Exploring Elastin-Like Polypeptide Tags and Mini-intein for Recombinant Protein Purification in *Leishmania tarentolae*

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Abstract

Recombinant technology has enabled the production of a wide range of proteins with applications in various fields, including reproduction, food supplements, and medicine. However, the high costs associated with producing and purifying recombinant proteins limit their widespread use in the veterinary field. Therefore, the development of alternative, low-cost strategies for production and purification is essential to address these challenges. The non-pathogenic eukaryotic protozoan *Leishmania tarentolae* has emerged as an affordable expression system for heterologous proteins, providing glycosylation profiles similar to those of higher eukaryotes. Despite this, the cost of recombinant protein purification remains a significant barrier to expanding the use of recombinant products in areas such as livestock.

In this study, we evaluate an unconventional purification tag, the elastin-like polypeptide (ELP), for purifying recombinant proteins secreted by *L. tarentolae*. We also explored the use of the mini-intein ΔI-CM as a cleavable linker between ELP and the target protein. This intein was found to undergo intracellular self-cleavage, precluding its use in secretion-based applications in this host. Despite this limitation, the ELP-mediated purification from the culture supernatant was successfully achieved with a high purity using 0.6 M ammonium sulfate at 25-30 °C. To our knowledge, this is the first report of ELP-based protein purification in a protozoan host, representing a promising tool with broad potential for applications in molecular biology, pharmaceutical development, and other fields that require high-purity proteins with an improved cost-to-benefit ratio.

1. Introduction

The application of biotechnology in animal health and production is increasingly expanding. In particular, biological molecules have extended their applications from vaccines for controlling infectious diseases[1], to enhancing productivity by modulating hormones or elements of the immune system[2], to immunocastration[3], reproductive purposes[4], parasite control[5], and several others. They can be obtained from natural sources using extraction methods or produced recombinantly in heterologous organisms. The use of recombinant proteins provides several advantages over protein mixtures obtained from animal extracts, including greater product homogeneity and safety. Recombinant technology also enables the production of proteins that do not exist in nature, such as optimized enzymes or single-chain antibodies, which are useful in diagnosis and treatment of certain diseases. However, the use of recombinant

products in animals remains limited, mainly due to the costs associated with the production processes[6].

Often, obtaining these recombinant products in an active form requires a eukaryotic expression and post-translational machinery. While the mammalian cell systems are effective, they can be costly, labor-intensive, and time-consuming, prompting the exploration of alternative expression platforms that may overcome these limitations. One interesting option is *Leishmania tarentolae*, a unicellular eukaryotic protozoan that parasitizes reptiles, particularly lizards. This protozoan is not pathogenic to mammals, including humans, and is characterized by its rapid growth rate *in vitro* and low culture costs[7]. Furthermore*, L. tarentolae* has eukaryotic protein-folding and post-translational modification machinery that enables it to produce complex proteins in an active form[8]. Several options for recombinant protein expression are available for *L. tarentolae*, including secretory or intracellular expression through constitutive (genome-integrated), inducible (genome-integrated or episomal), or cell-free (plasmid-based or PCR-based) expression systems[9]. These features make it an excellent choice as a heterologous expression system for eukaryotic protein production.

L. tarentolae has been used for the expression of eukaryotic proteins, typically along with chromatography-based purification systems[10]. When alternative expression systems, such as *L. tarentolae*, replace mammalian cells, the overall production costs decrease. However, purification processes can still account for 45 to 92% of the total production cost[11]. Therefore, to further reduce the overall costs of recombinant protein production, it is essential to minimize the expenditures associated with downstream processing. Traditional affinity-based purification methods are often too costly for industrial-scale applications, particularly for proteins that must be produced in large quantities at low cost. In this context, elastin-like polypeptide tag-based purification methods provide a cost-effective, non-chromatographic alternative.

Elastin-like polypeptides (ELPs) are repetitive synthetic polypeptides derived from the hydrophobic domain of tropoelastin. The most commonly used are composed of tandemly repeated blocks of (Val-Pro-Gly-X-Gly)*n*, where X can be any residue except proline (because proline destroys the inverse phase transition property of the ELP), and "*n*" represents the number of pentapeptide repeats in the polypeptide. ELPs exhibit rapid phase transition behavior at a specific temperature referred to as the inverse transition temperature (Tt)[12,13]. ELPs are structurally disordered and soluble in aqueous solutions below their Tt; however, intramolecular contacts between the nonpolar regions result in aggregation and, hence, precipitation above the Tt[14]. The transition of ELPs and their fusion proteins can also be isothermally triggered by reducing the Tt through the addition of salts from the Hofmeister series[15]. The temperature/salt-induced phase transition of ELP is reversible as it resolubilizes when the temperature is reduced below the transition temperature or when the salt concentration is decreased[16]. Over the years, the vast majority of ELP-fused proteins have been produced in *Escherichia coli*, with some reports of their expression in plants (*Nicotiana*), fungi (*Aspergillus nidulans*)[17], and yeast (*Pichia pastoris*)[18]. Notably, there are no studies of their use in any protozoa.

The removal of the purification tag is another downstream step that adds to production costs, for which the self-cleaving properties of inteins offer innovative solutions. Inteins (intervening proteins) are polypeptide sequences that are excised from precursor proteins through an autocatalytic process known as protein splicing. Through targeted mutations at specific residues, inteins were engineered to perform controlled cleavage reactions in response to stimuli such as pH changes or redox agents[19]. These engineered inteins allow for the purification of proteins without residual affinity tags, as the tag is removed during the purification process.

To date, most applications of intein-mediated purification have been carried out using *E. coli* as the expression system. Due to their sensitivity to temperature and pH, inteins often undergo premature self-cleavage when expressed at elevated temperatures (>32 °C) or exposed to acidic conditions (pH <7.4). This limitation has hindered their use in mammalian expression systems,

where such growth conditions are the rule and can reduce protein yields. Despite these challenges, inteins have been successfully used in other eukaryotic systems, such as insect cells[20] and plants[21], which grow at lower temperatures. Given that *L. tarentolae* grows optimally at 26 °C, it presents a promising alternative host for exploring intein-based strategies in the production of recombinant biomolecules.

In this study, we explore the use of ELP and the pH-inducible mini-intein ΔI-CM[22] as a purification system for heterologous protein expression in *L. tarentolae,* using enhanced green fluorescent protein (eGFP) as a model. To further assess its applicability, the system was also tested with bovine follicle-stimulating hormone (bFSH), the main exogenous hormone used for superovulation in cattle embryo transfer. Current commercial FSH products are derived from porcine or ovine pituitary extracts[23], and despite decades of research, no recombinant FSH has achieved widespread commercial adoption, largely due to cost constraints in livestock production[24]. Recently, *L. tarentolae* has been shown to produce glycosylated and biologically active bFSH[10], which in this work was purified from culture supernatant by immobilized metal ion affinity chromatography (IMAC).

