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cDNA Sequence Analysis and Expression of κ-Bungarotoxin from Taiwan Banded Krait[1]

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The cDNAs encoding κ-bungarotoxin was constructed from the cellular RNA isolated from the venom glands of *Bungarus multicinctus* by reverse transcription-polymerase chain reaction. A high degree of nucleotide sequence homology was observed between κ-bungarotoxin and other κ-neurotoxins. The κ-bungarotoxin was subcloned into the expression vector pET32a(+) and transformed into BL21(DE3) *E. coli* strain. The recombinant toxin was expressed as a fusion protein. Recombinant κ-bungarotoxin was separated from the fused protein by cleavage with CNBr and purified by reversed phase high performance liquid chromatography. In addition to κ-bungarotoxin, the cDNA fragment encoding κ_3-bungarotoxin was also found in the cDNA mixtures prepared from the cellular RNA of the venom glands of the same snake. This result suggests that the venom glands of Taiwanese *B. multicinctus* should secrete at least two kinds of κ-neurotoxins.

The venom of *Bungarus multicinctus* (Taiwan banded krait) contained several neurotoxic proteins including α-bungarotoxin, κ-bungarotoxins and β-bungarotoxins, which had been well studied by several groups (1,2). Among them, α-bungarotoxin and β-bungarotoxins are postsynaptic and presynaptic neurotoxins, respectively. κ-Bungarotoxins exhibits a potent effect on the neuronal nicotinic receptor, and blocks a transmission in several neuronal system in which α-bungarotoxin has no effect (3). Up to date, three κ-bungarotoxins including κ-bungarotoxin, κ_2-bungarotoxin and κ_3-bungarotoxin have been isolated from *Bungarus multicinctus* venom (4,5). Although cDNA fragments encoding κ_2- and κ_3-bungarotoxins had been constructed (6), the cDNA fragment encoding κ-bungarotoxin was not determined yet. In contrast to the finding that κ_2- and κ_3-bungarotoxins were isolated from the venoms collected in the Guangdong province of China (5), previous studies showed that κ-bungarotoxin was the only κ-neurotoxin from the venom of Taiwanese snakes (4). However, in the present study, the cDNAs encoding κ-bungarotoxin as well as κ_3-bungarotoxin were constructed from the cellular RNA isolated from the venom glands of one Taiwanese snake. It is evident that Taiwanese *Bungarus multicinctus* snake should secrete at least two kinds of κ-neurotoxins in its venom.

MATERIALS AND METHODS

Preparations of mRNA from venom gland. Cellular RNA was isolated from the snake (*Bungarus multicinctus*) venom glands which were stored in liquid nitrogen immediately after sarcrificed. Two deep frozen glands from one snake were homogenized to extract RNA by a guanidinium isothiocyanate/phenol chloroform isolation kit (Stratagene Ltd., USA).

PCR amplification and cloning. Two oligonucleotide primers of sense and antisense orientations based on the signal peptide and 3′-noncoding region of neurotoxins (6) with the forward sequence, 5′-ATGAAAACTCTGCTGCTGACCTTGGTG-3′ and the reverse one 5′-CTCAAGGAATTTAGCCACTCGTAGAG-3′ were synthesized.

RT-PCR was carried out with 100 μl reaction buffer containing 100 mM Tris-HCl (pH 8.3), 1 mM dNTP, 1μM antisense primer and 200 ng RNA template. In the reverse transcription, the cDNA was started with r*Tth* reverse transcriptase (5 U) and 2 μl of 10 mM $MnCl_2$ at 70°C for 15 min, and stopped the reaction by placing the tube on ice until needed. A 8 μl chelating buffer containing 50% glycerol (v/v), 100 mM Tris-HCl (pH 8.3), 1 M KCl and 7.5 mM EGTA/0.5% Tween 20 was added to the reaction. After addition of 8μl of 25 mM $MgCl_2$ and 1 μM sense primer, the amplification was proceeded on a thermocycler 94°C/45°C/72°C 1 min each, for a total 30 cycles.

