

High-level expression of fusion peptide Dybowskin-2CDY_a and hEGF in *E. coli* and its dual function

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Abstract: Dybowskin-2CDY_a (Dy2), with a broad antimicrobial spectrum and low hemolytic feature, is a newly discovered type of antimicrobial peptides from *Rana dybowskii*. In order to get a dual function peptide which inhibits bacterial growth and promotes cell proliferation, we cloned the gene of Dy2 and hEGF (human epidermal growth factor) into the prokaryotic expression vector *pET-30a(+)*. With isopropyl-β-D-thiogalactoside (IPTG) induction, a 13.7KDa peptide with a 6×His tag was highly expressed in the form of inclusion in *E. coli* BL21 (DE3). SDS-PAGE and western-blot confirmed the expression of the fusion peptide hEGF-Dy2. Under the optimized condition of 1.0mmol/L IPTG induction and incubation time 4h at 37°C, the yield of hEGF-Dy2 reached 30mg/L following purification on nickel-nitrilotriacetic acid (Ni-NTA) metal affinity chromatography matrices. The purified fusion peptide showed significant antibacterial activities against *Staphylococcus aureus*, *Escherichia coli* O157, *Pseudomonas aeruginosa* and proliferating activities on NIH3T3. These results indicated that the fusion peptide might have a good prospect in therapy of trauma and burns.

Keywords: Antimicrobial peptide; Dybowskin-2CDY_a; human epidermal growth factor; fusion expression; antibacterial activities; proliferating activities.

INTRODUCTION

Antimicrobial peptides (AMPs) generally composed of 12–60 amino acids and they are considered as an evolutionarily ancient component of the innate immune response against microbes in many organisms (Hancock, 1997; Hancock and Sahl 2006; Zasloff, 2002). AMPs exhibit a broad spectrum of activities against bacteria, fungi, parasites, viruses, and even some tumor cells at a concentration which is relatively nontoxic to eukaryotic cells, these facts make them attractive candidates as novel therapeutic agents (Conlon *et al.*, 2004; Xu *et al.*, 2013).

The widespread use of antibiotics improved the risk of bacterial resistance drastically, super bacteria of antibiotic resistance has already existed. Statistics shows that it would take 10 or more years to develop a new antibiotic, but a new generation of drug-resistant bacteria occurs within 2 years (Feng *et al.*, 2006). AMPs, unlike conventional antibiotics, are known to be less vulnerable to bacterial resistance (Xu *et al.*, 2013). Previously, we cloned and identified a novel antimicrobial peptides from the skin of *Rana dybowskii*, Dybowskin-2CDY_a (Dy2, SAVGRHSRRFGLRKRKH, GenBank number: ACF08009.1). The amino acid composition and sequence of Dy2 bear little resemblance to other frog antibacterial peptides found before. Dy2 is rich in arginine, has the feature of large quantities of positive charge, broad antimicrobial spectrum and low hemolytic activity (Jin *et al.*, 2009), these make it possible to be a peptide antibiotic.

Both basic research and clinical applications require high quality peptides to be readily available in a cost-effective manner. In general, isolation from natural sources is a labor intensive and time-consuming process, and therefore does not provide an efficient method to obtain peptides in large amounts. Chemical synthesis, although very efficient, is a complex and costly process (Andersson *et al.*, 2000). Fortunately, recombinant DNA technology provides an economical means for protein manufacture. Indeed, many antimicrobial peptides have been successfully obtained through recombinant production in various heterologous hosts (Ingham and Moore, 2007). Among the systems available for recombinant protein production, *Escherichia coli* has been the most widely used host which has many expression advantages including short cycle, low production cost, clear genetic background, high conversion efficiency and easy operation (Li and Chen, 2008). To prevent antimicrobial peptides from degradation in the process of expression and reduce the toxicity to the host cell, a big fragment is usually added to the N end of it, including thioredoxin and green fluorescent protein (Xu *et al.*, 2013; Xu *et al.*, 2007; Yu *et al.*, 2010).

