Simple bioseparations using self-cleaving elastin-like polypeptide tags

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We introduce a new method for the purification of recombinant proteins expressed in *Escherichia coli* using self-cleaving elastin-like polypeptide (ELP) fusion tags without the need for affinity chromatography or proteolytic tag removal. Using this method we obtained high purity, activity and reasonable yields for ten diverse target proteins.

Development of simple and reliable methods for protein purification is an important goal for both large-scale production of purified recombinant proteins and high-throughput proteomics studies of peptide libraries. Self-cleaving ELP tags consisting of repeating pentapeptides of VPGXG (X = any amino acid) fused to a controllable self-cleaving intein have the potential to be an essential tool in bioseparations. ELPs can be designed to spontaneously and reversibly fall out of solution when heated to 30–40 °C[1,2]. This phase transition temperature (T_t) is most sensitive to the composition and molecular weight of the ELP, but can also be affected by salt concentration and by the properties of accompanying fusion proteins[2–4]. The reversibility of the precipitation is distinct from simple irreversible aggregation or misfolding, and this property has now been used by several groups to purify ELPs and protein products fused to ELPs[1,2,5]. Another recent advance in the field of bioseparations has been the development of intein-fused affinity tags that inducibly self-cleave under mild conditions (room temperature and neutral pH)[6], thus providing new options for the purification of native recombinant proteins[7,8].

We have used a tightly controllable ELP tag with an engineered self-cleaving intein to create a simple, economical alternative to conventional methods of protein purification. In practice, the target protein gene is fused to a gene encoding a self-cleaving ELP-intein tag, and the resulting fusion is overexpressed in *E. coli*. The soluble ELP fusion precursor is then isolated from the insoluble cell debris by centrifugation at a temperature below the ELP T_t. The ELP-tagged fusion is then heated in the presence of salt to a temperature above its T_t, which causes the ELP tags to self-associate into an insoluble precipitate (Fig. 1a). It is important to note that the precipitation is limited to the ELP portion of the fusion, and does not affect the fusion partners. The precipitated ELP fusion can then be separated from the soluble cellular components in a second centrifugation step, and redissolved in a pH 6.0 buffer at low temperature.The intein self-cleavage reaction then releases the target protein from the ELP tag, which can be subsequently removed by an additional cycle of salt addition, heating and centrifugation. Thus, a highly purified native protein is produced from a total cell lysate via simple mechanical means, without chromatography (Fig. 1b).

We constructed a small library of ELPs of varying lengths and inserted it into the expression vector pET-21(+) under the control of

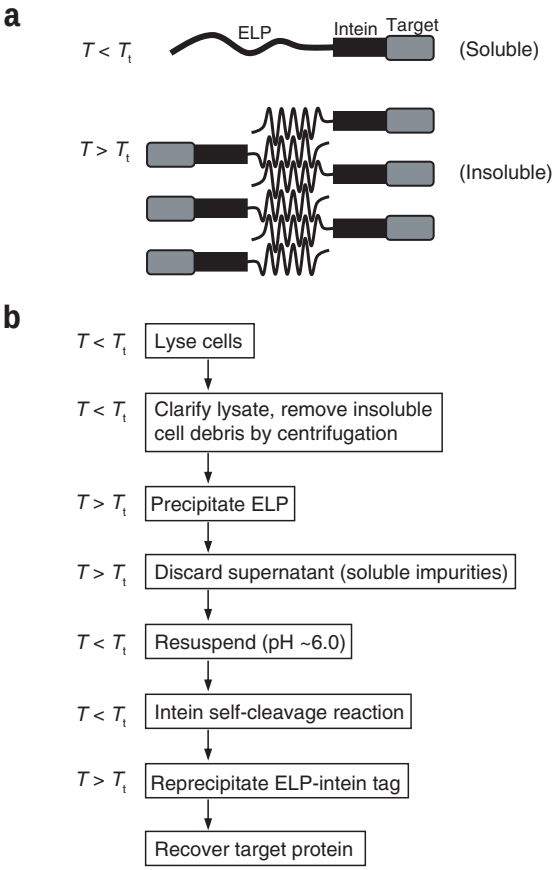

Figure 1 | Purification of ELP-intein–tagged protein. (**a**) The ELP-intein tag is designed to self-associate into an insoluble core upon heating to a temperature above the ELP T_t allowing the properly folded intein and target protein to be recovered by centrifugation. (**b**) Schematic of the target protein purification, described in detail in the text.

