Expression and purification of a cationic peptide hydrogelator in *Escherichia coli*

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Abstract

Hydrogel is a well-known material widely used in various biomedical applications. In this study, POP1, a cationic peptide, was designed to self-assembly in response to change in environmental pH, subsequently affording a hydrogel. Expression of a small cationic peptide in bacteria is known to be challenging due to its toxicity and difficulty in purification. Here, the recombinant gene of POP1 was cloned into a modified pET19b. Double-digestion with restriction enzymes and DNA sequencing revealed successful cloning. As expected, the growth curve of the induced host cells, *E. coli* BL21(DE3), showed mild toxicity. Therefore, various expression conditions were assessed to optimize peptide production. The results of peptide expression were monitored by acetic-urea PAGE. To isolate POP1 from bacterial proteins, during sonication step, pH of a solution was adjusted according to pI of POP1. High performance liquid chromatography (HPLC) equipped with C18 reversed-phase column was performed to purify the recombinant peptide. The mass of POP1 was confirmed by electrospray ionization mass spectrometry. In conclusion, this is a pilot study for production of peptide hydrogelator that was successfully expressed and purified without fusion protein in a common bacterial strain.

Introduction

Hydrogel is a hydrophilic material constructed either by covalent or physical cross-linking of its polymeric constituents, which can be synthetic or natural. Peptide-based hydrogel has gained more attention due to their biocompatibility and biodegradability[1]. In general, peptide hydrogel is designed to fold into an amphipathic secondary structure and further self-assemble to form hydrogel. Stimuli-responsive hydrogelation are directed by amino acid sequence and composition. For example, introduction of charged amino acid residue can control pH-responsiveness while modulation of hydrophobic content plays a role in temperature-responsiveness[2]. For peptide production, since chemical peptide synthesis is not environmental friendly, bacterial expression is an alternative option to produce high quantity of peptide hydrogelator. However, small amphipathic cationic peptides generally exhibit antibacterial activity causing inner and outer membrane disruption against both Gram-positive and Gram-negative bacteria[3,4]. In addition, small peptides are susceptible to degradation by bacterial protease. As a result, the expression and the purification of these peptides are challenging. To aid in purification, co-expression of the peptide with some fusion protein has been reported to improve the yield[5]. However, this adds extra steps to protein production process as fusion protein must later be removed. Our laboratory is interested in developing self-assembly polypeptide-based hydrogel for biomedical and agricultural applications. In this study, POP1, a cationic 66-amino-acid peptide, is designed *de novo* to act as hydrogelator under

alkaline pH. Here, we aimed to express POP1 using *E. coli* BL21(DE3) system under T7 promoter without any fusion protein.

Methodology

Construction of expression vector

To construct the expression vector encoding POP1 peptide, we followed a method reported by Farmer and Kiick[6]. Briefly, for seamless cloning, a pET-19b plasmid was modified by site-directed mutagenesis to remove *Sap*I restriction site. Subsequently, a concatemerized products encoding for POP1 was inserted between *Nco*I and *Bam*HI restriction site of the modified pET-19b. This plasmid was transformed into *E. coli* DH5α using electroporation method. After culturing overnight, ampicillin-resistant colonies were selected for plasmid extraction by alkaline lysis miniprep. The isolated plasmid was initially examined by a digestion reaction, containing *Nco*I and *Bam*HI, followed by agarose gel electrophoresis. Then, the nucleotide sequence was confirmed by DNA sequencing (Macrogen, Korea).

