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Expression and Purification of an Antimicrobial Peptide by Fusion with Elastin-like Polypeptides in *Escherichia coli*

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Abstract Different carrier molecules have been fused to antimicrobial polypeptides (AMPs) to facilitate recombinant protein expression and purification. Some of them have improved the stability of AMPs and reduced the toxicity to host cells, but most current strategies still have some problems to be solved such as poor yield, low purity, high expense, time-consumption, and difficulty in scaling-up. Here, we introduced the elastin-like polypeptides (ELPs) as a fusion partner to express an antimicrobial polypeptide halocidin18 (Hal18). By the reversible soluble–insoluble phase transition, 69 mg of the fusion protein were purified from 1 l of culture medium with the purity of nearly 95%. After cleavage with hydroxylamine, the ELP's tag was easily separated from Hal18 in the next round of inverse transition cycle and Hal18 (1.7 mg, ~1.9 kDa) was mainly found in the supernatant with a recovery of about 47% and purity of 60%. Antimicrobial activity showed that Hal18 had strong antimicrobial activity against *Escherichia coli* and *Micrococcus luteus* but weak activity against *Pichia pastoris*.

Keywords Antimicrobial polypides · Elastin-like polypeptide · Soluble expression · Halocidin · Fusion protein

Introduction

Antimicrobial peptides (AMPs) are amphiphilic, positively charged molecules, which have been used as novel antimicrobial agents in the fields of therapeutics, animal drugs, and food preservatives [1–4]. More than 500 AMPs have been reported (an online

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database of antibacterial peptides could be found at http://www.aps.unmc.edu/AP/main.php) [1]. Cationic AMPs have been isolated from a wide variety of organisms including plants, animals, and microbes in the past two decades. Isolation of AMPs from natural resources or chemical synthesis is not economically viable because it is time-consuming and its production is costly [5]. Accordingly, researchers have sought to mass-produce AMPs by recombinant means using different eukaryotic and prokaryotic expression systems [5–7]. In previous studies, the main obstacle of the heterogeneous expression came from toxicity to host cells, susceptibility to proteolytic degradation, and the small size of AMPs [8]. To overcome these difficulties, various fusion partners have been developed including glutathione S-transferase (GST) [9, 10], maltose-binding protein (MBP) [11], baculoviral polyhedrin (Polh) [7], and thioredoxin (Trx) [12, 13]. Although these studies showed that certain fusion molecules could greatly improve the stability of the target proteins in host cells, there is still no optimized method to meet the needs related to expression and purification at the same time. Therefore, a biological expression system with high expression level and simplified purification procedures for producing AMPs is desirable.

ELPs are artificial biopolymers containing repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly (VPGXG; ten VPGXG repeats is defined as one monomer or one ELP), which is derived from the characteristic repeat motif, VPGVG, found in native, mammalian elastin. In this sequence, the X can be substituted by any naturally occurring amino acid except Pro [14–16]. The solubility of ELPs depends on the temperature and the salt concentration of the solution. When the temperature is above the inverse transition temperature (Tt) of ELPs [17], ELPs aggregate and precipitate rapidly and completely. Below the Tt, ELPs are highly soluble in water. A reversible, soluble-to-insoluble phase transition occurs within a narrow temperature range (2–3 °C). Meyer et al. have previously showed that the environmental sensitivity and reversible solubility of ELPs were retained when ELPs were fused with other proteins, and the activities of the ELP fusion proteins were also retained after cycling through the inverse phase without denaturation [18–20]. This unique property allows us to separate the ELP fusion protein from other contaminating proteins and purify the ELP fusion protein easily from the supernatant by inverse transition cycle (ITC) procedures [21].

In this work, we synthesized the gene encoding an ELP with guest residues Val, Ala, and Gly in the ratio 5:2:3 and then oligomerized synthetic gene encoding 10 Val-Pro-Gly-Xaa-Gly repeats (the 10-mer or ELP10) up to 3, 6, and 9 times to create a series of genes as the fusion partner to express and purify the antimicrobial polypeptide.