The PCR products were cloned into pCRII vector according to the TA-cloning procedures (Invitrogen, San Diego, USA).

DNA sequencing. Sequence analysis was carried out according to the dideoxy method with a sequencing kit (Sequenase sequencing system, USB) labeling with [^{35}S]dATP (Amersham, > 1000 Ci/mmol). The reaction products were sequenced in 6% polyacrylamide gel

[1] The sequence data of cDNA for the precusor of Bungarus multicinctus κ-bungarotoxin has been deposited in the EMBL, GeneBank, and DDBJ nucleotide sequence databases under Acession No. Y08721.

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which was dried and exposed on a Kodak film for two days at room temperature.

Cloning and expression of cardiotoxins. Synthetic oligonucleotides were designed to produce a 236-bp amplified DNA fragment spanning the open reading frame of κ-bungarotoxin. Primer 1 introduced a 5′ *EcoR* V site and an in frame initiating Met codon preceding Arg-1 of κ-bungarotoxin,

EcoR V

5′-GATATCATGCGGACTTGCCTCATATCAC-3′.

AspIleMetArgThrCysLeuIleSer

Primer 2 was the reverse primer for RT-PCR amplification of κ-bungarotoxin. The PCR products were cloned into pCR II vector. The inserted DNA fragment was cut with *EcoR* V and *EcoR* I and ligated into the large fragment of *EcoR* V/*EcoR* I-cut pET32a(+). The entire sequence was confirmed by dideoxynucleotide sequencing.

The resulting plasmid pET-*Kappa* was transformed into *E. coli* strain BL21(DE3). Transformats were selected on LB-agar plates supplements with 50 μg/ml ampicillin. For induction of gene expresssion, *E. coli* BL21(DE3) cells containing pET-*Kappa* were grown at 37°C in LB medium containing 50 μg/ml ampicillin. After reaching an OD_{550} = 1.0, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM. The culture was induced for a period of up to 4 hr. The cells were harvested and lysed by ultrasonication.

Purification of recombinant κ-bungarotoxin. The recombinant κ-bungarotoxin was expressed as a fusion protein in *E. coli*. After ultrasonication, the mixture was then centrifuged for 30 min at 27000g to remove any remaining insoluble particles. The supernatant was dialyzed against three changes of 4 liters of deionized water, and subjected to lyophilization. The fusion protein was purified by His-Bind resin (Novogen Inc., USA) according to manufacturing's procedure, then treated with 400-fold molar excess of CNBr. The recombinant κ-bungarotoxin was further purified by HPLC on a SynChropak RP-P column (4.6 mm × 25 cm), and eluted with a linear gradient of 5 - 50 % acetonitrile for 70 min. The eluate was monitored at 235 nm.

SDS-PAGE analysis. SDS-PAGE was performed in 15 % acrylamide according to the method of Laemmli (7). Samples were denatured by boiling for 5 min in 0.125 M Tris, 4 % SDS in the presence of 4 % β-mercaptoethanol.

Sequence comparison of protein and homology search. In the comparison and analysis of the amino acid sequence from determined cDNA sequence, a software package (PC/GENE program, Stratagene Ltd., USA) was used for sequence alignment based on percent sequence identity.