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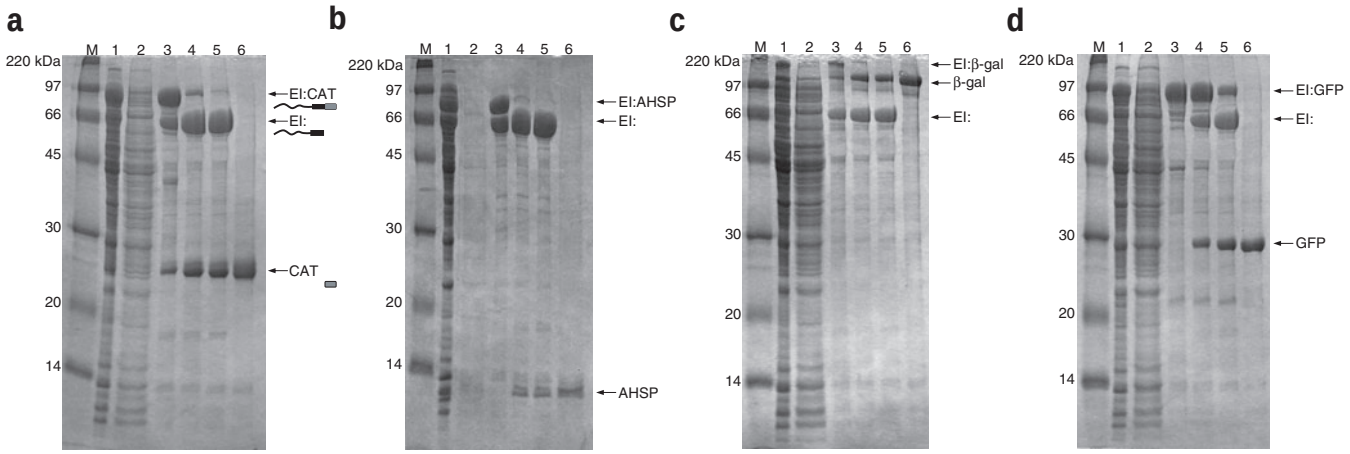

Figure 2 Purification of test proteins. (**a**) SDS-PAGE analysis of samples taken over the course of the CAT purification. Lane 1, cleared lysate; lane 2, supernatant after precipitation of the ELP fusion; lane 3, resolubilized ELP fusion at the beginning of the self-cleavage reaction; lanes 4 and 5, samples taken at 4 and 25 h of the self-cleavage reaction; lane 6, purified product. Lanes 1 and 2 were loaded at half the amount of lanes 3, 4 and 5, and lane 6 was loaded at ~1.5 times the amount of lanes 3, 4 and 5. (**b**–**d**) Purification results for three additional proteins: AHSP (**b**); β-galactosidase (β-gal; **c**); the green fluorescent protein (GFP; **d**). Gels are as described in **a**. Similar results were obtained for several additional test proteins (**Supplementary Fig. 3** online).

a T7 promoter (Supplementary Methods and Supplementary Fig. 1 online). We then used dynamic light scattering to evaluate the ability of each ELP to precipitate efficiently in fusion to the large and highly soluble NusA protein[9], and determined the approximate T_t in each case (data not shown). Ultimately we chose $(VPGXG)_{110}$ because it fulfilled our design objectives: a moderate transition temperature (below 30 °C) in high salt buffers, efficient precipitation above T_t regardless of the fusion context and the shortest possible length. We then used a previously reported cloning strategy[8] to insert other protein sequences immediately after the carboxy-terminal His-Asn dipeptide of the intein in the ELP-intein fusion. This strategy retains the conserved C-terminal amino acids necessary for the intein cleavage reaction, and allows the recovery of a native product protein with no additional amino acids.

Table 1 |Product protein quantification and activity assays.

Product protein (molecular weight)	Quantity of purified protein[a] (μg/ml)	Specific activity of purified protein	Specific activity reported in the literature	Percent recovery[b]	Purification fold[b]
α-hemoglobin stabilizing protein (AHSP) (12 kDa)	104.1 ± 9.1	Acceleration of rate of αHb oxidation by AHSP: 6.2	Acceleration of rate of αHb oxidation by AHSP: 5.8 (ref. 12)	NA[i]	NA
β-lactamase (29 kDa)	70.3 ± 5.1	317.8 units/mg[c]	240 units/mg (ref. 13)	26.5	14.7
β-galactosidase (β-gal) (116 kDa)	122.3 ± 10.9	358.5 units/mg[d]	250–1,200 units/mg[e]	>100[f]	45.0[f]
Catalase (80 kDa)	79.8 ± 7.8	703.3 units/mg[g]	275–1,486 units/mg (ref. 14)	29.6	14.6
Glutathione *S*-transferase (GST) (26 kDa)	118.0 ± 17.8	75.7 units/mg[h]	25–125 units/mg[e]	18.2	12.9
Green fluorescent protein (GFP) (27 kDa)	110.2 ± 6.1	511 nm fluorescence	511 nm fluorescence	21.5	10.5
Maltose binding protein (41 kDa)	46.4 ± 4.0	Binds maltose resin	Binds maltose resin	NA	NA
S-824 (12 kDa)	45.1 ± 5.2	Binds heme (412 nm)[15]	Binds heme (412 nm)[15]	NA	NA
Chloramphenicol acetyl transferase (CAT) (26 kDa)	96.3 ± 5.2	ND[i]	ND	ND	ND
NusA (55 kDa)	56.4 ± 2.7	NA	NA	NA	NA

