

18

Protein Purification by Inverse Transition Cycling

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INTRODUCTION, 329

OUTLINE OF PROCEDURE, 331

General, 331

Fusion protein design considerations, 334

Selection of the ELP tag and ITC conditions, 334

PROTOCOLS, 336

Fusion of an ELP moiety to a protein of interest, 336

Purification of ELP fusion proteins from cell lysate, 338

General transition cycling of ELP fusion proteins, 341

CONCLUSION, 343

REFERENCES, 343

INTRODUCTION

Elastin-like polypeptides (ELPs) are environmentally responsive biopolymers based on the elastin-derived pentapeptide repeat Val-Pro-Gly-Val-Gly. ELPs undergo a reversible phase transition termed an “inverse temperature transition” (Urry 1992, 1997). Below their transition temperature (T_t), the polypeptides are highly soluble in aqueous solutions. However, when the temperature is raised above T_t , the hydrated polypeptide chains hydrophobically collapse and aggregate, forming a separate, ELP-rich phase. The T_t of an ELP can be conveniently controlled at several different levels. For example, the T_t can be adjusted over a wide range of temperatures through control of the amino acid sequence. The transition can also be triggered isothermally by modulation of environmental conditions, in particular by the type and concentration of added co-solutes.

Significantly, ELP fusion proteins, which are produced by joining the gene encoding a protein of interest with an ELP gene segment, can also undergo a reversible phase transition similar to that of the free ELP (Meyer and Chilkoti 1999). Thus, the environmental responsiveness of ELPs

TABLE 1. Uses of ELP Fusion Proteins and ITC

Mode	Use
ELP fusion proteins per se	postexpression purification of recombinant proteins (scale-up: industrial quantities; scale-down: parallel, high-throughput purification of expressed gene libraries) separation of reactants (e.g., after protein labeling) enzyme recycling buffer exchange and concentration of the fusion protein
Non-covalent capture using ELP fusions	characterization of unknown binding partners (in serial or high-throughput format) determination of equilibrium binding and rate constants competitive immunoassays

can be easily imparted by genetic fusion to a protein of interest. This is useful because, when the transition is triggered, the fusion protein aggregates and can be collected by centrifugation or filtration. The transition is reversible, and therefore the pelleted ELP fusion protein can be re-solubilized after removal of the supernatant by dissolution in aqueous solution at a temperature less than the T_t . It is important to note that the fused target protein does not denature and precipitate when the phase transition is induced, but rather, it is the ELP that aggregates. For free ELPs, the aggregated phase contains up to ~60% water by weight (Urry et al. 1985), and the target protein apparently remains sufficiently hydrated to prevent its denaturation. Thus, an ELP tag genetically incorporated into a protein of interest provides a “molecular handle” to manipulate the protein, which allows easy and rapid purification, concentration, and buffer exchange. Potential applications of inverse transition cycling (ITC) are summarized in Table 1.

In an extension of this concept, ELP fusion proteins can also be used for non-covalent capture of affinity-binding partners from solution (D.E. Meyer and A. Chilkoti, in prep.). After binding, the non-covalent complex can be separated from solution upon triggering the ELP phase transition. This concept has many potential applications. First, non-covalent capture of molecules by an ELP fusion protein is a potentially useful tool to study protein–ligand interactions. For example, a protein of interest (antibody or antibody fragment, antigen, receptor, ligand, etc.) could be fused to an ELP and used to “fish out” an unknown binding partner from a solution-phase combinatorial library. After capture and purification, the complex can be interrogated by mass spectrometry or other spectroscopic techniques to identify the bound ligand. Second, capture of a labeled affinity partner from solution can also be used to determine equilibrium binding and rate constants, based on the quantification of the free and bound (i.e., captured) binding partner as a function of concentration and time. Third, non-covalent binding of a target analyte by an ELP fusion protein can also be used as the basis for competitive immunoassays for the quantification of analyte concentration in complex mixtures.

For each of these applications, the principle of ITC is used to selectively remove the ELP fusion protein or its non-covalent complex with a ligand from solution. Figure 1A shows a schematic of ITC, and Figure 1B shows typical SDS-PAGE results of protein purification by ITC from cell lysate. Initially, the ELP fusion protein is below its T_t and is soluble. The inverse temperature transition is triggered by increasing the solution temperature to above the T_t or by adding salt to depress isothermally the T_t to below the solution temperature, or by a combination of both. Upon triggering the transition, the fusion protein aggregates and can be separated from other molecules present in solution by centrifugation or filtration. The remaining soluble molecules are removed by decanting or pipetting off the supernatant. The purified fusion protein is then resolubilized at a temperature below its T_t in the buffer and volume of choice. The resolubilized fusion protein is often centrifuged (or filtered) a final time at a temperature below its T_t to remove any remaining insoluble matter that may have been trapped in the pellet along with the aggregated ELP fusion protein. Additional rounds of ITC can be undertaken until the fusion protein is purified to the desired degree.

