Simultaneous Phase Transition of ELP Tagged Molecules and Free ELP: An Efficient and Reversible Capture System

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In this paper, we demonstrate proof-of-principle for a method that allows selective recovery of molecules present at very low concentrations in complex mixtures. The method makes use of an elastin-like polypeptide (ELP) as a coaggregant for the capture of an ELP tagged recombinant protein present at concentrations as low as 10 pM, with a recovery higher than 90%. This coaggregation process was found to be independent of the concentration, at least up to 10 pM concentration of the ELP tagged protein. The coaggregation process is highly specific as was demonstrated by spiking crude cell lysate with the ELP tagged recombinant protein to a final concentration of 1 nM and recovering more than 80% of it to a high level of purity. The method should be particularly useful for high-throughput proteomic studies, where small amounts of poorly expressed proteins could be recovered for analysis by mass spectrometry. In a more general context, the concept presented in this paper provides a method that is highly efficient, specific, and fully reversible, which should render it useful in areas other than recombinant protein purification.

Introduction

Researchers working in a variety of fields often need to capture, in an efficient and highly specific manner, molecules that are present at very low concentrations in a very complex matrix. The common approach is to use affinity based systems, with the streptavidin−biotin (SA-B) interaction being the prime example of this approach due to its unmatched level of affinity.[1,2] There are, however, two main drawbacks associated with affinity based systems: the higher the affinity level between the target (e.g., B) and the ligand (e.g., SA), the more difficult it is to release the target molecule from the ligand; affinity systems are associated with the use of a surface (beads, resins, etc.) onto which the ligand is coupled. If a large surface area is used (such as that associated with polymeric beads), there is high potential for nonspecific and irreversible binding of either contaminating molecules or of the target. The objective of this work is to provide proof-of-principle for a method that has high capture efficiency, has full reversibility, and that exposes the sample to an absolute minimum surface area.

A growing number of reports have been made on the use of elastin-like polypeptides (ELPs) tags as fusion partners to recombinant proteins, which allows purification of the recombinant proteins without the use of chromatography. ELPs consist of repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly (VPGXG), where Xaa is any amino acid except proline.[3] ELPs are able to undergo a reversible inverse phase transition (RIPT), within a very narrow temperature range (∼2 °C).[3−5] Below a critical transition temperature (T_t), ELPs are soluble, whereas above this temperature, they become insoluble. Meyer and Chilkoti[3] observed that when ELP is fused to a recombinant protein, the ELP tag maintains the ability to undergo RIPT. This observation lead to the development of a very simple method for purification of recombinant proteins: inverse transition cycling[6] (ITC). The basis for ITC is to selectively aggregate ELP tagged proteins using an environmental stimuli (either increasing the temperature, adding NaCl, or a combination of both), so that the aggregates can be separated from other proteins present in the mixture, either by centrifugation[6−9] or by microfiltration.[10] These aggregates can be resolubilized by decreasing the temperature of the mixture and/or decreasing the ionic strength of the buffer in which the protein is present. This approach has been demonstrated with a variety of target proteins,[6−9] and in all cases, the target fusion protein was obtained with purity levels higher than 95%. This suggests minimal nonspecific binding of contaminant molecules to ELP based aggregates. This approach has also been shown to yield purified proteins in quantities that are similar with those obtained using oligohistidine tags.[6]

ITC has been demonstrated in the past with proteins that were expressed to high levels,[6−9] but poor expression levels are expected for toxic proteins, complex multidomain proteins, or in some cases of heterelogous protein expression. Later in this paper, we describe how for very low concentrations of ELP tagged proteins, ITC in its original format does not allow recovery of the proteins. In proteomics, massive numbers of proteins are expressed in parallel, and optimizing expression conditions for each one is not feasible. Mass spectrometry for protein studies requires protein concentrations as little as 0.1−1 pM.[10] We hypothesized that if an ELP tagged molecule is present in a solution at a very low concentration (even in the pM range) adding an excess amount of free ELP to the sample and inducing RIPT would form hybrid aggregates via the interaction of the ELP moieties of the two molecules. Coaggregation of free ELP and ELP tagged proteins has been reported for the purification of a fusion of a single-chain Fv of an anti-atrazine antibody to ELP,[11] and as a component of a competitive phase-separation immunoassay,[12] but no quantitative data was presented on the efficiency of coaggregation. In the work presented here, it is quantitatively shown that this coaggregation process is highly specific and efficient even when using very low concentrations of ELP tagged molecules. Especially noteworthy, this interaction is fully reversible.

