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David W. Wood[a]
[a] Department of Chemical and Biomolecular Engineering, Ohio State University, Columbus, OH, USA

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Non-Chromatographic Recombinant Protein Purification by Self-Cleaving Purification Tags

David W. Wood
Department of Chemical and Biomolecular Engineering, Ohio State University, Columbus, OH, USA

The genetic modification of recombinant proteins to include affinity tags can greatly simplify their purification. A key drawback to this technology has been the difficulty and expense associated with the removal of the tag from the purified product. In this work, we review the development and application of an engineered self-cleaving module that can be used to generate convenient self-cleaving tags. Further, we describe the use of this module with conventional affinity and non-chromatographic purification tags, which allow purification of native proteins through simple and inexpensive means. Limitations to these technologies are also discussed, along with some perspectives on the short-term future development of these methods.

Keywords elastin-like peptide tag; intein; non-chromatographic bioseparation; phasin tag; recombinant protein purification

INTRODUCTION

The production of purified recombinant proteins is a critical capability in many industries. These products include commodity proteins and enzymes, as well as research reagents and pharmaceuticals, where in each case the required purity of each product is typically dictated by its application. Each product is also subject to its own value considerations, which can force tradeoffs between cost and purity for a given target protein. In the case of pharmaceutical products, high purity is absolutely required and the value of the products is high; thus the associated purification processes can account for a large majority of the production cost. Conventional processes typically consist of several chromatographic steps, each of which must be individually optimized for each new target (Fig. 1a). For commodity enzymes, on the other hand, the required purity is substantially lower and in some cases crude extracts with little or no purification can suffice. A common feature of all these products is a desire for simpler and less expensive purification methods for their manufacture.

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Address correspondence to David W. Wood, Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, OH 43210, USA. E-mail: wood.750@osu.edu

In addition to the basic needs of product and process for recombinant enzymes, several drivers for new purification technology development are becoming increasingly important. In research, high-throughput methods are becoming more critical as genomic projects give rise to proteomic studies (1–3). The development of platform technologies to accelerate the development of new products is highly desirable for the pharmaceutical industry (4), while disposable technologies are becoming increasingly attractive as they can simplify cleaning validation and facilitate the approval of multiproduct facilities (5–7). Green processes are also drawing increased attention from many sectors for their use of renewable resources and their reduced carbon footprint (8,9).

A final, yet highly important short-term driver, which has gained quite a lot of attention over the past few years, is the unexpected and dramatic increase in process titers for mammalian cell culture processes. This increase, by close to two orders of magnitude in the production of antibodies and other glycoproteins in less than a decade, has created a substantial downstream bottleneck in the production of these biopharmaceutical products. This has greatly increased the impetus for developing high-capacity, low volume processes, and has generated interest in a wide variety of old and new technologies. Among these are both the "ABC" approaches, referring to "Anything But Chromatography," as well as new chromatographic approaches, including novel continuous processes and more effective modes for conventional separations (10–14).

Many of these issues can be very effectively addressed through the use of fusion tags for protein purification, which are very commonly used at laboratory scales (Fig. 1b). In these methods, the target protein is genetically modified to include a partner protein, or tag, which is expressed in fusion to the recombinant product to form a single bifunctional molecule. The function of the tag is to greatly simplify the purification method, allowing the target and tag to be co-purified using a very simple previously optimized method (15–17). The strength of tag technologies is that they are predictable, easy to optimize, and highly effective for the purification of virtually any

