ISSN : 0974 - 7435 Volume 10 Issue 5

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 10(5), 2014 [1035-1041]

Expression of antimicrobial peptide dybowskin-2CDYa and hEGF in *pichia pastoris* and characterization of its dual function

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ABSTRACT

Dybowskin-2CD*Ya (Dy2*) with broad spectrum antibacterial activity is a novel antimicrobial peptide identified from skin of the frog *Rana dybowskii*. In order to get a dual function protein with inhibiting bacterial growth and promoting cell proliferation, we explored the fusion expression of Dy2 and human epidermal growth factor (hEGF) by yeast expression system. The fusion gene was constructed and cloned into the expression vector *pPIC9K*, transformed to competent *P. pastoris* GS115. The expressed fusion protein was purified from the culture supernatant by Sephadex G-50 and followed by C18 reverse phased HPLC and indentified by SDS-PAGE and antibacterial activity assay. Antimicrobial activity assay showed that the recombinant fusion protein could inhibit the growth of a broad spectrum of bacteria, while displaying a significant promoting effect on proliferation and migration of NIH3T3 cell lines compared with the control group. These results indicated that the expressed fusion protein was capable of killing baterial and promoting proliferation of cells, which suggest that it has a good prospect in the therapy of trauma and burns.

KEYWORDS

Antibacterial peptide;
Dybowskin-2CDYa;
Human epidermal growth factor;
Fusion expression;
Dual function protein fusion.

INTRODUCTION

Antibacterial peptides secreted by amphibian skin granular glands with broad spectrum antimicrobial activity and other biological functions contribute to the first line of innate immunity defense are low molecular weight peptides composed of 10-50 amino acid residues and usually positively charged[1]. Dybowskin-2CDYa(Dy2) is a novel antibacterial peptide identified from *Rana dybowskii* (SAVGRHSRRFGLRKHRKH, GenBank number: ACF08009.1), and shows low conservation with other amphibian's antibacterial peptides[2]. It has the potential to be peptide antibiotics with rich arginine, positive charge, high pI (12.60) as well as strong ability to inhibits the growth of a variety of Gram-negative (*Pseudomonas aeruginosa, Escherichia coli*) and Gram-positive (*Staphylococcus aureus, Listeria monocytogenes*)[3].

Human epidermal growth factor (hEGF) is one of growth promoting factors. It is a mitogen for epithelial and mesenchymal cells and one of the most powerful cytokine involved in promoting cell proliferation and dif-

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ferentiation[4]. hEGF have been widely and effectively applied in treating skin burns, scald, cornea injury, and gastric ulcer[5].

Main measures for trauma and burns is to prevent infection and promote wound healing[6]. In order to get a dual function protein which could inhibit bacterial growth and promote cell proliferation, the coding sequence of Dy2 and hEGF connected by the cutting sequence (LVPRAS) of the thrombin protein was constructed by PCR method. We aimed for expressing the fusion protein which could have the dual function and be used to treat trauma and burns.

MATERIALS AND METHODS

Restriction enzyme *EcoR I*, *Not I*, T4 DNA ligase, *pMD19T* plasmid, DNA markers were all purchased from TAKARA (Dalian, China). Tris, acrylamide, bisacrylamide, low molecular protein marker were obtained from Sigma Chemical Company. The yeast expression vector *pPIC9K* and *Pichia pastoris GS115*(*his-*) were ordered from Invitrogen. *Escherichia Coli* JM109 was purchased from Promega. *Bacillus cereus*(*1.126*) was purchased from China General Microbiological Culture Collection Center(CGMCC), NIH3T3 cell was ordered from ATCC, *Entro-hemorrhaguc E.coli O157*, *Streptococcus pneumoniae*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Salmonella typhi*, *Acinetobacter haemolyticus bauamnniiÿE.Coli* EBSL were provided by National Institute on Drug Abuse of China. Sequencing and primer synthesis were done by TAKARA (Dalian, China). All other chemicals used were of analytical grades.

