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Purification of Recombinant Protein by Cold-Coacervation of Fusion Constructs Incorporating Resilin-Inspired Polypeptides

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ABSTRACT: Polypeptides containing between 4 and 32 repeats of a resilin-inspired sequence AQTPSSYGAP, derived from the mosquito *Anopheles gambiae*, have been used as tags on recombinant fusion proteins. These repeating polypeptides were inspired by the repeating structures that are found in resilins and sequence-related proteins from various insects. Unexpectedly, an aqueous solution of a recombinant resilin protein displays an upper critical solution temperature (cold-coacervation) when held on ice, leading to a separation into a protein rich phase, typically exceeding 200 mg/mL, and a protein-poor phase. We show that purification of recombinant proteins by cold-coacervation can be performed when engineered as a fusion partner to a resilin-inspired repeat sequence. In this study, we demonstrate the process by the recombinant expression and purification of enhanced Green fluorescent protein (EGFP) in *E. coli*. This facile purification system can produce high purity, concentrated protein solutions without the need for affinity chromatography or other time-consuming or expensive purification steps, and that it can be used with other bulk purification steps such as low concentration ammonium sulfate precipitation. Protein purification by cold-coacervation also minimizes the exposure of the target protein to enhanced proteolysis at higher temperature.

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KEYWORDS: coacervation; resilin; recombinant protein; purification; designed polypeptide

Introduction

The biotechnology and biopharmaceutical industries continue to produce a burgeoning array of therapeutic proteins, enzymes, and diagnostic reagents, produced as recombinant proteins in bacterial and eukaryotic cell expression systems. Laboratory studies on recombinant proteins in highly purified and well-characterized forms often utilize affinity-tag systems that share the following features: (a) one-step adsorption purification; (b) a minimal effect on tertiary structure and biological activity; (c) easy and specific removal of the tag to produce native-like protein; (d) simple and accurate assay of the recombinant protein during purification; and (e) applicability to a number of different proteins. Common systems currently used include, poly-His, poly-ARG, FLAG, glutathione *S*-transferase and Maltose-binding protein (reviewed by Terpe, 2003).

These affinity–purification systems work very well on a small scale, but are expensive for scale-up. Purification is likely to represent a major portion of the cost of biopharmaceutical production in bacteria and plants. For example, in commercial production of insulin in *E. coli*, chromatography accounts for 30% of operating expenses and 70% of equipment costs (Petrides et al., 1995). The development of novel purification methods that are simple and inexpensive are therefore likely to be of interest for scientific studies and for the bio-pharmaceutical industry.

Recently, novel purification strategies have been developed utilizing the coacervation properties of elastin. Elastin-like polypeptides (ELPs) have been shown to exhibit temperature-induced phase transitions, similar to those of tropoelastin and α-elastin (Reiersen et al., 1998; Vrhovski et al., 1997). Coacervation of soluble tropoelastin molecules and ELPs is characterized by thermodynamically reversible association as temperature is increased under appropriate ionic conditions, protein concentration and pH (Reiersen et al., 1998; Reguera et al., 2007; Vrhovski et al., 1997). This reversible temperature transition of ELPs has been exploited to purify recombinant proteins (Hassouneh et al., 2010; Meyer and Chilkoti, 1999). Typically, this procedure requires the constructs to be heated to around 40°C

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(Hassouneh et al., 2010; Yeo et al., 2011), which may not be ideal for heat labile or proteolytically sensitive proteins.

Resilin is an elastomeric protein found in insects with unique properties including high elasticity and resilience (Jensen and Weis-Fogh, 1962; Weis-Fogh, 1960). An interesting structural feature in the resilin protein from *Drosophila melanogaster* is that its N-terminal domain comprises multiple, very similar, 15 residue repeating sequences, all of which contain a Tyr-Gly-Ala-Pro (YGAP) sequence (Ardell and Andersen, 2001; Elvin et al., 2005). During studies on the isolation and purification of the recombinant resilin from the first exon of this *D. melanogaster* protein (Elvin et al., 2005), this polypeptide segment unexpectedly showed cold-coacervation, with a phase transition occurring when samples were held at 4°C (Elvin et al., 2005). Recently, a range of biophysical approaches have been used to study the solution characteristics of this resilin segment (Dutta et al., 2011). On heating from 0°C, an abrupt decrease in the hydrodynamic diameter, D_h, was observed at 6°C that corresponds with turbidity changes. This decrease in D_h was indicative of upper critical solution temperature (UCST) behavior, and the association and dissociation was found to be reversible without any hysteresis during temperature cycling (Dutta et al., 2011).