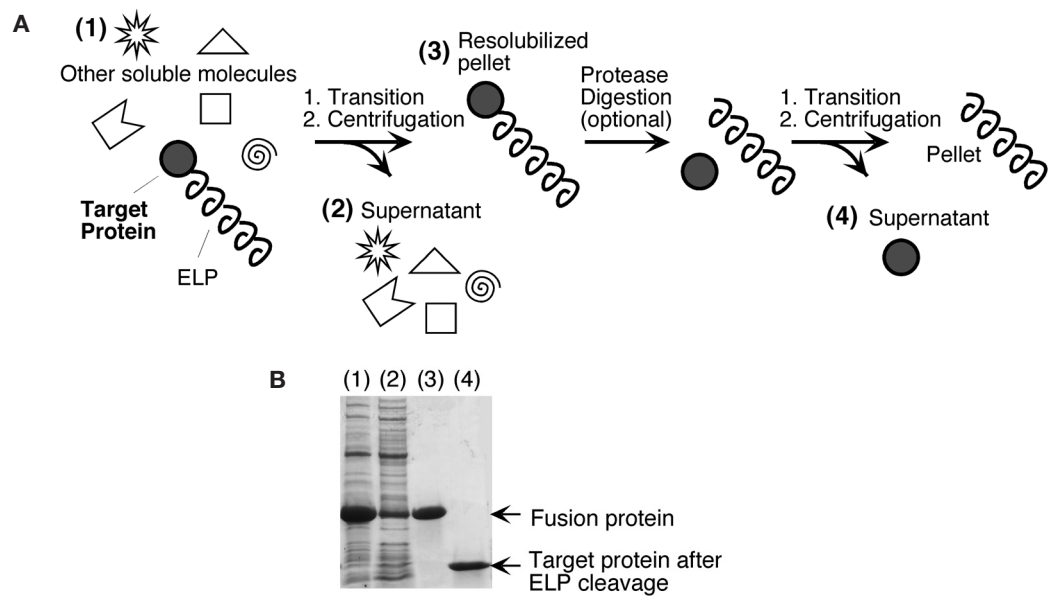


FIGURE 1. (A) Schematic of protein separation by inverse transition cycling (ITC). The ELP–target fusion protein is initially present in a complex mixture (e.g., cellular lysate, immunoassay sample solution). The ELP inverse transition is triggered by increasing the solution temperature and/or by adding NaCl, resulting in aggregation of the fusion protein. After centrifugation (or filtration), the supernatant (or filtrate) containing the contaminating soluble molecules is discarded, and the fusion protein is recovered by resolubilizing the pellet (or washing the filter) with buffer at a temperature below the T_t . If necessary, the target protein can be separated from the ELP by cleavage with a site-specific protease at a recognition site between the target protein and ELP, followed by a final round of ITC. (B) Typical SDS-PAGE results illustrating the purification of an ELP fusion protein from the lysate. Lane numbers correspond to the steps as labeled in A. Lane 1 is the soluble cell lysate with the overexpressed *E. coli* thioredoxin/ELP[V-20] fusion protein and contaminating *E. coli* proteins. Lanes 2 and 3 are the supernatant and pellet, respectively, after one round of ITC. Purified fusion protein is obtained in the pellet, while the contaminating *E. coli* proteins are discarded in the supernatant. Lane 4 shows purified thioredoxin after cleavage with thrombin and purification by ITC from the cleaved ELP tag.

ITC is technically simple, fast, economical, and requires no specialized equipment or reagents. Separations can be completed in minutes using standard laboratory centrifuges and NaCl. Furthermore, these advantages can be realized over a wide range of purification scales. For microliter-scale expression and purification, ITC can be employed in parallel for high-throughput applications. At the other extreme, because it avoids the expense associated with traditional chromatographic separations, ITC is also likely to be useful for the purification of single proteins at the gram-to-kilogram scale in industrial bioprocessing.

OUTLINE OF PROCEDURE

General

ITC purification is a convenient method for a variety of separations. Common to all these applications is the concept that genetic fusion of a protein of interest to an ELP imparts the environmental sensitivity of the ELP to the fusion protein. Triggering the ELP inverse temperature transition by changing environmental parameters, which are typically the solution temperature and ionic composition, allows the target protein to be removed selectively from solution.

A simple but important concept to remember when working with ELPs and their fusion proteins is that there is no unique and fixed T_t associated with a given construct. Rather, the T_t (i.e.,

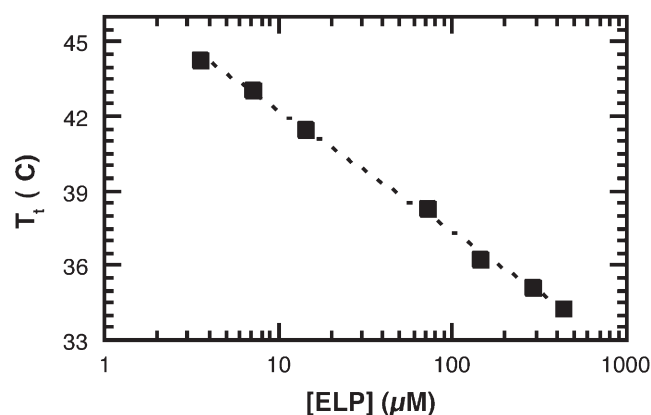


FIGURE 2. Effect of ELP concentration on the T_t . The T_t was determined for ELP[V₅A₂G₃-180] at each concentration in PBS as follows. The solutions were heated at a rate of 1°C per minute in a temperature-controlled spectrophotometer, and the optical density at 350 nm was measured as a function of temperature. The T_t was defined at the point of 50% maximal turbidity. The data show that, for a given ELP sequence and molecular weight, the T_t decreases logarithmically (*dashed line*) with increasing ELP concentration. For ITC applications, this effect is of practical importance for ELP fusion protein concentrations lower than ~100 μM, which are typical for ELP fusion proteins in the cellular lysate.

the temperature at which the transition occurs for given environmental conditions) is dependent on a number of factors. Note that, for the purpose of this discussion, reported T_t values are at an ELP fusion protein concentration of 25 μM in phosphate-buffered saline (PBS) unless otherwise specified.