Optimization of protein expression

To monitor the growth curve, the validated plasmid was introduced into *E. coli* BL21(DE3) by heat shock method. An inoculant in LB broth at the ratio of 1 to 100 (v/v) was incubated at 37°C while shaking at 220 rpm. After 3.5 h (OD_{600} ~0.6-0.8), IPTG was added to a final concentration of 1 mM followed by incubation at 37°C while shaking at 220 rpm. The culture was collected to monitor OD_{600} every 30 min. To produce protein, the plasmid was transformed into *E. coli* BL21(DE3)pLysS by electroporation method. Here, two tubes of culture were prepared where each tube contained 200 µL of the inoculant mixed with 20 mL of LB broth. Each tube was separately incubated until OD_{600} equal to 0.8 and 2.0, respectively, before addition of IPTG to a final concentration of 1 mM. The incubation was then continued at 37°C. Here, 1 mL of bacterial culture was collected before and after induction at 2, 4, 6 and 24 h to measure OD_{600}. After centrifugation at 12,000 rpm for 5 min, the cell pellet was sonicated in sample buffer (3 M urea, 5% (v/v) acetic acid, 10% (v/v) glycerol and 0.1% (v/v) methylene blue) at 20% amplitude for 1 min. The cell lysates were analyzed by acetic-urea polyacrylamide gel electrophoresis (AU-PAGE). Separation was performed at 150 volts for 100 min on a 15% polyacrylamide gel, prepared in the presence of 6 M urea, with 15% (v/v) acetic acid as running buffer. The protein was visualized by staining with 0.1% amido black solution. The same experiment was also repeated with 20°C-incubation after induction to investigate the effect of incubation temperature.

Optimization of recombinant peptide purification

An inoculant in LB broth at the ratio of 1 to 100 (v/v) was incubated at 37°C while shaking at 220 rpm until OD_{600} equal to 2.0. Then, IPTG was added to a final concentration of 1 mM followed by incubation at 37°C. After 24 h, the cell pellet was collected and resuspended in either 0.1% trichloroacetic acid (TFA) or buffer pH 8 (50 mM Tris-HCl and 1mM EDTA) before pulse-sonication at 30% amplitude at a pulse rate of 10-s on and 10-s off for 15 min followed by centrifugation at 12,000 rpm for 15 minutes. The pellet obtained from sonication with buffer pH 8 was further sonicated in 0.1% TFA at 30% amplitude as previously mentioned for 15 min. All the collected supernatants and pellets were lyophilized to fine powder. Each sample powder was dissolved in 8 M urea before analysis by AU-PAGE.

To remove bacterial proteins, pellet of IPTG-induced culture was sonicated in buffer pH 8 containing 0.5% Triton X-100 for 30 min. After centrifugation at 12,000 rpm for 30 min, the cell pellet was sonicated in buffer pH 8 for 10 min. This step was repeated once followed by centrifugation at 12,000 rpm for 30 min. The pellet was resuspended in 0.1% TFA and was sonicated for another 30 min. After centrifugation, the supernatant was lyophilized to fine powder, which later was dissolved in 0.1% TFA for purification. High performance liquid

chromatography (HPLC), equipped with C18 reversed-phase column, was performed to purify POP1, with a linear gradient of 0-100% solvent B over 50 min at a flow rate of 1 mL/min, where solvent A and B were 0.1% TFA in water and 0.1% TFA in 9:1 ratio of acetonitrile to water (v/v), respectively. The peptide elution was monitored at 220 nm. The molecular mass was identified by the Agilent 6420 Triple Quadrupole LC/MS System (Agilent, USA).

Results and Discussion

POP1, a 66-residue cationic peptide, was *de novo* designed to fold into 6-stranded ß-sheets connected by five ß-bulge turns. The sequence of POP1 is $MVK[(VK)_3VPDGT(KV)_3KPDGK]_2(VK)_3VPDGT(KV)_4$. Each ß-strand consists of alternating valine and lysine residues, a pattern with high ß-sheet propensity[7]. At physiological pH, POP1 should adopt random coil structure due to the electrostatic repulsion between protonated ε-amino group of lysyl side chain on the opposite ß-strand. Once the environmental pH changes from neutral to alkaline, this event alleviates the repulsive interaction leading to hydrophobic collapse of valine residues. As a result, folding of POP1 is triggered and hydrogelation occurs by self-assembly of peptide molecules to from fibrillar network. Similar pH-responsive folding and self-assembly mechanism was proposed for other peptide hydrogelators reported in literature and the concept was adopted to guide our design of POP1[3,5,7].

