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Towards the elimination of chromatography in protein purification: Expressing proteins engineered to purify themselves

Courtney Mee, Mahmoud Reza Banki, David W. Wood *

Department of Chemical Engineering, Princeton University, Princeton, NJ 08544-5263, United States

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Abstract

The development of affinity-tag fusion technology in the mid-1980s provided a simple and convenient method for the purification of arbitrary recombinant proteins through genetic engineering. This method has recently been enhanced by the introduction of self-cleaving affinity tags based on engineered self-splicing inteins. The ability of these tags to release native product proteins from fusion precursors, in response to simple temperature or pH changes, has increased the appeal of this method for large-scale applications. We have recently developed two simple, cost-effective and scaleable protein purification technologies that exploit the self-cleaving reaction in novel, non-chromatographic contexts. In the first system, small granules of polyhydroxybutyrate (PHB) are produced in the expression host, which then act as an affinity carrier for the subsequently expressed target protein. The protein is tagged with a self-cleaving PHB-binding protein, allowing simple purification of the native product by simple granule washing and tag cleaving. In the second system, the target protein is tagged with a reversibly precipitating, self-cleaving elastin-like protein, allowing it to be separated from contaminants by simple cycles of precipitation and washing. The self-cleaving reaction again delivers a fully native, substantially purified product. Tests on several target proteins for both technologies show high purity and activity at reasonable yields, while initial economic analyses of these methods indicate a potential to dramatically reduce the cost of protein purification. Thus, the introduction of self-cleaving purification tags has enhanced an existing technology while enabling new technologies, and is likely to have a profound effect on the future of bioseparations.
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1. Introduction

The advent of recombinant DNA technology has greatly simplified heterologous protein expression, accelerating the study of existing proteins and the production of new proteins with enhanced features. However, large-scale recovery and purification of these proteins remains both difficult and expensive [1,2], while demands for highly purified active proteins in areas of medicine, biocatalysis and emerging technologies are increasing rapidly. To keep up with the growing demand, protein purification methods must be developed to be efficient, reliable and cost effective and must not require individual optimization.

Thus, the discovery of improved purification methods has the potential to impact the bioseparations industry significantly, and is a driving force for our research into new bioseparation methods. This review describes two recently developed self-cleaving aggregation tags, each of which begins with the genetic manipulation of the target protein at the DNA level [3–6]. These methods are reliable and inexpensive at small-scale, and circumvent the need for empirical chromatographic process development or expensive affinity columns. In each case, the aggregation tag sequesters the target protein into an insoluble phase, allowing it to be purified by simple centrifugation and washing. Subsequent self-cleaving of the aggregation tag delivers a purified native target in a soluble phase for simple recovery. These methods have been used to purify several test proteins at reasonable yields, and are the subject of our current research for scale-up.

2. Conventional affinity tag technology

Beginning in the 1980s, recombinant DNA technology allowed the construction of affinity tagged proteins, which

* Corresponding author. Tel.: +1 609 258 5721; fax: +1 609 258 0211.
E-mail address: dwood@princeton.edu (D.W. Wood).

