

## Cloning, Direct Expression, and Purification of a Snake Venom Cardiotoxin in *Escherichia coli*

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The cardiotoxin analogue III (CTX III), isolated from the Taiwan cobra (*Naja naja atra*) venom, is a sixty-amino acid, all  $\beta$ -sheet protein. We report the direct expression of CTX III from its synthetic gene as inclusion bodies in *Escherichia coli*. The yield of the expressed protein is about 40 mg/liter of the culture. CTX III trapped as inclusion bodies is dissolved and refolded by the slow refolding technique. The refolded protein is purified by reverse phase high performance liquid chromatography. The purified and refolded CTX III sample is further characterized by SDS-PAGE, circular dichroism, two-dimensional NMR spectroscopy and haemolytic activity. To our knowledge, this is the first report of the direct expression and purification of snake venom cardiotoxins. © 1996 Academic Press, Inc.

Snake venoms are a mixture of many different proteins of which the cardiotoxins and the neurotoxins are the most toxic (1). These are small molecular weight (6500–7000), all  $\beta$ -sheet proteins with four disulfide bridges (2,3). These two toxin classes show a high degree of homology in their primary sequences (1). A comparison of the solution structures of neurotoxins and cardiotoxins shows that the overall folds of the two toxins are similar, with only subtle differences (4–6). Surprisingly, despite the gross similarities in their three dimensional structures, these two toxins exhibit drastically different biological activities (7). Unlike the neurotoxins, which bind to the acetylcholine receptor at the postsynaptic junction (1), cardiotoxins show a wide array of biological activities (8–10). In recent years, there has been an increased interest in understanding the structural basis for the broad spectrum of biological activities observed for cardiotoxins (11–13). However, the validity of these models could not be tested as no reliable structure-function relationship data on this class of proteins are available. Site-directed mutagenesis studies of cardiotoxins could provide an useful handle to probe the structure-function relationship of these toxins. In pursuit of this final objective, using a synthetic gene, we attempted to express cardiotoxin analogue III from the Taiwan cobra (*Naja naja atra*). In this communication, we report the first successful direct expression (without the fusion protein) and the purification of such a cloned CTX III.

### MATERIALS AND METHODS

Acetonitrile, urea, oxidised glutathione and  $\beta$ -mercaptoethanol were obtained from Sigma Co., USA. All the nucleotides required for synthesizing the oligonucleotide were also purchased from Sigma Co. Pre-stained molecular weight marker proteins kit was procured from Bio-Rad. As the pre-staining of the marker proteins involves the tagging of polysaccharide (information from the manufacturer's brochure) moiety(ies), the molecular weight of the marker proteins are correspondingly higher than expected. Taq DNA polymerase, *Nde* I and *Eco*R I enzymes were purchased from Promega. *Escherichia coli* BL21 (DE3) and pET21b were from Sigma. All other chemicals used in this study were of high quality analytical grade.

*Construction and expression of the ctx synthetic gene.* The gene encoding the CTX III protein, designated as *ctxC*, was constructed from 11 fragments of synthetic nucleotides. The oligonucleotides were synthesized on a Applied Biosystems

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380B synthesizer based on the amino acid sequence of CTX III(7). The translational codons of the synthetic gene are preferentially used in *E. coli* (14). In addition, an extra codon at the N-terminal was created for direct expression in *E. coli*. The assembly and the initial cloning of the *ctxC* gene into pGEM7 to obtain pGEM-CTX III were performed similar to that as described by Chang *et al* (15). The *ctxC* gene in the recombinant pGEM CTX III plasmid was amplified by the polymerase chain reaction (PCR) using, 5'-GGATCCAAGCTCATATGCTGAAATGCAA-3' and 3'-ACGTTGATCACTTAAGGAGCTCAGATCT-5' as primers. For the former primer, the two underlined bases were those mutated to create a new *Nde I* site. The latter primer retains the original *EcoR I* site. The *Nde I-EcoR I* fragment encoding 61 amino acids (Fig. 1) was cloned into the *Nde I* and *EcoR I* site of pET21b to obtain pCT3 using the approach reported by Lei *et al* (16). The identity of the cloned *ctxC* was confirmed by nucleotide sequencing. The expression of *ctxC* in pCT3 was controlled by the T7 promoter and lacI regulating element. BL21 containing *ctxC* expression plasmids was grown at 37°C in 5 litres of 2X LB medium supplemented with ampicillin (100 mg/L). When the OD<sub>600</sub> of the growing culture reached about 2.5, IPTG (30 mg/L) was added to initiate overexpression. The culture was allowed to grow for an additional 5 h and was harvested and submitted to sonication. The resultant protein patterns in the supernatant and the pellet were analysed by SDS-PAGE as described by Laemmli (17).