Halocidin18 (Hal18), a new AMP containing 18 amino acids (WLNALLHHGLN-CAKGVLA), is a subunit of halocidin isolated from *Halocynthia aurantium*. Hal18 showed greater antimicrobial activity than full-length halocidin against broad-spectrum antibiotic-resistant bacteria [22]. In this study, we employed different sizes of ELPs as fusion partners for the expression of the model AMP Hal18 to construct an advanced AMP purification system, which has advantages of technical simplicity, high purity, high recovery rate, low cost, and ease of scale-up.

Materials and Methods

Bacterial Strains

E. coli XL10-GOLD (Stratagene, La Jolla, CA) was used to construct the recombinant plasmids and assay the antimicrobial activity of purified recombinant Hal18 against a

representative gram-negative strain. *Escherichia coli* BL21 (DE3) (Novagen) was used as the host strain for the expression of the Hal18-ELP fusion proteins. *Micrococcus luteus* (CMCC28001) was used to assay antimicrobial activity of the purified Hal18 against a representative gram-positive strain. *Pichia pastoris* was used to assay antimicrobial activity of the purified Hal18 against a representative fungal strain.

Plasmid Construction

All plasmids were constructed using standard cloning procedures, and their sequences were confirmed by DNA sequencing. The hal18 gene, followed by a hydroxylamine cleavage site (encoding Asn-Gly, N-G), was generated from synthetic oligonucleotides containing optimized *E. coli* major codons (primerF: atcatggcctgaattgcgcgaaaggcgtgctggcgaatggc gaattcaaaggccta, primerR: caattcaggccatgatgcagcagcgcattcagccacatatgcgcgcgcttcggacc) and inserted into the pFastBac plasmid before the EcoRI site by a reverse PCR. The constructed plasmid was named pFH18. The DNA segments encoding different sizes of ELPs (~3ELPs, ~6ELPs, and ~9ELPs) were derived from the plasmids pET3E, pET6E, and pET9E(constructed in this work), respectively and inserted into pFH18 between the EcoRI and HindIII sites to generate the fusion genes (h3/6/9e, 0.56 kb, 0.9 kb, and 1.4 kb) and construct plasmids pFH3E, pFH6E, and pFH9E. To construct the expression vectors, the fusion genes h3/6/9e were derived by NdeI and HindIII and inserted into the same sites of plasmid pET23a, yielding the three recombinant plasmids pETH3/6/9E (Fig. 1). For quantitative comparison of protein expression levels and purification yields, the three resulting plasmids were finally transformed into *E. coli* BL21 (DE3) and each expressed and purified in parallel following standard procedures [20].

