A non-chromatographic protein purification strategy using Src 3 homology domains as generalized capture domains

Heejae Kim, Wilfred Chen[*]

Chemical and Biomolecular Engineering Department, University of Delaware, 150 Academy St., Newark, DE 19716, United States

ABSTRACT

Protein purification using inverse phase transition of elastin-like polypeptide (ELP) domains is a useful alternative to chromatography. Genetic fusions of ELP domains to various proteins have the ability to reversibly transition between soluble monomers and micron-sized aggregates and this has been used to selectively purify many ELP fusions. Affinity domains can enhance this technology by using specific protein binding domains to enable ELP mediated affinity capture (EMAC) of proteins of interest (POI) that have been fused to corresponding affinity ligands. In this paper, we highlight the use of Src homology 3 (SH3) domains and corresponding peptide ligands in EMAC that have differential binding affinities towards SH3 for efficient capture and elution of proteins. Furthermore, differences between capture and elution of a monomeric and a multimeric protein were also studied.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

The large number of available sequenced genomes have greatly expanded our capability to produce therapeutic proteins of interest (POI). However, large-scale purification of recombinant proteins remains a challenge and adds significant cost to their overall production and use. The gold standard for protein purification is column chromatography, which relies on modified resin particles that take advantage of differences in charge, hydrophobicity, or affinity towards ligands to separate proteins from each other and other biomolecules. In particular, affinity domains such as protein A (Hober et al., 2007; Huse et al., 2002), polyhistidine (Bornhorst and Falke, 2000), maltose binding protein (Nallamsetty and Waugh, 2007), and glutathione s-transferase (Harper and Speicher, 2011), have been utilized in conjunction with resins for their high specificity (Terpe, 2003). Using commercially available affinity ligand modified resin particles, column chromatography can be used to bind and elute POI to a high degree of purity. Although modified resins are highly accessible, column chromatography is limited because of high resin costs and slow volumetric throughputs that affect scale-up.

Elastin-like polypeptides (ELP) are oligomeric repeats of the pentapeptide sequence VPGXG (X is a guest amino acid residue except proline) and are non-chromatographic alternatives to using resin, because ELP exhibits reversible inverse phase transition from a highly water soluble state to an insoluble aggregate state (Meyer and Chilkoti, 1999; Urry, 1997). This thermally-responsive nature of ELPs has been exploited to selectively trigger the formation of micron-sized ELP aggregates that are easily separated from much smaller constituents in solution using centrifugation or filtration (Hassouneh et al., 2010). The reversible nature of ELP aggregation has been exploited for purification schemes by genetically fusing ELPs to POI (Banki et al., 2005; Hassouneh et al., 2010; Sun et al., 2014). The reversible phase transition property is retained as an ELP fusion, enabling reversible switching of the entire fusion protein between soluble monomeric and insoluble aggregate states for simple purification by centrifugation.

While ELP fusions have been expressed in hosts such as *E. coli* (Hassouneh et al., 2010), yeast (Sallach et al., 2009; Schipperus et al., 2009) and plants (Floss et al., 2010), ELP purification is highly dependent on ELP concentration, and low protein yields could decrease the effectiveness of ELPs (Meyer et al., 2001). Furthermore, the large size of ELP domains have to be carefully chosen so that the inverse phase transition or the fusion itself does not affect the activity of the protein of interest (Christensen et al., 2009). While the thermally responsive properties of ELP can be modulated by changing the guest residue (Urry, 1997) and there are empirical models to predict suitable sequences (Christensen et al., 2013) for the required transition temperature (T_t), these efforts could be tedious.

ELP mediated affinity capture (EMAC), on the other hand, utilizes a single ELP fusion to capture and purify various proteins (Liu
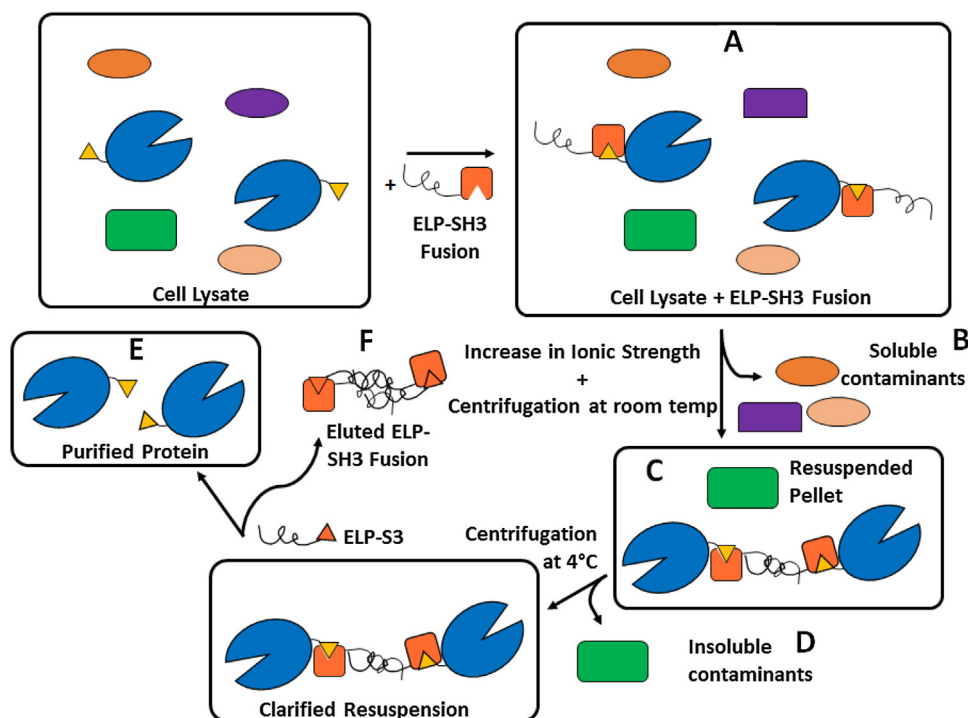
Fig. 1. Protein Purification Strategy. (A) Purified ELP-SH3 fusions are mixed with clarified lysates containing POIs fused to three repeating weak ligands (w3). (B) The ELP-POI complexes are precipitated by increasing the salt concentration and temperature. Subsequent centrifugation separates soluble contaminants from (C) aggregated ELP complexes. (D) Insoluble contaminants that co-precipitated during aggregation are removed using centrifugation at 4 °C. (E) POI is eluted from ELP-SH3 fusions by the addition of ELP-s3. (F) The ELP fusions can then be removed by inducing ELP aggregation.

et al., 2012; Stiborova et al., 2003), and has also been adapted for other biomolecules (Kostal et al., 2004) and metals (Kostal et al., 2001). EMAC uses specific protein binding pairs that act as protein capture domains to adapt ELP properties for the entire complex for affinity precipitation (Liu and Chen, 2013; Madan et al., 2013; Sheth et al., 2014). EMAC allows the use of small affinity tags as fusion partners and the expression of the POI in an optimal host that promotes folding and post-translational modifications of the POI, while allowing ELP to be hyper-expressed in *E. coli*. Ideally, the chosen protein binding pair expresses well as fusions in a variety of hosts to increase the flexibility of affinity precipitation for purification.