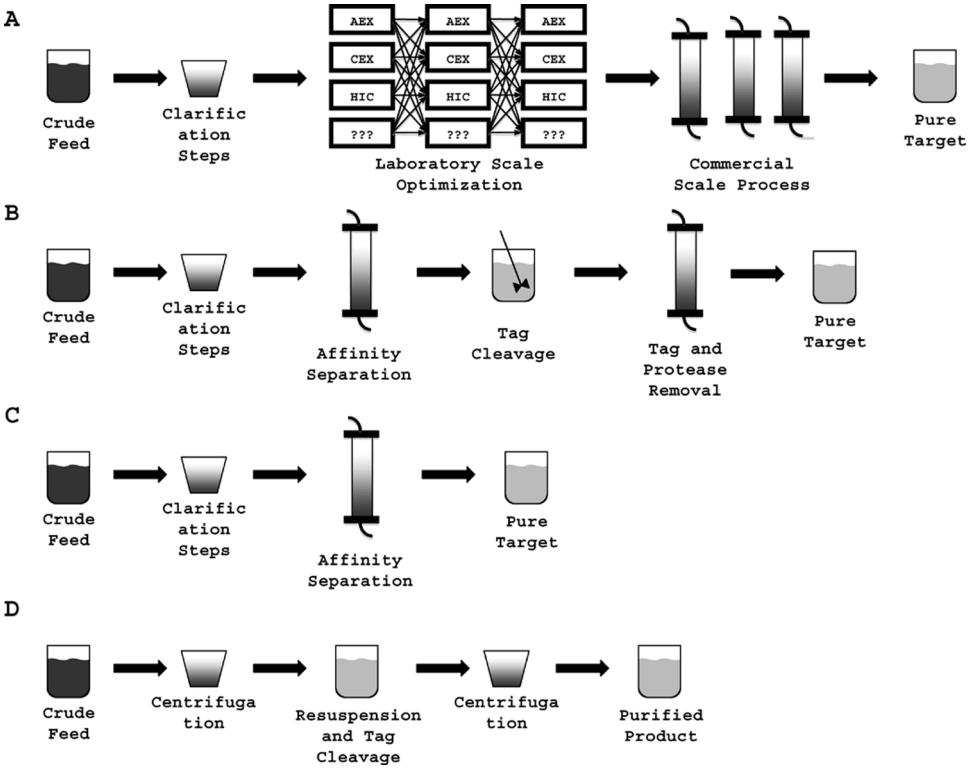

FIG. 1. Comparison of conventional and tag-based processes. (A) A conventional chromatographic process typically requires several chromatographic steps, each of which must be optimized at laboratory scale. In some cases (e.g., antibodies), platform methods can greatly simplify this optimization. The product is typically highly purified, often to a purity of greater than 99%. (B) A conventional affinity tag process does not require optimization, but requires separate steps for cleaving of the tag (typically by a protease enzyme), followed by a step to remove the cleaved tag and protease. The target is typically over 95% pure after these methods. (C) A self-cleaving tag method allows the tag to cleave on-column, allowing only the cleaved target to elute. Targets from these methods are also typically over 95% pure. (D) A non-chromatographic self-cleaving process can provide a reasonably pure product without a chromatographic step. Targets produced by these methods can range in purity from 75% to 95%.

product. Thus they lend themselves to the development of platform and high-throughput processes, and are therefore potentially very appealing to the pharmaceutical industry. A critical limitation of tag technology, however, is the presence of the tag attached to the purified target protein at the end of the process. This is not as critical with commodity and research enzymes, where only the activity of the product is relevant, but can be a substantial problem in pharmaceutical applications, where the tag may elicit an immune response in the patients receiving these products. Conventional tag removal typically requires the use of highly specific endopeptidase enzymes, which are typically cost-prohibitive for even the most lucrative products, and can result in additional difficulties with product destruction and regulatory approval. For this reason, tag technologies have not been largely used in the biopharmaceutical industry. Further, the use of conventional tags suggests an affinity separation, which can significantly increase the costs of commodity enzymes. Thus the appeal of tag methods can be limited in these applications as well.

We have sought to alleviate these difficulties through the development of several new capabilities in tag technology. The first of these is a type of self-cleaving affinity tag. In this case a conventional affinity tag is joined to a self-cleaving module, which cleaves itself from the target protein in response to mild changes in buffer pH or temperature. The tag and self-cleaving module remain fused to each other, and can easily be separated from the target during the process (Fig. 1c). The second innovation has been the incorporation of non-chromatographic purification tags, which allow target recovery by simple precipitation and centrifugation or filtration (Fig. 1d). The elimination of a conventional affinity separation when using these tags can greatly decrease their cost. Further, these tags can be combined with our self-cleaving modules to generate very simple methods for generating non-tagged target proteins at low cost. Although some difficulties remain with these technologies, they have the potential to provide significant new large-scale purification platforms for a wide variety of industries and products.

DEVELOPMENT OF INTEIN-BASED SELF-CLEAVING AFFINITY TAGS

Many issues associated with the use of affinity tags can be solved through the development of self-cleaving tags. In practice, a self-cleaving tag works the same as a conventional affinity tag, but has the major advantage of eliminating the requirement for proteolytic tag removal. This also allows the target protein to be cleaved from an immobilized tag, thus eliminating the need to purify the cleaved tag from the target in a separate step. In the past decade, several approaches have been used to generate self-cleaving modules of various types, which could be combined with conventional tags to make them self-cleaving (Table 1). Among these are engineered derivatives of the FrpC protein derived from *Neisseria meningitides* (18), the cell envelope sortase A transpeptidase from *Staphylococcus aureus* (19), and the MARTX toxin cysteine protease domain from *Vibrio cholerae* (20). An additional example is N^{pro} fusion technology, based on the self-processing N^{pro} module of classical swine fever virus (21,22). In this case, the tag drives the expressed fusion into an insoluble aggregate phase, and cleaves upon refolding. Although this does not follow the classical self-cleaving tag model, a significant purification of the precursor protein from aggregated inclusion bodies can be accomplished by simple washing. In each case, these self-cleaving technologies exhibit various advantages and limitations related to cost, complexity, efficiency, target protein modification, and premature cleaving during expression. As with any expression and purification process, the appropriate method for a given product will depend on the requirements of the product.

In our work we have focused on the development of self-cleaving modules based on engineered self-splicing proteins known as inteins (23). Short for "INtervening proteins," inteins are found as insertions in various host proteins in many diverse organisms (24–26). Once the host protein and nested intein are translated and fold, the intein excises itself in a process known as protein splicing. Further, because they are mobile genetic elements, the intein's splicing capability is self-contained and many inteins retain splicing activity when moved to foreign contexts (27). These features, combined with a full understanding of the splicing mechanism and its modulation by mutation, have made inteins tractable for many applications in biotechnology (28–35).

To create self-cleaving affinity or other purification tags, inteins can be modified such that splicing is suppressed and replaced by isolated cleaving at one or both of the intein termini. This is typically accomplished by the introduction of rational mutations within the intein at the N- and/or C-terminal splicing junctions (35–37). Thus, inteins can be generated that exhibit isolated cleaving at their N-terminus, which is generally triggered by the addition of thiol compounds (38), or at their C-terminus, which is generally triggered by changes in pH at a suitable temperature (39,40). In some cases, inteins can be generated that exhibit cleaving at both termini, which is also generally triggered by thiol compound addition (41). Very recent studies also suggest that rapid isolated cleaving can be induced at the N-terminus, in some cases by the addition of hydroxylamine (42), and other inteins with additional triggers are in development in several laboratories (43–46).

