

SUMO Mediating Fusion Expression of Antimicrobial Peptide CM4 from two Joined Genes in *Escherichia coli*

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Abstract Antibacterial peptide CM4 (ABP-CM4) is a small cationic peptide with broad-spectrum activities against bacteria, fungi, and tumor cells, which may possibly be used as an antimicrobial agent. To improve the expression level of CM4 in *Escherichia coli*, two tandem repeats of CM4 genes were cloned into the vector pSUMO to construct an expression vector pSUMO–2CM4. The fusion protein SUMO–2CM4, purified by Ni²⁺-chelating chromatography, was cleaved by hydroxylamine hydrochloride to release recombinant CM4. After the cleaved sample was re-applied to a Ni-IDA column, finally, about 48 mg recombinant CM4 was obtained from 1 L bacterial culture with no less than 96% purity, which was the highest yield of CM4 reported so far.

Introduction

The increasing resistance of bacteria and fungi to currently available antibiotics is a major concern worldwide, leading to enormous efforts to develop new antibiotics with new modes of actions [5]. One potential source of novel antibiotics is the antimicrobial peptides which are relatively small molecules that are less than 100 amino acids in

length and have a broad spectrum of antimicrobial activity. They serve as an ancient defense mechanism against pathogenic microorganisms that easily come in contact with the host through the environment [4, 11].

Cecropins are positively charged peptides that were originally isolated from the blood lymph of the giant silk moth (cecropins A and B) and, successively, from the small intestine of the pig (cecropin P1). Cecropins have the ability to form specific amphipathic alpha-helices which allow them to target nonpolar lipid cell membranes. Upon membrane targeting, they form ion-permeable channels subsequently resulting in cell depolarization, irreversible cytolysis and finally death [1]. ABP-CM4, an antibacterial peptide isolated from the hemolymph of the silkworm *Bombyx mori*, belongs to the cecropins family. It is a highly cationic peptide consisting of 35 amino acids and it kills bacteria, fungi, and cancer cells by permeabilizing the cell membrane without being toxic to mammalian cells [3, 12, 13]. Its wide range of activities provides the possibility that ABP-CM4 can be used as an antimicrobial and anticancer agent in the future.

Because of their natural destructive behavior toward microorganisms and relative sensitivity to proteolytic degradation, antimicrobial peptides are often produced by being fused to a fusion partner in heterologous hosts to neutralize their innate toxic activity and increase their expression levels. Many fusion partners have been used to express and purify antimicrobial peptides, including F4 fragment of PurF [7], thioredoxin [14], green fluorescent protein (GFP) [10], and protein PaP3.30 [8]. However, the effects of the affinity tag on different target proteins differ and no affinity tag appears to be generally superior for all recombinant proteins.

In this work, two tandem CM4 genes were fused to SUMO, and the monomer CM4 was obtained by cleaving

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the SUMO–2CM4 with hydroxylamine that specifically cleaves between the amino acid residues Asn–Gly. The SUMO fusion system and customized expression and purification protocol described here have further improved the efficiency and lowered the costs of producing antimicrobial peptide CM4.

Materials and Methods

Bacterial Strains, Vectors, and Enzymes

Escherichia coli DH5 α (maintained in our laboratory) was used for subcloning and plasmid amplification. *Escherichia coli* BL21 (DE3) (Novagen, USA) was used as the expression host. *P. aeruginosa* (ATCC 27853) and *Penicillium chrysogenum* (AS 3.564) were obtained from Institute of Microbiology Chinese Academy of Sciences (IMCAS). *E. coli* K₁₂D₃₁ was conserved by our laboratory. The linearized pSUMO vector with *Stu*I and *Hind*III restriction sites was purchased from LifeSensors (LifeSensors, Malvern, PA). All the restriction enzymes and T4 DNA ligase were purchased from Takara Biotech Co. Ltd (Dalian, China).

Construction of Expression Vectors

To obtain a gene encoding two tandem CM4 genes, two oligonucleotides pSUMO-F (5'-TAACGGCCGTTGGAA GATCTTTAAG-3') and pSUMO-R (5'-CCCAAGCTT CATTAGTTGATAGTGGCAGCCT-3') were chemically synthesized. In this primer pair, one hydroxylamine cleavage site (AACGGC) was added to the 5-terminal of pSUMO-F, *Hind*III (AAGCTT), and stop codons (TCATTA) site were introduced to the reverse primer pSUMO-R. Using this primer pair, the two tandem CM4 genes of interest were amplified from plasmid Trx–2CM4 that was constructed previously by Zhang's lab [15]. The purified PCR product was digested with *Hind*III, and ligated into the pSUMO plasmid at the corresponding restriction sites. The ligation mixture was transformed into *E. coli* DH5 α cells for verification by sequencing.

