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Full Length Research Paper

Fusion expression and high-level preparation of a glycine-rich antibacterial peptide (SK_{66}) derived from *Drosophila* in *Escherichia coli*

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SK_{66}, a derivative of the gene *cg13551* of *Drosophila* containing 66 amino acid peptide with N-terminal serine and C-terminal lysine, shows high antimicrobial activities. To obtain it in large amounts, the mature DNA fragment of SK_{66} was acquired from the pMD18-T-SK_{66} simple vector using PCR and then inserted into the Nco I and Xho I enzyme-cutting sites of pET-32a plasmid, the recombinant vector named pET-32a-SK_{66} was transformed into the competent cell *E. coli* BL 21. The fusion protein was expressed in soluble form under the optimized conditions at high level (more than 44% of the total proteins). Then the expressed product was purified by affinity binding chromatography with Ni-NTA, salt-out, freeze-dried. The SK_{66} was cleaved from the fusion protein by enterokinase, purified by using RP-HPLC and has strong antibacterial activity.

Key words: Antibacterial peptides, *Drosophila melanogaster*, fusion expression, preparation, SK_{66}.

INTRODUCTION

With the rapid development of levels of bacterial resistance to antibiotics, we find that resistant to most or all available agents has appeared in the clinic. Thus, the discovering of new drugs becomes more and more important. One potential source of novel antibiotics is the antibacterial peptides that were produced by insects. Insect immune organs, mainly the fatbody, can produce a variety of antibacterial proteins and peptides in response to injury and infection (Bulet et al., 1999). A few of these peptide antibiotics have entered clinical trials with mixed success to date (Andres et al., 2004).

Since it dose does not contain sufficient antibacterial peptide in the tissue, the amount of the peptide extracted was far from meeting the need. The use of recombinant expression methods might be introduced to obtain in a quantity sufficient to meet the need for it. To avoid the lethal effects on *Escherichia coli* host cells during its expression, many fusion partners have been used to express and purify antimicrobial peptides, including RepA protein (Zhang et al., 1998), F4 fragment of PurF (Pyo et al., 2004), thioredoxin (Barrell et al., 2004; Li et al., 2006), green fluorescent protein (GFP) (Skosyrev et al., 2003) and protein PaP3.30 (Rao et al., 2004).

We previously identified a new peptide, SK66, and showed its antibacterial activity which suggested SK66 is a candidate for a new antibiotic (Feng et al., 2009). In this paper, we describe in detail the over expression by using fusion partner thioredoxin, high-level preparation of a glycine-rich antibacterial peptideSK_{66} of *Drosophila* in *Escherichia coli*. It showed obvious antibacterial activity against *P. aeruginosa* by ager diffusion assay.

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Abbreviations: SK_{66}, 66-residue mature peptide with N-terminal serine and C-terminal lysine; **IPTG,** isopropyl β-D-thiogalactoside; **LB,** luria-bertini; **PCR,** polymerase chain reaction; **PVDF,** polyvinylidene difluoride; **EK,** enterokinase; **RP-HPLC,** reverse-phase high performance liquid chromatography.

#These authors contributed equally to this paper

MATERIALS AND METHODS

Strains, vectors and enzymes

E. coli strains DH5α (Novagen, USA) and BL21(DE*3)* (Novagen, USA) were used as the hosts for subcloning and expression, respectively. The pMD18-T simple vector (Takara) was used as the vector for subcloning of the PCR product of genes and pET-32a (Novagen, USA) was used for expression. T4 DNA ligase was purchased from Fermentas. Restriction enzymes were purchased from Takara (Japan) and used according to the recommendation of the supplier. Ni Sepharose 6 fast flow was purchased from GE healthcare. The enterokinase was purchased from Shanghai Kaiyang Biotechnology. Anti-His horseradish peroxidase (HRP)-conjugate kits (QIAGEN) were used for fusion protein detection. The gel purification kit was purchased from Qiagen (Germany).

