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cDNA Sequence Analysis and Expression of Cardiotoxins from Taiwan Cobra[1]

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The cDNAs encoding cardiotoxins I, III and N were constructed from the cellular RNA isolated from the venom glands of *Naja naja atra* by reverse transcription-polymerase chain reaction. A high degree of sequence homology was observed with the three cardiotoxins. The cardiotoxins were subcloned into the expression vector pET 20b(+) and transformed in BL 21(DE3) *E. coli* strain. The expressed protein was isolated from the inclusion bodies of *E. coli* and purified by reversed phase high performance liquid chromatography. The purified recombinant cardiotoxin III showed an immunoreactivity with anti-cardiotoxin III antibodies as revealed by immunoblot analysis. © 1996 Academic Press, Inc.

Cardiotoxins (cytotoxins) form the major toxic proteins found in cobra venom and they are made up of 60–63 amino acids residues in a single polypeptide chain cross-linked by four disulfide bonds (1). They are currently more than 50 determined sequences of cardiotoxins from varied species of elapid snakes (1). In contrast to another prominent group of structurally similar neurotoxins with well-established acetylcholine receptors and mode of action (2), cardiotoxins showed no defined cellular targets and diverse pharmacological functions (1). Although chemical modification studies give some clues, in part, to elucidate the structure-function relationship of cardiotoxins, the residues exclusively involved in the action of cardiotoxins remained to be fully resolved (1,3–6). In order to well-characterize the essential residues of cardiotoxins closely related to their actions, the effective and convenient way was to clone the structural gene encoding cardiotoxins which were subjected to site-directed mutagenesis studies. However, in contrast to extensive studies on the genetic structures of phospholipase A2 and postsynaptic neurotoxins from elapidae snake (7–9), limited studies had been reported on those of cardiotoxins. To the best of our knowledge, only one cardiotoxin gene has been cloned from *Naja naja sputatrix* (10). In the present studies, we constructed cDNA encoding cardiotoxin I, cardiotoxin III and cardiotoxin N from *Naja naja atra* by using reverse transcription-polymerase chain reaction (RT-PCR) from the cellular RNA isolated from venom glands of *Naja naja atra* (Taiwan cobra). The cDNA has been sequenced and expressed in *E. coli* as cardiotoxins. The expressed protein is purified by high performance liquid chromatography (HPLC), and reacts specifically with anti-cardiotoxin III antibodies.

MATERIALS AND METHODS

Preparations of mRNA from Venom Gland

Cellular RNA was isolated from the snake (*Naja naja atra*) 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).

[1] The sequence data of cDNA for the precursor of cardiotoxin I, III and N (*Naja naja atra*, Taiwan cobra) have been deposited in the EMBL Data Library under the accession numbers Z54226, Z54227 and Z54230, respectively.

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PCR Amplification and Cloning

Two oligonucleotide primers of sense and antisense orientations based on the signal peptide and 3′- noncoding region of neurotoxins (2) with the forward sequence, 5′- ATGAAAACTCTGCTGCTGACCTTGGTG-3′ and the reverse one 5′-CTCAAGGAAT TTAGCCACTCGTAGAGC-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 MnCl2 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 MgCl2 and 1 μM sense primer, the amplification was proceeded on a thermocycler 94°C/52°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 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 218-bp amplified DNA fragment spanning the open reading frame of cardiotoxin-III. Primer 1 introduced a 5′ *Nde* I site and an in frame initiating Met codon preceding Leu-1,

Nde I
5′-CATATGTTAAAATGCAATAAACTCGTTCCTT-3′.
MetLeuLysCysAsnLysLeuIlePro

Primer 2 was the reverse primer for RT-PCR amplification of cardiotoxins. The PCR products were cloned into pCR II vector. The inserted DNA fragment was cut with *Nde* I and *EcoR* I and ligated into the large fragment of *Nde* I/*EcoR* I-cut pET 20b (+). The entire sequence was confirmed by dideoxynucleotide sequencing.

The resulting plasmid pET-CTX-III 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 expression, *E. coli* BL21(DE3) cells containing pET-CTX-III were grown at 37°C in LB medium containing 50 μg/ml ampicillin. After reaching an OD_{550} = 1.0, isoprophyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. The culture was induced for a period of up to 4 hr. The cells were harvested and lysed by ultrasonication.

