Biochemistry and Biotechnology Research
Vol. 4(3), pp. 48-54, August 2016
ISSN: 2354-2136
Full Length Research Paper

Production of a new construct using intein-elastin like fusion protein for simple and non-chromatographic purification of the recombinant protein

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Accepted 18 August, 2016

ABSTRACT

Intein (INT) or internal protein is a self-cleavage splicing element used for the protein purification and simple bioseparation. INT in fused form to chitin binding domain (CBD) produce an affinity protein purification method for the recombinant proteins. On the other hand elastin-like protein (ELP) is a synthetic biopolymer consisting of the pentapeptide repeats of VPGXG (X is any amino acid except proline). ELP is a thermal responsive polypeptide and contains a reversible transition stage. For each ELP molecule a transition temperature (Tt) is determined so that aggregation can take place after temperature increases while it will solubilize after a decrease of temperature from Tt. The focus of this work is ELP-fused INT sequence design for simple purification of the recombinant protein. In this work ELP_{10} (150 bp) was synthesized and cloned into pBluescript. Then Elp_{60} (900 bp) was produced using recursive directional ligation (RDL) method. Finally, ELP_{60} was subcloned into pTXB1 (an INT-CBD expression vector) and ELP_{60} expression was confirmed using SDS-PAGE method. Additionally INT-ELP_{60} fusion proteins were purified using two sequential steps of inverse transition cycle (ITC). The data was shown; ELP_{60} was expressed and successfully purified in one step and non-chromatographic method. We propose that constructed pTX-INT-ELP can be used for simple and easy purification of the recombinant proteins.

Keywords: Intein, elastin liked protein, expression, Inverse transition cycling, purification.

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Abbreviation: INT, Intein; **ELP,** elastin like protein; **ITC,** inverse transition cycle; **CBD,** chitin binding domain; **Tt,** transition temperature.

INTRODUCTION

High-throughput protein purification is a critical step in the recombinant protein production. In protein purification numerous ways could be employed however affinity chromatography is an easy method for the one-step purification (Cabrita et al., 2006; Gottstein and Forde, 2002; Hage, 2006). Peptide tag is a powerful tool for affinity chromatography and purification of the recombinant proteins. His-tag is a widely used affinity tag employed for purification of the recombinant proteins (Waugh, 2005; Arnau et al., 2006; Zhao et al., 2013). But in therapeutic-protein and peptide expression, removal of his-tag is necessary in the protein production - purification at gram quantities by employing a timely and cost-effective procedure (Arnau et al., 2006). For this propose, a few protein expression systems have been developed for easy and simple protein purification (Li, 2011; Terpe, 2003). Self-cleavage intein (INT) tag is a new strategy for the high-throughput recombinant protein purification that does not require recombinant endoproteases for removing the tag. INT or internal protein is a protein splicing element that can catalyze the specific splicing reactions without using a separate protease. After INT-catalyzed protein splicing, the ligation of the flanking exteins (external protein) produce a mature protein (Lichty et al., 2005; Fong et al., 2010; Elleuche and poggeler, 2010; Pezza et al., 2004;

Gogarten et al., 2002; Banki and Wood, 2005). On the other hand, elastin like polypeptide (ELP) is a synthetic, palindromic and toxic-free biocompatible polymer. ELP consists of the pentapeptide repeats of VPGXG (X = any amino acid except Pro). It is a thermally responsive polypeptide that undergoes a returnable phase transition. For each ELP molecule a transition temperature (Tt) is determined according to molecular weight, X residue, salt concentration and pH. ELP conformation is immediately converted to aggregate or solubilized after increase or decrease of temperature from Tt, respectively (Li et al., 2001; Park and Won, 2009; Hassouneh et al., 2010; Ribeiro et al., 2009). Based on this property, scientists have developed a column-free recombinant protein purification procedure termed inverse transition cycling (ITC). ITC is a non-chromatographic protein purification method that is technically simple, fast economical, and requires no specialized equipment or reagents. ITC is reversible and can be repeated; therefore, the purity of the ELP fusion protein can be increased using multiple rounds of ITC and target protein can be segregated from its ELP tag either using specific proteolytic digestion or INT self cleavage splicing (Banki et al., 2005; Meyer and Chilkoti, 1999; Simnick, 2007; Lim et al., 2007; Floss et al., 2010). Also, ELP polymer library can be produced by using recursive directional ligation (RDL) method. Synthesis of sequences encoding ELPs by RDL is performed using the repeated oligomerization of a monomer sequence that encodes the monomer ELP, typically 5–10 pentapeptides (75-150 bp) (Meyer and Chilkoti, 2002; McDaniel et al., 2010).

