Communication

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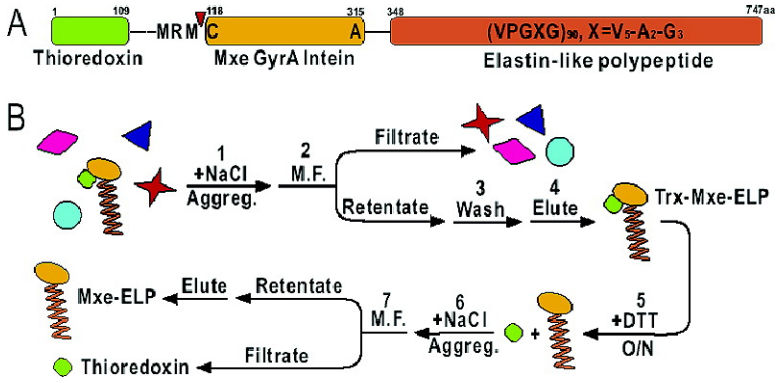

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Self-Cleavable Stimulus Responsive Tags for Protein Purification without Chromatography

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The need for simple methods to purify recombinant proteins is increasingly urgent and is driven by two technological imperatives. At the laboratory level, the advent of proteomics and high-throughput structural biology has created an urgent demand for simple methods to express and purify recombinant proteins. Although the use of affinity tags in combination with chromatographic separation has gained widespread use for purification of recombinant proteins, affinity tags have several problems for lab-scale purification of recombinant proteins: (1) they require chromatographic separation to achieve high purity, which limits their application in a high-throughput setting, and (2) when implemented in batch mode, the level of purity is typically insufficient for structural biology. At an industrial scale, an increasing number of protein and peptide therapeutics are in the development pipeline, and the emphasis on reducing their cost of production provides a strong impetus for the development of economical and large-scale protein purification methods. Chromatographic separation of proteins is, in general, difficult to scale up, and in the case of affinity chromatography, the cost of the resin is prohibitive at a large scale. Another significant problem with the use of most affinity tags is that a protease is usually required to cleave the target protein from the affinity tag. Three problems are associated with the use of a protease: (1) it adds additional cost to the purification process, (2) it adds an additional step to the purification process, because the protease needs to be separated from the target protein, and (3) due to their limited specificity, proteases can attack the target protein. The system described in this communication provides a general solution to these problems.

The first solution to these problems exploits the finding by Chilkoti and co-workers that elastin-like polypeptides (ELPs) retain their inverse phase transition behavior when fused to other proteins.[1] They showed that the phase transition behavior of ELP fusion proteins could be triggered by gentle heating of *Escherichia coli* lysate or by the addition of NaCl to aggregate the protein without nonspecific salting out or irreversible precipitation of other proteins. The aggregates were then recovered by centrifugation, isolated, and redissolved by reversing the phase transition to yield purified, and in all cases tested thus far, functionally active fusion proteins.[1,2] In this study, we have combined this purification method, inverse transition cycling, with self-cleaving inteins for protease-independent cleavage of the target protein to further expand the utility of this technology.

To demonstrate proof-of-principle of this methodology, thioredoxin (Trx) was fused at its C-terminus with a mini-intein from *Mycobacterium xenopi* GyrA gene (Mxe)[3] and an ELP[1,2] to create

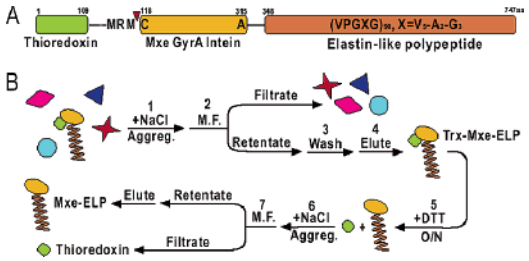

Figure 1. Scheme of tripartite self-cleaving environmentally responsive purification tag. (A) Triple fusion structure. Thioredoxin as the target protein was fused with Mxe GyrA mini-intein and elastin-like polypeptide. A peptide (MRM) was inserted before the N-terminal end of the intein sequence to control the cleavage activity. The C-terminal end of Mxe was modified (N → A) to block C-terminal cleavage or splicing. The arrow indicates the site of self-cleavage, which is induced by DTT. Numbers denote the amino acid sequence length. (B) Purification scheme. M.F. (microfiltration membrane), DTT (dithiothreitol), O/N (overnight incubation).

