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Functional expression of a *Drosophila* antifungal peptide in *Escherichia coli*

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Abstract

Drosomycin is a key effector molecule involved in *Drosophila* innate immunity against fungal infection. This peptide is composed of 44 residues stabilized by four disulfide bridges. As the first step towards the understanding of the molecular basis for its specific antifungal activity, rapid and efficient production of the wild-type peptide and its mutants is needed. Here, we report a pGEX system for high-level expression of recombinant Drosomycin. The fusion Drosomycin protein with a carrier of Glutathione *S*-transferase (GST) was initially purified by affinity chromatography followed by Enterokinase cleavage. The digested product was separated by gel filtration and reverse phase HPLC. Mass spectrometry and circular dichroism spectroscopy analysis revealed that the recombinant peptide has identical molecular weight and correct structural conformation to native Drosomycin. Classical inhibition assay showed clear antifungal activity against *Neurospora crassa* with the IC_{50} of 1.0 μM. Successful expression of the CSαβ-type antifungal peptide in *E. coli* offers a basis for further studying its functional surface by alanine scanning mutagenesis strategy. Also, our work should be helpful in developing this peptide to an antifungal drug.

Keywords: Drosomycin; CSαβ scaffold; Protein expression; Innate immunity

Drosophila innate immunity offers the first line of defense against various pathogens. Antimicrobial peptides, as key components of the innate immunity, widely exist in *Drosophila* hemolymph [1,2]. Of them, Drosomycin, regulated by the Toll signal pathway, is the first inducible antifungal peptides with unique structural and functional properties, which was originally isolated from 2000 1-day-old adult males of *Drosophila melanogaster* [3]. This molecule is a small cationic peptide composed of 44 residues stabilized by four disulfide bridges [3,4]. Drosomycin and plant defensins share about 30% amino acid sequence identity and thus comprise a fungus-specific defensin family with similar structural and functional features. Twelve years have passed since the discovery of Drosomycin [3], but its functional surface responsible for the interaction with fungal membrane is not yet established, which hampers its application in drug exploration. Mutational analysis of a plant defensin from *Raphanus sativus* (named Rs-AFP2) performed by Samblanx et al. has assigned its functional surface to two adjacent sites which are primarily located on some solvent-exposed loop and turn [5]. In reference with these data, Landon et al. proposed a putative surface for Drosomycin [6]. Another related study based on functional analysis of Drosomycin and its six isoforms in *D. melanogaster* suggested the essential role of the α-helix in antifungal activity [7] which sharply differs from that of Rs-AFP2. However, this conclusion appears not solid and may need to be re-evaluated in that these authors did not provide chemical and structural data (MS and CD)[1] to

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[1] *Abbreviations used:* GST, glutathione *S*-transferase; EK, enterokinase; MS, mass spectrometry; CD, circular dichroism; CSαβ, cysteine-stabilized α-helix and β-sheet; PCR, polymerase chain reaction; LB, Luria–Bertani broth; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; MEA, malt extract agar; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibition concentration.

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distinguish misfolded/unfolded with correctly folded peptides of their recombinant products [7]. The existence of components with non-native conformation has great impact on functional characterization. Thus, functional determinants and the mechanisms by which Drosomycin inhibits fungal growth remain unsolved.

In addition, from a structural perspective, Drosomycin shares similar folding architecture to a class of functionally unrelated peptides—scorpion sodium channel toxins, both classified into the CSαβ superfamily [8,9]. The secondary structure elements in this superfamily comprise one β-sheet of two or three antiparallel strands and one α-helix. The helix is linked to the third strand via two disulfide bridges with a conserved sequence motif CXXXC in the α-helix and CXC in the β-strand. On the basis of a combined analysis of sequence, structure and evolution, Zhu et al. have found a putative evolutionary link between them and suggested that Drosomycin might be an ancestor of scorpion sodium channel toxins [9]. However, the experimental evidence is lacking.