RESULTS AND DISCUSSION

Although the cDNAs encoding κ_1- and κ_2-bungarotoxins had been constructed six years ago (6), the nucleotide sequence encoding κ-bungarotoxin still remained to be determined. Comparative analyses on the determined cDNA sequences of κ_2- and κ_3-bungarotoxins show that the nucleotide sequence of signal peptide and 3′-untranslational sequence of κ-neurotoxins are highly conserved. Thus, two primers were designed from the conserved regions at the begining of the signal peptide and 3′-untranslational region which was in proximity to the stop codon of κ-neurotoxins, respectively. PCR amplification of the venom glands cDNA mixtures with the designed primers achieved the isolation of a PCR fragment estimated to be about 300 bp (Fig. 1). The DNA fragments were then subcloned by TA-cloning kit. More than 10 clones were selected for nucleotide sequencing. Two out of the selected clones had the same cDNA sequence, which deduced protein sequence corresponded to that of κ-bungarotoxin (Fig. 2). The deduced protein sequence is the same as the previous published sequence determined by protein sequencing technique (8). Moreover, it was found that one of the seclcted clones encoded κ_3-bungarotoxin. Nevertheless, the codon encoding Pro at position 7 of κ_3-bungarotoxin protein sequence was CCC rather than CCT noticed by Danse and Garnier (6). Phylogentic analysis on the nucleotide sequence of three κ-bungarotoxins using commercial software package (PC/GENE, CLUSTAL program) revealed that the evolutionary relationship of κ-bungarotoxin was more closer to κ_3-bungarotoxin than to κ_2-bungarotoxin. The nucleotide sequence of κ-bungarotoxin shares 93% and 96% homology with those of κ_2-bungarotoxin and κ_3-bungarotoxins (6), respectively. Previous studies showed that κ-bungarotoxin was the only one κ-neurotoxin isolated from the venom collected in Taiwanese area (4). Both κ_2- and κ_3-bungarotoxins were isolated from the venoms collected from different geographical region, the Guangdong province of China (5). However, the results of our studies indicated that the venom glands of Taiwanese snakes could produce at least two kinds of κ-neurotoxins, κ-bungarotoxin and κ_3-bungarotoxin.

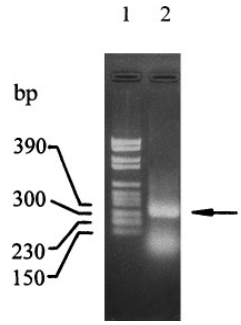

FIG. 1. Identification and size determination of PCR-amplified cDNA. Electrophoresis was carried out in 2% agarose gel. Lane 1, DNA molecular markers VI of 0.15 to 2.2 Kb (Boehringer Mannheim, Germany). Lane 2, the arrow indicates the PCR products of about 300 bp.

In order to subclone the κ-bungarotoxin into the expression vector, a new primer was designed to create a *EcoR* V site in the begining of the nucleotide sequences for encoding amino acid sequence of κ-bungarotoxin. The primer sequence 5′-GATATCATGCGGACTTGCCTCATATCAC-3′ (the underline indicated the *EcoR* V site) was synthesized for subcloning κ-bungarotoxin into the expression vector. Moreover, for preventing the formation of hairpin structures within the

primer, the bases A at positions 10 and 15 were replaced by C and T, respectively. The resulting amino acid sequences did not change at all. The PCR procedure was performed with *pfu* DNA polymerase. The amplified DNA was inserted into pCRII vector, then subcloned into the expression vector pET 32a(+) by digested with *EcoR* V/*EcoR* I. The κ-bungarotoxin was expressed as a fusion protein, and purified by His-Bind resin (Novogen Inc., USA). However, minor contaminants were found to be co-eluted with the recombinant protein (Fig. 3A). Since the toxin itself is devoid of Met, the Met residue preceding the Arg-1 of κ-bungarotoxin serves as a CNBr-cleavage site. The results of SDS-PAGE showed that the CNBr-cleavage products of fusion protein contained two major polypeptide fragments with apparent molecular weights corresponding to the fused protein and κ-bungarotoxin, respectively (Fig. 3A). The recombinant κ-bungarotoxin was then purified by HPLC on a SynChropak RP-P column (Fig. 3B). However, the purified toxin fraction was not homogeneity as revealed by N-terminal sequence determination. The major component has the expected N-terminal amino acid sequence as that of native κ-bungarotoxin. However, the minor ones have a short N-terminal amino acid extension relative to venom-derived κ-bungarotoxin, which was owing to that CNBr-cleavage did not exclusively occur at the Met preceding the first amino acid of the recombinant toxin and also occurred at Met residues of the C-terminal region of the fused protein. Whether the recombinant κ-bungarotoxin has the same biological activity as that of venom-derived toxin remains to be determined. However, the yield of the fusion protein is approximately 75 mg per liter culture, suggesting that this expression system may be beneficial to provide enough materials for future studies on the structure-function of κ-bungarotoxin.