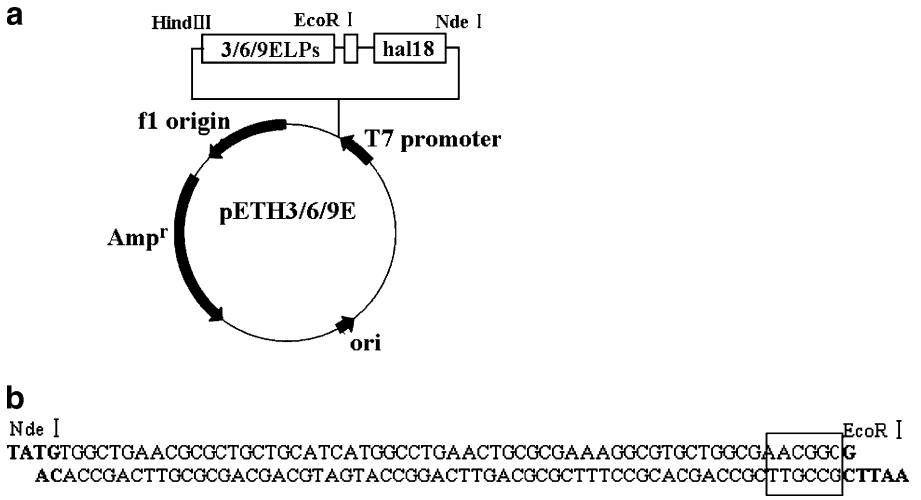

Fig. 1 Construction of the three recombinant plasmids pETH3/6/9E. **a** Gene map of the three 25 recombinant plasmids pETH3/6/9E. *3/6/9ELPs* three ELPs gene with different numbers of monomer, *hal18* halocidin 18-mer gene, *AmpR* ampicillin resistance gene, *ori* pBR322 origin. **b** Design of the nucleotide sequence for Hal18 peptide containing optimized *E. coli* codons. The hydroxylamine cleavage site (encoding Asn-Gly) is *boxed*; the NdeI and EcoRI sites are shown in *bold*

Media and Culture Conditions

For plasmid construction, *E. coli* XL10-GOLD was grown in Luria–Bertani (LB) medium with 100 μg/ml ampicillin and incubated at 37 °C and 250 rpm; for fusion protein expression, *E. coli* BL21 was grown in Terrific Broth (TB; 1 l of TB contains 12 g tryptone, 24 g yeast extract, 2.31 g potassium phosphate monobasic, 12.54 g potassium phosphate dibasic, and 4 ml glycerol) with 100 μg/ml ampicillin at 30 °C, 180 rpm. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a UV-visible spectrophotometer (UV-1601PC; Shimadzu). When the culture grew up to an OD_{600} of 0.5–0.6, IPTG was added to a final concentration 1 mM. After incubation at 30 °C for 5 h, culture samples were harvested for subsequent purification.

Purification of Hal18-ELP Fusion Protein

E. coli cultures were harvested by centrifugation (2,000×g, 4 °C, 15 min), then resuspended in 4 ml phosphate-buffered saline (1 l contains 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4, pH 7.4) per gram of cells (wet weight) and lysed by ultrasonic disruption at 4 °C. The lysate was centrifuged at 16,000×g at 4 °C for 15 min to remove insoluble matter. During the purification step, the ELPs phase transition was induced in the soluble cell lysate by increasing the NaCl concentration (1.5 M NaCl and 40 °C water bath for 30 min). The aggregated fusion protein was separated from solution by centrifugation at 16,000×g at 40 °C for 15 min. The supernatant was aspirated and discarded, and the pellet containing the fusion protein was dissolved in an equal volume of PBS at 4 °C by agitation. Any remaining insoluble matter was removed by centrifugation of 16,000×g at 4 °C for 15 min, and the supernatant containing the purified fusion protein was retained. Each step of ITC purification was monitored by SDS-PAGE, and the final protein concentration and purity were determined by BCA protein assay kit (Pierce) and reverse phase–high pressure liquid chromatography (RP-HPLC), respectively.

Cleavage and Recovery of Hal18

The purified fusion proteins were mixed with three volumes of hydroxylamine cleavage reaction buffer (0.22 M Tris base, 1.7 M hydroxylamine-HCl, 4.5 M guanidine-HCl, and 1% l-propanol, pH 9.0, with the final solution adjusted to pH 8.8) [23] and incubated at 55 °C for 24 h. The cleavage reaction samples were then dialyzed against PBS (pH 7.4). After dialysis, the ELP tag was removed by the next round of ITC. The cleavage products and purified Hal18 were analyzed by Tricine-SDS-PAGE [24]. The SDS-PAGE gels were stained by silver staining or Coomassie blue staining methods (ELP fusion proteins were not very easily stained by Coomassie blue). Hal18 was finally assayed and purifiied by RP-HPLC with the AKTA basic system (Amersham Biosciences) using a 4.6×100-mm Chromolith Performance RP18e column with a linear gradient of 20% to 40% acetonitrile containing 0.1% (*v*/*v*) trifluoroacetic acid at a flow rate of 3 ml/min for 15 min. Reactions were monitored at 215 nm. The molecular weight was analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) on a Voyager system 4095 mass spectrometer (Applied Biosystems).