For separation applications using ITC, the two most relevant factors affecting T_t are (1) the concentration of the ELP fusion protein and (2) the solution composition (in particular, types of ions and their concentrations). Examples of the effects of these two factors on the T_t for a given ELP construct are shown in Figures 2 and 3. Because the T_t decreases logarithmically with increasing concentration (Fig. 2), the effect of ELP concentration is of greatest practical importance at concentrations lower than ~100 μM. Increasing the NaCl concentration also decreases the T_t , and, as a rule of thumb, the T_t is depressed by ~15°C for each 1 M increase in NaCl concentration (although this effect could vary for ELPs with different fractions of charged residues, for example). The effect of ions on the T_t of ELPs in solution appears to follow the Hofmeister series (Cacace et al. 1997), and as shown in Figure 3, ions such as guanidinium can be used to increase the T_t , as well.

The T_t of an ELP also depends on its amino acid sequence and its molecular weight, as illustrated in Figure 4. The sequence dependence of the ELP T_t has been studied extensively by Urry and coworkers, who have found that substitution at the fourth residue of the Val-Pro-Gly-Val-Gly pentapeptide, termed the “guest residue,” with residues more hydrophilic than Val increases the T_t from that of the native sequence, whereas substitution with more hydrophobic residues reduces the T_t (Urry et al. 1991). These data allow the rational design of ELPs exhibiting a specific target T_t through selection of the type of guest residues and their relative frequency of substitution in the ELP sequence (Table 2). The effect of molecular weight is of greatest practical importance for short ELPs (e.g., less than ~50 pentapeptides in length) and becomes less important for higher molecular weights (Fig. 4). Here, the different ELP constructs are distinguished using the notation ELP[X₁Y_j-*n*], where the bracketed capital letters are the single-letter amino acid codes and the corresponding subscripts designate the frequency of each guest residue in the repeat unit, and *n* describes the total length of the ELP in number of pentapeptides. For example, ELP[V₅A₂G₃-180] is an ELP that has a repeating sequence of 10 pentapeptides with the guest residues Val, Ala, and Gly in a 5:2:3 ratio, respectively, and is 180 pentapeptides in length.

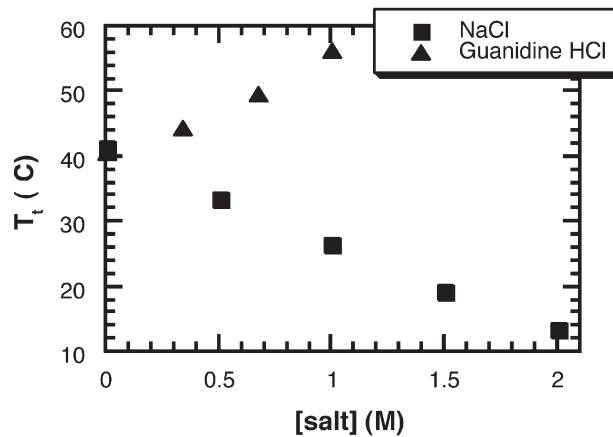


FIGURE 3. Effect of ions on the T_t . The T_t was determined for ELP[V₅A₂G₃-180] at 25 μ M in PBS supplemented with increasing concentrations of NaCl or guanidine HCl. (The T_t was defined as described in Fig. 2.) The data show that the T_t can be modulated by the ionic composition of the solution for a given ELP sequence, molecular weight, and concentration. This allows “fine tuning” of the T_t to a desired temperature during ITC.

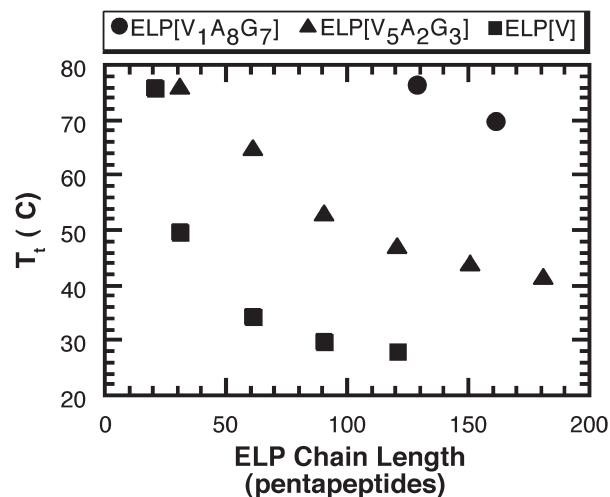


FIGURE 4. Effect of ELP sequence and length on T_t . The T_t was determined (as described in Fig. 2) in PBS for 25 μ M solutions of ELP with varied sequences and chain lengths. The T_t increases for a given molecular weight with increasing hydrophilicity of the guest residue in the ELP sequence (see text for sequence notation), and increases with decreasing molecular weight for a given sequence. The molecular-weight effect is most dramatically observed at lower chain lengths (e.g., less than \sim 50 pentapeptides). However, even ELPs with short chain lengths can be useful in ELP fusion protein applications because their relatively high T_t values can be functionally depressed by the addition of NaCl or by increasing the ELP concentration (9).