Cloning of the fragment of SK_{66} and Construction of fusion expression plasmid

According to the genes (GenBank accession no. *cg13551*), the forward primer 5′ ATACCATGGCTAGCCAGGTGGGCGACCTAGG 3′ introduced a NcoI site (underlined). The reverse primer was 5′ATACTCGAGTCACTTGATCTGCTGGTGGTGGAAC 3′ in which the underlined site was XhoI. A terminator was introduced before the XhoI site (bordered). The PCR reaction consisted of 2 min at 94℃, 30 cycles (each cycle: 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 30 s) and a final extension at 72℃ for 10 min. PCR products from a 2% agar gel were purified with the gel purification kit and ligated into the pMD18-T simple vector.

The pMD18-T-SK_{66} simple vector was digested with NcoI and XhoI and the 201 bp DNA fragment was purified and inserted between the NcoI and XhoI of pET-32a plasmid. The pET-32a-SK_{66} recombinant plasmid was transformed into the expression host *E. coli* BL21 (DE3).

Expression, western blot analysis and purification of the fusion protein

A single transformed BL21 (DE3) colony harboring the recombinant pET-32a-SK_{66} vector was used to inoculate 20 ml LB containing 1 mM ampicillin in a flask overnight shaking at 37℃. 1 ml of culture was transferred to a fresh 20 ml LB containing 1 mM ampicillin. The culture was grown with 260 rpm shaking at 37℃ until the OD (600 nm) reached 0.9. The culture was induced by addition of 0.8 mM isopropyl-b-D-thiogalactopyranoside. Before induction, 1 ml of culture was immediately removed as the zero time-point sample. 1 ml samples were removed after 3 h induction. At the same time, a single transformed BL21 (DE3) colony harboring the pET-32a vector was cultured in 20 ml LB containing 1 mM ampicillin as control.

Samples were heated at 95℃ for 5 min and then centrifuged at 10,000g for 5 min prior to 12% SDS-PAGE. After electrophoresis, gels were stained with coomassie brilliant blue R-250. Western blot analysis was also performed. Anti-hexahistidine horseradish peroxidase (HRP)-conjugate kits (QIAGEN) were used for immunodetection of the hexahistidine.

The cell pellet from 1 l of induced culture was resuspended in 15 ml binding buffer consisting of 20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4. The resuspended cell pellet was sonicated in an ice bath using a Branson sonifier 250 (Branson, Danbury, CT, USA) on output settings 400 W(5, 5 × 45). After 12000 g centrifugation for 10 mins, the supernatant was loaded into a column containing 5 ml of freshly prepared Ni sepharose 6 fast flow (GE healthcare) resin. The column was washed with 50 ml binding buffer, followed by 30 ml wash buffer consisting of 20 mM sodium phosphate, 0.5 M NaCl, 60 mM imidazole, pH 7.4. The protein was eluted with 30 ml elute buffer consisting of 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4. The materials corresponding to the elution peak was collected, desalted and freeze-dried. Finally we obtained fusion protein about 55 mg from 1 l of induced culture.

Enterokinase cleavage and RP- HPLC

To release K_{66} from the fusion protein, enterokinase (Invitrogen) was added to cleave the fusion protein according to the ratio of 25 U/mg protein after a reaction buffer: (500 mM Tris-Cl, 10 mM $CaCl_2$, 1% Tween20, pH 8.0) was added. The mixture was incubated at 37℃ for 2-4 h. The reaction product were dissolved in buffer A: ultrapure water containing 0.1% TFA, The ODS-C18 column was equilibrated with 10% B: acetonitrile containing 0.1% TFA. Elution was carried out at a flow rate of 1 ml/min with a linear gradient from 10% B (acetonitrile containing 0.1% TFA) to 40% B (acetonitrile containing 0.1% TFA) over 30min. The elution was monitored at 214 nm. The peak fractions were collected, freeze-dried and then resuspended in distilled water for 15% SDS-PAGE analysis and antibacterial assay.