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Experimental Section

Plasmids and Host Cells. To test the method described in this paper, we used a fusion of thioredoxin (Trx) and ELP (herein referred to as Trx-ELP) as a model protein. The plasmid that encodes for Trx-ELP used in this study was pTrx-ELP90, and its assembly is described elsewhere.[6,7] It is based on the pET-32b expression vector (Novagen, Madison, WI) and the fusion protein consists of thioredoxin, followed by a $(His)_6$ tag, a thrombin cleavage site and the ELP tag. The ELP tag in this plasmid is denoted as ELP [$V_5A_2G_3$-90], which comprises 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3. To obtain a plasmid that encodes for the ELP tag only, the plasmid that encodes the fusion protein was digested with *Nde*I (Fermentas), and the large fragment was separated by agarose gel electrophoresis and purified with DNA extract kits (Qiagen). The purified DNA fragment was then self-circulated under the catalysis of T4 DNA ligase (Fermentas) to generate the plasmid that encodes for the expression of the ELP without thioredoxin. This plasmid is called pELP90. The two vectors used in this study were transformed into *E. coli* strain BLR (DE3) competent cells (Novagen), using the $CaCl_2$ transformation method.

Protein Expression and Purification. The transformed cells were cultivated in Luria-Bertani media, supplemented with 100 μg/mL ampicillin. Shaker flasks were inoculated from a single colony and cultured overnight at 37 °C. It has been shown that expression of ELP and ELP-tagged proteins is greatly enhanced when the cells are not induced using isopropyl β-thiogalactopyranoside (IPTG) but rather when the cultivation period is extended,[7,13] and this approach was used in this study. An additional culture experiment was done with nontransformed *E. coli* BLR (DE3) strain, to provide cell lysate devoid of ELP or ELP tagged proteins. The cells were harvested by centrifugation at 5000*g*, 4 °C, for 10 min. The pellet was resuspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.2 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH7.4) in a volume ratio of 1:25 to the initial volume of culture medium. The resuspended cells were disrupted by introducing discontinuous ultrasonic pulses (VirTis Comp.) in an ice−water bath. The lysate was then centrifuged at 16 000*g*, 4 °C, for 10 min to remove insoluble particles. From these samples, Trx-ELP or ELP was purified using three rounds of ITC.[6] The ELP protein contained an $(His)_6$ tag and a thrombin cleavage site which were both removed by incubating (overnight) the sample with thrombin (Sigma) followed by another round of ITC to obtain pure ELP. The purity of the proteins was determined using SDS−PAGE followed by staining with Coomassie Brilliant Blue for Trx-ELP or with copper (Bio-Rad) for ELP. The concentrations of purified ELP and ELP-fused proteins were determined by measuring the UV absorbance at 280 nm and by using the extinction coefficients determined from the amino acid sequence (19 700 $M^{-1}cm^{-1}$ for Trx-ELP and 5690 $M^{-1}cm^{-1}$ for ELP). The molecular weights of Trx-ELP and ELP are 50 and 36 kDa.

Labeling of Trx-ELP with ^{125}I. Trx-ELP was radioiodinated by the iodine monochloride method.[15−17] To a tube containing 50 μL of glycine buffer (2 M glycine, 2 M NaCl, pH 8.8) and 50 μL of iodine monochloride reagent (3.3 mM ICl, 1.8 M NaCl), 5 μL of ^{125}I isotope was added, and the contents of the tube were mixed for 1 min. To this mixture was added 4 mg of Trx-ELP in 600 μL of PBS, supplemented with 40 μL of glycine buffer, and the mixture was allowed to react at room temperature for 2 min. This mixture was subjected to two rounds of microfiltration based ITC[14] to separate free radioactive iodine from Trx-ELP. This was achieved by adding 500 μL of 5M NaCl to induce aggregation of the Trx-ELP, filtering the solution through a 0.2μm Supor (Pall Corp.) disposable microfiltration cartridge, and collecting the filtrate. The aggregates retained in the membrane were washed with 500 μL of 2.5 M NaCl and then resolublized and eluted from the filter with 500 μL of PBS. This process was repeated twice. To ensure that no free radioactive iodine was present, gamma counting was done on the filtrate obtained in the second round of ITC. This filtrate accounted for 0.006% ± 0.0003% of the initial counts in the mixture before the first round of ITC; therefore, the amount of the radioactive free iodine left is negligible. The concentration of ^{125}I-labeled Trx-ELP was determined by measuring the UV absorbance of the sample at 280 nm. Gamma counting was performed using a Wizard 3 1480 Automatic Gamma Counter (Perkin-Elmer Life Sciences).