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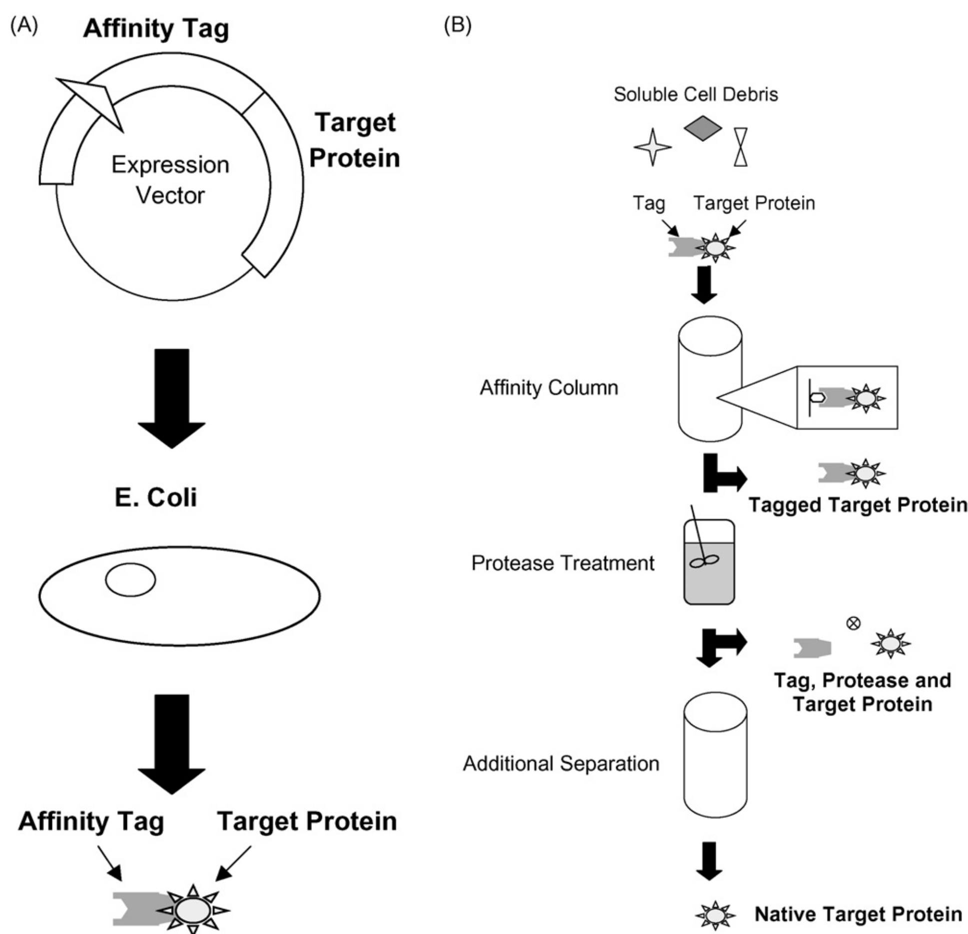

Fig. 1. (A) The production and purification of an affinity tagged target protein. A clone is constructed where the target protein gene is fused to an affinity tag gene in a suitable expression vector. In this example, the target is downstream from the affinity tag, but in principle, the tag can be on either end of the target. Expression of this gene fusion (in *E. coli* for example) results in the production of a two-domain fusion protein comprising the affinity tag and target separated by a suitable protease target sequence. Arrow symbol on the vector indicates an appropriate inducible promoter for protein expression. (B) A solution of soluble cell debris and the affinity tagged target protein is loaded onto an affinity column, where the fusion binds selectively to a ligand-functionalized resin. After contaminants are removed, the tagged target protein is eluted from the column. Treatment with protease cleaves the tag from the target protein in a batch step. An additional separation step is necessary to isolate the native target protein from the tag and protease.

provided a means to apply highly specific affinity separations to arbitrary target proteins [7–9] (Fig. 1A). In this method, conventional gene-fusion techniques are used to join the DNA encoding a target protein to the DNA encoding an affinity tag. Expression of the resulting gene fusion in an appropriate host cell yields a tagged target protein, which can be easily purified via specific binding of the affinity tag to an immobilized ligand (Fig. 1B). In most cases, the ligand is immobilized onto one of a number of commercially available resins, providing a ready means for the purification of the fusion protein [7,8]. Once the fusion is purified and recovered from the resin, treatment with protease cleaves the purified protein from the affinity tag [7,9,10]. The tag and protease are then removed in an additional separation step (Fig. 1B). Although conventional affinity technologies have greatly simplified protein purification at laboratory scale, the resins and buffers necessary for these processes are expensive. Furthermore, the additional expense of protease treatment can be prohibitive, especially at large-scale [11,12].

