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ORIGINAL ARTICLE

Expression and purification of antimicrobial peptide CM4 by N^{pro} fusion technology in *E. coli*

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Abstract Antimicrobial peptide CM4 is a small cationic peptide with broad-spectrum activities against bacteria, fungi, and tumor cells. Different strategies have been developed to produce small antibacterial peptides using recombinant techniques. To date, no efforts to obtain large quantities of active recombinant CM4 have been reported. In order to establish a bacterium-based CM4 production system, CM4 was cloned into pET28a and expressed with N^{pro} mutant (EDDIE) fusion. CM4 expressed as EDDIE are deposited as inclusion bodies. On in vitro refolding by switching from chemotropic to kosmotropic conditions, the fusion partner is released from the C-terminal end of the autoprotease by self-cleavage, leaving CM4 protein with an authentic N terminus. Purified CM4 was separated on Ni^{2+}-chelating chromatography column and cation-exchange chromatography column. Mass spectroscopic analysis indicated the protein to be 4132.56 Dalton, which equalled the theoretically expected mass. N-terminal sequencing of CM4 showed the sequence corresponded to the native protein. The recombinant CM4 exhibited the same antimicrobial and anti-tumor activity as reported previously. The expression strategy presented in this study allows convenient high yield and easy purification of recombinant CM4 with native sequences.

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Introduction

In the past decades, the increasing use of antimicrobial in humans, animals, and agriculture has resulted in many pathogens developing resistance to these powerful drugs and made many infectious diseases be increasingly difficult to treat (Greenwood 1998; Snary et al. 2004). Therefore, development of novel antimicrobial agents is still urgently needed. Antimicrobial peptides are an evolutionarily conserved component of the innate immune response, which is the principal defense system for the majority of living organisms ranging from prokaryotes to humans (Hancock 2001; Zasloff 2002). At present, more than 1,000 antimicrobial peptides have been found and a number of antimicrobial peptides were isolated and purified from various species such as mellitin and cecropin (Bulet et al. 2004).

CM4, an antibacterial peptide first isolated from the hemolymph of the silkworm Bombyx mori by our group (Zeng et al. 1989), belongs to the cecropins family. It is a highly cationic peptide consisting of 35 amino acids, and it shows strong activities against several bacteria and fungi (Li and Zhang 2007; Chen et al. 2008; Zhou et al. 2009). In addition, CM4 can kill some tumor cell lines, while exhibiting no toxicity toward normal human leucocytes at the same concentration (Jia et al. 1996; Zhang and Dai 1997). Its wide range of activities provides the possibility that CM4 can be used as an antimicrobial and anticancer agent in the future.

The biosynthesis of recombinant antimicrobial peptides in vivo is currently the most popular technique of preparing polypeptides. The *E. coli* expression system is still

the most commonly used because of its high level of expression, the relative simplicity of the DNA manipulations, and the short time required to produce product. However, alleviating the toxicity and proteolytic degradation of an expressed antibacterial peptide in the *E. coli* system and purifying the peptide easily are common problems. Many fusion partners have been used to express antimicrobial peptides, including RepA protein, F4 fragment of PurF, thioredoxin, green fluorescent protein (GFP), and protein PaP3.30 (Zhang et al. 1998; Skosyrev et al. 2003; Barrell et al. 2004; Pyo et al. 2004; Rao et al. 2004; Li et al. 2006). However, the fusion partners must be cleavaged by specific protease. These procedures lead to additional processing steps.

N^{pro} fusion technology is based on the autoprotease N^{pro} derived from classical swine fever virus or its mutant EDDIE as an N-terminal self-cleavage fusion tag (Stark et al. 1993; Clemens et al. 2007). Mutation of the N^{pro} protein to produce the engineered variant EDDIE led to profound improvement of aggregation behavior and cleavage activity. EDDIE exhibits the unique feature of autoproteolytic activity at its own C-terminus (Stark et al. 1993). Upon activation in its native form, it releases the fused target protein with a distinct N-terminus. The N^{pro} expression system represents a widely applicable tool for high-level expression of recombinant peptides and proteins without the need for chemical or enzymatic removal of the tag. In particular, toxic and difficult-to-express proteins have been expressed at high titers. Furthermore, authentic N-termini have been produced for all investigated fusion partners (Stark et al. 1993).

