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Rapid and simple purification of elastin-like polypeptides directly from whole cells and cell lysates by organic solvent extraction

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Abstract

Elastin-like polypeptides (ELP) are a well-known class of proteins that are being increasingly utilized in a variety of biomedical applications, due to their beneficial physicochemical properties. A unifying feature of ELP is their demonstration of a sequence tunable inverse transition temperature (T_i) that enables purification using a simple, straightforward process called inverse transition cycling (ITC). Despite the utility of ITC, the process is inherently limited to ELP with an experimentally accessible T_i . Since the underlying basis for the ELP T_i is related to its high overall hydrophobicity, we anticipated that ELP would be excellent candidates for purification by organic extraction. We report the first method for rapidly purifying ELP directly from whole *E. coli* cells or clarified lysates using pure organic solvents and solvent mixtures, followed by aqueous back extraction. Our results show that small ELP and a large ELP-fusion protein can be isolated in high yield from whole cells or cell lysates with greater than 95 % purity in less than 30 min and with very low levels of LPS and DNA contamination.

TOC image

1. Introduction

Elastin-like polypeptides (ELP) represent a class of proteins derived from mammalian elastin, also commonly referred to as tropoelastin (TEL). TEL is composed of many

[†]Footnotes relating to the title and/or authors should appear here.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].

Conflicts of interest

The authors declare no competing financial interest

repeating hydrophilic and hydrophobic domains, with the pentapeptide unit VPGVG being the most prevalent hydrophobic unit.^{1,2} Synthetic ELP are based on a related pentapeptide repeat, VPGXG, where X is a guest residue that may be any amino acid except proline. The hallmark feature of ELP is their intrinsic lower critical solution temperature (LCST) in water, where heating the solution to a certain threshold temperature drives hydrophobic collapse and reversible ELP coacervation. Urry et al. established the biophysical understanding of this so-called inverse transition temperature (Tt).^{3–6} This critical temperature is highly dependent on numerous intrinsic factors, including ELP molecular weight and guest residue hydrophobicity, and external factors, such as pH, ELP concentration, and salt composition.

In addition to the appealing feature of a tunable Tt, ELP offer several additional benefits that make them highly attractive materials in biomedical and therapeutic applications.⁷ For example, they can be imbued with unique functionality, such as targeting peptides or sites for chemical modification via genetic manipulation. Additionally, ELP have shown beneficial stealth-like and pharmacokinetic properties comparable to PEGylation,⁸ while being biodegradable⁹ and nonimmunogenic.¹⁰ A variety of applications, including targeted delivery mediated by targeting peptides^{11,12} and local hyperthermia^{13–15}, size-controlled nanoparticle formation¹⁶, small molecule drug^{17–19} and vaccine^{20,21} delivery vehicles, tissue engineering,^{22–24} anti-fouling coatings^{7,25}, and underwater adhesives²⁶ have been reported using ELP constructs.

As an alternative to commonly used affinity or size-exclusion chromatography methods, ELP can be uniquely purified non-chromatographically by exploiting their inherent Tt. ELP can also be utilized as a purification tag for fusion protein isolation. The Chilkoti group pioneered this relatively simple purification process, known as inverse transition cycling (ITC), wherein a clarified lysate containing an ELP or ELP-fusion is transitioned above its Tt, isolated from the soluble contaminants by centrifugation, re-dissolved in cold aqueous solution, and centrifuged again to remove insoluble contaminants.^{27–30} Repeating this cycle multiple times increases ELP purity. In many cases, a highly pure ELP can be isolated in less than a day by performing 2 – 5 rounds of ITC; however, each round also diminishes the total yield of the purified material.

Despite the vast number of publications describing ITC as an ELP purification method, the process has severe inherent limitations in some cases. For example, non-protein macromolecular contaminants, such as nucleic acids, can be problematic for the ITC process and are commonly co-purified with the target ELP.^{27,28,31} Although a nucleic acid precipitation step with branched polyethylenimine (bPEI) is often effective before ITC, it increases the processing cost and time, as well as being incompatible with certain ELP constructs.^{31,32}

In addition, the utility of ITC is limited only to ELP exhibiting convenient transition temperatures. Some constructs in this range still do not transition strongly and, subsequently, purify poorly by ITC. This limit is primarily governed by the overall hydrophobicity and molecular weight of the ELP construct. For very hydrophobic guest residues (or fusion proteins) and high molecular weight ELP, the Tt falls below 20 °C and becomes

experimentally difficult. Low-expressing ELP, hydrophilic inclusions, and very low molecular weight ELP that exhibit transition temperatures too high to reasonably reach in aqueous solution, even when including salts to lower the T_t, are examples of other experimentally challenging cases. Since ITC, due to its unique specificity and simplicity, is typically the preferred method for ELP purification, therefore, ELP found in the literature are rarely at the extremes of hydrophobicity/hydrophilicity or molecular weight ranges.

Non-chromatographic protein purification methods, other than ITC, are rare. Purification by organic extraction; however, has been utilized for a handful of proteins with unique physicochemical properties. Of particular interest, Yakhnin and coworkers recognized an opportunity for exploiting the highly hydrophobic properties of green fluorescent protein (GFP) and described the first extraction method for GFP purification from bacterial cell lysates.³³ This simple two-step method was further optimized for GFP and a number of similar fluorescent variants, offering 70 % recovery and purity exceeding 95 % in less than an hour.³⁴

Similarly, Sandberg and coworkers recognized the hydrophobicity of tropoelastin and developed a complex extraction method for recovering soluble TEL from tissue samples.³⁵ While other methods are typically preferred today for TEL recovery,³⁶ this procedure has been used to extract elastin from bovine tissues^{37,38} and a modified method successfully purified TEL from *E. coli* lysates.³⁹ Using a mixture of n-propanol and n-butanol, an estimated yield of 90 % TEL was recovered from lysate, with minimal non-target protein contamination. Although effective and relatively fast, this process still takes several hours to complete.

Inspired by the successful extractions of GFP and TEL, we hypothesized that intrinsically hydrophobic ELP and ELP-fusions may be rapidly isolated in high purity via organic solvent extraction. Further, due to the destabilizing nature of organic solvents on bacterial cells (e.g., *E. coli*), we anticipated that a single step could simultaneously lyse the expression cells and selectively extract the target ELP without nucleic acid contamination. Herein, we describe a set of organic extraction procedures for rapid recovery of extremely pure ELP and an ELP-fusion directly from whole cells and cell lysates with little or no contamination by nucleic acids or lipopolysaccharides.

