Purification of *Escherichia coli* RNA polymerase using a self-cleaving elastin-like polypeptide tag

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Abstract: A self-cleaving elastin-like polypeptide (ELP) tag was used to purify the multisubunit *Escherichia coli* RNA polymerase (RNAP) via a simple, nonchromatographic method. To accomplish this, the RNAP α subunit was tagged with a self-cleaving ELP-intein tag and coexpressed with the β, β′, and ω subunits. The assembled RNAP was purified with its associated subunits, and was active and acquired at reasonable yield and purity. To remove residual polynucleotides bound to the purified RNAP, two polymer precipitation methods were investigated: polyethyleneimine (PEI) and polyethylene (PEG) precipitation. The PEG procedure was shown to enhance purity and was compatible with downstream ELP-intein purification. Thus, this simple ELP-based method should be applicable for the nonchromatographic purification of other recombinant, *in vivo*-assembled multisubunit complexes in a single step. Further, the simplicity and low cost of this method will likely facilitate scale up for large-scale production of additional multimeric protein targets. Finally, this technique may have utility in isolating protein interaction partners that associate with a given target.

Keywords: ELP; intein; RNA polymerase; multisubunit enzyme; purification

Introduction

Multisubunit enzymes catalyze many important physiological processes, including transcription, chemical synthesis, and nutrient transport. The mechanism of action of many protein complexes is frequently probed *in vitro*, thus requiring significant amounts of purified, active protein. Historically, multisubunit enzymes have been purified from endogenous sources using a variety of complex purification methods.[1–7] Whereas the purified endogenous preparations are usually properly folded and associated, their purification requires several steps, is time consuming, and product recovery is typically very low. Alternately, multisubunit enzymes can be produced using recombinant DNA technology. In many cases, each subunit is overexpressed and purified separately, and the enzyme complex is subsequently reconstituted *in vitro* from the purified subunits.[8–10] Although the resulting yields are

Abbreviations: CAT, chloramphenical acetyltransferase; ELP, elastin-like polypeptide; PBS, phosphate buffered saline; PEG, polyethylene glycol; PEI, polyethyleneimine; RNAP, RNA polymerase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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generally higher than those obtained from endogenous sources, these methods also require several purification steps, followed by a potentially difficult renaturation step.

Affinity chromatography, including the use of affinity tags, has proven highly successful for the purification of multisubunit enzymes, however, these methods also exhibit some limitations. Among these is the potential for dissociation of the target complexes during elution from the affinity matrix, and unexpected nonspecific cleavage within the target protein by protease during protease-mediated affinity tag removal. Although new methods based on gentle immunoaffinity,[11] and self-cleaving chitin-binding tags have been successfully used to purify protein complexes, including *Escherichia coli* RNA polymerase (RNAP),[12,13] they still rely on expensive chromatographic methods.

A recent alternative to traditional affinity tag chromatography has been reported that exploits the reversible precipitation properties of elastin-like polypeptides (ELP).[14–16] In this case, the ELP tag reversibly precipitates upon gentle warming, allowing simple recovery of the tagged target. This system has been combined with engineered inteins to yield self-cleaving ELP-intein tags, which eliminate the need for proteolytic removal of the ELP tag from the purified target.[14,15] The simplicity and low cost of the self-cleaving ELP-intein method offers an attractive means for purifying assembled multisubunit enzymes in a single step.

This work describes the successful purification of *in vivo*-assembled recombinant *E. coli* RNAP using our previously developed ELP-intein method.[14,17] The *E. coli* RNAP can cycle between two different forms: the catalytically competent core enzyme which is composed of two α subunits associated with the β, β′, and ω subunits, and the σ-associated holoenzyme, which is required to initiate transcription at promoter elements.[18,19] Non-chromatographic purification of recombinant core RNAP was accomplished by fusing the α subunit to a self-cleaving ELP-intein tag and co-overexpressing it with the β, β′, and ω subunits. This was followed by ELP precipitation to isolate and purify the intact recombinant core RNAP. The endogenous host σ^{70} promoter specificity subunit copurified with the core enzyme to yield σ^{70}-associated holoenzyme, which is highly indicative of the "gentleness" of the ELP-intein procedure. In addition, we investigated the ELP-intein method for compatibility with polyethyleneimine (PEI) and polyethylene glycol (PEG) precipitation steps to remove nonspecifically bound polynucleotides from the purified RNAP preparation. Finally, the activity and purification factor of the RNAP preparation was quantified using a nonspecific ribonucleotide incorporation assay.

Results

Purification of E. coli *core RNAP*

In general, the ELP-intein purification method allows a tagged target protein to be selectively precipitated by a mild temperature shift, allowing soluble impurities to be removed.[14–16] The temperature is then lowered and the tagged target is resuspended in a cleaving buffer, causing the ELP-intein tag to self-cleave. The cleaved tag can then be easily separated from the target protein by a subsequent precipitation step.[14] In this work, recombinant *in vivo*-assembled core RNAP was purified by fusing a self-cleaving ELP purification tag to the α subunit and coexpressing it with the β, β′, and ω subunits. The ELP-intein-α fusion protein and the β, β′, and ω subunits were co-overexpressed in the *E. coli* strain BLR (DE3) at 15–18°C. As in previous work,[14] the cells were lysed and the lysate clarified at 4°C and pH 8.5 to recover the soluble, tagged target protein. The tagged α subunit was then purified by selective ELP precipitation using 0.4*M* ammonium sulfate (final concentration) at room temperature. The tagged target was then redissolved in phosphate buffered saline (PBS), pH 6.2 at 4°C. The shift in pH to 6.2 accelerates the cleavage reaction, allowing it to proceed to completion at room temperature overnight. A final 0.4*M* ammonium sulfate (final concentration) precipitation step was used to remove the cleaved ELP-intein tag, leaving the native, purified RNAP in the soluble fraction.