To our knowledge, this is the first report of ELP-based purification in a protozoan host. These findings provide a proof of concept and broaden the potential of *L. tarentolae* as a versatile expression platform, with promising implications for biopharmaceutical manufacturing and other biotechnological applications in resource-limited settings.

2. Materials and Methods

2.1 Plasmid construction

The generation of the expression vectors was outsourced to GenScript. The coding sequence for the enhanced green fluorescent protein (eGFP) was cloned into two commercial vectors that allow its secretion, the constitutive expression vector pLEXSY-Hyg2 (Jena Bioscience), and the inducible, episomal, and bicistronic expression vector pLEXSY_IE-blecherry4 (Jena Bioscience), which simultaneously expresses the protein of interest and the Cherry fluorescent protein as reporter gene. A 30-repeat ELP sequence was inserted into both vectors, followed by a spacer linker sequence, the mini intein ΔI-CM[22], when applicable, and the eGFP sequence. The ELP and ΔI-CM sequences used correspond to those from the pET/EI-CAT vector developed by Banki and collaborators[25] (AddGeen #71461), while the eGFP sequence was obtained from the peGFP-C3 vector. For the expression of bovine follicle stimulant hormone (bFSH), it coding sequence was obtained from the pLEXSY-Hyg2_FSH vector[10], fused to 110-repeat ELP sequence and cloned in pLEXSY_IE-blecherry4 (Jena Bioscience). To improve the secretion efficiency, the native signal peptide present in the commercial vectors was replaced with the signal peptide from *Leishmania mexicana* acid phosphatase, which has demonstrated higher efficiency in recombinant protein secretion[26]. A 6-histidine tag was added to the N-terminus of the eGFP and bFSH coding sequences for detection purposes.

2.2 Cell culture, transfection and clone selection

For the pLEXSY_IE-blecherry4 inducible vector, the *L. tarentolae* LEXSY-Host-T7-TR strain (Jena Bioscience) was cultured at 26°C in brain heart infusion (BHI) medium (37 g/L) supplemented with penicillin (50 U/ml), streptomycin (50 µg/mL), hygromycin (100 µg/mL), nourseothricin (100 µg/mL), and hemin (5 µg/mL) (Sigma–Aldrich). Cells were transfected with pLEXSY_IE-blecherry4_bFSH by nucleofection using the Amaxa Nucleofector 2b (program U033) and the Amaxa Basic Parasite Nucleofector kit 1 (Lonza) according to the manufacturer's

instructions. Selection of the transgenic population was performed by culturing the transfected cells in supplemented BHI medium containing bleomycin (100 µg/mL, Jena Bioscience). After 24 h, the cells were harvested by centrifugation (2000 *g*, 10 min at room temperature), resuspended in fresh medium and spread over a 0.45 µm nitrocellulose membrane filter (Millipore) placed on agar (10 g/L prepared in 1 M HEPES, pH 7.5) plates containing supplemented BHI, bleomycin (100 µg/mL) and 10% fetal bovine serum. The plates were sealed and incubated upside-down at 26°C. Approximately one week later, individual colonies appeared, and nitrocellulose membranes were transferred to a fresh agar plate containing 10 µg/mL tetracycline. Two days later, 12 single colonies displaying an intense pink coloration (Cherry protein) were transferred to 1 mL of supplemented BHI medium (24-well plate) and incubated for 24 h at 26°C in the presence of 10 µg/mL tetracycline. Next, Cherry and eGFP expression was quantified by measuring fluorescence (λex/em = 590/610 nm and λex/em = 480/510 nm, respectively) in a Varioskan Flash spectrofluorimeter. The clone with the highest dual fluorescence signal was selected for further studies. The clone producing ELP-FHS was selected based on Cherry expression.

For the pLEXSY-Hyg2 constitutive vector, the *L. tarentolae* LEXSY-Host-P10 strain (Jena Bioscience) was cultured at 26°C BHI medium (37 g/L) supplemented with penicillin (50 U/ml), streptomycin (50 µg/mL), and hemin (5 µg/mL) (Sigma–Aldrich). Cells were transfected as previously described, with the following exceptions: hygromycin (100 µg/mL) was used as the selection antibiotic, induction was not required, and only eGFP expression was analyzed.

2.3 Protein expression

Cells from a static culture in the exponential growth phase (4–8 × 10^7 cells/mL) were diluted 1:5 in fresh, supplemented BHI medium and incubated for 24 h at 90 RPM and 26°C. Then, the cultures were diluted again 1:5 in fresh medium, and incubation resumed under the same conditions. When the cell density reached 6–7 × 10^7 cells/mL, tetracycline was added (10 µg/mL) to induce protein expression, and incubation was extended for an additional 72 h. Samples were collected at 24, 48, and 72 h post-induction to evaluate Cherry and eGFP expression. For the constitutive expression system, induction was not required, and only eGFP expression was analyzed.

2.4 Inverse transition cycling (ITC) of ELP fusion proteins

Ammonium sulfate [$(NH_4)_2SO_4$] was added to the culture supernatant containing ELP fusion proteins (M1) at a 1:1 ratio. Samples were incubated for 15 min at 25°C, 30°C, or 37°C to induce the ELP phase transition and promote aggregation of the fusion protein. The aggregated ELP fusion protein was then separated from soluble contaminants (M2) by centrifugation at 10,000 × *g* for 10 min at room temperature (for large volumes, ultrafiltration with a 0.2 µm filter may be used instead of centrifugation). The pellet was resuspended in a cold, low-salt buffer (50 mM Tris, 2 mM EDTA, pH 8.8) and centrifuged again at 4°C ("cold spin"). The resolubilized ELP fusion protein remained in the supernatant (M3), while insoluble contaminants formed the cold spin pellet (CS). A schematic representation of the procedure is shown in Figure 2A.

*2.5 M*ass *spectrometry*

The bands were excised from the Coomassie-stained gel and processed for identification by mass spectrometry (MS) at the Biochemistry and Analytical Proteomics Unit (UByPA) from the Institut Pasteur de Montevideo, Uruguay. In-gel digestion of selected protein bands was performed overnight at 37 °C by incubation with trypsin (Sequencing grade, Promega). Peptide extraction was performed with 0.1% trifluoroacetic acid (TFA) in 60% acetonitrile. Samples were vacuum-dried, resuspended in 0.1% TFA, and desalted using C18 OMIX tips (Agilent). Peptides

were analysed on a nano-HPLC (UltiMate 3000, Thermo) coupled to a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive Plus, Thermo). For analysis of supernatant proteins, an elution gradient from 1% to 35% B was applied. LC-MS/MS data analysis was performed in accordance with the PatternLab IV software (http://www.patternlabforproteomics.org). The proteome from *L. tarentolae* was downloaded from UniProt (April 2021) (http://www.uniprot.org). A target-reverse database including the 127 most common contaminants was generated using PatternLab's database generation tool.