2. Materials and Methods

2.1. Materials

Material suppliers are reported in the ESI.

2.2. ELP expression

Plasmids encoding V12, V24, and CryS96⁴⁰ were generously provided by the MacKay lab. ELP constructs were expressed in BL21(DE3) *E. coli*, using methods similar to those previously published.⁴¹ Competent BL21 cells were transformed with ELP plasmid constructs using standard heat shock methods and plated on selective agar. Individual colonies were chosen for expression and used to inoculate 5 mL of LB in 50 mL conical tubes. This starter culture was grown in an incubator/shaker at 300 rpm and 37 °C for 18 h;

the cells were then pelleted by centrifugation at $6,000 \times g$ for 10 min, resuspended in TBPV (Terrific Broth supplemented with 10 mM proline and 100 mM valine), and transferred to a total of 333 mL TBPV in a 2 L flask. Primary cultures were shaken at 300 rpm and 37 °C; induced cultures had 2 mM IPTG added to the TBPV in the primary cultures. After 24 h, cells were collected by centrifugation at $6,000 \times g$ for 15 min and excess media was decanted. Cell pellets were flash frozen and stored at -80 °C until further work-up.

2.3. Cell Lysis

2.3.1 Conventional physical and chemical lysis—Following expression, cells were lysed using standard procedures for physical or chemical lysis. For physical lysis, cells were thoroughly resuspended in pure water (4 mL per 1 g wet pellet weight) and incubated, on ice, with lysozyme (50 – 500 µg/mL) for 30 – 60 min with periodic mixing. Then, the cell suspension was probe tip sonicated, on ice, with a 1/8" microtip and a 30 % duty cycle for 15 min. One of two lysis buffers were used for chemical lysis: homemade buffered urea³¹ or commercial B-PER (Thermo Fisher Scientific). For the urea method, cells were thoroughly resuspended in lysis buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris, pH 8.0; 4 mL per 1 g wet pellet weight) and incubated at 20 °C for 1 h on a shaker. The B-PER method was followed according to manufacturer instructions, resuspending the cell pellet with lysozyme (0.1 mg/mL) in 4 mL B-PER per 1 g wet pellet weight and a 15 min incubation with shaking at 20 °C. After lysis, regardless of method, insoluble debris was pelleted by centrifugation at $15,000 \times g$ for 15 min and decanted to obtain clarified lysate for use in subsequent steps.

2.3.2. Combined lysis and purification by organic solvent extraction—For the combined lysis and purification approach, cell pellets were thoroughly resuspended in the organic solvent extractant of choice, similarly to the methods previously described. The rest of the lysis and purification procedure was patterned after the organic extraction purification method for cell lysates and is described in the section on organic extraction from whole cells.

2.4. ELP Purification

2.4.1. Inverse Transition Cycling—Target ELP were subjected to one or more rounds of ITC using the general method of Meyer, *et al.*²⁷ wherein the ELP was precipitated at 30 °C (construct dependent), pelleted by centrifugation at elevated temperature, and re-dissolved at 20 °C.

2.4.2. Organic solvent extraction from cell lysates—ELP were purified at 20 °C using an organic extraction similar to that described for GFP.^{33,34,42} For all small scale screening, 100 µL of clarified lysate was used for extraction using the following general steps at 20 °C. Initially, a non-optimized extraction method (Scheme 1A) was performed. In a 1.5 mL microcentrifuge tube, 60 µL of 5 M NaCl and 500 µL of a saturated (NH₄)₂SO₄ solution were combined as an aqueous salt solution, followed by 400 µL of the organic extractant laid over the top. Clarified lysate (100 µL) was then added to the organic phase and the mixture was immediately vortexed for 5 – 10 s prior to centrifugation at $8,000 \times g$ for 5 min. After centrifugation, the upper organic phase containing the target protein was selectively recovered by pipetting, taking care to avoid the aqueous subphase and

precipitated interphase. A similar procedure was followed when separating the process into two steps by first extracting the lysate with only organic solvent, adding the aqueous salt solutions to the recovered organic phase, and again recovering the upper organic phase after centrifugation (Scheme 1B). Larger scale extractions were performed using the same relative volumes.

2.4.3 Organic extraction from whole cells—The process for extraction directly from whole cells (pelleted cells with the supernatant removed) is patterned after that used for extraction from cell lysates. For this direct extraction procedure, target protein was extracted at 20 °C with 4 mL of organic extraction solvent per 1 g wet pellet. The samples were then thoroughly vortexed, bath sonicated for 1 – 5 min, and periodically mixed for 15 – 60 min. In cases including added salts in the process, aqueous salts were added after the cell pellet was resuspended in organic solvent. After the incubation period, insoluble cellular debris was pelleted at $15,000 \times g$ for 15 min and the extraction solvent was recovered.

2.5. Target protein recovery methods

2.5.1 Back-extraction from organic solvent—Protein samples in organic solvent were concentrated and recovered in water by back-extracting at 20 °C in a centrifuge tube. Typically, a small amount of water (10 – 50 % of the volume of lysate used in the initial extraction) was first added to the protein solution and mixed thoroughly. Then, 2 – 5 volumes of anti-solvent (usually ethyl acetate or diethyl ether) was added to the solution until it became cloudy before brief centrifugation to facilitate phase separation. In most cases, the lower aqueous phase was approximately equivalent to the volume of water initially added. Finally, the upper organic phase was removed, leaving the remaining aqueous subphase that contained the target protein.

2.5.2 Alternative recovery methods—A variety of evaporative strategies were used to assess the efficiency of ELP recovery. For passive evaporation, samples (typically in organic solvent) were either placed on an orbital shaker in a 37 °C environment or placed under a focused stream of air or N₂ at ambient temperature until dry. Alternative methods employed rotary evaporation or lyophilization (~100 mTorr). Lyophilized samples were first back-extracted into water, then flash frozen without the addition of cryoprotectants, and loaded into a lyophilizer and processed until all the water was removed. Regardless of the recovery method used, samples were re-dissolved in water prior to analysis.

2.6. Electrophoretic analysis

2.6.1 SDS-PAGE—Target proteins were separated via SDS-PAGE to identify purification efficiency and provide initial confirmation of protein identity by molecular weight, compared to a molecular weight ladder Bluestain 2 (Gold Bio). Proteins were electrophoresed through a 15 % resolving gel with a 5 % stacking gel in a Tris-glycine buffer system. Samples were prepared using a 2X loading solution consisting of stacking gel buffer, glycerol, SDS, BME, urea, and bromophenol blue and incubated for 5 min at 95 °C; electrophoresis was carried out at 180 V for 45 – 60 min at 20 °C.

