

Research Article

Chromatography Purification of The Promising Biomaterial: Elastin-Like Protein Genetically Expressed in *Escherichia coli*

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Abstract

Inverse transition cycling is the most popular purification method for elastin-like polypeptides, a promising biomaterial with high biocompatibility and functional properties. However, this mean depends on the high concentration of elastin-like protein in solution, large molecule weight or high-salt concentration. To overcome the defects of inverse transition cycling, we successfully developed a chromatographic method to effectively purify the recombinant elastin-like proteins expressed in *Escherichia coli* BL21(DE3), with formulation of MGRS ((VPGVG)10S)5 and a transition temperature higher than 40°C. Ion-exchange chromatography was carried out to remove the most charged proteins from cell lysis prior to hydrophobic proteins were isolated using reverse phase chromatography. A maximum quantity of 303.92 ± 10.17 mg per liter of culture was obtained for the recombinant elastin-like proteins and the purity of the recombinant ELP50 was more than 95%.

Keywords: Elastin; Expression; Inverse Transition Cycles; Reverse Phase Chromatography; Purification

Abbreviations:

| | | |
|------|---|---------------------------------------|
| M | : | Methionine |
| G | : | Glycine |
| R | : | Arginine |
| S | : | Serine |
| V | : | Valine |
| P | : | Proline |
| A | : | Alanine |
| ELPs | : | Elastin-Like Polypeptides or Proteins |
| Tt | : | Temperature Transition |
| RDL | : | Recursive Directional Ligation |
| ITC | : | Inverse Transition Cycles |

| | | |
|----------------|---|---|
| SDS | : | Sodium Dodecyl Sulfonate |
| IPTG | : | Isopropylβ-D-Thiogal-Actopyranoside |
| SDS-PAGE | : | Sodium Dodecyl Sulfonate Polyacrylamide Gel Electrophoresis |
| CV | : | Column Volumes |
| CIP | : | Cleaning in Place |
| PBS | : | Phosphate- Buffered Saline Solution |
| RPC | : | Reverse Phase Chromatography |
| MW | : | Molecular Weight |
| <i>E. coli</i> | : | <i>Escherichia coli</i> |

Introduction

Elastin-Like Polypeptides (ELPs), a frontier biomaterial with high biocompatibility and biodegradability, is a kind of artificial polymers comprising of repeats of VPGXG or VAPGXG (X termed as guest residue can be any amino acids other than

P) derived from the natural elastin of cattle or human. Urry et al. firstly synthesized ELPs and reported the fundamental characterization of inverse temperature transition (T_t) for ELPs [1]. ELPs are soluble at temperature below the T_t and insoluble when the temperature exceeds T_t . Benefited from Recursive Directional Ligation (RDL) [2], a molecular technique to develop protein-based molecules through gene recombination with precise sequence control, various ELPs were created with keen interest for basic/practical research in regenerative medicine and tissue engineering, such as treating articular cartilage damage, ocular tissue engineering or mimicking extracellular matrix scaffolds [3-5]. Drug and therapeutic peptide delivery was another attractive concern of ELPs based on temperature transition characterization which could be triggered by local hyperthermia to actively target to damaged tissue or troublesome solid tumors [6].

Most of the reported ELPs were purified using Inverse Transition Cycles (ITC) based on temperature regulation to precipitate target ELPs at the temperature $\geq T_t$ and throw away the contaminating proteins back and forth. Comparison to chromatographic purification approaches for protein, ITC is highly regarded because it's a non-chromatographic method without requirement of special equipment to implement and easy to scale-up. ITC seems to be a good alternative means for ELPs purification. However, the reversible phase transition behavior of ELPs greatly depends on large molecular weight, high concentration of ELPs in the solution, composition of the amino acids constituting ELPs, or high salt concentration [7-9]. To purify ELPs using ITC, high concentration of ELPs up to 20-100 mg/mL was required without other auxiliary condition while the recombinant ELPs could be produced with an efficiency of 500 mg/L of the bacterial culture [3]. Some reports showed that ITC could be fulfilled at lower concentration of ELPs with help of higher salt (e.g. Sodium chloride) concentration to decrease the T_t [9]. However, regular high-salt stimulation is harmful for centrifuge and the purified protein must be desalted by dialysis or gel chromatography. Further, it has been reported many promising ELPs with $T_t \geq 40^\circ\text{C}$ [2,10-12]. When the T_t is higher than 40°C , the working temperature must be maintained higher than T_t during centrifugation and solid-liquid separation, but that is difficult to operate and likely results in shortening machine lives as the working temperature of general centrifuge is no more than 40°C . In these cases, a new method is imperative to develop for the ELPs with $T_t \geq 40^\circ\text{C}$ even for some ELPs with lower T_t . Here, we reported an effective purification method based on chromatography.