In this study, we tried to use the autoproteolytic function of N^{pro} from classical swine fever virus to produce CM4 in *E. coli*. CM4 expressed as EDDIE fusions are deposited as inclusion bodies. On in vitro refolding by switching from chemotropic to kosmotropic conditions, the fusion partner is released from the C-terminal end of the autoprotease by self-cleavage, leaving the target protein with an authentic N-terminus.

Materials and methods

Strains, plasmids, culture medium, and enzymes

Plasmid pET30a-N^{pro} mutant EDDIE (pET30a/EDDIE) was donated by Dr. Bernhard Auer (Institute of Biochemistry, University of Innsbruck, Austria). *E. coli* DH5a was used as the host for subcloning and plasmid amplification. *E. coli* BL21(DE3) was used as the host for expression of heterogonous protein. *E. coli* $K_{12}D_{31}$ was used in antimicrobial assay of CM4. pET28a (Novagen, USA) was used as the expression plasmid. T4 DNA ligase, restriction enzymes NdeI, SpeI, and SalI were purchased from Takara (Dalian, China).

Construction of EDDIE-CM4 fusion expression plasmid

Two primers P1(5′-GGACTAGTTGCCGTTGGAAGATC-3′)and P2(5′-ACGCGTCGACTTAGATAGTGGCAG-3′) were designed to amplify the CM4 gene. To allow the amplified fragment to be ligated into the pET30a/EDDIE vector and facilitate cleaving of the fusion protein, The primer P1 incorporated a SpeI site containing the cystein 168 of the autprotease site and the primer P2 incorporated a SalI site and a translational termination codon at their 5′-terminus. After CM4 gene was ligated into pET30a/EDDIE vector. The resulting plasmid, pET30a/EDDIE-CM4, was transformed into *E. coli* DH5a, and positive clone was sequenced to ensure that the coding sequence was correct. In addition, because His tag is beneficial for the fusion protein separated by Ni^{2+}-chelating affinity chromatography, EDDIE-CM4 gene fragments digested by NdeI and SalI were subcloned into pET28a and the resulting plasmid pET28a/His-EDDIE-CM4 was transformed into *E. coli* BL21(DE3) to express of heterogeneous protein. The scheme for the construction of expression vector is illustrated in Fig. 1.

Expression and purification recombinant fusion protein

Plasmid pET28a/His-EDDIE-CM4 was transformed into *E. coli* BL21(DE3), and the bacteria were grown at 37°C in 50 ml LB medium (1% Bacto-tryptone, 0.5% yeast extract, and 85 mM NaCl) with 50 μg/ml kanamycin. When the OD_{600} reached 0.6, 1 mM Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was subsequently added and the culture was incubated at 37°C for 5 h. To purify His-EDDIE-CM4, bacteria were harvested by centrifugation at 8,000*g* for 10 min at 4°C and then suspended in 10 ml washing buffer (50 mM Tris–HCl, 5 mM EDTA, and 1% Triton X-100, pH 8.0). After repeated rounds of sonication, the solution was centrifuged at 12,000 g and then the supernatant was decanted and the precipitate pellet was solubilized in 2.5 ml of denaturation buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM imidazole, and 8 M urea, pH 8.0), The cellular debris were removed by centrifugation at 4°C for 15 min at 10,000*g*. The supernatants were applied to Ni^{2+}-IDA His-bind resin and bound His-EDDIE-CM4 was recovered according to the manufacturer's instructions.

Optimization of refolding conditions

To enhance refolding efficiency of inclusion bodies, some key factors that affect the cleavage rates of His-EDDIE-CM4 were optimized, such as pH, salt concentrations, and