3. Self-cleaving affinity tags

During the 1990s, affinity tag purification technology was further simplified through the development of self-cleaving affinity tags [13–16], made possible through the discovery of a naturally occurring self-splicing protein element. A 1990-study of the VMA1 gene from *Saccharomyces cerevisiae* identified an embedded protein sequence within the VMA1 enzyme [17]. In a process known as protein splicing, this intervening protein sequence, or intein, removes itself and ligates the flanking segments, or exteins, to produce two separate protein products [18]. More than 200 intein sequences have been discovered to date, and about a third have demonstrated splicing capabilities in the laboratory [19]. Inteins are self-contained and many have been shown to splice in both foreign host cells as well as non-native host proteins. In general, these inteins can splice in many systems and require no cofactors, thus making them attractive for a number of applications in biochemical research [20].

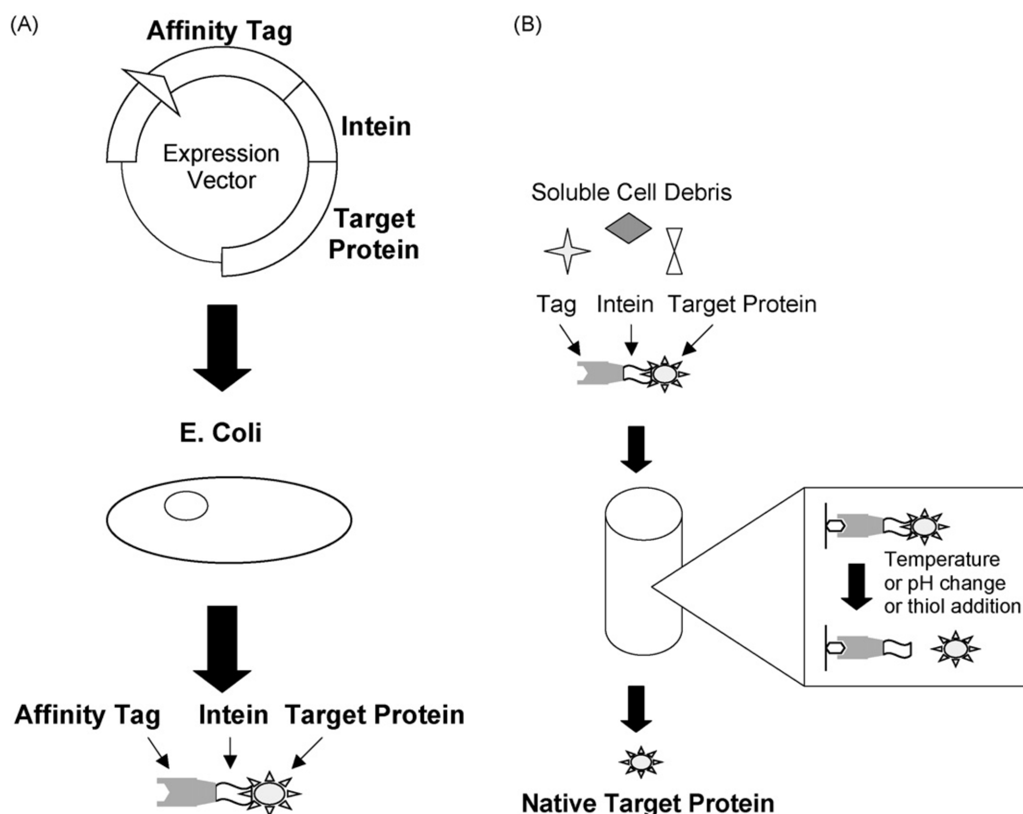

Fig. 2. (A) The production and purification of an intein-mediated affinity-tagged target protein. A clone is constructed with the target protein downstream from the affinity tag and intein. The location of the affinity tag and target protein relative to the intein can vary depending on the system used. As with conventional methods, expression of this gene fusion results in a tripartite fusion protein comprising the conventional affinity tag, a self-cleaving intein, and the target protein. (B) Soluble cell debris and the affinity tagged target protein are loaded onto an affinity column. Once the precursor is isolated from contaminants, cleavage of the intein is initiated. Target proteins cloned downstream from the intein (at the intein C-terminus) are typically cleaved by changes in pH or temperature [15,16], although thiol-induced C-terminal cleaving is used in the IMPACT-CN system [14]. Targets cloned upstream from the intein (at the intein N-terminus) are typically cleaved by thiol addition [13,16]. Once cleaved, the native target protein is recovered from the column while the affinity tag remains attached to the resin.