Materials and Methods

Reagents

SP Sepharose XL resin, Q-sepharose fast flow resin, Source 3 ORPC resin and Sephacryl S-100 HR were the products

of GE Healthcare (Pittsburgh, USA). Protein marker (Thermo Scientific™ PageRuler Prestained Protein Ladder, Art. No. 26616), Isopropyl β -D-1-Thiogalacto- Pyranoside (IPTG), tris base, Sodium Dodecyl Sulfonate (SDS) and other analytical grade reagents was purchased from Xi'an Zhou Dingguo Biotech.Co.Ltd, China.

Vector and Strain

The strain used as expression host cells is *Escherichia coli* (*E. coli*) BL21(DE3) retained by the lab of tissue engineering, college of life sciences, Northwest University, China. The gene coding for elastin-like protein ELP50 with formulation of MGRS ((VPGVG)₁₀S)₅ was obtained using RDL technique (Figure 1) [2] and the expression vector pET28a (+)-ELP50 was constructed and analyzed by routine molecular methods [13].

Expression of Recombinant ELP50

A single colony of *E. coli* strain BL21 (DE3) transformed by pET28-ELP50 was selected and cultured overnight at 200 rpm and 37°C in a rotary shaker in 50 mL LB medium (1% NaCl, 1% tryptone, 0.5% yeast extract) with addition of 50 $\mu\text{g/mL}$ kanamycin. Thereafter, the overnight culture was inoculated into 950 mL TB medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol) with 50 $\mu\text{g/mL}$ kanamycin and subpackaged into twenty 500 mL Erlenmeyer flasks with working volume of 100 mL. The cells were cultivated at 37°C and 200 rpm. Isopropyl β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 100 $\mu\text{g/mL}$ to induce the recombinant ELP50 expression while the cell density reached to OD₆₀₀ value up to 0.6, meanwhile the culture temperature was down regulated to 30°C and the rotate speed was increased to 250 rpm. After 4 h IPTG-induced expression, the culture was collected for purification as well as aliquot for sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of recombinant ELP50

Purification of recombinant ELP50 was performed on the AKTA Explorer100 chromatographic system (Amersham Pharmacia Biotech, USA) with the ultraviolet detection system.

Cell lysis:

The culture was centrifuged at 10000 rpm for 15min to harvest transgenic BL21 (DE3) cells. After washing twice with 20 mM Tris-HCl (pH8.0), per gram of the wet cell pellet was added 10 mL 20 mM Tris-HCl (pH8.0) supplemented with 0.5 mg/mL lysozyme and 0.1 mg/mL DNaseI. The cell suspension was cooled and completely lysed by sonication at 4°C with 15 s pulses-10 s break operation. Insoluble debris was removed from total cell lysis by centrifugation at 10000 rpm for 30 min. The pH value of the supernatant was adjusted to 3~4 using HCl with gentle agitation. The solution was centrifuged again at 10000 rpm for 30 min, the supernatant was collected

and the pH value was regulated to 8~10 using 1 M Tris-HCl.

Cation exchange chromatography:

After 1.5 Column Volumes (CV) of wash with buffer B (20 mM phosphate buffer, 0.75 M NaCl, pH5.7, PB), the column was equilibrated with 2CV of buffer A (20 mM PB, pH5.7) and adjusted the detector to zero. The cleared lysate was loaded onto SP Sepharose XL resin and flow washing was conducted with buffer A until the absorbance decreased to the baseline, the flow-through fraction containing the target ELP50 was collected and the pH value was regulated to 8.0 with 1 M Tris base. After 1.5CV of contaminants elution with buffer B, the process of Cleaning in Place (CIP) was carried out according to the operation manual provided by manufacturer. Flow velocity was 30 mL/min duration the whole chromatographic process.