METHODS

Gene amplification and construction of *pPIC9K-HDy2*

Dy2 gene was first identified from the skin of *Rana dybowskii* and cloned into plasmid *pMD19T* in our laboratory[2]. The coding sequence of mature peptide was obtained by the amplification of vector *pMD19T-Z73* (containing *Dy2* cDNA) with primers of Z73-1 and Z73-2. PCR reaction system were of 0.2 mL tubes in a final volume of 50 μL containing 5 μL 10×PCR buffer, 0.12 mM dNTPs, 0.4 μM of Z73-1 and Z73-2, 1 U Pfu Taq polymerase and 10 ng *pMD19T-Z73*, and the reaction condition was of 94 °C for 5 min, 30 cycles of 94°C for 30 s, 55 °C for 30s and 72 °C for 30s, and then 72 °C for 5 min. The recovered product was named $Dy2_{mp}$, which had the sequence of thrombin cleavage site at 5' end and *Not I* restriction site sequence at 3' end. The coding sequence of *hEGF* was constructed by Overlap Extension PCR[18] using the primers Ph1-Ph4, which were designed according to the cDNA sequence of the mature peptide *hEGF*(GenBank number: JQ346088.1). Part sequences of 4 primers were complementary in order to construct a complete sequence of hEGF named $hEGF_{50}$ with *EcoR I* at 5' end and the sequence of thrombin cleavage site at 3' end. After that, another amplification was performed by primers E1 and Z73-2 with the template of $hEGF_{50}$ and $Dy2_{mp}$ under the same conditions above. The recovered product was named *HDy2* which was digested and connected with *pPIC9K* using *EcoR I* and *Not I* sites at the end of HDy2. The constructed *pPIC9K-HDy2* was transformed into *E. coli* JM109 by electroporation and screened on lysogeny broth (LB)-ampicillin plates. Several bacterial colonies on agar plates were randomly chosen and cultured, and then plasmids were extracted which used as template for the amplification by universal primers of 5' α-factor (GAC TGG TTC CAA TTG ACA AGC) and 3'AOX (GCA AAT GGC ATT CTG ACA TCC). The presence of the *HDy2* gene was confirmed by the size of PCR production and DNA sequencing. The sequence of primers, the engineered restriction site, complementary sequence and termination codon were shown in TABLE 1.

Expression of HDy2

pPIC9K-HDy2 was linearized by *SacI* and transformed into spheroplasts of *P. pastoris* GS115 by electroporation system(MicroPulser, BIO-RAD, USA)at the conditions of 1500V, 25uF, 200&!, 3.8mS and 0.2cm cuvette. In order to select positive clones with multiple copies of the expression vector, Clones were planted onto RD plates containing G418 at concentrations of 0-20 mg/ml, and clones growing at the highest concentration of G418 were picked and

TABLE 1 : The primers sequence for antibacterial peptide HDy2 gene cloning

Primers	Sequence (5'-3')
Ph1	5'-AATAGTGACTCTGAATGTCCCCTGTCCCACGATGGGTACTGCCTCCATGATGGTG-3'
Ph2	5'-CATACTTGTCCAATGCTTCAATATACATGCACACACCATCATGGAGGCAGTACC-3'
Ph3	5'-TGAAGCATTGGACAAGTATGCATGCAACTGTGTTGTTGGCTACATCGGG-3'
Ph4	5'-CCACCACTTCAGGTCTCGGTACTGACATCGCTCCCCGATGTAGCCAACAACACAG-3'
E1	5'- CCGGAATTCAATAGTGACTCTGAATGTC -3'
E2	5'-AGAACCTCTTGGAACCAAGCGCAGTTCCCACCACTTCAGGTCTC- 3'
Z73-1	5'-TTGGTTCCAAGAGGTTCTTCTGCAGTAGGAAGACATAG - 3'
Z73-2	5' -ATAGTTTAGCGGCCGCTTAATGTTTTCTGTGTTTTC -3'

Single underlined bases are complementary sequences of the primer Ph1 and Ph2 , Ph3 and Ph4; double underlined bases are complementary sequences of the primer Ph2 and Ph3; The hatched section shows the restriction enzyme cutting site (*EcoR I* and *Not I*); the thrombin cleavage site are shown in bold; Wavy line bases show the position of the termination codon.

screened for expression. Selected clones were incubated at 28°C in 100ml of BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% YNB, 1% glycerol and 2×10^{-4}% Biotin) in a shake flask to $OD_{600} \approx 8$, and cells were harvested by centrifugation at 8000g for 10min and resuspended in 2L BMMY medium in a fermentor. Fermentation was maintained at 28°C, pH6.0, 30%-40% dissolved oxygen and added methanol to 1% every 24 h. Culture supernatants were got by centrifuging at 8000g, 4°C for 10 min, and the fusion protein were analysed by 10% SDS-PAGE and Coomassie Blue Staining[19].