Proteins from other insects, including those from mosquito, *Anopheles gambiae* (Lyons et al., 2007), flea, *Ctenocephalides felis* and buffalo fly, *Haematobia irritans exigua* (Lyons et al., 2011) all contain repeating sequences that include the YGAP motif. These proteins have been subsequently produced from recombinant constructs, and all show cold-coacervation.

We have previously described various multimeric constructs based upon the repetitive motifs observed in the *D. melanogaster* resilin gene (cg15920), and a similar gene from *A. gambiae* (BX619161) that contains many sequence similarities, including YGAP repeats, but whose function has not yet been confirmed. Given that the consensus repeat motifs included in these various multimeric constructs were first generated in study of resilin, we have chosen to name these recombinant products as resilin-inspired polypeptides (RIPs). We also demonstrated that these recombinant RIPs were easily purified, with a final step of cold-temperature coacervation to form RIP-enriched (200–300 mg/mL) coacervates (Lyons et al., 2007). This cold-temperature coacervation appears, at present, to be unique to resilin and resilin-like proteins, and to the designed RIPs.

In the present study, we have examined whether multiple copies of the *A. gambiae* consensus repetitive motif, AQTPSSQYGAP, can be employed as a fusion-tag for co-expression and purification by cold-coacervation of another protein of interest. In this study, enhanced green fluorescence protein (EGFP) (Zhang et al., 1996) was used as a model. We predicted that this cold-coacervation approach may provide a novel means of purifying recombinant proteins, and could eliminate the need for affinity chromatography. Once purified, the protein of interest can be enzymatically cleaved from the RIP tag and the contaminating RIPs removed by a second round of cold-coacervation.

Materials and Methods

Construct Design

Constructs have previously been made containing 4, 8, 16, or 32 copies of the 11 residue *A. gambiae* consensus motif AQTPSSQYGAP in the expression vector pETMCS1. This consensus motif was based upon the repetitive domains of the *A. gambiae* (African malaria mosquito) resilin-like gene, and a recursive gene construction method was used, as previously described (Lyons et al., 2007). These constructs were digested with *Sna*BI and *Hin*dIII, and the liberalized vector was retained and purified.

The compatibility of the blunt-ended restriction enzymes *Sna*BI and *Sma*I has previously been exploited in generating these constructs (Lyons et al., 2007), and is used in adding the engineered thrombin-cleavage domain and EGFP sequence. EGFP (Zhang et al., 1996) was engineered to contain *Sma*I and *Hin*dIII restriction enzyme sites at the N- and C-termini, respectively (Fig. 1). The gene was amplified from an existing vector using the following primers: EGFP_for (5′ACCCGGGCTGGTTCCGCGTGGCTCTATGGTGAG CAAGGGCGAGGA3′) and EGFP_rev (5′CAAGCTTGATCACCTTATACGTACTTGTACAGCTCGTCCATGC3′). The forward primer also contained a thrombin cleavage site, LVPR^GS, upstream of the EGFP sequence (Fig. 1). Phusion DNA polymerase (New England Biolabs, Ipswich, MA) was used for PCR following the manufacturer's recommendations. PCR reactions were performed using a PCT200 thermocycler (MJ Research, St. Bruno, Canada). The reaction profile was 94°C for 3 min, 10 cycles of 94°C for 10 s, annealing at 55°C for 20 s and extension at 72°C for 1 min, followed by 25 cycles of 94°C for 10 s, annealing at 65°C for 20 s and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. PCR products were cloned into pGEM-T Easy (Promega, Alexandria, NSW, Australia), transformed into DH5α and grown on Luria–Bertani (LB) agar plates containing 20 mM glucose and 100 μg/mL ampicillin. Plasmid preparations were performed as per manufacturer's instructions (Qiaprep® miniprep kit; Qiagen, Doncaster, VIC, Australia), and sequences were confirmed with M13 forward and reverse

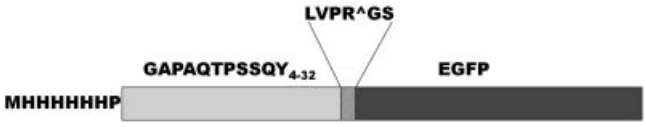

Figure 1. Schematic representation of the construct made in this study.

primers using BIG DYE 3.1 terminator mix on an ABI 3130xl Sequencer (PE Applied Biosystems, Foster City, CA). Sequence files were analysed using Sequencher™ 4.1 (GeneCodes, Ann Arbor, MI).