Cationic peptides with amphipathic helical or ß-sheet structure has been reported to introduce pores on bacterial membrane[4]. The examples include LL-37, defensin, and protegrin. Cationic peptide hydrogelators has also been reported to exhibit antibacterial activity when used as hydrogel scaffold[8]. As predicted, toxicity of POP1 to bacterial host cells was confirmed when optical density at 600 nm of transformed BL21(DE3) cultures was measured (**Figure 1A**). The growth rate of non-induced and induced bacterial culture was initially comparable after induction with 1 mM IPTG. However, 1-h after induction, the induced culture had lower growth rate than the non-induced culture even though the cell density of the induced culture gradually increased over time. This result suggested that POP1 exhibits mild toxicity to BL21(DE3).

Expression of toxic peptide is typically challenging, particularly for a small cationic peptide like POP1. To overcome the toxic response, either induction at high cell density or incubation at low temperature was recommended. Here, to optimize protocol for peptide production, *E. coli* strain was switched to BL21(DE3)pLysS to prevent leaky expression. Then, we compared the expression level between the induction at optical density of 0.8 and that of 2.0 both at 20°C and 37°C. To monitor peptide production, the cell pellet was collected at 2, 4, 6 and 24 h after induction with 1 mM IPTG. AU-PAGE was adopted to detect the presence of POP1 since Tris-glycine and Tris-tricine SDS-PAGE failed to detect the presence of a low molecular weight cationic peptide. Histone was used as a protein marker with lowest molecular weight of ~ 11.3 kDa. **Figure 1B** indicated the presence of protein (arrow) with molecular weight that is approximate to POP1, which is 7.15 kDa, when expression was induced at OD_{600} = 0.8. Interestingly, this band was not clearly observed in cell lysates of other conditions i.e. induction at OD_{600}= 2.0 and incubation at 20°C (data not shown). As a result, the optimal condition for expression was induction at OD_{600} ~ 0.8 with 2 h of incubation at 37°C.

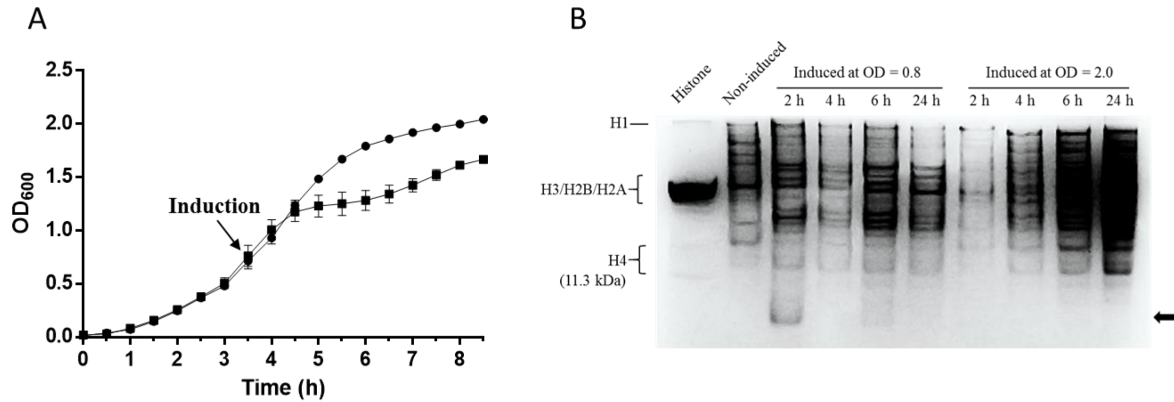

Figure 1. A) Growth curve of BL21(DE3) after induction with 1 mM IPTG. Arrow indicates the point of induction. OD_{600} of non-induced (•) and induced (■) culture was monitored every 30 min. The error bars represent the standard deviation of three independent experiments. B) AU-PAGE of cell lysate after induction at 37°C. Histone was used as a reference marker. Arrow indicates the presence of proteins with molecular weight that was approximate to that of POP1.