Samples taken throughout the RNAP ELP-intein only purification procedure [Figs. 1 and 2(A)] were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Bands due to the α (36.5 kDa), β (150.6 kDa), and β′ (155.1 kDa), and σ^{70} (70.2 kDa) subunits are clearly visible in the ELP-intein only purified product (Fig. 3, lane 5). As only the α subunit was tagged, the presence of the other four subunits in the purified product indicates their copurification (Fig. 3, lane 5). Further, the absence of uncleaved precursor after 16 h of cleavage (Fig. 3, lane 4) indicated that cleavage proceeded to completion. Although this method is clearly capable of purifying the multisubunit *E. coli* RNAP, and presumably other multisubunit complexes, RNAP is known to nonspecifically bind polynucleotides. These polynucleotides interfere with many promoter-specific assays, and they are therefore commonly removed by one of two precipitation methods. To further assess the capabilities of the ELP-intein method for RNAP purification, it was combined with each of these polymer precipitation methods and RNAP sample purity was evaluated.

Removal of residual bound polynucleotides from the RNAP preparations

The presence of bound polynucleotides in the ELP-intein only purified RNAP preparation [Figs. 2(A)

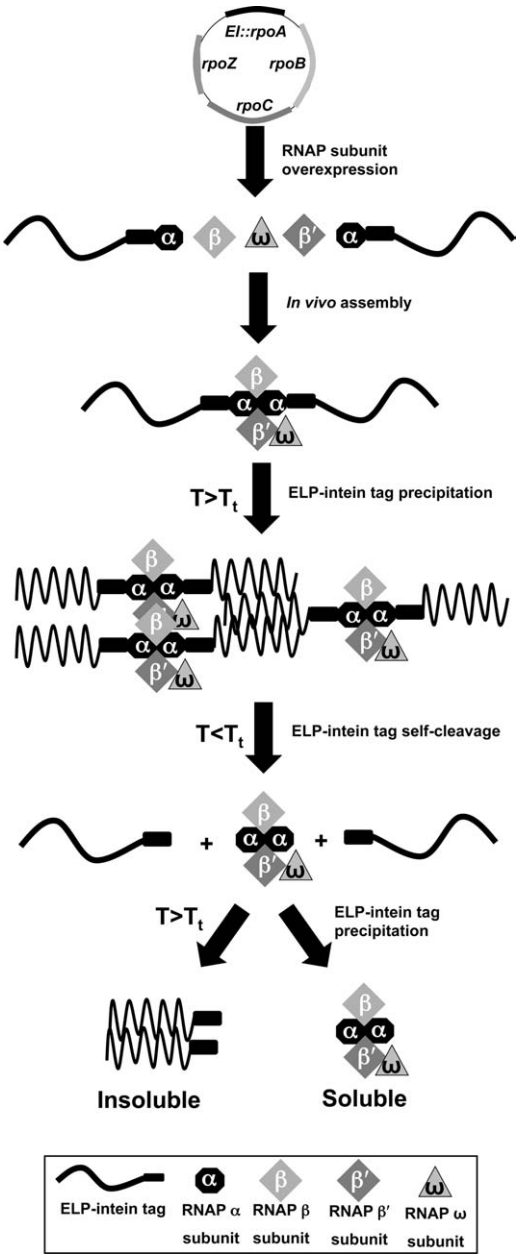

Figure 1. Schematic illustrating the *in vivo*-assembly and purification of recombinant *E. coli* RNAP using self-cleaving ELP-intein precipitation. The gene for the RNAP α subunit was fused to the DNA encoding the ELP-intein tag. In step 1, the ELP-intein-α fusion precursor is coexpressed with the β, β′, and ω subunits in a single host cell. In step 2, the ELP-intein-tagged α subunits associate with other RNAP subunits *in vivo*. In step 3, the ELP-intein tagged complex is recovered and precipitated *in vitro* by raising the temperature (*T*) above the ELP transition temperature (T_t), thus permitting purification by simple centrifugation.[14] In step 4, the temperature of the sample is lowered below T_t and the pH is shifted to initiate the ELP-intein tag self-cleavage reaction, releasing the purified RNAP. In step 5, the mixture is raised above T_t and the insoluble ELP-intein tag is fractionated from the soluble purified enzyme complex.

and 3, lane 5] was confirmed by electrophoretic analysis of the preparation (data not shown). Therefore, two polymer precipitation steps, one using PEI and the other PEG, were tested for compatibility with the ELP-intein system [Fig. 2(B,C)]. These methods exploit the salt-dependent dissociation of polynucleotides from RNAP, allowing their separation from the enzyme complex via selective polymer precipitation at high salt concentrations. This step is generally preceded by a polynucleotide precipitation at lower salt concentrations to separate the polynucleotide-bound RNAP from other proteins, thus enhancing the purity of the RNAP.