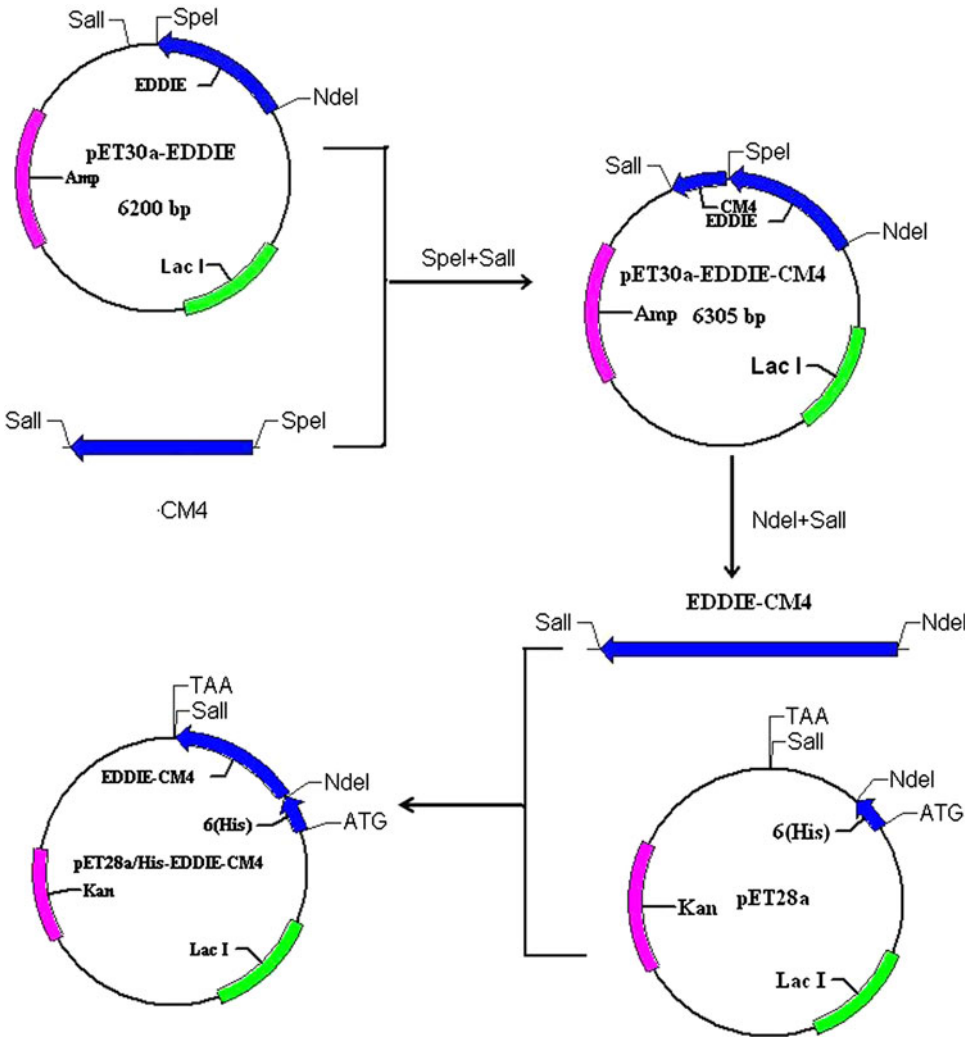

Fig. 1 The schematic representation of the fusion constructions of pET28/His-EDDIE-CM4

refolding temperature. His-EDDIE-CM4 inclusion bodies were rapidly diluted 1:50 into 1 ml refolding buffer (different pH, salt concentrations) and incubated at different temperature without string. SDS–PAGE assay was performed to analyse the cleavage rates of the fusion protein. The images of gels were scanned by Gel-Doc 2000 gel documentation system (Bio-Rad, USA) and analyzed by using QuantityOne software,Version 4.4.0 (Bio-Rad, USA).

Release and purification of His-EDDIE-CM4

Purified His-EDDIE-CM4 inclusion bodies (400 mg) were diluted 1:20 into 2l the optimized refolding buffer and incubated at appropriate temperature without string. After refolding, the insoluble sample was filtered through 0.45 μm membrane. The soluble sample was ultrafiltrated (at 20°C) in a bench-top system (Milliopre Labscale TM TFF System, Millipore Corporation, USA) with a cut-off of 10 kD membrane (Millipore Corporation, USA) to about half of its initial volume. Then the ultra-filtrated sample was purified on Ni^{2+}-IDA His-bind resin and unbound CM4 were recovered according to the manufacturer's instructions. Recombinant CM4 was further purified on a 5-ml CM Sepharose HP column (Amersham Pharmacia, USA). 20 ml of refolded protein was injected onto the column, which was previously equilibrated with 6 column volumes of 20 mM PBS, pH 6.0. CM4 was eluted from the column with a 10-volume linear gradient from 20 mM PBS, pH 6.0 to 20 mM PBS, 1 M NaCl, pH 6.0 at the rate of 1 ml/min. Absorbance at 280 nm was monitored with a UV detector connected to a data acquisition package. Experiments were carried out at 4°C and CM4 containing peaks were identified by Tricine SDS–PAGE analysis.

