Engineering a Recyclable Elastin-like Polypeptide Capturing Scaffold for Non-Chromatographic Protein Purification

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Previously, we reported a non-chromatographic protein purification method exploiting the highly specific interaction between the dockerin and cohesin domains from Clostridium thermocellum *and the reversible aggregation property of elastin-like polypeptide (ELP) to provide fast and cost-effective protein purification. However, the bound dockerin-intein tag cannot be completely dissociated from the ELP-cohesin capturing scaffold due to the high binding affinity, resulting in a single-use approach. In order to further reduce the purification cost by recycling the ELP capturing scaffold, a truncated dockerin domain with the calcium-coordinating function partially impaired was employed. We demonstrated that the truncated dockerin domain was sufficient to function as an effective affinity tag, and the target protein was purified directly from cell extracts in a single binding step followed by intein cleavage. The efficient EDTA-mediated dissociation of the bound dockerin-intein tag from the ELP-cohesin capturing scaffold was realized, and the regenerated ELP capturing scaffold was reused in another purification cycle without any decrease in the purification efficiency. This recyclable non-chromatographic based affinity method provides an attractive approach for efficient and cost-effective protein purification.* © 2013 American Institute of Chemical Engineers *Biotechnol. Prog.*, 29:968–971, 2013
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Introduction

In the search of a simple, scalable, and cost-effective downstream protein purification method, the coupling of elastin-like polypeptides (ELP) and the self-cleaving intein with a target protein[1] has become a highly attractive system for non-chromatographic protein purification. This ELP-intein fusion strategy has been successfully applied for the purification of various recombinant proteins expressed in *Escherichia coli*[2,3] and transgenic plants[4] with good production yield and protein purity. However, the target protein must be expressed as a fusion protein to ELP-intein and the ELP tag cannot be reused after intein cleavage. To make this technique more cost-effective, there is a need to develop an improved process with ELP recycling. We reported recently a new non-chromatographic protein purification method based on the high-affinity cohesin (Coh)–dockerin (Doc) interaction.[5] Direct purification and recovery of Doc-intein tagged proteins was achieved using the thermo-responsive ELP-cohesin capturing scaffold.[5] After intein cleavage, the target protein was separated from the ELP-Coh–Doc-intein complex by an additional cycle of precipitation. This approach not only increases the overall expression level by using a smaller dockerin tag, but also provides the possibility of recycling the ELP scaffold by disrupting the Coh–Doc interaction for additional cycles of purification.

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Since the Doc domain requires two calcium ions to maintain its structural integrity,[6] removal of the calcium ions by disodium ethylenediaminetetraacetate (EDTA) can potentially dissociate the Coh–Doc interaction for regeneration. However, the interaction between the Coh–Doc pair from *Clostridium thermocellum* employed in the previous study cannot be fully disrupted because of the extremely high binding affinity (10^{-9} M).[7] Demishtein et al.[8] designed a series of truncated dockerin variants of CelS from *C. thermocellum* in which the calcium-coordinating function was partially impaired. It has been shown that the binding of one particular mutant Doc(Δ16), which has 16 N-terminus residues deleted, with the cohesin domain can be completely reversed in the presence of EDTA while retaining a similar binding affinity. We demonstrate here the use of this truncated Doc(Δ16) in place of the original full length dockerin domain in our protein purification design for efficient protein purification and ELP scaffold recycling (Figure 1). Chloramphenicol acetyl transferase (CAT) was chosen as the target model protein to be purified in this study.

Materials and Methods

Strains and plasmids

All procedures for DNA manipulation were performed according to standard methods.[9] The high fidelity Phusion DNA polymerase (Thermo Scientific, Odessa, TX) was used for polymerase chain reaction (PCR) amplification using a