Purification of HDy2

Culture supernatants were filtered through 0.45um nitrocellulose filter, and then EDTA was added to a final concentration of 10μM to inhibit the degradation of the HDy2 by metal-dependent proteases. The filtrate was concentrated to 10% of original volume by Rotary Vacuum Evaporator at 40°C and then loaded onto Sephadex G25(GE, Healthcare) column (2.6cm× 28cm) for desalting. The column was pre-equilibrated with distilled water containing 0.02% NaN_3 and then eluted with the same solution at a flow rate of 3ml/min. The eluent was monitored by absorbance at 280nm, and elution peak were collected and lyophilized, and then diluted to the concentration of 5mg/mL in 0.1% (V/V) TFA solution applied to RP-HPLC semi-preparative C18 column (10mm×150mm, 10μm, Beckman, USA). The column was eluted with the following condition at a flow rate of 1mL/min: 0min, 100%A (A: 0.1% Trifluoroaceic Acid, V/V in dH2O); 0-40min, 0%-40% B(B:0.1% Trifluoroaceic Acid, V/V in methanol). The eluent was monitored by absorbance at 214 nm. The peak fraction with antibacterial activity was lyophilized and its purity was examined by SDS-PAGE.

Antibacterial activity of HDy2

The purified HDy2 were screened for their antibacterial activity by using the cup-plate agar diffusion method[7]. Bacterium were cultured at 37°C in LB liquid medium, harvested in exponential phase (OD_{600}: 0.6-0.8), centrifuged at 8000g for 10min and diluted to 10^7 CFU/mL by normal saline solution, and then mixed to the 50°C sterile nutrient agar (0.5%, V/V) by gentle shaking. The Oxford cups (φ=8 mm) were placed onto the agar medium and filled with 100μL (0.2mg/mL) purified HDy2.

Cell proliferation assay of HDy2

In order to test the influence of HDy2 concentration and incubation time on NIH3T3 cell proliferation, NIH3T3 cells were seeded into 96-well plate(1.6×10^3/well) and cultured in DMEM medium (10% FBS, 5% CO2, 37°C and 24h), and then HDy2 was added into each wells to final concentration of 37.5μg/mL, 18.75μg/mL, 9.38μg/mL, 4.69μg/mL, 2.34μg/mL. 15 well cell were prepared for each concentration, and 5 were taken to test the proliferation rate by MTT method every 24 hours[9]

Wound healing assay in vitro

NIH3T3 cells were seeded into 6-well plate (1.0×10^6/well, 10% FBS in DMEM, 5%CO_2, 37°C),

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and a linear wound was generated in the monolayer with a sterile 200µl plastic pipette tip, and cells were washed twice to remove detached cells and debris. Six separate wounds were generated[9]. Size of wounds were observed and analyzed by software TScratch at 0h, 6h, 24h and 48h after treatment by HDy2.

Statistical analysis

The data (mean ± SD) of all the experiments were subjected to statistical analysis by one-way ANOVA(n=5 for Cell proliferation assay and n=6 for Wound healing assay. $P<0.05$ was considered to be statistically significant).