[a]Yield of protein from a shake-flask cell culture with approximate dry cell weight of 5.65 ± 1.9 mg/ml. [b]Purification fold and percent recovery were calculated from the relative activity of the total lysate before purification and the activity of the purified protein. [c]One unit of β-lactamase hydrolyzes 1.0 μmole of nitrocefin per minute at 25 °C. [d]One unit of β-galactosidase hydrolyzes 1.0 μmole of *o*-nitrophenyl β-D-galactopyranoside (ONPG) to *o*-nitrophenol and D-galactose per min at 25 °C. [e]Range of activities of commercially available enzymes available from Sigma-Aldrich. [f]Recovery and purification fold were overestimated owing to lower activity of this enzyme in fusion. [g]One unit of catalase decomposes 1.0 μmole of H_2O_2 per minute at pH 7.0 at 25 °C. [h]One unit of GST conjugates 1.0 μmole of 1-chloro-2,4-dinitrobenzene (CDNB) per minute at pH 6.5 at 25 °C. [i]NA, not applicable; ND, not determined.

We purified chloramphenicol acetyl transferase (CAT) using an ELP-intein-CAT (EI:CAT) fusion and followed the purification by SDS-PAGE analysis (Fig. 2a). Growth in Terrific Broth medium at 18 °C for 48 h without induction allowed a buildup of the EI:CAT fusion in *E. coli* BLR cells. After recovery and gentle disruption in a pH-8.5 buffer at 4 °C (Supplementary Methods online), we used centrifugation to separate the insoluble cell debris from the soluble lysate components (Fig. 2a, lane 1). To reduce the ELP transition temperature, we added NaCl to the cleared lysate to a 1.5 M final concentration. We then warmed the sample to 30 °C for 10 min and centrifuged at the same temperature for 5 min to pellet the ELP precipitant. SDS-PAGE analysis of the discarded supernatant solution indicated an absence of the EI:CAT fusion, suggesting nearly full recovery of the fusion (Fig. 2a, lane 2). We then resuspended the translucent EI:CAT pellet at 4 °C, in a low-salt pH-6.0 buffer to solubilize the ELP protein and initiate the self-cleavage reaction. Analysis of the resuspended material indicates near-complete recovery and solubility of the ELP fusion (Fig. 2a, compare lanes 1 and 3). Additional tests on this and other proteins indicated that there was no detectable insoluble material remaining after this resolubilization step (Supplementary Fig. 2 online). For efficient cleavage of the intein, we subsequently incubated the ELP fusion at 18–22 °C. Analysis of samples taken at 4 and 25 h indicated that EI:CAT cleaves over time to yield the ELP-intein (EI:) tag and the cleaved CAT product protein (Fig. 2a, lanes 4 and 5). Upon completion of the cleavage reaction, we added NaCl as before and heated the sample to 30 °C for 10 min. The precipitated EI: tag was then separated by centrifugation, and the purified CAT protein was recovered in the supernatant. We then reduced the salt content of the purified product by dilution and ultrafiltration (Fig. 2a, lane 6). In addition to CAT, we purified several other proteins of various sizes in a similar manner, and evaluated them for yield, recovery and activity when possible (Fig. 2b–d, Table 1 and Supplementary Fig. 3 online).

Over the course of this work, we tested several conditions for cell growth and protein expression. We noted that the presence of the ELP fusion protein generally slowed growth, and our attempts to induce high levels of expression over a few hours (as is typical when using the T7 promoter) were not effective. We hypothesize that this is due to the large number of amino acid repeats in the ELP, $(VPGXG)_{110}$, although media supplemented with glycine and valine did not change the growth and expression levels noticeably (data not shown). However, growth at 18 °C with leaky expression from the T7 promoter yielded strongly overexpressed product after 24 h, with maximum expression typically occurring at around 48 h. This result is consistent with previously reported observations by other investigators[5,10]. For the aforementioned reasons, as well as to suppress premature intein cleavage, we chose 18 °C for 48 h without induction in Terrific Broth as the standard growth condition for all the samples. We anticipate that expression may be improved in the future by using optimized expression strains and growth conditions to suit specific target proteins.

A strength of this purification method is that the conditions over which it is effective are quite broad, thus providing great flexibility in its implementation. The ELP transition temperature can be adjusted using salt concentration whereas the intein cleaving reaction can take place over a wide range of conditions. Some optimization will be required for new, uncharacterized products on a case-by-case basis, as is true of any purification method. The prototype presented here aims to simplify protein purification by eliminating the cost and complexity associated with column operation. Although the reduction in cost is somewhat offset by the long induction time and large ELP tag, these issues are minor when taken in the context of conventional protein expression and purification. For example, the additional expression time required for this system represents only a small percentage increase in a typical process time. In most of the cases we have shown, the intein cleaving reaction is essentially complete in 4–10 h, making it competitive with any conventional chromatography process, and the shake-flask yields we report are reasonable in comparison to those obtained using previously reported ELP strategies[10] and conventional affinity methods[11]. Furthermore, the simple mechanical recovery of precipitated ELP fusion protein suggests a variety of means for scale-up, including tangential-flow microfiltration or continuous centrifugation. Alternately the method might be used in a robotic system to purify protein libraries for screening. The simplicity and self-contained nature of this system promise a breakthrough in the production of purified recombinant proteins in research and industrial enzymes for commercial use.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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