Chemical Synthesis of Control Hal18 Peptide

Standard Hal18 was chemically synthesized with an automated solid-phase peptide synthesizer (Pioneer; Applied Biosystems; provided by Shanghai Biotech Bioscience &

Technology) and purified to near homogeneity by RP-HPLC. Each peak was identified by MALDI-TOF mass spectrometry.

Antimicrobial Activity Assays

The antimicrobial activity of Hal18 was assayed by using radial diffusion assay and MIC assays against *E. coli* XL10-GOLD, *M. luteus*, and *P. pastoris*. Cells were cultured overnight in LB or YPD medium to an OD_{600} of 0.3, and 1% (*v*/*v*) culture was rapidly added and dispersed into warm LB containing 1% agar. The mixture was then plated on 90 mm petri dishes. For the radial diffusion assay, 10 μl (200 mM) of solution containing recombinant Hal18 and the control H9E (1.3 mg/ml) in PBS was added into a piece of filter paper in the bacteria plate and then incubated at 37 °C for 16 h; 200 μl (200 mM) samples and controls were added into an oxford cup in the *P. pastoris* plate and then incubated at 30 °C for 48 h. For the MIC assay [7], the sample peptide and the chemically synthesized control Hal18 (200 mM) were dissolved in PBS and then serially double diluted to 2 mM and added to an equal volume of a test cell culture (10^5CFU/ml) with LB medium in a 96-well tissue culture plate. The plate was incubated at 37 °C overnight, and the lowest sample concentration yielding no bacterial growth was identified as the MIC.

Results

Expression of Recombinant Fusion Protein

To investigate the effect of ELP size on protein expression and purification level of Hal18, we constructed three plasmids pETH3/6/9E and transformed them into an *E. coli* strain BL21 (DE3). Each of the transformants was expressed and purified in parallel. The three fusion proteins were purified from the cell lysate through one round ITC at the same moderate condition (40 °C, 1.5 M NaCl) and were analyzed by SDS-PAGE gel. SDS-PAGE assay results demonstrated that H6E and H9E were successfully expressed (reaching up to 30% of total cell proteins, data not shown) and their molecular masses were 26 kDa and 37 kDa, respectively, as predicted. The results showed that the product of H6E was higher than H9E (Fig. 2a), which indicated that the length of the ELPs has dramatic effects on the fusion protein products. However, H3E was not successfully expressed since the OD_{600} of the culture was decreased drastically while the culture was induced. Based on previous reports [19, 20], we predicted that the Tt of H6E should be higher than that of H9E and H6E aggregation would occur at a higher concentration of salt than H9E at same temperature. Therefore, H9E was chosen for further research.

Purification of Recombinant Protein H9E

To find the optimal purification conditions for H9E, the phase transition profile of H9E was investigated at different temperature and NaCl concentrations. T_d was defined as the temperature when the solution becomes turbid at a given NaCl concentration. The curve (Fig. 2b) shows that controlling the NaCl concentration range from 0 to 1.5 M allowed T_d to be tuned over a 40 °C range and thereby, provides a means for isothermal triggering of the transition. The T_d decreases with increasing NaCl concentration. When the salt concentration was up to 1 M, H9E began to aggregate at room temperature. Optimal ITC condition of 1.5 M NaCl in a 40 °C water bath for 30 min was determined, which was