Anion exchange chromatography:

Q-sepharose fast flow resin was washed with 1.5 CV of buffer D (20 mM Tris-HCl, 0.75 M NaCl, pH8.0), then the column was equilibrated with 2 CV of buffer C (20 mM Tris-HCl, pH8.0) buffer and adjusted the detector to zero. The pH8.0 flow-through solution from cation exchange chromatography was loaded and washed with buffer C until the absorbance dropped to the baseline, the flow-through fraction containing the target ELP50 was collected. After 1.5 CV of contaminants elution with buffer D, the CIP process was implemented as above. Flow velocity was 30 mL/min in whole process.

Reverse phase chromatography (RPC):

Buffer E (20 mM Tris-HCl, 70% ethanol, pH8.0) was employed to wash the Source 30RPC resin prior to column equilibrium with 2 CV of buffer C (20 mM Tris-HCl, pH8.0) and zero regulation of detector. The flow-through fraction collected from anion exchange chromatography was loaded onto the RPC resin and the absorbance was modulated to baseline with buffer C. After contaminants elution with 1.5 CV of 55% buffer E, the target protein ELP50 was eluted with 2 CV of 70% buffer E and collected. The CIP process was performed subsequently after contaminants elution with 2CV of 100% buffer E. Flow velocity was 30 mL/min.

Gel chromatography:

After pretreatment and zero-regulation with flow washing of 1.5 CV of buffer F (20 mM PB, 125 mM NaCl, pH7.0), the solution collected from RPC was loaded onto the gel chromatography column and eluted with buffer G, the fraction containing the target protein ELP50 was collected. Then the column was treated with CIP protocol.

Finally, the solution containing ELP50 was dialyzed against ultrapure water at 4°C in dialysis membranes with a 4 kDa cut off (Spectra Por7, MWCO1000) for 48 h with intermittent water

change. After dialysis, samples were lyophilized.

All purification steps were monitored by SDS-PAGE according to standard protocol. The content and yield coefficient of ELP50 were automatically calculated by machine based on peak area.

MALDI-TOF-MS Analysis

The molecular weight of purified ELP50 was determined on an AXIMA Performance instrument (Shimadzu, Tokyo, Japan). The data acquisition was implemented in positive ion linear mode with a nitrogen laser of 98. The matrix is 10 mg/mL α -cyano-4-hydroxy-cinnamic acid dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid. Two milligrams of purified recombinant ELP50 was dissolved in 100 μ L deionized water and 1 μ L of this sample was spotted on to a stainless target plate, after then 1 μ L matrix solution was mixed with sample and drying at room temperature for analysis.

The experiments from ELP50 expression to MALDI-TOF-MS Analysis were repeated 3 times.

Thermal turbidimetry

It was defined as the temperature at which the derivative of the OD350-versus- temperature curve reached its maximum [3]. Lyophilized recombinant ELP50 was respectively dissolved in double distilled water and phosphate-buffered saline solution (PBS, NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, pH 7.4) at a concentration of 700 μ g/mL. The solution of ELP50 was placed into the 96-well plate with the volume of 100 μ L per well. It were determined by measuring the turbidity at 350 nm between 0°C and 50°C at 1°C/5 min scan rate, Data were collected on a Synergy™ 2 Multi-Mode microplate reader (BioTek, Vermont, USA).