Antibacterial assays of SK_{66}

Bacillus subtilis (ATCC 9372) was used. A plate growth inhibition assay was performed as follows. First, 5 ml of LB 0.5% agar me=-dium containing 1×10^7 logarithmic-phase bacterial cells were poured on 1.5% agar LB medium in a 9 cm petri dish. After cooling, 5 µl of the sample containing 5 µg of protein were dropped on the gel and the plates were incubated at 37℃ for *B. subtilis* (ATCC 9372) over night.

RESULTS AND DISCUSSION

Construction of the fusion expression plasmid

A 204 bp containing a NcoI site at 5'end and an XhoI site at 3'end was obtained using PCR with primers SK_{66}F and SK_{66}R and then subcloned into the pMD18-T simple vector. The recombinant clone vector pMD18-T- SK_{66} simple vector and pET-32a were digested with NcoI and XhoI (data not shown). The target fragments were successfully inserted between the *Nco*I and *Xho*I of pET-32a. The open reading frame of the fusion protein Trx-SK_{66} encodes a peptide of 223 amino acids, which consisits of a Trx Tag, a 6 His Tag, a S Tag, a 66 residue mature peptide SK_{66} to reflect its N-terminal serine and C-terminal lysine and a EK. The recombinant vector pET-32a-SK_{66} was confirmed by sequencing (Figure 1).

Expression and preparation of the fusion protein Trx-SK_{66}

After induction, the recombinant cells were harvested and whole-cell lysates were analyzed by 12% SDS-PAGE. Compared to the control of BL21 (DE3)/pET-32a, recombinant expression products revealed an about 25.63 kDa target band at the expected position after staining with

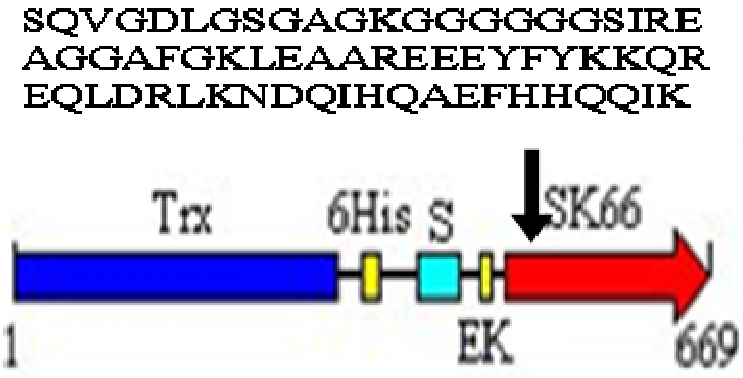

Figure 1. The deduced amino acid sequence of SK_{66} and the schematic structure of the Trx-SK_{66}.