TABLE 2. Examples of ELP Sequences

Construct	Monomer sequence ^a	T_t ($^{\circ}$ C) ^b
ELP[V]	(VPGVG) ₅	30
ELP[V ₅ A ₂ G ₃]	(VPGVG) ₂ VPGAG VPGGG(VPGVG) ₃ VPGGG VPGAG VPGGG	40
ELP[V ₁ A ₈ G ₇]	(VPGVG)VPGAG(VPGGG VPGAG) ₇	70

^aPolypeptide sequence encoded by the monomer gene.

^bApproximate transition temperature for high-molecular-weight ELPs (i.e., greater than 100 pentapeptides) at 25 μ M concentration in PBS.

The T_t of an ELP with a particular sequence and molecular weight can also be affected by the target protein to which it is fused. Proteins with a large fraction of solvent-exposed hydrophobic surface area can depress the T_t of a fused ELP. In one example, the protein tendamistat, with a calculated exposed hydrophobic surface area of 53%, caused a $\sim 26^\circ\text{C}$ reduction in T_t (Meyer and Chilkoti 1999). Most soluble proteins (>90% in a survey of 100 proteins from the Protein Data Bank), however, have a calculated exposed hydrophobic surface area of less than 30%. Based on experimental observations, fusion to proteins with this degree of exposed hydrophobic surface area should have an insignificant effect on the ELP T_t (less than a 5°C change relative to free ELP).

Fusion Protein Design Considerations

A gene for a typical ELP fusion protein includes three components: (1) the gene of the target protein of interest, (2) an intervening peptide linker sequence, and (3) the ELP gene. Typically, the gene of the fusion is constructed in three sequential steps. If the gene of interest (encoding the target protein) is already available in an expression vector, this vector can be modified using a synthetic oligonucleotide cassette to include the endonuclease recognition sequence for ELP insertion and codons for a peptide linker sequence. An ELP gene segment can then be cloned into the ELP insertion site as described below in the Protocols section, completing the assembly of the gene for the ELP fusion protein. This last step can be completed in parallel, if desired, to create a library of different ELPs fused to the same target protein.

Alternatively, if a number of different target proteins are to be studied, it may be preferable to synthesize a generic expression vector containing an ELP tag into which the genes of the various target proteins can be cloned. This can be achieved by first modifying an expression vector, adjacent to a multiple cloning site, to introduce the ELP insertion site and to encode for the linker sequence. Next, the selected ELP gene segment is cloned into the ELP insertion site. The vector is then ready for the parallel cloning of a library of target genes into the multiple cloning site adjacent to the ELP.

The peptide linker sequence can be designed to incorporate several useful features. First, it can provide a spacer between the target protein and ELP. This may help to retain functional activity of the target protein if the ELP is fused near the active site of the folded protein. Second, the linker sequence can encode for a protease recognition site so that the ELP can be cleaved from the target protein after ITC purification. Thrombin, with a Leu-Val-Pro-Arg|Gly-Ser recognition sequence, has been used successfully with ELP fusion proteins (Meyer and Chilkoti 1999). Factor Xa protease has also been used with ELP fusion proteins (McPherson et al. 1992), and other proteases may also be useful. Finally, a secondary purification tag (e.g., a His₆ tag) can be included between the protease recognition site and the target protein for purification after cleavage from the ELP.

There is no clear rationale for the choice of constructing amino- or carboxy-terminal ELP fusions, and this decision may depend on the properties of the target protein. If the target protein is known to express at a high level, it may be preferable to fuse the ELP to the carboxyl terminus. (ELP incorporated at the amino terminus could interfere with proper folding of the target protein, in which case a carboxy-terminal ELP fusion may be preferable because it is translated after synthesis of the target protein is completed.) On the other hand, an amino-terminal ELP tag has been observed to increase the soluble expression of a poorly expressed target protein (Meyer and Chilkoti 1999). This may be because an amino-terminal ELP aids folding of the target protein by hydrophobic interactions with the nascent target protein chain or by sterically preventing intermolecular aggregation of folding intermediates of the target protein. Given all of these considerations, it may be best to try both configurations.

Selection of the ELP Tag and ITC Conditions

The implementation of ITC can be optimized in terms of fusion protein expression and separation efficiency for a given target protein by control of ELP amino acid sequence, ELP molecular

weight, and solution conditions (temperature and NaCl concentration). This may be important for some applications, such as large-scale production of a target protein where maximization of yield is an important goal. Alternatively, a generic protocol has been developed that may not be optimal for all proteins, but should provide acceptable separations for a wide variety of proteins. This approach would be more useful, for example, in high-throughput expression and purification of a large number of different proteins with unknown properties.