Purification of Recombinant Cardiotoxin

The recombinant cardiotoxin was found to be exclusively appear in the inclusion bodies of *E. coli.* After ultrasonication, the insoluble pellet was resuspended in 8 M urea solution by vigorous stirring and intermittent sonication at 600 W over a period of 30 min. 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 lyophilized proteins were dissolved in 1 ml of 6 M guanidine-HCl (pH 8.0) and reduced with 2% β-mercaptoethanol. The reaction was allowed to proceed at 37°C for 2 hr, then the reaction mixtures were desalted by passage through a Sephadex G-25 column equilibrated with 0.1 M acetic acid. The recombinant cardiotoxin was further purified by HPLC on a SynChropak RP-P column (4.6 mm × 25 cm), and eluted with a linear gradient of 25–50% acetonitrile for 70 min. The eluate was monitored at 235 nm.

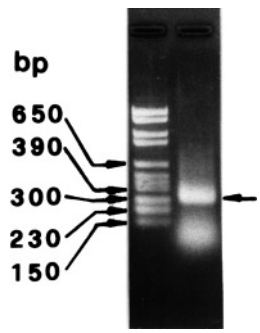

FIG. 1. Identification and size determination of PCR-amplified cDNA. Electrophoresis was carried out in 2% agarose gel. Lane 1: DNA molecular markers VI of 0.15 to 2.2 Kb (Boehringer Mannheim, Germany). Lane 2: The arrow indicates the PCR reaction products of about 280 bp.

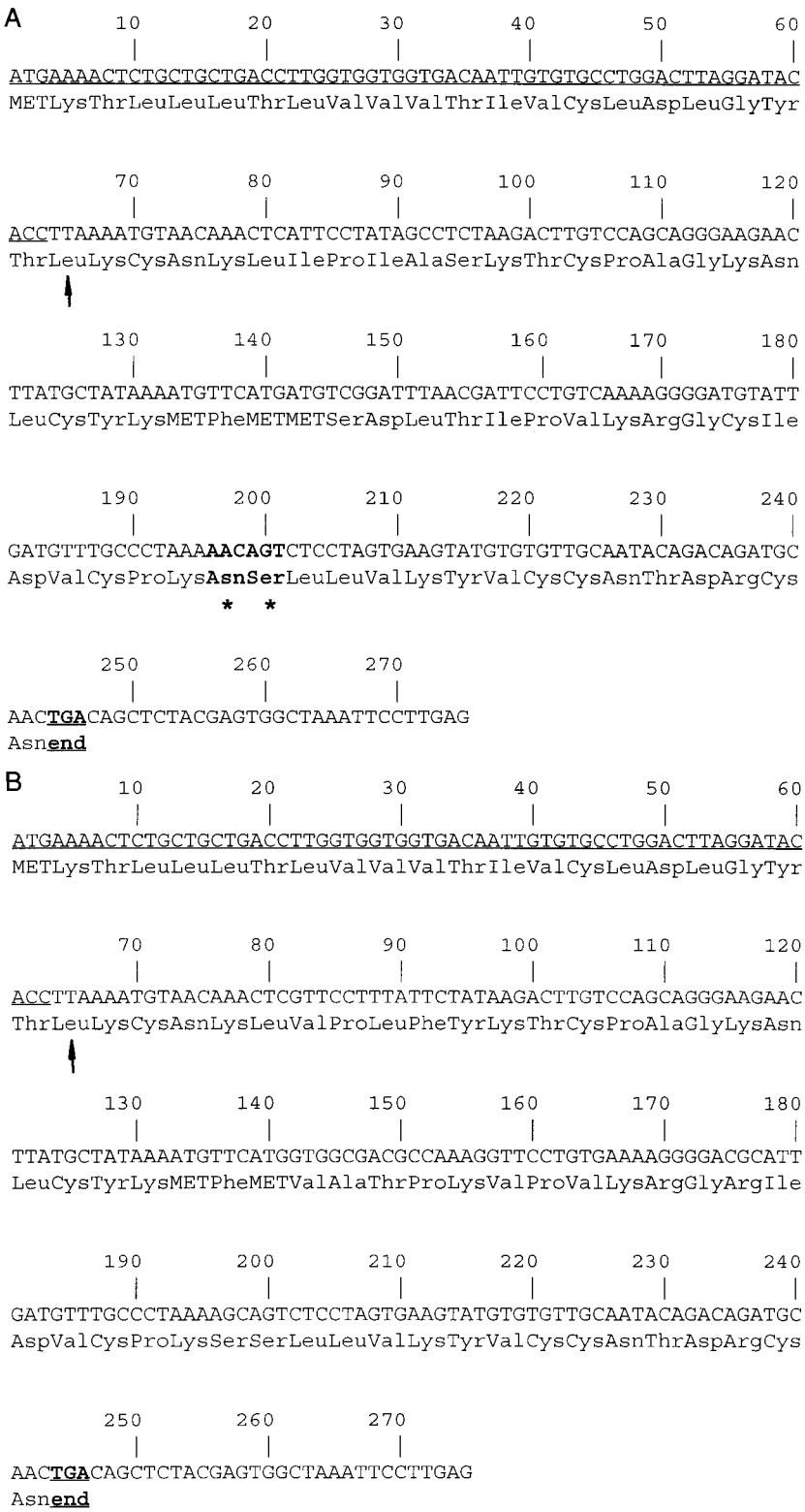

FIG. 2. Legend on facing page.