Capture of Trx-ELP by Coaggregation with Free ELP in PBS. Free ELP and a mixture of labeled and unlabeled Trx-ELP were added to a counting vial containing 500 μL of PBS. To the vial was added an equal volume of 5 M NaCl to induce aggregation at room temperature. For all experiments, the final concentration (in 1 mL) of free ELP was 20 μM and for Trx-ELP, and the concentration ranged from 1 μM to 10 pM. To maximize the accuracy in gamma counting, for total concentrations of Trx-ELP higher than 1 nM, the labeled protein was 1 nM and the rest was un-labeled, and for total Trx-ELP concentration equal to or less than 1 nM, and all of the Trx-ELP was labeled. To separate the Trx-ELP and ELP coaggregates, the samples were centrifuged at 16 000*g*, for 5 min, at room temperature. The supernatant was transferred to another tube and the pellet was resolubilized with 500 μL of PBS. Measurement of the radioactivity of the initial mixture (before NaCl addition), the supernatant, and the resolublized pellet allowed the recovery percentage to be calculated. The time limits for counting were set to 1 min for the tests in which the Trx-ELP concentration ranged from 1 μM to 1 nM, 5 min for Trx-ELP at 100 pM, and 10 min for Trx-ELP at 10 pM. To amplify the radioactivity counts at very low Trx-ELP concentrations (10 pM), a sample with a total volume equal to 10 mL (as opposed to 1 mL) was used. To reduce the loss of Trx-ELP due to nonspecific binding onto the surface of pipet tips, the experiments were designed to minimize the number of pipetting steps. Each experiment (at each concentration) was repeated three times, and the average and standard deviation was calculated from the results. A set of control experiments was conducted similarly but using bovine serum albumin (BSA) at a final concentration of 20 μM instead of free ELP.

Capture of Trx-ELP from Cell Lysate. To 10 mL of cell lysate obtained from an overnight 250 mL culture of the nontransformed host cells were added free ELP and labeled Trx-ELP. To this sample was added 10 mL of 5 M NaCl to induce aggregation (high salt conditions). The final concentrations of ELP and Trx-ELP in the 20 mL samples were 5 μM and 1 nM, respectively. For each round of ITC, two centrifugation steps (16 000*g* for 5 min at room temperature) were done. After centrifugation using high salt concentrations (high salt spin), the supernatant containing soluble contaminants was removed, and the pellet was resuspended in 10 mL of cold PBS, followed by an additional centrifugation step (low salt spin). The pellet obtained after centrifugation with PBS contained particulate contaminants and the supernatant contained the resolublized free ELP/Trx-ELP mixture, which was transferred to a new tube. The same procedure was used for the second and third rounds of ITC. Radioactivity levels were measured for the initial sample before the addition of NaCl and for the samples from each purification step in every round of ITC. The radioactivity observed in the resuspended pellet represents the amount of protein that was recovered. The amount of protein lost was determined by adding the radioactivity obtained for the supernatant produced in the high salt conditions spin and the radioactivity obtained for the pellet from the low salt spin. After three rounds of ITC, 1 unit thrombin (Sigma) was added to the sample containing Trx-ELP and ELP. After incubation (overnight) at room temperature, one additional round of ITC was preformed to separate the Trx from the ELPs. The supernatant, containing the Trx, was desalted and concentrated by centrifugation using an ultrafiltration membrane filter with a cutoff MW of 3 kDa (Pall Corporation). The purity of the final sample was assessed by 15% SDS−PAGE, and stained with Coomassie Brilliant Blue.

Results and Discussion

To determine the capture efficiency of the coaggregation process, ITC purified Trx-ELP was labeled with ^{125}I. To a tube