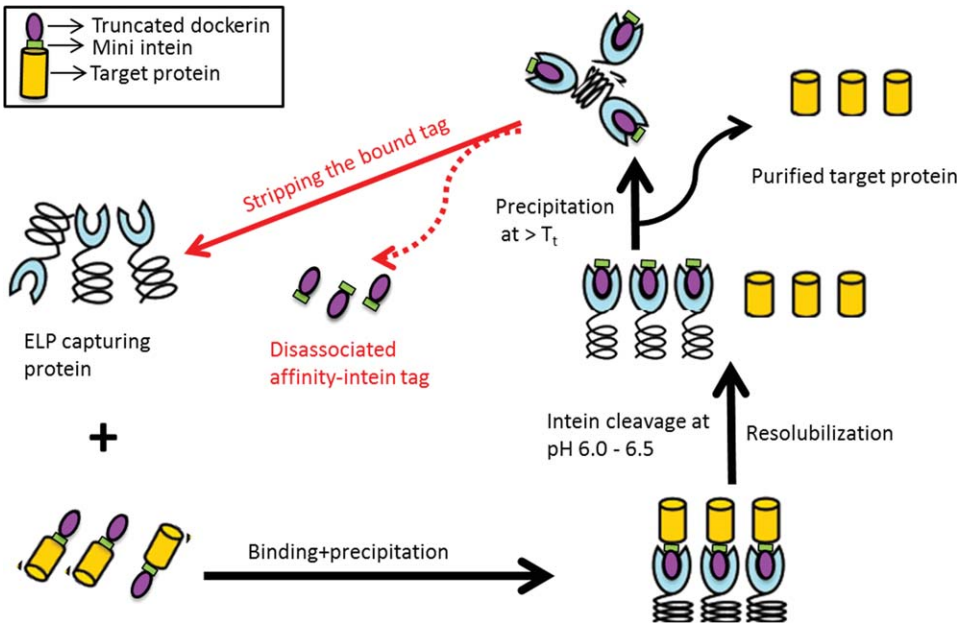

Figure 1. A schematic of the non-chromatographic affinity purification approach involving the regeneration and recycling of the ELP capturing scaffold. Insert: a picture of the fusion target protein.

S1000™ Thermal Cycler (Bio-Rad, Hercules, CA). *E. coli* strain DH5α was used as the host for genetic manipulations.

To construct Doc(Δ16)-intein-CAT (D(Δ)I-CAT), the gene encoding for the truncated dockerin domain from *C. thermocellum* with 16 N-terminal residues deleted was obtained by PCR from the *celS* gene[10] using the forward primer 5′-GGA ATTCCATATGGGCAAGAGATATGTTTTGAGATCAGG-3′ and the reverse primer 5′-GCGGAGCTCACGTTCTTGT ACGGCAATGTATC-3′. The amplified fragment was cloned into *Nde*I and *Sac*I linearized plasmid pET21(+)-EICAT,[2] resulting in a new plasmid pET21(+)-D(Δ)ICAT coding for D(Δ)I-CAT with the engineered *Mycobacterium tuberculosis* (Mtu) recA mini-intein in between.

Expression of recombinant proteins

The expression and purification of the ELP[KV_8F-40]-CohCT (ELP-CohCT) capturing scaffold was done as previously described.[5] *E. coli* strain BL21(DE3) (F^- *ompT gal dcm lon* $hsdS_B(r_B^-\ m_B^-)$ λDE3) expressing D(Δ)I-CAT were grown in Luria-Bertani (LB) medium supplemented with 1.5% glycerol, 20 mM $CaCl_2$, and 100 μg/mL ampicillin at 37°C until OD_{600} reached 1.2–1.5. Protein expression was induced by 400 μM IPTG at 20°C. Cells were harvested and resuspended in the binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$) followed by sonication.

Binding, cleavage, and regeneration

Cell lysates containing D(Δ)I-CAT were incubated with purified ELP-CohCT for 1 h at room temperature in the binding buffer. After incubation, NaCl was added to a final concentration of 2 M and the mixture was heated at 37°C for 10 min and centrifuged for 15 min at 15,000 rpm at the same temperature. The pellet was resuspended in ice-cold cleaving buffer (1× phosphate buffered saline (PBS), 40 mM Bis-Tris, pH 6.2, supplemented with 10 mM $CaCl_2$) and the sample was incubated at room temperature overnight for the cleavage reaction. Once the cleavage reaction was completed, another thermal cycle was used to precipitate the ELP complex and the final purified product left in the supernatant was transferred into a fresh tube. The precipitated ELP complex was resolubilized in 50 mM ice-cold EDTA solution. After the pellet was fully solubilized, the mixture was incubated at room temperature for 4 h. Another thermal cycle was used to precipitate the regenerated ELP-CohCT scaffold and the supernatant containing the disassociated Doc(Δ16)-intein portion was removed. The salt in the purified product was removed by dialysis in the binding buffer using 6,000–8,000 Dalton MWCO Dialysis Membrane (Spectrum, Rancho Dominguez, CA). For each purification step, 20 μL of the protein sample was loaded onto a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel for analysis. The dilution factors for all the samples have been normalized so that protein loading in each lane of the SDS-PAGE gel corresponds to an equal amount of the starting material.

Protein quantification and activity assays

The concentrations of protein samples collected during purification and the final purified samples were measured using the Bradford method. CAT activity was measured in reaction with chloramphenicol in the presence of acetyl coenzyme A followed by the reaction with 5,5′-dithio-bis (2-nitrobenzoic acid) (DNTB) (Sigma, St. Louise, MO). The increase in A_{412nm} was monitored at 25°C using a temperature controlled spectrophotometer with a CPS-controller (Shimadzu).