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        10         20         30         40         50         60
ATGAAAACTCTGCTGCTGACCTTGGTGGTGGTGACAATCGTGTGCCTGGACTTAGGATAC
METLysThrLeuLeuLeuThrLeuValValValThrIleValCysLeuAspLeuGlyTyr

        70         80         90        100        110        120
ACCAGGACATGCCTCATATCACCTTCTTCTACCCCTCAGACATGTCCAAATGGGCAGGAC
ThrArgThrCysLeuIleSerProSerSerThrProGlnThrCysProAsnGlyGlnAsp

       130        140        150        160        170        180
ATATGCTTTCTAAAGGCTCAGTGTGATAAATTCTGTTCCATCAGAGGACCTGTAATCGAA
IleCysPheLeuLysAlaGlnCysAspLysPheCysSerIleArgGlyProValIleGlu

       190        200        210        220        230        240
CAAGGATGTGTTGCTACCTGCCCTCAATTTAGATCCAATTATAGATCTCTTCTCTGTTGC
GlnGlyCysValAlaThrCysProGlnPheArgSerAsnTyrArgSerLeuLeuCysCys

       250        260        270        280        290
ACAACAGACAATTGCAACCACTAACTCTACGAGTGGCTAAATTCCTTGAG
ThrThrAspAsnCysAsnHisEND
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FIG. 2. Nucleotide and deduced protein sequences of the precusor of κ-bungarotoxin. The nucleotide sequence of 290 base pairs is shown above the amino acid sequence of 87 residues including a signal peptide of 21-amino acid residues. The mature κ-bungarotoxin of 66 residues starts at Arg.

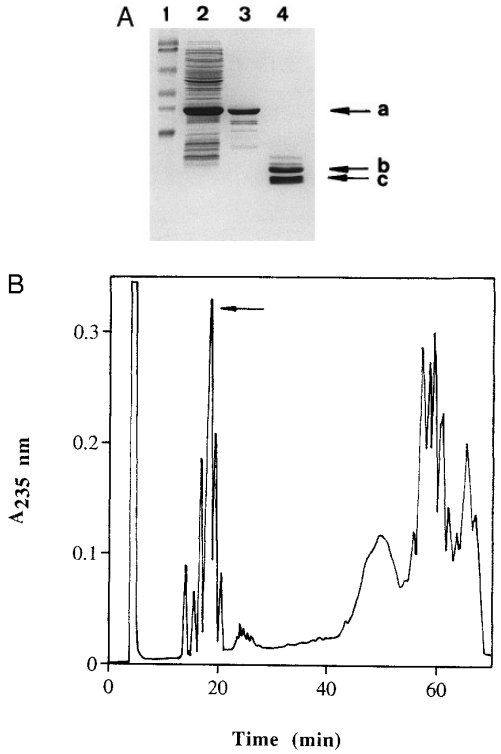

FIG. 3. SDS-PAGE analysis and purification of recombinant κ-bungarotoxin. (A) SDS-PAGE analysis of recombinant κ-bungarotoxin Lane 1, Molecular markers (prestained SDS-PAGE standards, BIO-RAD, Hercules, USA): phosphorylase (107,000), BSA (76,000), ovalbumin (52,000), carbonic anhydrase (36,800), soybean trypsin inhibitor (27,200) and lysozyme (19,000); lane 2, the induced extract of *E. coli* containing plasmid pET-*Kappa;* lane 3, the fusion proteins purified by His-Bind resin; lane 4, CNBr-cleavage products of the fusion proteins. Arrows a, b, and c represent fusion protein, fused protein, and recombinant κ-bungarotoxin, respectively. (B) The CNBr-cleavage products were applied on a SynChropak RP-P column, equilibrated with 0.1% TFA, and eluted with a linear gradient of 5-50% acetonitrile for 70 min. The flow rate was 0.8 ml/min and the eluate was monitored at 235 nm. The arrow indicates the fraction containing recombinant κ-bungarotoxin, and the fused protein was eluted at an acetonitrile concentration higher than 40% (v/v).

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