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METHODS AND PROTOCOLS

A dual ELP-tagged split intein system for non-chromatographic recombinant protein purification

Changhua Shi · Qing Meng · David W. Wood

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Abstract Self-cleaving elastin-like protein (ELP) tags provide a very promising tool for recombinant protein purification. With this method, the target protein is purified by simple ELP-mediated precipitation steps, followed by self-cleavage and removal of the ELP tag. Unfortunately, however, inteins usually experience some level of pre-cleavage during protein expression, which can significantly decrease final yields. In this study, we solve this problem by splitting the intein into two ELP-tagged segments. Each segment is incapable of pre-cleavage alone, but the assembled segments release the target protein rapidly when assembled in vitro. The result is the very tight control of the tag cleaving reaction, combined with the simplicity of the ELP purification method. Using this system, we successfully purified four different sizes of target proteins with final yields comparable to or higher than our original contiguous intein–ELP system. Further, we demonstrate a streamlined split intein method, where cells expressing the tagged intein segments are combined prior to cell lysis, allowing the segments to be co-purified in a single reaction mixture.

Keywords Elastin-like protein · Split intein · Protein purification · Self-cleaving tag

Introduction

Inteins are protein domains that can self-excise from precursor proteins while splicing together the flanking precursor segments with a native peptide bond (Perler 2002; Paulus 2000; Gogarten et al. 2002). By mutating specific amino acids near the splice junctions, inteins can be converted into self-cleaving elements. In these modified inteins, cleavage takes place at one or both splice junctions and is typically controlled by pH or thiol reagents (Mathys et al. 1999; Chong et al. 1997). Many conventional affinity tags have been successfully combined with inteins to generate self-cleaving affinity tags, including chitin-binding domain (Evans et al. 1999) and maltose-binding protein (MBP) (Xu et al. 1999). In most of these cases, however, the engineered inteins usually exhibit some level of pre-cleavage in vivo, which can significantly impact the yield and productivity of the purification process (Cui et al. 2006; Banki et al. 2005). High levels of pre-cleavage are protein dependent and are increased at higher expression temperatures, which decrease the appeal of intein technology at large scale.

An important recent advancement has been the combination of elastin-like proteins (ELPs) with self-cleaving inteins to provide a rapid and simple non-chromatographic method for protein purification (Banki et al. 2005; Ge et al. 2005). In this case, the ELP tag selectively precipitates under mild conditions, allowing simple purification of the fused target protein. Although this method is convenient, inexpensive, and highly effective, it also suffers from limitations arising from premature cleaving in vivo during protein expression. Split inteins, whose activities depend on recognition and assembly of two complementary fragments, offer a potential solution to this problem (Gogarten et al. 2002; Wu et al. 1998).

Using available structural information and the rapidly cleaving ΔI-CM intein (Van Roey et al. 2007; Derbyshire et al. 1997; Wood et al. 1999; Banki et al. 2005), we have generated a highly efficient self-cleaving system for use with ELP tags. The engineered ΔI-CM mini-intein was split into a 110-amino acid N-terminal fragment and a 58-amino acid C-terminal fragment. Each fragment is fused to an ELP tag, while the C-terminal fragment is also fused to the target protein of interest (Fig. 1a). Thus, the target protein fused to

C. Shi · Q. Meng (✉)
Institute of Biological Sciences and Biotechnology,
Donghua University, Shanghai 201620,
People's Republic of China
e-mail: mengqing@dhu.edu.cn

C. Shi · D. W. Wood (✉)
Department of Chemical and Biomolecular Engineering,
Ohio State University, Columbus, OH 43210, USA
e-mail: wood.750@osu.edu