Results and Discussion

The expression of ELP50

In our previous work, the expression vector pET28a (+)-ELP50 of elastin-like protein ELP50 with formulation of MGRS ((VPGVG)10S)5 was constructed in which the amino acids R and S at N-terminal of ELP50 was derived from the recognition sequence of *Bg*III meanwhile the hybridized recognition sequence GGATCT of *Bg*III and *Bam*HI introduced an additional S residue per 10 repeats of VPGVG when ELP-encoding gene was elongated by RDL strategy (Figure. 1). Before production and purification of ELP50, three repeats of preparative experiments were carried out in 500 mL shake flasks with 100 mL TB medium for ELP50 expression. A decrease in the culture temperature from 37°C to 30°C after IPTG induction was adjusted to benefit the production and accumulation of the recombinant proteins. Using these conditions, $27.8\text{g} \pm 3.5$ fresh biomass was harvested from 1 L

culture solution after 4 h of IPTG induction. The SDS-PAGE analysis indicated that the gene coding for ELP50 could be expressed effectively when the BL21 (DE3) cells transformed by pET28a (+)-ELP50 was induced 4 h with IPTG (Figure 2, lane 1). Duration the induction, the recombinant ELP50 was accumulated in soluble form rather than inclusion body within the cytoplasm of host cells (Figure 2, lane 2), which has been determined in our previous work that the recombinant ELP50 was accumulated as soluble protein in *E. coli* BL21 (DE3) [13].