The PEI precipitation step was adapted from a previously described method for isolating endogenous *E. coli* RNAP.[20] The cell lysate was mixed with PEI at a low salt concentration to precipitate polynucleotides with the bound RNAP. The resulting pellet was then resuspended at a high salt concentration to dissociate RNAP from the insoluble polynucleotides, which were removed via a final centrifugation step. The resulting supernatant, containing ELP-intein-tagged RNAP, was then mixed with solid ammonium sulfate to precipitate and concentrate the remaining proteins in the sample. Finally, the ELP-intein-tagged RNAP was redissolved in wash buffer and 0.4*M* ammonium sulfate was added to induce ELP precipitation. After incubation at higher temperature to precipitate the ELP-intein-α subunit precursor, the mixture was centrifuged to separate the tagged protein from the remaining soluble contaminants. However, no ELP precipitant was observed after incubation and centrifugation, and SDS-PAGE analysis indicated that the ELP-intein-α subunit precursor remained in the supernatant. Further, a range of ammonium sulfate precipitation concentrations was tested to selectively precipitate the ELP-intein-α precursor, but it was observed that ammonium sulfate precipitation was not ELP-selective; all the proteins in the sample precipitated equally in a salt concentration-dependent manner. Notably, when ELP-intein purification preceded the polynucleotide removal step, PEI addition also failed to yield a precipitate. Therefore, it is clear that the ELP-intein methodology is incompatible with PEI-mediated enrichment of RNAP preparations.

The PEG precipitation step was derived from a previously described small-scale endogenous RNAP purification method.[21] As with PEI, polynucleotides with bound RNAP were precipitated from the PEG solution at a low salt concentration, separating the RNAP-polynucleotide complex from many cellular components. A buffer with a high salt concentration was then used to "release" the RNAP from the polynucleotides, followed by a centrifugation step to fractionate soluble RNAP from the precipitated polynucleotides. This was followed by the ELP-intein purification method to further enhance the purity of

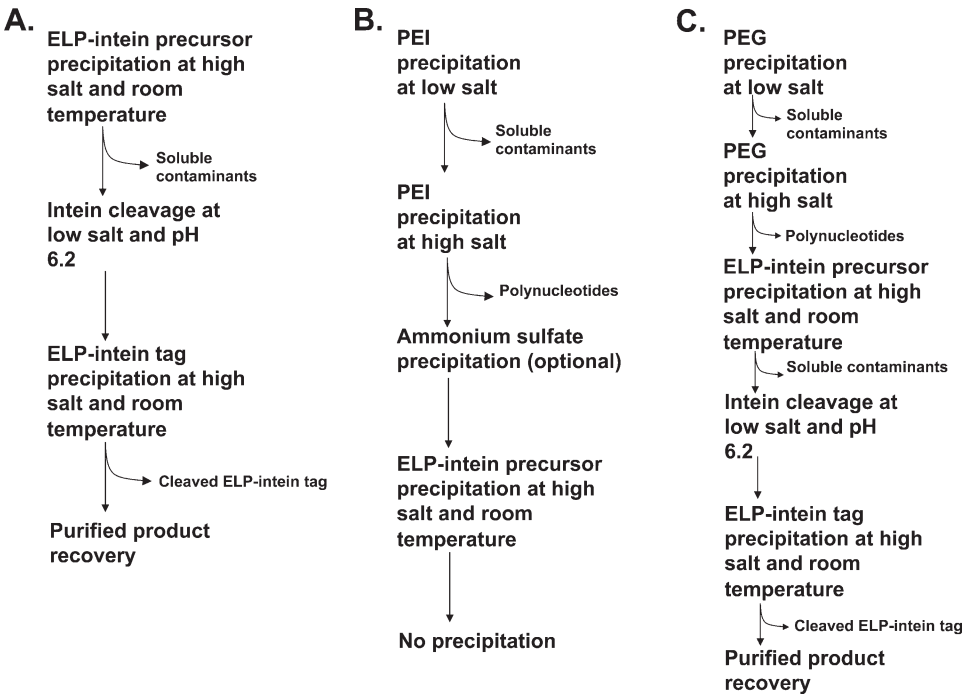

Figure 2. Schematic illustrating the strategies and outcomes for column-free purification of *E. coli* RNAP by self-cleaving ELP-intein tags. (A) ELP-intein purification method without a polymer precipitation step. (B) ELP-intein purification preceded by PEI precipitation to remove residually bound polynucleotides. (C) ELP-intein purification preceded by PEG precipitation to remove associated polynucleotides.

the RNAP preparation. The purified RNAP was analyzed by SDS-PAGE, where it was observed that bands corresponding in size to the core enzyme subunits were clearly present, along with a band due to the σ^{70} subunit [Fig. 4(A), lane 1]. In addition, a faint band attributed to the ω subunit (10.2 kDa) could be observed in the purified product [Fig. 4(A), lane 1], and its identity was unequivocally confirmed by Western blot with anti-ω antibodies [Fig. 4(B), lane 1]. Thus the ELP-intein purification results with PEG paralleled those from the ELP-intein only purification without PEG, which suggests that PEG precipitation does not inhibit subsequent ELP precipitation or intein cleaving, and is thus compatible with the ELP-intein purification method. In addition, it appears that the final purity of RNAP is enhanced when the PEG polynucleotide precipitation step precedes the ELP-intein method [compare Fig. 3, lane 5 with Fig. 4(A), lane 1]. An unexpected result observed with the PEG-ELP-intein method was an apparent increase in the total mass of protein in the sample going from purified precursor to purified product (Table 1, part B). The expected result would be a significant decrease in mass arising from the removal of the ELP-intein tag and any uncleaved precursor, as is observed in the ELP-intein only method (Table 1, part A). Although the source of this anomaly is not clear, we hypothesize that it may be an artifact of the residual PEG in the purified precursor. The final yields of the RNAP