In this study we selected human epidermal growth factor (hEGF) as the fusion partners of Dy2 expression, which is a mitogen for epithelial and mesenchymal cells and one of the most powerful cytokine involved in promoting cell proliferation and differentiation (Zeisberg and Kalluri, 2004). hEGF have been widely and effectively applied in treating skin burns, scald, cornea injury, and gastric ulcer (Değim *et al.*, 2011). We aim to express and get the fusion peptide hEGF-Dy2 in cost-effective manner, and it

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should have an antibacterial and proliferating function and could be used to treat trauma and burns and solve their low efficacy in clinic.

MATERIALS AND METHODS

Restriction enzyme *Kpn* I, *Not* I, T4 DNA ligase, the prokaryotic plasmid *pET-30a(+)*, DNA markers were all purchased from TAKARA (Dalian, China). Tris, acrylamide, bisacrylamide, low molecular protein marker were obtained from Sigma Chemical Company. *E. coli* JM was purchased from Promega. NIH3T3 cell was ordered from ATCC, competent *E. coli* BL21 (DE3) cells was purchased from Tiangen biotechnology company. *Staphylococcus aureus*, *Streptococcus pyogenes*, *E. coli* O157 were provided by National Institute on Drug Abuse of China. Small plasmid extraction kit, DNA gel recovery kit, PCR cleaning kit were of AXYGEM products. Sequencing and primer synthesis were done by TAKARA (Dalian, China). His-tag antibody and goat anti-mouse IgG(H+L) HRP were from Kangwei biotechnology company. Ni-NTA agarose column (Histrap FF) was from GE Company. All other chemicals used were of analytical grades.

Gene Amplification and Connection of hEGF and Dy2

The coding sequence of hEGF and Dy2 was designed based on the codon preference of *E. coli*, and Dy2 was linked to the 3'-end of hEGF, and they were linked by the coding sequence of enterokinase cleavagesite (DDDDK). Thrombin cleavagesite (LVPRNS) was added to the N'-end of hEGF, and *Kpn* I restriction sites and protection bases were at 5' end of the fusion protein, and *Not* I restriction sites and protection bases were at 3' end. The length of the fusion peptide gene was 271bp. According to the principle of SOE-PCR, eight long primers (Phd1 - Phd8) and two short primers (Phd - up and Phd - down) were synthesized (The primers sequence are shown in table 1). SOE-PCR amplification was performed with 10 μ L 5 \times PCR buffer, 1 μ L PrimeSTAR HS DNA Polymerase (2.5U/ μ L), 4 μ L dNTP Mixture (2.5 mM each), 0.1 μ L Phd1 - Phd8 (20 μ M), 32.2 μ L ddH₂O. The reaction was 94 $^{\circ}$ C for 4min, 5 cycles of 98 $^{\circ}$ C for 10s, 65 $^{\circ}$ C for 10s and 72 $^{\circ}$ C for 30S, 5 cycles of 98 $^{\circ}$ C for 10s, 55 $^{\circ}$ C for 10s and 72 $^{\circ}$ C for 30S, and a final extension of 72 $^{\circ}$ C for 5 min. Then 1 μ L Phd-up (20 μ M) and 1 μ L Phd-down (20 μ M) were added, the second reaction condition was 94 $^{\circ}$ C for 4min, 30 cycles of 98 $^{\circ}$ C for 10s, 55 $^{\circ}$ C for 10s and 72 $^{\circ}$ C for 30s and a final extension of 72 $^{\circ}$ C for 5 min

Construction of Recombinant *pET30a(+)-hEGF-Dy2* Vector

The purified *hEGF-Dy2* fragment and the *pET30a(+)* plasmid were digested with *Kpn* I and *Not* I (37 $^{\circ}$ C, 4h), and was ligated with T4 DNA ligase (350U/ μ L, 16 $^{\circ}$ C, 12h), and then transformed into *E. coli* JM109 competent cells by heat-shock (42 $^{\circ}$ C, 90s). Positive colonies resistant to kanamycin on a Luria-Bertani (LB) plate were selected

and the presence of *hEGF-Dy2* gene was confirmed by PCR and DNA sequencing.