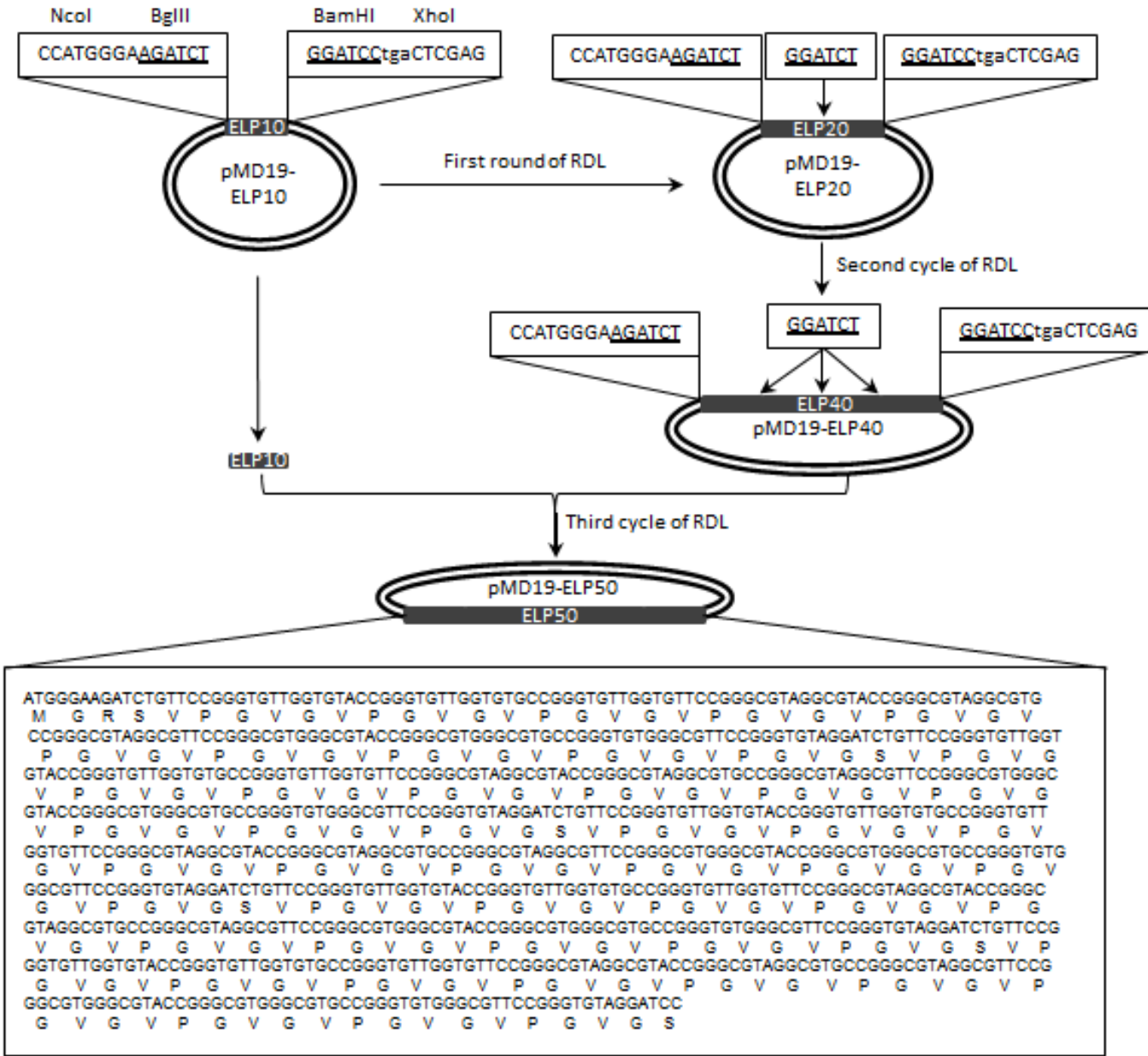


Figure 1: The recombinant ELP-multimer-coding gene was elongated with RDL strategy.

Chromatographic purification of ELP50

The harvested cells were suspended in 20 mM Tris-HCl (pH8.0) and lysed by sonication on ice bath. The cell debris along with insoluble proteins was precipitated from the cytoplasmic soluble fraction through centrifugation as the target ELP50 was soluble in cell lysis (Figure 2, lanes 2). The soluble fraction was then loaded onto cation exchange chromatographic column of SP Sepharose XL resin which was washed with buffer A and the flow-through fraction was collected. The chromatographic analysis indicated that target ELP50 existed in the flow-through fraction P1 (Figure 3A) and visualized by SDS-PAGE (Figure 2, lane 3). In this step, 4448.97 ± 240.98 mg protein including 906.47 ± 16.70 mg ELP50 was collected from the initial total protein (Table 1). These results showed the recombinant ELP50 could not be absorbed onto the resin through cationic exchange. The pH value of the solution containing ELP50 collected from cation exchange chromatography was regulated to 8.0 with 1M Tris base and uploaded onto anion exchange chromatography of Q-sepharose fast flow resin and washed with buffer C, by which the flow-through fraction contained 740.89 ± 23.06 mg ELP50 was collected and SDS-PAGE analysis indicated that the portion of P1 flow-through fraction (Figure 3B, the lane 4 of Figure 2 and Table 1).

| The collected fraction | Volume (mL) | Total proteins (mg) | ELP50 (mg) | Yield coefficient (%) |
|--------------------------------|--------------------|----------------------|--------------------|-----------------------|
| Cation exchange chromatography | 273.67 ± 25.70 | 4448.97 ± 240.98 | 906.47 ± 16.70 | 90.66 ± 0.85 |
| Anion exchange chromatography | 271.67 ± 20.84 | 935.96 ± 45.81 | 740.89 ± 23.06 | 74.08 ± 0.92 |
| Reverse phase chromatography | 109.67 ± 4.51 | 446.44 ± 20.16 | 402.03 ± 14.05 | 40.20 ± 0.75 |
| Gel chromatography | 273.33 ± 18.04 | 317.91 ± 10.91 | 303.92 ± 10.17 | 30.39 ± 0.52 |

Table 1: Purification effect of chromatographic manipulation.

The flow-through fraction collected from anion exchange chromatography was loaded onto the RPC resin. After contaminants elution with 1.5 CV of 55% buffer E, the column was eluted with 2 CV of 70% buffer E, the on-line monitoring (Figure 3C) showed the target protein ELP50 existed in the second eluting peak (Figure 3 P2) and was visualized by SDS-PAGE (Figure 2, lane 5). The elution buffer contained 402.03 ± 14.05 mg ELP50 corresponding to the second eluting peak was collected and loaded onto the gel chromatography column with which the buffer was replaced by PBS. SDS-PAGE analysis (Figure 2, lane 6) and on-line monitoring (Figure 3D) indicated that the prepared ELP50 has high purity $\geq 95\%$. The results of chromatographic purification revealed that the recombinant ELP50 could not bind onto the cationic or anionic column through ion-exchange while lots of charged contaminating proteins was exchanged onto the resin and then was eluted as impurity. These results were corresponding to the amino acid composition of recombinant ELP50. ELP50 was genetically constructed and composed of the repeats of VPGVG, within which the sequence was lack of charged amino acid residues. The predominant residues were V, P and G which contributed to the hydrophobicity of ELP50. However, when the RPC was carried out, the proteins lack of charged residues including ELP50 were absorbed onto the immobile phase and some polar components were washed away using strong-polar buffer C. Further, some weak-polar proteins were eluted away by 55% of buffer E. The target ELP50 could be isolated from other polypeptides and was collected when the RPC column was eluted with 70% buffer E (Figure 3 C). These results indicated that the ion-exchange especially anionic exchange was valuable for the purification of recombinant ELP50