Previously we have shown the use of dockerin and cohesin as the interaction pair for EMAC (Liu and Chen, 2013). Although effective, dockerin and cohesin fusions typically suffer from lower expression levels (Barak et al., 2005). In this work, we hoped to improve upon this technology by using smaller binding ligands that potentially have less impact on expression to the POI. One ideal candidate is the well-characterized Src homology 3 (SH3) domain that has been used in many protein immobilization and scaffolding applications (Dueber et al., 2009; Ramirez et al., 2013). In nature, SH3 domains, across many different prokaryotes and eukaryotes, interact with a diverse array of proline-rich motifs, making this strategy potentially host-cell independent (Li, 2005; Nguyen et al., 2000; Posern et al., 1998). Of interest are two motifs with well-defined differential binding affinities, PPPALPPKRRR (strong ligand) and PPPVPPRR (weak ligand) (K_d = 0.1 and 10 μM, respectively), that can be used for binding and competitive displacement (Dueber et al., 2007).

The strong and weak ligands are ideal for EMAC because of their simplicity, length, and specificity toward the cognate SH3 domain (Li, 2005). In this study, we not only devise a new strategy to capture the POI using an ELP-SH3 fusion, but also exploit the difference in binding affinities between the two ligands to elute the POI after purification (Fig. 1). The successful purification of two

distinct model POIs, a monomeric enhanced cyan fluorescence protein (ECFP) and a homodecameric methanol dehydrogenase (MDH) from *Bacillus methanolicus* (Arfman et al., 1997), is used to show versatility in this approach for proteins of multiple origins.

2. Materials and methods

All primers were purchased from IDT (Coralville, IA). All ingredients for culturing media were purchased from Fisher Scientific (Pittsburgh, PA), all ingredients for SDS-PAGE were purchased from BIO-RAD (Hercules, CA), all enzymes related to DNA manipulation and cloning were purchased from New England Biolabs (Ipswich, MA), and all other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

2.1. Strains and cloning

Escherichia coli NEB5α (New England Biolabs, Ipswich, MA) [*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*] was used as the host for all genetic manipulations and plasmid maintenance. *E. coli* strain BLR (DE3) (EMD Millipore, Madison, WI) [*F⁻ ompT hsdS$_B$(r$_B$⁻ m$_B$⁻) gal dcm (DE3) Δ(srl-recA)306:Tn10 (Tet^R)*] was used as the production host for all proteins. *E. coli* cultures were grown in Luria-Bertani (LB) media (10.0 g/L tryptone, 5.0 g/L yeast extract, 10.0 g/L NaCl) supplemented with 50 μg/mL Kanamycin.

Gene fragment encoding the SH3 domain was amplified from pI-SH3-GFP (a gift from Prof. Zhilei Chen, Texas A&M University) via PCR, using primers SH3-F1 and SH3-R1 and inserted into pELP–CFP-Zif268 (Blackstock et al., 2015) using *Sac*II and XhoI to create pELP-SH3. Further repeats of SH3 domains were inserted using primer pairs SH3-F2 and SH3-R2 using sites SalI and SacII, and SH3-F3 and SH3-R3 using sites EcoRI and SalI to generate pELP-2rSH3

and pELP-3rSH3, respectively. Positive clones were selected and transformed into BLR for expression.

Three repeats of the strong ligand sequence (PPPALPPKRRR) were cloned into pET24a-ELP[KV8F-40] using the SLIC method (Li and Elledge, 2012). Both ligand sequences, separated by 3 glycine-serine repeats acting as linker sequences between ligand repeats, were synthesized using overlapping oligonucleotide. 20 bp of homology to the plasmid adjacent to the SacII and XhoI sites were included in the insert as overhangs. These oligonucleotides were mixed at equimolar ratios, incubated at 98 °C for 30 s, and then allowed to cool to room temperature. pET24a-ELP[KV8F-40] cut with SacII and XhoI, purified, and then treated with T4 polymerase without any dNTPs for 30 min at room temperature and then quenched with dATP. This processed plasmid was incubated with the insert and transformed into NEB5α to repair gaps and nicks in the plasmid. The resulting plasmid, pET24a-ELP[KV8F-40]-s3, was transformed into BLR for expression.

The binding motif consisted of the following: three tandem repeats of the weak ligand (PPPVPPRR) gene sequence separated by 3 glycine-serine pairs between each weak ligand followed by a $(G_4S)_3$ linker. The entire gene sequence was synthesized using overlapping oligonucleotides with overhangs corresponding to NdeI and BamHI. These oligonucleotides were mixed at equimolar ratios, incubated at 98 °C for 30 s, and then allowed to cool to room temperature. The resulting product was treated with T4 Polynucleotide Kinase and inserted into pET24a using the NdeI and BamHI sites, yielding pET24a-w3 (w3 referring to three tandem repeats of the weak ligand). Sequences for CFP and methanol dehydrogenase (MDH) from *Bacillus methanolicus* were amplified using PCR and either CFP or MDH sequence were ligated using the EcoRI and XhoI sites on pET24a-w3. The resulting plasmids, pET24a-w3CFP-his6 and pET24a-w3MDH-his6, were transformed into NEB5α and positive clones were subsequently transformed into BL21 (DE3) for expression.

2.2. Protein expression

All proteins were expressed in Terrific Broth (TB) media (12 g/L tryptone, 24 g/L yeast extract, 0.4% (v/v) glycerol, 9.4 g/L potassium phosphate monobasic, 2.2 g/L potassium phosphate dibasic) supplemented with 50 μg/mL kanamycin. ELP fusions expressed using overnight leaky expression. In summary, cultures were induced with overnight preculture from a colony isolated from a plate to an OD600 = 0.05 and grown for 16 h at 37 °C in a shake flask.

w3CFP and w3MDH expressing cultures were innoculated with an overnight culture from a single colony to an OD600 = 0.05. The culture was allowed to grow at 37 °C in a shake flask until OD600 = 0.6, at which point the culture was supplemented with 200 μM IPTG and grown overnight (16 h) at 20 °C.