Following electrophoresis, proteins were stained with Coomassie Brilliant Blue G-250 using standard procedures. Briefly, for rapid Coomassie staining, gels were rinsed with water and soaked in a mixture of 40 % MeOH and 10 % HOAc for one min. Next, the gels were immersed in Coomassie stain solution (0.25 % w/v Coomassie Brilliant Blue G-250, 40 % MeOH, 10 % HOAc), microwaved for ~25 sec in the stain solution, and incubated for 10 min with gentle rocking. Gels were de-stained in deionized water or de-stain solution (40 % MeOH, 10 % HOAc) until sufficient contrast was apparent. Finally, gels were typically imaged using a BioRad Chemidoc Touch imaging system.

2.6.2 Agarose gel electrophoresis—Agarose gel electrophoresis was used to identify the presence of contaminating nucleic acids that were not removed in the various protein purification methods. In brief, 0.5 % agarose gels were cast in TBE buffer (pH 8.7) with a 1:10,000 dilution of nucleic acid detection reagent GelRed (Biotium). Samples were prepared with a 5X sample loading buffer containing TBE and glycerol; electrophoresis was conducted at 20 °C for 45 min at 80 V. After electrophoresis, gels were imaged with a BioRad Chemidoc Touch imaging system.

Additionally, agarose gel electrophoresis was used to separate and analyze proteins in cell lysates and extracts based on differing isoelectric points. Briefly, samples were prepared in 5X loading solution consisting of glycerol in TBE buffer and loaded in 1 % agarose gels cast in TBE buffer (pH 8.7). Electrophoresis was conducted at 20 °C for 1 h at 80 V. Prior to Coomassie staining, gels were fixed in 25 % IPA and 10 % HOAc with gentle rocking for 1 h. Then, the gels were soaked in Coomassie stain solution (0.06 % w/v Coomassie Brilliant Blue R-250 and 10 % HOAc) until dark bands were visible; typically, staining took up to 4 h. Finally, gels were imaged in a BioRad Chemidoc Touch imager.

2.7. Alcohol dehydrogenase assay

Chaperone activity was measured using the alcohol dehydrogenase (ADH) aggregation assay.⁴⁰ The kinetics of aggregation were monitored at 360 nm using a spectrophotometer equipped with a temperature controlled plate holder, Synergy Neo (Biotek). Aggregation of ADH (100 µg/300µL, Sigma-Aldrich) was monitored at 48 °C for 2 h. Addition of CryS96 fusion protein was used to reduce the total ADH aggregation in the samples based on chaperone activity.^{40,44}

2.8. Lipopolysaccharide analysis

Analysis of lipopolysaccharide (LPS) content in the extracted protein samples was performed using a method described by Zhu and colleagues.⁴⁵ Briefly, SDS PAGE analysis was performed as previously described and the gels rinsed with water for 1 min. Then, the gels were oxidized in 100 mL of an HIO₄ solution (30% EtOH, 10% HOAc, 0.7% HIO₄) for 10 min before rinsing for 5 min twice in H₂O. Silver stain solution (0.2% AgNO₃) was then used to develop the gels for 5 – 8 min or until bands were apparent. The staining was stopped using a 10% HOAc solution. Commercial lipopolysaccharide (LPS; Sigma-Aldrich) was serially diluted and used as a standard for visualization when comparing with extracted samples

3. Results and Discussion

3.1. Screening ELP extractability from cell lysates

Although relatively few examples exist for hydrophobic protein extraction from complex mixtures, the work conducted with GFP^{33,34} and TEL^{35,39} provided a starting point for the feasibility study of ELP extraction. GFP and TEL are both highly water-soluble and hydrophobic, like ELP, and this physicochemical similarity is critical for proteins intended for organic solvent extraction. Two common metrics for quantifying protein hydrophobicity are grand average of hydropathy (GRAVY)⁴⁶ and aliphatic index.⁴⁷ GRAVY scores account for all amino acids in a protein (Table 1), with scores ranging from -4.5 to +4.5, where +4.5 is the most hydrophobic. Aliphatic index focuses on the impact of only a handful of amino acids (isoleucine, leucine, valine, and alanine), compared to the rest of the amino acids in each protein (Table 1). The lowest possible aliphatic index score is zero, representing a very hydrophilic protein, while very hydrophobic proteins may achieve a score that slightly exceeds 100. Even though GFP and TEL are both regarded as quite hydrophobic proteins and extractable in organic solvent, the two differ greatly in their hydrophobicity scores (Table 1). Notably, the GRAVY metric indicates GFP as hydrophilic, while the aliphatic index indicates GFP as moderately hydrophobic and similar to TEL. Since both proteins are organic solvent extractable, we reasoned that the aliphatic index might be a better general indicator of ELP extractability since it focuses specifically on the number of hydrophobic residues. Nevertheless, other metrics may prove useful for ELP, especially due to their inherently repetitive sequence, where the only variability typically arises in the guest residue or within the fusion protein domain (if present).

Even though TEL is relatively large and moderately hydrophobic, it shares a common sequence component with many ELP designs. The successful extraction of TEL led us to believe that organic solvent extraction could be a highly effective alternative purification for accessing the difficult to purify categories of ELP, especially since lower molecular weight polymers are generally more soluble than their higher molecular weight counterparts.^{48,49} Because TEL is known to contain patches of lysine, we anticipated that ELP containing hydrophilic guest residues or fusion peptides may also be extractable. Additionally, since organic extraction favors more hydrophobic molecules, we anticipated that more hydrophobic constructs would be most amenable to purification by organic solvent extraction.

Our strategy for purifying ELP by organic solvent extraction is depicted in Scheme 1 with the original non-optimized method (Scheme 1A; Figure S4A) inspired by the procedure for GFP extraction.³⁴ When the lysate is introduced into the vessel containing a salt-saturated lower aqueous phase and an organic upper phase, the dissolved proteins are either organic soluble and remain in the upper phase or become precipitated by the salts and/or organic solvent. Centrifugation expedites the eventual three phase partitioning, where insoluble material mostly settles between the two layers, forming a solid interphase, and the extracted proteins are easily recovered from the upper organic extractant layer.