Colonies containing EGFP were digested with *Sma*I and *Hin*dIII, and the insert was purified by gel extraction. The *Sma*I/*Hin*dIII digested EGFP fragment was then ligated into the *Sna*BI/*Hin*dIII digested vector. As an expression and purification control, the EGFP fragment was also cloned into *Sma*I/*Hin*dIII digested pET MCS1 to create an EGFP only construct. Sequence integrity of all constructs was confirmed by sequencing as described above.

Expression of Recombinant Proteins

Plasmids were transformed into the *rne131 E. coli* strain BL21Star™ (DE3)/pLysS (Invitrogen, Mount Waverley, VIC, Australia) and selected on LB plates containing 20 mM glucose, 100 μg/mL ampicillin, and 34 μg/mL chloramphenicol. Overnight cultures of bacterial cells were grown in LB medium containing 20 mM glucose, 100 μg/mL ampicillin, and 34 μg/mL chloramphenicol, and subsequently used to inoculate 1 L cultures of ZYP-5052 media for auto-induction of protein expression (Studier, 2005). Expression was performed in standard 2 L baffled flasks in flat-bed incubators. Following 4 h growth at 37°C and 20 h growth at 28°C, cells were collected by centrifugation (10,000*g* for 20 min at 4°C) and stored at −80°C prior to extraction.

Extraction of Soluble Proteins

Cell pellets were thawed and resuspended in lysis buffer (50 mM Tris–HCl, pH 7.2, 10 mM benzamidine HCl, 10 mM EDTA, 1% Triton X-100, 10 mM 2-mercaptoethanol), and homogenized using an UltraTurrax T25 (IKA, Staufen, Germany). Treated cells were re-frozen at −80°C and then thawed to promote cell lysis. Cell disruption was completed using a 180 W Ultrasonics™ A180 sonicator (Melbourne, Australia) with a 10 mm ultrasonic probe. The cell suspension was sonicated (30 s, three times) on an ice water bath. The soluble protein fraction was recovered in the supernatant following centrifugation at 20,000*g* for 30 min at 4°C. To remove DNA contamination, polyethyleneimine solution at pH 8.0 was added to the cleared supernatant to 0.5% (w/v) final concentration. After 30 min at 4°C the solution was centrifuged at 8,000*g* for 15 min, and the clarified supernatant collected.

Coacervation of RIP-Tagged EGFP Proteins

For coacervation, protein solutions were held on ice for 1 h, or were held in a cold room at 4°C for 4 h.

Concentration of RIP-Tagged EGFP Proteins

For preparation of protein concentrates of each of the expressed constructs, ammonium sulfate (AS) (SigmaUltra, Sigma–Aldrich, St. Louis, MO) was added slowly to the supernatant with mixing to final concentrations of 10%, 15%, 20%, 25%, 30%, or 40% saturation. Precipitated proteins were retained following centrifugation at 8,000*g* for 30 min, resuspended in sterile phosphate-buffered saline (PBS) to volumes equivalent to the original supernatant, dialysed and compared by SDS–PAGE, fluorescence analysis and UV absorbance at 280 nm.

Thrombin Cleavage of RIP-Tagged EGFP Protein

Cleavage of the fusion proteins was performed using either soluble thrombin (27-0846-01, GE Healthcare, Rydalmere, NSW, Australia) or a Thrombin CleanCleave™ kit (Sigma–Aldrich), both according to the manufacturer's instructions. All cleavage reactions were carried out at room temperature for 16 h with continuous mixing. Cleavage was terminated by adding phenylmethanesulfonylfluoride to 1 mM final concentration. Supernatants were subject to cold-coacervation or were precipitated in an appropriate concentration of AS as determined above.