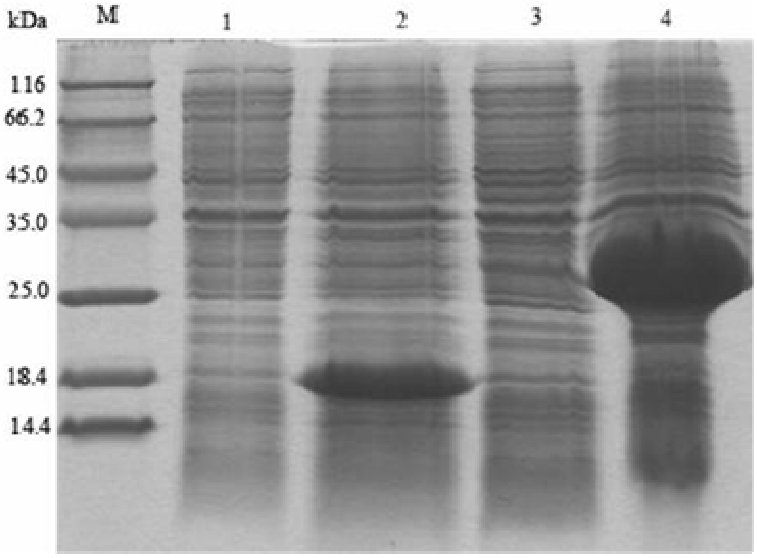

Figure 2. 12% SDS–PAGE analysis of Trx- SK_{66} expression in *E. coli* BL21(DE3). M: protein molecular weight marker; Lane 1, uninduced bacterial lysate containing pET32a+ expression vector; lane 2, bacterial lysate containing pET32a+ expression vector after IPTG induction for 3 h.; lane 3, uninduced bacterial lysate containing pTrx expression vector; lane 4, induced bacterial lysate containing pTrx expression vector after IPTG induction for 3 h.

coomassie brilliant blue R-250 (Figure 2). Densitometry analysis using quantity one software showed that the fusion protein reached more than 44% of the total protein. In addition, the protein was further confirmed by western blot analysis with anti-hexahistidine horseradish peroxidase (HRP)-conjugate kits. The lysate was mixed with nickel-NTA resin to allow the His-tagged recombinant protein to bind to the nickel. After extensive washing of the His bind resin column with binding buffer containing 50 mM imidazole, the contaminating proteins were successfully removed and the fusion protein Trx-SK_{66} was eluted from the column with 500 mM imidazole (Figure 4, lane 1).

Enterokinase cleavage and preparation of the SK_{66} by RP- HPLC

The Trx-SK_{66} was divided into an about 17.27 kDa Trx and an about 7.26 kDa SK_{66} by enterokinase cleavage (Figure 4, lane 2). The SK_{66} was successfully separated from reaction product by RP-HPLC (Figure 3,). These results were further confirmed by 15% SDS-PAGE (Figure 4). And the 13 mg SK_{66} was obtained from per liter cul-

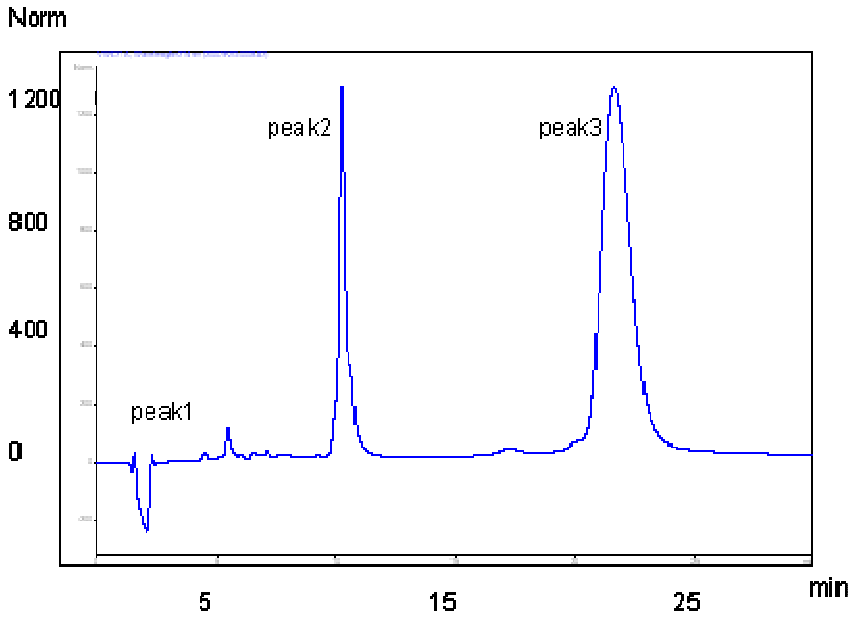

Figure 3. Elution profile of fusion protein cleaved by enterokinase on RP-HPLC with a ODS-C18 column.