Cultures were sedimented using centrifugation at 4000 × g for 5 min at 4 °C, and the pellet was suspended in 1x PBS (Phosphate Buffered Saline) to an OD600 = 20. The cell suspension was sonicated to release protein and clarified using centrifugation at 15,000 × g for 15 min at 4 °C. w3CFP and w3MDH clarified cell lysates were mixed with protease inhibitor cocktail (EMD Millipore, Billerica, MA), aliquoted and stored at 4 °C. ELP fusions were immediately purified using ITC.

2.3. Purification using inverse phase transition cycling (ITC)

ELP aggregation and subsequent phase change can be triggered by an increase in either ionic strength or temperature. It is our recommendation to induce the aggregation by titrating in ammonium sulfate at room temperature. All aggregations were induced using a max of 0.5 M $(NH_4)_2SO_4$. Aggregation can be monitored visually or using a spectrophotometer by measuring absorbance at 350 nm.

The presence of micron-sized particles induces a visible change in turbidity. The turbidity is highly ELP concentration dependent, and care should be taken to limit both the ionic strength and centrifugation force, to prevent irreversible precipitation.

In general, ELP-fusions were incubated with a max of 0.5 M $(NH_4)_2SO_4$, and the well-mixed solution was centrifuged at 12,000 × g for 10 min at room temperature. The supernatant was aspirated out of the vessel and the pellet was resuspended with 1xPBS and incubated on ice to resolubilize the aggregates. Resuspended pellets were centrifuged at 12,000 × g for 5 min at 4 °C, which completes one full cycle of ITC.

2.4. ELP protein concentration

ELP-SH3 fusion concentrations were determined after purification by measuring A280 and converting to protein concentrations using empirically determined extinction coefficients. (ELP-SH3, ε^{-1} = 1.16 (mg/mL) cm; ELP-2rSH3, ε^{-1} = 1.30 (mg/mL) cm; ELP-3rSH3, ε^{-1} = 1.65 (mg/mL) cm). ELP-s3 concentration was determined by measuring A260 and converting to protein concentrations using the empirically determined extinction coefficient of ε^{-1} = 4.28 (mg/mL) cm.

2.5. CFP assay

All CFP fluorescence measurements were taken using a BioTek Synergy 2 plate reader. Samples were excited at a wavelength of 435 nm and the fluorescence emission was measured at 485 nm.

2.6. Purification of ELP proteins through inverse transition cycling

All ELP-SH3 and ELP-s3 proteins were purified using at least two rounds of ITC. During each cycle, the protein pellet was concentrated during the resuspension step. Purified ELP-SH3 fusions were mixed with clarified lysate of the POI expression (Fig. 1A) and incubated at room temperature while rotating for 30 min. $(NH_4)_2SO_4$ concentration was increased dropwise until turbidity was observed, from a 3 M $(NH_4)_2SO_4$ stock solution. No more than 0.5 M $(NH_4)_2SO_4$ was added to each sample. Samples were gently mixed and centrifuged at 12,000 × g for 10 min at room temperature (Fig. 1B). The supernatant was removed and cold 1x PBS was added to the pellets. The pellet and buffer were mixed using gentle pipetting while incubating on ice for 30 min. After the aggregates were dislodged and dissolved into monomers, the sample was centrifuged at 12,000 × g for 5 min at 4 °C (Fig. 1C).

Purified ELP-s3 was added to resuspended samples and gently mixed using a pipette. The mixture was incubated on a rotator at room temperature for 30 min. $(NH_4)_2SO_4$ concentration was increased dropwise until turbid to a maximum concentration of 0.5 M $(NH_4)_2SO_4$. The turbid mixture was then centrifuged at 12,000 × g for 10 min at room temperature. The supernatant, which contains the purified protein, was aspirated and stored separately (Fig. 1E). The ELP-SH3 fusion/ELP-s3 pellet was resuspended for analysis.

2.7. Low pH release of strong ligand from SH3 domains

Strong ligand can be released from SH3 domains using a 0.1 M Sodium Citrate pH 4.7 (regeneration buffer). The ELP-SH3/ligand complex was aggregated using 0.5 M $(NH_4)_2SO_4$ and centrifuged at 12,000 × g for 10 min at room temperature. After discarding the supernatant, the pellet was resuspended in the regeneration buffer and incubated on ice for at least 10 min ensuring that the protein has been completely resuspended and deaggregated. The solution was then centrifuged at 12,000 × g for 5 min at 4 °C to remove insoluble aggregates. The resulting supernatant was separated, the
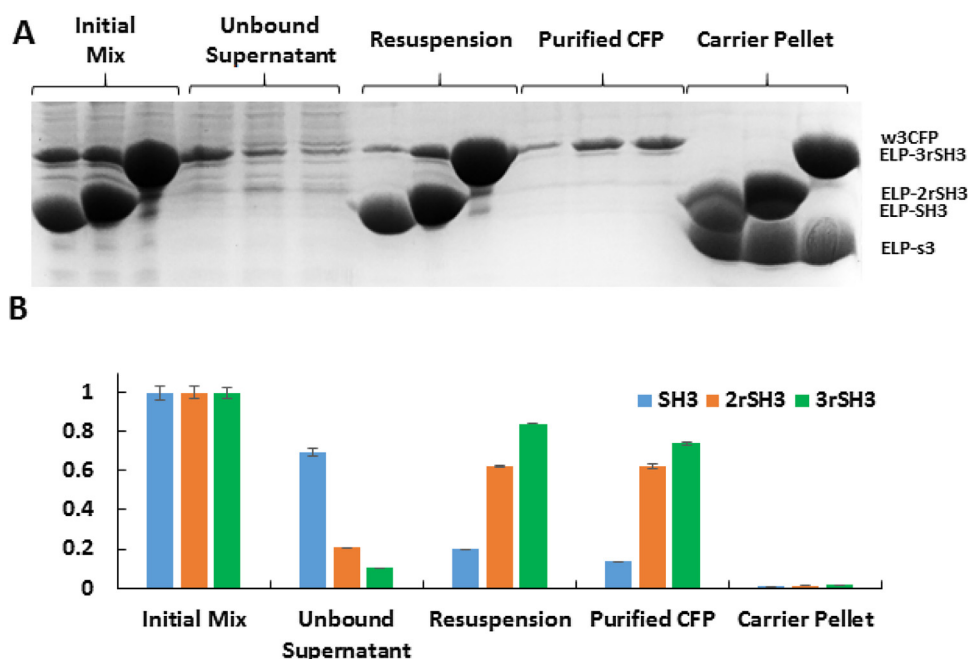
Fig. 2. Purification of w3CFP fusions. (A) Coomassie stained SDS-PAGE analysis of w3CFP pull-down using ELP-SH3, ELP-2rSH3, ELP-3rSH3 constructs and release of w3CFP from ELP-SH3/w3CFP using ELP-s3, (B) Normalized ECFP fluorescence of all purification steps.