Supported by these facts, it is a crucial step to obtain enough amount of pure Drosomycin and its mutants for studying the functional surface information and its evolutionary role in the origin of scorpion sodium channel neurotoxins. Here, we report an efficient method for the first time in the expression of Drosomycin using GST-fusion system in *E. coli*. The recombinant peptide showed strong antifungal activity against *Neurospora crassa* and has no difference from native Drosomycin in the chemical and structural properties as revealed by the MS and CD data.

Materials and methods

Materials

Drosophila melanogaster was kindly provided by Hubei University (Wuhan, China). pGEX-6P-1 is a product from Amersham Biosciences. *E. coli* DH5α and *E. coli* BL21(DE3) strains were purchased from Tiangen Biotech (Beijing, China). *N. crassa* was purchased from Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). All primers used in this study are synthesized by SBS Genetech (Beijing, China), and listed here (dT3AP: 5′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTT TTTTT-3′;DrW-F:5′-ATGGATCCGATGACGATGACAAG GACTGCCTGTCCGGAAGA-3′; DrW-R: 5′-ATGTCG ACTTAGCATCCTTCGCACCAGCA-3′; pGEX 5′-GG GCTGGCAAGCCACGTTTGGTG-3′).

Preparation of total RNA and the first-strand cDNAs

Drosophila melanogaster adults were ground into fine powder in liquid nitrogen. The TRIZOL reagent (SBS Genetech, Beijing) was used to prepare total RNA according to the supplier's instructions. Total RNA was reverse-transcribed into the first-strand cDNAs using RT-PreMix kit and a universal oligo(dT)-containing adaptor primer (dT3AP) from SBS Genetech (Beijing, China).

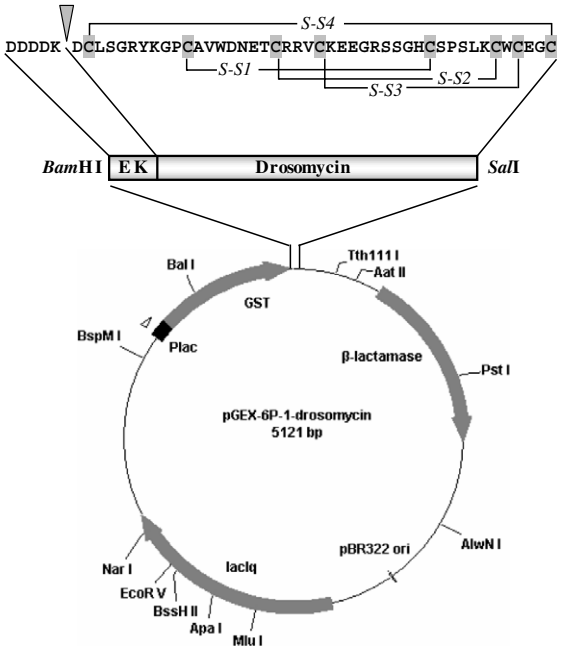

Fig. 1. Construction of pGEX-6P-1-Drosomycin expression vector. The cDNA encoding the mature Drosomycin was inserted into *Bam*HI and *Sal*I sites of pGEX-6P-1 with an EK cleavage site at the 5′ end of the cDNA. Triangle indicates the cleavage site of EK and S-S1-4 represent four disulfide bridges.

Construction of recombinant expression vector

To construct pGEX-6P-1-Drosomysin expression vector, we employed PCR strategy to amplify the first-strand cDNAs using primers DrW-F and DrW-R. To facilitate correct in-frame with the vector, we introduce a *Bam*HI site (underlined) and codons of enterokinase (EK) cleavage site (dotted) at the 5′ end of the forward primer DrW-F and a *Sal*I site (underlined) and a stop codon (boldfaced) at the 5′ end of the reverse primer DrW-R. The PCR product was digested by *Bam*HI and *Sal*I and ligated into pGEX-6P-1 (Fig. 1). Finally, the recombinant plasmid was transformed into *E. coli* DH5α competent cells and positive clones were confirmed by DNA sequencing using the primer pGEX 5′.