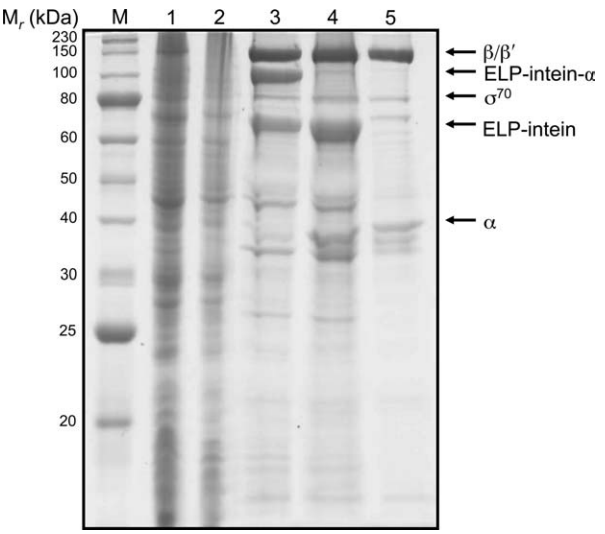

Figure 3. Optimized ELP-intein purification of *E. coli* RNAP. Lane 1, 52 μg of clarified cell lysate; lane 2, 26 μg of supernatant after precipitation of the ELP-intein-α fusion protein; lane 3, 8 μg of resuspended sample at the beginning of the self-cleaving reaction; lane 4, 8 μg of sample at the end of the self-cleaving reaction, and Lane 5, 5 μg of the final purified product. The gel was calibrated with molecular mass standards (lane M) ranging from 230 to 20 kDa; numbers at the side of the image indicate the molecular mass values of the standards in kDa and arrows indicate bands due to ELP-intein-α precursor, ELP-intein, α, β, β′, and σ^{70}. The band due to the ω subunit is faint and not clearly visible.

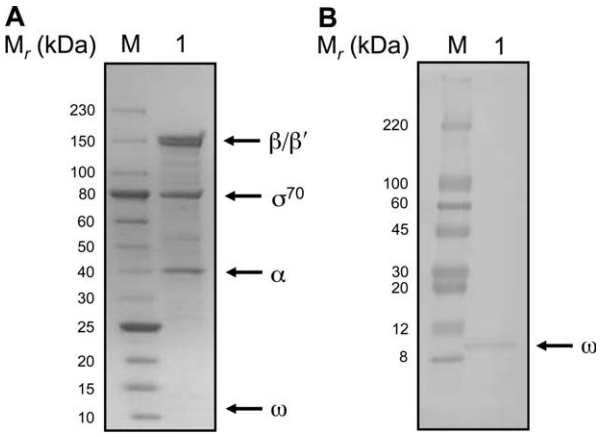

Figure 4. Detection of the ω subunit in the PEG-ELP-intein purified RNAP preparation. (A) An image of an SDS-PAGE gel loaded with PEG-ELP-intein purified RNAP. (B) Western blot analysis of the PEG-ELP-intein purified RNAP preparation using anti-ω antibodies. The gels were calibrated with molecular mass standards (lanes M) ranging from 230 to 10 kDa for SDS-PAGE analysis and 220 to 8 kDa for Western blot analysis; numbers at the side of the images indicate the molecular mass values of the standards in kDa and arrows indicate bands due to the α, β, β′, ω, and σ^{70} subunits.

preparations were 18 mg/L (0.6 mg per gram wet cell paste) for the ELP-intein only purification preparation and 0.9 mg/L (0.03 mg per gram wet cell paste) when both the PEG precipitation and the ELP-intein procedure were combined. The majority of this loss in product was due to insufficient ELP precipitation after polynucleotide removal. It has been hypothesized that polynucleotides aid ELP precipitation, and its removal may hamper precipitation of ELP-bound products. Regardless, the ELP-intein system is capable of efficiently purifying RNAP at reasonable yield if polynucleotide removal is not required.

Finally, an aliquot of the PEG-ELP-intein purified RNAP preparation was probed by agarose gel electrophoresis and $A_{260\ nm}/A_{280\ nm}$ analyses to assess polynucleotide purity. It was observed via agarose gel electrophoresis that the additional PEG step substantially reduces the amount of residually bound polynucleotides compared to the ELP-intein only step, although significant DNA contamination remains (data not shown). This was confirmed by measurements of the $A_{260\ nm}/A_{280\ nm}$ ratios for the different preparations. Specifically, the ELP-intein only ratio was 1.76, corresponding to approximately 50% protein and 50% nucleic acids, while the $A_{260\ nm}/A_{280\ nm}$ ratio for PEG-ELP-intein samples was 1.32, corresponding to approximately 90% protein and 10% nucleic acids. Lastly, if either the PEI or PEG precipitation steps followed the ELP-intein purification, removal of polynucleotides failed.