Expression of hEGF-Dy2 and optimization of expressing condition

The recombinant plasmid *pET30a(+)-hEGF-Dy2* was transformed into *E. coli* BL21(DE3) and positive colonies were identified by PCR T7 5'(TAATACGACT CACTATAGGG) and 3' (TGCTAGTTATTGCT CAGCGG). The reaction condition was as follows: 94 $^{\circ}$ C for 5min, 30 cycles of 94 $^{\circ}$ C for 30s, 55 $^{\circ}$ C for 30s and 72 $^{\circ}$ C for 30s and a final extension of 72 $^{\circ}$ C for 5 min. The identified colony was inoculated in 5mL LB medium supplemented with antibiotic kanamycin (50 μ g/mL) and incubated at 37 $^{\circ}$ C, 180rpm. Approximately 0.5mL overnight culture was inoculated into 50mL LB medium and continued to culture in same conditions. When OD600 value of the culture reached 0.6-0.8, 1.0 mM IPTG was added to induce the expression of hEGF-Dy2. The expressed fusion peptide was analyzed by 12% SDS-PAGE and Western Blot. Expressing condition of the fusion peptides was optimized based on culture time, temperature and IPTG concentration.

Large scale expression renaturing and purification of hEGF-Dy2

Following induction, cells were cultured in 1L fermenter under the optimized condition. The culture was centrifuged at 8,000rpm for 5 min, the cell pellet was washed twice by 20mL phosphate-buffered saline (PBS) and then resuspended in 20mL solution A (50 mM Tris - Cl, 1 mM EDTA, 100 mM NaCl, 1% TritonX-100, pH8.5), frozen and thawed four times by liquid nitrogen, disrupted with sonicator on ice and spun down at 12000rpm for 10 min. The precipitate was respectively treated by ultrasonic cleaning in solution B (50mM Tris-Cl, 1mM EDTA, 100mM NaCl, 1% TritonX-100, 2M, pH8.5), solution A (50mM Tris-Cl, 1mM EDTA, 100mM NaCl, 1% TritonX-100, pH8.5), solution C (50mM Tris-Cl, 1mM EDTA, 100mM NaCl, 1% TritonX-100, 2M guanidine hydrochloride, pH8.5). Finally the pellet (inclusion body) was dissolved in solution D (50mM Tris-Cl, 1mM EDTA, 100mM NaCl, 1% TritonX-100, 10mM DTT, 2mM sodiumdeoxycholate, 8M urea, pH8.5) at room temperature for 2h and centrifuged at 12,000rpm for 10 min. The supernatant was placed into dialysis tubing and renatured at 4 $^{\circ}$ C for 36h in the gradient buffer (50mM Tris-Cl, 100mM NaCl, 6M/4M/2M urea, 1% glycine, 5% glycerin, 0.2% PEG, pH8.5) and transferred to binding buffer (50mM Tris-Cl, 150mM NaCl, 10mM imidazole, pH8.5). The fusion peptide was purified using Ni-NTA agarose column according to the manufacturer's instruction (Histrap FF, GE Company). The fusion peptide was eluted with the buffer (50mM Tris-Cl, 150mM NaCl and 300mM imidazole, pH8.5), the eluate was analyzed by 12% SDS-PAGE and then desalted and freeze-dried.

Antibacterial activity assay of expressed hEGF-Dy2

Antibacterial activity of expressed hEGF-Dy2 was tested by Colony Forming Unit Assay. The bacteria (*Staphylococcus aureus*, *E. coli* O157, *P. aeruginosa*) were cultivated in LB medium to mid-log phase, centrifuged at 8000g for 10min and diluted to 5×10^5 CFU/mL by PBS. Assays were performed in the volume of 500 μ l PBS containing 10^5 cells/mL and 500 μ l different concentrations of hEGF-Dy2, incubated for 16 h at 37°C, 180rpm. Then bacteria were diluted to appropriate concentration, 10mL diluted broth was evenly applied on LB agar plates and incubated overnight at 37°C. The colony was counted and compared to the control plate.