To establish a baseline for the feasibility of ELP purification by organic extraction, we chose six ELP constructs varying in molecular weight, hydrophobicity (aliphatic index), and

presence of a non-ELP element as a fusion (Table 1; Sequence information in Supplemental Data). V12 and V24 were selected because their low molecular weight places them near the lower limit of what can be reasonably purified by ITC due to their expected high T_f values. Three novel triblock ELP constructs (S12-K₄-S12, V12-K₄-V12, and V24-K₄-V24) were also designed with two ELP blocks flanking an internal polar water-solubilizing domain containing four lysines that are in the molecular weight and hydrophobicity range that could produce lower T_f values. CryS96, an ELP fusion with the α B-crystallin peptide, was chosen as a representative high molecular weight ELP fusion with a hydrophilicity similar to TEL. CryS96 has been previously purified by ITC and characterized MacKay and coworkers.⁴⁰ Since the crystallin domain has chaperone activity, it further serves as a basis to judge whether protein activity is retained after organic solvent extraction.

Because TEL is known to be extractable in a mixed solvent system of propanol and butanol,^{35,39} we selected seven organic solvents of varying polarity for extraction efficiency screening. Each solvent was tested on its own and in a 1:1 (v:v) combination with the other solvents, resulting in the evaluation of 28 extractants (Table 2). These extractants were then used to extract each of the three ELP containing solubilizing domains from clarified lysates, using the simplified extraction procedure (Scheme 1). The recovered organic extract was dried by evaporation as previously for TEL,³⁵ reconstituted in water equal to the original lysate volume, and analyzed by SDS-PAGE.

Extraction screens of V12-K₄-V12 (Figure 1), S12-K₄-S12 (Figure S1), and V24-K₄-V24 (Figure S2) show successful extractions of each construct with a variety of different organic solvent extractants. The most prominent band in each lysate and extracted sample corresponds well to the expected target molecular weight for each ELP construct, based on the understanding that ELP migration is known to deviate by up to 20 % from the calculated molecular weight.^{30,50,51} Notably, extraction efficiency was not universal for a given extractant across all three ELP.

ELP enrichment and recovery were highly variable for the different ELP-extractant combinations. The target ELP was enriched with 20 of the 28 combinations for V12-K₄-V12, while extraction of V24-K₄-V24 and S12-K₄-S12 was more selective. The general contaminant profile for a given extractant was similar and unaffected by the ELP of interest. Blends containing nBuOH and EtOAc were extremely selective for ELP, whereas MeOH and its blends were more promiscuous. Of the 28 extractants, only a few exhibited a good balance between yield and purity for all ELP, with nBuOH:EtOH standing out as the ideal universal extractant.

Initial screens with smaller constructs such as V24 using 1:1 blends of nBuOH:IPA, nBuOH:Ace, and nBuOH:ACN were explored since these worked well for the V12-K₄-V12 construct of similar molecular weight. Although detection of this very low molecular weight V24 protein is difficult using standard stains, extraction with nBuOH:IPA (Figure S3) appeared successful. More rigorous analysis would be needed to fully assess V24 and V12 extraction efficiency, particularly since the latter was even more challenging to resolve and identify via SDS-PAGE (data not shown).

From the initial extraction screens, we hoped to discern extractability relationships between ELP hydrophobicity and solvent polarity. We anticipated that S12-K₄-S12 would be most readily extracted by more polar solvents, like MeOH, while the more hydrophobic V12-K₄-V12 and V24-K₄-V24 constructs would be more efficiently extracted in less polar solvents, like IPA. Unfortunately, we were unable to discern any clear trends relating ELP and solvent properties, even when considering additional solvent properties and multiple metrics for ranking solvent polarity (Table S2). A larger and more diverse ELP library may be required to potentially uncover any existing relationships between ELP extractability and solvent polarity.

3.2. Optimizing extraction from lysates

After identifying the best extractants from the initial screen and subsequent optimization, we wanted to further optimize the extraction process to maximize yield and purity. The initial screens used a single lysate:extractant ratio (1:4 v/v) which was effective, but potentially sub-optimal for achieving the highest yield with minimal solvent. Additionally, even though aqueous NaCl and (NH₄)₂SO₄ were necessary in the published method for GFP extraction, we anticipated that they may be unnecessary additions for process improvement, since many of the most effective extractant blends already phase separated from the aqueous medium and precipitated non-target proteins on their own. Excluding these salts would simplify the process and reduce additional downstream processing for their removal.

We interrogated the effects of varying lysate:extractant ratios and the absence of added salts on extraction efficiency using the preferred extractant blends for V24-K₄-V24 and analyzed the extracts by SDS-PAGE (Figure S5). In the absence of salts, the original 1:4 lysate:extractant ratio provided target enrichment with all extractants, although the extracts were less pure than previously seen with salt-containing aqueous phases. Lower lysate:extractant ratios (1:2) were equally effective, indicating that an even lower extractant volume might be used without a loss in recovery. It is important to note; however, that the ideal lysate:extractant ratio may vary as a function of construct type, extractant composition, and lysate concentration. When including NaCl and (NH₄)₂SO₄, there appeared to be no risk of increasing the lysate:extractant ratio, although yield was consistently reduced at the expense of increased purity. Some extractant blends, namely 1:2 nBuOH:EtOH and 1:2 nBuOH:Ace (Figure S5B & C), did show evidence that too much extractant may inhibit, rather than boost, target and contaminant protein extractability in the absence of added salts. Consequently, caution should be taken when choosing the appropriate lysate:extractant ratio.

Having established the ideal lysate:extractant ratio and a need for NaCl and (NH₄)₂SO₄ salts to reach high purity, we sought to further improve the extraction efficiency of V24-K₄-V24 by screening the blend ratios of the leading extractant blends across the range of 4:1, 2:1, 1:1, 1:2, and 1:4 (v/v). As shown in Figure S6, each of the four extractant blends with nBuOH showed a similar trend and resulted in extremely pure ELP. As expected, since pure nBuOH was ineffective, all the 4:1 ratios of nBuOH with the other solvents significantly reduced the amount of ELP extracted. Reducing the nBuOH content increased ELP recovery, but too little butanol again reduced efficiency, as was especially visible with

nBuOH:ACN mixtures. From this data, a 1:2 ratio was identified as ideal for nBuOH blended with IPA, EtOH, and Ace; the original 1:1 ratio was optimal for nBuOH:ACN.