For general implementation, an ELP of moderate length (~36 kD) with a T_t of ~54°C (e.g., ELP[V₅A₂G₃-90]) has been found to be useful for a range of target proteins. The T_t of this ELP is high enough to withstand a downward shift induced by fusion to a hydrophobic target protein, yet low enough so that the T_t can be reduced to 30–35°C with the addition of only moderate amounts of NaCl (1–2 M). Use of this (or a similar) ELP tag is recommended for initial, pilot ITC purification of a new target protein. For poorly expressed target proteins, for which the T_t is too high or for which no phase transition is observed due to low concentration, the soluble lysate can be spiked with free ELP prior to purification by ITC. The addition of free ELP reduces the T_t by the concentration effect and helps to ensure efficient capture of the aggregated proteins by centrifugation.

Complete optimization of ITC may require empirical testing of different ELP tags for a given target protein. Because the expression yields of a target protein, *E. coli* thioredoxin, have been found to increase with decreasing ELP tag length (Meyer and Chilkoti 1999), the goal is to determine the shortest ELP tag that will still allow efficient purification at moderate temperatures and NaCl concentrations. This can be achieved using test expression cultures of the target protein fused to a small library of ELPs (e.g., three or four constructs with the ELP tag ranging in size from ~10 kD to ~35 kD). One ELP at the low end of this range, the ~9 kD ELP[V-20] tag, has been successfully used for purification of thioredoxin by ITC, and provided a fourfold increase in target protein yield versus a control 35-kD ELP tag (Meyer et al. 2001). However, the inverse transition of the fusion protein with the 9-kD tag had a more complex dependence on temperature (Meyer et al. 2001), and furthermore, some target proteins failed to exhibit an inverse temperature transition when fused to tags of this size (D.E. Meyer and A. Chilkoti, unpubl.). From a design perspective, because the T_t increases with decreasing molecular weight, smaller tags require the incorporation of a greater fraction of hydrophobic guest residues to keep the T_t low enough such that ITC can be performed at conditions that do not denature the target protein. At the other end of the ELP molecular-weight range (e.g., the 35-kD ELP[V₅A₂G₃-90]), all fusion proteins tested to date, albeit limited in number, have been successfully purified by ITC. Larger ELP tags may also protect the reversibility of the temperature-induced aggregation by limiting intermolecular contact of the target protein in the aggregated state.

For postexpression purification, a target T_t above 37°C is typically selected so that the fusion protein remains soluble *in vivo* during culture, while allowing the inverse temperature transition to be triggered by only a small increase in temperature or NaCl concentration in the soluble cell lysate. This selection of the T_t is based on the assumption that expression of the ELP in its stable state is likely to result in a higher yield of properly folded target protein. However, this assumption has not yet been tested in detail, and it is possible that a T_t below the culture temperature may be preferable because formation of an aggregated ELP phase within the cell may protect the target protein from degradation by intracellular proteases.

For ITC applications involving the noncovalent binding of a target molecule to an ELP fusion protein, the effect of ITC conditions on binding affinity must also be considered. A T_t slightly above (e.g., ~5°C or less) the incubation temperature of the reaction allows separation of the complex from solution by only a modest increase in temperature (0–5°C) and/or a modest increase in NaCl concentration (0–300 mM NaCl). Any decrease in affinity due to ITC performed under these conditions is likely to be negligible. On the other hand, larger swings in temperature or ionic strength could lead to undesired shifts in binding equilibrium, resulting in release of the target molecule prior to capture by ELP aggregation.

Protocol 1

Fusion of an ELP Moiety to a Protein of Interest

Genes for ELPs and for ELP fusion proteins can be readily synthesized using standard methods of molecular biology. A number of approaches for synthesizing ELP genes have been described in the literature (McPherson et al. 1996; McMillan et al. 1999; Meyer and Chilkoti 1999). A protocol for producing ELP fusion proteins using genes generated by one of these methods is presented below. At the time of writing, genes encoding ELPs as described in this protocol are available free of charge from the authors for noncommercial research purposes. Note that this protocol serves only as an example, and the DNA amounts, fluid volumes, etc., can be successfully varied from those shown here.

MATERIALS

Buffers and Reagents

Calf intestinal alkaline phosphatase (CIAP)
Enzyme buffers
10× Ligase buffer
Restriction enzymes
T4 DNA ligase

Vectors

Expression vector containing the target protein gene that has been modified to include a unique restriction endonuclease recognition site for ELP insertion
Vector containing an ELP gene, which is flanked by *Pfl*MI (at the 5′ end of the gene) and *Bgl*I (at the 3′ end) recognition sequences, which are both cleaved to form 3′ single-stranded GGC extensions

Special Equipment

Gel Electrophoresis and PCR
Agarose gel electrophoresis equipment
Agarose gel extraction kit
PCR purification kit

METHOD

Preparation of ELP Insert

Due to their repetitive nature, larger ELP genes produced by gene oligomerization are not effectively amplified using PCR.

1. Digest ~4 μg of the ELP plasmid DNA to completion with *Pfl*MI and *Bgl*I.
2. Purify the ELP fragment by agarose gel extraction (commercial kits are available for this purpose). The ELP gene fragment size in base pairs is the number of pentapeptides encoded mul-

multiplied by 15 (3 bases per amino acid \times 5 amino acid residues per pentapeptide). Elute in 30 μ l of H₂O and store frozen at -20°C until further use.