Genetically engineered mini-inteins that possess mutations at their intein-extein junction residues have allowed isolated N and C-terminal cleaving activities to be generated [21]. Combining affinity tags with these inteins has allowed the development of self-cleaving tags for use with arbitrary product proteins [13–16]. In practice, a gene encoding a self-cleaving intein is inserted between the genes encoding the target protein and a given affinity tag, and this fusion is expressed in an appropriate host cell (Fig. 2A). Using the specific binding of the affinity tag to an immobilized ligand matrix, as in traditional affinity technologies, the resulting tripartite precursor protein is purified. The intein is then induced to cleave off the target protein by either thiol addition, small shifts in pH and/or increases in temperature (Fig. 2B). A significant advantage is the ability to cleave the tag while it is still bound to the column, thus eliminating subsequent steps required for removal of the protease and cleaved tag. This method is the basis for the commercially available IMPACT-CN system from New England Biolabs [13,14], which is now commonly used for the recovery of highly pure and active proteins.

It is clear that intein-mediated purification systems alleviate some the problems associated with conventional affinity fusion technologies. Most significantly, the need for protease treatment and its accompanying removal step are completely eliminated by this technology, thus eliminating a major expense associated with this technique. However, conventional intein systems do not address another economic issue that hinders the implementation of this technology in large-scale processes: the high cost of the appropriate affinity resin is still unavoidable and perhaps prohibitive in large-scale commercial systems [11].

4. Self-cleaving aggregation tags

Despite the success of self-cleaving affinity-fusion purification methods, the need for expensive affinity columns remains prohibitive at large-scale. Available affinity resins can cost upwards of $1000/l of bed volume depending on the ligand and tag, and in almost all cases are substantially more expensive than conventional chromatographic resins. Some of these resins, furthermore, have low binding capacity for the tagged fusion protein, which also contributes to increases in process scale and cost [11,12,22]. Thus, researchers are driven to discover protein purification methods that eliminate the need for affinity columns completely. One approach is to combine C-terminal cleaving inteins with previously developed aggregation tags to develop powerful, simple and inexpensive purification methods