Extractions in the presence of high concentrations of NaCl and $(\text{NH}_4)_2\text{SO}_4$ have been well-studied for GFP.^{33,34,42} Since the data in Figure S5 show greater recoveries in the absence of added salts, we hypothesized that aggressive salting out of contaminating proteins may also be non-specifically entraining the desired ELP. This phenomenon could be especially problematic at the relatively high protein concentrations present under these conditions. On the other hand, the presence of salts significantly improved sample purity and could not be completely excluded. As an alternative strategy, we chose to split the one-step method into two sequential steps (Scheme 1B) to first extract the target ELP in high yield and then remove contaminating proteins from this ELP-enriched solution in a second extraction step with added salts. Using an optimized extraction blend for each of the three triblock ELP, we investigated this optimized two step method in two different ways after performing the initial salt-free extraction and compared them to the original one step extraction (Figure 2). Using optimized method A (Figure 2A), we added the salt solution directly to the same tube containing pelleted debris and the extracted ELP solution supernatant. For optimized method B (Figure 2B), we transferred only the ELP extract solution to a new tube containing the salt solution.

The extraction solvent, dictated by the ELP construct of interest, clearly plays a key role in identifying the most productive extraction method (Figure 2). For IPA extraction of S12-K₄-S12 (Figure 2C), purity and yield were completely unaffected by the extraction method used. For V12-K₄-V12 and V24-K₄-V24 (Figure 2C & D), the same extractant (1:2 nBuOH:IPA) generated similar patterns of sample purity. The non-optimized extraction method provided highly enriched and relatively pure ELP, while salt omission resulted in lower purity. Greater variation in extraction yield was expected based on the extraction method employed; however, this was only the case for V24-K₄-V24. The optimized two step methods A and B performed equivalently, while drastically improving purities to 95 %. We infer from these findings that the protein content of the debris pelleted in the initial extraction step cannot be readily resolubilized when the salt solution is added in the second step. Thus, optimized method A in a single tube is preferred due to higher throughput and less potential sample loss because there are fewer sample transfers.

3.3. Post-extraction ELP recovery

Traditionally, the main benefit of working with ELP is their unique capacity to undergo a sequence-dependent aggregation and isolation via the ITC method. This property makes the recovery of ELP from aqueous solutions very convenient compared to conventional protein isolation methods. However, there are limitations to ITC purification, such as the occurrence of ELP sequences with transition temperatures that are impractical. Due to the demonstrated solubility of ELP in various organic solvents, several alternatives are available for recovering the construct from solution (Table S1). These methods have different benefits and limitations depending on the properties of the ELP. Based on our experience, the primary challenge that may be encountered is that, in some instances, the recovered ELP does not dissolve readily in either H₂O or the organic solvent it was extracted with (data not shown). Although we

have not thoroughly investigated these occurrences, we have observed that rotary evaporation to dryness caused the most problems, whereas passive evaporation was nearly always successful.

Back-extraction is a rapid and attractive alternative recovery method, since the target protein always remains in solution and re-dissolution issues are bypassed. Back-extraction from organic solvent into aqueous solution was a critical step in the optimized extraction of GFP (specifically, nBuOH was added as anti-solvent to drive GFP from ethanol and into the aqueous layer).³⁴ In contrast, we found that nBuOH was often useful as an ELP extractant. Consequently, our back-extraction process (Scheme 1D) required a different anti-solvent that was miscible with the chosen extractant, immiscible with water, and does not extract the target ELP. We employed broad extractant screens to identify potential anti-solvents for our ELP constructs. Using V24-K₄-V24 as an example, we successfully back-extracted this ELP from 1:2 nBuOH:IPA into water by adding EtOAc as anti-solvent (Figure 3). The rapid back-extraction process appeared to be lossless in comparison to the original extraction process, thus suggesting that this method presents an opportunity to concentrate the protein in an aqueous acceptor medium via a rapid and simple process. Additionally, subsequent washes with anti-solvent did not affect ELP recovery. Following this same approach, we also back-extracted S12-K₄-S12 and V12-K₄-V12 using EtOAc; we also had mixed success when using other anti-solvents such as diethyl ether (data not shown).

Next, we evaluated the functional properties (e.g., T_f) of the back-extracted ELP by testing the temperature transition behavior of V24-K₄-V24 (i.e., between 35 – 50 °C, depending on the protein and salt concentrations). After organic extraction from lysate or whole cells, drying by passive evaporation or lyophilization, and re-solubilization in water, these ELP retain their ability to reversibly transition when heated and cooled. Following back-extraction, V24-K₄-V24 was able transition with gentle heating (~ 40 °C) and re-solubilize when cooled to 20 °C (Figure 4). This reversible thermal transitioning demonstrates that the basic ELP functional character has not been altered throughout the extraction and back-extraction processes.

3.4. ELP extraction from whole cells

To explore the full potential of the organic extraction method for ELP purification, we adapted the procedure for target protein extraction directly from whole cells, rather than clarified cell lysates. By combining cell lysis with ELP extraction in a single step, the overall process of recovering pure protein from cell culture would be accelerated, greener, and minimize opportunities for protein degradation during isolation. Since many of the organic extractant blends should have the propensity to disrupt bacterial cells and liberate the desired ELP in a single, combined extraction step (Scheme 1C & Figure S4B), we anticipated that this approach could provide superior lytic ability and yield compared to traditional methods and commercial reagents.

V24-K₄-V24 was selected for direct extraction of cell pellets using two of the most efficient extraction solvents for this construct, 1:2 nBuOH:IPA and 1:2 nBuOH:Ace. In both cases, extraction with a 60 min incubation was performed with and without a follow-up salting-out step. Following lysis, all samples were dried and reconstituted with water to the original

volume, to allow direct comparisons of the lysis methods. The reconstituted samples were analyzed by SDS-PAGE and compared to clarified lysates generated from three standard lytic procedures: probe tip sonication, a commercial nonionic detergent solution (B-PER), and buffered urea (Figure 5A).

Both extractant blends were highly effective in recovering a very large amount of V24-K₄-V24. The extracted samples were also extremely pure and showed only a single band of the expected molecular weight, indicating > 95 % purity. Added salts again reduced the yield as seen before with clarified lysate extraction. No detectable contaminants were observed in the absence of salt; therefore, there are no obvious benefits in going beyond simple organic solvent extraction. Since the cell pellet contains significantly less water than the corresponding cell lysate, we believe that the specific composition of the organic extractant becomes even more important than before. Thus, the extractant blend may be more selective for ELP and have less tolerance for contaminating proteins, even in the three traditional lysis methods looked very similar to one another, with only slight variations in ELP recovery observed. Importantly, absence of additional salts, appeared that a greater quantity of highly pure ELP was recovered by direct organic extraction, compared to the relatively impure ELP liberated by probe tip sonication or B-PER. Based on these experiments, we conclude that traditional lysis methods may be incomplete for ELP release from cells, such that the use of these methods may reduce overall ELP yield from the start. A more quantitative analysis; however, is needed to validate these findings.