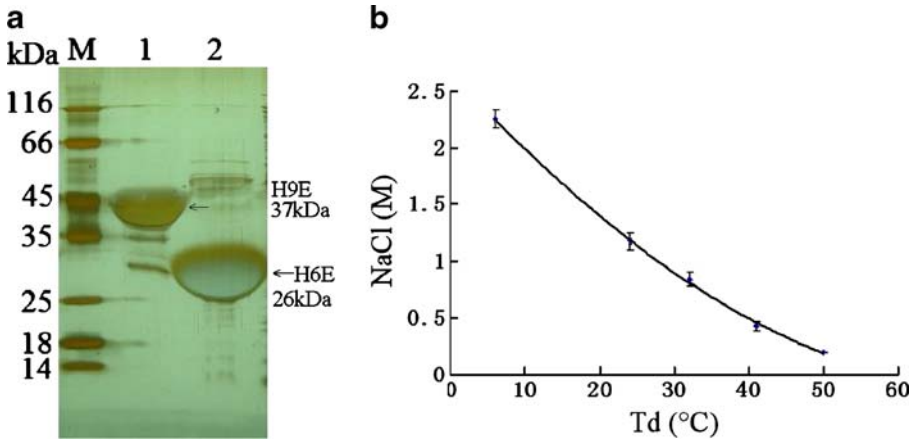

Fig. 2 **a** SDS-PAGE analysis of the products of one round of ITC procedure from the supernatant. *M* molecular weight markers, *1* H9E, *2* H6E. **b** Td as a function of NaCl concentration for H9E (1.3 mg/ml) in 50 mM phosphate buffer, pH 8.0. All experiments were done in triplicate

different from the isothermal condition originally selected for H9E aggregation. Under this optimal purification condition, H9E was purified from the supernatant by two rounds of ITC; each round of recovery production was assayed by SDS-PAGE (Fig. 3), and the purity of the last round production was determined by RP-HPLC. The results indicated that two rounds of ITC allowed H9E purification to nearly 95%.

Cleavage and Recovery of Hal18

Hal18 was liberated from its ELP fusion partner 9ELPs by cleavage with hydroxylamine at a recognition site (N-G) located between Hal18 and the ELP tag. After cleavage, purity of Hal18 was improved by another round of ITC aimed at removing the ELPs tag and any

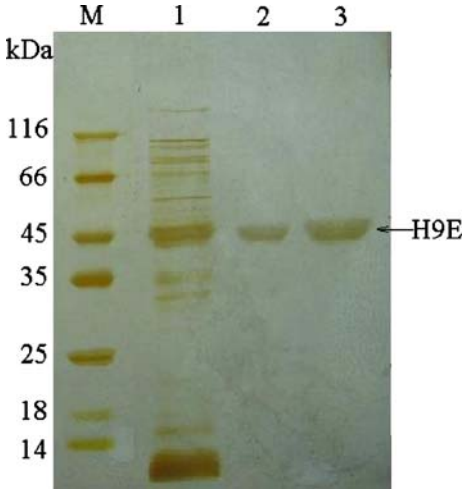

Fig. 3 SDS-PAGE analysis of each stage of H9E purification. *M* molecular weight markers, *1* supernatant of cell lysate, *2* one round of supernatant, *3* two rounds of supernatant

remaining uncleaved H9E. Tricine-SDS-PAGE analysis results showed about~2 kDa mass of Hal18, which was almost identical to that of the synthetic version (1.93 kDa). It demonstrated that purified Hal18 and ELP tags were successfully separated from each other (Fig. 4). Total protein product weights of purification step and cleavage step were obtained by BCA protein assay method. The final Hal18 recovery is shown in Table 1.

Antimicrobial Activity of Hal18

Antimicrobial activity of purified Hal18 was determined by using the radial diffusion assay. It showed strong antimicrobial activity toward *E. coli* and *M. luteus* and weak antifugal activity toward *P. pastoris*, which has not been reported. By contrast, no antimicrobial