Thermo raw files were searched against the database using the Comet search engine applying the following parameters: fully specific trypsin as proteolytic enzyme allowing up to 2 missed cleavages; methionine oxidation as variable modification and a precursor mass tolerance of 40 ppm. Peptide spectrum matches were filtered using the Search Engine Processor (SEPro), using stringent FDR criteria set at 3% at spectrum level, 2% at peptide level, and 1% at the protein level[27].

2.6 Western blot

Proteins from supernatants or cell extracts were separated by 12% SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (GE). The membranes were blocked overnight in PBS containing 5% (w/v) skim milk and 0.1% (v/v) Tween 20 (PBS-M-T). After washing, membranes were incubated overnight at 4°C with anti-GFP rabbit polyclonal antibody (Chromotek pabg1) or anti-HisTag mouse polyclonal antibody (Genscript A00186) diluted 1:1000 and 1:500 in PBS-M-T, respectively. Following additional washes, membranes were incubated for 45 min at room temperature with a peroxidase-conjugated anti-rabbit antibody (Invitrogen 31460, diluted 1:40000) or anti-mouse antibody (Millipore Ap130p, diluted 1:2000). Peroxidase activity was visualized by chemiluminescence using the Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo), following the manufacturer's instructions. All washing steps consisted of 3–4 rinses in PBS with 0.1% (v/v) Tween 20, 5 min each at room temperature with gentle shaking (100 RPM).

2.7 Fluorimetry

Fluorescence from eGFP (λex/λem = 488/510 nm) and Cherry (λex/λem = 590/610 nm) was measured in samples (200 µL/well) loaded into F-bottom chimney 96-well plates (Greiner), using a Varioskan™ Flash spectrofluorometer (Thermo Scientific).

2.8 Recovery Yield Calculations

For purification from culture supernatants, fluorescence values were corrected by subtracting the background signal of BHI medium. After purification, the fluorescence of the M3 (resolubilized) fraction was corrected by the concentration factor, calculated as the difference between the original and final sample volume. Recovery percentages were calculated assuming that the corrected fluorescence in the supernatant represents 100% of the produced protein, and the corrected M3 fluorescence represents the recovered fraction.

3. Results

3.1. Production of eGFP fused to ELP tag

The expression vector pLEXSY_IE-blecherry4 was used to express an N-terminal ELP-tagged enhanced green fluorescent protein (eGFP) **(Figure 1A)**. This vector allows for the inducible expression and secretion of the protein into the culture medium. Additionally, the plasmid co-expresses the Cherry fluorescent protein, which remains in the cytosol of the cell, facilitating the

selection of highly expressing clones. Following transfection of *L. tarentolae*, one clone was selected as outlined in section 2.2.

The expression kinetics of eGFP and Cherry reporter proteins were evaluated. Fluorescence intensity measurements indicated that a significantly higher accumulation of the ELP-eGFP was achieved 72 h post-induction **(Figure 1B-C)**. Additionally, ELP-eGFP expression was confirmed by Western blot analysis using an anti-GFP antibody, revealing a predominant band at the expected size (~40 kDa), and a smaller band corresponding to free eGFP lacking the ELP tag (~26 kDa). No additional degradation products were detected under any of the conditions tested **(Figure 1D)**.

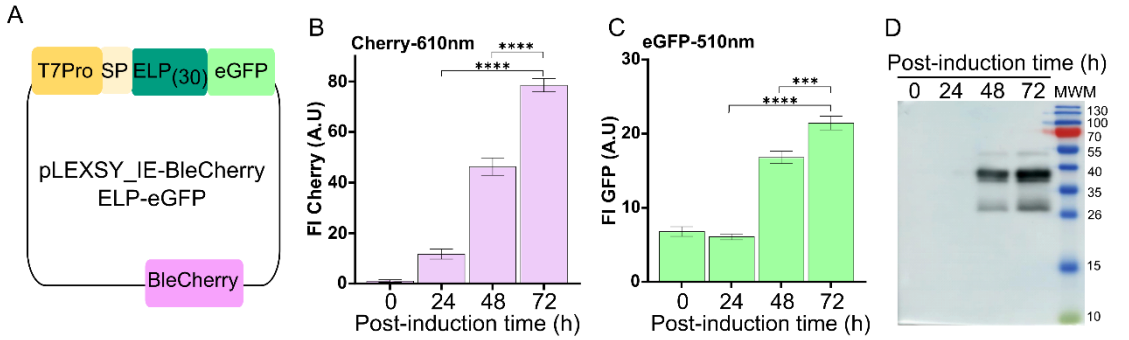

Figure 1. (A) Diagram and main components of the pLEXSY_IE-BleCherry4 construct used to express ELP-eGFP. T7Pro: T7 promoter regulated by a Tet operator; SP: secretion signal peptide; $ELP_{(30)}$: elastin-like polypeptide tag (30 pentapeptide repeated); eGFP: enhanced green fluorescent protein; BleCherry: bleomycin resistance gene and Cherry fluorescent protein gene. **(B-D)** Culture samples from induced cells (expressing pLEXSY_IE-BleCherry_ELP-eGFP) were collected at 0, 24, 48, and 72 h post induction and were analyzed by: **(B)** Cherry fluorescence intensity (FI, λex/λem = 490/610 nm); **(C)** eGFP fluorescence intensity (FI, λex/λem = 488/510 nm), and **(D)** Western blot (supernatant samples) with anti-GFP antibody. Revealing ELP-eGFP (~40 kDa), and free eGFP (~26 kDa). FI is expressed as arbitrary units (A.U). Plots display the mean ± standard deviation of three biological replicates. The statistical analysis was performed via one way ANOVA followed by Tukey's multiple comparisons test, ***P = 0.0001 and, ****P < 0.0001.

3.2. ELP-eGFP purification by Inverse Transition Cycling

To purify the ELP fusion from the culture medium, Inverse Transition Cycling (ITC) was employed **(Figure 2A)**. The effect of different $(NH_4)_2SO_4$ concentrations (0.4, 0.6, 0.8 and 1 M) and temperatures (25, 30 and 37°C) on the ITC process was evaluated **(supplementary figure 1).** The results indicate that this construct does not undergo significant precipitation changes under the evaluated conditions. While the precipitated fraction tends to increase up to 0.8 M of $(NH_4)_2SO_4$, then drops at 1 M—likely due to reduced resolubilization rather than lower precipitation. Although the results from varying incubation temperatures are similar, 30°C showed slightly better outcomes. Therefore, the precipitation condition at 30°C with 0.6 M $(NH_4)_2SO_4$ was selected for scaling-up the process. The efficiency was evaluated by SDS-PAGE, revealing a major band at ~40 kDa and a minor one at ~55 kDa **(Figure 2B**, bands 2 and 1, respectively). This minor band had already been identified as a marginal component in the culture supernatant through Western blot analysis. It should be noted that, as expected, the 26 kDa band corresponding to tag-free eGFP is not recovered **(Figure 1D)**.