The aim of this study is a new construct production using intein-elastin like fusion protein for simple and non-chromatographic purification of the recombinant protein. For this purpose, cloning, expression and purification of ELP_{60} into pTXB1 were carried out.

EXPERIMENTAL

ELP oligomerization

ELP $[VPGV(G/A)G]_{10}$ (150 bp) monomer sequence encoding a 50 amino acid was synthesized and cloned into *EcoRV* site of pBluescript. For oligomerization and the next subcloning, *PflMI* and *BglI* restriction sites were designed at 5′ and 3′ ends of ELP_{10} sequence, respectively (Figure 1).

RDL method was used to produce five ELP polymer libraries. First, recombinant vector containing ELP_{10} (named pBlue-ELP_{10}) was digested with *PflMI* and *BglI* to excise the sequence encoding ELP_{10}. Also, pBlue-ELP_{10} was linearized with *PflMI* and enzymatically dephosphorylated with calf intestinal alkaline phosphatase (CIP). After cleanup, the sequence encoding ELP_{10} and linear pBlue-ELP_{10} were ligated and transformed into *E. coli* cell strain of XL1-Blue. Therefore, ELP_{20}, ELP_{30}, ELP_{40}, ELP_{50} and ELP_{60} were produced using 5 rounds of RDL (Figure 2). Successful oligomerization was confirmed using double-digestion of pBlue-ELP with *PflMI* and *BglI* and following gel electrophoresis. Finally, ELP_{60} sequence was confirmed by DNA sequencing.

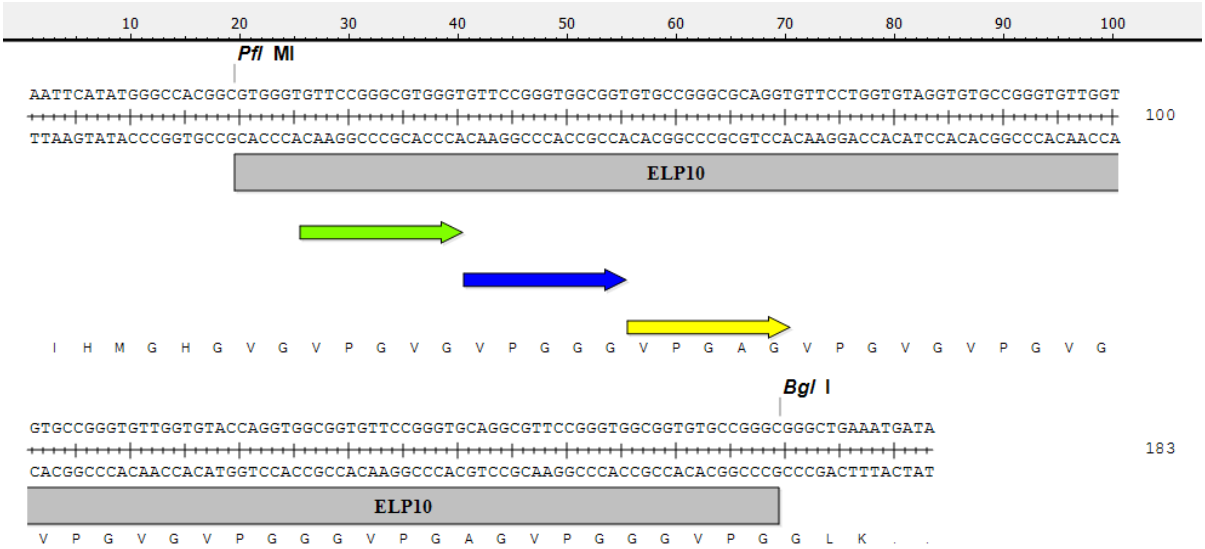

Figure 1. Nucleotide and amino acid sequences of ELP_{10}.