Our focus has been on the development of an intein-based self-cleaving module for use in industrial bioseparations, ideally to be used in the pharmaceutical industry. This intein would require rapid isolated cleaving activity for the purification process, but would also require minimal size and highly controllable activity to maximize efficiency of upstream expression. Further, the cleaving reaction itself must be inexpensive and robust, and involve equipment and buffers similar to those already found in the bioprocess industries. Finally, this intein should cleave cleanly, without the requirement for any modifications to the target protein.

To generate these characteristics, we started with the *Mycobacterium tuberculosis* RecA (Mtu) intein (47). This intein exhibits a canonical splicing mechanism, and was one of the first to be discovered and shown to splice outside of its native context (48). Thus the required modifications for generating isolated cleaving were relatively straightforward, and we chose to introduce isolated pH-sensitive C-terminal cleaving through mutation of the initial intein residue from cysteine to alanine (40). Like most inteins, the Mtu intein consists of a splicing domain with an inserted endonuclease domain (Fig. 2a). Because the endonuclease domain is not required for splicing or cleaving, our first step was to delete this domain based on a model prediction of the intein interdomain boundaries (49,50). Unfortunately, the resulting mini-intein, referred to as the ΔI intein, exhibited low activity and stability, making it unsuitable for the proposed applications (49). To remedy this, we employed a directed evolution strategy, where the intein was fused to a thymidylate synthase reporter enzyme (51). Depending on the configuration of the fusion, rapid splicing or cleaving of the intein would rescue auxotrophic *E. coli* strains expressing the fusion precursor. Random mutagenesis of the intein, coupled to genetic selection using the TS reporter, allowed us to isolate two new intein mutants (Fig. 2b). The first is a stable, rapidly splicing mini-intein mutant, known as ΔI-SM (Splicing Mutant), while the second is a rapidly cleaving mini-intein mutant, known as ΔI-CM (Cleaving Mutant) (40).

The ΔI-CM mutant exhibits several characteristics that make it attractive for applications in bioseparations. First and most importantly, it exhibits rapid and controllable cleaving in response to small changes in pH and

TABLE 1
Comparison of primary characteristics of cleaving methods

Cleaving method	Cleaving trigger	Primary advantages	Primary disadvantages	References
Conventional Protease	Protease Enzyme Addition, incubation times and temperatures depend on specific proteases used.	Well-established, with many optimized options available. No premature cleaving.	Expensive. Can require additional amino acids on the target protein. Can cleave target unexpectedly. Cleaving performance can be unpredictable.	(15–17,96,97)
N-cleaving intein	Thiol compounds, M2-mercaptoethanesulfonic acid (MESNA), hydroxylamine, thiophenol, β-mercaptoethanol, 1,4-dithiothreitol (DTT), free cysteine, all typically at 30 mM to 100 mM concentration. Incubation at 4°C to 37°C for 2 hours to overnight.	Most commonly used, many proteins purified. Very little premature cleaving. Acceptable cleaving rate at variety of temperatures.	Requires high concentrations of thiol compounds for cleaving. Difficult for disulfide bonds in proteins. Expensive at large scale.	(38,39,94,95, 98–104)
C-cleaving intein	pH below 7.0, incubation at room temperature to 37°C for 4 to 24 hours (higher temperatures accelerate cleaving).	Simple cleaving method, generally safe for most proteins. Most inexpensive and flexible cleaving trigger. Upstream affinity tag can aid solubility.	Substantial premature cleaving *in vivo* during expression. Very slow cleaving at low temperature.	(39,40,52)
N+C cleaving intein	Same as N-cleaving inteins.	Very little premature cleaving.	Requires dialysis of N-terminal peptide after cleaving.	(41)

Neisseria meningitides FrpC protein (N-cleaving)	10 mM Ca^{2+}, incubation at 4°C to 37°C, 4 to 8 hours (higher temperatures accelerate cleaving).	Very little premature cleaving during expression. Very fast cleaving upon Ca^{2+} addition.	Requires addition of Asp residue to C-terminus of target protein. May require EDTA and DTT in purification buffers.	(18)
Staphylococcus aureus sortase A transpeptidase (C-cleaving)	5 mM Ca^{2+}, 10 mM Gly3, incubation at 25°C for 4 to 6hours.	Fast cleaving. Cleaving module appears to increase solubility of target protein.	Requires addition of Gly residue to N-terminus of target protein. Some premature cleaving. Potential for unwanted cleaving in target.	(19,105,106)
Vibrio cholerae MARTX cysteine protease domain (N-cleaving)	50 to 100 mM inositol hexakisphosphate ($InsP_6$) at 4°C to room temperature for 1–2 hours.	Fast cleaving. Cleaving module appears to increase solubility of target protein.	Requires 1 to 4 residues to be added to C-terminus of target protein. Small potential for unwanted cleaving in target.	(20)
Clasical Swine Fever Virus Self-processing N^{pro} Module	*In vitro* refolding of fusion protein under kosmotropic conditions.	Inclusion bodies allow substantial purification by simple washing. High-level expression of target. Protects target from protease. Decreases impact of toxic targets. Wide variety of N-terminal amino acids on target is allowed.	Efficient refolding procedure for target must be available.	(21,22,107)