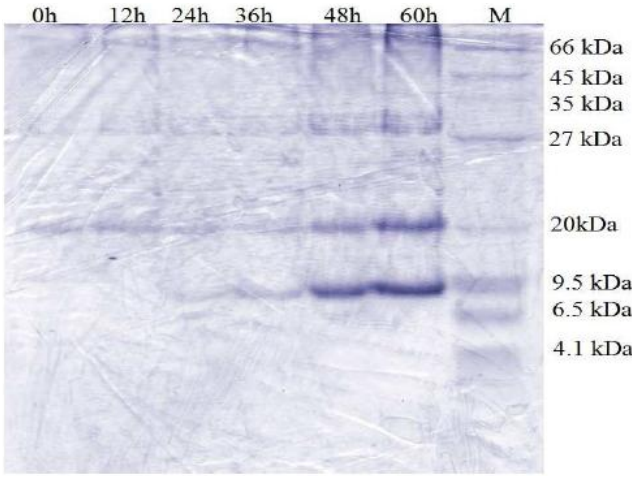
RESULTS

Gene clone and construction of expression vector *pPIC9K-HDy2*

The entire coding region of the fusion protein HDy2 was obtained by PCR amplification using 8 primers shown in TABLE 1. Amplified sequences were cloned into *E. coli* JM109 and confirmed by DNA sequencing. Then the HDy2 gene was connected into the yeast expression vector *pPIC9K*, and the inserted gene was confirmed by PCR and sequencing. Agarose electrophoresis appeared a 450bp band as expected and the sequencing result showed sequences and the ORF was consistent with designed. Positive yeast strains with HDy2 gene were selected by G418 and one was randomly selected for expressing the target protein.

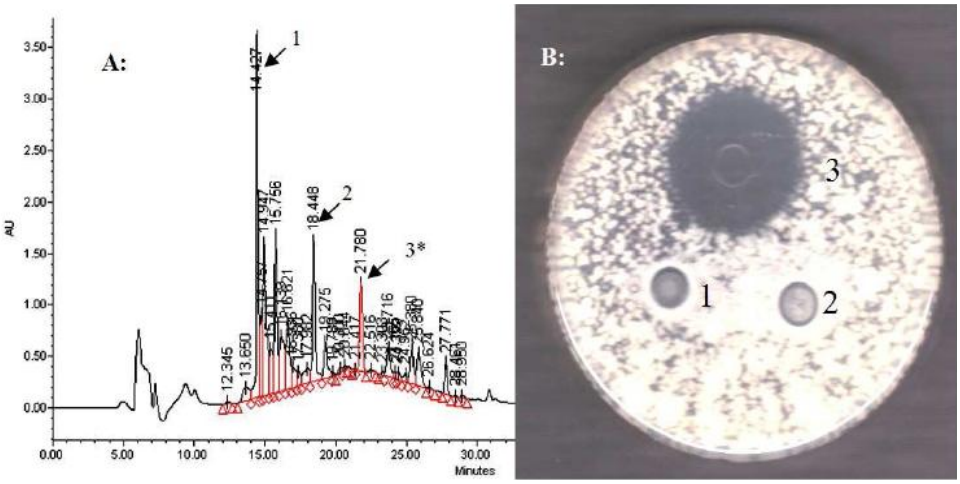
Expression of HDy2

Expression of the fusion HDy2 was investigated by detecting the presence of the recombinant protein in the supernatant of the cultured *P. pastoris*. The result showed that an approximately 8.8kD band was present in the supernatant of *P.pastoris* precipitated by TCA-acetone method after 24-60h and the target protein was not found in the 0-12h broth (Figure 1).



Samples were collected at 12h intervals after 0.5% methanol induction. 8.8 KD expression product was detected after 24h methanol induction. M:low molecular weight marker; 0-60h: fermentation time of recombinant *P. pastoris*.

Figure 1 : SDS-PAGE analysis of recombinant HDy2 expression at different induction times



The column (C18, 10mm×150mm, 5µm, Beckman, USA) was eluted with the following condition at a flow rate of 1ml/min: 0min, 100%A (A: 0.1%(V/V) Trifluoroaceic Acid (TFA) in dH2O); 0-40min, 0%-40% B(B:0.1%(V/V) Trifluoroaceic Acid (TFA) in methanol). The eluent was monitored by absorbance at 214nm and three mainly peak were collected(A). Only peak 3 exhibited antibacterial activity(B).