These bands were excised and subjected to peptide mapping via trypsin digestion followed by mass spectrometry analysis. Notably, the ELP sequence lacks cleavage sites for trypsin or other common proteases, preventing the detection of ELP-derived peptide fragments. The analysis revealed the presence of eGFP in both samples, with an amino acid sequence coverage of 97% and 84%, respectively **(Figure 2C)**.

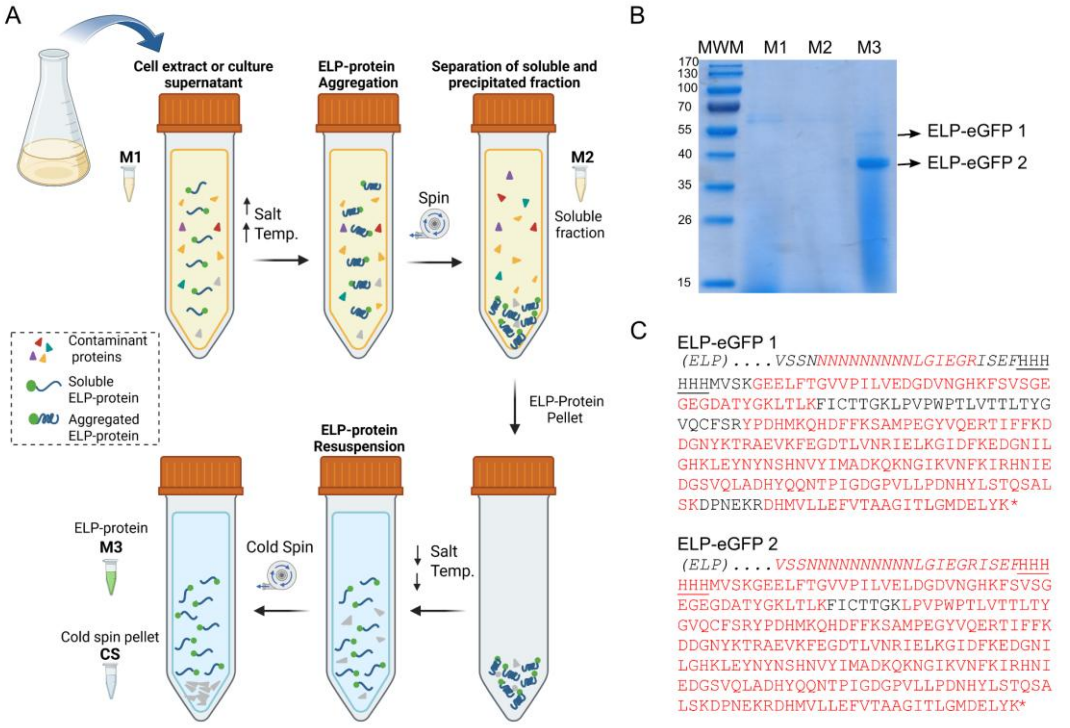

Figure 2. (A) Schematic representation of the inverse transition cycling (ITC) purification method for ELP fusion proteins. The temperature is increased, and salt, $(NH_4)_2SO_4$, is added to the culture supernatant (M1) containing the ELP fusion protein to induce a phase transition, leading to its aggregation. The aggregated ELP fusion protein is then separated from soluble contaminants (M2) by centrifugation at room temperature ("Spin"). This pellet is subsequently resuspended in a cold, low-salt buffer and centrifuged at 4°C ("Cold Spin"). The solubilized ELP fusion protein remains in the supernatant (M3), while insoluble contaminants that do not resolubilize form the cold spin pellet (CS). Schema created using BioRender (https://BioRender.com/5ml1v0l). **(B)** ELP-eGFP purification process analyzed by SDS-PAGE: culture supernatant (M1), soluble (M2), and precipitated fraction (M3) after the ITC with 0.6 M ammonium sulfate and 30°C for 15 min. **(C)** Mass spectrometry analysis of bands of interest (ELP-eGFP1: 55 kDa, and ELP-eGFP2: ~40 kDa) excised from SDS-PAGE. The eGFP sequence, preceded by a 6×His tag (underlined), and a spacer sequence (italicized), is shown. Peptides identified in each band are highlighted in red.

3.3. Effect of precipitation time and resolubilization conditions on ELP recovery

The secretion of recombinant proteins into the culture medium provides several advantages that include, the post-translational processing (if required) of the target protein, the avoidance of cell lysis, and a lower amount of contaminants when compared to the protein-rich intracellular environment. However, secreted proteins are often highly diluted in the culture supernatant, which can reduce the efficiency of ELP-mediated precipitation[28]. Since a substantial portion of the protein of interest remained in the soluble fraction (M2), we aimed to improve recovery by increasing the incubation time under precipitation conditions. After 15 min, 3% of ELP-eGFP was recovered in the precipitated fraction, whereas extending the precipitation to 1 hour increased recovery to 7%. Both fluorescence measurements and Western blot analysis confirmed an increase in ELP-eGFP precipitation with the longer incubation time **(Figure 3A-B)**.

To enhance the recovery of ELP-eGFP from the precipitated fraction (M3), we optimized the resuspension conditions. Precipitates were obtained by $(NH_4)_2SO_4$ precipitation at 0.6 M and incubation at 30°C for 15 min. These precipitates were then resuspended in a cold buffer (50 mM Tris, 2 mM EDTA, pH 8.8) and different conditions were evaluated. We tested extending the

resuspension time from 10 min on ice to overnight at 4°C. In addition to prolonged cold incubation, we applied sonication in a water bath for 10 min to aid protein disaggregation, and added the reducing agent dithiothreitol (DTT) to disrupt disulfide bonds that contribute to aggregation. Fluorescence emission analysis of eGFP revealed increased recovery under all tested conditions compared to the 10 min on-ice control **(Figure 3C)**. Overall, these optimized methods nearly doubled the amount of soluble ELP-eGFP recovered. Sonication and DTT did not enhance solubility beyond the improvement achieved with extended cold incubation alone

3.4. Use of exogenous ELP as a precipitation facilitator

Although extending precipitation time improves ELP recovery, it should be noted that extended incubation at 25–30°C can compromise the stability of certain proteins. To address this issue, we explored the use of an exogenous elastin-like polypeptide (ELP) as a carrier to improve protein precipitation[29].

An exogenous ELP with 110 repeats was produced in *E. coli* and then purified using ITC. The use of this exogenous ELP at concentrations of 5, 10, and 50 µM, along with 0.6 M $(NH_4)_2SO_4$ at 25°C, increased the percentage recovery of ELP-eGFP from 3% to 12% **(Figure 3D-E)**. Among the tested concentrations, 5 µM exogenous ELP appeared to be the most effective.