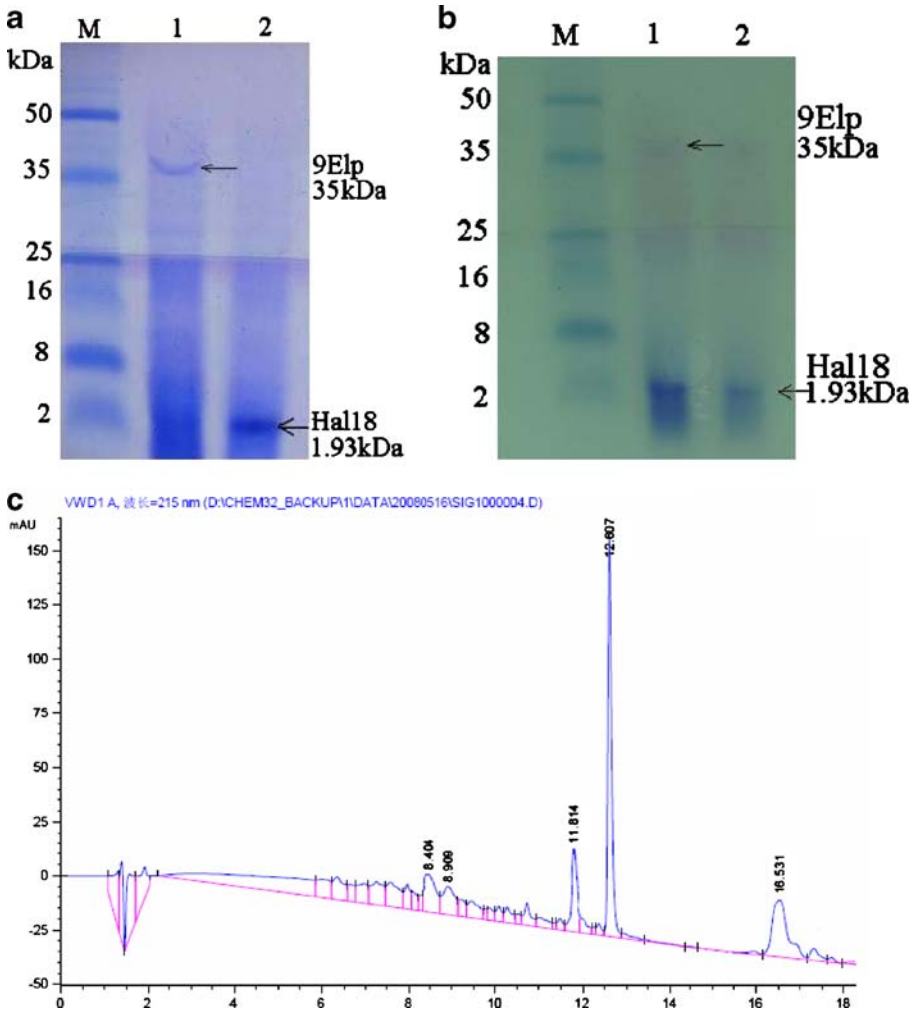

Fig. 4 Tricine-SDS-PAGE assays of the purity of Hal18 cleavage and recovery from H9E. *M* molecular weight markers, *1* hydroxylamine cleavage sample, *2* recovery of Hal18 (*arrow*) after dialysis and another round of ITC. **a** Wash the page in wash buffer for half an hour. **b** Wash the page in wash buffer for 1 h. **c** RP-HPLC assays of the purity of Hal18 that separated from H9E

Table 1 Summary of recovery of Hal18.

Step	Total protein[a] (mg)	Amt of Hal18 (mg)	Recovery (%)	Purity (%)[c]
Two rounds of ITC	69	3.6[b]	100	95
Cleavage, dialysis, and centrifugation	2.8	1.7	47	60

[a] Determined by BCA protein assay kit

[b] Calculated from the difference in the estimated molecular masses of Hal18 and 9ELPs

[c] Purity was detected by RP-HPLC analysis method

activity was detected in the fusion protein H9E and the distilled water controls (Fig. 5). These results confirmed that Hal18 was present in the soluble fraction after dialysis and centrifugation. The radial diffusion assay results also indicated that gram-positive bacteria were more sensitive to Hal18 than gram-negative bacteria. A MIC assay was done to quantitatively investigate the antimicrobial activity of recombinant Hal18. MICs to *E. coli* and *M. luteus* of Recombinant Hal18 recovered by RP-HPLC were 20 and 8 mM, respectively, which were the same as the chemical synthesis of control Hal18 peptide.