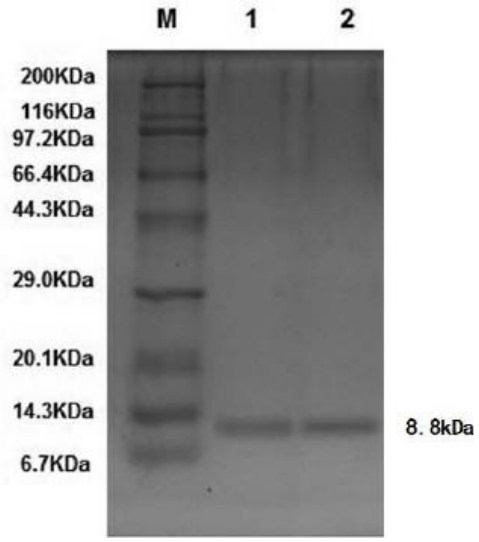
Figure 2 A: Purification of HDy2 by RP-HPLC with a C18 cloumn chromatography. B:Antibacterial activity of the mainly peaks shown in A

Purification of recombinant HDy2

Concentrating and desalting were needed before the RP-HPLC. Collected main peaks from RP-HPLC and examined the antibacterial activity. The result showed that the main peak 3 (retention time was 21.78min, and peak area ratio was 6.32%) had the antibacterial activity and other peaks were inactivity(Figure 2). Total protein of fermentation supernatant was 79 mg/mL detected by BCA method. The relative content of the recombinant protein was 6.32% by area normalization method and the expression quantity approximately 0.30 mg/mL though theoretical calculations. The main peak 3 was collected and lyophilized, and SDS-PAGE analysis result revealed a single band of about 8.8kD (Figure 3).

Antibacterial activity of HDy2

Bacterial inhibition zone method was applied to observe the antibacterial activity of HDy2, the results showed that HDy2 has different antibacterial activity against tested strains. The bacterial inhibitory effect on *Staphylococcus aureus* was the strongest (φ=22.0mm) and *Streptococcus pneumoniae* was the weakest(φ=8.4mm). The detail result was shown in TABLE 2.



Peak$_{21.78}$ collection of RP-HPLC chromatography was lyophilized, dissolved in distilled water and subjected to SDS-PAGE analysis. A single band of 8.8 kD was shown in the gel. M: low protein marker. 1,2: purified recombinant HDy2

Figure 3 : SDS-PAGE of purified recombinant HDy2

Proliferation activity of HDy2 on NIH3T3 cell

The proliferation rate of NIH3T3 cells was increased by HDy2 in the range of 18.75-37.50μg/mL at 24h and 72h group. But at 48h, proliferation rate were increased in 9.38-18.75μg/mL. The peak value all appeared in 18.75μg/mL at each time group, and the proliferation rate in 18.75μg/mL was significantly increased in comparison with control at 48h time group($P<0.01$) (Figure 4). When the concentration further increased or decreased, the proliferation rate did not add further.

Promoting cell migration of HDy2

Effect of HDy2 on migration of NIH3T3 cells was measured by wound healing assay. The speed of wound healing depends on the rate of cell migration. Based on the statistics analysis, the results showed that the wound closure rate of experimental group (18.75μg/mL) was more significantly improved than the control group at the time of 24h and 48h ($P<0.01$). Other high and low concentrations and longer culture time have been tested which didn't show much effect on cell proliferative property of fusion protein indicating the optimum concentration required to achieve significant cell proliferation.

DISSCUSSION

Antimicrobial peptides with broad-spectrum antibac-

TABLE 2 : Antimicrobial activity of HDy2 (0.20 mg/mL)

Tested strains	Result	Tested strains	Result
Bacillus cereus	++	*Klebsiella pneumonia*	+
Entro-hemorrhaguc E.coli O157	+	*Salmonella typhi*	++
Streptococcus pneumoniae	±	*Acinetobacter haemolyticus bauamnnii*	++
Staphylococcus aureus	+++	*E.coli* EBSL	+

The judgment standard of the anti-bacterial effect was as follow: judged to be negative(-) if the diameter of bacterial inhibition ring=8mm; 8mm<φ<10mm as critical value(±); 10mm<φ<15mm as weakly positive(+); 15mm<φ<20mm as positive (++); φ>20mm as strong positive (+++) .