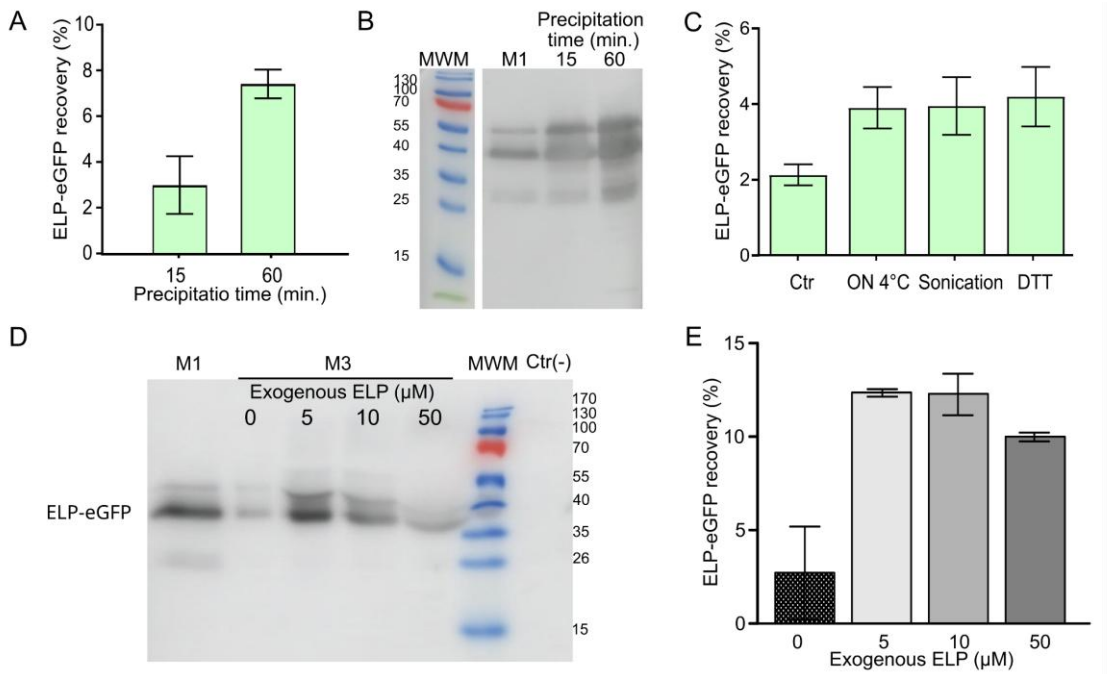

Figure 3. Evaluation of ELP-eGFP recovery and resuspension under different precipitation and treatment conditions. (A–B) Effect of precipitation time on ELP-eGFP recovery. Precipitation was performed with 0.6 M $(NH_4)_2SO_4$ at 25°C for either 15 or 60 min. **(A)** Quantification of ELP-eGFP in the precipitated fraction by eGFP fluorescence (λex/λem = 488/510 nm), expressed relative to the corresponding supernatant. **(B)** Western blot analysis of ELP-eGFP in the supernatant (M1) and precipitated fractions using an anti-GFP antibody. **(C)** Optimization of ELP-eGFP resuspension from the precipitated fraction. Recovery was quantified by eGFP fluorescence after resuspension under the following conditions: control (Ctr, 10 min on ice), overnight (ON) at 4°C, sonication in a water bath (10 min), and addition of 1 mM DTT. All samples were initially precipitated with 0.6 M $(NH_4)_2SO_4$ for 15 min at 25°C. Data represent the mean ± standard deviation (SD) from replicate. **(D–E)** Effect of exogenous ELP on ELP-eGFP precipitation. Precipitation was performed with 0.6 M $(NH_4)_2SO_4$ for 15 min at 25°C in the presence of increasing concentrations of exogenous ELP (5 µM, 10 µM, and 50 µM). **(D)** Western blot of the supernatant (M1) and precipitated fractions (M3), probed with an anti-GFP antibody. Negative control (Ctr–) corresponds to 50 µM

exogenous ELP without ELP-eGFP. **(E)** Quantification of ELP-eGFP recovery in the precipitated fractions (M3) by fluorescence (λex/λem = 488/510 nm). Data represent the mean ± SD from replicates.

3.5. ELP-bFSH purification by Inverse Transition Cycling

After successfully purifying the model protein eGFP using ELP-mediated precipitation, we applied the same strategy to purify a protein of veterinary relevance, the bovine follicle stimulant hormone (bFSH). We constructed an inducible expression vector encoding bFSH chimera previously produced in *L. tarentolae*[10]. To enhance precipitation efficiency, the bFSH chimera was fused to an extended ELP tag consisting of 110 pentapeptide repeats **(Figure 4A)**. Induction kinetics were monitored using the co-expressed Cherry fluorescent reporter **(Figure 4B)**. Despite successful induction, bFSH expression in the culture supernatant remained very low and was barely detectable by Western blot after extended exposure times **(Supplementary Figure 2)**. These low protein levels suggest that the expression of bFSH with a 110-repeat ELP tag is challenging for *L. tarentolae.*

To assess the enrichment of the ELP-bFSH fusion protein, the culture supernatant was treated with 0.6 M $(NH_4)_2SO_4$ at 30 °C for 15 min. The resulting fractions were analyzed by Western blot. As shown in **Figure 4C**, the ELP-bFSH fusion protein, undetectable in the untreated supernatant (M1), was enriched in the precipitated fraction (M3) following ITC. In M3, the expected ~73 kDa ELP-bFSH band was observed, along with additional bands of lower apparent molecular weight, which may correspond to truncated ELP tags **(Figure 4C)**.

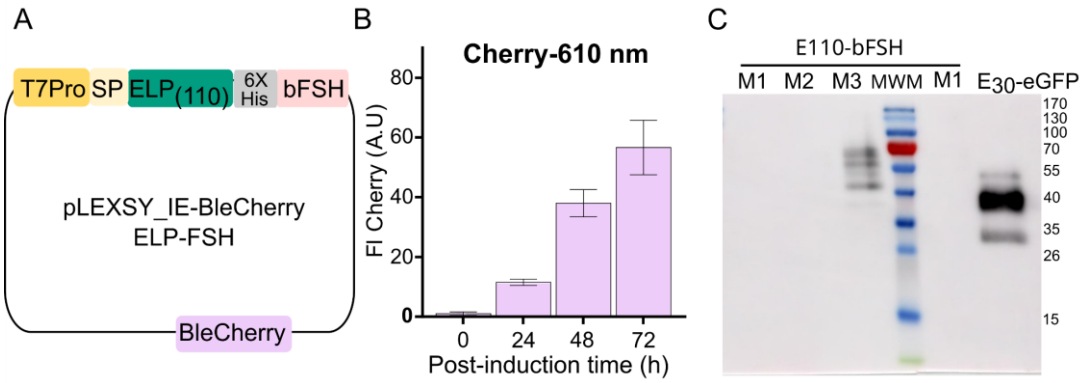

Figure 4. (A) Diagram and main components of the pLEXSY_IE-BleCherry4 construct used to express ELP-bFSH. T7Pro: T7 promoter regulated by a Tet operator; SP: secretion signal peptide; $ELP_{(110)}$: elastin-like polypeptide tag (110 pentapeptide repeated); 6xHis: histidin-Tag; bFSH: bovine FSH (9); BleCherry: bleomycin resistance gene and Cherry fluorescent protein gene. **(B)** Culture samples from induced cells (expressing pLEXSY_IE-BleCherry_ELP-bFSH) were collected at 0, 24, 48, and 72 h post induction and were analyzed by Cherry fluorescence intensity (FI, λex/λem = 590/610 nm). FI is expressed as arbitrary units (A.U) and plot displays the mean ± standard deviation of three biological replicates. **(C)** Western blot analysis of ITC precipitation of ELP-bFSH. The supernatant (M1), non-precipitated (M2), precipitated fractions (M3), and supernatant from ELP-eGFP (E30-eGFP) as a reference control, probed with an anti-His antibody.