ELP_{60} cloning

An INT-CBD expression vector, pTXB1 (Biolabs, N6707) was used for ELP_{60} expression. For pTX-ELP_{60} production, ELP was amplified using forward (F-XB) and reverse (R-XB) primers containing *AgeI* and *BamHI* cut sites, respectively (Table 1). Because of the GC rich and palyndromic context of ELP sequence, PCR reaction was performed with the modified protocol, with 3 s annealing time at 60.3°C. Then, PCR product and pTXB1 vector were digested with *AgeI* and *BamHI*. After cleanup, ELP_{60} was ligated into the linearized pTXB1 and transformed into *E. coli*. The presence of ELP_{60} in pTX-ELP_{60} vector was confirmed using co-digestion with

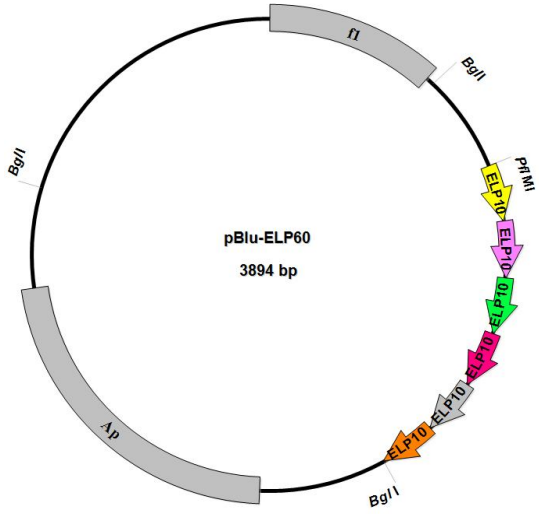

Figure 2. Overview of ELP oligomerization by RDL.

Table 1. Primers used for subcloning ELP60 into pTXB1.

Primer	Sequence	Size (bp)
F-XB	5′-GGTACCGGTCGAATTCATATGGGCCAC-3	27
R-XB	5′-GGAGGATCCAAAGCTTATCATTTCAGCC-3′	28

BamHI and *AgeI* enzymes.

ELP_{60} expression and purification

The produced expression plasmid, pTX-ELP_{60}, was transformed into susceptible *E. coli* strain ER2566. ER2566 is a preferred T7 expression host. Bacteria were grown in 5 ml of Luria-Bertani broth containing ampicillin (50 µg/ml) at 37°C. Bacteria were induced using 1 mM IPTG in the OD_{600} = 0.5. Purification of INT-ELP_{60} was performed by two rounds of the hot and cold spin (ITC method). For this purpose, the *E. coli* lysate (recombinant ER2566) was prepared after 10 cycles of sonication in phosphate buffer saline (PBS) with 1M NaCl and centrifugation at 12000×g for 15 min. Then, lysate was heated at 37°C about 30 min and pellet containing ELP was isolated using centrifugation (hot spin). In the cold spin step precipitated ELP was cooled about 30 min to 4°C. This procedure was repeated in two rounds and the sample collection was completed for SDS-PAGE analysis.

RESULTS

ELP_{60} oligomerization and construction of expression vector

ELP_{60} synthesis was performed using RDL method as described by Meyer and Chilkoti (2002). After 5 rounds of RDL, five libraries were produced from pBlue-ELP_{10} monomer. Produced vectors were analyzed on agarose gel 1% (Figure 3A). After each step of RDL, a 150 bp fragment was added into ELP sequence and the result was confirmed using double digestion with *PfimI* and *BglI* and observation of 300, 450, 600, 750 and 900 bp fragments on agarose gel (Figure 3B). New vector (pBlue-ELP_{60}) was used for subcloning of the ELP_{60} sequence into pTXB1.

For this, ELP_{60} encoding sequence was amplified using F-XB and R-XB primers. The 900 bp PCR fragment was digested by *AgeI* and *BamHI* restriction enzymes (Figure 4A) and cloned within linearized pTXB1 vector containing the T7 promoter (Figure 4B). *AgeI* and *BamHI* co-digestion of recombinant vector confirmed the presence of ELP_{60} in pTX-ELP_{60} (Figure 4C). Therefore, ELP_{60} was fused to downstream of INT in expression vector pTXB1.