$(NH_4)_2SO_4$ concentration was adjusted to 0.5 M, and allowed to come to room temperature. The turbid mixture was centrifuged at $12,000 \times g$ for 10 min at room temperature. The supernatant, which exclusively consists of SH3 ligand tagged protein, was separated from the pellet, which exclusively consists of the ELP-SH3. The supernatant was adjusted to a neutral pH using 1.5 M Tris-HCl pH 8.8. The resulting ELP-SH3 pellet was resuspended in 1x PBS pH 7.4 and used for a subsequent purification cycle.

3. Results and discussion

3.1. Production of weak ligand-tagged POIs and ELP-SH3 capture proteins

The two peptide ligands were chosen based not only on their prior use *in vivo* scaffolding applications (Dueber et al., 2009), but also on their adaptability as a multivalent design to increase binding specificity and affinity (Dueber et al., 2007; Sethi et al., 2011; Tomlinson et al., 2000). We chose three tandem repeats of the weak ligand as the binding motif for our EMAC purification scheme based on literature suggesting that this is the minimum number to provide good cooperative binding capabilities (Dueber et al., 2007). The purification tag used in this investigation consists of three tandem repeats of the weak ligand (w3) and a short non-interacting linker region serving as the N-terminus, in-frame fusion to the POI. Both POIs, CFP and MDH, used in this study showed minimal impact to expression when fused to the binding ligands, accounting greater than 20% of the total protein in the cell lysate (Fig. S1A).

Although the binding of SH3 domains to corresponding binding ligands has been demonstrated, there was uncertainty concerning the impact of ELP driven aggregation, solution conditions, and centrifugation on the SH3/ligand binding stability. In addition, it was uncertain whether a corresponding increase in the number of SH3 binding domains in the capture protein is necessary to ensure tight binding. To address this question, three different ELP fusions (ELP-SH3, ELP-2rSH3, and ELP-3rSH3) containing either 1, 2 or 3 tandem repeats of the SH3 domain, respectively, were constructed

for testing. All three fusions were highly expressed and were easily purified using two cycles of inverse phase transition (Fig. S1B). The high solubility of ELP-SH3 fusions even after multiple ITCs indicates high salt tolerance of SH3 domains and negligible premature aggregation, which could negatively impact SH3 functionality.

3.2. Purification of weak ligand-tagged POIs by ELP-SH3 fusions

Initially, an excess amount of ELP-SH3 fusion, a ratio of 5:1 to w3CFP, was used to ensure efficient protein capture. The feasibility of using ELP-SH3 fusions as the capture protein as w3CFP was specifically pulled out from the clarified *E. coli* cell lysate as indicated by SDS-PAGE (Fig. 2A). The step-by-step purification efficiencies were better quantified using CFP fluorescent measurements. The three different ELP fusions exhibited markedly different binding efficiencies with the 2rSH3 and 3rSH3 fusions captured over 70% of w3CFP compared to only 20% for ELP-SH3 (Fig. 2B).

These results suggest that multi-valent binding is essential in achieving the required high binding efficiency, however, the difference in capture efficiency is much greater between ELP-SH3 and ELP-2rSH3 and the affinity increase seems to diminish as the number of SH3 tandem repeats increases. It is interesting to note that the overall capture efficiency varied only slightly for 3rSH3 beyond a 3:1 ratio and never truly reached 100% (Fig. S2). This suggests that a large fraction of the w3CFP is likely truncated and cannot be sequestered by the ELP fusions.