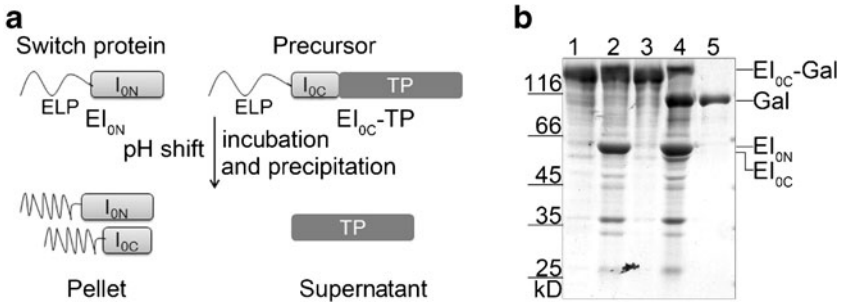

Fig. 1 Illustration of the dual ELP-tagged split intein system. **a** Schematic representation of the dual-tag system as described as in the text. **b** Purification of the β-galactosidase by the dual ELP-tagged split intein system. *Lane 1*, purified β-galactosidase target tagged with ELP and C-terminal segment of split mini-intein at 0 h; *lane 2*, mixture of tagged β-galactosidase precursor and switch protein at 0 h; *lane 3*, purified β-galactosidase target tagged with ELP and C-terminal segment of split mini-intein after 12 h of incubation at room temperature; *lane 4*, mixture of tagged β-galactosidase precursor and switch protein after 12 h of incubation at room temperature; *lane 5*, purified target protein after precipitation of the ELP-tagged intein segments

the C-terminal intein fragment and ELP tag becomes the "precursor protein," while the N-terminal intein fragment becomes the "switch protein." The cleaving activity of the truncated intein in the precursor protein is tightly repressed, even when the precursor is expressed at 37 °C, but is restored by addition of the switch protein. We further observed that overall cleavage efficiency is significantly higher when the precursor and switch protein are purified together than when they are purified separately and recombined. Although the cleaving efficiency of this dual ELP-tagged split intein system is slightly lower than that of the *cis*-cleaving ΔI-CM intein, there is no pre-cleavage in vivo, leading to final yields that are comparable to or higher than the contiguous intein. Furthermore, our study suggests that the recognition and association of the intein fragments is strong, even though there is a large ELP tag fused to each intein segment. Importantly, this dual ELP–intein technology has the potential to be applied in many other systems, including yeast and mammalian cell expression hosts commonly used in biopharmaceutical manufacturing.

Materials and methods

Plasmid construction

Plasmids encoding target proteins tagged with the C-terminal intein fragment and ELP purification tag (ELP-I_{0C}-TP) were constructed by deletion of the N-terminal 110 amino acids from our original ΔI-CM intein plasmids, pET/EI-GFP (GFP, green florescent protein), pET/EI-Gal (β-galactosidase), pET/EI-Lac (β-lactamase), and pET/EI-MBP (maltose-binding protein) (Banki et al. 2005). Further, a GSGS glycine–serine linker was introduced between each intein fragment and its ELP tag. To create the precursor protein expression plasmids, two primers, 5′-GGTCGAATTCGGCAGTGGATCCCGCGTGCAGGCGCTCGCGGATGCC-3′ and 5′-GTGCATGTTGTGTACAACAAC-3′, were used to amplify the sequence corresponding to the last 58 amino acids of the ΔI-CM mini-intein (corresponding to amino acids 383–441 of the full length *Mtu* RecA intein). The PCR products were digested with *Eco*RI and *Bsr*GI and ligated into the original full-size intein expression plasmids, resulting in pET/EI_{0C}-GFP, pET/EI_{0C}-Gal, pET/EI_{0C}-Lac, and pET/EI_{0C}-MBP. To create the ELP-tagged switch protein expression plasmid, the N-terminal 110 amino acids of the ΔI-CM mini-intein were amplified by two primers: 5′-GATTTCAGAATTCGGCAGTGGATCCGCCCTCGCAGAGGGCACTCGGATC-3′ and 5′-ACCTCTAGATCAGCCAGACGCCGGAATCGGCGC-3′. The PCR products were digested with *Eco*RI and *Xba*I and ligated into the pET/ELP backbone plasmid, replacing the original intein-target protein fusion to result in the switch protein plasmid, pET/EI_{0N}.