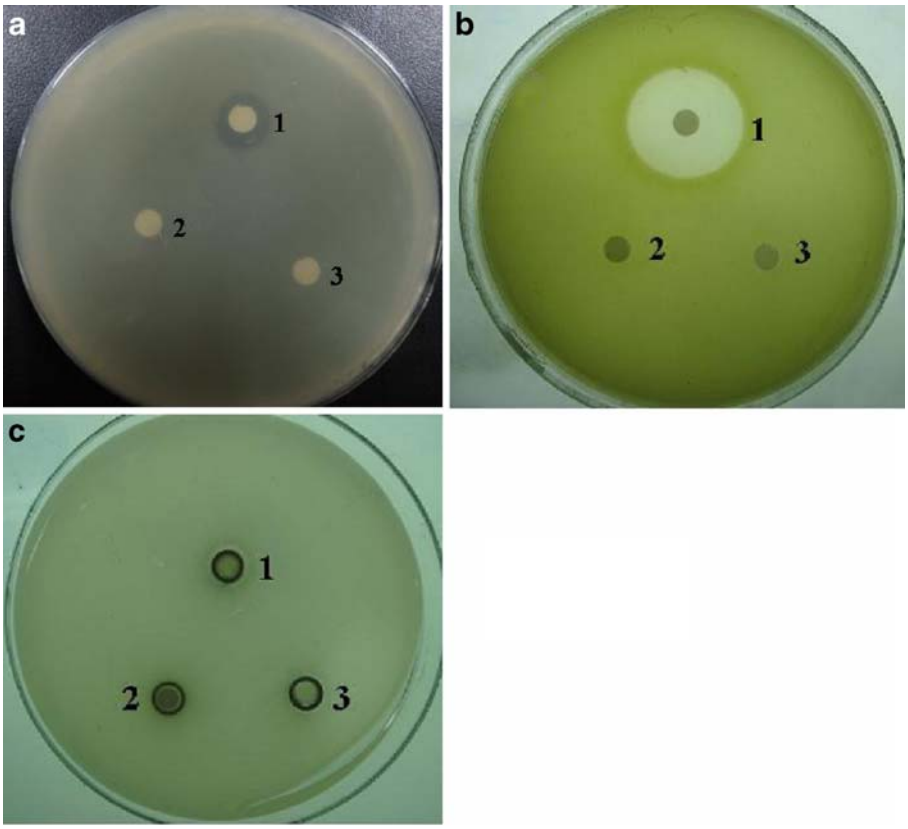

Fig. 5 Hal18 antimicrobial activities assays against **a** *E. coli*, **b** *M. luteus*, and **c** *P. pastoris*. *1* purified Hal18, *2* H9E, *3* **a** and **b** were distilled water as a negative control, while **c** is the cleavage sample (H9E) after dialyzed

Discussion

Production of antimicrobial polypeptide at a large scale for the commercial development is a significant challenge. The expression of heterogeneous proteins in bacteria is by far the simplest and most inexpensive means to produce large amount of product of interest. However, it is difficult for small peptides to be expressed in engineered bacteria at high levels and be recovered from the expression systems. Several biological expression systems have been developed by fusing the antibiotic peptide with a partner protein with anionic properties to avoid the toxicity of the products to the host cells.

Here, we sought to identify a more beneficial fusion partner for AMP expression. This partner (a) is about more than ten times larger than the target AMP, (b) can protect the AMP from proteolytic degradation, (c) is nontoxic to the host bacterium, (d) is soluble, and (e) is easily purified. We identified an elastin-like polypeptide that satisfies these criteria.

Comparing with other fusion tags such as GST, MBP, Polh, and Trx et al., ELPs have advantages in the expression and purification of AMP. First, the expense on the chromatographic resins and equipment is eliminated. For all we know, the early stages of other methods need immobilized metal affinity binding chromatography or HPLC [23, 25–28] and their later stages are time-consuming and not economic. The potentially lower cost and ease of scale-up of this method is likely to prove attraction for large-scale (in the grams to kilograms range) purification of antimicrobial polypeptides. Second, this method is fast and simple with only a few short centrifugation or filtration steps followed by reconstituting the purified protein in a buffer of low ionic strength; the separation and recovery conditions are mild, requiring only a modest change in temperature or ionic strength. Third, because protein purification is usually the limiting step in structure/function studies and in screening of proteins in pharmaceutical development, we believe that the ability to simultaneously purify a large amount of proteins by inverse transition purification is likely to be extremely useful for laboratory-scale purification (in the microgram to milligram range) of proteins [19]. Finally, Carlson et al. have clearly demonstrated that ELP tags, in some cases, are better than oligohistidine fusion in such aspects as the retention of functional activity, better solubility, and significantly easier scale-up [18].