The higher binding efficiency afforded by the multivalent interaction suggested that this strategy was an ideal platform for the purification of homomultimers. Previously, our lab has shown that only a small fraction of monomers in a multimeric structure must be bound to ELP in order to confer the reversible phase transition properties of ELP to a multimeric protein (Chen et al., 2015). To investigate this effect for EMAC, the w3 tag was fused to each subunit of a homodecameric MDH from *B. methanolicus* (Arfman et al., 1997). While the number of repeats of tandem SH3 domains fused to the ELP still had a large effect on the purification efficiency, the amount of ELP used to pull-out MDH could be reduced (Fig. 3A).
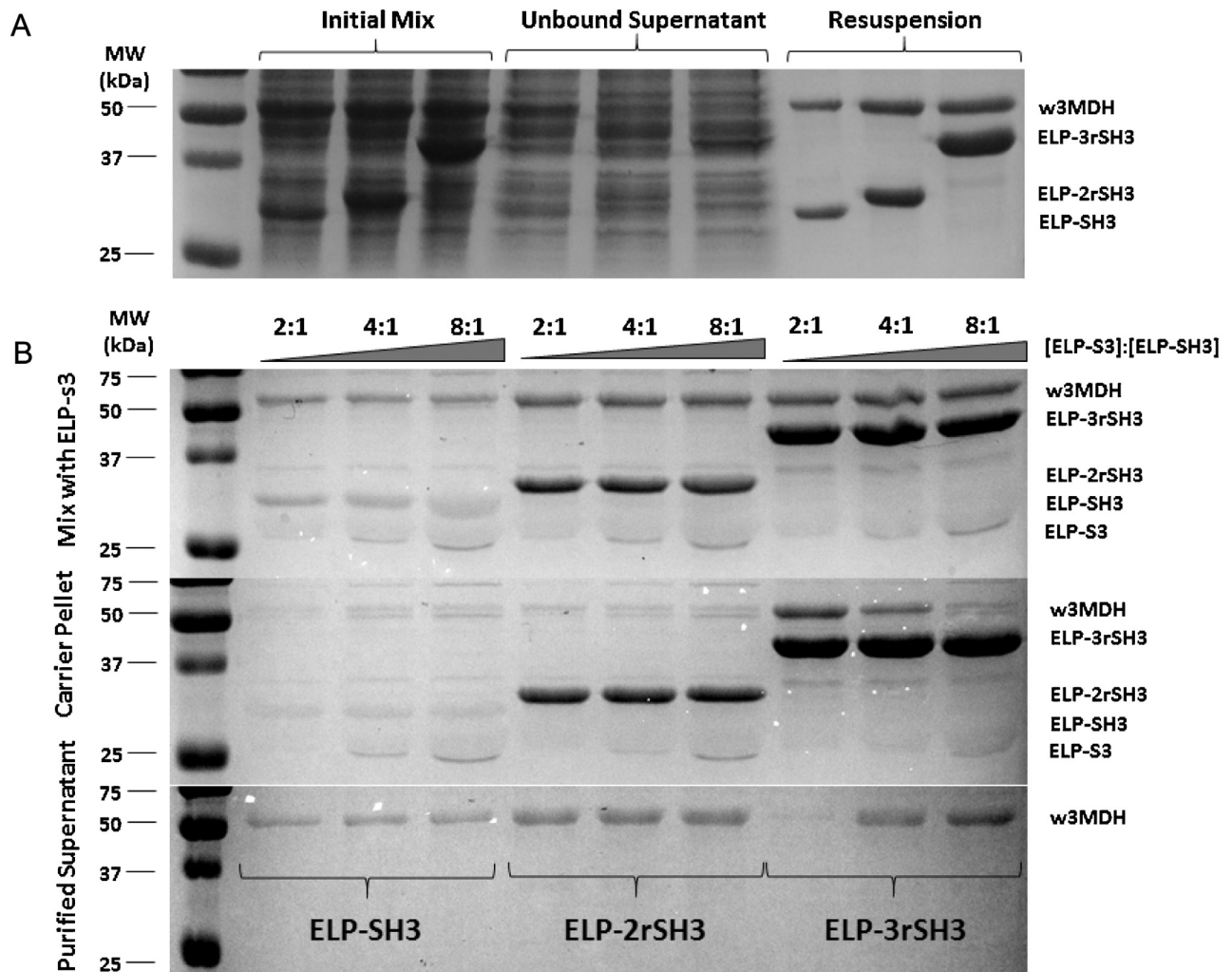
Fig. 3. Coomassie stained SDS-PAGE analysis of w3MDH purification. (A) Pull-down of w3MDH using ELP-SH3, ELP-2rSH3, ELP-3rSH3, (B) Elution of w3MDH using ELP-s3 from purified ELP-SH3/w3MDH complex.

Using a [ELP-SH3 fusion]:[w3MDH monomer] ratio of 2:1 (Fig. 3A), both ELP-2rSH3 and ELP-3rSH3 were able to pull-out almost all of w3MDH from the lysate while ELP-SH3 only captured about half of the w3MDH in the lysate.

3.3. Elution of bound POIs by competitive displacement

The 100-fold difference in binding affinity between the strong (s3) and w3 binding motifs provides a convenient way to separate the POI from the capture ELP fusions by competitive displacement. Elution of the POI is dependent on preferential binding of the SH3 domain to s3, causing the ELP-SH3 fusions to release the POI. Inducing ELP aggregation (Fig. 1F) will selectively remove the bound complexes from the POI (Fig. 1E).

To test this idea, ELP was fused to three tandem repeats of the strong ligand (ELP-s3) (Fig. S1B). Purified w3CFP/ELP-SH3 fusion complexes were mixed with ELP-s3 at a 2:1 [ELP-s3]:[ELP-SH3] ratio to sequester the ELP-SH3 fusions from w3CFP. All three ELP-SH3 fusions were effectively displaced by ELP-s3 (Fig. 2A), however, the overall yield was higher for the 2rSH3 and 3rSH3 fusions as the pull-down efficiency was better (Fig. 2B).

Much like the capture stages of the purification, the elution profiles for the decameric w3MDH were also different from that of the monomeric w3CFP. While both ELP-2rSH3 and ELP-3rSH3 were

effective in capturing w3MDH from lysate using a capture ratio of 2:1 [ELP]:[POI], eluting w3MDH from ELP-3rSH3 required a higher concentration of ELP-s3 than when ELP-2rSH3 was used. Effective elution from ELP-2rSH3 was achieved using a 2:1 ratio of [ELP-s3] to [ELP-SH3] as compared to a much higher 8:1 ratio for ELP-3rSH3 (Fig. 3B).
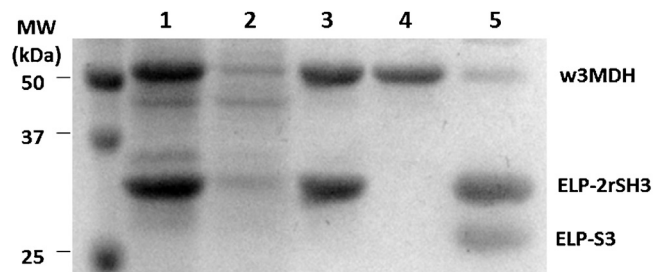
3.4. Optimization of w3MDH purification using ELP-2rSH3

For practical application, it is crucial to minimize the use of capturing ELP-SH3 fusion to reduce the amount of ELP-s3 needed to elute the POI. Despite similar w3MDH capture capabilities of ELP-2rSH3 and ELP-3rSH3, the considerably better elution profile for ELP-2rSH3 makes it more optimal to use ELP-2rSH3 to minimize the total amount of ELP needed.