Cell proliferation activity of hEGF-Dy2 on NIH3T3 cells

NIH3T3 cells in the logarithmic growth phase were plated into a 96-well plate (2000 cells / well) and incubated in DMEM medium (10% FBS) at 5% CO₂, 37°C for 24h. The culture medium was replaced with 200 μ l fresh complete medium containing serial dilutions of fusion peptide hEGF-Dy2, and the control was without fusion peptide. Cells were divided into 24h, 48h and 72h groups, and proliferation activity was assayed by MTT method accordingly (Sen *et al.*, 2010).

Wound healing assay in vitro

NIH3T3 cells were seeded into 6-well plate (1.0×10^5 /well, 10% FBS in DMEM, 5% CO₂, 37°C), and a linear wound was generated in the monolayer with a sterile 200 μ l pipette tip, then the cells were washed twice to remove detached cells and debris (Gebäck *et al.*, 2009). Sizes of wounds were observed and analyzed by software TScratch at 0h, 6h, 12h, 24h and 48h after treatment by 75 μ g/mL hEGF-Dy2.

STATISTICAL ANALYSIS

All data are presented as mean \pm SD from three or more experiments. The results were evaluated by one-way ANOVA. $P < 0.05$ was considered significant.

RESULTS

Cloning and construction of pET30a(+)-hEGF-Dy2

The coding region of hEGF and Dy2 were amplified by SOE-PCR with 10 primers shown in table 1. Purified hEGF-Dy2 DNA and vector pET30a(+) were subjected to digestion with *Kpn* I and *Not* I. The expressing plasmid pET30a(+)-hEGF-Dy2 was constructed and cloned into *E. coli* JM109. DNA sequencing result showed that hEGF-Dy2 sequence was consistent with the designed one and the open reading frame was correct after connection.

Expression and optimization of hEGF-Dy2

The recombinant pET30a(+)-hEGF-Dy2 was successfully transformed into *E. coli* BL21(DE3) and induced by IPTG. SDS-PAGE and Western Blot of total bacterium lysate showed that the fusion peptide hEGF-Dy2 was successfully expressed (fig. 1). After 4h induction, hEGF-Dy2 reached the maximum in the time optimization test and plate used, although it was also expressed in a very low level in 0h induction (fig. 2a). The IPTG concentration optimization test showed the peptide expression was highest when induced by 1mM IPTG (fig. 2b). In the temperature optimization test the yield of hEGF-Dy2 was the maximum at 37°C (fig. 2c). Thereafter 1.0mM IPTG and 4h culturing at 37°C were conditions applied in the following experiment. We tested that hEGF-Dy2 was expressed in the form of inclusion body (fig. 2c).

Table 1: Primer sequences for the clone of hEGF and Dy2

Primers	Sequence (5'-3')
Phd1	5'- CGG <u>GGTACCCTGGTTC</u> CGCGTAATAGTGACAGTGAATGTCCGCTGAGTCATGACG-3'
Phd2	5'-CAATATACATACAAACACCGTCATGCAGACAATAACCGTCATGACTCAGCGGAC-3'
Phd3	5'-GTGTTTGTATGTATATTGAAGCACTGGACAAATATGCATGTAATTGTGTTGTTG-3'
Phd4	5'-TCAGGTCACGATACTGACAACGTTCCACCAATATAACCAACAACACAATTACATG-3'
Phd5	5'-GTCAGTATCGTGACCTGAAATGGTGGGAAGTGCCTCTGGACGACGACGACAAGA-3'
Phd6	5'-GCAGACCAAAACGACGACCATGACGACCAACTGCACTCTTGTCTCGTCGTCGTC-3'
Phd7	5'-GTCGTCGTTTTGGTCTGCGTAAACATCGTAAACATTAAGCGGCCGCTAAACTAT-3'
Phd8	5'- <u>ATAGTTTA</u> GCGGCCGCTT AATGTTTACGATGTTTACGCAG-3'
Phd-up	5'- CGG <u>GGTACCCTGGTTC</u> CGCGT-3'
Phd-down	5'- <u>ATAGTTTA</u> GCGGCCGCTT AAT-3'

Restriction enzyme sites (*Kpn* I and *Not* I) are shown in bold; protection bases are in the box; the dotted underline bases are enterokinase recognition sites; single underlined bases are thrombin recognition sites; double underlined bases are termination codon.