We further evaluated the extracted V24-K₄-V24 material by running the extracted and lysed samples on agarose gels followed by Coomassie R-250 staining (Figure S12B). Since this ELP has a calculated isoelectric point of 10.2, while the vast majority of *E. coli* proteins have a PI below 10,^{52–54} we expected that the majority of proteins would migrate toward the anode, whereas the target ELP would migrate toward the cathode under the mildly basic electrophoresis conditions employed (pH 8.7). As anticipated, a large amount of protein was detected on the anode side of the well for each of the standard lysis methods, while the extracted samples did not show any protein in this area. The only protein detected in the extracted samples was found to migrate toward the cathode and showed reversible thermal transitioning behavior (Figure 4), thus providing further evidence that the extracted protein is an ELP.

During the direct extraction process from whole cells, a very rapid visible change occurred within the first few minutes – a grainy, spongy-looking mass began to settle to the bottom of the tube. This observation, along with the expectation that the lytic and extraction processes would be very rapid, led us to shorten the extraction from 60 to 15 min to provide a more direct comparison to the B-PER procedure. Additionally, we compared the direct extraction process to cells that had been grown under IPTG induction, attempting to increase the target protein yield (Figure 5B & Figure S7).

As expected, the data in Figure 5B show equivalent extraction efficiency with nBuOH:IPA at 15 min, as compared to 60 min; we expect this process could be shortened even further to just a few minutes. Extraction with nBuOH: Ace (Figure S7) was also effective with the 15 min incubation. Although reports have indicated exceptionally high yields for ELP

production under IPTG induction,³¹ the yield in our case was significantly lower for an equivalent cell mass. The reduced yield may be due to ELP entrainment within inclusion bodies. Alternatively, the stretch of lysine residues in our construct may become cytotoxic when rapidly produced. Nonetheless, extractants identified as ideal candidates from lysate screens typically also worked very well for direct extraction from whole cells. Although we encountered only few instances where lysate extractant success did not translate well to whole cells, other ELP requiring other extractants may have different results. Since the lytic effects of many solvents, especially simple alcohols, are well-known and often extremely potent in releasing cellular contents,⁵⁵ we believe that these cases may be easily remedied without significantly altering ELP solubility by spiking in a very small amount of this solvent class.

3.5. Extracting the ELP fusion protein, CryS96, from whole cells

Next, we investigated the extraction of an ELP fusion,^{41,56–61} specifically a relatively hydrophilic and functional ELP, CryS96 (Table 1).⁴⁰ Since the aliphatic index of CryS96 (58.6) is most similar to S12-K4-S12 (47.7), we used the results of the previous extraction screens to select a handful of extractant blends most likely to extract CryS96.

Our initial screen of CryS96 extractability was conducted using a previously purified solution of CryS96. These encouraging results led us to use the same set of solvents for extracting CryS96 directly from whole cells (Figure 6). Of these selected extractants, pure EtOH provided the best balance of yield and purity, although other extractants, such as pure IPA (Figure 6) and 1:1 nBuOH:EtOH (data not shown) also worked well. The T_f of the EtOH extracted/EtOAc back-extracted CryS96 construct was the same as reported previously (data not shown).⁴⁰ This successful extraction of an ELP-fusion protein is an important milestone in showing the versatility of ELP and ELP-fusion extraction with organic solvents.

3.6. Functional evaluation of extracted CryS96 by alcohol dehydrogenase activity assay

Previous reports of CryS96 activity from MacKay and coworkers have shown that ITC purified CryS96 is an effective chaperone protein.⁴⁰ Using the alcohol dehydrogenase activity assay a 70% reduction in aggregation was seen with 333 μ M CryS96.⁴⁰ Based on initial screens, pure EtOH extraction followed by EtOAc back extraction generated high yield and purity (Figure 6). Addition of 333 03bcM EtOH extraction-purified CryS96 led to a 61% decrease in ADH aggregation (Figure 7), in excellent agreement with the activity reported by CryS96 that was purified by ITC. These results suggest that organic solvent extraction of ELP does not disrupt their functional activity. Further investigation of varying blends and ratios of organic extractions is warranted.

3.7. Scale-up of ELP extractions from lysates and whole cells

Liquid-liquid extraction (LLE) is a scalable purification technique routinely employed in industrial processes. For large-scale production of biomolecules, LLE offers numerous economic and process benefits over chromatographic purification⁶² and fits into continuous production schemes, including bioreactors. To be considered viable for scaled up production,

ELP purification by organic extraction must maintain a high level of efficiency. As an initial step, we briefly explored extraction scalability from lysates and whole cells.

Using V24-K₄-V24 lysates, we performed large-scale extractions (35 mL lysate) with 1:2 nBuOH:IPA using the non-optimized method shown in Scheme 1A. Although the extraction was highly effective in recovering a large amount of ELP, some minor protein contamination remained (Figure S8). Since our earlier experiments showed that salts could reduce protein contamination (Figure S5), we attributed these observations to slow and/or inefficient mixing such that the contaminants were not properly salted out. We expect that the optimized extraction procedure (Figure 2) could minimize contaminants in larger scale lysate extractions.

Finally, we investigated extractions of S12-K₄-S12, V12-K₄-V12, and V24-K₄-V24 with 1:1 EtOH:Ace, 1:2 nBuOH:EtOH, and 1:2 nBuOH:IPA, respectively, on the 2.5 g scale using whole cells. All three ELP were successfully extracted at this scale (Figure S9). S12-K₄-S12, for example, shows an equivalent yield and purity at both small (0.2 g) and large (2.5 g) scales. Similarly, whole cell extraction of CryS96 was equally efficient, regardless of scale, when extracting with IPA or EtOH (Figure 6B). Based on these collective preliminary results for scaling extractions more than 10-fold from lysates and whole cells, we believe that organic solvent extraction is a viable, valuable, and rapid process for scalable ELP purification.