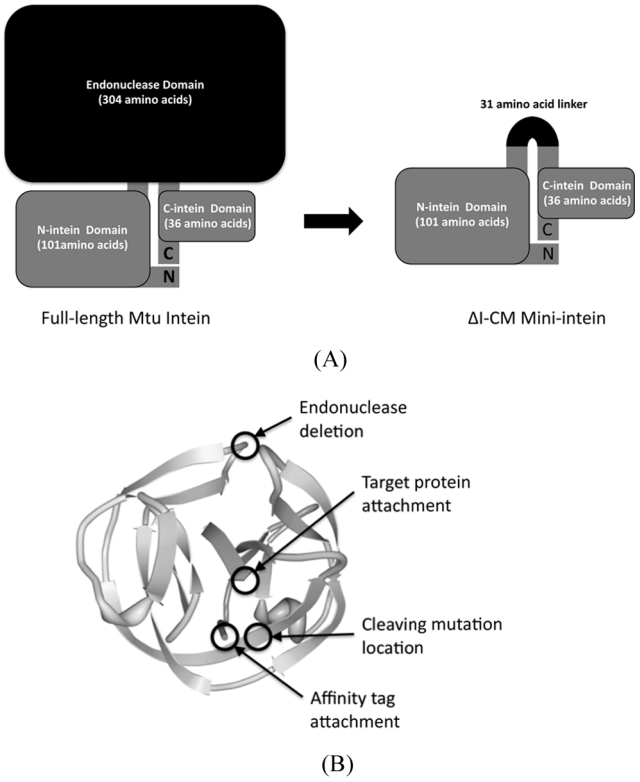

FIG. 2. Construction and structure of the ΔI-CM mini-intein. (A) The ΔI mini-intein was first constructed through a deletion of the central endonuclease domain of the *Mycobacterium tuberculosis* RecA intein. The first nine and last twenty-two amino acids of the endonuclease were left as a linker to stabilize the resulting ΔI intein (49). Subsequent mutagenesis introduced an aspartic acid to glycine mutation at residue 150 of the mini intein (420 of the original full-length intein), which accelerated C-terminal cleaving (40). (B) Crystal structure of the splicing domain of the Mtu RecA intein, showing the locations where the affinity tag and target protein are attached, as well as the modifications associated with its development. (PDB structure 2IN8) (108).

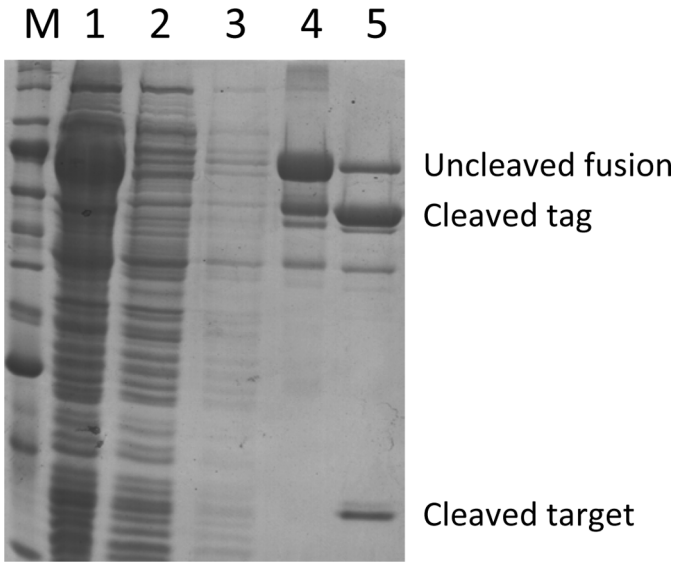

FIG. 3. SDS-PAGE analysis of self-cleaving of a Maltose Binding Protein (MBP) tag. In this case, and target protein is recombinant human acidic fibroblast growth factor (aFGF) and the tag is maltose-binding protein. The tagged precursor protein (Uncleaved Fusion) was purified by a standard affinity separation, eluted from the column, and allowed to cleave overnight at room temperature. The products are the Cleaved Target (aFGF), and Cleaved Tag (MBP-intein fusion). Lanes: M=Molecular weight markers; 1 = Clarified cell lysate; 2 = Column loading flowthrough; 3 = Column wash; 4 = Eluted precursor protein; 5 = Cleavage products after 24 hours at pH 6.5 and room temperature.

temperature. Specifically, cleaving is strongly suppressed at pH 8.5 and low temperature (4°C), allowing the precursor protein to be purified over several hours or days under these conditions. At pH 6.0 to 6.5 and higher temperatures, cleaving is rapid and can typically be completed overnight at room temperature or within a few hours at 37°C (Fig. 3). Overall, a combination of temperature and pH can be used to modulate the self-cleaving rate by a factor of approximately 10,000 fold, depending on the target protein (52). These features make this intein especially attractive for proteins produced in *E. coli*, where the intracellular pH is approximately 7.5 and relatively rapid expression can take place at temperatures as low as 12°C to 15°C. Under these conditions it is fairly simple to acquire a high yield of uncleaved precursor protein for purification.