3.6. Evaluation of the ΔI-CM mini-Intein as an auto-releaser of the ELP-tag in the L. tarentolae system.

We generated inducible and constitutive expression constructs where the ΔI-CM sequence was positioned between ELP and eGFP (ELP-ΔICM-eGFP) **(Figure 5A)**. Western blot analysis of the culture supernatants from the corresponding cell lines transfected with these constructs revealed the presence of eGFP lacking the ELP tag. This result suggests that the fusion protein

undergoes premature cleavage **(Figure 5B)**. To determine whether intein cleavage occurred at intracellular level, we analyzed cell lysates from the cell line with constitutive expression of the transgene by Western blotting. The presence of a prominent band corresponding to untagged eGFP in the cell lysates **(Figure 5C)** indicates that the construct undergoes highly efficient cleavage within the cells and not upon secretion to the culture medium.

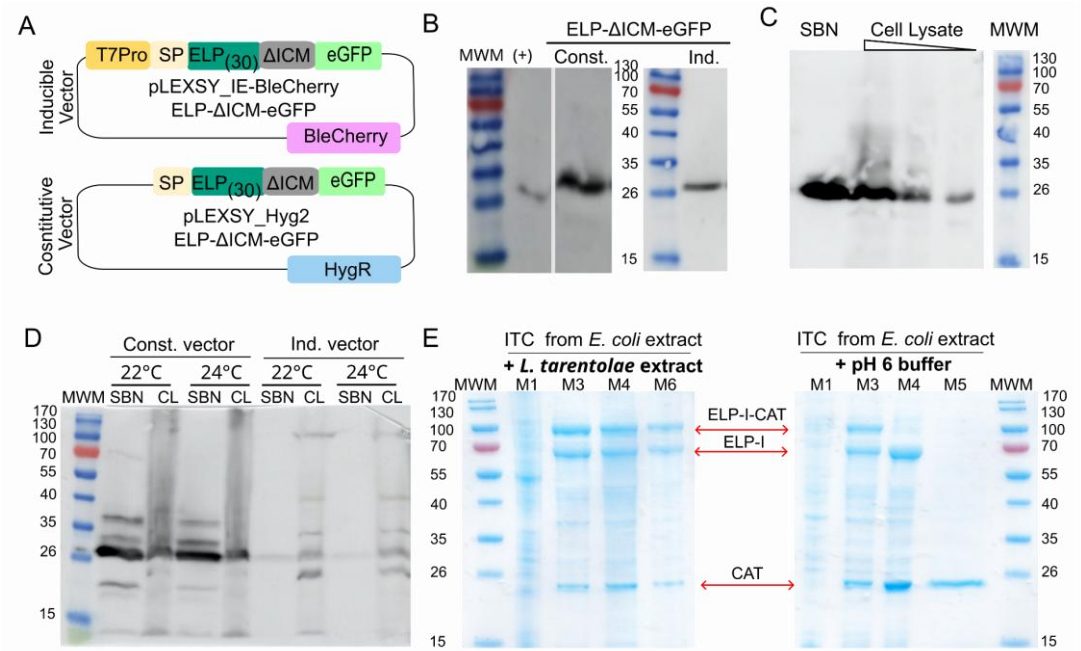

Figure 5. Western blot and SDS-PAGE analysis of ELP_ΔI-CM_eGFP expression and intein cleavage. (A) Diagram and main components of the inducible vector (pLEXSY_IE-BleCherry4) and the constitutive vectors (pLEXY_Hyg2) used to express ELP-ΔICM-eGFP. T7 Pro: T7 promoter regulated by a Tet operator; SP: secretion signal peptide; $ELP_{(30)}$: elastin-like polypeptide tag (30 pentapeptide repeated), ΔI-CM: mini intein, HygR: hygromycin resistance gene; and BleCherry: a fusion of bleomycin resistance and the Cherry reporter protein. **(B)** Western blot of culture supernatants from constitutive (Const.) and inducible (Ind.) *L. tarentolae* ELP-ΔICM-eGFP clones, with free-eGFP as a reference control (+). **(C)** Western blot of supernatant (SBN) and increasing amounts of cell lysate from the constitutive *L. tarentolae* ELP-ΔICM-eGFP clone. **(D)** Effect of decreasing the culture temperature (24°C, and 22°C) on ELP-ΔICM-eGFP production in supernatants (SBN) and cell lysates (CL). **(E)** SDS-PAGE analysis of the effect of *L. tarentolae* cell extract on the cleavage of the ΔICM intein in the recombinant ELP-ΔICM-CAT protein (E-I-CAT) produced in *E. coli*. Red arrows indicate the expected molecular weights of free CAT, CAT fused to ELP-ΔI (E-I-CAT), and free E-I. M1: cell extract, M3: precipitated fraction, M4: precipitated fraction incubated for 18 h with either *L. tarentolae* extract (left panel) or at pH 6 (right panel), M5: soluble fraction after precipitating ELP in fraction M4 (free CAT), and M6: insoluble fraction after precipitating ELP in fraction M4.

The proteolytic activity of the ΔI-CM intein is induced by a decrease in pH and an increase in temperature[22]. In our experiments, the pH of the culture medium remained stable above 7.3, leaving culture temperature as the potential factor for modulating intein activity. To evaluate its effect on autoproteolysis, the culture temperature was reduced from the standard 26°C to 24°C, and 22°C. Microscopic examination confirmed a satisfactory cell motility at all tested temperatures, although growth was progressively slower at lower temperatures. Western blot analysis of supernatant and cell lysate samples showed bands with higher molecular weight than untagged eGFP, as well as free eGFP **(Figure 4D)**. A similar profile was observed in cultures grown at 16°C and 18°C **(Supplementary Figure 3)**. These results suggest that lowering the culture temperature by at least two degrees led to a partial decrease in intein cleavage efficiency; however, free eGFP remained the predominant species.