Analytical Procedures

Protein expression and purity was assessed by SDS–PAGE analysis (Laemmli, 1970) using precast 4–12% gradient Bis–Tris gels (Invitrogen). Electrophoresis was performed at 180 V for 40–80 min, using PageRuler™ Plus prestained protein ladder (Fermentas, Scoresby, Australia). Gels were stained with Coomassie brilliant blue R-250 for 1 h, and destained at room temperature. The SDS–PAGE image was analyzed by Band Leader 3.0 image analysis software to determine the purity of each protein.

Fluorescence was used as a measure of relative levels of recombinant protein. Cell lysates or preparative samples were transferred into black 96-well plates (Greiner Bio-One, Wemmel, Belgium) and measured using a BMG POLARstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany) in fluorescence intensity mode. EGFP fluorescence was detected using 485 nm excitation and 520 nm emission measurement.

Protein concentrations were estimated by measuring UV absorbance at 280 nm using a UV–visible spectrophotometer.

Prior to thrombin cleavage, proteins were analyzed by matrix-assisted laser desorption/ionization mass spectrometry time-of-flight mass spectroscopy (MALDI-TOF MS) to confirm sizes and purity of recombinant proteins. This was performed as previously described (Lyons et al., 2007), using a Voyager DESTR mass spectrometer with data acquisition performed using Voyager Instrument Control Panel v5.10 and analyzed using VoyagerData Explorer v4.0 software (Applied Biosystems).

Results

Expression and Extraction of Recombinant Proteins

Following the cloning of the EGFP sequence into each of pETMCS1 only control vector and An4-, An8-, An16-, and An32-containing constructs, sequence integrity was verified.

After expression and extraction, recombinant protein was found in the clarified soluble fractions from all preparations relative to the pETMCS1 vector only control. Levels of expression were measured by either SDS–PAGE or fluorescence measurements; both methods gave comparable values. The expressed products were readily identified in the SDS–PAGE gels (Fig. 2A). The multi-step process that was used to extract the recombinant proteins was effective, and fluorescence analysis indicated that there was no significant loss of recombinant proteins throughout the extraction and clarification processes (data not shown).

EGFP alone gave the highest expression and An32-EGFP the lowest expression. Of the various RIP-EGFP constructs, An16-EGFP was consistently the most highly expressed at approximately 60% of the expression observed for EGFP alone (Fig. 2B).

Examination of the SDS–PAGE gel (Fig. 2A) also showed aberrant gel mobilities, with the RIP-containing recombinant proteins consistently running slower (larger) than expected.

Precipitation and Coacervation of RIP-Tagged Recombinant Proteins

Purification by coacervation alone can be achieved (data not shown), but this is not a quantitative process and coacervation yields depend upon the concentration of the fusion protein. In this study, cold-coacervation could be best demonstrated using protein samples that had previously been isolated using AS precipitation. Low concentrations of AS have previously been shown to almost exclusively precipitate the RIPs (Lyons et al., 2007, 2011). AS precipitations were performed on clarified soluble protein over a range of AS saturations (10–40%). Recombinant protein was precipitated at 20% AS for An16-EGFP, and An32-EGFP, while 30% AS was needed for An4_EGFP and An8_EGFP precipitation (Fig. 3). EGFP alone did not precipitate until at least 40% AS was used, which is consistent with previous studies investigating salt precipitation of EGFP protein (McRae et al., 2005). The absence of fluorescence at lower concentrations of AS indicates no precipitation of EGFP (Fig. 3).

Following resuspension of the precipitate in 1/10 volume of PBS and dialysis overnight against an excess of PBS, the enriched protein was stored on ice for 1 h. After 30 min a coacervate was observed in An8-EGFP, An16-EGFP and An32-EGFP, resulting in two distinct phases as shown for An16-EGFP in Figure 4A. When observed using a Safe Imager™ blue-light illuminator (Invitrogen) the coacervate was observed to fluoresce much more strongly that the upper phase suggesting an enrichment of the recombinant protein (Fig. 4B). This coacervate was determined spectrophotometrically by absorbance at 280 nm to have a concentration of 240 mg/mL, while the upper layer was 30 mg/mL.