the tripartite fusion Trx−Mxe−ELP (Figure 1A). We chose the mini-intein from the *M. xenopi* GyrA gene that has an Asn → Ala mutation at the C-terminus of the intein for N-terminal cleavage.[3] The amino acids Met-Arg-Met were added before the Mxe intein sequence to control the cleavage reaction.[4] The ELP used in the fusion consists of 90 repeats of the pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is Val, Ala, and Gly in the ratio of 5:2:3, respectively, as reported previously.[1,2,5,6]

This protease-free and chromatography-free protein separation process is remarkably simple, as shown schematically in Figure 1B. NaCl is added to crude cell lysate at room temperature to trigger the ELP phase transition, which results in the aggregation of the ELP fusion protein to form micrometer-sized particles.[5,6] The mixture is then filtered through a 0.2-μm membrane, resulting in the retention of the ELP fusion aggregates and elution of other *E. coli* proteins and macromolecules. Next, the aggregates are re-solubilized and recovered by passing water through the membrane, which reverses the phase transition and results in dissolution of the aggregated fusion protein. We chose microfiltration, as opposed to centrifugation,[1] to recover the aggregated fusion protein to simplify the purification process to the level associated with DNA "minipreps". Dithiothreitol (DTT, 20 mM) is then added to self-cleave the Mxe GyrA intein, releasing Trx from the Mxe−ELP tag. Another round of inverse transition cycling is performed to isolate thioredoxin from the Mxe−ELP fusion. The purified thioredoxin is then desalted and concentrated by using an ultrafiltration centrifugation filter with a MW cutoff of 3 kDa (Pall Corp).

SDS-PAGE analysis for each step of the purification process is shown in Figure 2A. The cell lysate from nontransformed cells (lane 1) and transformed cells expressing Trx−Mxe−ELP after induction

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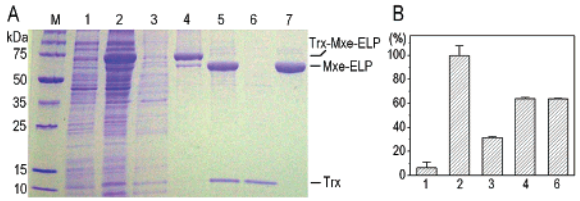

Figure 2. Purification by the self-cleaving stimulus responsive tag. (A) SDS-PAGE of each step of the purification and self-cleavage process. Lane 1, crude extract of untransformed cultured cells; lane 2, crude extract of cultured cells expressing the tripartite fusion Trx−Mxe−ELP; lane 3, *E. coli* proteins in the filtrate from a 0.2-μm microfiltration membrane; lane 4, Trx−Mxe−ELP fusion (72.6 kDa) eluted with water from the membrane; lane 5, overnight cleavage with 20 mM DTT; lane 6, thioredoxin (12.6 kDa) filtered through a 0.2-μm membrane; lane 7, eluted Mxe−ELP tag (60.0 kDa); lane M, molecular mass marker proteins. (B) Thioredoxin activity assay for each stage of purification (lanes 1−4, 6 as in part A) by insulin reduction assay. Values were normalized to the activity in the cell lysate.

with IPTG (lane 2) were obtained by ultrasonic cell disruption followed by centrifugation and precipitation of nucleic acids with polyethylenimine. Addition of NaCl to a final concentration of 1.67 M NaCl triggered the ELP phase transition, and the fusion proteins aggregated to form micrometer-sized particles that resulted in a visibly turbid solution.[5,6] After filtration, other proteins passed through the membrane (lane 3), but the aggregated Trx−Mxe−ELP fusion was retained on the microfiltration membrane, as seen from the absence of the band corresponding to the fusion protein in the SDS-PAGE of the filtrate. When the high salt buffer was switched to water, the resolubilized ELP fusion was eluted out from the membrane as soluble protein (lane 4). The two bands in lane 4 are due to partial in vivo cleavage of the Trx−Mxe−ELP, resulting in a fraction of ELP−intein fusions that do not contain Trx. After incubation with 20 mM DTT overnight, Trx was released from the Mxe−ELP tag (lane 5). A second round of inverse transition cycling resulted in the separation of the Trx (lane 6) from the ELP fusion tag (lane 7). A quantity of 50 mg/L of the Trx−Mxe−ELP fusion protein was obtained, as determined by UV−vis (using a calculated extinction coefficient[7] for Trx equal to 13 940 M^{-1} cm^{-1}). The first round of inverse transition cycling provided ∼90% purity; we note that greater levels of purity (≥95%) can be easily obtained by simply repeating the cycling procedure several times, as shown previously by Chilkoti and co-workers.[1,2] Taking into account that 25% of the total amount of the fusion produced was cleaved in vivo, the total expression level of the Trx−Mxe−ELP fusion protein was estimated to be ∼70 mg/L. When Trx−ELP was expressed without the intein, the expression level was found to be 80 mg/L, consistent with a previous study,[2] indicating that there is only a small decrease in the yield by incorporating the intein in the construct.

