The cDNA encoding a novel three loop protein was cloned from cellular RNA isolated from the venom gland of *Bungarus multicinctus multicinctus* by RT-PCR. The mature protein has 82 amino acid residues. It shared only 25–38% similarity with some cardiotoxins and did not have sequence similarity with neurotoxins, while its cDNA was about 70% similar to both the cDNAs encoding neurotoxins and the cDNAs encoding cardiotoxins.

**Key words:** *Bungarus multicinctus multicinctus*; three loop protein; cDNA cloning; sequence analysis

The venom of the Taiwan banded krait, *Bungarus multicinctus multicinctus* contains several neurotoxins including α-bungarotoxin, β-bungarotoxins, and κ-bungarotoxins, which have been thoroughly investigated. α-Bungarotoxin binds with high affinity to the muscle nicotinic acetylcholine receptor and induces flaccid paralysis of skeletal muscles, while κ-bungarotoxins have a potent effect on the neuronal nicotinic receptor and block the transmission in several neuronal systems. In addition to the pharmacologically known neurotoxins, some neurotoxin-like proteins have also been isolated from *Bungarus multicinctus multicinctus*. There is no identified cardiotoxin from it. Cardiotoxins affect heart muscle and cause considerable cardiovascular depression. Although the venom of the Taiwan banded krait has well been studied, the venom of the Chinese continental banded krait is rarely studied. In this investigation, the cDNA encoding a novel member of the three loop protein family was cloned from the venom gland of the Chinese continental banded krait.

The total RNA was prepared from one venom gland of a Chinese continental banded krait by a guanidinium isothiocyanate/phenol chloroform isolation kit (Promega, USA). The two strands of 18s and 28s RNAs were intact as identified by 1% formaldehyde denatured gel electrophoresis. The spectrophotometric analysis showed that OD260/280 was 1.8 and OD260/230 was 2.0. The synthesized oligo(dT)25 was used to convert reverse transcript mRNA into the single strand cDNA by the M-MLV reverse transcriptase in order to get the versatility of cDNA mixtures.

Comparative analysis of the analyzed cDNA sequences of α-neurotoxins, κ-neurotoxins, and cardiotoxins from Elapidae and Hydrophiidae venoms showed that the nucleotide sequences of the 5′,3′-untranslated regions and signal peptide coding region were highly conserved. The plus and minus degenerate primers were designed from these highly conserved regions. The plus primer was 5′-AGATGAAAACCTCG(C/T)TGCTG(A/T)CCTTGG-3′ and the minus primer was 5′-GGATGGTCCATGATC(T/G)GA(T/G)GAGAAGCA-A-3′. PCR amplification of the above prepared cDNA mixtures with the designed primers and the Taq DNA polymerase (Promega, USA) allowed the isolation of PCR fragments estimated to be about 350 bp by 2% agarose electrophoresis. The DNA fragments were then purified by low melting point agarose and cloned into the pGEMT-vector (Promega, USA). The white clones were screened by PCR and ApaI/PstI restriction endonuclease digestion. Then the positive clones were selected for nucleotide sequencing. From analysis of the sequenced cDNAs on PC/GENE 6.8 (IntelliGenetics Inc., Switzerland), the cDNA encoding a novel three loop protein was cloned from *Bungarus multicinctus multicinctus* (Fig. 1). The deduced precursor comprised a 21-amino-acid signal peptide and a following 82-amino-acid mature protein with ten cysteine residues. The signal peptide, rich in hydrophobic amino acid residues, is similar to those of short chain neurotoxins, κ-neurotoxins, and cardiotoxins. The residue substitutions are always among the hydrophobic amino acids, which would not affect the hydrophobicity of the signal peptides. Although the signal peptide is very close to those of short chain neurotoxins, κ-neurotoxins, and cardiotoxins, the mature protein is dissimilar to all the known neurotoxins and cardiotoxins retrieved from the EMBL and GenBank libraries. Generally, the long neurotoxins consist of 70–74 amino acid residues and cardiotoxins contain about 60 amino acid residues. However, the novel protein comprises as many as 82 amino acid residues. Its PI value is predicted to be 7.4 and its molecular mass is approx. 9 kDa.

The novel protein has as many as 7 tyrosine residues, 9 glycine residues, and 9 serine residues. It also contains a polar tail fragment rich in serine, which is different from the tail fragments rich in basic residues in long neurotoxins. The structurally invariant residues common to all short chain neurotoxins, long chain neurotoxins, and cardiotoxins include Cys3, 17, 24, 45, 49, 60, 61 and 66;
Gly20; Tyr25; Gly44; Pro50 and Asn67.9 The corresponding positions in this protein are 3, 17, 24, 50, 55, 69, 70, 75, 20, 25, 50, 58, and 76 respectively. All these amino acid residues are also conserved with the exception that the site 76 is Gly instead of Asn in this novel protein. The eight critical cysteine positions are strictly conserved, suggesting that the novel protein might indeed have a three loop conformation similar to those of neurotoxins and cardiotoxins.10 The novel protein has a 6-amino-acid insertion in the second loop and a 2-amino-acid insertion between Cys55 and Pro58 as compared with α-bungarotoxin and κ-bungarotoxin (Fig. 2). The fifth disulfide bond situated in the first loop in the protein differs from that positioned in the second loop in α-bungarotoxin and κ-bungarotoxin. We previously identified two long neurotoxin homologues in which the fifth disulfide bond is also located in the first loop.11 Nevertheless, the cysteine positions of the fifth disulfide bond (Cys6-Cys11) in the novel protein are different from those (Cys6-Cys11) in the two long neurotoxin homologues. The functionally significant residues in neurotoxins contain Lys27, Trp29, Asp31, Arg37, Gly38, and Lys53 (the corresponding site in the protein is 62).12 Apart from Trp29, all the other functionally important amino acid residues are not conserved in the protein, indicating it would not display neurotoxicity.

The comparative analysis of nucleotide sequences showed that the cDNA encoding the novel protein shared about 70% similarity with those of short chain neurotoxins, long chain neurotoxins, κ-neurotoxins, and cardiotoxins. However, the comparative analysis of amino acid sequences found that the novel protein shared only 25–38% similarity with some cardiotoxins from different species and did not have homology with neurotoxins (Fig. 2). This phenomenon may be explained that these mature protein coding regions evolve more rapidly than the other regions for diverse functions. To get more insight into the evolution of the protein, the molecular evolutionary analysis was done on PC/GENE 6.8 (Intelligenetics Inc., Switzerland) and undertaken on the known nucleotide sequences from Bungarus multicinctus multicinctus (Fig. 3). The cDNA sequences encoding cobratoxin and cardiotoxin 3 from Naja naja atra
of which is different from that of the long neurotoxins. It has no homology with neurotoxins and its amino acid composition is also dissimilar to that of neurotoxins. However, it shares approx. 25–38% similarity with some cardiotoxins from different species, although its amino acid composition is different from that of the cardiotoxins.

References