ELP_{60} production and purification

The ER2566 cells containing the pTX-ELP_{60} were induced for INT-ELP_{60} fusion protein expression. Because the molecular weight of ELP_{60} and INT was 32 kDa and 28 kDa, respectively, the INT-ELP60 fusion protein was observed about 60 kDa on the SDS-PAGE gel (Figure 5A). ELP_{60} in fusion form (INT-ELP_{60}) was purified using two rounds of ITC (hot spin and cold spin) with about 90% purity. After the hot spin or cold spin in any round of ITC, the sample was analyzed by SDS-PAGE technique (Figure 5B). The macroscopic reverse

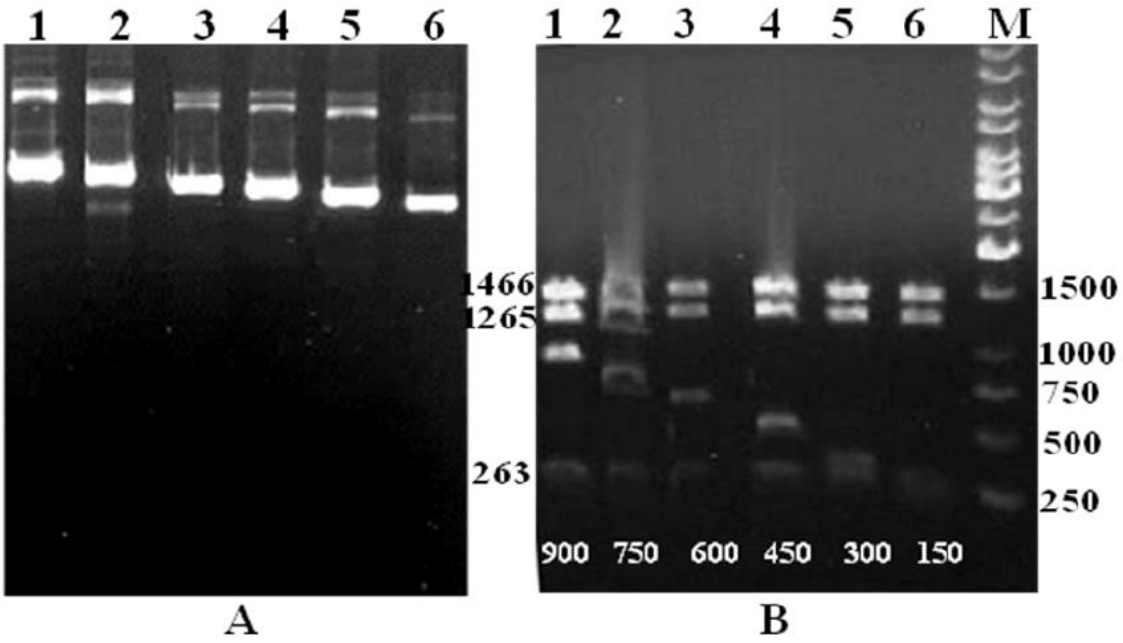

Figure 3. Electrophoresis analysis of ELP polymer libraries produced using RDL method. A) Undigested plasmids and B) *PflMI*/*BglI* co-digested plasmids; (lane 1) plasmid containing ELP_{60}, (lane 2) plasmid containing ELP_{50}, (lane 3) plasmid containing ELP_{40}, (lane 4) plasmid containing ELP_{30}, (lane 5) plasmid containing ELP_{20}, (lane 6) plasmid containing ELP_{10}, (M) DNA marker (1kb).