Using the optimized purification regimes for each protein as described above, the volumetric productivity (milligrams of recombinant protein per liter of culture) varied

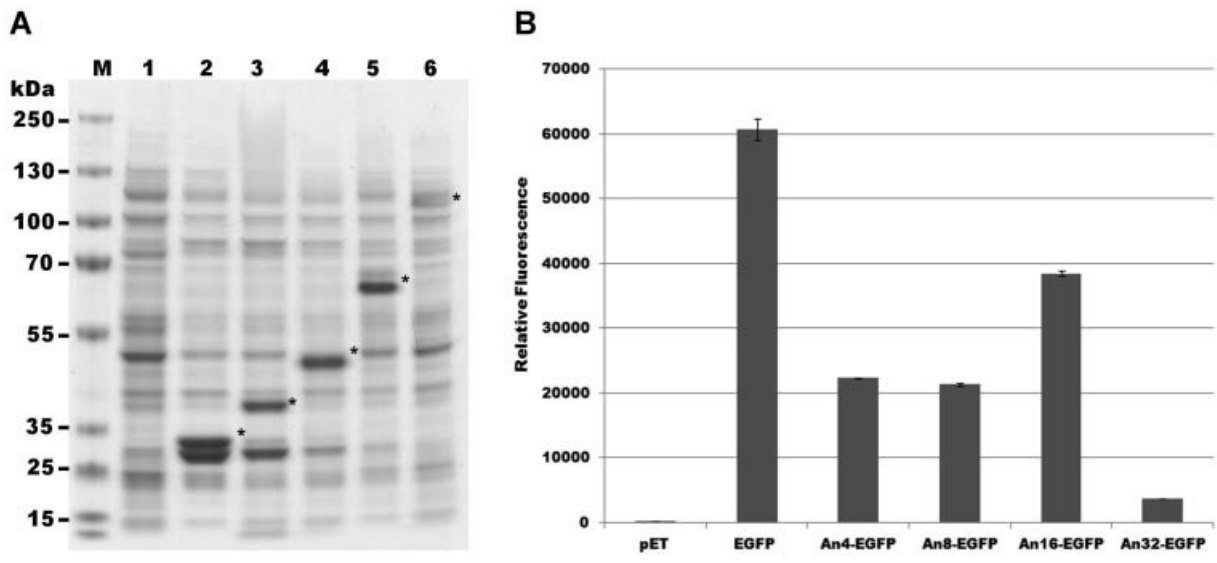

Figure 2. **A**: SDS–PAGE analysis of expressed soluble protein fractions. **Lane 1**: the pETMCS1 control vector, (**Lane 2**) EGFP only, (**Lane 3**) An4-EGFP, (**Lane 4**) An8-EGFP, (**Lane 5**) An16-EGFP, and (**Lane 6**) An32-EGFP. Recombinant proteins are marked *. (**B**) The relative fluorescence (in arbitrary units) of soluble protein fractions from each of these six constructs.