N-terminal sequence and mass spectroscopic analysis of CM4

Following SDS–PAGE electrophoresis of the purified CM4, the resolved protein was transferred on a polyvinylidene fluoride (PVDF) membrane. The appropriate

PVDF band was subsequently sequenced using Edman degradation with the aid of an Applied Biosystems Model Procise automated protein sequenator 491 (Applied Biosystems, USA). Stepwise liberated phenylthiohydantoin (PTH) derivatives were identified using an online 120A high-performance liquid chromatography (HPLC) system equipped with a PTH C18 (2.1 × 220 mm; 5 μm particle size) column. HPLC analysis of the recombinant CM4 was carried out on a HPLC system (Agilent 1100 LC, USA) using a C18 reverse-phase column; the sample was eluted using a gradient of water and acetonitrile over a period of 36 min. Absorbance was monitored at 280 nm. The peak centered at 5.6 min was collected and lyophilized. The lyophilized material was dissolved in Milli-Q water, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy was performed on Applied Biosystems Voyager DE Pro (ABI, USA).

Antimicrobial activity assay

Antimicrobial activity of recombinant CM4 was detected using a radial diffusion assay. Briefly, $K_{12}D_{31}$ was grown to the mid-logarithmic phase and washed. Approximately 2×10^6 cfu/ml was incorporated into a thin (1.2 mm) agarose underlay gel that contained 1% (wt/vol) agarose. A gel punch was used to make evenly spaced wells. For the assay, 50 μl of solution containing purified CM4 with concentration of 0.5 mg/ml in 20 mM PBS was added to each well. After the plates were incubated for 12 h at 37°C, the diameter of the clear zone surrounding each well was measured to evaluate the antimicrobial activity. In the meantime, PBS buffer were also detected as the negative control. The above assays were performed in triplicate.

K_{12} D_{31} growth curve was also tested. About 2×10^6 cfu/ml *E. coli* $K_{12}D_{31}$ growing in log phase was inoculated into 1 ml of LB medium. Purified recombinant CM4 was added at various concentrations, and PBS buffer was also added as negative control. After shaking for 12 h (37°C, 220 rpm), bacterial growth was determined by measuring OD_{600}.

Cancer cell viability assay

The SHG-44 human glioma cell line was obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. SHG-44 cells were cultured in DMEM medium till mid-log phase, then seeded in 96-well plate at a density of 1×10^4 cells per well in 100 μl medium. After 24 h of incubation, SHG-44 cells were treated with 10, 20, 30, 40, and 50 μg/ml CM4 for 48 h. After treatment, 10 μl of 5 mg/ml MTT was added and the cells were incubated further for 4 h at 37°C. The supernatant was discarded and 100 μl of DMSO was added to each well. The mixture was shaken on a mini shaker at room temperature for 10 min and the spectrophotometric absorbance was measured by Multiskan Spectrum Microplate Reader (Thermo) at 570 and 630 nm (absorbance 570 nm, reference 630 nm). Triplicate experiments were performed in a parallel manner for each concentration point and the results were presented as mean ± SD. The net $A_{570\ \mathrm{nm}} - A_{630\ \mathrm{nm}}$ was taken as the index of cell viability. The net absorbance from the wells of cells cultured with 0.1% DMSO was taken as the 100% viability value. The percent inhibition of the treated cells was calculated by the following formula:

$$\begin{aligned}\%\ \text{Inhibition} &= \left[(A_{570\,\mathrm{nm}} - A_{630\,\mathrm{nm}})_{\text{control}}\right. \\ &\quad \left. -(A_{570\,\mathrm{nm}} - A_{630\,\mathrm{nm}})_{\text{treated}}\right] \\ &\quad /(A_{570\,\mathrm{nm}} - A_{630\,\mathrm{nm}})_{\text{control}} \times 100\%.\end{aligned}$$

Results

Construction of His-EDDIE-CM4 fusion expression plasmid

After PCR amplification, the target DNA fragments (147 bp) were successfully obtained and then subcloned into the prokaryotic expression vector pET30a. The results of restriction digestion showed that the target fragment had been successfully inserted into the vector, and the following DNA sequencing also validated that the EDDIE gene and CM4 coding sequences were combined into one open reading frame (data not shown). To facilitate purification of the fusion protein by affinity chromatography, EDDIE-CM4 gene was digested by NdeI and SalI and subcloned into plasmid pET28a. The final determination was confirmed by digestion with restriction endonucleases and sequencing. The presence of a poly-histidine tag at the N-terminal of the product ensures simple purification and detection. The construction of the plasmid is shown in Fig. 1.