Results and Discussion

Construction and expression of target proteins using the truncated dockerin domain

The truncated Doc(Δ16) was generated by deleting one of the calcium-binding loops of the full length dockerin domain of CelS from *C. thermocellum*, starting from Asp2 (the first residue of the calcium-binding loop) to Lys18 in the middle of the first α-helix.[11] The truncated Doc(Δ16) domain was

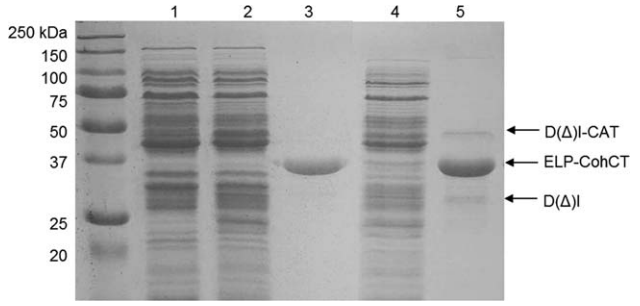

Figure 2. SDS-PAGE analysis of samples taken over the course of ELP-Coh and D(Δ)I-CAT binding experiments.

Lane 1, cell lysates of *E. coli* BL21 harboring pET24(a) as a control; Lane 2, D(Δ)I-CAT total cell lysates; Lane 3, purified ELP-Coh; Lane 4, supernatant after binding and precipitation of the ELP complex; Lane 5, resolubilized ELP-Coh/D(Δ)I-CAT complex before intein cleavage.

Table 1. The CAT Enzyme Activity During Each Purification Step

Step	Enzyme Activity (U/ml)
Clarified cell lysate	3838.2
Soluble unbound fraction	1347.1
Purified precursor, before cleavage (D(Δ)I-CAT)	2211.8
Purified product (CAT)	1948.7

Samples were diluted to the same volume of the clarified cell lysate for direct comparison.

used to replace the dockerin domain from our previously constructed DocCT-intein-CAT fusion[5] to generate Doc(Δ16)-intein-CAT (D(Δ)I-CAT). Protein expression was carried out as described previously.[5] In addition to the mature D(Δ)I-CAT, two smaller bands corresponded to the prematurely cleaved D(Δ)I and CAT proteins of roughly 25 kDa and 27 kDa, respectively, were detected (Figure 2, Lane 2). Premature intein cleavage during expression is a common phenomenon observed in many cases,[3] and could be minimized by decreasing the induction temperature at the cost of lowering expression.

Protein purification using the truncated dockerin domain

The binding affinity of the truncated dockerin has been reported to be 10^{-8} M,[8] which is one order of magnitude lower than that of the full-length dockerin domain (10^{-9} M). To test whether this decrease in affinity could affect the ability of the truncated dockerin to function as an effective affinity tag, purification of D(Δ)I-CAT using the same ELP[KV_8F-40]-CohCT (ELP-CohCT) capturing scaffold[5] was investigated. After 1 h incubation with ELP-CohCT, all the full-length D(Δ)I-CAT and the cleaved D(Δ)I were captured and co-precipitated from the rest of the cell lysates after one cycle of thermal precipitation (Figure 2, Lane 5), while the premature cleaved CAT was left in the supernatant (Figure 2, Lane 4). The complete removal of the D(Δ)I-CAT from the cell lysates (Figure 2, Lanes 2 and 4) suggested that the lower binding affinity of the truncated dockerin tag did not compromise the binding efficiency with the cohesin domain. About 58% of the CAT activity was recovered after a single binding and precipitation step (Table 1) with the activity loss due to premature intein cleavage. Purified CAT was further obtained after inducing cleavage of the ELP-Coh-D(Δ)I-CAT complex in the pH 6.2 buffer (Figure 3A, Lane 3), and the cleaved D(Δ)I tag that was still bound to ELP-CohCT (Figure 3A, Lane 2) was removed by another cycle of thermal precipitation.

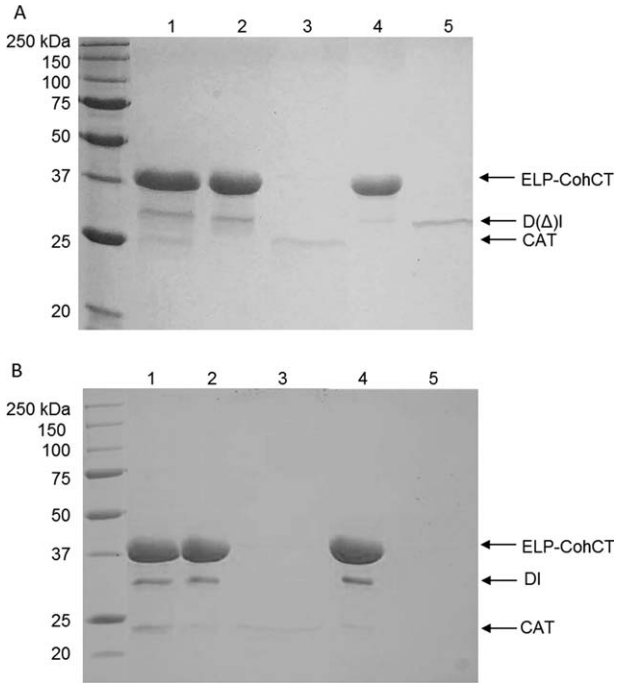

Figure 3. A: SDS-PAGE analysis of the ELP-Coh/D(Δ)I-CAT complex over the course of intein cleavage and CAT purification.