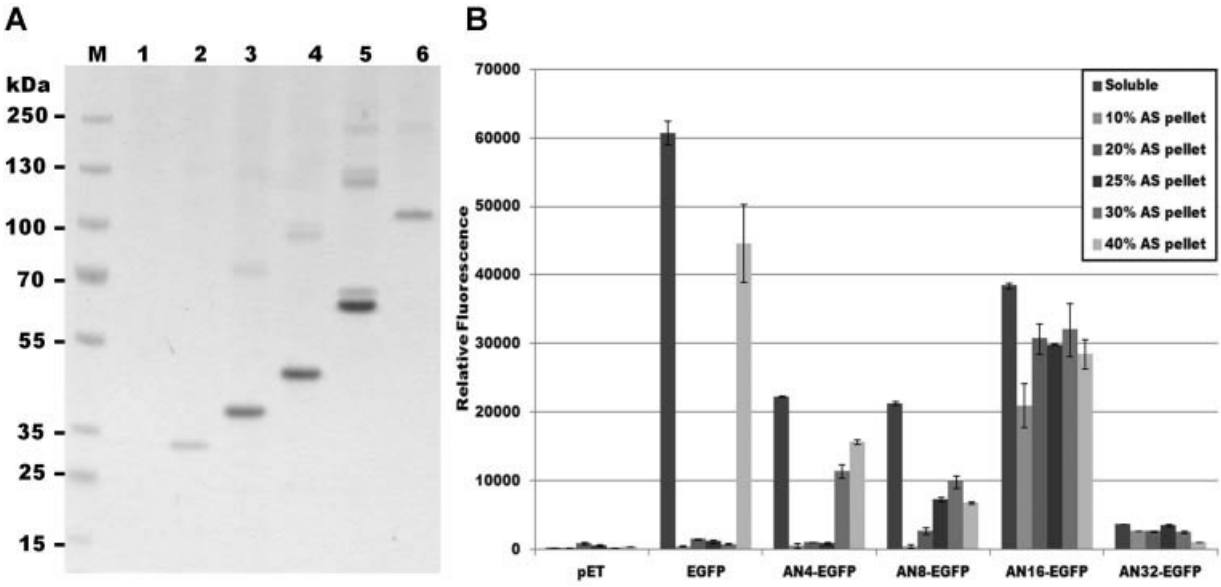

Figure 3. **A**: SDS–PAGE analysis of purified proteins following precipitation by AS saturation (40% for **lanes 1–2**, 30% for **lane 3**, and 20% for **lanes 4–6**) and resolubilisation in PBS. Lane 1: The pETMCS1 control vector, (Lane 2) EGFP only, (Lane 3) An4-EGFP, (Lane 4) An8-EGFP, (Lane 5) An16-EGFP, and (Lane 6) An32-EGFP. **B**: The relative fluorescence (in arbitrary units) for resuspended pellets of each recombinant construct obtained after precipitation at various AS saturations (0–40%). Initial soluble protein, 10% AS pellet, 20% AS pellet, 25% AS pellet, 30% AS pellet, and 40% AS pellet.

significantly for each construct. Concentrations of purified recombinant proteins recovered from batch cultures were determined spectrophotometrically by absorbance at 280 nm. The EGFP only control expressed at 185 mg/L, An4-EGFP at 70 mg/L, An8-EGFP at 110 mg/L, AN16-EGFP at 115 mg/L, and AN32-EGFP at 14 mg/L.

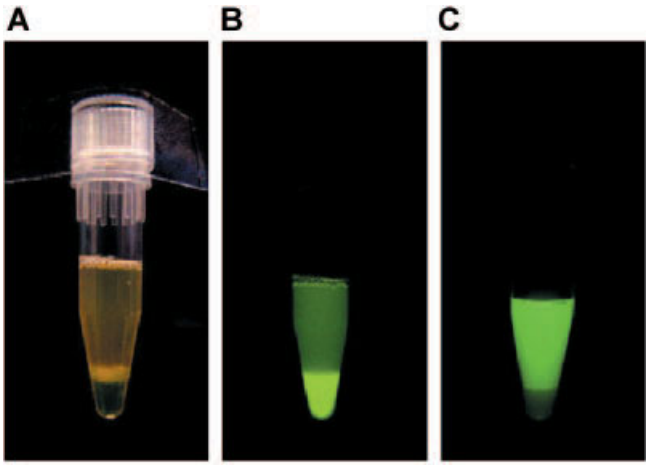

Figure 4. Expressed An16-EGFP, resuspended and dialyzed following isolation using 20% AS precipitation. **A**: Biphasic appearance under white light of protein solution at 30 mg/mL in the upper phase and at 240 mg/mL in the lower, coacervate phase. **B**: The same sample observed under blue light illumination. **C**: The same sample observed under blue light illumination following thrombin cleavage.

Thrombin Digestion

"In silico digestion" of the various recombinant products with thrombin using the PeptideCutter tool (http://ca.expasy.org/tools/peptidecutter/) predicted a single thrombin cleavage site within each recombinant protein. The predicted cleaved EGFP fragment was at 27 kDa, while the predicted cleaved RIP fragments varied in size from 5.9 to 36.4 kDa.

Experimentally, An16-EGFP was initially digested with soluble thrombin (27-0846-01, GE Healthcare) at 10 units thrombin per mg fusion protein. Following overnight incubation cleavage was observed. This was illustrated by blue-light illumination, where the lower coacervate layer minimally fluoresced while the upper phase fluoresced intensely (Fig. 4C). The upper phase consisted of liberated EGFP, while the AN16 component formed a coacervate as before, but with reduced fluorescence. SDS–PAGE analysis showed that the cleavage of EGFP from An16 was incomplete. Soluble thrombin contaminants within the solution made it difficult, however, to get good purification, as judged by SDS–PAGE gels (data not shown).