Figure 2B shows the corresponding Trx activity at each purification step, which was determined by the insulin reduction assay.[11] The ∼25% activity of Trx (normalized to the activity level in cell lysate as being 100%) observed in the filtrate (lane 3) is due to the endogeneous Trx encoded by the *E. coli* genome (not transformed host, lane 1) and due to premature in vivo release of Trx from the fusion protein during culture, which accounts for the majority of the Trx activity in the filtrate, as also seen in lanes 1 and 3 of Figure 2A. After filtration, the activities in the filtrate (lane 3) and of the eluted Trx−Mxe−ELP fusion (lane 4) account for 100% of the activity observed in the cell lysate, showing that activity was not lost during purification. The activity of Trx after cleavage and a second round of inverse transition cycling (lane 6) was experimentally indistinguishable from the activity of the Trx−Mxe−ELP fusion that was eluted from the microfiltration membrane in the first round of purification. In a control experiment with Trx−ELP (without the intein), we observed that capture and recovery of the fusion protein through the microfiltration membrane was greater than 95% (data not shown), indicating that this simple purification method allows remarkable recovery of the target protein.

In summary, we have demonstrated that it is possible to purify an ELP fusion protein by a simple microfiltration procedure and that the target protein can be cleaved from the ELP tag using an intein, thus obviating the need for expensive proteases and their removal. The primary limitation of this methodology, the premature in vivo cleavage of the fusion protein, can be solved, we believe, by the use of ligand-activated inteins.[8,9] The attractive features of this approach for lab-scale purification are its extraordinary technical simplicity and low cost: the only reagents and equipment required to purify milligram levels of protein at the lab bench are NaCl and DTT, a 0.2-μm membrane filter, and a syringe. We believe this methodology could also be easily scaled up for kilogram-level purification of peptide and protein therapeutics. Furthermore, because this procedure eliminates the need for proteases for isolation of the target protein, it is attractive for structural biology, where contaminating levels of the proteins interfere with structure determination, and for industrial applications, where the cost of the protease can represent a significant cost of the final product. The simplicity of the self-cleaving inverse transition cycling procedure demonstrated here is reminiscent of DNA minipreps and should liberate biologists from the tedium and complexity of protein purification by making it eminently feasible to simultaneously purify multiple proteins on the laboratory bench without recourse to expensive reagents and equipment.

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Supporting Information Available: Experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Meyer, D. E.; Chilkoti, A. *Nat. Biotechnol.* **1999**, *17*, 1112−1115.
(2) Trabbic-Carlson, K.; Li, L.; Kim, B.; Chilkoti, A. *Protein Sci.* **2004**, *13*, 3274−3284.
(3) Evans, J. T. C.; Benner, J.; Xu, M. Q. *Protein Sci.* **1998**, *7*, 2256−2264.
(4) Evans, J. T. C.; Benner, J.; Xu, M. Q. *J. Biol. Chem.* **1999**, *274*, 18359−18363.
(5) Meyer, D. E.; Trabbic-Carlson, K.; Chilkoti, A. *Biotechnol. Prog.* **2001**, *17*, 720−728.
(6) Meyer, D. E.; Chilkoti, A. *Biomacromolecules* **2002**, *3*, 357−367.
(7) Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989**, *182*, 319−326.
(8) Buskirk, A. R.; Ong, Y.-C.; Gartner, Z. J.; Liu, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10505−10510.
(9) Skretas, G.; Wood, D. W. *Protein Sci.* **2005**, *14*, 523−532.
(10) Telenti, A.; Southworth, M.; Alcaide, F.; Daugelat, S.; Jacobs, W. R., Jr.; Perler, F. B. *J. Bacteriol.* **1997**, *179*, 6378−6382.
(11) Holmgren, A. *J. Biol. Chem.* **1979**, *254*, 9627−9632.

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