Determination of RNAP activity

The activities of the ELP-intein only and PEG-ELP-intein purified RNAP preparations were evaluated using a transcription assay based on the incorporation of radiolabeled RNA nucleotides into salmon sperm DNA transcripts.[22] In this assay, catalytically competent RNAP nonspecifically associates with the tailed ends of salmon sperm DNA generated by sonication, producing RNA transcripts with incorporated radioactive RNA nucleotides. After termination, the reaction mixture is spotted and dried on DEAE cellulose paper, which binds to the ends of RNA transcripts whereas unincorporated RNA nucleotides are washed away. Scintillation counts of the washed DEAE cellulose paper are used to compare RNAP test samples to positive and negative controls.

Both the ELP-intein only and PEG-ELP-intein RNAP preparations showed significant increases in specific activity throughout the course of the purification (Table 1). The specific activity for RNAP purified by the ELP-intein only method increased by over 750-fold through the purification procedure (Table 1, part A). Although specific activity is expected

Table I. *Specific Activity of ELP-Intein and PEG-ELP-Intein Purified E. coli RNAP*

Sample	mg Protein[a]	Units	Specific activity (U/mg)	Yield (%)[b]	Purity[c](%)
(A) ELP-intein purified *E. coli* RNAP					
Clarified lysate	4.2[a]	5.1	1.2	100	0.09
Purified precursor	0.12[a]	34.1	284	669	22
Purified product	0.076[a]	70.4	926	1380	71
(B) PEG-ELP-intein purified *E. coli* RNAP					
Clarified lysate[d]	8.1[e]	30.5	3.8	100	0.3
Purified precursor	0.021[e]	17.7	843	58	65
Purified product	0.039[e]	37.1	951	122	73

[a] From 0.1 g wet cell paste starting material.
[b] Yield is calculated relative to measured RNAP activity of clarified cell lysate. Presence of unlabeled nucleotides in the lysate may have led to subsequent calculated yields of greater than 100% (see text).
[c] Purity was determined by specific activity comparisons to that of a commercial *E. coli* RNAP (USB Corporation) positive control standard.
[d] Sample taken after PEG precipitation, before ELP-intein purification.
[e] From 1 g wet cell paste starting material.

to increase upon purification, it is likely that unlabelled nucleotides present in the cell lysate samples inflated these observed increases. Because the assay only detects incorporation of radioactive nucleotides, excess unlabeled nucleotides in the clarified cell lysate would dilute the concentration of radioactive nucleotides in the assay, and thus artificially lower the measured RNAP activity for this sample. In subsequent samples, the concentration of unlabeled nucleotides would be much lower, which would greatly increase the relative incorporation of labeled nucleotides for the same level of RNAP activity, thus causing the relative increases in specific activity and calculated yield to be overstated. This anomaly is also apparent in Table 1, part B, where the specific activity of the released RNAP increased by 250-fold when the ELP-intein purification was performed after an initial PEG precipitation step.

Discussion

Chromatographic purification of target proteins is often expensive, time consuming, and requires multiple steps. Affinity methods using self-cleaving inteins have simplified protein purification by allowing highly specific binding and purification of the tagged target, followed by protease-free release of the target from the resin-bound affinity tag. However, the high cost of traditional affinity resins can limit the attractiveness of these methods at industrial scale. Recently, the reversible aggregation properties of ELP have been used as an inexpensive alternative to conventional affinity tag methods.[14–16] This work describes the single step purification of recombinant, *in vivo*-assembled *E. coli* RNAP using a previously reported self-cleaving ELP-intein purification tag.[14] Furthermore, it was confirmed that this method is compatible with PEG precipitation to remove most nonspecifically bound polynucleotides. Thus this method provides a simple, economical alternative to previously reported single-step RNAP purification methods involving immunoaffinity chromatography[11] or the intein-based IMPACT-CN chromatography system.[12,13]

PEG precipitation for removal of bound polynucleotides was found to be compatible with ELP-intein purification. Although this step significantly decreases the amount of contaminating nucleic acids associated with the purified RNAP, it is apparent that a chromatographic step is still required for complete removal of nucleic acids from RNAP preparations. This can be accomplished by simply passing the purified sample over either DEAE cellulose[21] or a similar anion-exchange column. Several other rapid purification methods for RNAP, such as PEI or PEG precipitation,[20,21] also yield preparations with residual polynucleotide contamination, and thus also require a similar chromatographic polynucleotide removal step.[19,22] Therefore this requirement is not unique to any precipitation-based method, including the ELP-intein method. Regardless, the method of polynucleotide removal can be selected based on the purity and process economics needs of a particular experiment.

PEI precipitation was also tested and found to be incompatible with the ELP-intein purification method. This might result from interactions between the charged PEI molecules and the ELP moiety, which may interfere with the ELP-water interactions that drive ELP precipitation. In addition, when either the PEI or PEG precipitation methods were used after ELP-intein purification, the RNAP-polynucleotide complex failed to precipitate. We hypothesize that this is due to a decrease in the concentration of cellular polynucleotides in the lysate as a result of the ELP-intein purification, which would decrease the overall ability of the bound polynucleotides to precipitate.