To further optimize the capture, we investigated the effect of [ELP-2rSH3]:[w3MDH] on w3MDH recovery (Fig. 4A). Even a 1:1 [ELP-2rSH3]:[w3MDH] capture ratio still resulted in over 80% recovery, while the efficiency drops significantly to 60% for 1:1.5 [ELP-2rSH3]:[w3MDH] (Fig. 4B). From the result, the optimal capture ratio is likely somewhere between 2:1 and 1:1. We used a 1.5:1 [ELP-2rSH3]:[w3MDH] capture ratio and a 2:1 [ELP-s3]:[ELP-2rSH3] elution ratio in an optimized purification of w3MDH from cell, resulting in an overall recovery of 90% (Fig. 5).
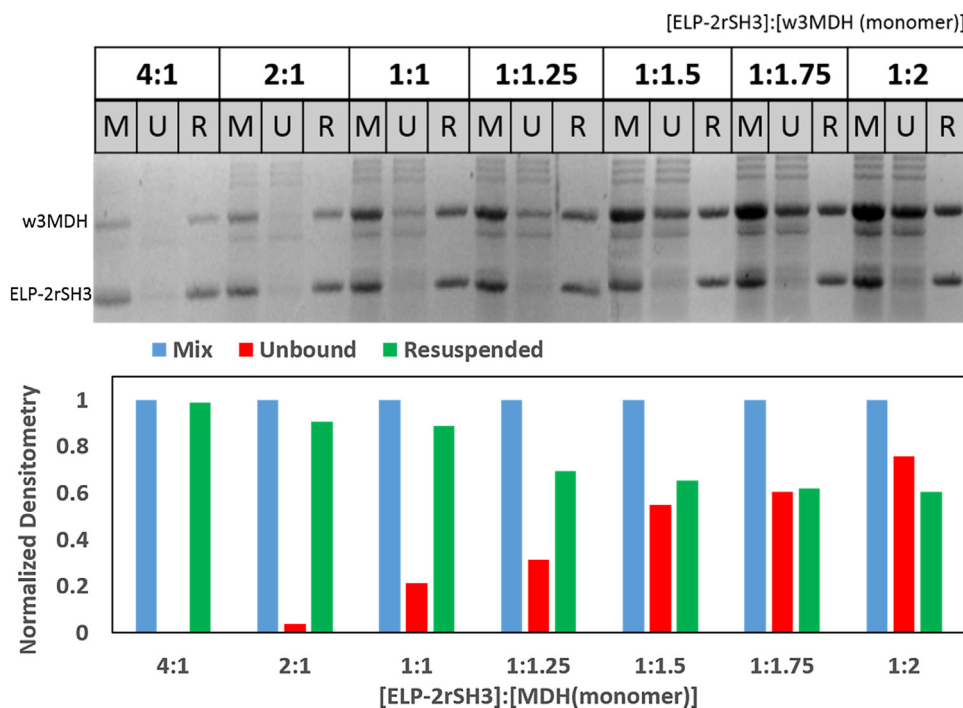
Fig. 4. w3MDH capture and elution optimization with ELP-2rSH3. (A) Coomassie stained SDS-PAGE analysis of w3MDH pull down using ELP-2rSH3 at various w3MDH: ELP-2rSH3 ratios. ELP-2rSH3 was kept constant at 10 μM while w3MDH (monomer) concentration was varied. M = Mix, U = Unbound, R = Resuspension (B) Normalized densitometry of the pull-down experiments. All w3MDH band intensities were normalized to the initial mixture w3MDH band intensity at each capture ratio.

Fig. 5. Coomassie stained SDS-PAGE of purification fractions of optimized purification of w3MDH using an ELP-2rSH3:w3MDH ration of 1.5:1. (1) Initial mix, (2) Unbound supernatant, (3) Resuspended ELP-2rSH3/w3MDH complex, (4) Released w3MDH, (5) Pelleted ELP-2rSH3/ELP-s3.

3.5. Recycling of ELP-SH3

We next investigated the possibility of recycling the ELP-SH3 capture domain for subsequent purification. The pH dependent nature of SH3/ligand binding provides a simple way to recycle the ELP-SH3 capture domain. After the target protein is successfully eluted from ELP-SH3 fusions, ELP-SH3 and strong ligand can be separated by incubating the complex in 0.1 M sodium citrate buffer pH 4.7 (regeneration buffer). To minimize protein degradation, the highest pH that adequately separates SH3 domains from the strong ligand should be used and we predict that this optimal pH will be different for every protein.

As a proof of concept, CFP genetically fused to the strong ligand (CFP-s1) was specifically pulled out of lysate using ELP-SH3. Subsequently, the resulting complex was resuspended in the regeneration buffer (Fig. 6). Triggering the aggregation of ELP-SH3 using ITC separates the two proteins from each other. On top of this, the recovered ELP-SH3 was reused for further pull-down of w3CFP from lysate, thus opening up the possibility of a recycle strategy. This pH triggered release has also been successful using ELP-2rSH3 and ELP-3rSH3 (data not shown). While pH can be used to directly elute POI from SH3 domains, this strategy would be highly dependent on the pH stability of target proteins. Although the current purification scheme makes it difficult to separate the capture domain and the displacement ligand since both are fused to ELP, this can be easily alleviated by replacing the ELP domain on the strong ligand with a different moiety such as the maltose binding domain.