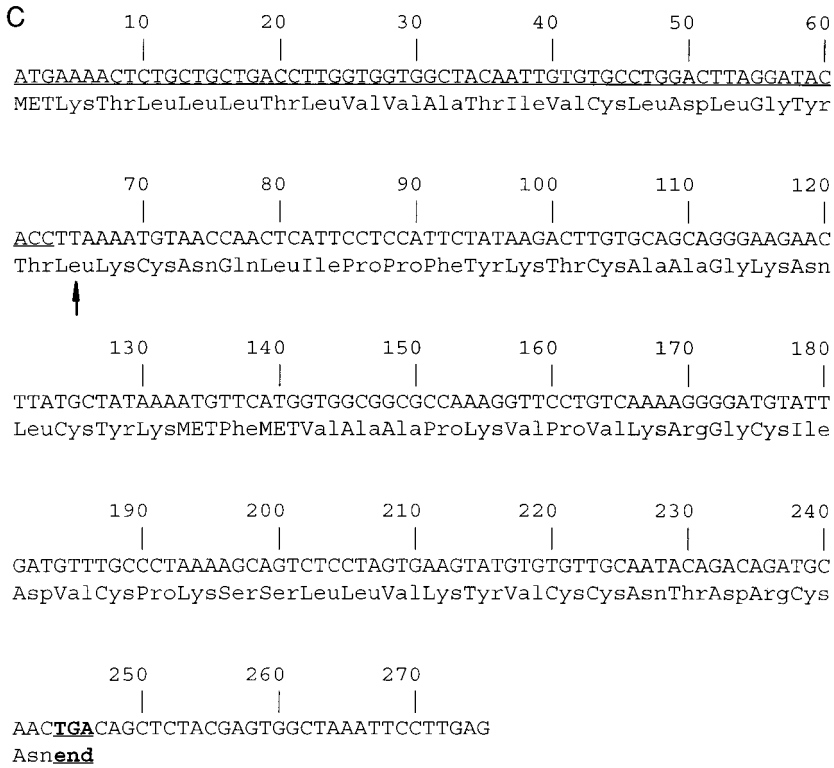

FIG. 2. Nucleotide and deduced protein sequences of the precursor of cardiotoxins I, III and N. The nucleotide sequence of 275, base pairs is shown above the amino acid sequence of 81 residues including a signal peptide of 21-amino acid residues. The mature cardiotoxins I (A), III (B) and N (C) of 60 residues starts at Leu. Residues at positions 45 and 46 of cardiotoxin I indicated by asterisks (*) are the two amino acids which show discrepancy with the published sequence determined by protein sequencing.

Gel Electrophoresis and Immunoblotting of Cardiotoxin

SDS-PAGE was performed in 15% acrylamide according to the method of Laemmli (11). Samples were denatured by boiling for 5 min in 0.125 M Tris, 4% SDS in the presence of 4% β-mercaptoethanol. After SDS-PAGE, the protein on the gel was electrophoretically transfered to the Immobilon PVDF membrane according to the method of Towbin *et al.* (12). The immunoreactive protein was visualized by immunological analysis using anti-cardiotoxin III antibodies and stained with horseradish peroxidase conjugated Protein A.

RESULTS AND DISCUSSION

In our initial purpose, we want to clone the gene encoding cobrotoxin, a neurotoxin from Taiwan cobra. Therefore, comparative analysis on the determined cDNA sequences, such as *Dendroaspis angusticeps* toxin 2, *Dendroaspis angusticeps* fasciculin 1, *Laticauda semifasciata* erabutoxin a, *Aipysurus laevis* toxin b and *Aipysurus laevis* toxin d (7), was undertaken. It was found that the nucleotide sequence of signal peptide and 3′-untranslational sequence of neurotoxins showed a high conservation. Thus, several forward and reverse primers from the 5′- and 3′- untranslational regions were designed for amplification the cDNA encoding cobrotoxin. Among them, two primers were designed from the conserved regions at the beginning of the signal peptide and 3′- untranslational region which was in proximity to the stop codon of neurotoxins, respectively. PCR amplification of the venom cDNA mixtures with the designed primers achieved the isolation of a PCR fragment estimated to be about 280 bp (Fig. 1). The DNA fragments were then subcloned by TA cloning kit. More than 30 clones were selected for nucleotide sequencing. Unfortunately, no