Expression and purification of His-EDDIE-CM4 fusion protein

Escherichia coli BL21(DE3) cells harboring pET28a/His-EDDIE-CM4 were induced by IPTG, and the expression of the His-EDDIE-CM4 protein was analyzed by SDS–PAGE with Coomassie brilliant blue R-250 staining. A predominant protein of 30 kDa, corresponding to the expected molecular size of His-EDDIE-CM4 was observed (Fig. 2, lane 2). Most of the His-EDDIE-CM4 protein

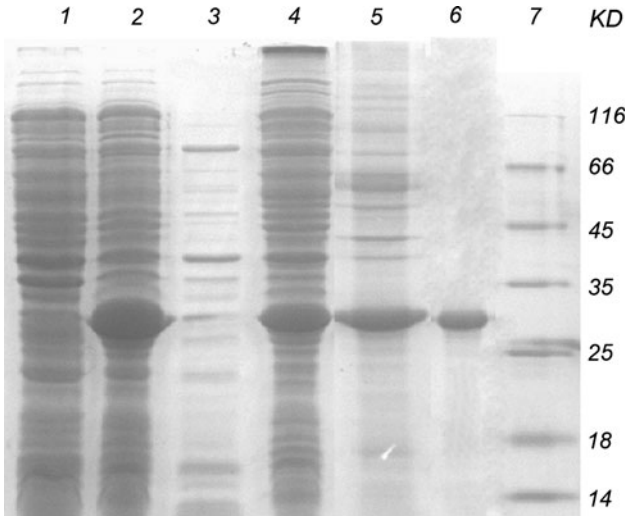

Fig. 2 SDS–PAGE analysis of recombinant His-EDDIE-CM4 expressed in *E. coli* BL21. *Lane 1* crude cells extracts of uninduced *E. coli* BL21 containing pET28/His-EDDIE-CM4; *lane 2* crude cells extracts of induced *E. coli* BL21 containing pET28/His-EDDIE-CM4; *lane 3* sonicated supernatant of induced *E. coli* BL21 containing pET28/His-EDDIE-CM4; *lane 4* inclusion bodies of induced *E. coli* BL21 containing pET28/His-EDDIE-CM4; *lane 5* inclusion bodies dissolved with 8 M urea; *lane 6* purified inclusion bodies on Ni^{2+}-IDA His-bind resin; *lane 7* protein molecular weight marker

retained in the insoluble fraction (Fig. 2, lane 4). After washing with 2 M urea, the inclusion bodies were dissolved in solubilizing buffer (8 M urea, 50 mM Tris–HCl, 25 mM DTT, pH 7.5). About 462 mg of fusion protein was obtained from 1 l of culture medium. After denaturation, His-EDDIE-CM4 protein was purified in a single purification step on a Ni^{2+}-IDA His-bind resin. The result of SDS–PAGE showed that the fusion protein His-EDDIE-CM4 could be eluted efficaciously from the column with elution buffer containing 100 mM imidazole and reached an apparent purity of 95% (Fig. 2, lane 6).

Optimization of refolding conditions of His-EDDIE-CM4

The cleavage rates of His-EDDIE-CM4 were initially determined by screening an array of pH, salt concentrations, and refolding temperature in 1.5 ml eppendorf tube. As shown in Table 1, pH and salt concentrations play an important role in the cleavage rates of His-EDDIE-CM4. When pH and salt concentrations were 8.5–9.5 and 500 mM, respectively, the cleavage rates and solubility of His-EDDIE-CM4 were higher than other conditions. At the same time, when refolding time was 3 days, the cleavage rates of His-EDDIE-CM4 were next to 50% (Fig. 3). In our study, the buffer also contained 0.2 M Arginine, 5% Glycerol, and Tween-20 in order to prevent them from forming aggregate through hydrophobic interaction. In summary, 500 mM NaCl, 20 mM Tris, 2 mM EDTA, 5% Glycerol, 0.2 M Arginine, 10 mM DTT, 0.01% Tween-20, and pH 8.5–9.5 were chosen as optimal condition.