Protein expression and purification

All protein expression experiments were performed in the *Escherichia coli* strain BLR(DE3) (F − *omp*T *hsd*SB(rB−, mB−) *gal dcm* (DE3)). Transformed cells containing the expression plasmids were cultured in 5 ml Luria broth media supplemented with 100 μg/ml ampicillin overnight at 37 °C. Overnight cultures were diluted 1:100 (*v*/*v*) into Terrific broth media (12 g tryptone, 24 g yeast extract, 2.31 g KH_2PHO_4, 12.54 g K_2HPO_4 per liter) supplemented with 100 μg/ml ampicillin in shake flasks. The cells were then incubated at 37 °C for another 3–4 h, to an OD_{600} of ~0.8 and induced with isopropyl β-D-1-thiogalactopyranoside at either 37 °C for 3 h or 25 °C for 24 h.

Protein purifications were performed according to our previously reported method (Fong et al. 2009). Briefly, harvested cells were resuspended at 20 % (*w*/*v*) in low-salt lysis buffer (10 mM Tris–HCl, pH 8.5, 2 mM EDTA) and lysed by sonication using two to four pulses of 20 s each at 0.5 W RMS. The cell lysate solutions

were clarified by centrifugation at 2,100×*g* and 4 °C for 10 min. The ELP fusion proteins were precipitated by adding ammonium sulfate to a final concentration of 0.4 M and recovered by centrifugation at room temperature for 6 min at 1,600×*g*. The precipitated fusion proteins were then redissolved in low-salt lysis buffer and precipitated again to completely remove remaining contaminant host cell proteins. The purified fusion proteins were then resuspended in a low-pH cleavage buffer (PBS supplemented with 40 mM Tris–Bis, pH 6.2, 2 mM EDTA). The purified C-terminal fusion precursors (EI_{0C}-TP) were mixed with the purified N-terminal switch protein (EI_{0N}) at a molar ratio of 1:2 and incubated at room temperature. For the cleaving kinetics assay, cleaving reaction samples at different time points were stopped by adding sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer heating to 100 °C for 10 min. For the streamlined one-step purification, *E. coli* cells expressing the N-terminal switch protein (EI_{0N}) and the C-terminal fusion proteins (EI_{0C}-TP) were mixed at a ratio of 2:1 (*w*/*w*) before lysis and purified using the above method. In each case, samples were taken over the course of incubation and analyzed using SDS-PAGE.

Protein activity assays for green fluorescent protein, β-galactosidase, β-lactamase, and maltose-binding protein are shown elsewhere (Banki et al. 2005; Fong et al. 2009). Protein concentrations were determined by Bradford protein assays (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard. The cleavage efficiencies were analyzed by scanning the densitometry (ImageJ 1.46r, http://imagej.nih.gov/ij) of the precursor and the cleavage product bands on SDS-PAGE. The cleavage efficiency was calculated according to the following equation:

$$\text{Cleavage efficiency} = \frac{[\text{Cleavaged target protein}]}{[\text{Cleavaged target protein}] + [\text{Uncleaved precursor}]}$$

All assays were performed in triplicate.

Results

Two-step purification

The switch protein (EI_{0N}) and precursor (EI_{0C}-Gal) were initially expressed, purified separately, and then mixed together to induce *trans*-cleavage in vitro. As expected, the C-terminal fragment was observed to lose cleaving activity in vivo, leading to high-level expression of uncleaved EI_{0C}-Gal precursor protein. Both the tagged target protein and switch protein were purified using two cycles of ELP precipitation, using 0.4 M ammonium sulfate to induce precipitation at room temperature. As shown in Fig. 1b, no prematurely cleaved tag appears on the gel (Fig. 1b, lane 1), and no cleavage products can be detected after 12 h of incubation at room temperature in the absence of switch protein (Fig. 1b, lane 3).

The purified precursor and switch proteins were then mixed and incubated for 12 h at room temperature, and the reactions products were analyzed via SDS-PAGE (Fig. 1b, lane 4). After incubation and cleaving, the cleaved EI_{0C} tag and switch protein were separated from the β-galactosidase target protein by an additional ammonium sulfate precipitation (Fig. 1b, lane 5). As expected, the intein segments assembled and cleaved, allowing the β-galactosidase target protein to be efficiently purified. Notably, the observed intensity of the cleaved tag (EI_{0C}) band appears lower than the target protein band (Gal) on Coomassie-stained SDS-PAGE. We attribute this disparity to the high concentration of glycine in the ELP tag, which has been shown to have low staining efficiency relative to arginine, tyrosine, lysine, and histidine (de Moreno et al. 1986).