Before purification by chromatography technique, the bacterial proteins must be removed to improve separation efficiency. POP1, a lysine-rice peptide, has calculated pI of 10.48 with ~36% lysine and ~36% valine contents. According to the design, we anticipated that at basic pH, lysyl side chain would be deprotonated, which reduces its solubility leading to peptide precipitation. On the other hands, at acidic pH, POP1 should remain unstructured and freely dissolve in solution due to the repulsion from protonated lysine residues. With this hypothesis, repeated homogenization in alkaline buffer followed by that in acidic buffer should efficiently remove bacterial proteins. To test this hypothesis, cell pellet was either directly sonicated in 0.1%TFA or sequentially sonicated in buffer pH 8 followed by 0.1% TFA as illustrated in **Figure 2A**. AU-PAGE in **Figure 2B** revealed that the peptide with Mw ~ 7 kDa (arrow) was observed in the supernatant both from sonication in 0.1% TFA (S) and sequential sonication in the buffer pH 8 followed by 0.1% TFA (S2). As predicted, this band was only slightly observed in acid-insoluble protein (P1), basic-soluble protein (S1) and basic-insoluble protein sonicated in 0.1% TFA (P2). This result proves our hypothesis and the buffer pH 8 was sufficient to precipitate POP1 from solution, which imply that pK_a of lysyl side chain was perturbed. In this study, we decide to adopt sequential sonication method to maximize bacterial protein removal even though sonication of cell pellet in 0.1% TFA (S) or sequential sonication in buffer pH 8 followed by 0.1% TFA (S2) shows no difference in protein pattern in AU-PAGE.

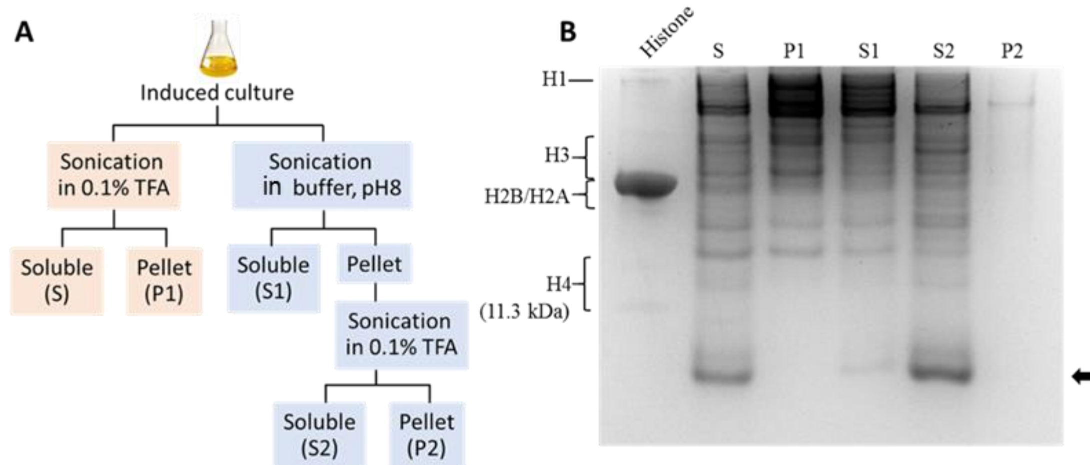

Figure 2.A) A schematic of sonication steps of cell lysates in acidic and alkaline solutions. B) AU-PAGE of cell lysate after sonication at different pH. Histone was used as a reference marker.

Purification of peptide and small protein typically employs reversed-phase HPLC (RP-HPLC). To monitor the elution pattern, the crude peptide solution was subjected to 30-kDa MWCO filter to further remove bacterial protein. The flow-through was analyzed by reversed-phase HPLC using linear gradient of 0 – 60% solvent B in 30 min, **Figure 3A**. The ESI-MS chromatogram indicated that the peptide eluted at ~ 29 min was POP1. The observed m/z values were corresponding to the calculated m/z including 1,789.73, 1,431.98, 1,193.49, 1,023.13, 895.37, 795.99, 716.49 and 651.45 which are +4, +5, +6, +7, +8, +9, +10 and +11 charge state, respectively, **Figure 3B**. This HPLC gradient was adopted for routine purification of POP1, which currently being characterized for folding and self-assembly mechanism.