As there are no prior reports of proteins fused to the ΔI-CM intein being expressed in *L. tarentolae*, we investigated whether an intracellular component of this protozoan could trigger intein cleavage. To test this, we examined the effect of an *L. tarentolae* cell extract on a recombinant E-I-CAT protein (chloramphenicol acetyltransferase (CAT) fused to a 110-repeat ELP and the ΔI-CM intein) produced in *E. coli*. The E-I-CAT protein present in the *E. coli* extract was purified by ITC, and the resulting precipitate was divided into two fractions. One was resuspended in the *L. tarentolae* cell extract at pH 8.8 **(Figure 4D, left panel)**, and the other in a buffer at pH 6, which is optimal for intein cleavage **(Figure 4D, right panel)**. After 18 h of incubation at room temperature **(M4, Figure 4D)** no cleavage was observed in the *L. tarentolae* extract: the intensity of the E-I-CAT band remained unchanged, and no increase in E-I or free CAT was detected relative to fraction M3. In contrast, clear signs of cleavage were observed at pH 6, as E-I-CAT level decreased and the amount of E-I and free CAT increased **(Figure 4D)**.

4. Discussion

The expansion of the bioactive molecule market has driven the need for cost-effective and efficient methods for the production and purification of recombinant proteins—particularly in the agro-veterinary sector, where profit margins are often limited[6]. In this study, we aimed to evaluate a non-conventional purification system based on the use of an elastin-like polypeptide (ELP) tag fused to the mini-intein ΔI-CM for protein expression in *Leishmania tarentolae*. As this method does not rely on chromatographic systems, it offers a more economical and scalable alternative, making it particularly attractive for large-scale industrial applications.

In this study, we explored the use of the mini-intein ΔI-CM as a cleavable linker between ELP and eGFP in *L. tarentolae*, assessing both constitutive and inducible expression systems. Western blot analyses revealed that eGFP was primarily free of the ELP-tag, both in the supernatants and in the cell lysates, indicating that intein-mediated cleavage occurred efficiently inside the cells under standard culture conditions.

Although previous reports have noted some degree of *in vivo* intein cleavage during expression[25], the high efficiency observed at the cultivation temperature of *L. tarentolae* was unexpected. Attempts to suppress cleavage by reducing culture temperature (to 24°C, or 22°C) had only a modest effect, suggesting that the proteolytic activity of ΔI-CM in this protozoa may be activated through an alternative mechanism, potentially involving host-specific factors. This hypothesis was refuted based on the results obtained upon incubating a ELP_ΔI-CM_CAT fusion protein produced in *E. coli* with a *L. tarentolae* cell extract, which revealed no cleavage of the recombinant protein. While these results argue against the presence of a soluble, extractable host's molecule(s) responsible for intein activation, it remains possible that intracellular conditions, such as compartment-specific environments or redox gradients, may promote intein cleavage. Indeed, during secretion, the protein may transit through acidic compartments that could promote cleavage. Although the pH of *Leishmania* organelles has not been reported, studies in mammalian cells indicate that the trans-Golgi network and secretory granules exhibit a pH near 6[30]. If true, our data suggests that this is a very efficient process. Based on this finding, ΔI-CM does not appear suitable for secretion-based applications using *L. tarentolae* as an expression host.

Regarding the use of ELP, we demonstrated the successful expression and purification of an ELP-tagged eGFP fusion protein in *L. tarentolae* using an inducible secretion system. Western blot analysis revealed a predominant band corresponding to the full-length ELP-eGFP fusion protein. Importantly, no major degradation products were observed, indicating the overall stability of the recombinant protein in the culture medium.

Purification by ITC, capitalized on the unique phase transition behavior of ELPs, combining 0.6 M $(NH_4)_2SO_4$ and a temperature of 30°C was sufficient to induce selective aggregation of the fusion protein. Despite the low protein concentration in the culture medium, ITC enables the recovery of the fusion construct, as confirmed by SDS-PAGE . In addition to the expected 40

kDa band, a band of approximately 55 kDa was also observed. The ELP sequence used was designed to avoid post-translational modification sites and lacks lysine residues that could be oxidized by lysyl oxidase, thus preventing the formation of desmosine-type cross-links. Therefore, a plausible explanation for the presence of this minor ~55 kDa band is that the ELP tandem repeats have been amplified during the integration of the construct into 18S rRNA loci of the *L. tarentolae* genome. In *Leishmania sp*., unlike in *E. coli*[31], homologous recombination and gene amplification occur at a high rate[32,33]. Since *L. tarentolae* contains multiple copies of these 18S genes, it is possible that multiple ELP sequences were integrated into the genome of the same clone. Mass spectrometry analysis revealed the presence of eGFP in both bands, while ELP-derived peptide fragments are not detected since the ELP sequence lacks cleavage sites for trypsin or other common proteases.

However, recovery remained modest (~3%), compared to the 21.5% recovery obtained for ELP-eGFP expressed at high levels in *E. coli* and purified using ITC, likely due to the high dilution of ELP-eGFP in the *L. tarentolae* supernatant. Several authors have proposed the addition of free ELP as a co-precipitation "carrier" to overcome this issue[29,34]. We adopted this strategy and observed a fourfold increase in recovery of ELP-eGFP when exogenous ELP was added, reinforcing the idea that low ELP concentration in the supernatant is a limiting factor. Our findings are consistent with those of Christensen et al.[29], who reported 3- to 6-fold increases in recovery of ELP-tagged proteins when using free ELP as a co-precipitant. The mechanism by which exogenous ELP improves precipitation may involve the formation of heterotypic aggregates that enhance the aggregation of the tagged protein.

To further evaluate the applicability of this system to a protein of veterinary interest, we fused a bovine FSH chimera to an extended ELP tag containing 110 pentapeptide repeats. Although induction was successful, bFSH expression levels in the culture supernatant remained very low and were barely detectable by Western blot. This suggests that the expression of bFSH fused to a large ELP tag may pose challenges for *L. tarentolae*, possibly due to overloading of the secretory machinery or issues related to protein stability. Future studies on the bFSH-ELP chimera should be focused on optimizing recombinant protein expression levels and on testing shorter ELP tags. Despite these low expression levels, ITC purification enabled specific enrichment of the ELP-bFSH fusion protein. A band corresponding to the full-length fusion protein was observed in the precipitated fraction, while it was undetectable in the untreated supernatant. Additional bands of lower apparent molecular weight were also detected, likely corresponding to truncated ELP-bFSH variants. These results demonstrate that ITC can recover target proteins even at low expression levels, and highlights the compromise between improved precipitation efficiency and the challenges associated with expressing longer ELP repeats.

A comparative analysis by Banki *et al.*[35] evaluated conventional affinity-based purification methods against alternative strategies, including ELP tagging. The study examined both the technical performance and the large-scale economic implications of these systems, focusing on raw material costs. Their analysis demonstrated that ELP-based purification can reduce costs by at least 11-fold compared to His-tag affinity chromatography. Overall, our results validate *L. tarentolae* as a suitable host for producing secreted ELP-tagged proteins and demonstrate that combining ELP-tagged secretion with ITC and carrier-assisted precipitation constitutes an effective low-cost purification strategy.