as it could effectively have eliminated the charged peptides and other charged impurities although they could not absorb ELP50 directly. As for RPC, the locking phase is hydrophobic and can absorb various nonpolar proteins via hydrophobic interactions. When the RPC column was eluted with weak polar mobile phase of buffer F, proteins as well as other components with different hydrophobicity were eluted from the RPC resin successively and separated effectively.

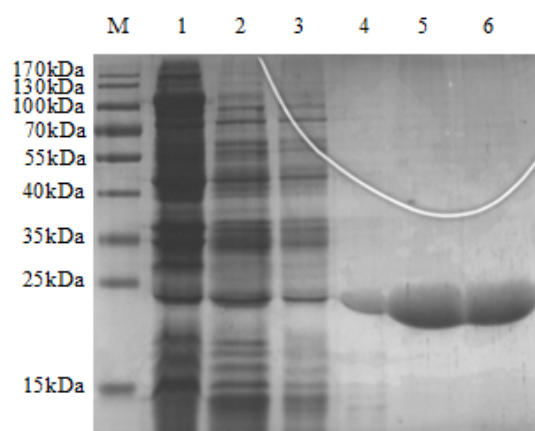
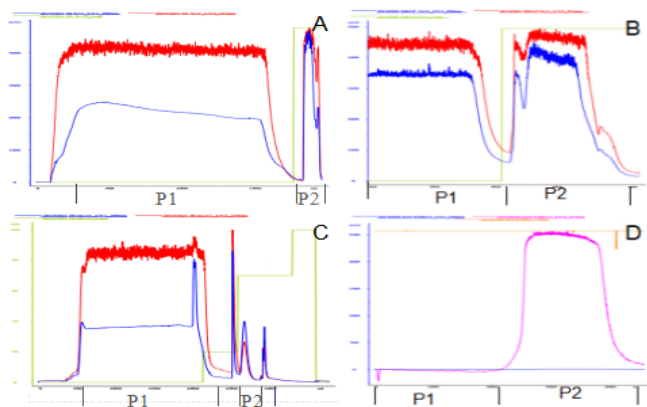


Figure 2: Separation efficiency of ELP50 visualized by SDS-PAGE. All samples of protein were derived from fermentation culture. M: protein marker; lane1: total protein; lane2: the supernatant of cell lysis; lane3: Flow-through fraction of cation exchange chromatography; lane4: Flow-through fraction of anion exchange chromatography; lane5: Separation efficiency on reverse phase chromatographic resin; lane6: Separation efficiency on gel resin.



Figures 3(A-D): On-line monitoring showed the separation efficiency of ELP50. A: Separation efficiency on cation exchange resin; B: Separation efficiency on anion exchange resin; C: Separation efficiency on reverse phase chromatographic resin; D: Separation efficiency on gel resin; P1: flow-through fraction; P2: eluted fraction.

The buffer system of collected solution containing ELP50 was changed into PB through gel chromatography (Figure 3 D) and the desalinated solution containing ELP50 was lyophilized and a maximum quantity of 303.92 ± 10.17 mg per liter of culture was obtained for ELP50 (n=3). MALDITOF-MS Analysis showed the molecular weight (MW) of purified ELP50 was 21046.22 Da (Figure 4). This value was in accordance with the theoretical MW of ELP50 although was smaller than the calculated values of 23.7kDa derived from the migration distance, which might result from the structure of ELP50 and the electrophoresis system.