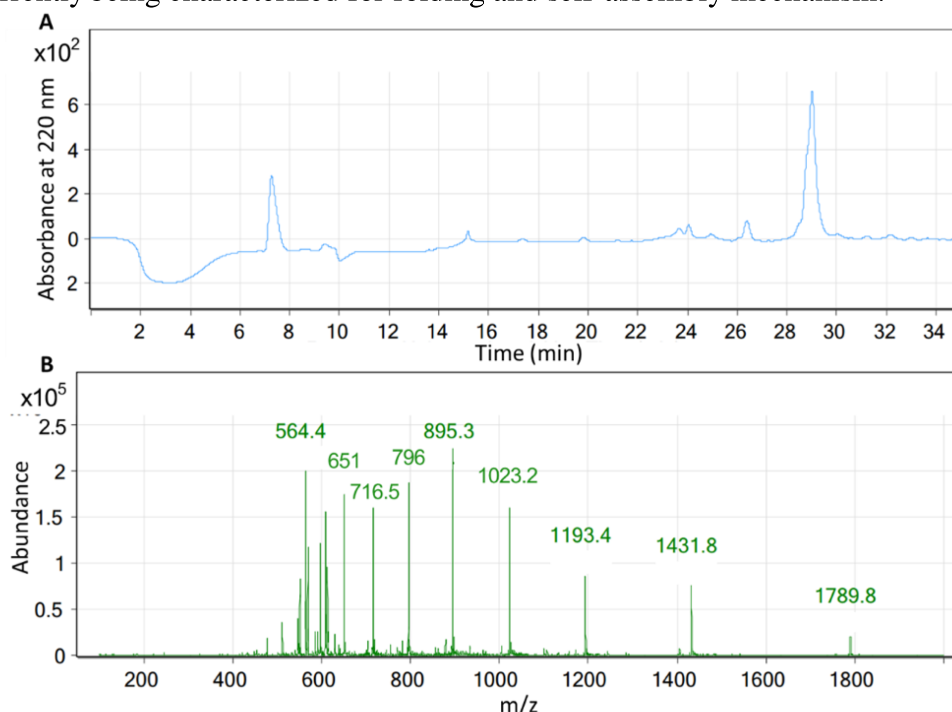

Figure 3. A) HPLC chromatogram of crude polypeptide. B) ESI-MS chromatogram of HPLC elution at ~29 min.

Conclusion

POP1 is a small cationic peptide that was designed to fold into 6-stranded ß-sheet connected by ß-bulge turns. Folding and self-assembly of POP1 can be triggered by change in environmental pH to afford hydrogel. In this work, *POP1* was cloned into a modified pET19-b to express in BL21(DE3)pLys using T7 promotor expression system. Despite the toxicity to host cell, POP1 was successfully expressed by induction at 37°C. Partial removal of bacterial proteins was accomplished by sonication in basic buffer followed by sonication in 0.1% TFA. POP1 was further purified by RP-HPLC and its identity was confirmed by ESI-MS. In conclusion, we successfully expressed and purified POP1, a cationic peptide hydrogelator, from *E. coli* without any fusion protein.

References

1. Tan H, Marra KG. Materials. 2010;3:1746-1767.
2. Schloss AC, Williams DM, Regan LJ. Adv Exp Med Biol. 2016;940:167-177.
3. Salick DA, Kretsinger JK, Pochan DJ, Schneider JP. J Am Chem Soc. 2007;129(47):14793-14799.
4. Bahar AA, Ren D. Pharmaceuticals. 2013;6(12):1543-1575.
5. Sonmez C, Nagy KJ, Schneider JP. Biomaterials. 2015;37:62-72.
6. Farmer RS, Kiick KL. Biomolecules. 2005;6:1531-1539
7. Schneider JP, Pochan DJ, Ozbas B, Rajagopal K, Pakstis L, Kretsinger J. J. Am. Chem. Soc. 2002;124(50):15030-15037.
8. Omardien S, Brul S, Zaat SAJ. Front Cell Dev Biol. 2016;4:111.

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