Avoid heating or vortexing the sample during gel extraction.

Vector Preparation

3. Digest ~ 1 μ g of the expression vector plasmid DNA with the appropriate restriction endonuclease that specifically cleaves at the ELP insertion site.

The ELP insertion site is a recognition site for a restriction endonuclease that (1) is unique to the expression vector and (2) creates a nonpalindromic overhang compatible with the ELP insert upon cleavage (e.g., 5'-GGC-3'). The insertion site must be positioned such that it is in frame with the coding sequence of the fusion protein, and stop codons must be included downstream from the insertion site. Examples of restriction enzymes that are compatible with this particular system include: *AlwNI*, *BglI*, *BstI*, *BstAPI*, *DraIII*, *MwoI*, *PflMI*, and *SfiI*. Introduction of the ELP insertion site can be achieved by cassette mutagenesis of the expression vector that already contains the gene of the target protein.

Confirm the plasmid digestion by agarose gel electrophoresis.

It is critical that the target protein vector be fully digested. Incomplete digestion can produce an overwhelming background of wild-type vector upon transformation.

If digestion of the plasmid DNA is incomplete, add more enzyme and/or allow the reaction to proceed longer until the vector is fully linearized. If applicable, heat-inactivate the restriction enzyme for 20 minutes at 65°C , or as suggested by the supplier.

4. Dephosphorylate the linearized vector (e.g., using 1 unit of CIAP per pmole of vector for 1 hour at 50°C). Heat-inactivate the CIAP for 10 minutes at 75°C , or as suggested by the supplier.
5. Purify the linearized, dephosphorylated vector (e.g., using a commercial PCR purification kit). Elute in 30 μ l of sterile H₂O and store frozen at -20°C .

Ligation

6. Set up the ligation reaction by adding 5 μ l of vector (~ 150 ng); 10 μ l of the purified insert (molar insert to vector ratio of ~ 5 – 10 :1); 2 μ l of 10 \times ligase buffer, 1 μ l of T4 DNA ligase (1 unit/ μ l), and 2 μ l of sterilized H₂O (20 μ l total reaction volume). Incubate for 2–4 hours at 16°C .
7. Transform into an *E. coli* strain (or other host). A noninducible, *recA*⁻ *E. coli* strain is recommended.
8. Spread on agar plates with antibiotic selection, and allow colonies to grow overnight.
9. Screen colonies using colony PCR for smaller ELP genes (note that due to their repetitive nature, larger ELP genes produced by gene oligomerization are *not* effectively amplified using PCR) or plasmid minipreps followed by diagnostic digests for larger ELP genes. Expect 50–90% of colonies to harbor the fused ELP gene.

A high wild-type background can be due to incomplete digestion of the vector and/or to incomplete dephosphorylation. Direct transformation of the vector without ligation can be used to test the completeness of the restriction digest. If this test produces significantly fewer colonies (e.g., tenfold less) than does ligation with the ELP insert, then incomplete digestion of the vector is not the problem. In this case, ligation and transformation of the vector without adding the ELP insert can be used to determine the extent of self-ligation allowed by incomplete dephosphorylation of the vector. Again, the number of colonies without insert should be much less than for ligation with the ELP insert.

Also expect some dimer or trimer inserts for small ELP gene inserts (e.g., less than ~ 300 bp), typically in less than 10% of the colonies.

10. Transform the expression vector encoding the ELP–target protein fusion into a suitable expression host (*recA*⁻ recommended for *E. coli* expression).

Protocol 2

Purification of ELP Fusion Proteins from Cell Lysate

This protocol is useful for the purification of ELP–target fusion proteins after expression. Briefly, the cells are lysed, DNA is precipitated, and insoluble cellular debris is removed to yield the soluble cell lysate. The ELP–target fusion protein is then selectively aggregated and removed from solution by centrifugation at a temperature above T_t . After dissolution of the pelleted fusion protein in the buffer and volume of choice, any remaining insoluble material is removed by a final round of centrifugation at a temperature below the ELP T_t .

MATERIALS

Buffers and Reagents

Additional buffer

NaCl

Expression culture harvested and resuspended in low-salt buffer (e.g., Tris or phosphate buffered at pH 7–7.5 with 20–150 mM NaCl) in $\sim 1/20$ of the culture volume.

Polyethylenimine, 10% aqueous solution (Optional, see box below step 3).

METHOD

1. Lyse the cells. Any lysis method should work, although ultrasonic disruption may help to break apart and resolubilize any ELP aggregates that have formed during culture. If sonication is used, lyse on ice to ensure that the solution temperature remains below the ELP T_t (e.g., $\sim 5^\circ\text{C}$ or more lower than the T_t). One protocol for preparation of bacterial lysates is provided in Chapter 4.
2. Centrifuge the lysed cell suspension at $\sim 20,000g$ for 15–20 minutes at $\sim 4^\circ\text{C}$. Remove the supernatant to a new centrifuge tube and discard the pellet, which contains insoluble cellular debris.
3. *Optional:* Add polyethylenimine to a final concentration of 0.5% and mix gently. The solution should turn white due to coprecipitation of the polyethylenimine and the DNA present in the suspension. Incubate for 10–20 minutes on ice, occasionally mixing the suspension gently. Centrifuge at $\sim 20,000g$ for 15–20 minutes at 4°C . Remove the supernatant to a new centrifuge tube and discard the pellet, which contains precipitated DNA and insoluble cellular debris.