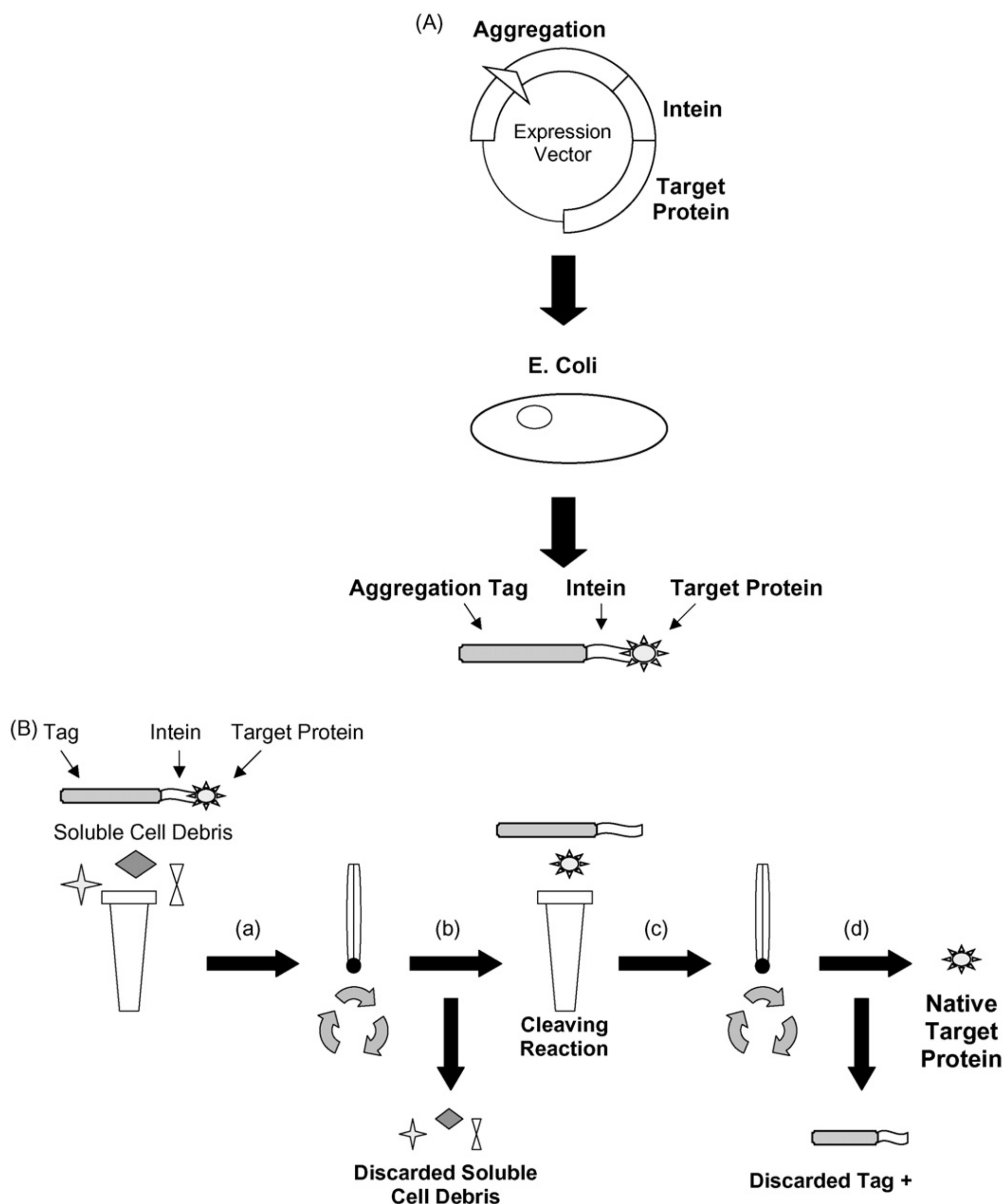

Fig. 3. (A) The production of an intein-mediated aggregation tagged target protein. A clone is constructed with the target protein downstream from the aggregation tag and intein. (B) Purification of target proteins with the use of a self-cleaving aggregation tag. (a) Soluble cell debris and the aggregation-tagged target protein are subjected to heat and/or centrifugation depending on the specific method [3–6]. (b) The supernatant, containing the soluble cell debris, is removed and the pelleted precursor is resuspended in a pH 6.0 buffer to initiate the cleaving reaction [3,4]. Alternately, the cleaving reaction can be induced by thiol addition [5,6]. (c) The resulting sample undergoes another round of heat and/or centrifugation as before for each method to separate the cleaved tag from the soluble product. (d) The pellet containing the cleaved tag and intein is then discarded. The resulting supernatant contains the purified native target protein.

[3–6]. These systems utilize tripartite fusions comprising one of two aggregation tags, combined with a self-cleaving intein, fused to a given target protein (Fig. 3A). This fusion precursor is over-expressed in *E. coli* [3,4,6] or *Ralstonia eutropha* [5] and can then be isolated and purified by cycles of centrifugation and resuspension in coordination with the established cleavage reaction (Fig. 3B).