Our developed intein can be combined with virtually any conventional affinity tag, and initially we worked with the maltose binding protein (MBP) affinity tag (40,52). This

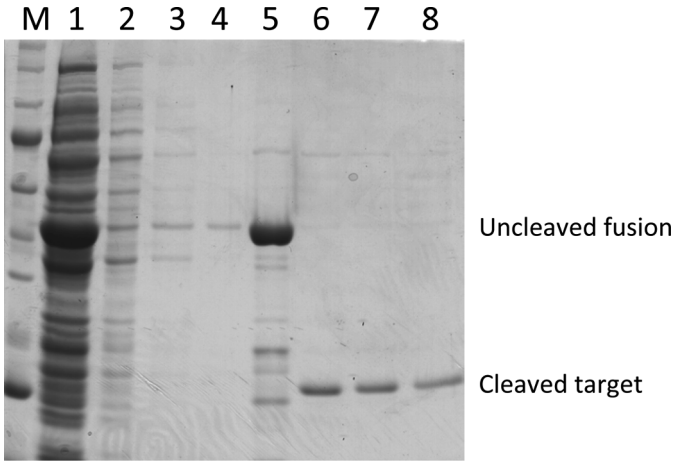

FIG. 4. SDS-PAGE analysis of Green Fluorescent Protein (GFP) purification by self-cleaving chitin tag. Tagged precursor protein is bound to a chitin column, while cellular contaminants are washed away. Cleaving of the column-bound precursor is induced by a pH shift, allowing the cleaved GFP product to be eluted directly from the column. Lanes: M=Molecular weight markers; 1 = Clarified lysate; 2 = Column loading flowthrough; 3–4 = Column wash; 5 = Resin sample (pre-cleaving) with bound, tagged target protein; 6–8 = Cleaved product (GFP) eluting from column after overnight incubation at pH 6.5 and room temperature.

work demonstrated that this intein expresses well in *E. coli* at high yield, and that premature cleaving of the intein during expression can be minimized by expression at low temperature without a major loss of productivity. Further, the intein worked well with the maltose binding protein, and did not affect the solubility of the fused target protein. Indeed, the ability of the maltose binding protein to enhance the solubility of the target was not compromised in any cases. Initial attempts at on-column cleaving were also highly successful, suggesting that this intein may have substantial utility in future bioseparations strategies. Most recently, we have moved to a stronger chitin-binding protein tag, which provides a highly purified product after on-column cleaving from a chitin resin (Fig. 4). A remaining drawback, however, is the continued requirement for affinity resins and apparatus for the purification process itself. To remedy this, we explored two new strategies based on non-chromatographic separation tags.

PHB-BINDING PHASIN TAGS

Oil shortages during the 1970s and 1980s spurred great interest in the production of plastics from renewable carbon resources. One of the key discoveries was the ability of several organisms to produce polyhydroxyalkanoates from simple sugars (53). The most common of these is polyhydroxybutyrate (PHB), which can serve as a biodegradable plastic for several conventional applications. Most importantly, PHB can be produced in a large variety of non-native organisms, including *E. coli* and several other common recombinant protein expression hosts (54,55). In *E. coli*, PHB expression can be easily introduced through the introduction of three enzymes, and the expressed PHB appears as macroscopic granules within the *E. coli* cytoplasm (56,57). The macroscopic size and relatively high density of these particles suggests utility as an affinity carrier, where a tagged protein might bind selectively to the PHB granules and be recovered and purified by simple centrifugation or filtration.

A known class of proteins with specific affinity for PHB particles is the phasins, which were discovered during the mid 1990s (58–61). These proteins have many characteristics that suggest their use as an affinity tag. They are relatively small and have specific affinity for PHB granules. Further, they can be expressed in many cell types, and have even been used in the past to bind fused target proteins to PHB particles (60,62). Despite this potential, their use as an affinity separation method had not caught on, largely due to the fact that many host proteins also bind nonspecifically to the hydrophobic PHB surface. Therefore the recovery of the phasin-tagged protein, by treatment with mild detergent for example, also typically released contaminant host cell proteins.

The incorporation of an intein with a phasin tag effectively solves the difficulties associated with non-specific binding of contaminants (57,63). This is due to the simple fact that the intein cleaving reaction selectively releases only the tagged target from the surface of the granules. The relatively mild cleaving conditions further ensure that the contaminants are not released as the intein cleaves (Fig. 5). Finally, the incorporation of three tethered phasin proteins in each tag provides strong multivalent binding, allowing multiple washes for increased purity with high recovery of the target protein (57). The result is an effective phasin-PHB strategy, where the target can be easily purified using simple non-chromatographic means (64,65).

To simplify this strategy, we have generated an *E. coli* strain that incorporates the PHB-synthesis enzymes on a separate plasmid. This strain is made competent, and then transformed with an expression vector containing a phasin-intein-tagged target protein gene. The cells are grown for 30 hours in lactate-supplemented growth medium at 37°C to accumulate PHB granules, and then can be induced by IPTG addition. The tagged target protein accumulates inside the cells, where it binds to the PHB granules. The cells are then recovered and lysed (typically by sonication) in a pH 8.5 buffer to suppress cleaving, and the large and relatively dense PHB granules are recovered via centrifugation or filtration (Fig. 5). The granules can be washed several times for increased purity, although some yield losses can occur as the phasin-tagged protein leaches from the surface of the granules. Once the granules and associated tagged target proteins have been purified, the intein tag is induced to cleave by resuspension of the