3.8. Analysis for the presence co-extracted contaminants

3.8.1. Nucleic acid analysis—Nucleic acid contamination is a prevalent issue when ELP are purified by ITC; however, this problem was not anticipated for the organic solvent extraction procedure. Using V24-K₄-V24 lysate as a test case, we analyzed each of the 28 organic solvent extraction combinations via agarose gel electrophoresis using GelRed to stain for nucleic acids such as dsDNA, ssDNA, and RNA (Figure S10). For comparison, we included a sample purified by three rounds of ITC. Notably, ITC-purified V24-K₄-V24 still has a considerable amount of nucleic acid contamination and would require an additional step of adding branched polyethylenimine (bPEI) to precipitate the nucleic acids.^{27,29} Although often effective, the additional time and resources can lead to complications and reduce the recovery of certain ELP constructs.³¹

For most organic extractants, there was no detectable nucleic acid contamination arising from the extraction process, except for extraction solvents containing MeOH and the Ace:ACN combination. The nucleic acid contaminants in the MeOH and Ace:ACN cases are likely plasmid DNA based on their comparative migration relative to control DNA. Even though a handful of samples contained contaminating nucleic acids, the most promising extractants for isolating nucleic acid-free ELP lacked MeOH and contained nBuOH, the latter serving to prevent carryover (Figure S11).

Having demonstrated that organic extraction from cell lysates typically excluded nucleic acid carryover, we similarly evaluated contamination when extracting directly from whole extractions and lyses of V24-K₄-V24 to agarose gel electrophoresis (Figure S12A). All three standard lysis methods showed a considerable amount of nucleic acid contamination, as

anticipated. Regardless of the extractant or salt composition used; however, no nucleic acids were detectable using the direct extraction method from whole cells. Based on these results, we expect that other extractant blends will also exclude nucleic acid contamination.

3.8.2. Lipopolysaccharide analysis—We continued to evaluate the scope of contamination in ELP extracts by assaying for LPS content using the method reported by Zhu and colleagues⁴⁵ that does not require any further separation operations. In brief, we ran SDS-PAGE gels as previously described and then rinsed them with H₂O for 1 min. As the data in Figure S13 shows, there appears to be substantially less than 31 ng LPS content in a 50 µg sample of V12-K₄-V12 extract. Taken together, we infer from these findings that organic solvent extraction can eliminate both LPS and nucleic acid contamination in ELP isolates.

3.9. Toward understanding the ELP extraction mechanism

We initially expected amphiphilic ELP to be amenable to organic extraction because of their relative hydrophobicity and hydrophilicity as reported for GFP; however, the ELP extraction mechanism appears to be more complex. Based on an analysis of our initial screens of three similar ELP variants, we have not been able to discern any clear trends between the various rankings of solvent polarity and relative ELP hydrophobicity (Table S2 and Table 1 respectively). Additionally, no obvious connection existed between other solvent properties, such as hydrogen bonding potential.

When considering ELP extractability for all the organic solvents used in the screens, nBuOH clearly stands out among the other alcohols. We originally chose nBuOH because it precipitated non-target *E. coli* proteins during TEL extraction³⁹. It had also been reported in the GFP extraction literature as a means to efficiently push the ethanol-solubilized GFP into an aqueous phase, whereas tBuOH promoted GFP precipitation.³⁴ Based on this background information, we expected that moderate amounts of nBuOH would not solubilize ELP and might serve as a useful back-extractant.

For the three triblock ELP assessed, pure nBuOH was unable to extract any target ELP, whereas pure IPA achieved successful extraction in two of the three cases. The lower molecular weight alcohols, EtOH and MeOH, were able to extract all three ELP; however, they also tended to co-extract a number of other proteins. When used in combination with other solvents, nBuOH had the lowest promiscuity toward co-extraction of contaminating proteins, while IPA blends showed comparable results.

Even though nBuOH was poor at extracting ELP on its own, we were surprised by the high ELP selectivity it provided when used in combination with other solvents. To gain further insight into this high extraction performance, we investigated the effect of butanol isomerism on ELP extractability. Working with V24-K₄-V24 lysates, we compared nBuOH, sBuOH, and tBuOH in combination with the other alcohols (Figure 8).

Unexpectedly, extraction efficiency and selectivity appear to be unaffected by the butanol isomer employed. When combined with IPA or EtOH, 50 % butanol in the organic extractant phase is sufficient to eliminate extraction of non-target proteins without a noticeable change

in yield, regardless of the butanol isomer used. Extraction yield and selectivity are similar for 1:1 butanol:MeOH mixtures, whereas these qualities deteriorated at 1:2 and 1:4 butanol:MeOH ratios. The combination of butanol with IPA was the most effective in providing both high yield and purity with a minimal amount of butanol. Butanol:EtOH mixtures were also quite effective; however, an increasing amount of low molecular weight protein contamination occurred with decreasing butanol content.

These data with simple alcohol extractants demonstrate the influence of all butanol isomers in equally increasing extractant selectivity without sacrificing yield. Further, for V24-K₄-V24 extraction, these data further show that increasing alcohol molecular weight and/or decreasing polarity plays a role in the extraction mechanism and increases selectivity for ELP over non-ELP proteins. This trend is expected to be dependent on the ELP of interest. Additional studies with a broader set of ELP and higher molecular weight alcohols may provide additional insights into the extraction mechanism.

In addition to the ability of butanol to increase extraction selectivity, this solvent also enhanced extraction yields when blended with other solvents. For example, neither nBuOH nor ACN was able to extract to any appreciable extent the three lysine-containing ELP from lysates. The combination of nBuOH and ACN; however, showed a moderate-to-large amount of extracted ELP for each variant. Although most obvious for nBuOH:ACN, we also observed this effect when using nBuOH:IPA, IPA:EtOAc, and IPA:ACN for V24-K₄-V24 extraction (Figure S2). The peculiar behavior of butanol toward ELP extractability enhancement remains unclear from the current data, although we expect that the ELP may exist in the organic phase as a micro- or nanoemulsion mediated by solvent-solvent interactions and residual water from the lysate or cells. Studies investigating the underlying basis for the success of these mixed solvent extractants will be needed to guide the rational selection of organic solvents for extracting a particular ELP construct. More focused mechanistic studies would need to be done for greater understanding of this phenomenon just as the recent findings for the unique effects of guanidinium salts on both preventing and causing hydrophobic collapse of ELP.⁶³

4. Conclusions

Organic extraction has been demonstrated as an extremely rapid and efficient method for the scalable purification of ELP directly from whole cells or cell lysates, requiring less than 30 min in either case. We have shown that the optimized extraction process is highly efficient in removing contaminating nucleic acids, non-target proteins, and lipopolysaccharides in a single step and may be optimized for ELP sequences differing widely in molecular weight, hydrophobicity, or the presence of non-ELP fusions. In the case of direct extraction from whole cells, it is possible to recover more isolated target protein than the amount of crude target protein liberated by some standard lysis methods. Utilizing organic extraction, we anticipate that many other ELP constructs, especially those difficult to obtain by traditional methods, will become accessible by expression in bacteria without the need for purification tags. The ability to retain biological activity with CryS96 shows that organic extraction is an alternative means for protein purification while retaining the functionality of the fusion domain.