5. The PHB system

To eliminate the need for an affinity resin, a naturally occurring granular material was sought that could be easily produced in various expression systems. The likely candidate, polyhydroxyalkanoic acid (PHA), is very well studied and is believed to provide a means for certain bacterial strains to store carbon in the absence of oxygen, nitrogen or phosphorus [23,24]. Polyhydroxybutyrate (PHB), the most common PHA polymer, has the chemical composition $–[O–CH(CH_3)CH_2CO]_n–$, and is synthesized through the action of three well-characterized enzymes [25,26]. PHB is typically produced in a granular form in the cytoplasm, with particles sizes on the order of one micron [25,27,28]. Further, PHB granules have been successfully produced in a variety of expression hosts, including many bacterial and yeast systems [29–32] in addition to transgenic plant cells [33–35].

The size and density of PHB granules allows their easy recovery from cell lysate through a variety of mechanical processes, such as centrifugation or filtration, making PHB an ideal candidate for purification technology. Finally, small proteins known as phasins are available with strong and specific affinity for PHB granules [36–38], and it has been demonstrated that PHB can act as an effective affinity carrier for phasin-tagged fusion proteins [36,39,40].

By combining PHB granules and self-cleaving phasin tags, an economically beneficial alternative to traditional affinity-based purification methods has been developed [4,5]. As described in the method by Banki et al. [4], PHB granules and a phasin-intein-tagged protein are coproduced from two plasmids in *E. coli* cells, where the tagged protein becomes attached to the granules through the phasin tag. The cells are lysed, and the granules and bound target protein are purified through a series of centrifugation and resuspension steps. An induced pH shift initiates cleaving of the purified PHB-bound precursor, resulting in a mixture of insoluble PHB-intein components and the soluble target protein. A final round of centrifugation removes the granules and bound tags, allowing the recovery of the purified target protein from the supernatant.

Research on several target proteins indicates reasonable product yields at small-scales, which are competitive with those of conventional affinity fusion technologies. This method has also been shown by Barnard et al. to be effective in *R. eutropha*, where PHB granules are produced naturally [5]. In *Ralstonia*, overexpression of the target fusion is induced by simple phosphate limitation, while the facultative anaerobe character of this strain allows extraordinarily high cell densities in fermentation. The effectiveness of the PHB method, its expected scale-up potential and its potential for use in a variety of protein expression systems is substantial. The easy mechanical recovery of PHB granules in the self-contained system alleviates much of the economic strain of traditional affinity-fusion purification technologies [11].

6. The ELP system

Much like the PHB system, the Elastin-like Polypeptide (ELP) system provides effective protein purification at reasonable yields without the need for an affinity column. Elastomeric polypeptides consisting of the basic gene unit VPGVG were first studied in 1992 [41]. The unique thermally-induced phase transition behavior of this biopolymer made it an ideal candidate for protein purification research. ELPs comprised of repeat motifs of VPGXG, where X is any amino acid with the exception of proline, are highly soluble at room temperature, but fall out of solution at a transition temperature of around 30–40 °C [41–45]. The exact transition temperature (T_t) of a given ELP is dependant on a number of factors including chain length, concentration, and the types and concentrations of ‘X’ residues. In general, increased chain length or salt concentration lowers the T_t, allowing some latitude in optimization of ELP-based processes. Further, previous investigators have shown that ELP tags can be used to purify fused target proteins in an active form, thus indicating that the precipitation of the tag does not inactivate the target [43,44,46].

