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Purification of recombinant proteins from *E. coli* at low expression levels by inverse transition cycling

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A crucial and challenging problem in proteomics is purification, identification, and characterization of proteins, some of which are expressed at very low levels. The preferred method for purification of low abundance proteins exploits multiple affinity purification tags on a single recombinant protein e.g. tandem affinity purification (TAP) [1]. However TAP is both experimentally lengthy, involving many sequential binding, washing and elution steps and costly, requiring two different and expensive resins to recover the purified recombinant protein. Hence processing large amounts of cell lysate makes it prohibitively expensive especially for scale-up.

Here we present a new and simple strategy to purify soluble recombinant proteins from *E. coli* at a protein concentration that approaches the limit of a single protein molecule per cell. This method utilizes the unique aggregation properties of elastin-like polypeptides (ELPs) to capture recombinant fusion proteins composed of a target protein and an ELP tag from cell lysate. ELPs are artificial, genetically encodable polypeptides composed the repeating pentapeptides sequence VPGXG, where the guest residue (X) can be any naturally occurring amino acid except Pro [2,3]. ELPs exhibit a unique reversible inverse phase transition behavior; below a critical transition temperature (T_t) ELPs are highly soluble in aqueous solution, however at temperatures even a few degrees Celsius above T_t, ELP will undergo a solubility-insolubility phase transition, leading to aggregation of the polypeptide [4]. The T_t of an ELP is a function of a number of variables including identity and stoichiometry of the guest residue, molecular weight, ELP and salt concentration in aqueous solution [5–9].

The environmental sensitivity and reversible solubility of ELPs are retained when expressed as recombinant fusions with proteins. This feature can be exploited for non-chromatographic purification of recombinant proteins by inverse transition cycling (ITC) [10]. In ITC, an ELP fusion protein is selectively separated from other contaminating biomolecules in cell lysate by the sequential and repeated steps of aggregation, centrifugation, and resolubilization of the fusion protein [10,11]. A number of different proteins have been purified by this method using either centrifugation [11–18] or micro-filtration [19]. The direct purification of ELPs has also been extended to the capture of native proteins by ELP-tagged capture reagents [14–17].

Proteins expressed at the level of micrograms per liter of culture are difficult to purify by chromatography because of the losses associated with non-specific and irreversible adsorption of the target protein to chromatography resins as well as the relative increase in contamination from host proteins that are non-specifically adsorbed to the chromatography resin are subsequently eluted with the target protein. We demonstrate a new variant of ITC that solves

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this problem by the addition of excess free ELP to cell lysate to efficiently drive the phase transition at low concentrations of ELP fusion proteins. This simple modification of ITC enables the purification of ultra-low concentrations of ELP fusion proteins (defined as < 100 μg soluble expressed protein per liter of culture) from *E. coli* lysate with high purity and good yield. In addition, the purification process from lysing the cells to pure protein takes less than one day with culture volumes ranging from 1 to 6 liters of bacteria growth.

To demonstrate the feasibility of purifying ultra-low levels of expressed proteins, we investigated the purification of three ELP fusion proteins: thioredoxin (Trx), blue fluorescence protein (BFP), and chloramphenicol acetyltransferase (CAT) that were each fused at their C-terminus to ELP[$V_5A_2G_3$-90], a 36 kDa polypeptide containing 50% valine, 20% alanine, and 30% glycine at the guest residue position. Both unlabeled and ^{14}C-labeled ELP fusion proteins were separately expressed from *E. coli* at moderately high levels (20–200 mg/L culture), and purified to homogeneity (as assessed by SDS-PAGE) by ITC under standard conditions prior to the experiments reported in this study.

To determine if the addition of free ELP as a co-aggregant could efficiently capture and purify ELP fusion proteins from solution, a proof-of-principle experiment was performed by mixing 5 μM ELP with three different ^{14}C-labeled, purified ELP fusion proteins (Trx-ELP, BFP-ELP, and CAT-ELP) at two different concentrations (1 μg and 10 μg fusion protein in 10 ml PBS). The concentration of free ELP of 5 μM was chosen because preliminary experiments indicated that this concentration optimally balanced recovery of Trx-ELP, as measured by thioredoxin reductase activity [20], with ease of filtration. Concentrations higher than 5 μM increased the filtration pressure without increasing the recovery of Trx-ELP.