Expression and initial purification of fusion protein

The constructed vector was transformed into *E. coli* BL21(DE3) for protein expression. After incubation at 37 °C overnight in Luria–Bertani broth (LB) plate containing 100 μg/mL ampicillin, single colony was inoculated into 10 ml ampicillin-containing LB medium and then incubated at 37 °C overnight with shaking at 200 rpm. Ten milliliters of the overnight cell suspension was added to a flask containing 1 l of ampicillin-containing LB and incubated with shaking at 37 °C until OD_{600} reached 0.7. Expression of fusion protein was induced with 1.0 mM IPTG. Cells harvested after 4 h were

centrifuged at 5000*g* for 10 min and the pellet was suspended in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) with 1/20 volume of initial culture. After sonication and subsequent centrifugation, DTT was added into the supernatant of the cell lysates to a final concentration of 1 mM. The sample was then loaded on glutathione–Sepharose 4B beads (Probe, Beijing), pre-equilibrated three times with PBS, to bind for one hour by gently shaking at room temperature (25 °C). To remove unbound proteins, the beads were washed twice with PBS and once with 50 mM Tris–HCl, pH 7.3. Finally, the GST-fusion proteins bound on glutathione beads were eluted by 50 mM Tris–HCl, pH 8.0 containing 10 mM GSH. The eluate was analyzed by SDS–PAGE.

Gel filtration and reverse-phase HPLC

The fusion protein was cleaved by EK from sinobio Biotech Co. Ltd (Shanghai, China) at 37 °C for 3 h with 1 U enzyme per milligram fusion protein. Released recombinant Drosomycin (r-Drosomycin) was separated from GST-tag using Sephadex G-50 Medium (Pharmacia. Bead size: 50–150 μm) in a column of 16 × 500 mm (column bed volume: 100 ml), which was pre-equilibrated with 3 bed volumes of 20 mM NH_4Ac, pH 6.8. Fractions were collected and analyzed by SDS–PAGE. The fraction containing r-Drosomycin was lyophilized for the last purification by reverse-phase HPLC on C18 column (Agilent Zorbax 300SB-C18, 4.6 × 150 mm, 5 μm) which was previously equilibrated with 0.05% TFA in water. Elution was carried out using a linear gradient of 0–50% acetonitrile within 40 min with a flow rate of 1 ml/min. Effluent was monitored by measuring the absorbance at 225 nm using Diode Array and Multiple Wavelength Detectors (Agilent Tech, Waldbronn, Germany). Fractions eluted from 13 to 25 min of retention time were collected for antifungal assay and SDS–PAGE. Active fraction with the expected molecular weight was further analyzed by CD and MS.

Determination of protein concentration

Protein concentration was determined according to the Bradford method [10].

Mass spectrometry

The mass spectra of r-Drosomycin were obtained on a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization source with a spray voltage of 4.50 kV. The