In 2005, two groups demonstrated that protein purification using a self-cleaving ELP tag is possible using similar techniques to the PHB purification strategy [3,6]. In this method, the desired protein is genetically fused to a self-cleaving ELP-intein tag and overexpressed in *E. coli*. Unlike the PHB system, in which affinity interactions bind the phasin portion of the tripartite precursor to solid granules, the ELP system relies on self-association of the ELP tags for phase partitioning. Following cell lysis, centrifugation at a temperature below T_t allows separation of the insoluble cell debris from the soluble cell lysate, which includes the soluble ELP-intein tagged protein. Raising the temperature to T_t in the presence of salt induces precipitation of the ELP fusion, allowing it to be separated from the remaining soluble cell debris through another round of centrifugation. Once purified, the ELP fusion precursor is re-solubilized at a temperature below T_t in a pH 6.0 buffer. This initiates self-cleaving of the ELP-intein portion, which can then be removed by another cycle of salt addition, heating above T_t, and centrifugation. The soluble, native product protein is then recovered in the final supernatant.

Like the PHB system, the ELP system allows the simple purification of active proteins from cell lysate and eliminates the need for expensive chromatography. The ELP system, however, generally results in higher protein yields and is potentially a more flexible system due to the fact that PHB expression is not required for the separation. This system performs under a broad range of conditions, including salt concentration to vary T_t and a wide range of pH and temperatures to influence the cleavage reaction. Further, the use of ELP tags with thiol-induced cleaving inteins has also been shown by Ge et al. that have the potential to alleviate premature cleavage of the target in some systems [6]. The simplicity of this self-contained system also suggests a variety of scale-up processes for the recovery of the precipitated fusion, such as filtration or continuous centrifugation.

7. Future prospects

The PHB and ELP systems provide simple and economical means for purification of recombinant proteins. Reliable at small scale, they have been shown to effectively isolate a variety of arbitrary proteins and both exhibit a wide flexibility in buffer choice and temperature conditions. Most importantly, the ELP and PHB systems are competitive with conventional purification methods in overall processing time and cost [11]. Notably, a rough economic evaluation has illustrated a potential 125-fold decrease in annual materials cost for the PHB and ELP purification methods in comparison to the maltose-binding protein fusion with proteolytic tag removal (the New England Biolabs pMal fusion system) and an 11-fold decrease in comparison with the His-tagged purification without tag removal [11]. In comparison to the commercially available IMPACT-CN system, the intein-mediated ELP and PHB methods demonstrate up to a 13-fold decrease in materials cost, due primarily to higher yields and the elimination of the affinity resin and associated costs.

These processes promise an exciting economical alternative to conventional purification techniques at large scale as well as for applications in high-throughput proteomic research. Methods of scale-up could potentially include tangential flow fil-

tration or a continuous centrifugation process. With tangential flow filtration, process time is a concern, as removing contaminants from the system can be difficult due to cake formation and other typical membrane-associated difficulties. We have also observed that the concentration of ELP can strongly effects its solubility, which can lead to precursor losses as some dissolution takes place during processing (unpublished results). Continuous centrifugation, on the other hand, requires a larger capital investment initially, but may be more cost effective over time. Since both of the self-cleaving aggregations tag methods are quite novel, where small-scale proof-of-principle experiments have only been published within the past year, little work has been completed in characterizing their scale-up potential and optimization. This will clearly be an exciting area of research over the near future.

The PHB and ELP intein-mediated systems are also ideal candidates for use in a variety of expression systems. Research into their application in mammalian cells could open a world of possibilities for protein purification for pharmaceutical use. These two systems could further be simplified with the development of improved inteins, with faster and more controllable cleaving rates. Indeed, this may be necessary for their use in mammalian systems, where expression conditions are likely to induce premature cleaving of pH-controlled tags, while induction of cleaving by thiol might cause misfolding of complex proteins by reducing their disulfide bonds [47]. However, genetic selection systems can help to identify suitable mutant inteins with desirable characteristics, and research into the development of more controllable inteins is ongoing in several groups [48–50]. The simplicity and potential for scale-up and high-throughput applications make the intein-mediated PHB and ELP systems ideal for future research projects, and may ultimately have a substantial impact in bioseparation technology in applications ranging from laboratory to manufacturing scales.

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