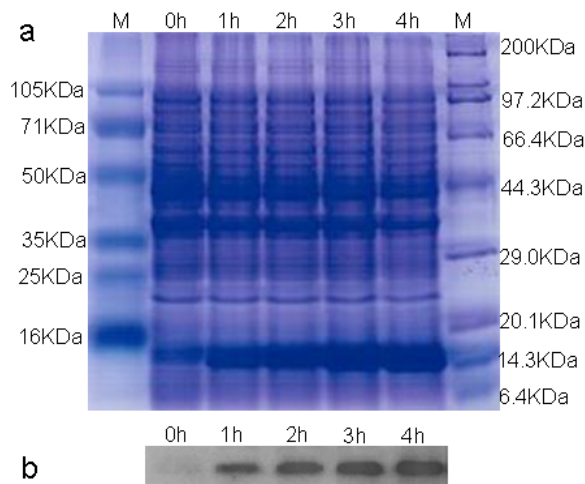


Fig. 1: 12% SDS-PAGE and Western blot analysis of recombinant hEGF-Dy2 expression at different induction times

a. Total protein samples of bacterium lysate were collected at 1h intervals after 1.0mM IPTG induction. The expression product of 13.7KD was detected in the analysis and it was expressed in zero hour of the induction, although it was little. Lane M(left): Pretained standards (Mid range, 2-105KD); Lane M(right): Premixed Protein Marker (Broad, 6.5-200KD); Lane 0h, 1h, 2h, 3h, 4h: Bacterium induction products of the corresponding time. b. Western Blot assay of recombinant bacterium protein samples after induced at 0-4h

Purification of recombinant hEGF-Dy2

Bacteria culture containing fusion peptide was collected and treated. The inclusion body was dissolved, renatured and then purified by a Ni-NTA agarose column in one step, and peak appeared when the concentration of

imidazole was 300mM. The elution was then desalted using Sephadex G-25 and lyophilized for further analysis. About 30mg of recombinant hEGF-Dy2 was recovered from 1L of bacterial culture. SDS-PAGE analysis showed a single band (fig.3).

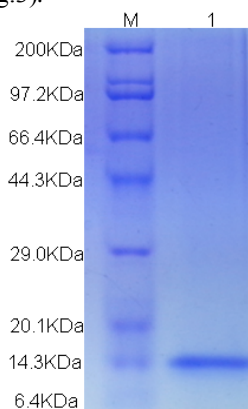


Fig. 3: 12 % Tricin-SDS-PAGE analysis of purified recombinant hEGF-Dy2

Lane M: Premixed Protein Marker(Broad,6.5-200kDa); Lane1: Purified hEGF-Dy2 after affinity chromatography.

Antibacterial activity of purified recombinant hEGF-Dy2

Colony Forming Unit Assay showed that purified recombinant hEGF-Dy2 had significant antibacterial activities against different bacteriastrains, *Staphylococcus aureus*, *E. coli* O157 and *P. aeruginosa* (fig. 4), where there is a dose dependent effect between concentrations of the fusion peptide and antibacterial activity. Half maximal inhibitory concentration (IC50) for *S. aureus* was 14.34µg/mL, for *E. coli* O157 was 15.93µg/mL, and for *P. aeruginosa* was 14.75µg/mL.