Table 1 Optimization of refolding conditions of His-EDDIE-CM4

pH	4 °C				25°C			
	6.5	7.5	8.5	9.5	6.5	7.5	8.5	9.5
500 mmol/l	0.065[a,b]	0.063	0.127	0.195	0.080	0.040	0.034	0.033
400 mmol/l	0.050	0.048	0.069	0.151	0	0	0	0
300 mmol/l	0.030	0.024	0.108	0.156	0	0	0	0
200 mmol/l	0.009	0.006	0.025	0.052	0	0	0	0
100 mmol/l	0	0	0	0	0	0	0	0

[a] The rate of cleavage was calculated according to the proportion of EDDIE-CM4 and EDDIE

[b] Protein concentration was determined by Nanodrop 1000 (Themer) according to extinction coefficient of proteins

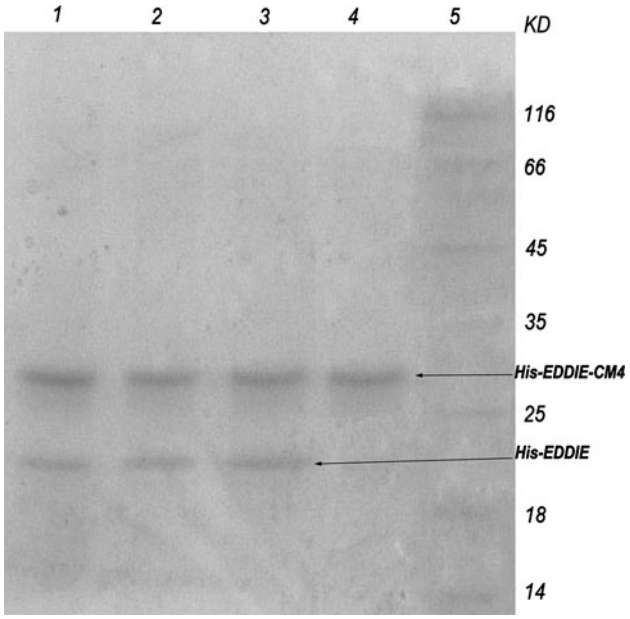

Fig. 3 Cleavage rates of inclusion bodies at different refolding time. *Lane 1* samples after self-cleavage at 4°C for 24 h; *lane 2* samples after self-cleavage at 4°C for 48 h; *lane 3* samples after self-cleavage at 4°C for 72 h; *lane 4* His-EDDIE-CM4 before self-cleavage; *lane 5* protein molecular weight marker

Purification of recombinant CM4

Supernatants were loaded onto an affinity column. His-EDDIE-CM4 and His-EDDIE proteins binded to Ni-NTA agarose. CM4 protein that unbinded to agarose was collected.

Refolded CM4 was further purified by CM-Sepharose ion exchange chromatography with most of the CM4 tightly bound to the column. CM4 were obtained after elution with a linear gradient of NaCl (0.05–1 M; Fig. 4). The purity at this stage was approximately 90% as revealed

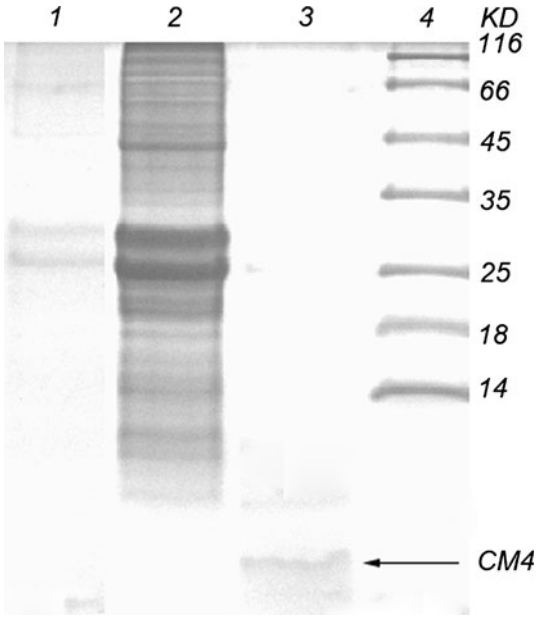

Fig. 4 Refolding and purification of CM4. *Lane 1* autocleavage of His-EDDIE-CM4 in 2l of the refolding buffer; *lane 2* the concentrated protein after ultra-filtrated with 10 kDa membrane; *lane 3* the purified CM4 after cation exchange chromatography; *lane 4* molecular weight protein marker

Table 2 Isolation of recombinant CM4 from EDDIE-CM4 fusion protein (Based on 1l of bacterial culture. The wet weight of cells is 1.5 g)