Using the ELPs as the fusion partner yielded 69 mg of the fusion protein in *E. coli* from 1 l culture. This yield was higher in comparison with the use of Polh, which yielded only 29.91 mg of the fusion protein [7], but much lower comparing with the recent report using Trx (416 mg/l) [25]. Based on the report [29], the yield of the ELPs fusion protein has the potential to be increased ten times. We will conduct this experiment at a later time. Additionally, we inserted the fusion gene h9e to the pPIC9k and constructed the recombinant plasmid pPICH9E and then transformed it to the *P. pastoris GS115*. After induction by methanol, we purified 448 mg H9E from 1 l culture.

Usually fusion proteins are cleaved chemically or enzymatically to release the protein. For this study, we used hydroxylamine to cleave H9E at the Asn-Gly junction between the fusion partners. The maximum cleavage rate and minimum number of side chain modifications can be achieved under optimal hydroxylamine cleavage conditions. Several conditions were suggested for the hydroxylamine cleavage reaction (2 M urea, 2 M hydroxylamine-HCl, 0.1 M Tris base, pH 9.0, at 45 °C [30]; 0.5 M hydroxylamine-HCl, pH 8.65, at 45 °C [7]; and 0.22 M Tris base, 1.7 M hydroxylamine-HCl, 4.5 M guanidine-HCl, and 1% 1-propanol, pH 9.0, at 55 °C [31]). The first two conditions [7, 30] resulted in lower cleavage yields (about 30%). Therefore, we employed the last one [31]. Optimization experiments showed that the cleavage of H9E fusion proteins in freshly prepared buffer was relatively efficient, whereas, comparable cleavage in a guanidine-HCl-free or urea-

containing buffer was ineffective (50%). This result was similar to the previous report [31] in which cleavage yields with guanidine-HCl and urea as the denaturant were about 96% and 38%, respectively.

At first, we anticipated that the purity of Hal18, which was obtained by the last round of ITC for the removal of the ELPs tag, was at least more than 80%. However, the RP-HPLC results showed that it only reached to 60%. We think that this impurity might come from the following three contaminants: (a) uncleaved fusion protein and the ELPs tag, (b) undesired cleavage product, since hydroxylamine can cleave Asn-Gly peptide linkages under relatively harsh conditions and also Asn-Leu and Asn-Ala peptide linkages and the recombinant Hal18 also contained a Asn-Ala peptide linkages, and (c) dimerization of Hal18, which occurs when it contains cysteines. We speculate that there are several ways to improve the purity of Hal18. More rounds of ITC could possibly reduce the uncleaved fusion protein. Jang et al. have demonstrated that the dimerization of α-helical peptides via interdisulfide bond between the substituted Cys residues might be a powerful modification to strengthen the activity of a monomeric antimicrobial peptide [32]. Though hydroxylamine has high cleavage efficiency (up to 96%), some unexpected cleavage may happen at the same time. Also, we found that it is not suitable for large-scale production to be exposed to the harsh cleavage conditions required for hydroxylamine and dialysis. This problem may be solved by enzymatic cleavage such as TEV protease, Enterokinase, and Factor Xa et al. Most of the proteases allow fusion target protein in the downstream steps (a) to retain its native N-terminus without any unwanted amino acid residues on their amino termini; (b) to retain its activity under a variety of reaction conditions; (c) to be capable of cleaving fusion proteins at a wide pH range with the presence of various detergents and denaturants; and (d) also easily removed by nickel affinity chromatography [33]. In summary, the use of ELPs as a fusion partner for a model AMP, Hal18 enabled stable expression and effective purification of a milligram of antimicrobial polypeptide.

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