The inverse phase transition was triggered by adding NaCl to a final concentration of 3 M, which was chosen because it is high enough to drive the phase transition when this ELP is present at a concentration of 5 μM [5]. In control experiments, the free ELP was omitted to investigate the effect of the ELP co-aggregant on recovery of the ELP fusion proteins. After triggering the ELP phase transition by the addition of NaCl, all fusion protein solutions were filtered through 0.2 μm syringe filters to capture the ELP aggregates, and were washed with 10 ml of a high-salt buffer. The aggregates were then dissolved by reversing the phase transition by injection of 2.0 ml low-salt PBS buffer and eluted from the filters. The radioactivity in each sample was measured by scintillation counting and is reported in counts per minute (CPM). The percent recovery for each fusion for all experimental conditions was calculated from a log-log calibration curve of the CPM *versus* the mass of fusion protein.

Fig. 1 shows that the addition of free ELP results in a 7–50 fold greater capture of fusion protein as compared to the control (ITC performed without free ELP). The recovery of the fusion proteins with the addition of free ELP ranged from 45% ± 11% to 93% ± 20%. This significant increase in fusion protein capture likely results from more efficient retention of the mixed ELP/ELP fusion protein on the filter because the added ELP not only lowers the T_t due to the increase in ELP concentration [5], but also likely promotes the formation of large aggregates that are more easily retained by the membrane filter. As a parenthetical note, although it may appear that the amount of ELP fusion protein capture *without* the addition of free ELP increases with decreasing fusion protein concentration, this elevation in the apparent % capture is merely an artifact of the detection limit in β-counting in that background radioactivity constitutes a higher fraction of the total radioactivity at the lower fusion protein concentration.

In order to determine how the ELP co-aggregation technique would perform in purifying a poorly expressed ELP fusion protein from *E. coli* culture, the soluble *E. coli* lysate from 1 L of culture (~17 mL) was separately spiked with 1 μg of ^{14}C-labeled Trx-ELP, BFP-ELP, and CAT-ELP fusion protein, which corresponds to a concentration of ~1 nM ELP fusion protein

in the soluble lysate. By measuring the optical density of the *E. coli* culture at 600 nm prior to cell harvest, and assuming that OD_{600} = 1 corresponds to $8x10^8$ cells/ml, 1 μg of the fusion proteins corresponds to an expression level of 2–3 ELP fusion protein molecules per *E. coli* cell.

Capture from lysate was carried out as described previously with the following minor modifications. First, because of the very high concentration of *E. coli* biomolecules in the soluble lysate, a prefiltration step of the lysate was necessary to prevent clogging of the filter. After addition of the ELP fusion protein and NaCl (3 M final concentration), the lysate was raised to room temperature and filtered through a 0.45 μm membrane to remove non-specifically aggregated protein. The filtered lysate was then chilled on ice, free ELP was added, and the lysate was warmed to room temperature to trigger the phase transition. The aggregates were captured by filtration through a 0.22 μm membrane, and were washed with 150 mL PBS + 3 M NaCl to remove contaminants. The aggregates were then dissolved by reversing the phase transition by injection of 3 mL PBS, and the soluble ELP fusion protein was eluted from the membrane with PBS buffer. The eluted protein was then further purified by 5 rounds of ITC utilizing centrifugation (in place of filtration) to purify the fusion protein from any remaining *E. coli* proteins. The number of ITC rounds to obtain pure protein was verified by SDS-PAGE (data not shown). Control experiments without added ELP were omitted as recovery from buffer (Fig. 1) indicated that capture without added ELP does not result in the recovery of detectable amounts of ELP fusion protein. Fig. 2 shows that 62 ± 12, 31 ± 4, and 19 ± 7 % of the 1 μg of Trx-ELP, BFP-ELP, and CAT-ELP fusion proteins, respectively, were recovered with the addition of free ELP.