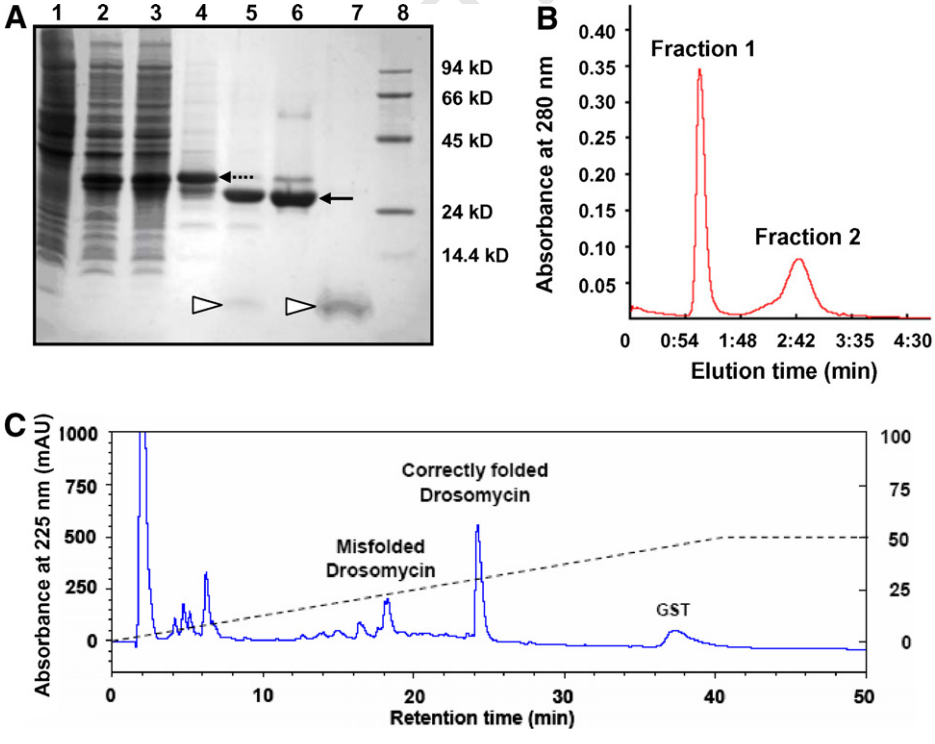

Fig. 2. Expression and purification of r-Drosomycin. (A) Tricine SDS–PGAE showing the expression and purification of r-Drosomycin. Lane 1: total cell extract of *E. coli* carrying pGEX-6P-1-Drosomycin without IPTG; lane 2: IPTG-induced total cell extract; lane 3: supernatant from cell lysate prepared by sonication; lane 4: fusion protein by affinity chromatography; lane 5: fusion protein cleaved by EK; lanes 6 and 7: the first and second fractions separated by gel filtration; lane 8: molecular weight marker. Dotted arrow: fusion protein; Solid arrow: GST; Triangle: r-Drosomycin. (B) Gel filtration showing the separation of Drosomycin from other proteins. (C) Reverse-phase HPLC chromatogram showing the separation of the second fraction from the gel filtration. The C18 column was equilibrated with 0.05% TFA and the purified proteins were eluted from the column with a linear gradient from 0% to 50% acetonitrile in 0.05% TFA.

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heated capillary was maintained at 200 °C at a voltage of 30 V. Calculation was carried out using Bioworks 3.1 provided by the manufacturer.

CD spectroscopy

CD spectra were recorded on a JASCO J-715 spectropolarimeter (Japan) at a protein concentration of 0.45 mg/mL dissolved in water. Spectra were measured at 25 °C from 250 to 190 nm by using a quartz cell of 1.0 mm length. Data were collected at 1 nm intervals with a scan rate of 200 nm/min. The CD spectra measure was performed by averaging three scans. Secondary structure content was estimated by JASCO CD standard analysis.

Antifungal assays

Neurospora crassa was incubated on 1× MEA (malt extract agar) plate at room temperature (25 °C) for one week. Spores were harvested and suspended in sterile water with an OD_{595} of 0.5. Six milliliters of 1× MEA-containing 0.8% agarose was mixed with 50 µl spores suspension and poured into one Petri dish of 9.0 cm in diameter. Holes with a diameter of 2 mm were punched into the medium, filled with 2 µl of sample each hole. Fungus was incubated in dark at 26 °C for 12–24 h.

Liquid growth inhibition assay

Spores suspended in 1× MEA with the OD_{595} of 0.1 were dispensed in aliquots of 80 µl into wells of a microplate containing 20 µl of either water or a series of diluted samples (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 µM) in a 96-well microplate. After incubation for 24 h at 26 °C in dark, the growth of *N. crassa* was evaluated by measuring the culture absorbance at 595 nm using a Microplate Reader (Perlong tech, Beijing). The experiment was repeated twice. Growth inhibition was tested by measuring culture OD_{595} treated with a series of concentration r-Drosomycin. IC_{50} was determined as peptide concentration which can lead to 50% of growth inhibition.