To optimize the cleavage reaction time, the *trans*-cleaving kinetics of the dual ELP system was studied. The cleavage reaction was prepared as before, and at different time points, the reaction was stopped by adding SDS-PAGE loading buffer and heating the sample. The cleavage rate was highest during the initial 3 h but stopped after 7 h at room temperature with a final cleavage efficiency of approximately 75 % (Fig. 2a). The cleaving rate constant (K_{obs}) was calculated according to the pseudo-first-order reaction equation, $P=P_0(1-e^{-kt})$ (Nichols et al. 2003). K_{obs} is determined to be 0.65 h^{-1}, which was comparable to a previously reported *trans*-cleavage K_{obs} induced by small peptides and faster than the ΔI-CM mini-intein, although the total cleavage efficiency was lower (Volkmann et al. 2009; Wood et al. 2000).

To demonstrate general applicability of our dual ELP-tagged split intein system, three additional proteins of various sizes (green fluorescent protein, β-lactamase, and maltose-binding protein) were also purified (Fig. 2b). All three additional target proteins could be purified similarly to the β-galactosidase target protein, where each exhibited no pre-cleavage in vivo but cleaved rapidly in the presence of the switch protein. Notably, the final cleavage efficiencies of the smaller target proteins (GFP and β-lactamase) were lower than those of the larger target proteins (β-galactosidase and MBP) (Table 1). We hypothesize that larger, highly soluble target proteins aid the folding and increase the

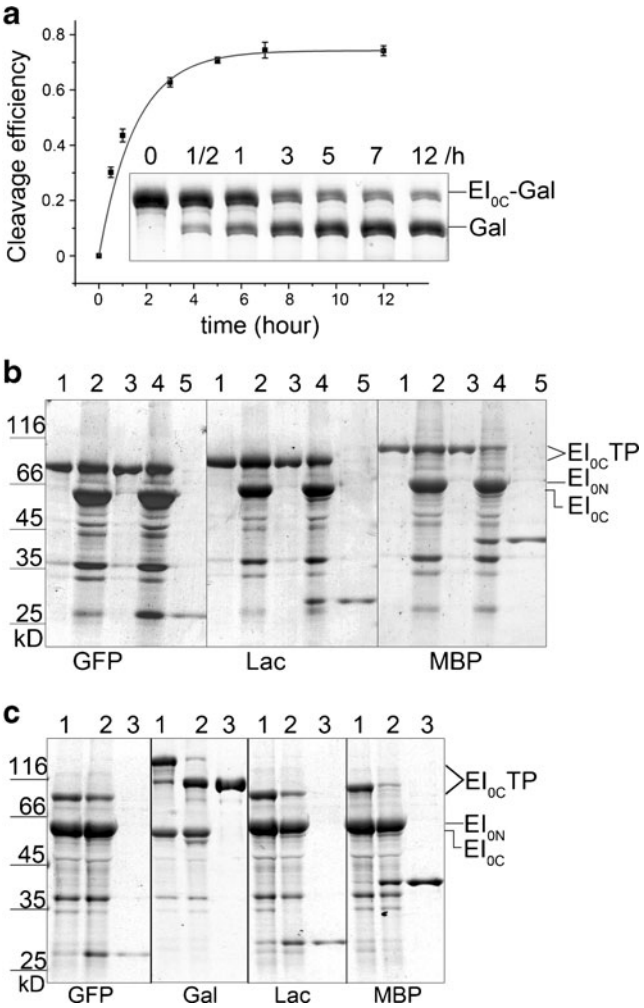

Fig. 2 Optimization of the dual ELP-tagged split intein system. **a** The cleavage kinetics of the dual ELP-tagged split intein system at room temperature for the β-galactosidase target protein. **b** Purification by two-step method. *Lane 1*, purified precursor proteins tagged with ELP and C-terminal intein segment (EI_{0C}-TP) at 0 h; *lane 2*, mixture tagged target protein (EI_{0C}-TP) and switch protein (EI_{0N}) at 0 h; *lane 3*, purified precursor proteins tagged with ELP and C-terminal intein segment (EI_{0C}-TP) after 7 h of incubation at room temperature; *lane 4*, cleavage reaction products after 7 h of incubation at room temperature; *lane 5*, purified target protein after precipitation of cleaved EI_{0C} tag and EI_{0N} switch protein. **c** Purification by one-step method. *Lane 1*, mixture tagged target protein (EI_{0C}-TP) and switch protein (EI_{0N}) at 0 h; *lane 2*, cleavage reaction products after 7 h of incubation at room temperature; *lane 3*, purified target protein after precipitation of cleaved EI_{0C} tag and EI_{0N} switch protein

stability of the intein fusion protein, which can enhance the cleavage reaction.