To our knowledge, the occurrence of other small molecule and metabolite contaminants in ITC purified ELP has not been investigated. A variety of these contaminants may be carried along by the extraction process reported herein. Indeed, the contaminant profile is expected to be greatly dependent on the extractant employed. Numerous simple techniques such as gel filtration or dialysis are available for removing these low molecular weight contaminants. A second extraction, such as aqueous back-extraction or an additional organic extraction might also be effective in removing certain classes of contaminants. Alternatively, a round of ITC may be the most expedient method for removing a broader range of small molecules.

Although the newly-described method of purifying ELP by organic extraction has proven successful in many cases, additional experimentation is needed fully understand the mechanism of this process and a more in-depth analysis of the contaminant profile. The extraction mechanism is likely to depend on the specific combination of ELP and extractant. Employing a larger library of ELP constructs and organic solvents may be useful in understanding the extraction mechanism such that one could rationally predict extractability by relating ELP and solvent properties.

At present, the most prudent approach to employing this extraction method for other ELP constructs would focus on the following points:

- Perform a broad solvent screen to identify the most useful extractants that retain function for downstream applications.
- The ideal extractant depends on the application needs – a balance between yield, purity, and contaminant profile must be established.
- Avoid using promiscuous solvents like MeOH and explore additional solvents, as necessary.
- Extraction from whole cells is most likely the best option, especially for labile proteins susceptible to enzymatic degradation. This is also a simpler process and does not require optimizing cell lysis conditions.
- The order of the processing steps and timing also deserve attention to achieve the highest consistency in extraction outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
SDS-PAGE analysis of V12-K₄-V12 extraction screen using organic extractants listed in Table 2. S = protein MW standards, L = clarified lysate.

Figure 2.

SDS-PAGE analysis of ELP extractions from lysate using (A) optimized method A and (B) optimized method B for (C) S12-K₄-S12, (D) V12-K₄-V12, and (E) V24-K₄-V24. L = clarified lysate; “+” = non-optimized method with salt; “-” = non-optimized method without salt.

Figure 3.
SDS-PAGE analysis of V24-K₄-V24 back-extraction with EtOAc compared to the original organic extract. L = lysate; A = aqueous subphase; O = organic phase; W = organic wash of A; E = extraction prior to back-extraction

Figure 4. Thermal transition behavior of V24-K₄-V24 back-extracted after extraction from whole cells with 1:2 nBuOH:IPA. The arrow indicates the aqueous sub-phase recovered for thermal transitioning.

Figure 5.

(A) SDS-PAGE analysis of 60 min direct V24-K4-V24 extraction from whole cells. S = MW standards, P = probe tip sonication lysate, B = B-PER lysate, U = urea lysate, BA = 1:2 nBuOH:IPA, BF = 1:2 nBuOH:Ac, “-” = without salt, “+” = with salts. (B) Direct extraction of V24-K4-V24 after 15 and 60 min treatment with 1:2 nBuOH:IPA. 60-I = IPTG-induced cell culture.

Figure 6.

SDS-PAGE analysis of (A) direct CryS96 extraction from whole cells (0.2 g) with pure ethanol. L = clarified lysate. (B) Scale-up of CryS96 extraction from whole cells.

Figure 7. Alcohol dehydrogenase activity assay. Time dependent optical density at 360 nm indicated ADH aggregation upon heating at 48 °C. ADH (black, control) and ADH + Crys96 protein (gray, 333 μ M CryS96) were measured every 45 s for 2 h. Data indicates mean absorbance measured at 360nm (n=3).

Figure 8. SDS-PAGE analysis of butanol isomer extraction efficiency in solvent blends with (A) IPA, (B) EtOH, and (C) MeOH for recovering ELP V24-K₄-V24 from cell lysates. Blend ratios (v/v) are as indicated for each solvent combination. L = lysate.

Scheme 1.

Procedures for (A) non-optimized and (B) optimized lysate extraction, (C) whole cell lysis/extraction, and (D) aqueous back-extraction.

Table 1

Properties of Selected ELP and Other Hydrophobic Proteins.

Construct ID	Class	MW (kDa) ^d	pI ^a	GRAVY ^b	Aliphatic Index ^c
GFP ^d	—	27.8	6.30	-0.681	71.6
TEL ^e	Multiblock	61.4	10.6	0.691	90.7
V12	Monoblock	5.10	5.49	1.16	114
V24	Monoblock	10.0	5.49	1.18	115
S12-K ₄ -S12	Triblock	12.1	10.2	-0.149	47.7
V12-K ₄ -V12	Triblock	12.4	10.2	0.673	95.3
V24-K ₄ -V24	Triblock	22.2	10.2	0.911	104
CryS96	Monoblock-fusion	40.8	6.76	0.158	58.6

^aMolecular weight and isoelectric point were calculated based on the sequence, excluding the methionine start codon.⁴³

^bCalculated using the expression: GRAVY = Sum of all hydrophathy values ÷ Number of amino acids

^cCalculated using the expression: Aliphatic Index = $X(\text{Ala}) + 2.9X(\text{Val}) + 3.9[X(\text{Ile}) + X(\text{Leu})]$.

^dBased on the sequence of GFPuv-his8

^eTEL represents the main chain of human elastin, excluding the 26 amino acid signaling peptide.

Table 2

Organic Solvent Extractants Screened for ELP Isolation.^{†‡}

ID	Solvent 1	Solvent 2	ID	Solvent 1	Solvent 2
A	IPA	–	CD	EtOAc	EtOH
AB	IPA	nBuOH	CE	EtOAc	MeOH
AC	IPA	EtOAc	CF	EtOAc	Ace
AD	IPA	EtOH	CG	EtOAc	ACN
AE	IPA	MeOH	D	EtOH	–
AF	IPA	Ace	DE	EtOH	MeOH
AG	IPA	ACN	DF	EtOH	Ace
B	nBuOH	–	DG	EtOH	ACN
BC	nBuOH	EtOAc	E	MeOH	–
BD	nBuOH	EtOH	EF	MeOH	Ace
BE	nBuOH	MeOH	EG	MeOH	ACN
BF	nBuOH	Ace	F	Ace	–
BG	nBuOH	ACN	FG	Ace	ACN
C	EtOAc	–	G	ACN	–

[†] All two-solvent extractant blends were mixed at a 1:1 (v/v) ratio.

[‡] Solvents are arranged by increasing polarity index, where A is least polar, and G is most polar.