Result

To construct pGEX-6P-1-Drosomycin expression vector, we amplified a cDNA fragment of 166 bp using primers DrW-F and DrW-R. Following digestion with *Bam*HI and *Sal*I, we ligated it into pGEX-6P-1 vector (Fig. 1). The

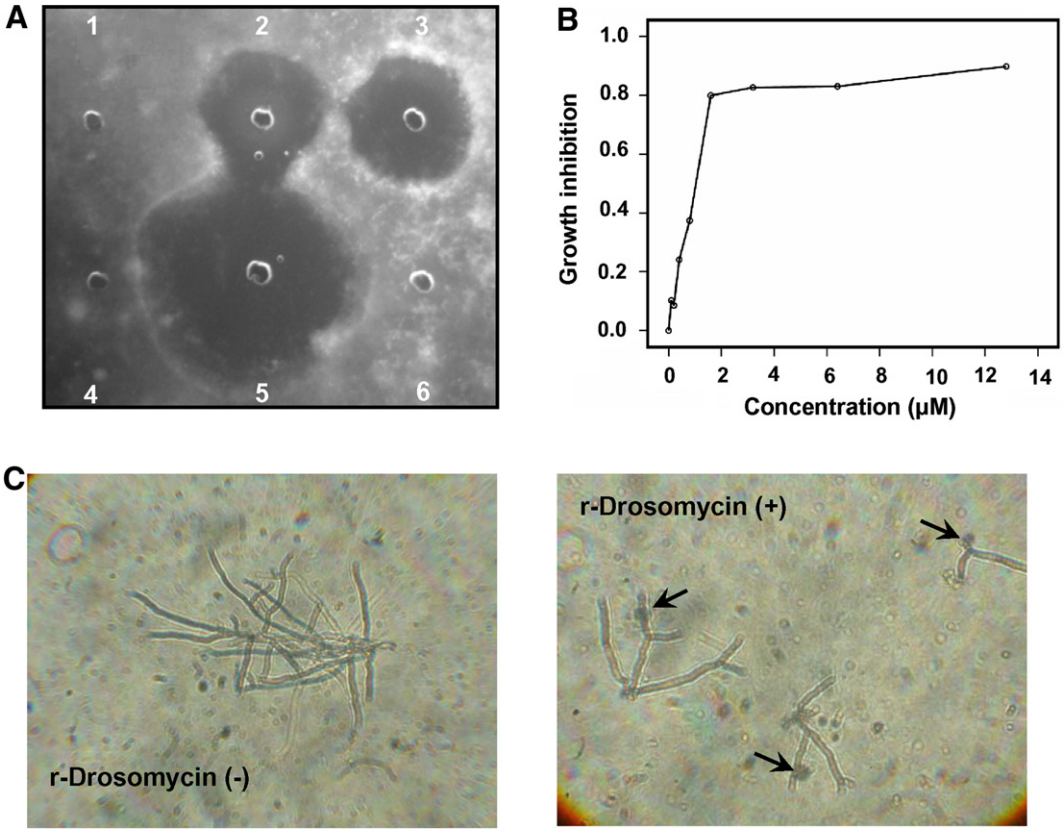

Fig. 3. Functional characterization of r-Drosomycin. (A) Inhibition zone assay showing the effect of r-Drosomycin on *N. crassa*. (1) Fusion protein; (2) fusion protein treated by enterokinase; (3) the second fraction of gel filtration; (4 and 5) misfolded and correctly folded r-Drosomycin identified by RP-HPLC; (6) water (control); (B) Growth inhibition of r-Drosomycin against *N. crassa*; (C) Partial lysis of *N. crassa* hyphae by r-Drosomycin. Photomicrographs were taken after 24 h of incubation of a *N. crassa* spore suspension in 1XMEA in the absence of r-Drosomycin (left), and in the presence of 2 µM peptide (right). Arrows indicate sites of hyphal lysis.

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recombinant plasmid was transformed into *E. coli* DH5α and its sequence was confirmed by DNA sequencing. Drosomycin was expressed in *E. coli* BL21(DE3) as a GST C-terminal fusion. This strategy can significantly improve the solubility of Drosomycin in that the fusion product of 31 kDa is primarily expressed in soluble form. The expression and purification results are shown in Fig. 2.