Fusion Protein Expression

The pSUMO–2CM4 plasmid that had been constructed was transformed into competent *E. coli* BL21 (DE3). Two colonies were picked and cultured in 3 mL LB medium with vigorous shaking (220 rpm) at 37°C to a density of ≈ 0.6 absorbance unit. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) (0.5 mM) was then added to induce the expression of the recombinant protein at 37°C for 3 h.

Purification of SUMO Fusion Protein

The pellet from 200 mL culture was resuspended in 15 mL binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, and 10 mM PMSF, pH 8.0), and lysed on ice by sonication at 400 W for 100 cycles (4 s working, 8 s free). The supernatant of the cell lysate resulting from centrifugation at 12,000 $\times g$ at 4°C for 20 min was applied to a Ni²⁺-chelating column. After extensive washing with binding buffer, the fusion protein was eluted with five column volumes of elution buffer (20 mM Tris, 500 mM NaCl, and 250 mM imidazole, pH 8.0). The peak fractions containing the fusion protein were pooled and dialyzed overnight at 4°C against phosphate-buffered saline (PBS).

Cleavage of SUMO Fusion and Purification of ABP-CM4

The lyophilized fusion protein was resuspended in cleavage buffer [6] (2 M hydroxylamine hydrochloride, 0.2 M Tris–HCl, pH 9.0) to release the target protein. The pH value of the solution was adjusted to 9.0 with 5 M NaOH. After 4 h incubation at 45°C, the reaction was terminated by lowering the temperature and adjusting the pH with HCl to below 8.0. The cleavage mixture was desalted by dialysis in PBS at 4°C and purified by another affinity chromatography step on Ni²⁺-IDA-agarose to remove His-tagged carrier and undigested fusion proteins. The purified proteins were checked on Tricine/SDS-PAGE and the samples were stored at –80°C for activity assay.

Assay of Antimicrobial Activity

The antibacterial activities of purified recombinant CM4 was tested by the microbroth dilution method [9] using *E. coli* K₁₂D₃₁, *Penicillium chrysogenum* and *P. aeruginosa*. Briefly, 10⁵–10⁶ colony forming units (CFU)/mL suspended in 100 μ L growth medium was mixed with 50 μ L recombinant peptide solution in a microtiter plate well with three replicates for each test solution. The 20 mM Tris solution (pH 8.0) was added as the negative control. Microbial growth was assessed by measuring the optical density at 600 nm after 10 h incubation at 37°C. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which there was no change in optical density.

Results

Plasmid Construction and the Expression of ABP-CM4 Fusion Protein

The construct for two tandem CM4 genes expression, containing a His-tag for affinity purification, is depicted in

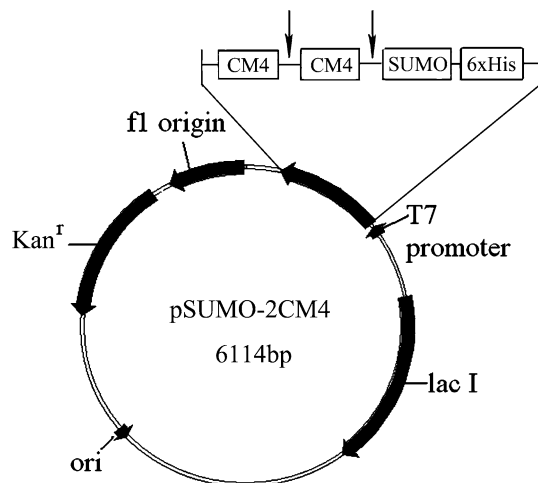


Fig. 1 Schematic representation of the expression vector pSUMO-2CM4. 2CM4 was expressed as a fusion protein with the SUMO. To facilitate peptide cleavage from the fusion protein, hydroxylamine cleavage sites, Asn–Gly (indicated by a vertical arrow), are created between the CM4, CM4 and SUMO

Fig. 1. The correct construction was transformed into the expression host *E. coli* BL21 (DE3), and subjected to a pilot expression test. As shown in Fig. 2, there was an obvious protein band after IPTG induction, and colony 1 was chosen for the induction experiment, and the fusion protein was efficiently produced in a soluble form induced by 0.5 mM IPTG at 37°C.