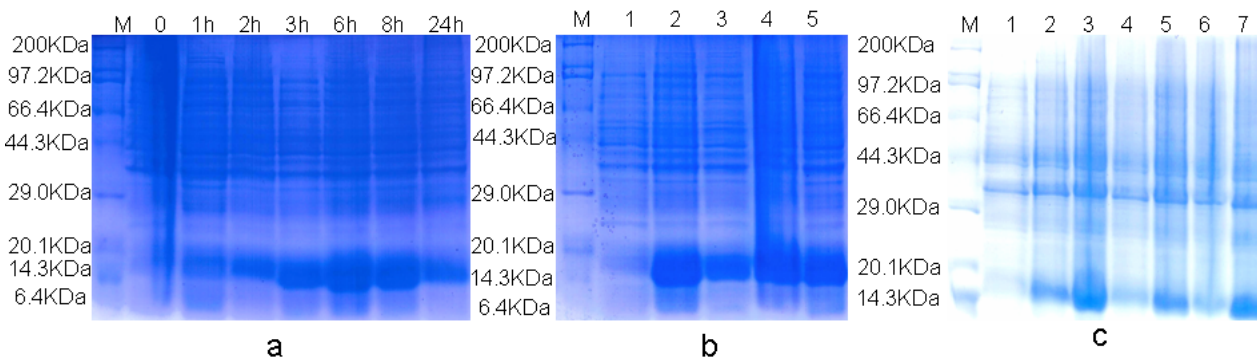


Fig. 2: 12% SDS-PAGE analysis of expression condition optimization test of recombinant hEGF-Dy2

Lane M: Premixed Protein Marker (Broad, 6.5-200kDa). A: Protein samples collected from recombinant bacteria (1mM IPTG induction at 37°) in different culture times of 0h, 1h, 2h, 3h, 4h, 6h, 8h, 24h, and hEGF-Dy2 yield reached the maximum at 4h. b: Protein samples collected from recombinant bacteria (cultured 4 hour at 37°) in different IPTG induction concentrations (lane 1-5: 0, 1, 0.4, 0.7, 1.2 mM IPTG), and hEGF-Dy2 yield was highest in 1mM IPTG. C: Protein samples collected from recombinant bacteria (1mM IPTG induction and cultured 4 hour) in different culture temperature (Lane1: without induction; Lane 2, 4, 6: Expressed products in bacterium supernatant at 37°, 27°, 33°; Lane 3, 5, 7: Expressed products in bacterium precipitate at 37°, 27°, 33°, and we could see that hEGF-Dy2 yield was the maximum at 37° and hEGF-Dy2 was expressed in inclusion body.

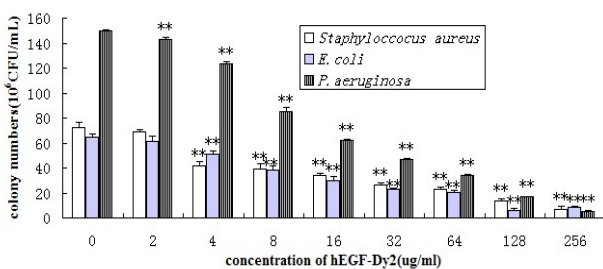


Fig. 4: Antibacterial activity of purified recombinant hEGF-Dy2

Antibacterial activity of different concentrations of hEGF-Dy2 on *Staphylococcus aureus*, *E. coli* O157 and *P. aeruginosa* was tested by colony forming unit assay. Certain concentration of bacteria and hEGF-Dy2 was mixed and cultured for 16h, and applied on LB agar plates, incubated overnight at 37°C. Finally the colony was counted and compared to the control group without hEGF-Dy2. ** $P < 0.01$

Proliferation activity of recombinant hEGF-Dy2 on NIH3T3 cells

Proliferation assay of NIH3T3 cells was performed with MTT method. The result showed increased proliferation by purified recombinant hEGF-Dy2 in the range of 9.38 μ g/mL-75 μ g/mL ($P < 0.01$), and it reached the highest values at 75 μ g/mL at 72h (fig. 5).