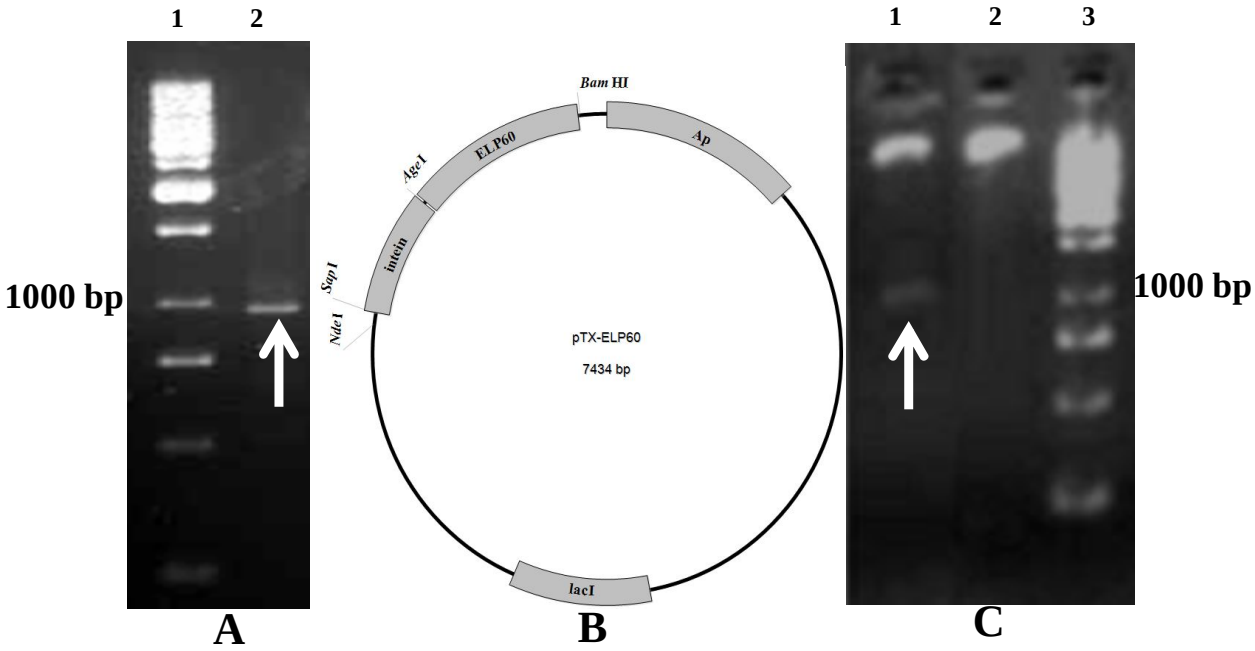

Figure 4. A) *AgeI* and *BamHI* co-digested ELP_{60}; (lane 1) 1 kb DNA ladder, (lane 2) digested and purified PCR product. B) Schematic construction of the pTX-ELP_{60}. C) *AgeI* and *BamHI* co-digested pTX-ELP_{60}; (lane 1) digested plasmid, (lane 2) undigested plasmid, (lane 3) 1 kb DNA ladder.

aggregation of insoluble ELP_{60} was observed in the first round of ITC (Figure 5C). These results confirmed successful function of the produced ELP_{60}.

DISCUSSION

Elastin-derived polypeptides have numerous applications

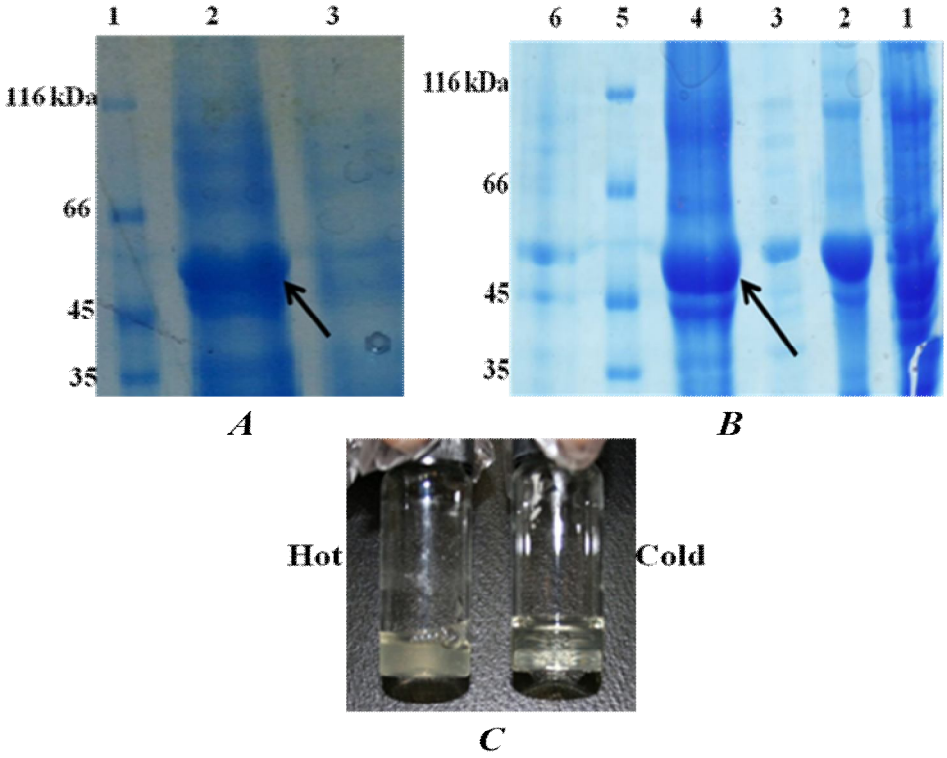

Figure 5. A) Expression of INT- ELP_{60} fusion protein; (lane 1) protein size marker, (lane 2) ER2566 *E. coli* containing pTX-ELP_{60} at 4 h after induction and (lane 3) before induction. B) Purification analysis of INT-ELP_{60} using two repeat ITC; (lane 1) The soluble cell lysate, (lane 2) the re-suspended pellet from the second ITC, (lane 3) the supernatant from the second ITC, (lane 4) the re-suspended pellet from the first ITC, (lane 5) the molecular size marker (lane 6) the supernatant from the first ITC. C) The macroscopic image of INT-ELP_{60} reverses aggregation using ITC.