Purification of SUMO-2CM4 Fusion Protein

As described above, Ni-IDA resin was used for fusion protein purification. Most of the proteins without 6×His tags were removed from the Ni-IDA resin using washing buffer containing 20 mM imidazole, and the 6×His-tagged SUMO-2CM4 (about 23.5 kDa) was eluted using elution buffer containing 250 mM imidazole with more than 90%

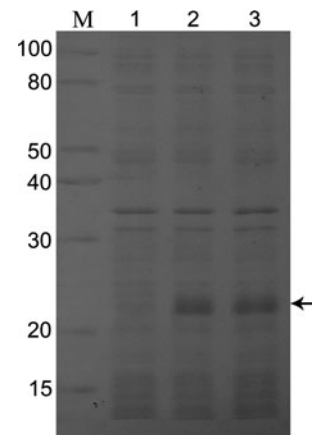


Fig. 2 Analysis of expressed fusion protein by SDS-PAGE. Lane M molecular mass marker, Lane 1 negative control, Lanes 2, 3 colony 1, 2 induced by 0.5 mM IPTG. The arrow indicated the location of the recombinant fusion protein

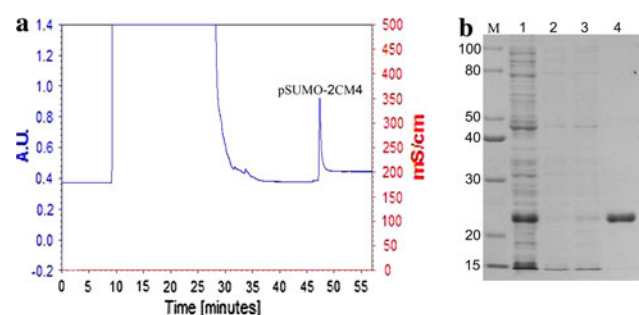


Fig. 3 Purification of SUMO-2CM4 fusion protein. **a** Ni-IDA affinity chromatography of fusion protein by using LPDataView (BIORAD). **b** The eluted fusion protein showed about 90% or more purity by electrophoretic analysis with 12% SDS-PAGE as analyzed by the BandsScan software (BioMarin Pharmaceutical Inc, UK). Lane M molecular weight marker, Lane 1 supernatant of cell lysate, Lane 2 flow-through, Lane 3 wash, Lane 4 elution

Table 1 Isolation of recombinant ABP-CM4 from pSUMO-2CM4 fusion protein

Purification step	Total protein (mg)	Fusion protein	Amount of rABP-CM4 (mg)	Purity (%) ^d
Sonicated supernatant	2015 ^a	280 ^b	80 ^c	NA
After affinity chromatography	NA	252 ^b	72 ^c	90
After affinity chromatography	60 ^a	NA	54 ^c	NA
Dialysis	NA	NA	48 ^a	>96

Estimations are based on 1 L of bacterial culture

NA not applicable

^a Determined using a Bradford assay

^b Percent of SUMO-2CM4 fusion protein from total proteins was estimated by SDS gel scanning

^c The amount of ABP-CM4 was calculated as a fraction of SUMO-2CM4 fusion protein

^d Purity of protein or peptide was estimated by SDS gels scanning as analyzed by the BandsScan software (BioMarin Pharmaceutical Inc, UK)

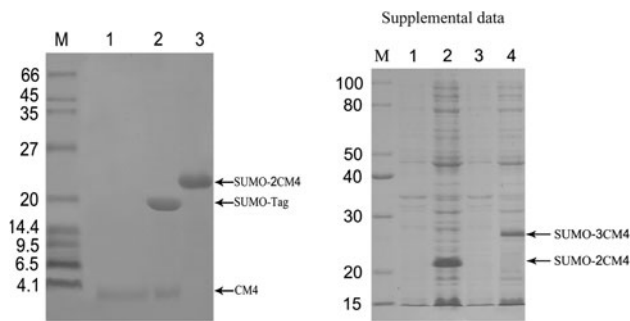


Fig. 4 Tricine/SDS-PAGE analysis of SUMO–2CM4 fusion protein cleaved by hydroxylamine and recombinant CM4 purification. *Lane M* low molecular weight marker, *Lane 1* purified recombinant CM4 by Ni-IDA, *Lane 2*: mixture of SUMO–2CM4 fusion protein by hydroxylamine cleavage, *Lane 3* purified SUMO–2CM4 fusion protein

purity (Fig. 3). About 252 mg fusion protein can be obtained per liter of bacterial culture (Table 1).