Purification step	nPro-CM4 (mg)	CM4 (mg)	Purity (%)[b]
Inclusion bodies	462[a]	/	>95
After refolding[c]	199[a]	20[a]	/
After ultrafiltration concentration	/	10[a]	>80
After Ni^{2+} affinity and IEX chromatography	/	6[a]	>90

[a] Protein concentration was determined by Nanodrop 1000 (Themer) according to extinction coefficient of proteins

[b] Purity of protein or peptide was estimated by SDS-PAGE stained by Coomassie blue

[c] The rate of solution of inclusion bodies was 43% in 2L refolding buffer

by Tricine SDS–PAGE analysis. Details of the purification of CM4 are summarized in Table 2. We obtained 6 mg of the pure CM4 protein from 1 l of the culture.

N-terminal amino acid sequence analysis

In order to confirm its authenticity, the CM4 protein was subjected to N-terminal amino acid sequencing, according to the sequencing result; the first 12 amino acids of N-terminal of CM4 were R-W-K-I-F-K-K-I-E-K-V-G, the same as the native CM4 sequence. On the other hand, the result of amino acid composition analysis indicated that the amino acid composition of the CM4 was also in accordance with the anticipated composition (data not shown). Moreover, the molecular weight of cleaved CM4 analyzed by MALDI-TOF mass spectroscopy was 4,132.56 Dalton, which was similar to the theoretical molecular weight of CM4 (4,135.68 Dalton).

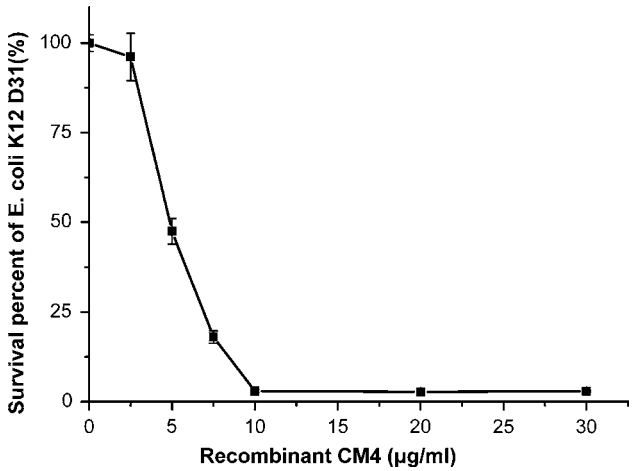

Fig. 5 *E. coli* $K_{12}D_{31}$-inhibition curve in liquid culture. *E. coli* $K_{12}D_{31}$ was used as the sensitive strain. Triple experiments were carried out, and the average values and variations (*error bars*) are presented

Bioactivity assay of recombinant CM4

To examine the antimicrobial activity of recombinant CM4, it was added into the culture of the sensitive *E. coli* strain. The concentration killing-curve is shown in Fig. 5. The growth of *E. coli* $K_{12}D_{31}$ was dramatically suppressed with the increasing concentration of recombinant CM4.

Antimicrobial activity of the purified recombinant CM4 was also detected with a radial diffusion assay. As shown in Fig. 6, the purified CM4 showed a high bactericidal activity against K_{12} D_{31} (spot 1), while no inhibition zone was seen around the spots of negative control (spot 2). It was obvious that the recombinant CM4 was bioactive and very effective in killing the sensitive strain.

To test the effect of CM4 on the proliferation of SHG-44 cells, the cells were treated with different concentrations of CM4. After 48 h of incubation, the cell viability was measured by MTT assay. CM4 treatment significantly inhibited the growth of SHG-44 cells; the number of viable cells decreases as the concentration of CM4 increases (Fig. 7). The effect of CM4 treatment is statistically significant when compared with the control group ($P < 0.05$).