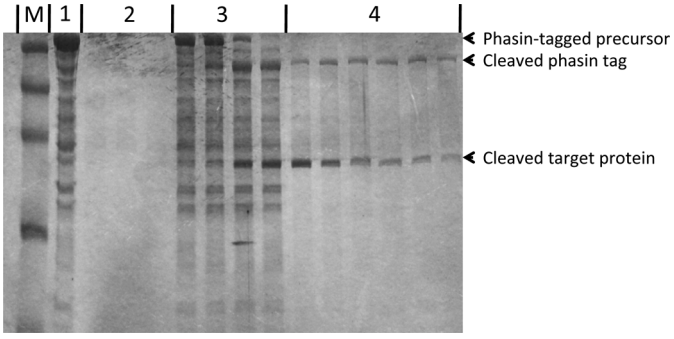

FIG. 5. SDS-PAGE analysis of phasin-intein tag protein purification by diafiltration. In this case the target protein is Maltose Binding Protein (MBP). The cell lysate was diluted in pH 8.5 buffer to allow washing of the PHB granules by crossflow diafiltration on a microporous membrane; the macroscopic PHB granules are retained by the membrane while smaller contaminant molecules are removed. After washing with six process volumes of buffer, the pH was shifted to 6.5 and the cleaved MBP product was collected as it passed through the membrane. Gel sections: M=Molecular weight markers; 1 = Cell lysate with precursor protein; 2 = Filtrate Filtrate during wash at pH 8.5; 3 = Retentate during cleaving reaction at pH 6.5; 4 = Filtrate during cleaving reaction at pH 6.5. Note that the majority of the contaminant proteins remain associated with the PHB granules in the retentante (gel section 3), although leached phasin tag is evident in the purified material (gel section 4).

granules in a pH 6.5 buffer. The cleaving reaction is not affected by the phasin tags, and can usually be completed at room temperature overnight, or more quickly at 37°C. Several proteins have been successfully purified using this method, and yields are generally reasonable for shake-flask expression.

Despite the simplicity and convenience of this method, several limitations have been observed. Perhaps the most significant is that the solubility of the target protein is not increased by the addition of the phasin tag, and in some cases the tag may actually decrease the soluble yield of the target protein. Thus, the method is best suited to well-expressed and highly soluble proteins, and may be less effective for the purification of more difficult targets. A second difficulty is that the phasin tag can leach from the surface of the granules after cleaving is complete, and appear as a contaminant in the final product (Fig. 5). The use of a triple phasin tag significantly decreases this problem, but the development of more strongly binding tags will increase the appeal of this method as well. Further, the available surface area of the granules limits the overall yield of the target protein. Although the granules are small and have a reasonably high surface area, their morphology puts a physical limit on possible yield. Finally, as mentioned above, long induction times are required for granule formation, and premature cleaving by the tag can significantly decrease the yield of the tagged target. Many of these limitations will likely be solved with some metabolic optimization of the expression strains, and the incorporation of second-generation inteins with more tightly controlled cleaving activity. It remains to be seen whether this method will be effective in other types of expression hosts, including transgenic plants and yeast cells. Many of these have been shown to be tractable for PHB production, although data on intein behavior in these systems is limited.

ELASTIN-LIKE POLYPEPTIDE TAGS

Elastin-like polypeptides (ELPs) are repetitive protein sequences that are analogous to elastin found in animal tissues (66–68). Studies of elastin and elastin-like polypeptides led to the discovery that these proteins undergo inverse phase transitions in response to temperature changes. At high temperatures, ELPs assemble into a solid phase and precipitate (69). Interestingly, this transition is reversible, making it distinct from heat-induced aggregation. More importantly, the specific conditions under which the phase transition occurs can be tightly controlled by modifying the size and composition of the ELP sequence, as well as the salt content of the associated solution (70–72). These characteristics suggest many potential applications for ELPs in medicine and biotechnology (73–77). Notably, they also suggest that ELPs might be used in the generation of self-associating, non-chromatographic purification tags, where reversible precipitation of the ELP tag could be used to selectively recover an ELP-tagged target. Further, because ELPs are simple proteins, they can be encoded on a DNA plasmid and used similarly to a conventional affinity tag. Indeed, this capability has been shown in previously reported work (78–81).

An additional advantage of the ELP tag is that there are now several papers on the design of ELP proteins for various transition behaviors, and it is possible to rationally design ELP sequences for specific transition temperatures in a given salt buffer (70,76,80). Additional ELPs have also been designed that react more strongly to salt concentration by incorporating charged residues in the guest positions described above (82). Finally, it has been shown that ELPs can be expressed in a wide variety of host cells, including plant and mammalian cells, and ELP tags can enhance the expression of recombinant proteins in transgenic plants while facilitating the purification of the tagged targets (83–85).

Combining an ELP tag with an intein generates an effective self-cleaving non-chromatographic purification tag (86,87). In our work, we have used a 550 amino acid ELP composed of 110 repeats of the pentapeptide valine-proline-glycine-Xaa-glycine, where the Xaa residues are made up of a mixture of valine, alanine and glycine in a ratio of 5:3:2. This composition has been shown by previous workers to produce inverse transitions at reasonable temperatures and salt concentrations, making the expressed ELP attractive for applications in protein purification (81). To facilitate the use of the self-cleaving ELP-intein tag, we have constructed an expression vector where the T7 promoter drives expression of the ELP-intein-target protein. This allows convenient and efficient expression of the tagged target in a variety of *E. coli* strains.