in biotechnology and biomedicine (Chow et al., 2008; MacEwan and Chilkoti, 2010; Zhang et al., 2015) such as controlled release of drugs (Walkera et al., 2012; MacEwan and Chilkoti, 2014), tissue engineering (Nettles et al., 2010; Annabi et al., 2013) and recombinant protein purification (Hassouneh et al., 2010; Ribeiro et al., 2009; Banki et al., 2005; Meyer and Chilkoti, 1999). Purification of recombinant proteins is a difficult process involving sequential chromatography steps that are different from one protein to another protein. Recombinant techniques that are used for pharmaceutical protein purification are exponentially increasing (Sodoyer, 2004; Durocher and Butler, 2009). Inexpensive and efficient protein purification methods are necessary to determine the expression and purification strategies. The affinity peptide tag approach is a novel strategy for protein purification that can achieve a high purity of protein using a single-step column. Nevertheless, in some cases such as pharmaceutical protein purification, the presence of the affinity tag is unwanted and tag deletion is very important (Arnau et al., 2006; Charlton and Zachariou, 2011). Therefore, selection of an efficient strategy is essential for high throughput purification of recombinant protein without additional amino acid in target protein. The use of INT-fused elastin like polypeptide is a new method for one step and non-chromatographic protein purification. Simplicity and reduced cost are two advantages of this strategy. In this method, target protein is purified using hot and cold spins according to the lower critical solution temperature (LCST) of ELP that is named the transition temperature (Tt). In conclusion, using ITC procedure and after INT-thiol cleavage, target protein could be produce without additional chromatography (Meyer and Chilkoti, 2002; Fong and Wood, 2010; Tian and Sun, 2011; Fong et al., 2009; Christensen et al., 2007).

On the other hand, large-scale economic and material cost comparisons have shown INT-ELP fusion tag is better than other affinity tags such as His-tag. So, the thermal responsive and self-cleaving aggregation tag technology reduces the material cost up to 11-fold in comparison with the His-tag purification method (Banki and Wood, 2005).

The INT-ELP_{60} fusion tag constructed in this work can be used as the affinity tag to non-chromatographic recombinant protein purification. This strategy is a smart choice for the production of recombinant pharmaceuticals,

the same as previous investigations (Banki et al., 2005; Fong and Wood, 2010; Tian and Sun, 2011; Fong et al., 2009; Christensen et al., 2007; Wu et al., 2006; Hassouneh et al., 2012). The role of the ELP in the fusion protein on the expression level and the purified protein efficiency was reported by Hassouneh et al. (2012). In this report, protein-ELP or ELP-protein orientation has shown different translation or expression. The protein-ELP expression was higher than ELP-protein. Herein, ELP_{60} was used in downstream of the multiple cloning sites (MCS) and the INT sequence (MCS-INT-ELP). Also, the ITC purification confirmed reversible aggregation of produced ELP_{60} fused to INT tag from pTX-INT-ELP_{60} system. In ITC, NaCl is the best salt for ELP precipitation. So that, up to 3M NaCl is used in the first ITC round to trigger isothermal transition of the ELP. Salt concentration decreased in the subsequent round (Hassouneh et al., 2012). Here, the phosphate buffer saline (PBS) with 1M NaCl was used for two ITC rounds.

Conclusions

It is shown the INT-ELP_{60} fusion tag is a functional self-cleaving affinity tag that can be effective in protein isolation and purification. Wood et al first used ELP_{110}-INT for bioseparation of recombinant protein [19, 37]. Here, however we have produced INT-ELP orientation and reduced ELP length to 60, which can be used for recombinant pharmaceutical protein purification.

ACKNOWLEDGMENTS

We would like to thank the research council of Malek Ashtar University of Technology for the financial support of this investigation.

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Citation: Masoumeh L, Mehdi Z, Reza SA, Morteza RS, 2016. Production of a new construct using intein-elastin like fusion protein for simple and non-chromatographic purification of the recombinant protein. Biochem Biotechnol Res, 4(3): 48-54.