In summary, we have demonstrated a new variant of the ELP fusion tag technique to purify ultra-low levels of ELP fusion proteins (15–20 pmoles of protein in a liter of culture, which corresponds to 2–3 protein molecules per cell) by the addition of an excess of free ELP to facilitate the selective aggregation of the fusion protein from cell lysate. These results are notable for the following reasons. The facile and inexpensive purification of the ultra-low level of proteins by this methodology is, to our knowledge, unprecedented, and cannot, we believe, be matched by competing techniques such as affinity chromatography. ELP fusion protein capture is also notable because 20–60% of the fusion protein was captured at the lower limit of 1 μg of protein (Fig. 2), and the amounts of the recovered proteins are in the picomole range from a liter of culture, which provides enough protein for downstream analysis by mass spectrometry [21–23]. Furthermore, because of the detection limits of scintillation counting for ^{14}C radioactivity, we were unable to test the lower limits of this technique, but we see no inherent limitations in purifying even lower concentrations of ELP fusion proteins by this methodology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ELP elastin-like peptide

Trx thioredoxin

BFP blue fluorescent protein

CAT chloramphenicol acetyltransferase

TAP tandem affinity purification

ITC inverse transition cycling

T_t critical transition temperature

CPM counts per minute

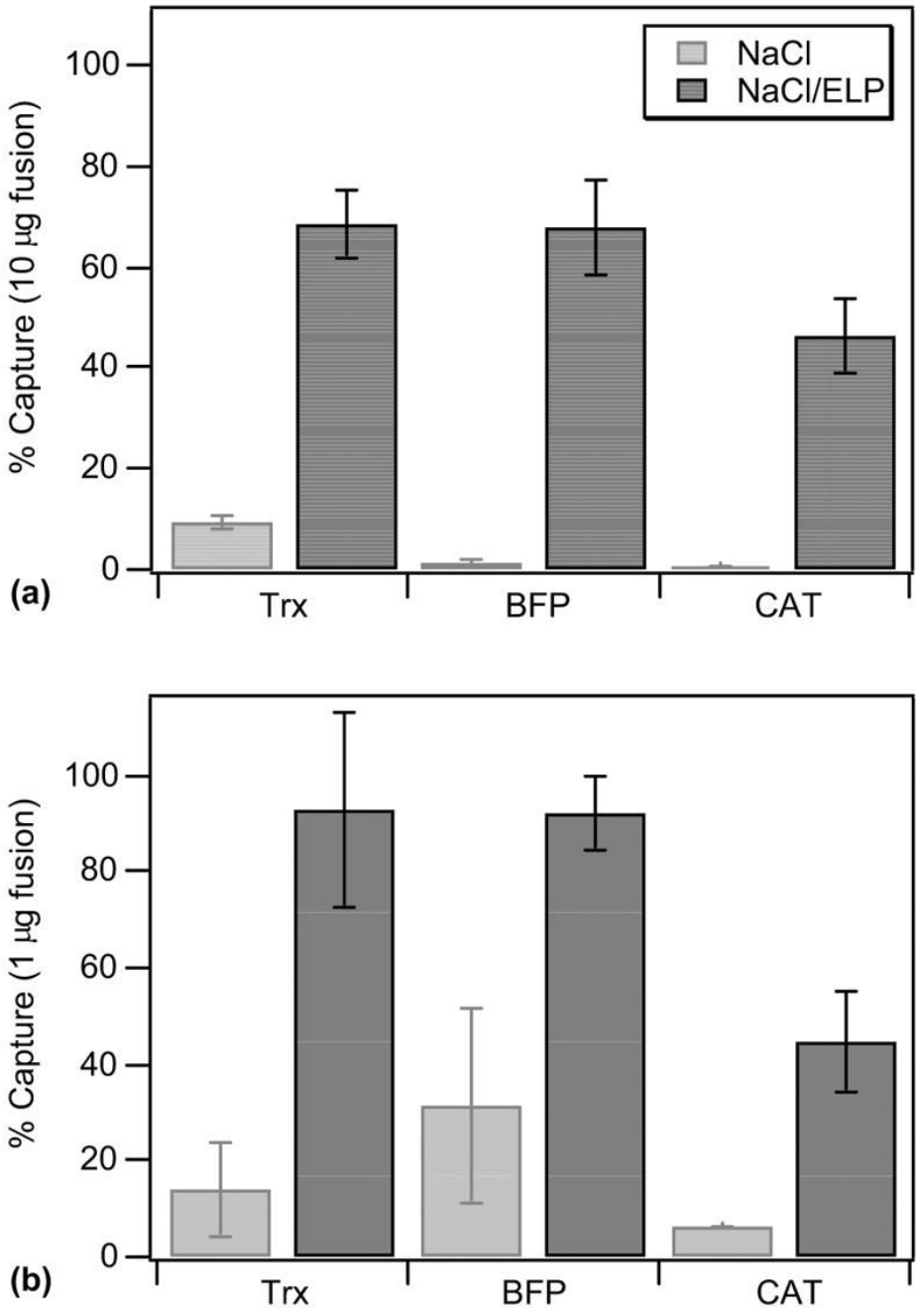

Fig. 1.

Percentage recovery of (a) 10 μg (~20 nM fusion protein) and (b) 1 μg (~2 nM fusion protein) ELP fusion proteins from 10 ml of PBS solution with and without the use of 5 μM free ELP. Error bars reflect the first standard deviation for at least three replicates of each sample.

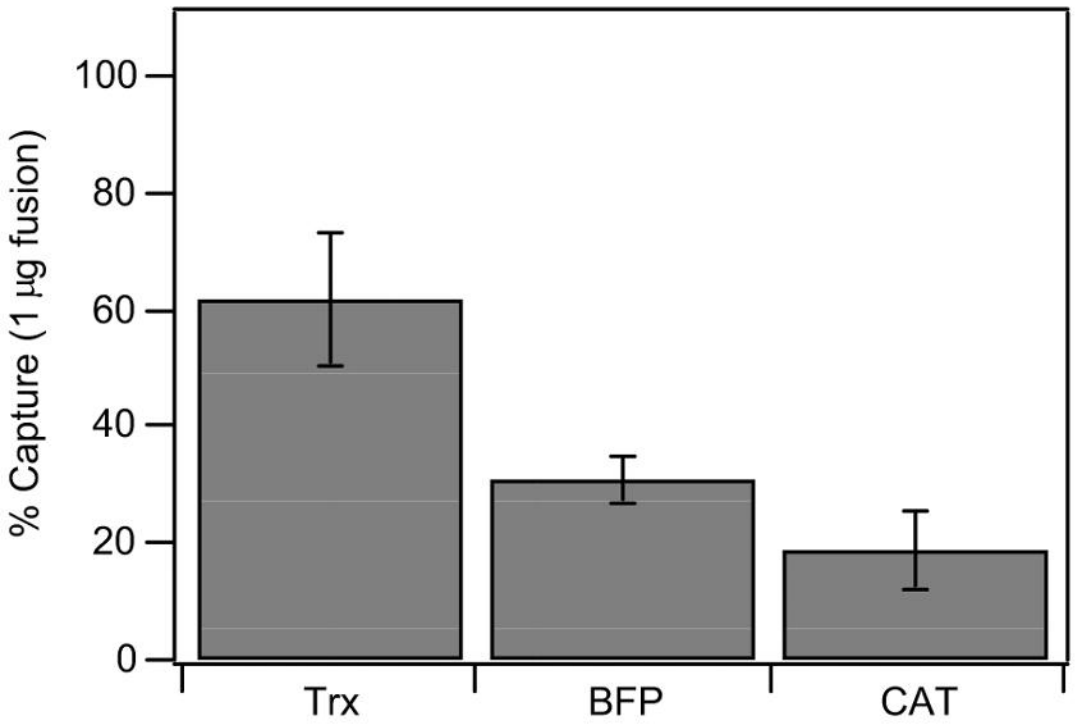

Fig. 2.

Percent recovery of 1 μg ^{14}C labeled Trx-, BFP-, and CAT-ELP fusion proteins from soluble *E. coli* lysate using 5 μM free ELP to facilitate fusion protein capture. Error bars reflect the first standard deviation from 3 replicates of each fusion protein. The mass of fusion protein added to soluble lysate (1 μg) is reflective of the concentration expected for 2–3 expressed molecules per *E. coli* cell.