonable selection of each tag and tag removal approach, which simplifies the expression and purification process and enables better application of target proteins.

8. Prospective

In the future, solubility tags will tend to be designed as non-interfering tags. Furthermore, careful consideration should be given to tag design for the influence between tags and proteins or conflicts among induced conditions (e.g., inteins and aggregating tags). Researchers should further explore expression systems and optimized conditions for novel solubility tags and controllable aggregating tags. Moreover, co-expression of enzymes should be explored further for large-scale applications. Overall, combining different functional tags and tag removal strategies will facilitate more convenient, efficient, and high-yield protein expression and purification.

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