By using the GST affinity chromatography, we obtained 10 mg GST-Drosomycin per liter of cell culture. The EK-cleaved fusion protein was further purified and desalted using Sephadex G-50 and two fractions were collected. SDS–PAGE analysis confirmed that r-Drosomysin was present in the second fraction. Thus, this fraction was further purified by RP-HPLC using C18 column, which produced several peaks between 13 and 25 min of retention time (Fig. 2C). Samples corresponding to these peaks were collected, together with other samples, for inhibition zone assays to test their antifungal activity. Clear inhibition zones were observed in three samples including EK-digested fusion protein, the second fraction by gel filtration, and HPLC-purified material collected at 24–25 min of retention time (Fig. 3A). On the contrary, the fusion protein and the HPLC sample collected at 13–23 min of retention time showed no inhibition effects on *N. crassa*. However, this later HPLC sample showed identical molecular weight to the active component of 24–25 min of retention time as checked by SDS–PAGE (data not shown). It thus appears that these components represent unfolded r-Drosomycin lacking of native conformation. The final yield of HPLC-purified active peptide is about 0.3 mg/L. Mass spectrometry analysis determined its exact molecular weight being 4889.0 Da which completely matches the theoretical value (4889.1 Da) (Fig. 4A).

Next, we undertook classical liquid growth inhibition assay to quantitatively evaluate the antifungal activity of r-Drosomycin. The result shows a strong activity against *N. crassa* with an IC_{50} of 1.0 μM (Fig. 3B) which is compatible with 0.6 μM of native Drosomycin against *N. crassa* [3]. Similarly, we also observed a partial lysis of *N. crassa* hyphae caused by r-Drosomycin at a concentration of 2 μM in which the treated hyphae extruded cytoplasmic material (Fig. 3C).

Strong antifungal activity indicates that the r-Drosomycin may adopt a native conformation which is well supported by our CD data. The CD spectrum of r-Drsomycin shows a typical curve of CSαβ peptides with a positive maximum at 190 nm and a negative minimum at 206 nm (Fig. 4B). Using these CD data, we estimated the content of secondary structure elements in r-Drosomysin by JASCO CD standard analysis. Result shows that the recombinant peptide contains 17.4% α-helix and 24.3% β-sheet which is highly compatible with the contents in the native peptide (25% and 22.7%, respectively) based on the calculation of NMR structure [11] by DSSP (http://bioweb.pasteur.fr/seqanal/interfaces/dssp.html). This confirms that r-Drosomycin retains the native structural conformation.