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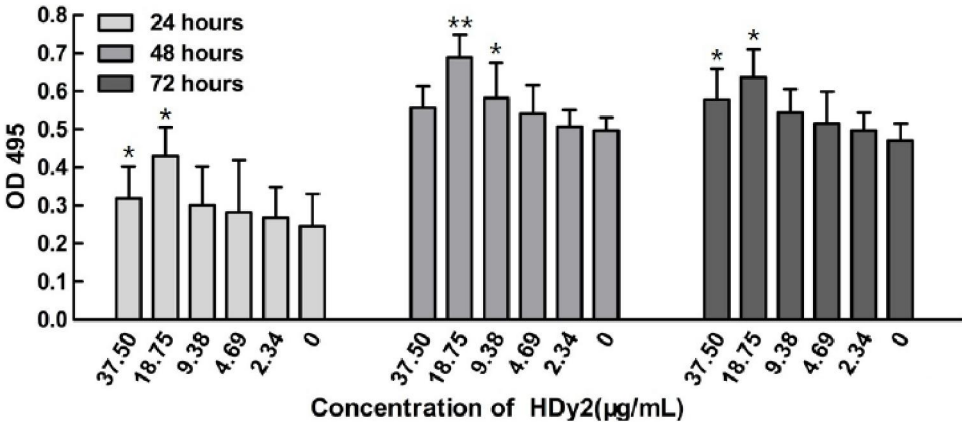
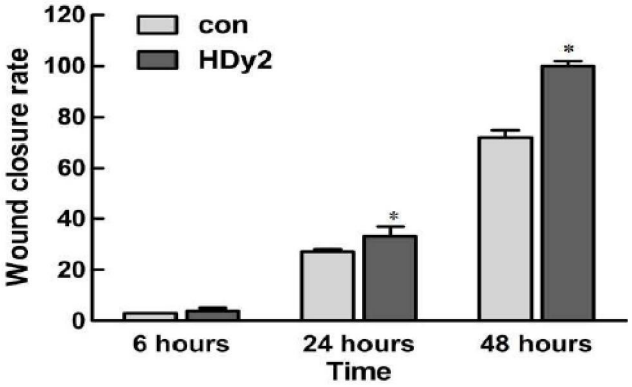


NIH3T3 cells were cultivated in DMEM medium and exposed to HDy2 (0μg/mL-37.50μg/mL). Proliferation of NIH3T3 cell was analyzed by MTT method at 24h, 48h and 72h. The group without HDy2 (0μg/mL) was taken as control. * P<0.05, ** P<0.01

Figure 4 : MTT result showed effect of HDy2 on NIH3T3 cell proliferation at different concentrations and incubation time

terial and antifungal abilities have a much clinical prospect as external antimicrobial agents for burns and other wound[10]. Chalekson' results showed that the survival rates of D2A21-treated rats were significantly higher than control by the rat model of Skin Wounds with *Pseudomonas aeruginosa* infection, and moreover the eschar in the experimental group showed no bacterial growth, while the control group showed numerous bacteria[11,12]. Szabo et al. found that antibacterial peptide A3-APO was effective against systemic infections *of Escherichia coli* in different mouse models[13].

Human epidermal growth factor (hEGF), a major cytokine involved in wound healing, has been applied in wound therapy[14,5]. So we designed the fusion protein HDy2 which should increase the molecular weight of Dy2 to prevent its degradation during expression,



Experimental group had higher wound closure rate than control group at 24h and 48h and the difference was significant (*$P<0.05$). Con, control group; HDy2,experimental group(18.75μg/mL HDy2)

Figure 5 : Effect of HDy2 on migration of NIH3T3 cells with wound healing assay

because the low molecular weight peptides with their potential toxicity to the host were difficult to achieve high level of expression[15]. It could be dual function of prevent infection and promote wound healing.

The result of this paper showed that expressed fusion protein HDy2 did not affect Dy2' antibacterial activity. This phenomenon suggested that the activity domain of Dy2 was not disturbed by fusing hEGF in its N-terminal, which was different from that antimicrobial peptides would be inactivated by adding 3 amino acids[16].

To test hEGF' activity in the expressed fusion protein, the effect of HDy2 on proliferation and migration of NIH3T3 was studied by the method of MTT assay and wound-healing assay. The later is simple, inexpensive, and one of the earliest developed methods to study directional cell migration in vitro[17]. The result demonstrated that proliferation and migration of NIH3T3 were significantly enhanced by HDy2. Accordingly, by inhibiting infection and promoting fibroblast proliferation and migration, the fusion peptide HDy2 are respected to be applied for treating acute and chronic wound, especially for that with infection.

ACKNOWLEDGMENT

Authors gratefully acknowledge the financial support of this study by Doctor Supporting Foundation of Liaoning University and National nature science foundation of China (31071916).

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