In this case, where there was incomplete cleavage, some An16-EGFP was present in the upper phase. The lower coacervate layer, however, contained predominantly An16 suggesting that the self-associating, cold-coacervating polypeptide excludes other proteins, including An16-EGFP, from the coacervate.

To reduce thrombin contamination following cleavage, we used the Thrombin CleanCleave™ kit (Sigma–Aldrich), after diluting the recombinant proteins to 1 mg/mL for

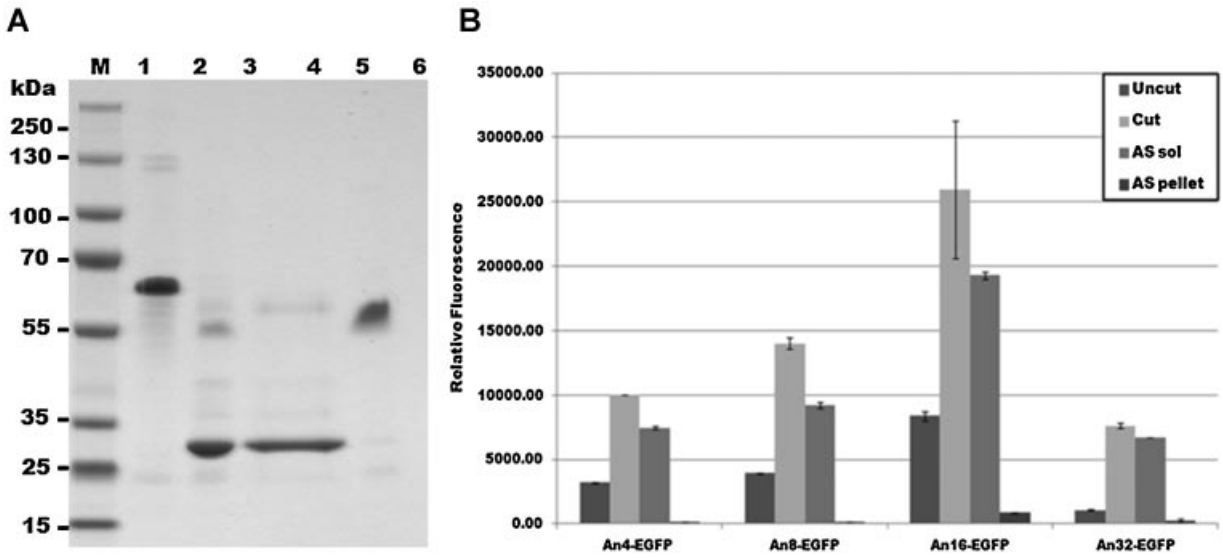

Figure 5. SDS–PAGE evaluation of thrombin digestion of An16-EGFP. **Lane 1**: Undigested An16-EGFP and (**Lane 2**) Thrombin-digested An16-EGFP, with the released EGFP running at 27 kDa.

digestion. Following overnight incubation with the thrombin–agarose suspension at 24°C with gentle agitation, the thrombin–Agarose resin was removed following the manufacturer's instructions. SDS–PAGE analysis (Fig. 5) showed that the cleavage of EGFP from An16 was reasonably complete and that there was little thrombin contamination. MALDI-TOF MS confirmed the cleavage of the An16-EGFP protein.

SDS–PAGE analysis and fluorescence measurement was used to quantify the cleavage efficiency. Following cleavage of the recombinant protein, fluorescence increased several fold, suggesting that either the RIP fusion tag incompletely quenches EGFP fluorescence or concentration-dependent fluorescence quenching is occurring. Cleavage efficiency was determined as the percentage fluorescence of recovered, uncleaved protein compared to total fluorescence of the uncut protein. An32-EGFP had the lowest cleavage efficiency at 75% cleavage, followed by An16-EGFP at 89% cleavage, and An8-EGFP and An4-EGFP both at 95% cleavage.