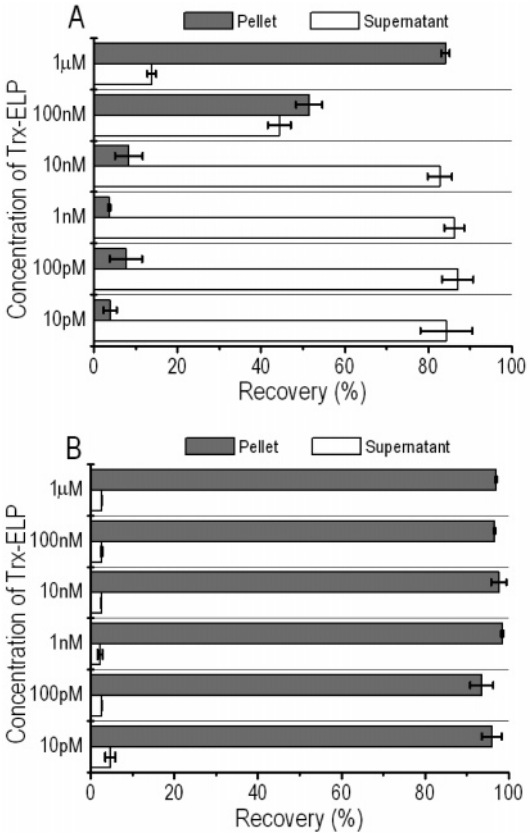

Figure 1. Percent of the initially labeled Trx-ELP that is associated with the supernatant (white bars) and resuspended pellet (gray bars) as a function of the Trx-ELP concentration, after one ITC cycle. (A) BSA at a concentration of 20 μM was used as the background for all experiments; (B) free ELP was supplemented to a concentration of 20 μM for all experiments. Error bars represent the gamma counting error and the standard deviation associated with three different experiments.

containing a mixture of free ELP and labeled Trx-ELP in PBS (500 μL) was added an equal volume of 5 M NaCl to trigger RIPT at room temperature. For all experiments, the final concentration (after NaCl addition) of free ELP was 20 μM and for Trx-ELP, and the concentration ranged from 1 μM to 10 pM. After centrifugation, the supernatant was transferred to another tube, and the pellet was resolubilized in 500 μL of cold PBS. Gamma counting was done for the initial sample containing free ELP and labeled Trx-ELP (before salt addition), for the supernatant and for the tube with the resolubilized pellet. A set of control experiments, using BSA at a concentration of 20 μM, instead of free ELP, was conducted to clearly identify the need for addition of free ELP as a coaggregant to recover Trx-ELP when present at very low concentrations. The results of the control experiments with BSA supplementation are presented in Figure 1A. The percent recovery is ~80% for the highest Trx-ELP concentrations (1 μM), an observation consistent with previous reports.[18] The percent recovery decreases sharply as the concentration of fusion protein decreases, with virtually no recovery in the 1 nM to 10 pM concentration range. As shown in Figure 1B (addition of free ELP to a final concentration of 20 μM) coaggregation allows recovery of more than 95% of Trx-ELP when the fusion protein concentration was varied from as much as 1 μM to as little as 10 pM. For all samples, the supernatant contained less than 5% of the total Trx-ELP added to the initial sample. It is remarkable that the capture efficiency of this process is independent of the Trx-ELP concentration, to a level of 10 pM when free ELP is used as the coaggregant. These results clearly demonstrate that the method could be used for a wide range of concentrations at which a target fusion is present: from high concentrations, where RIPT would allow recovery of the target without ELP supplementation, to extremely low concentrations, where coaggregation is required to recover the target. It is not possible, with the techniques we used (gamma counting), to determine the lowest concentration of Trx-ELP that can be recovered using coaggregation. A different set of experiments were done using a 0.2 μm membrane to capture the coaggregates formed during phase transition, instead of centrifugation. These experiments consistently generated percent recoveries of ~70% of the target protein, these values being lower than those shown in Figure 1B. After retaining the aggregates in the membrane, using 2.5 M NaCl, the membrane was washed with cold PBS, and the radioactivity associated with the PBS after passage through the membrane only accounted for ~1% of the initial amount of labeled protein. This means that ~30% of the initial labeled protein was irreversibly bound to the membrane. By using a membrane, the samples were exposed to a large surface area associated with the filter, which resulted in high levels of nonspecific and irreversible binding to the membrane. This shows the need to reduce the surface area to which the sample is exposed in order to minimize losses.