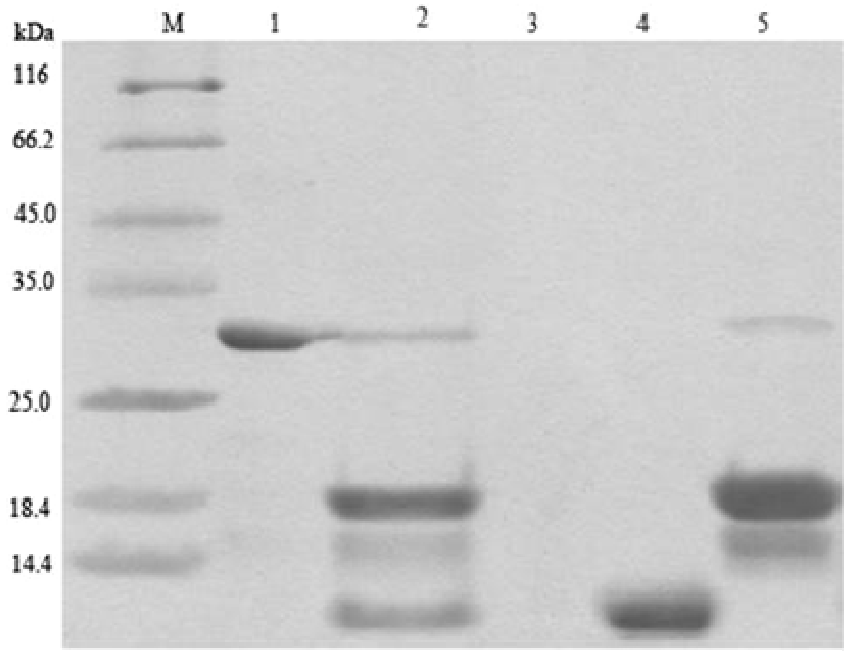

Figure 4. 15% SDS–PAGE analysis of purified fusion protein and SK_{66} M: protein molecular weight marker; Lane 1, Purified fusion protein. Lane 2, fusion protein cleaved by enterokinase. Lane 3, peak 1. Lane 4, peak 2. Lane 5, peak 3.

ture. Through agar diffusion assay, the SK_{66} displayed obvious antibacterial activity against *B. subtilis*, but the fusion protein showed no antibacterial activity against *B. subtilis* (Figure 5).

Conclusion

We constructed an efficient system for the expression and purification of SK_{66} in *E. coli* by the pET-32a vector.

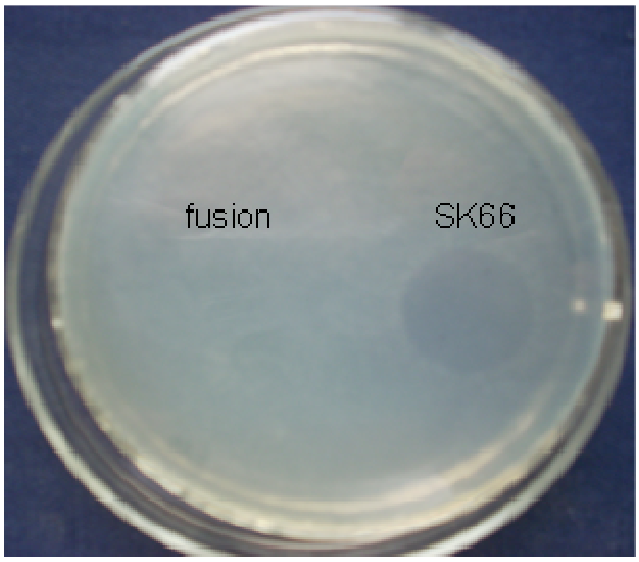

Figure 5. Antibacterial assay of SK_{66} against *B. subtilis* (SK_{66}; Fusion protein).

The recombinant strain should be a convenient source for preparing mature peptide in large scale and appears to be of potential interest for commercial purpose.

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