The main objective of the work was to assess the compatibility of ELP-mediated purification with *L. tarentolae* secretion, rather than to fully characterize the biochemical properties of the purified proteins. Although further optimization is needed to improve recovery yields, these findings represent an important proof of concept and expand the potential of *L. tarentolae* as a versatile expression platform. Beyond the specific system described here, this work contributes to the broader effort of developing affordable and scalable biomanufacturing technologies. Such

advances are particularly relevant for the veterinary and biopharmaceutical sectors, where cost-efficient expression systems can facilitate local production, reduce dependence on extractive sources, and accelerate access to essential bioproducts.

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CRediT authorship contribution statement

Lucía Bassetti: Writing – original draft, Methodology, Investigation. Martina Crispo: Writing review & editing. Mariela Bollati-Fogolín: Writing – review & editing. Sergio Pantano: Writing – review & editing. Marcelo A. Comini: Writing – review & editing, Supervision, Data curation. Cecilia Abreu: Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data cura tion, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Material

Exploring Elastin-Like Polypeptide Tags and mini-intein for Recombinant Protein Purification in *Leishmania tarentolae*

Supplementary Figure 1.

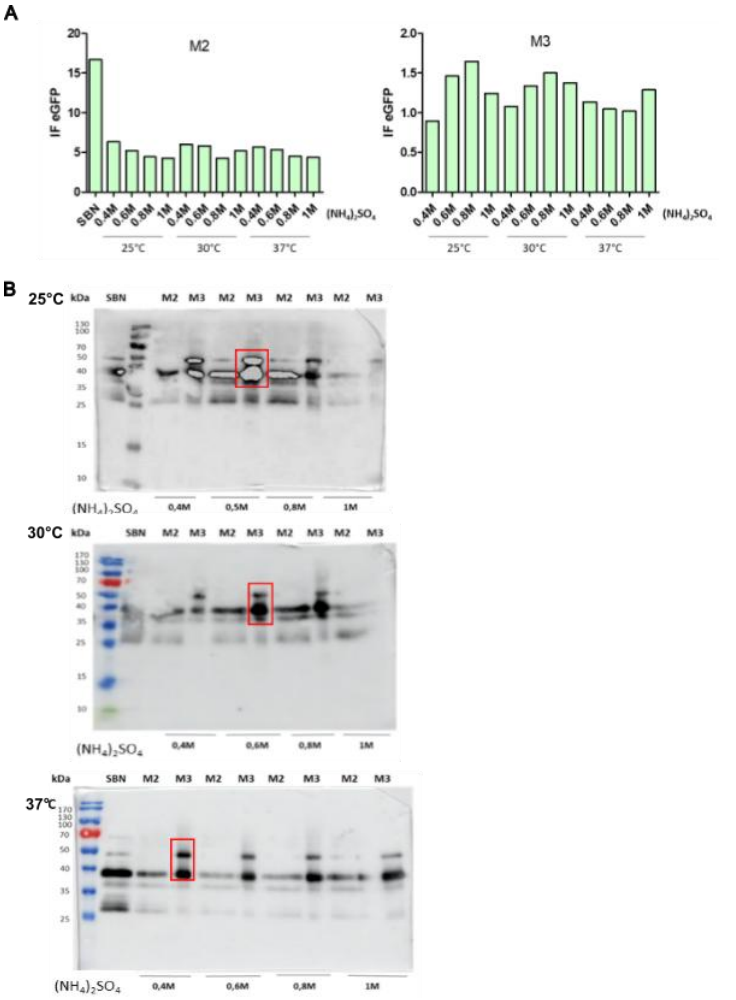

Figure S1. Evaluation of ELP-eGFP precipitation under different conditions by **(A)** Fluorometry and **(B)** Western blot. Samples from fractions M2 and M3 were analyzed following 15 min precipitation cycles of ELP-eGFP using varying concentrations of $(NH_4)_2SO_4$ (0.4, 0.6, 0.8, and 1 M) at different temperatures: 25°C, 30°C, and 37°C. SBN: culture supernatant; M2: soluble fraction after ELP precipitation; M3: insoluble fraction after ELP resolubilization; MWM: molecular weight marker.

Supplementary Figure 2.

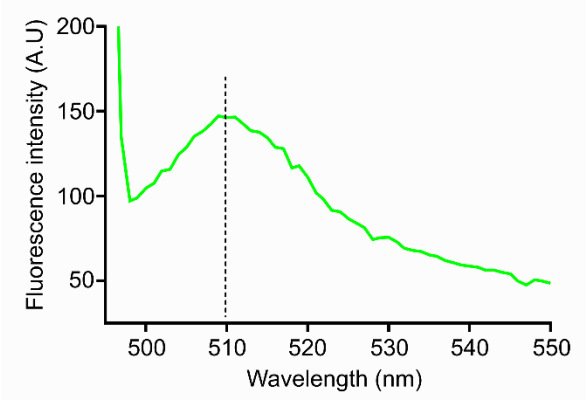

Figure S2. Fluorescence emission spectrum of ELP-eGFP purified by inverse transition cycling (ITC) using 0.6 M ammonium sulfate at 30 °C for 15 min. The precipitated ITC fraction (M3) was excited at 488 nm, and emission was recorded from 495 to 550 nm using a 5 nm slit width. The peak detected at 495 nm corresponds to residual excitation light, while the characteristic eGFP emission maximum is observed at ~510 nm. A.U= arbitrary units.

Supplementary Figure 3.

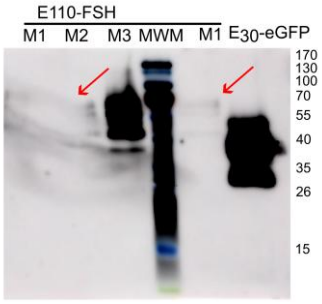

Figure S3. Western blot of ITC precipitation process of ELP-bFSH. The supernatant (M1), non precipitated (M2), precipitated fractions (M3), molecular weight marker (MWM), and supernatant from ELP-eGFP (E30-eGFP) as a reference control, probed with an anti-His antibody. This Western blot is over-exposed to evidence fade ELP-bFSH bands in M1 and M2 fractions (red arrows).

Supplementary Figure 4.

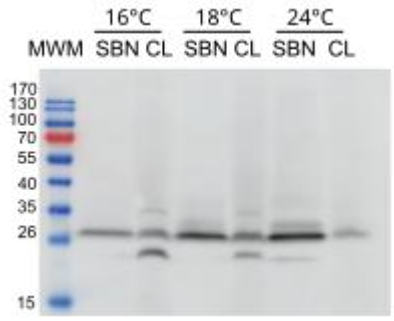

Figure S4. Effect of decreasing the culture temperature (24°C, 18°C and 16°C) on ELP-ΔICM-eGFP production in supernatants (SBN) and cell lysates (CL) from the constitutive *L. tarentolae* ELP-ΔICM-eGFP clone. Molecular weight marker (MWM).