One-step purification

Although the split intein system successfully suppressed pre-cleavage during protein expression in vivo, the requirement for two separate purifications is likely to increase the cost of production. Therefore, a one-step method was developed that involves purification of the switch protein and precursor together in a single reaction. This is accomplished by expressing the tagged target and switch proteins in separate cell lines as before, but then mixing the cells before lysis, thus allowing the target and switch protein to be purified together. Since the ΔI-CM intein is pH and temperature sensitive, it is possible to assemble and purify the two intein fragments at high pH without the pre-cleavage and induce cleavage of the assembled complex using a low-pH buffer.

As expected, there were no significant cleavage bands in the mixed precursors, and all four of the target proteins could be purified successfully at high yield using a single purification reaction of the assembled target and switch proteins. Notably, however, the final cleaving efficiencies of all four target proteins were higher than when they were purified using the two-step method (Fig. 2c, Table 1). As with the two-step purification method, the cleavage efficiencies were also observed to be higher for larger target proteins than the smaller ones. After 7 h of incubation at room temperature under cleaving conditions, the cleavage efficiency of GFP reached 55 %, which is almost three times the two-step purification. Remarkably, the ultimate cleavage efficiencies of the large test proteins (β-galactosidase and MBP) were higher than 90 %. Further, the yields of the target proteins purified by the one-step method also increased, with the yields of β-galactosidase increasing from 114.1 μg/ml (total purified protein/shake flask fermentation volume) using the two-step method to 352.9 μg/ml using the one-step method (Table 1).

Activity assay of the target protein

To explore whether the dual ELP tag purification methods will affect the activity of the final products, the target protein activities were assayed (Table 1). The activities of the target proteins from the two-step and one-step purification methods were similar and were close to the specific activities observed in previous reports (Banki et al. 2005; Fong et al. 2009).

Stability of the split intein in vivo

A significant drawback of the contiguous ΔI-CM intein is the requirement for low-temperature expression to minimize premature cleaving in vivo. This requirement can decrease expression efficiency in microbial hosts and has effectively prevented the use of pH-controlled inteins in mammalian cells. To explore the potential application of the dual ELP-tagged split intein system in mammalian expression hosts, we expressed the precursor and switch proteins for 3 h at 37 °C and analyzed whole cell lysates via SDS-PAGE. To compare suppression of premature cleavage during expression, the contiguous ΔI-CM intein was used as a control. With the dual ELP-tagged split intein system, no pre-

Table 1 Summary of the purification results from two different methods in the dual-tag split intein system

Products	Cleavage efficiency (%)		Yield (μg/ml)		Activity (units/mg)	
	Two steps	One step	Two steps	One step	Two steps	One step
GFP	19.6±5.5	55.3±8.2	40.6±4.5	63.2±7.9	17,335±1,080	18,053±1,010
Gal	68.8±1.1	90.5±4.2	114.1±19.6	352.9±15.5	721±185	769±190
Lac	59.3±2.0	70.1±5.3	51.8±11.9	61.3±4.1	1,526±173	1,633±105
MBP	78.5±2.7	97.3±1.2	55.1±3.9	95.0±6.2	Binds maltose resin	

cleavage is observed via SDS-PAGE, while the premature cleavage of the full size intein was 10–30 % depending on the target protein (Fig. 3). When the expression time was extended to 12 h at 37 °C, no premature cleavage products were observable with the split intein. Further, target proteins expressed for short and long times could be similarly purified by the one-step and two-step methods (data not shown).