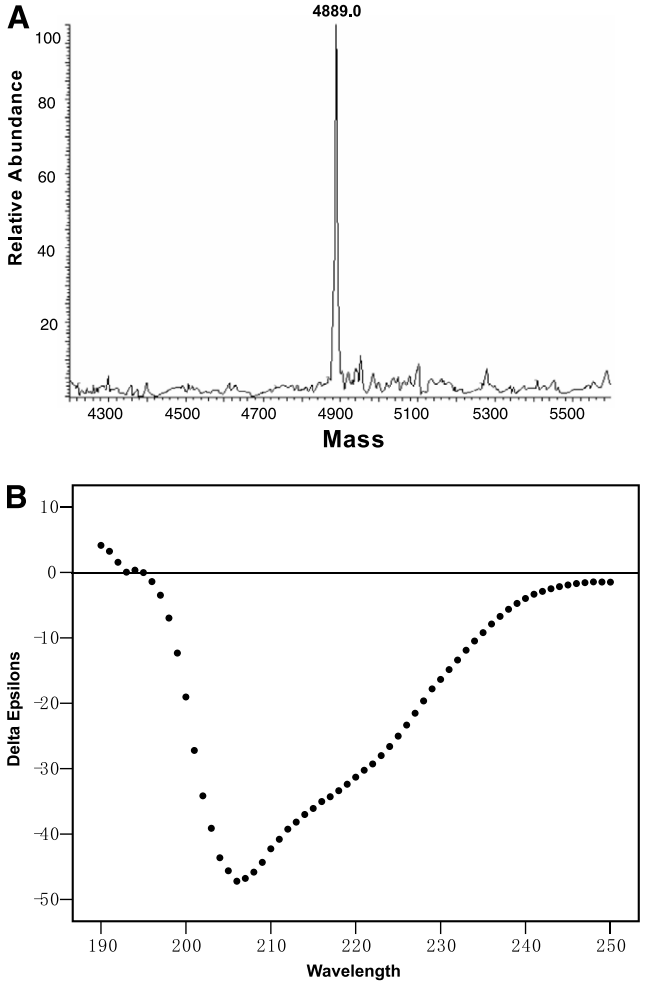

Fig. 4. Identification of r-Drosomycin by mass spectrometry (A) and circular dichroism spectra (B). The CD spectra of r-Drosomycin from 190 to 250 nm. Δε corresponds to the variation of molar amino acid residue absorption expressed in M^{-1} cm^{-1}.

Discussion

Peptides with the CSαβ motif constitute a superfamily with rather diverse bioactivities, ranging from plant sweet-tasting proteins to antimicrobial defensins and animal neurotoxins [9]. Structurally, these peptides are composed of one α-helix and one β-sheet of 2–3 strands stabilized by 3–4 disulfides. Although they provide an ideal model for studying structure, function and evolutionary relationship of polypeptides, one of the most important problems that have hampered advances in this field is the difficulty in obtaining correctly refolded recombinant peptides. In recent years, this case starts to change due to several successful expressions of scorpion neurotoxins and defensin in *E. coli*. In most cases, the production of these peptides frequently yields an inactive protein, aggregated in the form of so-called inclusion bodies, which needs further denaturation and refolding with various parameters difficult to optimize [7,12–14]. An alternative approach is to develop yeast expression system to overcome this limitation. For example, Samblanx et al. for the first time successfully

obtained recombinant radish antifungal peptide (Rs-AFP2) and various mutants using this system [5]. Previous studies have shown the GST-fusion system not only facilitates efficient purification, but also helps improve solubility of disulfide-rich peptides [15]. Here, we took advantage of these features to obtain enough soluble r-Drosomycin in *E. coli* and performed detailed evaluation of its structure and function. Introducing an EK cleavage site at the N-terminus of Drosomycin has apparent advantage in that it can generate a native molecule without any extra amino acid in its N-terminus. This has been confirmed by the mass spectrum analysis. Combined analysis of CD and functional data revealed that r-Drosomycin exhibits identical features with the native peptide in conformation and activity.

For the production of Drosomycin in *E. coli*, final purification by HPLC is needed as this step clearly discerned with folded and unfolded products. This will facilitate to obtain pure native-like peptide for fine structure and functional analysis. In our experiment, the unfolded Drosomysin shows no antifungal activity which in fact decreased the final yield of r-Drosomysin. Application of a GSH redox buffer to promote disulfide shuffling may be useful to increase the ratio of correctly folded product [17].

Our observation that soluble GST-Drosomycin shows no toxicity to *N. crassa* hints a possible steric hindrance effect of large GST protein that might shield the functional surface of Drosomysin or block the binding of Drosomysin to fungal membrane. Given GST-fusion peptide is increasingly used to solve 3D structure of some fused peptides [16], our fusion product could be useful for studying the functional surface of Drosomycin.

Considering unique roles of Drosomycin in insect innate immunity and invertebrate neurotoxin origin, successful expression of this native peptide undoubtedly paves the way to these related studies. At present, by using this expression system, we have obtained a recombinant engineering peptide in which a functional domain of a scorpion sodium channel toxin was grafted into the Drosomycin scaffold. The grated molecule loses the antifungal activity and transfers its targets to animal sodium channels (Yuan and Zhu, unpublished data). This provides another perspective for the use of Drosomycin scaffold to develop new drugs by our grafting strategy.

Acknowledgments

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