Precipitation of nucleic acids with polyethylenimine prior to induction of the ELP inverse transition is recommended because some nucleic acids present in the soluble lysate can pellet with the ELP in the centrifugation steps of ITC, particularly when higher salt concentrations are used (e.g., greater than ~ 1.5 M NaCl). If the polyethylenimine precipitation step is omitted, separation from nucleic acids can still be achieved using additional rounds of ITC.

4. Increase the solution temperature and/or add NaCl to trigger the inverse transition.

The solution temperature and NaCl concentration required to trigger the transition depend on the ELP sequence and molecular weight, the fusion partner, and ELP concentration. The goal is to lower the ELP T_t at least 3–5°C below the solution temperature during centrifugation. An effective T_t of 25–30°C (achieved using NaCl concentrations in the 0.1–2 M range) and centrifugation at 35–40°C typically works well, although higher temperatures may be used if the target protein is thermally tolerant. However, fusion to the ELP can reduce the thermal denaturation temperature of the target protein, and, thus, care must be taken not to exceed this temperature during ITC. If irreversible precipitation of the fusion protein is observed during ITC, consider using a different combination of temperature and NaCl concentration to prevent denaturation of the target protein, which is a separate event from the ELP transition. When working with a new construct for which the precise T_t is unknown, it is helpful to increase the temperature and/or NaCl (e.g., in 5°C or 500 mM increments) slowly until the transition is observed in the cell lysate. If solid NaCl crystals are used, dissolve them gently by inverting the tube. Do not vortex vigorously, because the ELP fusion protein can aggregate at the air–water interface of bubbles and be subsequently lost when the supernatant is decanted.

The solution should become turbid because of aggregation of the ELP.

Before proceeding to the centrifugation (step 5), it is essential that (1) the ELP fusion protein has undergone its transition and (2) the solution temperature is equal to or less than the centrifugation temperature. If both of these conditions are met, the ELP fusion protein will remain aggregated during the centrifugation and therefore will be captured. If, on the other hand, the centrifugation temperature is lower than the precentrifugation solution temperature, some or all of the ELP fusion protein may resolubilize and remain in the supernatant.

If no turbidity is observed after increasing the temperature and/or adding salt, confirm expression levels by SDS-PAGE. Although not generally required, spiking of additional free ELP (e.g., to a concentration of ~10–25 μM) with a matched T_t may be necessary for poorly expressed fusion proteins. If the protein is highly overexpressed but turbidity is still not observed, it may be necessary to use an ELP tag either with a lower T_t or one with a greater molecular weight (for examples, see Table 2 and Fig. 4).

5. Centrifuge at a temperature above the T_t at ~15,000g for 5–10 minutes to pellet the aggregated ELP fusion protein.

Typically this centrifugation is completed at a temperature in the range of ~25–40°C.

6. Discard the supernatant, which should be clear, and resuspend the pellet in a buffer of choice that is at a temperature below T_t .

The pellet is often brown or off-white during the first round of purification from cell lysate but is typically translucent in later rounds of ITC. The pellet can be resuspended by working aggressively with a pipettor (a 1000- μl pipettor with disposable tips is recommended). Place the pipette tip near the pellet, and repeatedly withdraw and expel the solution over the pellet. The aggregated ELP fusion protein is also typically adherent to the centrifuge tube, and therefore the tube walls should be washed thoroughly. A period of soaking (again, at a temperature below T_t) can help in the resolubilization. Mechanical scraping of the pellet and tube walls may also be helpful. Ensure that the pellet has been entirely resuspended. During the first round of ITC from the cell lysate, the solution typically remains turbid after resuspension of the pellet due to insoluble contaminants that will be removed in step 7. However, for later rounds of the ITC, the solution is generally clear after dissolution of the pellet.

The pellet can be resuspended in any desired volume (~1/50 to 1/10 of the lysate volume is recommended, depending on the expression level of the target protein).

7. Centrifuge at ~20,000g for 10–15 minutes at 4°C to remove any remaining insoluble matter. Retain the supernatant, and discard any pellet that is formed.

For the first round of purification from cell lysate, the pellet from this cold centrifugation step can be relatively large. However, it typically does not contain significant amounts of the ELP fusion protein.

8. Repeat steps 4–7 at least one additional time.

Typically, two rounds of transition cycling provide a high level of purity, although additional rounds may be undertaken if desired. Also note that a lower salt concentration can generally be used for the second round because the transition temperature is lowered by the significantly increased ELP fusion protein concentration achieved in the first round.

9. *Optional:* If a protease recognition site has been incorporated in the primary amino acid sequence between the ELP and the target protein, free target protein can be obtained by incubating the purified ELP fusion protein with the protease to cleave the ELP tag.