Like many proteins,[14] RNAP is active when the α subunit is tagged with the ELP-intein tag, suggesting that the enzyme co-purifies by association of the untagged subunits with the ELP-intein-α fusion protein. RNAP retained its activity through the PEG and ELP-intein purification steps and its specific activity was enhanced by upwards of 250-fold when both the PEG and ELP-intein steps were employed, and by upwards of 750-fold when the ELP-intein step alone was used. The lower improvement in specific activity for the PEG-ELP-intein method was due to the fact that the starting material for the ELP-intein purification had already been partially purified by PEG precipitation. Regardless, with or without PEG precipitation, the final ELP-intein product purity was enhanced significantly (Table 1), and visual inspection of the SDS-PAGE data indicated that the RNAP subunits purified in roughly stoichiometric amounts. Further, comparison of the specific activities of the purified samples to that of a commercially available control preparation suggests that the ELP-intein purified RNAP is roughly 70% pure (Table 1). This level of purity is roughly consistent with visual inspection of SDS-PAGE gels of the purified product [Figs. 3, lane 5 and Fig. 4, lane 1]. The agreement between these measurements suggests that the purified RNAP, although not completely pure, has retained nearly full activity through the purification procedure. Thus the procedure is gentle enough to substantially purify this target with very little loss in activity. Although subsequent chromatographic purification is required to completely remove residual polynucleotides, this method has utility as a simple, rapid and scaleable method for the purification of RNAP.

The successful column-free purification of *E. coli* RNAP suggests the potential for ELP-intein technology to be used for the purification of other multisubunit complexes. While the yields reported here are

low, these results demonstrate proof of concept for the purification of a multimeric protein at very small scale. Increases in scale are trivial with this method, and will likely have a positive impact on yield. It is also expected that yields will improve with other target complexes and subsequent process optimization. For example, with products where polynucleotide contamination is not a concern, the PEG precipitation step can be omitted, which substantially increased product yield in this work. Furthermore, these results suggest that the ELP-intein system could be used to identify interaction partners for other ELP-intein-tagged target proteins, and yield their complexes in a native state to facilitate downstream biochemical analysis. This was observed here when endogenously expressed σ^{70} copurified with the recombinant α, β, β′, and ω subunits. It is also likely that σ^{32} and other RNAP-binding proteins also copurified, and these may possibly account for some of the unidentified bands in Figures 3 and 4. A possible limitation to this application is that any effects of salts on the stability of the complex will likely influence the effectiveness of the technique. Ultimately, this work demonstrates a simple, inexpensive method to purify multisubunit *E. coli* RNAP, and further expands the known capabilities of the ELP-intein purification method.

Materials and Methods

Plasmids

The T7-dependent RNAP overexpression plasmid pEcRNAP1[23] encodes the α, β, β′, and ω subunits in a polycistronic operon (Fig. 5). Using pEcRNAP1 as a template, the gene encoding the RNAP α subunit (*rpoA*) was amplified by the polymerase chain reaction (PCR) using primers (sense: 5′-GGCGGCTGTA CACAACATGCAGGGTTCTGTGACAGAGTTT-3′ and antisense: 5′-GGCGGCAAGCTTCCATGGTCTGTTTC CTCAGTCGCTGACAAG-3′). The resultant PCR product was digested with BsrGI and HindIII restriction endonucleases and cloned between the BsrGI and HindIII sites of pET/ELP-intein:CAT,[14,17] replacing CAT with *rpoA* and thus constructing the ELP-intein-α cassette in pET. Plasmid pET/ELP-intein-α was cleaved with NdeI and NcoI and the DNA encoding the ELP-intein-α fusion protein was cloned between the NdeI and NcoI sites of pEcRNAP1, creating pEcrpo(EI-)ABCZ (Fig. 5).

Overexpression and purification

Plasmid pEcrpo(EI-)ABCZ was transformed into *E. coli* BLR (DE3) cells and transformants were selected in the presence of 100 μg/mL ampicillin. To co-overexpress the ELP-intein-α tagged fusion protein and the other core RNAP subunits, 200 mL of Terrific Broth (TB; 1.2% [w/v] tryptone, 2.4% [w/v] yeast extract, 17 m*M* KH_2PO_4, 72 m*M* K_2HPO_4) supplemented with 100 μg/mL ampicillin was inoculated with a 1:100 dilution of a fresh overnight culture grown in 5 mL of liquid Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin. The TB culture was grown to mid exponential phase ($A_{600\ nm}$ ~ 0.5–0.7; approximately 4 h) at 37°C and recombinant protein overexpression was induced by the addition of 1 m*M* isopropyl β-D-thiogalactopyranoside. Cells were grown for an additional 12–18 h at 18°C post induction and were harvested by centrifugation at 4000 × *g* for 10 min at 4°C. Approximately 1 g of wet cell paste was resuspended in 1.7 mL of pH 8.5 lysis buffer (10 m*M* Tris-HCl [pH 8.5], 2 m*M* ethylenediaminetetraacetic acid [EDTA], 0.1 mg/mL lysozyme) and stored at −20°C. To purify ELP-intein tagged core RNAP, cells containing overexpressed protein were thawed at room temperature and lysed by sonication (4–8 pulses of 7 sec each at a power setting of 0.3–0.5 W RMS). The resulting cell lysates were clarified by centrifugation and purified by one of the three methods outlined in Figure 2.