Lane 1, the ELP-Coh/D(Δ)I-CAT complex after intein cleavage; Lane 2, resolubilized ELP-Coh/D(Δ)I complex after precipitation; Lane 3, purified CAT in the supernatant after precipitation; Lane 4, regenerated ELP-Coh capturing scaffold after stripping the bound D(Δ)I with 50 mM EDTA; Lane 5, stripped D(Δ)I tag left in the supernatant after precipitation. B: ELP-Coh/DI-CAT complex over the course of intein cleavage and CAT purification. Lane 1, the ELP-Coh/DI-CAT complex after intein cleavage; Lane 2, resolubilized ELP-Coh/DI complex after precipitation; Lane 3, purified CAT in the supernatant after precipitation; Lane 4, resolubilized ELP-Coh capturing scaffold after stripping with 100 mM EDTA; Lane 5, supernatant after EDTA stripping and thermal precipitation.

Regeneration of ELP-CohCT for subsequent reuse

To recycle the ELP-CohCT capturing scaffold, the bound D(Δ)I tag was removed by the addition of 50 mM EDTA to chelate the Ca^{2+} ion that is required to maintain the structural integrity of the dockerin domain. After incubation, virtually all the D(Δ)I tag was dissociated and separated from ELP-CohCT after precipitation (Figure 3A, Lanes 4 and 5). These results indicated that the truncated dockerin domain retained high affinity toward its cohesin partner, yet its binding could be reversed by the calcium-chelating agent EDTA. In contrast, virtually no regeneration was observed when a similar purification and regeneration scheme was performed using the original full length dockerin domain (Figure 3B). This result supports that the difference in binding affinity is the key in providing the ability for regeneration without any compromise in the capturing efficiency. This is consistent with a previous study, in which the truncated dockerin-bearing protein could be eluted from the Coh-Doc column using EDTA.[11]

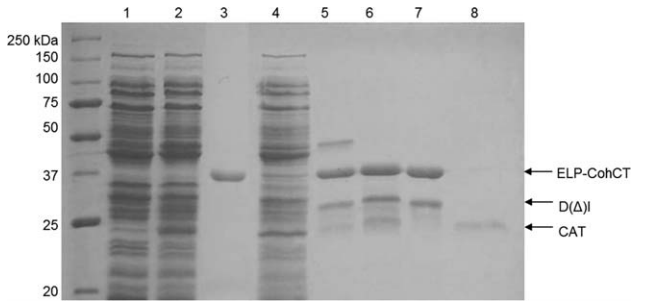

Figure 4. Reuse of the ELP-Coh capturing scaffold for a second cycle of CAT purification.

Lane 1, cell lysates of *E. coli* BL21 harboring pET24(a) as a control; Lane 2, D(Δ)I-CAT total cell lysates; Lane 3, regenerated ELP-Coh after EDTA stripping; Lane 4, supernatant after binding and precipitation of ELP complex; Lane 5, resolubilized ELP-Coh/D(Δ)I-CAT complex before intein cleavage; Lane 6, the ELP-Coh/D(Δ)I-CAT complex after intein cleavage; Lane 7, resolubilized ELP-Coh/D(Δ)I complex after precipitation; Lane 8, purified CAT in the supernatant.

To demonstrate the ability to reuse the regenerated ELP-CohCT capturing scaffold, another purification cycle was performed using the same cell lysates containing D(Δ)I-CAT. Virtually the same level of D(Δ)I-CAT (56%) was captured by the regenerated ELP-CohCT capturing scaffold as compared with the original purification step. Highly purified CAT was again obtained after intein cleavage (Figure 4, Lane 8). The ability to recycle the ELP-CohCT capturing scaffold while retaining almost 100% functionality makes this technology much more economical for large-scale practical applications.

Conclusions

In this work, a truncated Doc(Δ16) domain was used to enable the effective regeneration of the ELP-CohCT capturing scaffold without compromising the binding efficiency. The ease of regeneration using EDTA and the subsequent reuse of the ELP-CohCT capturing scaffold for purification without any decrease in the protein recovery efficiency make this non-chromatographic system very cost-effective for protein purification.

Acknowledgments

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