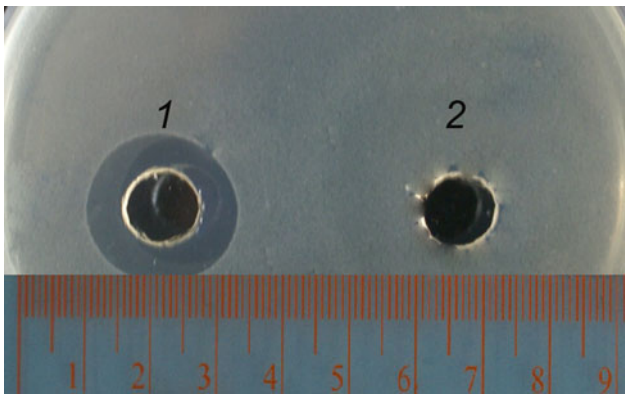

Fig. 6 Antibacterial activity of recombinant CM4 against *E. coli* $K_{12}D_{31}$ by agar well diffusion assay. 1, 50 µl of 500 µg/ml CM4; 2, PBS as the negative control

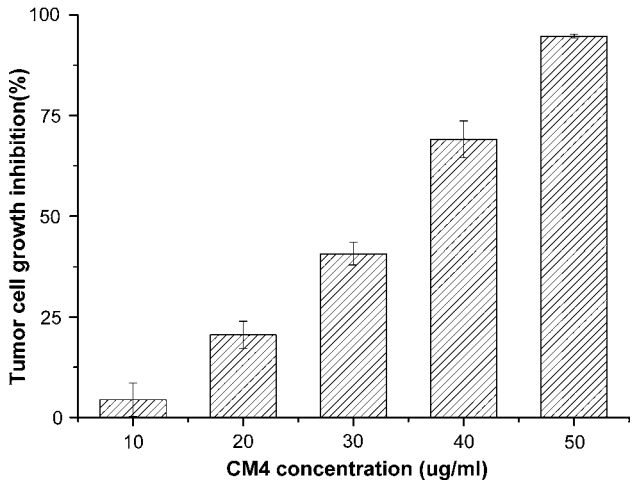

Fig. 7 Inhibitory effect of CM4 on the cell proliferation of SHG-44 cells. The results shown were the mean of three parallel experiments (triplicate wells) for each concentration point. Results were presented as the mean ± SD. The group of PBS added was used as the negative control

Discussion

Production of peptide for preclinical and clinical evaluation often requires multigram quantities. However, researchers are always troubled to express antibacterial peptidesis in *E. coli* for its antibacterial activity. Nowadays, Several expression systems (Inteins and thioredoxin) have been developed by fusing the antibiotic peptide with a partner protein or with anionic properties to avoid the toxicity of the heterologous protein to the host cells and the degradation of the products by innate protease (Lee et al. 1998; Fang et al. 2002). In our previous work, the strategy of fusing CM4 with the thioredoxin (TrxA) proved to be efficient to achieve high-level soluble expression in *E. coli* (Zhou et al. 2009). Cleavage of the expression system needs adding hydroxylamine hydrochloride into cleavage buffer. In addition, there is a glycine attached to the N-terminus of CM4 after cleavage.

Recombinant product with special respect to the N-terminus is important for proteins used in medical applications. N or C-terminal fusion tags must be removed either enzymatically with specific proteases or by the use of intein self-cleavage systems, since incomplete cleavage by methionine aminopeptidase may lead to intolerable microheterogeneity of the product and may even change the product's functionality and stability (Rene et al. 2009; Boix et al. 1996). These procedures lead to additional processing steps. N^{pro} system which is the N-terminal autoprotease N^{pro} can produce peptides and proteins with authentic N termini. The N^{pro} show several distinct features in protein expression(Clemens et al. 2007). Based on the above knowledge, we applied N^{pro} system to express CM4 fusion protein in *E. coli*. In the present work, CM4 gene was fused with N^{pro} gene for the purpose of eliminating the intrinsic antibacterial activity. The high percentage of target fusion protein in total proteins was obtained in *E. coli*. The expression level of CM4 fusion protein was greatly improved when conventional culture was effectively induced at a suitable growth phase with an optimal IPTG concentration at 37°C. Target protein CM4 was released by N^{pro} system autoproteolysis in vitro refolding and then was further purified by ion exchange chromatography. Activity assay showed recombinant CM4 had good antimicrobial and antitumor activity.

Overall, our protocols for preparation of recombinant CM4 described in this article offer an alternative way of producing large quantities of antibacterial peptides. However, the cleavage rate of His-EDDIE-CM4 fusion protein reached only 50%, which was caused by the first amino acid of CM4. How to enhance the cleavage rate of His-EDDIE-CM4 fusion protein will be further studied. We believe that the advantages of the protocols presented here are high expression yields and low-cost purification procedures. These results presented here provide a simple and reliable strategy for generating large quantities of active CM4 and should serve as a solid starting point for developing a method to express antibacterial peptides on a large scale.

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