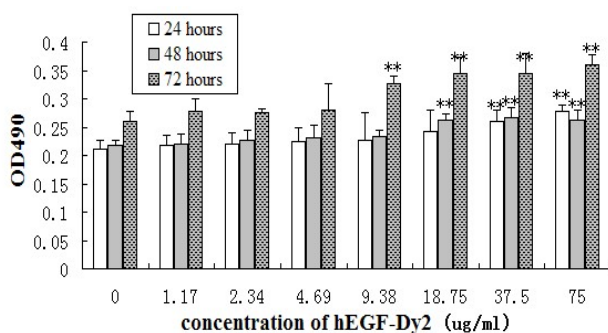


Fig. 5: MTT test result showed effect of hEGF-Dy2 on NIH3T3 cell proliferation

NIH3T3 cells were cultivated in DMEM medium and exposed to hEGF-Dy2 (0 μ g/mL-75 μ g/mL). Proliferation rate of NIH3T3 cells were analyzed by MTT method at 24h, 48h and 72h. The group without hEGF-Dy2 (0 μ g/mL) was taken as control. ** $P < 0.01$

Promoting migration activity of recombinant hEGF-Dy2 on NIH3T3 cells

Effect of recombinant hEGF-Dy2 on migration of NIH3T3 cells was assayed by Wound Healing Assay *in vitro*. The time needed for wound healing depends on the cell migration rate. The result showed that the wound closure rate of experimental group (75 μ g/mL) was significantly ($P < 0.01$) increased at 24h and 48h comparing to the corresponding control groups (fig. 6).

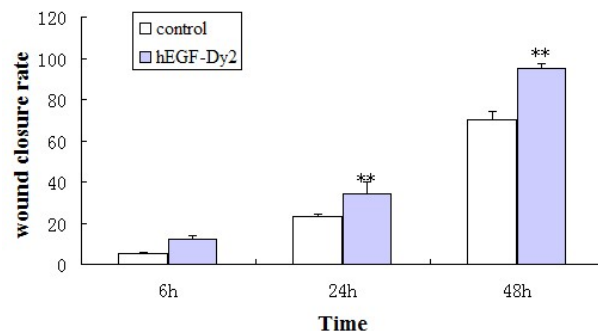


Fig. 6: Effect of hEGF-Dy2 on migration of NIH3T3 cells with wound healing assay *in vitro*.

NIH3T3 cells were seeded into 6-well plate, and a linear wound was generated in the monolayer. After hEGF-Dy2 (75 μ g/mL) was added to experimental group, and the size of wounds was observed and analyzed by software TScratch at 0h, 6h, 24h and 48h. Experimental group had higher wound closure rate than control group (without hEGF-Dy2) at 24h and 48h and the difference was highly significant (** $P < 0.01$).

DISCUSSION

To produce polypeptides by recombinant DNA technology has allowed a widespread use by obtaining larger quantities of highly bioactive but inexpensive AMPs (Li 2011). In recent years, many reports on methods of antimicrobial peptide expression in *Escherichia coli* and rapid purification, and demonstrated great variations of the yield from 0.3mg/L to 346mg/L, and most of which was lower than 20mg/L (Li, 2011). The expression of AMP Dybowskin-2CAMA was much lower in the yeast expressing system *Pichia pastoris* (Jin *et al.*, 2013). In this study we were able to obtain 30mg/L of recombinant fusion peptide hEGF-Dy2 with optimized expression condition, which was much higher than previous studies and should benefit further research on mechanisms, efficacy and safety.

AMPs with their broad-spectrum antibacterial and antifungal abilities have a much clinical prospect as external antimicrobial agents for burns and other wounds (Ahmad *et al.*, 2012). Clinical trials have been conducted or are in progress for a number of applications, such as skin infections, thrush, diabetic ulcers, and secondary infections related to cystic fibrosis (Hancock and Sahl 2006; Melo *et al.*, 2006). Antibacterial peptide A3-APO was effective against systemic infections in different mouse models (Szabo *et al.*, 2010). Human epidermal growth factor (hEGF), a major cytokine involved in wound healing, has been applied in wound therapy (Barrientos *et al.*, 2008; Değim *et al.*, 2011). In this study the purified hEGF-Dy2 demonstrated strong antimicrobial activity against different kinds of bacteria *Staphylococcus aureus*, *E. coli* O157 and *P. aeruginosa* and had cell proliferation and migration activity on NIH3T3 cells, which indicated that the fusion peptide would have a good prospect in the therapy of trauma and burns.

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