4. Conclusion

While the SH3 domain and its corresponding binding ligands have been widely used for protein scaffolding, their use for high-affinity protein purification has never been explored. In this study, we exploit the 100-fold difference in affinity between the strong (s3) and weak peptide (w3) ligands for the SH3 domain as a simple non-chromatographic scheme for protein capture and elution. Incorporation of a thermally responsive ELP module to the SH3 domain enables easy recovery of target proteins by affinity precipitation and allows POI expression under optimal conditions. Although this approach works for the monomeric CFP, the overall purification scheme is better suited for multimeric proteins because multivalent binding makes the pull-down of POI easier without substantially hindering elution. In addition, the choice of using SH3 binding ligands, which are regularly found in across many species as recognition domains during signal transduction, gives credence to the fact that these binding ligands can function in a variety of media and in the presence of a variety of contaminants. Furthermore, although we chose to demonstrate this technology using only N-terminal fusions of the purification motifs, there is no reason a C-terminal fusion will function in a different fashion. Lastly, recycling of SH3 domains can be achieved by using a low pH regeneration buffer to allow the decoupling of SH3 domains from binding ligands, and this can potentially lower the overall amount of ELP-SH3 protein for purification.
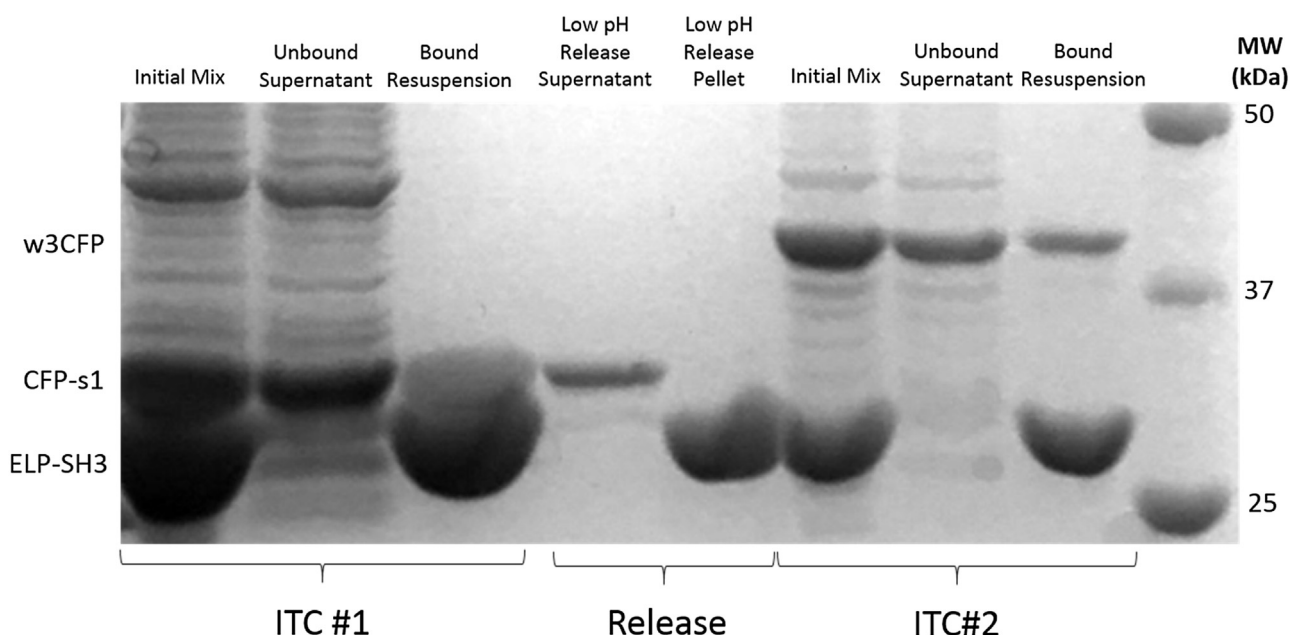
Fig. 6. Low pH release of binding ligands from ELP-SH3 domains. A regeneration buffer at pH 4.7 can be used to effectively separate strong ligand tagged proteins (CFP-s1) from ELP-SH3 domains. This regenerated ELP-SH3 can be used for further purification of w3CFP.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2016.07.016.

References

Arfman, N., Hektor, H.J., Bystrykh, L.V., Govorukhina, N.I., Dijkhuizen, L., Frank, J., 1997. Properties of an NAD(H)-containing methanol dehydrogenase and its activator protein from *Bacillus methanolicus*. Eur. J. Biochem. 244, 426–433.

Banki, M.R., Gerngross, T.U., Wood, D.W., 2005. Novel and economical purification of recombinant proteins: intein-mediated protein purification using in vivo polyhydroxybutyrate (PHB) matrix association. Protein Sci. 14, 1387–1395, http://dx.doi.org/10.1110/ps.041296305.

Barak, Y., Handelsman, T., Nakar, D., Mechaly, A., Lamed, R., Shoham, Y., Bayer, E.A., 2005. Matching fusion protein systems for affinity analysis of two interacting families of proteins: the cohesin-dockerin interaction. J. Mol. Recognit. 18, 491–501, http://dx.doi.org/10.1002/jmr.749.

Blackstock, D., Sun, Q., Chen, W., 2015. Fluorescent protein-based molecular beacons by zinc finger protein-guided assembly. Biotechnol. Bioeng. 112, 236–241, http://dx.doi.org/10.1002/bit.25441.

Bornhorst, J.A., Falke, J.J., 2000. Purification of proteins using polyhistidine affinity tags. Methods Enzymol. 326, 245–254.

Chen, Q., Sun, Q., Molino, N.M., Wang, S.-W., Boder, E.T., Chen, W., 2015. Sortase A-mediated multi-functionalization of protein nanoparticles. Chem. Commun.: Camb. 51, 12107–12110, http://dx.doi.org/10.1039/c5cc03769g.

Christensen, T., Amiram, M., Dagher, S., Trabbic-Carlson, K., Shamji, M.F., Setton, L.A., Chilkoti, A., 2009. Fusion order controls expression level and activity of elastin-like polypeptide fusion proteins. Protein Sci. 18, 1377–1387, http://dx.doi.org/10.1002/pro.157.

Christensen, T., Hassouneh, W., Trabbic-Carlson, K., Chilkoti, A., 2013. Predicting transition temperatures of elastin-like polypeptide fusion proteins. Biomacromolecules 14, 1514–1519, http://dx.doi.org/10.1021/bm400167h.

Dueber, J.E., Mirsky, E.A., Lim, W.A., 2007. Engineering synthetic signaling proteins with ultrasensitive input/output control. Nat. Biotechnol. 25, 660–662, http://dx.doi.org/10.1038/nbt1308.

Dueber, J.E., Wu, G.C., Malmirchegini, G.R., Moon, T.S., Petzold, C.J., Ullal, A.V., Prather, K.L., Keasling, J.D., 2009. Synthetic protein scaffolds provide modular control over metabolic flux. Nat. Biotechnol. 27, 753–759, http://dx.doi.org/10.1038/nbt.1557.

Floss, D.M., Mockey, M., Zanello, G., Brosson, D., Diogon, M., Frutos, R., Bruel, T., Rodrigues, V., Garzon, E., Chevaleyre, C., Berri, M., Salmon, H., Conrad, U., Dedieu, L., 2010. Expression and immunogenicity of the mycobacterial Ag85B/ESAT-6 antigens produced in transgenic plants by elastin-like peptide fusion strategy. J. Biomed. Biotechnol. 27434, 6, http://dx.doi.org/10.1155/2010/274346.

Harper, S., Speicher, D.W., 2011. Protein chromatography, methods in molecular biology. In: Clifton, N.J. (Ed.), Methods in Molecular Biology. Humana Press, Totowa, NJ, http://dx.doi.org/10.1007/978-1-60761-913-0.

Hassouneh, W., Christensen, T., Chilkoti, A., 2010. Elastin-like polypeptides as a purification tag for recombinant proteins. Curr. Protoc. Protein Sci. 11, http://dx.doi.org/10.1002/0471140864.ps0611s61 (Chapter 6, Unit 6).

Hober, S., Nord, K., Linhult, M., 2007. Protein A chromatography for antibody purification. J. Chromatogr. B. Anal. Technol. Biomed. Life Sci. 848, 40–47, http://dx.doi.org/10.1016/j.jchromb.2006.09.030.