Discussion

In this work, we applied a dual ELP-tagged system based on the split ΔI-CM intein to successfully resolve premature cleavage of self-cleaving intein tags during protein expression in vivo. The N-terminal fragment of the original ΔI-CM intein was removed and separately expressed with an ELP tag, resulting in a switch protein for activation of the *trans*-cleavage reaction. This change allows the cleavage reaction to be tightly repressed during protein expression in vivo but still provides rapid, pH-sensitive cleavage in vitro during the protein purification process. Because both fragments are tagged with the ELP protein, the method provides a ready means for removing the cleaved tag and switch protein once the purification process is complete. Finally, the high stability of the tagged precursor protein at 37 °C suggests that this method may eventually be applied to expression of complex glycoproteins in mammalian host cells.

It was unexpected that the cleaving efficiencies and the final yields from the one-step purification method were significantly higher than the two-step method. We hypothesize that chaperon proteins in the cell lysates may attribute to this. The active site of the ΔI-CM intein is surrounded by two large β-sheet rings, which include sequences from the N-terminal and C-terminal segments (Fig. 4a). Although the N-terminal fragment dominates this structure (Van Roey et al. 2007), removal of the C-terminal fragment would result in significant destabilization of the remaining β-sheet ring (Fig. 4b). This hole cannot close to form a functional cleaving element without the complementary fragment, so the open core of each intein segment provides an opportunity for chaperons to bind the unfolded intein segments, protecting them from irreversible aggregation (Song et al. 2012). Once assembled, the intein sheds the chaperons and other nonspecifically associating proteins, yielding more highly purified precursor proteins. The cleaving reaction is then controlled by pH and temperature, similarly to the contiguous ΔI-CM intein. Separate purification of the N-terminal and C-terminal intein segments, on the other hand, causes the chaperons to reach into the central hole of each intein

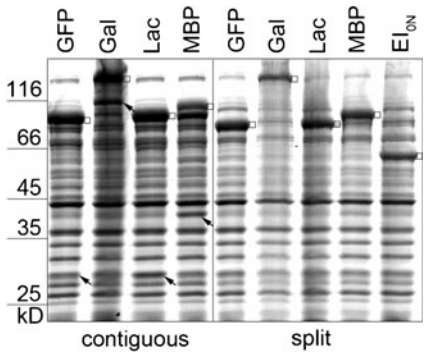

Fig. 3 Stability of the ELP-tagged target protein during expression in vivo. The *left panel* (*contiguous*) shows target proteins tagged with ELP and the full ΔI-CM mini-intein. The *right panel* (*split*) shows targets tagged with ELP and the C-terminal segment of the split mini-intein. Cells were induced to express protein at 37 °C for 3 h, and whole cell lysates were directly resolved via SDS-PAGE. Target proteins are indicated at the top of each lane. Uncleaved precursor proteins are indicated by *empty squares*, while cleaved target proteins are indicated by *arrows*. The EI_{0N} switch protein is shown at *far right*

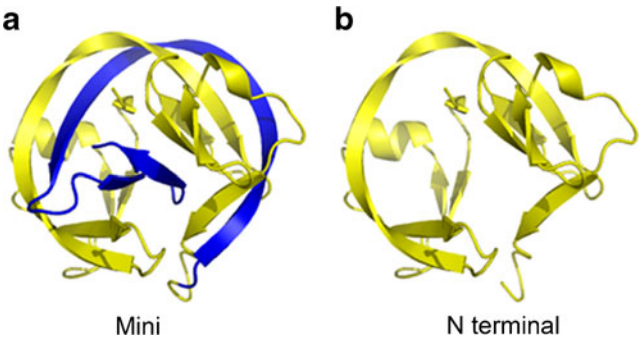

Fig. 4 The structure of the ΔI-CM mini-intein (Protein Data Bank ID#: 2IN0), derived from the full-length *Mtu* RecA intein through deletion of the endonuclease domain. **a** The N-terminal (*yellow*) and C-terminal (*blue*) segments are highlighted, showing their relative contribution to the overall β-sheet structure. **b** The N-terminal fragment of the split ΔI-CM mini-intein is shown

fragment without the complementary fragment, leading to increases in apparent impurities in the purified precursor and switch proteins and decreasing the overall efficiency of the assembly and cleaving reactions. This effect is reflected by the purity of the precursors purified by the two methods (Fig. 2b, c), where the precursor and switch proteins purified using the single-step method are visibly more pure than those purified with the two-step method. The sizes of the tag and target protein also have an effect on the stability of the split intein segments, where larger target proteins appear to further stabilize the unassembled intein segments, leading to more efficient cleavage and higher yields.