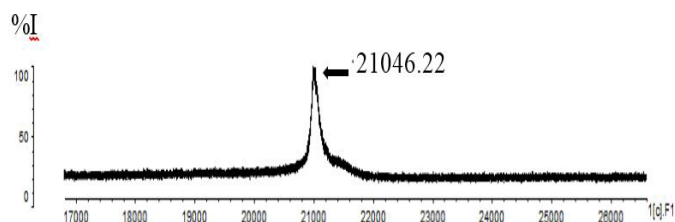


Figure 4: Molecular weight analysis of recombinant ELP50.

The inverse Transition Temperature (T_t) of the purified ELP50 was measured as thermal-sensitive characteristic was the most attractive property of elastin-like proteins. When the temperature is lower than T_t , ELPs dissolves in solution. On the contrary, ELPs will aggregate when the temperature is higher than the T_t and can be precipitated by centrifugation. This property possesses obvious superiority for protein purification because unwished peptides will have retained in the supernatant while ELPs aggregate to precipitate at the temperature higher than T_t , or some heat-labile proteins cannot redissolve at the temperature lower than the T_t of ELPs. For the recombinant ELP50, the T_t was $\approx 41^\circ\text{C}$ in PBS buffer

and $\approx 45.09^\circ\text{C}$ in deionized water with the concentration of $700 \mu\text{g/mL}$ (Figure 5). The phase transition property of ELP50 was in accordance with that ELPs with $T_t \geq 40^\circ\text{C}$ [8]. The recombinant ELP50 in cell lysis condensed 20 folds did not aggregate when the temperature was set at 45°C until the temperature was increased up to 60°C and the phase transition property could not be observed after 3 runs of ITC (data was not showed). The ITC manipulation at 60°C was difficult to carry out as not only the operating temperature range (-20 – 40°C) of centrifuge used in laboratory or industry, but also ELPs would re-dissolve very quickly once the temperature is lower than T_t due to the transition of ELPs from soluble to insoluble form in a very narrow range of temperatures [14]. Furthermore, the ELPs aggregation at the temperature $\geq T_t$ not only depends on the hydrophobic interaction of peptides which will result in co-aggregation of contaminant peptides. In order to obtain ELPs with high purity from fermented culture for biomedical application, it's essential to develop effective purification method for ELPs.

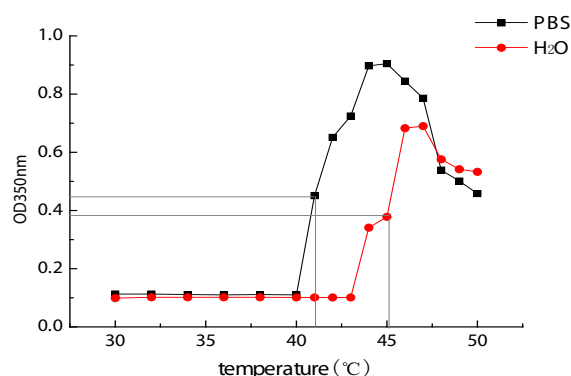


Figure 5: ITC analysis of ELP50 with the concentration of $700 \mu\text{g/mL}$.

Conclusion

We successfully employed chromatography displacing ITC to effectively separate the recombinant ELP50 with $T_t \geq 40^\circ\text{C}$ from the cell culture of engineered *E. coli*, in which ion-exchange chromatography was carried out to remove the most charged proteins and other polar impurity from cell lysis and hydrophobic proteins were separated using RPC. A maximum quantity of 303.92 ± 10.17 mg per liter of culture was obtained for ELP50 (n = 3) and the purity of the recombinant ELP50 was more than 95%.

Declaration of interest

All authors have nothing to disclose.

Submission Declaration and Verification

All authors approved to submit this manuscript to your journal for publication consideration and promised that the results were our initial work and not under consideration for publication elsewhere.

Author Contributions

Hongmin Li and Jihong Cui designed the experiments and wrote the manuscript. Yun mao, Sili Wei and Shasha Yang finished the purification experiments, wrote the draft and created the figures. Qi Wei cloned the plasmid and fulfilled the expression of ELP50. X Xie provided language help. The work was performed in the lab of FL Chen. All authors read and approved the final manuscript.

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