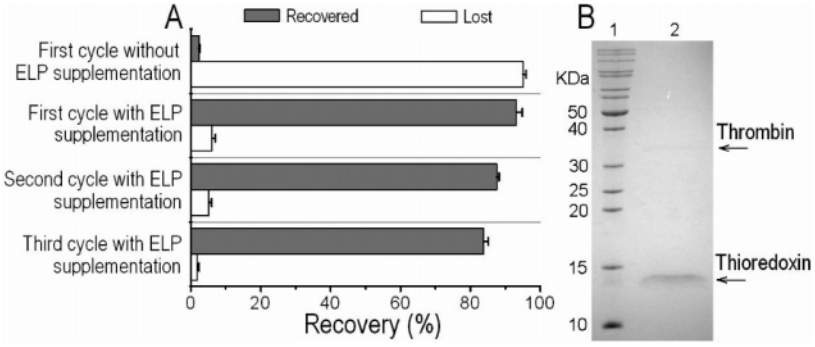

Figure 2. Capture of Trx-ELP at a concentration of 1 nM, from cell lysate supplemented with free ELP at a concentration of 5 μM. (A) Percent of the initially labeled Trx-ELP (in the initial sample prior to any ITC cycle was done) that is associated with the resuspended pellet (gray bars) and the summation of the radioactivity (white bars) in the supernatant from the first centrifugation step of ITC (high salt spin) and pellet from the second centrifugation step (low salt spin) of the same ITC cycle. Three ITC cycles were done, and the recovery percentages were calculated based on the initial sample in the experiment as being 100%. As the control, no free ELP was supplemented to the cell lysate containing Trx-ELP at 1 nM. Error bars represent the gamma counting error and the standard deviation associated with four different experiments. (B) SDS−PAGE for the sample obtained after 3 cycles of ITC, overnight cleavage with thrombin, followed by one additional cycle of ITC to separate the Trx from the ELP tag. The only other visible band corresponds to thrombin (37 kDa).

The efficiency of the coaggregation process was evaluated, in terms of percent recovery and purity of the final product, using cell lysate (obtained from nontransformed host cells) as the background matrix in which the capture is done. To 10 mL of cell lysate were added free ELP and labeled Trx-ELP. To induce coaggregation, 10 mL of 5 M NaCl were added to start the first round of ITC. The final concentrations of ELP and Trx-ELP in these 20 mL samples were 5 μM and 1 nM, respectively. Two additional ITC cycles were performed, and the reported percent recovery for all samples was calculated with respect to the radioactivity present in the sample before the first ITC cycle was performed. After 3 cycles of ITC, more than 80% of the labeled Trx-ELP was recovered (Figure 2A). It is important to note that for each ITC cycle both the percentage of recovered and lost Trx-ELP go down. Since recovery of Trx-ELP is less than 100% for each ITC cycle, the total mass of Trx-ELP decreases in consecutive ITC cycles. Because percent recoveries were calculated with respect to the radioactivity of the sample at the very beginning of the experiment, before any ITC cycle was done, both the percent recovered and percent lost decrease reflecting loss of protein as more ITC cycles are performed. A control experiment was conducted with cell lysate, using Trx-ELP at a final concentration of 1 nM, but without addition of free ELP as a coaggregant. As shown in Figure 2A, more than 95% of the labeled Trx-ELP is lost in the supernatant of the first round of ITC, proving that free ELP supplementation is essential to enable recovery of the Trx-ELP at this low concentration. The SDS−PAGE gel (Figure 2B) obtained for this experiment reveals that after three ITC cycles and overnight cleavage with thrombin, Trx was obtained to a high level of purity (thrombin was the only other protein detected in the gel, resulting in a very faint band). Coaggregation results not only in high capture efficiency, but also is a highly specific process. The ultimate limit of this method is still to be determined and alternative detection techniques must be used to determine what this limit is.

Although affinity based systems have a large surface area associated with the matrix onto which the ligand is coupled, the surface area that is required for coaggregation based capture, as described in this paper, is only that associated with the walls of the vial that contains the sample. This should minimize the potential for contamination due to nonspecific binding and loss of target molecules due to irreversible binding. The set of experimental conditions at which RIPT occurs can be manipulated using various combinations of temperature, salt concentration, and total ELP concentration. Also, ELP tags with different lengths and sequences[18] have been developed that should allow the use of coaggregation for desired sets of experimental conditions. One important aspect of ELPs is that single conjugation points, via incorporation of a lysine or a cysteine residue, can be engineered into the ELP sequence, allowing site-specific coupling of molecules with ELP tags. A possible application of this method is conjugating a library of nucleic acids to ELP, and using the coaggregation process as part of the "in vitro selection"[19,20] scheme to enrich for aptamers. In the initial rounds of selection, the aptamers account for a small percentage of the molecular population being screened. As such, they should be recovered with the maximum level of efficiency possible. We believe that the general concept demonstrated in this paper is applicable to more than capture and purification of proteins present at very low levels and has potentially wide ranging applications in many diverse areas.

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