For purification methods that included PEI precipitation, cell lysates were clarified by centrifugation at 7000 × *g* for 45 min at 4°C. A 10% (v/v) solution of PEI (pH 7.9) was slowly added to the supernatant with constant stirring to a final concentration of 0.35% (v/v). The mixture was stirred for an additional 5 min at 4°C and then centrifuged at 5000 × *g* for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 5.3 mL of TGED buffer (10 m*M* Tris-HCl [pH 7.9], 5% [v/v] glycerol, 0.1 m*M* EDTA, 0.1 m*M* DTT) supplemented with 0.5*M* NaCl. The mixture was then stirred for 5 min at 4°C, and centrifuged at 5000 × *g* for 15 min at 4°C.[20] The supernatant was discarded and the pellet was resuspended in 5.3 mL of TGED buffer supplemented with 1*M* NaCl. The mixture was stirred for 5 min at 4°C, centrifuged at 5000 × *g* for 30 min at 4°C, and the supernatant was collected. Solid ammonium sulfate was added to the eluted sample to a final saturation of 55% (350 g/L) and the mixture was stirred for an additional 20 min at 4°C. The precipitate was pelleted by centrifugation at 7000 × *g* for 45 min at 4°C and the pellet was resuspended in 500 μL wash buffer (10 m*M* Tris-HCl [pH 8.5], 2 m*M* EDTA, 0.1 m*M* DTT) and mixed with an equal volume of 0.8*M* ammonium sulfate to initiate the selective ELP precipitation. The sample was incubated for 10 min at room temperature, and precipitated precursor was pelleted by centrifugation at 14,000 × *g* for 6 min at room temperature, then resuspended in 400 μL of pH 6.2 cleaving buffer (PBS supplemented with 40 m*M* Bis-Tris-HCl [pH 6.2], 2 m*M* EDTA). These samples were incubated overnight (16–18 h) at room temperature to induce intein cleavage. After cleavage, the sample was mixed with an equal volume of 0.8*M* ammonium

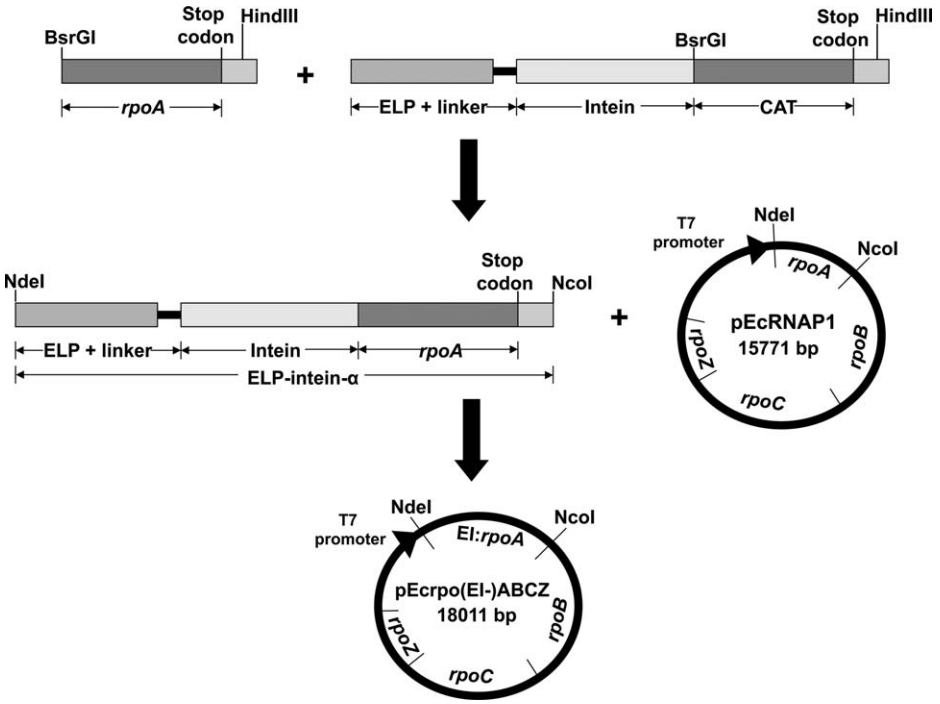

Figure 5. Schematic illustrating the construction of the plasmid pEcrpo(EI-)ABCZ. The plasmid pEcrpo(EI-)ABCZ encodes the tagged ELP-intein-α fusion precursor (encoded by *EI:rpoA*), β (encoded by *rpoB*), β′ (encoded by *rpoC*), and ω (encoded by *rpoZ*) subunits of *E. coli* RNAP under control of a single T7 promoter and terminator.

sulfate, incubated for 10 min at room temperature, centrifuged at 14,000 × *g* for 6 min at room temperature, and the supernatant was recovered.

For purification procedures that included PEG precipitation, the lysate was clarified by centrifugation at 14,000 × g for 6 min at 4°C, followed by the addition of solid NaCl to a final concentration of 0.5*M*. The 10 mL sample was mixed with 14 mL of volume of solution D (17% [w/v] PEG 6000, 157 m*M* NaCl, 10 m*M* DTT) at 4°C.[21] After stirring for an additional 10 min, the mixture was centrifuged at 7000 × *g* for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 mL of solution E (2.0*M* NaCl, 10 m*M* Tris-HCl [pH 7.9], 10 m*M* DTT), centrifuged at 9000 × *g* for 10 min at 4°C and the supernatant was collected for ELP purification as described above. In Figure 4, ELP precipitation was induced as in previous work[14] with 1.5*M* NaCl (final concentration) at 37°C instead of 0.4*M* ammonium sulfate at room temperature.