We initially expressed ELP-intein-target protein fusions in the *E. coli* strain BLR (Novagen). This strain was chosen because it has a knockout of the *rec*A gene, which substantially eliminates recombination of introduced DNA sequences, and it can be used with the T7 promoter for leaky or high-level expression of our tagged target (86). These features optimize expression of the fusion gene while decreasing any instability of the highly repetitive ELP sequence. Our initial experiments showed that overexpression of the tagged precursor by conventional IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction was ineffective, and we hypothesized that this was due to the highly repetitive nature of the ELP sequence, which would likely deplete several amino acid pools within the host cell (Fig. 6). Therefore we chose to accumulate the target protein fusion through leaky expression from the T7 promoter at low temperatures (15 to 18°C) over 24 to 48 hours. Depending on the product protein, this strategy typically yields over 100 milligrams of purified, cleaved target per liter of shake flask with very little premature cleaving

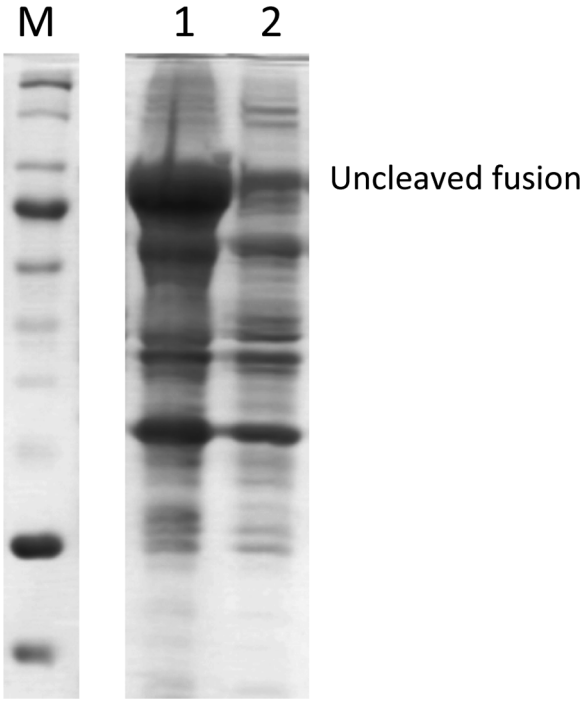

FIG. 6. SDS-PAGE analysis of uncleaved ELP-intein fusion expression in the presence and absence of IPTG. Lanes: M=Molecular weight markers; 1 = Soluble tagged protein from overnight expression at 18°C in absence of IPTG; 2 = Soluble tagged protein from overnight expression at 18°C in presence of 0.3 mM IPTG.

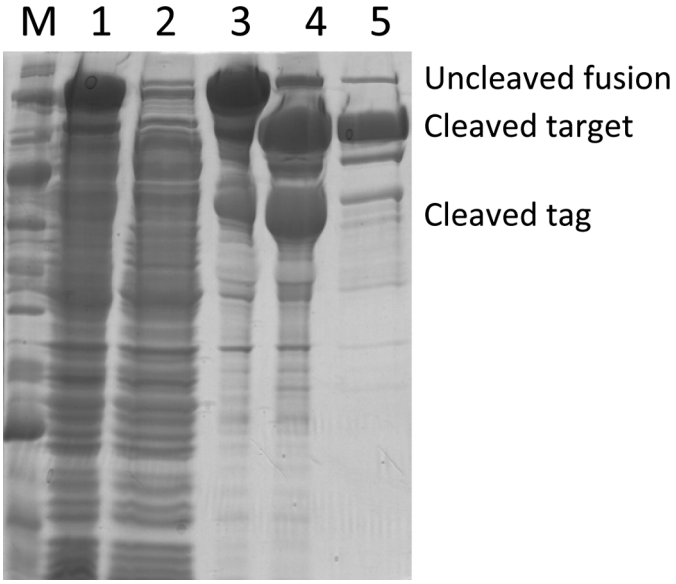

FIG. 7. SDS-PAGE analysis of β-galactosidase purification by ELP-intein tag. Lanes: M=Molecular weight markers; 1 = Clarified cell lysate; 2 = Soluble contaminants after salt addition; 3 = Resuspended tagged precursor protein; 4 = Cleaved target and tag after cleaving overnight at pH 6.5 and room temperature; 5 = substantially purified target protein after final precipitation of cleaved ELP-intein tag.

(86). Lysis of the cells, followed by thermal precipitation of the tagged target protein in 1.5 molar sodium chloride at 30°C to 40°C, depending on salt concentration, recovers the substantially purified uncleaved precursor. The cleaving reaction is then induced by a shift to pH 6.5 and incubated at room temperature overnight, followed by a final thermal precipitation of the cleaved ELP-intein tag. The cleaved, purified product protein is then retrieved from the soluble fraction of this final precipitation, while the cleaved tag is left in the pellet (Fig. 7). Ultimately we have used this method to purify over 20 target proteins of various types, and have shown in several cases that the presence of the ELP tag can enhance the yield of active and properly folded target protein (86,88–91).

In more recent work, we have enhanced this method, and have applied it to additional host cells. An important recent advance was the discovery that salts other than sodium chloride can be more effective in precipitating the ELP tag. Specifically, an examination of the Hoffmeister salt series suggested that ammonium sulfate may be ideal, and allows the precipitation steps of the purification to take place at a concentration of 0.4 molar salt and room temperature (18°C to 20°C) (92). As is obvious, the lower precipitation temperature is more attractive for heat-labile products, and decreases the energy requirements and complexity of the method. We have noted in some cases, however, that the use of ammonium sulfate can increase non-specific precipitation of contaminant proteins as well, which can decrease the purity of the final product.