Discussion

In this study we have shown the utility of RIPs as fusion tags for the expression and purification of recombinant proteins. The RIP constructs in this study were designed based on the repetitive sequence domains found in resilins (Ardell and Andersen, 2001; Elvin et al., 2005) and in a range of other insect proteins that have related sequences (Lyons et al., 2007, 2011). Although these other proteins may not have the same in vivo functional properties (Andersen, 2010; Lyons et al., 2011), their structural characteristics lead to similar cold-coacervation properties. These RIPs are currently the only class of proteins that have been demonstrated to possess low-temperature coacervation properties. This allows concentration of recombinant RIP-tagged proteins to upward of 200 mg/mL simply by placing the solution on ice.

These RIPs had previously been expressed as soluble polypeptides in *E. coli*, with constructs containing up to 64 repeats of the 11 residues *A. gambiae* consensus motif having been expressed (Lyons et al., 2007). Although expression levels were variable, the construct with 16 repeats of 11 residues (An16) produced a soluble and stable protein at greater than 400 mg/L using the Studier method for auto induction of protein expression (Lyons et al., 2007; Studier, 2005).

Coacervation has been used previously as a purification strategy, most notably with ELP-tags (Meyer and Chilkoti, 1999). These tags are useful in that they will coacervate when heated to a specific phase transition temperature, usually around 40°C (Meyer and Chilkoti, 1999). This temperature can be sensitive to the composition and molecular weight of both the ELP and accompanying fusion proteins. Unlike these ELP systems, the RIP constructs coacervate at low temperature, providing an advantage in reducing the likelihood of heat denaturation and minimizing any non-specific protease digestion of the expressed recombinant protein.

In the present study, SDS–PAGE and fluorescence measurement have been used to track purity and quantity of protein extracts. While the proteins appear to be pure, additional investigation is needed to accurately assess levels of *E. coli* protein contaminants. In SDS–PAGE gels, the mobility of RIP containing bands was slower than expected from their calculated molecular weights, which had been confirmed by MALDI-TOF MS, confirming previous

observation (Lyons et al., 2007). EGFP alone, however, migrated at approximately 27 kDa, as expected. Similar changes to mobility have been observed with other proteins, typically those with unusual compositions, such as glycoproteins, insect cuticle proteins, including those with a high Ala content (Andersen et al., 1995) and collagens, with a low hydrophobic residue and high Gly content (Hayashi and Nagai, 1980).

The coacervation process is most affected by the relative size of fusion tag. Effective coacervation with EGFP as the fusion protein only occurred with An16 and An32 based constructs, so it is preferable that the RIP component is the larger, dominant part of the fusion construct. The present study suggested that the size of the RIP component should be equal to or greater than the other fusion component. Thus, for example, An16 would be suitable for small to medium sized peptides or proteins, typically 5–30 kDa, with larger AN-constructs possible if required. This also suggests that a better understanding of this sequence–function relationship could lead to other RIP-tag structures with improved properties.

Thrombin cleavage was selected as a simple, readily available system. Further optimization of the thrombin cleavage process to approximate complete cleavage was not performed in the present study, as optimum conditions will depend on the other, non-RIP, protein in the construct. Removal of proteases following cleavage is an important consideration, but a range of commercially available products exist that avoid this contamination, such as the Thrombin CleanCleave™ kit (Sigma–Aldrich) employed within this study. Proteases may not be suitable for all applications, particularly larger scale production where an alternative system such as an intein insertion (Banki and Wood, 2005) may be more appropriate. Alternatively, in some cases cleavage may not be required. For example, a polymeric nano-particle construct could be formed through photo-crosslinking via the Tyr residues in the RIP segments (Elvin et al., 2005; Fancy and Kodadek, 1999).

The present study represents a proof of concept study only to highlight the potential application of RIPs as purification tools. Clearly significant further optimization of the system, including characterization of different types of RIPs, is required before this approach becomes a practical addition to the repertoire of purification systems available for biotechnology and biopharmaceutical industries. Reducing the time for purification and increasing purified yields would greatly improve the attractiveness of this technique, and might be achieved through characterization of different types of RIPs or improved understanding of the coacervation process. Results presented clearly demonstrate that this simple RIP system has the potential to produce high purity, concentrated solutions of protein without the need for affinity chromatography or other time-consuming and expensive downstream purification steps. The process is also compatible with other purification procedures. In the present study, coacervation was enhanced using low (20%) ammonium sulfate concentrations. Our study has shown that cold–coacervation is applicable to *E. coli* expression systems, but it is also expected that it would work well in other eukaryotic cell expression systems.

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