In cases when the PEI or PEG steps were omitted, cell lysates were clarified by centrifugation at 14,000 × *g* for 6 min at 4°C and mixed with an equal volume of 0.8*M* ammonium sulfate. The ELP precipitation and cleavage steps where then conducted as described above. Samples collected throughout each procedure were analyzed by SDS-PAGE.

Western Blotting

The anti-ω subunit polyclonal antibody, provided by Richard L. Gourse (The University of Wisconsin-Madison), had significant cross-reactivity to other *E. coli* RNAP subunits. Therefore it was precleared against an *E. coli* ω-knockout strain lysate using the following protocol. A single freshly-struck colony of the *E. coli rpoZ* knockout strain JV554 *rpoZ::kan* (also provided by provided by Richard L. Gourse)[24] was inoculated into 50 mL of LB media supplemented with 30 μg/mL kanamycin and grown at 37°C overnight. Cells were harvested by centrifugation at 6,000 × *g* for 20 min. The cell pellet was resuspended in 1 mL of resuspension buffer (50 m*M* Tris-HCl [pH 7.5], 2% [w/v] SDS), boiled for 5 min, and placed on ice. To the lysate, 9 mL of 50 m*M* Tris-HCl (pH 7.5) was added, followed by 0.2 mL of polyclonal anti-ω subunit antibody. The solution was incubated at 4°C for 36 h, then centrifuged at 10,000 × *g* for 15 min and the supernatant collected.

Protein samples were resolved by SDS-PAGE on a 4–20% (w/v) gradient polyacrylamide Tris-glycine gel, and transferred to a nitrocellulose membrane using the iBlot system (Invitrogen). The membrane was blocked for 1 h at 4°C in blocking buffer (2% [w/v] fish skin gelatin (FSG), 1 mM EDTA in PBS). Precleared primary antibody (1.5 mL) was added to 20 mL of dilution buffer (0.5% [w/v] FSG, 1 m*M* EDTA in PBS) and the membrane was incubated an additional 2 h at 4°C. The membrane was washed three times for 5 min each wash with 20 mL of dilution buffer. The secondary antibody, anti-rabbit IgG-horseradish peroxidase conjugate (Sigma-Aldrich), was diluted 1:10,000 in 20 mL of dilution buffer and added to the membrane, and the membrane was shaken for 2 h at room temperature.

Finally, the membrane was washed three times for 5 min each wash using 20 mL of dilution buffer and developed with 1 mL of undiluted (0.05% [w/v] 3,3′,5,5′-tetramethylbenzidine (TMB) solution (Sigma-Aldrich).

Determination of the yield and activity of purified RNAP

The yield of RNAP was determined using the Bio-Rad protein assay kit (Bio-Rad), which is based on the Bradford method.[25] The specific activity was determined for samples collected throughout the purification procedure using a modified version of a previously described method.[26] In each case, 60 μL reactions were prepared containing either 10 μL of in-process ELP purification sample, or purified *E. coli* RNA polymerase positive control (USB Corporation), or BC50 buffer (10 m*M* Tris-HCl [pH 7.5], 5 m*M* $MgCl_2$, 50 m*M* KCl, 1 m*M* DTT, 1 m*M* phenylmethanesulfonyl fluoride [PMSF]) only negative control. Each of these was mixed with 7 μg of sonicated salmon sperm DNA in 5 μL BC50 buffer and incubated for 30 minutes at 30°C. After 30 min, 10 μL of nucleotide mix (0.15 m*M* ATP, 0.15 m*M* GTP, 0.15 m*M* CTP, 0.0015 m*M* UTP, 0.15 m*M* [α-^{32}P]UTP, 20 U RNasin (Fisher), 10 m*M* $MgCl_2$, 5% [w/v] PEG 8000) was added to each reaction. The reactions were incubated an additional 45 min at 30°C, after which each reaction was spotted onto a 0.5 inch x 0.5 inch square of DE 81 diethylaminoethyl (DEAE) cellulose paper (Whatman) and dried under a heat lamp. The DEAE cellulose squares were washed three times with 0.2 M Na_2HPO_4 buffer, followed by two washes with deionized water; 5 min each wash, and a final wash with 95% (v/v) ethanol. The DEAE cellulose paper squares were dried under a heat lamp and the amount of incorporated radioactive nucleotide was measured in a liquid scintillation counter (Perkin-Elmer, Tri-Carb 2800 TR).

One unit of RNAP incorporates 1 nmol of ribonucleoside triphosphate into RNA in 10 min at 37°C. The σ^{70}-associated endogenous *E. coli* RNAP holoenzyme positive control reaction contained 10 units of enzyme. To convert data obtained from the scintillation counter into units; the signal from the buffer only reaction was subtracted from the signal obtained for the RNAP positive control reaction to determine the amount of radioactive nucleotide incorporated by 10 units of enzyme in this assay setup; all subsequent activity determinations were based on this calculation. The background was subtracted from the signal for each sample, and the resulting counts were converted into units using the data from the positive control.

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