Huse, K., Böhme, H.-J., Scholz, G.H., 2002. Purification of antibodies by affinity chromatography. J. Biochem. Biophys. Methods 51, 217–231.

Kostal, J., Mulchandani, A., Chen, W., 2001. Tunable biopolymers for heavy metal removal. Macromolecules 34, 2257–2261, http://dx.doi.org/10.1021/ma001973m.

Kostal, J., Mulchandani, A., Chen, W., 2004. Affinity purification of plasmid DNA by temperature-triggered precipitation. Biotechnol. Bioeng. 85, 293–297, http://dx.doi.org/10.1002/bit.10890.

Li, M.Z., Elledge, S.J., 2012. SLIC: a method for sequence- and ligation-independent cloning. Methods Mol. Biol. 852, 51–59, http://dx.doi.org/10.1007/978-1-61779-564-0_5.

Li, S.S.-C., 2005. Specificity and versatility of SH3 and other proline-recognition domains: structural basis and implications for cellular signal transduction. Biochem. J. 390, 641–653, http://dx.doi.org/10.1042/BJ20050411.

Liu, F., Chen, W., 2013. Engineering a recyclable elastin-like polypeptide capturing scaffold for non-chromatographic protein purification. Biotechnol. Prog. 29, 968–971, http://dx.doi.org/10.1002/btpr.1757.

Liu, F., Tsai, S.-L., Madan, B., Chen, W., 2012. Engineering a high-affinity scaffold for non-chromatographic protein purification via intein-mediated cleavage. Biotechnol. Bioeng. 109, 2829–2835, http://dx.doi.org/10.1002/bit.24545.

Madan, B., Chaudhary, G., Cramer, S.M., Chen, W., 2013. ELP-z and ELP-zz capturing scaffolds for the purification of immunoglobulins by affinity precipitation. J. Biotechnol. 163, 10–16, http://dx.doi.org/10.1016/j.jbiotec.2012.10.007.

Meyer, D.E., Chilkoti, A., 1999. Purification of recombinant proteins by fusion with thermally-responsive polypeptides. Nat. Biotechnol. 17, 1112–1115, http://dx.doi.org/10.1038/15100.

Meyer, D.E., Trabbic-Carlson, K., Chilkoti, A., 2001. Protein purification by fusion with an environmentally responsive elastin-like polypeptide: effect of polypeptide length on the purification of thioredoxin. Biotechnol. Prog. 17, 720–728, http://dx.doi.org/10.1021/bp010049o.

Nallamsetty, S., Waugh, D.S., 2007. A generic protocol for the expression and purification of recombinant proteins in *Escherichia coli* using a combinatorial His6-maltose binding protein fusion tag. Nat. Protoc. 2, 383–391, http://dx.doi.org/10.1038/nprot.2007.50.

Nguyen, J.T., Porter, M., Amoui, M., Miller, W.T., Zuckermann, R.N., Lim, W.A., 2000. Improving SH3 domain ligand selectivity using a non-natural scaffold. Chem. Biol. 7, 463–473.

Posern, G., Zheng, J., Knudsen, B.S., Kardinal, C., Müller, K.B., Voss, J., Shishido, T., Cowburn, D., Cheng, G., Wang, B., Kruh, G.D., Burrell, S.K., Jacobson, C.A., Lenz, D.M., Zamborelli, T.J., Adermann, K., Hanafusa, H., Feller, S.M., 1998. Development of highly selective SH3 binding peptides for Crk and CRKL which disrupt Crk-complexes with DOCK180, SoS and C3G. Oncogene 16, 1903–1912, http://dx.doi.org/10.1038/sj.onc.1201714.

Ramirez, M., Guan, D., Ugaz, V., Chen, Z., 2013. Intein-triggered artificial protein hydrogels that support the immobilization of bioactive proteins. J. Am. Chem. Soc. 135, 5290–5293.

Sallach, R.E., Conticello, V.P., Chaikof, E.L., 2009. Expression of a recombinant elastin-like protein in pichia pastoris. Biotechnol. Prog. 25, 1810–1818, http://dx.doi.org/10.1002/btpr.208.

Schipperus, R., Teeuwen, R.L.M., Werten, M.W.T., Eggink, G., de Wolf, F.A., 2009. Secreted production of an elastin-like polypeptide by *Pichia pastoris*. Appl. Microbiol. Biotechnol. 85, 293–301, http://dx.doi.org/10.1007/s00253-009-2082-9.

Sethi, A., Goldstein, B., Gnanakaran, S., 2011. Quantifying intramolecular binding in multivalent interactions: a structure-based synergistic study on Grb2-Sos1 complex. PLoS Comput. Biol. 7, e1002192, http://dx.doi.org/10.1371/journal.pcbi.1002192.

Sheth, R.D., Jin, M., Bhut, B.V., Li, Z., Chen, W., Cramer, S.M., 2014. Affinity precipitation of a monoclonal antibody from an industrial harvest feedstock using an ELP-Z stimuli responsive biopolymer. Biotechnol. Bioeng. 111, 1595–1603, http://dx.doi.org/10.1002/bit.25230.

Stiborova, H., Kostal, J., Mulchandani, A., Chen, W., 2003. One-step metal-affinity purification of histidine-tagged proteins by temperature-triggered precipitation. Biotechnol. Bioeng. 82, 605–611, http://dx.doi.org/10.1002/bit.10609.

Sun, Q., Madan, B., Tsai, S.-L., DeLisa, M.P., Chen, W., 2014. Creation of artificial cellulosomes on DNA scaffolds by zinc finger protein-guided assembly for efficient cellulose hydrolysis. Chem. Commun. 50, 1423–1425, http://dx.doi.org/10.1039/c3cc47215a.

Terpe, K., 2003. Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. Appl. Microbiol. Biotechnol. 60, 523–533, http://dx.doi.org/10.1007/s00253-002-1158-6.

Tomlinson, M.G., Lin, J., Weiss, A., 2000. Lymphocytes with a complex: adapter proteins in antigen receptor signaling. Immunol. Today 21, 584–591, http://dx.doi.org/10.1016/S0167-5699(00)01716-3.

Urry, D.W., 1997. Physical chemistry of biological free energy transduction as demonstrated by elastic protein-based polymers. J. Phys. Chem. B 101, 11007–11028, http://dx.doi.org/10.1021/jp972167t.