Attempts to express ELP-intein tagged targets in mammalian cells, which would be ideal for the production of antibodies and other complex glycoproteins, have been partially successful. The ELP tagged targets are produced effectively, but premature cleaving by the intein has led to unacceptable yield losses, as would be expected from the conditions of most mammalian cell culture processes (unpublished results). In the yeast strain *Pichia pastoris*, the ELP gene was initially unstable and tagged target proteins were difficult or impossible to express. We have addressed this problem through the re-engineering of the ELP gene for minimal repetition and lower GC nucleotide content. Although the re-engineered ELP gene is now stable in *Pichia* (manuscript in preparation), high-level expression of tagged, secreted target proteins has proven difficult. Collaborative work on expression of ELP tagged targets in other hosts is ongoing. In *E. coli*, we have noted that conventional IPTG induction with the ELP tag is effective in some cases, and surprisingly seems to be more dependent on the host strain and target protein than the ELP tag. Therefore we now recommend examining alternate expression strains, including those with native RecA activity, as well as IPTG induction when expressing new proteins.

Despite the power and simplicity of the ELP purification method, it still suffers from a few limitations. One of these is simply the large size of the tag itself, which is ultimately discarded at the end of the process. At over 700 amino acids, this tag is likely to create a significant metabolic burden on the expressing cells, and this will undoubtedly require additional raw materials to be added to the culture medium. In cases where the value of the purified product is low (e.g., commodity enzymes), and therefore the expense associated with fermentation and protein expression is significant, this may have a substantial impact on the cost of the purified product. Perhaps the most significant limitation of the ELP system is that the precipitation and recovery of the tagged target by centrifugation does not deliver as high purity as conventional affinity separations using tags and columns. This is primarily due to the co-precipitation of various contaminant proteins, as well as simple entrainment of the contaminants during centrifugation. Although the purity can be increased through the use of several precipitation steps and careful washing, yield losses will increase as a result. The most significant limitation at this point, however, is premature cleaving of the tag during expression in hosts other than *E. coli*. Thus the development of a more highly controllable intein would be a significant advance for this method.

FUTURE PERSPECTIVES

Self-cleaving affinity and purification tags have the potential to impact several areas in biotechnology and biomanufacturing. Their greatest strength is that they naturally lend themselves to high-throughput and platform processes, which makes them attractive for both research and large-scale production of a wide variety of proteins. Indeed it is this aspect of conventional tags that has made tag technologies ubiquitous in biology and chemistry laboratories throughout the world. The creation of a practical and reliable self-cleaving capability for these tags represents a major advance to this technology, and holds the potential to push these methods out of the laboratory and into large-scale applications in many biotechnology industries. To fully realize this potential, however, several additional developments must be made.

Perhaps the most important goal for self-cleaving tags is that the cleaving of the tags must be made more controllable. Ideally, the tags would show little or no cleaving during expression in a given host, but cleave very rapidly, and at low temperature, when desired. Although evolutionary methods can tune self-cleaving inteins to be generally faster or slower, these methods have not yet delivered an intein with an optimal combination of cleaving rates and conditions. Indeed, there may be no single optimal intein for all applications, potentially requiring individualized inteins to be developed for various expression hosts. Thiol-induced inteins, as well as some non-intein cleaving modules are currently the closest to the ideal, but each have their own limitations relative to other strategies (Table 1).

For large-scale and highly cost-sensitive applications, the focus will inevitably shift towards the purification tags and their associated processes. These tags must provide an opportunity to minimize buffer usage and equipment volume, while providing inexpensive chemistries and simple methods. Non-chromatographic tags eliminate the need for large packed beds and their associated scale limitations, but they have disadvantages as well. Primarily, entrainment or co-precipitation of contaminants decreases the purity of the target proteins. Although repeated precipitation and washing can alleviate this issue, these approaches will increase the complexity of the process and decrease yield. Because chromatography relies on specific partitioning of the tagged target onto an immobilized solid phase, which can then be washed extensively with minimal yield losses, it can provide very high yield and purity. This advantage suggests that a significant goal may be the development of cheaper affinity resins that can bind strongly at high capacity. This would provide high purity products, with the proven track record of chromatography, but at low cost and with the convenience of self-cleaving tags. The ability to regenerate these affinity resins efficiently will also decrease their cost of use, while also increasing their appeal as a general platform.

Issues surrounding the use of self-cleaving affinity tags in pharmaceutical applications are generally associated with validation of tag removal. These include both the actual cleavage of the tag from the target, and the complete removal of the cleaved tag from the final product. The ability to induce self-cleaving of a column-bound tag would seem to simplify these issues: the cleaved tag and any uncleaved target protein would remain bound to the column, and only the cleaved target would be recovered. A sufficiently strong affinity tag would thus effectively eliminate any chance of the tag or tagged target from contaminating the cleaved product. Although it is seemingly simple, this method would still require full validation, which will require significant investment before its adoption. This can be a critical weakness in the highly conservative pharmaceutical industry, where already established and proven methods are highly attractive from a regulatory standpoint. Perhaps ironically, increased interest in Quality by Design in the pharmaceutical industry may create a strong advocacy for self-cleaving tag methods. Because these methods are platform-oriented, they lend themselves to predictability in performance.

As with any new technology, widespread adoption of self-cleaving purification tags will require stepwise demonstration and validation of their capabilities. Published work in several laboratories has demonstrated their efficacy at laboratory scale in a variety of configurations, and they are being increasingly examined for larger-scale processes

(65,93–95). These studies suggest that various self-cleaving strategies can substantially decrease the consumable costs for affinity separations, but additional costs associated with specific products and processes in specific industries remain to be seen.

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