The solubility of the ELP fusion protein can be significantly greater than the solubility of the free target protein. Therefore, ensure that the protease cleavage step is performed at a concentration within the solubility limits of the free target protein. If the protein concentration is too high, addition of the protease and subsequent cleavage of the ELP can cause irreversible precipitation of the liberated target protein. Also note that, unless a secondary purification is performed, the protease remains with the purified target protein. Although not currently commercially available, the use of ELP-tagged proteases could solve this problem by enabling the simultaneous removal of both the protease and the cleaved ELP tag from solution by ITC, leaving only the purified target protein in the retained supernatant.

A final round of ITC can then be used to remove the free ELP, retaining the purified, free target protein in the supernatant.

10. Purified ELP fusion proteins can often be stored frozen in water at -80°C until use. Use a small aliquot to test for denaturation of the target protein after freezing; if the protein denatures, storage at -20°C in a 50% glycerol solution may be necessary.

Protocol 3

General Transition Cycling of ELP Fusion Proteins

This protocol is useful for buffer exchange, increasing the concentration of an ELP fusion protein in solution, and for general separation applications (e.g., purification from coreactants after chemical reaction or labeling, recovery of ELP–enzyme fusions after catalysis). It is also useful for the study of protein interactions through the capture of affinity-binding partners.

MATERIALS

Buffers and Reagents

Buffer
Purified ELP fusion proteins (typically in the micromolar concentration range)
NaCl

Additional Equipment

Centrifuge and centrifuge tubes
Pipettor (1000 ml) with disposable tips

METHOD

1. Add NaCl and/or increase the solution temperature to induce ELP aggregation.

The solution temperature and NaCl concentration required to trigger the transition depend on the ELP sequence and molecular weight, the fusion partner, and ELP concentration. The goal is to lower the ELP T_t at least 3–5°C below the solution temperature during centrifugation. An effective T_t of 25–30°C (achieved using NaCl concentrations in the 0.1–2 M range) and centrifugation at 35–40°C typically work well, although higher temperatures may be used if the target protein is thermally tolerant. However, fusion to the ELP can reduce the thermal denaturation temperature of the target protein, and thus, care must be taken not to exceed this temperature during ITC. If irreversible precipitation of the fusion protein is observed during ITC, consider using a different combination of temperature and NaCl concentration to prevent denaturation of the target protein, which is a separate event from the ELP transition. If solid NaCl crystals are used, dissolve them gently by inverting the tube. Do not vortex vigorously, because the ELP fusion protein can aggregate at the air–water interface of bubbles and be subsequently lost when the supernatant is decanted.

Before proceeding to the centrifugation (step 2), it is essential that (1) the ELP fusion protein has undergone its transition and (2) the solution temperature is equal to or less than the centrifugation temperature. If both of these conditions are met, the ELP fusion protein will remain aggregated during the centrifugation and therefore will be captured. If, on the other hand, the centrifugation temperature is lower than the pre-centrifugation solution temperature, some or all of the ELP fusion protein may resolubilize and remain in the supernatant.

The solution will become visibly turbid for concentrations of $\sim 1\text{--}5\ \mu\text{M}$ and greater. Because ITC may not be effective for concentrations lower than this, spiking the solution with additional free ELP (e.g., to a concentration of $\sim 10\text{--}25\ \mu\text{M}$) with a matched T_t may be necessary.

2. Centrifuge at a temperature above the transition temperature at $\sim 15,000g$ for 5–10 minutes.
3. Discard the supernatant, and resuspend the pellet in cold buffer of choice.

The pellet can be resuspended by working aggressively with a pipettor (a 1000- μl pipettor with disposable tips is recommended). Place the pipette tip near the pellet, and repeatedly withdraw and expel the solution over the pellet. The aggregated ELP fusion protein is also typically adherent to the centrifuge tube, and therefore the tube walls should be washed thoroughly. A period of soaking (again, at a temperature below T_t) can help in the resolubilization. Mechanical scraping of the pellet and tube walls may also be helpful. Ensure that the pellet has been entirely resuspended. The solution is generally clear after dissolution of the pellet.

The pellet can be resuspended in a volume of choice to achieve a desired concentration.

4. Centrifuge cold (4°C , $\sim 15,000g$, 5–10 minutes) to remove any remaining insoluble matter. Keep the supernatant and discard any pellet. In many instances, no pellet may be observed; however, it is good practice to always perform this cold centrifugation step after resuspending the pellet from the warm centrifugation step.
5. For additional rounds of ITC, repeat steps 1–4 as desired.

CONCLUSION

Purification of recombinant proteins using ELP fusion tags and ITC is a simple and powerful method for the purification of recombinant target proteins from cell lysate after expression. By eliminating traditional chromatographic purification methods, ITC provides substantial savings in time and expense. Furthermore, it is also useful for wide-ranging bioseparation applications following initial purification of a recombinant protein. Because it can be used to capture non-covalently a ligand specific to the target protein from solution, ITC is also useful for the study of protein–ligand interactions and forms the basis for competitive solution immunoassays. For all of these applications, ITC is flexible enough to allow optimization for a specific protein of interest using different ELP tags and solution conditions, yet is general enough to be useful for high-throughput applications involving different proteins with varied physicochemical properties. In conclusion, ITC is a new bioseparation technology that should have a significant impact on biotechnology and the biological sciences.

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