Cleavage and Purification of Recombinant CM4

The SUMO–2CM4 protein was competently cleaved with hydroxylamine hydrochloride, confirmed by checking the proteins on a Tricine/SDS-PAGE (Fig. 4, lane 2). After the cleaved sample was re-applied to a Ni-IDA column to subtract 6×His-tagged SUMO and undigested fusion protein, final purified CM4 (about 3.9 kDa) was obtained, and Tricine/SDS-PAGE results indicating that CM4 had been purified successfully with more than 96% purity (Fig. 4, lane 1). Table 2 shown the comparison of the Sumo fusion partner with Trx or Intein fusion partners in production of recombinant CM4. Finally, a purified recombinant CM4 was obtained at a yield of 48 mg/L (Table 1).

Table 2 Comparison of the Sumo fusion partner with Trx or Intein fusion partners

Fusion protein	Time cost (days)	Molecular weight of fusion tag (kDa)	Yield (mg/l)
Trx–nCM4	4–5	18	1.2–12
Intein–CM4	3	54	4.2
Sumo–2CM4	2	10	48

Table 3 The minimal inhibitory concentration of recombinant CM4 to selected microorganisms

Microorganisms	Minimum inhibitory concentration (uM)	
	Recombinant CM4	Native CM4
<i>E. coli</i> K ₁₂ D ₃₁	12	12
<i>P. chrysogenum</i>	22	20
<i>P. aeruginosa</i>	20	20

Antimicrobial Activity Assays

The antimicrobial activity of the purified recombinant CM4 was evaluated by determining its MICs against selected microorganisms. In this study, the recombinant CM4 had similar antimicrobial properties to the synthetic CM4. The results were shown in Table 3.

Discussion

It has been reported that it is difficult to express cationic antibacterial peptides in engineered bacteria because such peptides are highly toxic to the host bacteria cells and sensitive to intracellular proteases. Fortunately, there problems are somewhat alleviated when expressed with a fusion partner. SUMO is superior to commonly used fusion tags in enhancing expression and solubility [2]. We hypothesize that the attachment of a highly stable and compact SUMO structure to the N-terminus of the CM4 will facilitate correct protein folding and enhance solubility and expression.

In previous report, expression of the recombinant ABP-CM4 has been successfully achieved by constructing the vectors of pET32–nCM4 ($n = 1, 2, 3, \dots, 8$). It was found that expression levels are not directly proportional to the degree of multimerization. The strains BL21 (DE3)/pET32a–2CM4 or BL21 (DE3)/pET32a–3CM4 could express a higher level of recombinant CM4 than any others [15]. So two and three tandem repeats of CM4 genes were cloned into the vector pSUMO to construct an expression vector pSUMO–2CM4/pSUMO–3CM4. However, in SUMO-mediated peptide expression system, pSUMO–2CM4 could express a higher level of recombinant CM4 than pSUMO–3CM4 (shown in Fig. 4, Supplemental data). So we selected pSUMO–2CM4 for the large-scale expression of recombinant CM4.

In this work, we used hydroxylamine to cleave the fusion protein. Although, there is one additional amino acid (glycine) that attaches at the N-terminus of CM4 after cleavage, our experiments (Table 3) showed that the additional glycine has little effect on characteristics of CM4. One possible reason for this is that glycine is the simplest amino acid and is asymmetric. The side-chain of it is a hydrogen atom which permits a greater degree of conformational flexibility and has the least conformational space of any other amino acid residue. So the conformations of the other amino acids of the peptide are not significantly affected.

In summary, the SUMO fusion system and customized expression and purification protocol described here have greatly improved the efficiency and lowered the costs of producing ABP-CM4. This method has two major

advantages over the Trx or Intein fusion systems developed earlier [3, 15]: (1) the protein is purified quickly (2 days vs. 3–5 days); (2) compared with the size of Trx (18 kDa) or Intein tag (54 kDa), the small size of SUMO tag (10 kDa) results in a higher stoichiometric ratio of target polypeptide in fusion protein and, thereby, an increase in polypeptide yields (Table 2). Thus the new protocol provides a higher yield of ABP-CM4 with less time and effort.

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