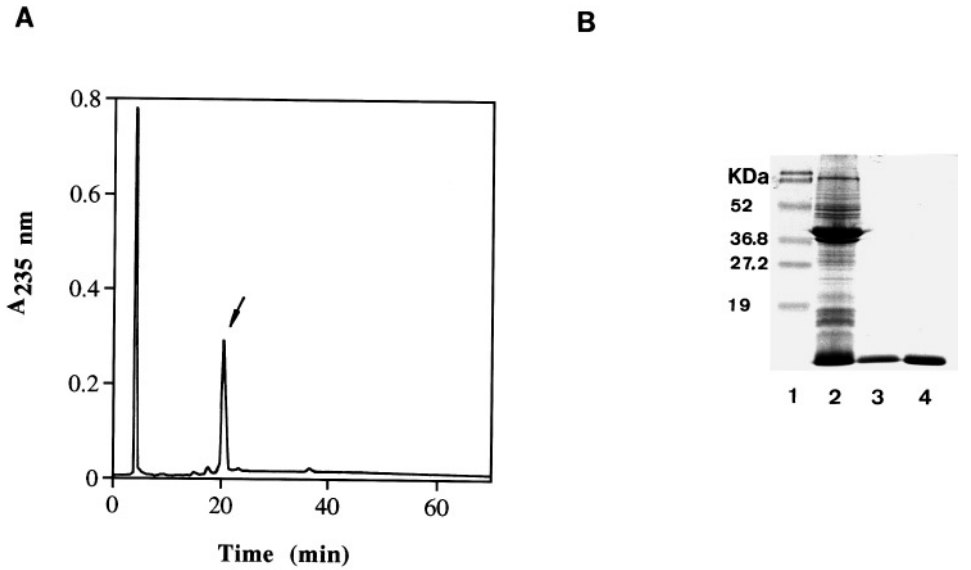

FIG. 3. Purification and SDS–PAGE analysis of recombinant cardiotoxin III. (A) The reduced recombinant cardiotoxin III was applied on a SynChropak RP-P column, equilibrated with 0.1% TFA and eluted with a linear gradient of 25–50% acetonitrile for 70 min. The flow rate was 0.8 ml/min and the eluate was monitored at 235 nm. The arrow indicates the recombinant cardiotoxin III. (B) SDS–PAGE analysis of recombinant cardiotoxin III. Lane 1: Molecular markers; Lane 2, The proteins in inclusion bodies; Lane 3, Purified recombinant cardiotoxin III from Fig. 3A; Lane 4, native cardiotoxin III from Taiwan cobra venom.

one out of the selected clones had the deduced amino acid sequences corresponded to cobrotoxin. However, the deduced amino acid sequences revealed that the cDNAs encoding cardiotoxin I, cardiotoxin III and cardiotoxin N, respectively (Fig. 2A, B and C). In addition to cardiotoxin I, the deduced protein sequence are the same as the previous published sequence determined by protein sequencing technique (13). The residues at positions 45 and 46 in the published sequence of cardiotoxin I were shown to be an Ser and Asn, which showed to be in reverse positions instead (Fig. 2A).

In order to subclone the cardiotoxins into the expression vector, a new primer was designed to create a *Nde* I site in the beginning of the nucleotide sequences for encoding amino acid sequence of cardiotoxins. For example, the primer sequence 5′-CATATGTTAAAATGCA-ATAAACTCGTTCCTT-3′ (the underline indicated the *Nde I* site) was synthesized for subcloning cardiotoxin III into the expression vector. Moreover, for preventing the formation of hairpin structures within the primer, the bases T and C at positions 15 and 18 were replaced by C and T, respectively. The resulting amino acid sequences did not change at all. The PCR procedures were performed with *pfu* DNA polymerase. The amplified DNA was inserted into pCRII vector, then subcloned into the expression vector pET 20b(+) by digested with *Nde* I/*EcoR* I. The expressed cardiotoxin III exclusively appeared in the inclusion bodies of *E. coli*, but did not show any hemolytic activity observed with native cardiotoxin from snake venom. Recombinant cardiotoxin III was dissolved in 6 M guanidine-HCl and reduced with β-mercaptoethanol. The reduced toxin was then purified by HPLC on a SynChropak RP-P column (Fig. 3A). The purified recombinant cardiotoxin III was homogeneity as revealed by SDS-PAGE (Fig. 3B). Immunoblot analysis indicated that the recombinant cardiotoxin showed an immunoreactivity with anti-cardiotoxin III antibodies (data not shown). Refolding of the purified recombinant cardiotoxin III is now in progress according to the procedure described by Wong *et al.* (14).

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