Compared to the contiguous intein, there are many advantages of our dual ELP-tagged split intein system. In contiguous systems, pre-cleavage of pH-controlled inteins in vivo is unavoidable, even at low expression temperatures. Spontaneous cleavage in vivo decreases final yields and can increase product costs, especially for valuable proteins used in laboratory studies or in industrial applications. This difficulty is compounded by the fact that optimal expression temperatures in many host cells tend to be fairly permissive for intein cleaving, leading to further increases in premature cleaving. Thus, a critical advantage of the dual ELP-tagged split intein system is the complete repression of pre-cleavage during in vivo expression, even at 37 °C for 12 h. The purified precursor and switch proteins are also stable in vitro, which will expand the application of self-cleaving intein tags. A significant impact will likely be in the area of mammalian cell expression, where expression hosts require a higher expression temperature and lower pH and have therefore exhibited unacceptable pre-cleaving with contiguous pH-triggered intein tags (data not shown).

The dual ELP-tagged split intein system also shows many advantages over other reported *trans*-cleavage purification systems. In a previous report, Volkmann et al. developed a *trans*-cleavage system, where cleaving was induced through the addition of a small peptide (Volkmann et al. 2009). Although it partially resolved the pre-cleavage of a continuous inteins in vivo, this system requires an expensive peptide and an additional step for removing the small peptide. These two factors cannot be ignored when applying the split intein purification system on a large scale. In the new dual ELP-tagged split intein system, both the precursor and the switch protein can be purified at low cost using ELP purification, and removal of the switch protein and cleaved tag can also be accomplished through simple precipitation. An additional split intein purification system was constructed by Lu et al. (2011), where the affinity between two segments of a split intein was used as part of the purification process. In this case, one intein segment was fused to a tag and bound to an affinity matrix, while the other intein segment was fused to the target protein. Recognition and binding of the two intein fragments induces cleaving, while affinity between the two segments keeps them both bound to the column and provides a highly purified target protein. The capability of this system is limited, however, by the ability of the two intein fragments to recognize each other and fold, and binding affinity between the segments is typically not sufficient to prevent some intein segment from being co-eluted with the target protein. However, our system is based on recovery of the catalytic center between the two separate intein fragments in solution, which improves recognition and cleaving capability. Further, conventional affinity tags and resins were used in both of the former methods, which will additionally increase the cost of the purification process. The non-chromatographic ELP tag used in our *trans*-cleavage system has many advantages in protein purification (Meyer et al. 2001; Meyer and Chilkoti 1999), including low-cost and easy scale-up.

Although the final cleavage efficiency of the dual ELP-tagged split intein system is slightly lower than the full-size intein, the finally yield is comparable to or higher than our original full-size intein (compare Table 1 to (Banki et al. 2005)). Because there is no pre-cleavage in vivo, even with extended expression at 37 °C, more tagged precursor can be harvested than in the contiguous intein system. In other previously reported *trans*-splicing or *trans*-cleaving systems, there is often no tag or only a small tag between the two intein fragments. In this system, we applied a relatively large ELP tag between the two intein fragments and showed that they can successfully recognize each other and recover pH-sensitive cleaving activity similar to the contiguous intein. Indeed, the enhanced ability of the intein segments in this system to recognize each other and assemble may be partly attributable to fusion with the highly soluble ELP tag. The result is stabilization of the intein fragments, which allows subsequent assembly at high efficiency.

Although the ELP tag was studied here, it is likely that other suitable affinity tags might show similar performance if applied in this system. Furthermore, because of the tight control of the cleavage reaction in *trans*, this system might also be applied to the mammalian and other eukaryotic expression systems that require higher expression temperatures. Thus, our new dual ELP-tagged split intein system is likely to expand the application of this economical and convenient self-cleavage tag.

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Conflict of interest The authors declare that they have no conflict of interest.

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