**Dissolution of the inclusion bodies.** The pellet on centrifugation was dissolved in 0.1 M Tris-HCl (pH 8.7) containing 2%  $\beta$ -mercaptoethanol and 8 M urea at room temperature. The process of dissolution of the pellet was allowed to continue for at least 3 h.

**High performance liquid chromatography (HPLC).** All HPLC runs were carried out on a semi-preparative reverse phase C<sub>18</sub>  $\mu$ -Bondapak column using 0–60% gradient of acetonitrile containing 0.1% trifluoro acetic acid over a time period of 60 minutes. The HPLC runs were carried out on a Hitachi machine (Model L-400). The eluted proteins were detected using 280 nm absorbance.

**Refolding.** The samples obtained from HPLC runs were dried using a speed-vac (Savant Co., USA). The dried powder comprising the denatured or partially structured CTX III was redissolved in 0.1 M Tris-HCl (pH 8.7) containing 8 M urea and 0.15 M  $\beta$ -mercaptoethanol and subjected to slow refolding against 0.1 M Tris-HCl (pH 8.0) containing 10 mM oxidized glutathione for about 36 h.

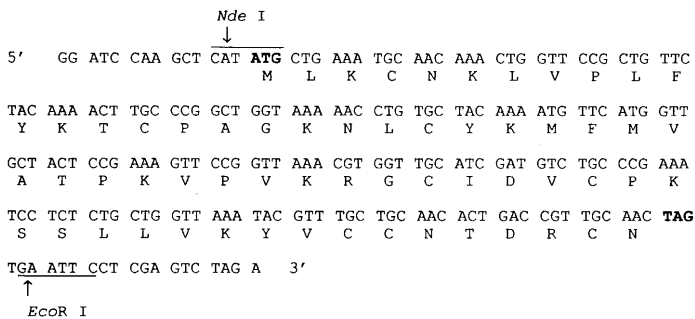
**Circular dichroism.** All circular dichroism measurements were carried out at 25°C on a Jasco J720 spectropolarimeter using 0.02 cm pathlength cell.

**Two dimensional proton NMR.** Spectra were recorded at 25°C and pH 6.8. on a Bruker DMX-600 spectrometer. All the spectra were obtained by dissolving appropriate amounts of the lyophilised refolded CTX III (expressed protein) in a mixture of 90% H<sub>2</sub>O and 10% D<sub>2</sub>O. Spectra were acquired with 2048 data points in t<sub>2</sub> and 502 points in t<sub>1</sub> dimension. The Nuclear Overhauser Enhancement (NOE) connectives for the secondary structure were obtained using the main-chain directed (MCD) approach (18).

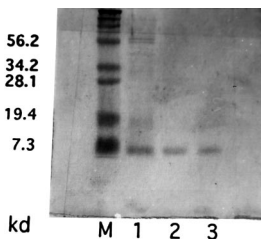
**Haemolytic activity.** Freshly collected rabbit blood in sodium citrate buffer was washed in isotonic NaCl solution. The cell suspension was centrifuged to get a clear supernatant. The washed cells were resuspended in the same buffer for haemolysis. Aliquots of the washed cells were then added and diluted to the desired CTX concentration to a final volume of 1 mL at 37°C. After the desired incubation period with gentle agitation, aliquots were pipetted out, centrifuged for 1 min at 14,000 rpm in a microcentrifuge. The absorbance of the supernatant was then measured at 540 nm for the determination of the lysis of rabbit red blood cells. All measurements were carried out with appropriate controls.

## RESULTS AND DISCUSSION

CTX III expressed in *E. coli* is trapped as insoluble inclusion bodies. We find that it is feasible to dissolve the CTX III trapped in the inclusion bodies using the denaturant buffer (0.1 M Tris-HCl,

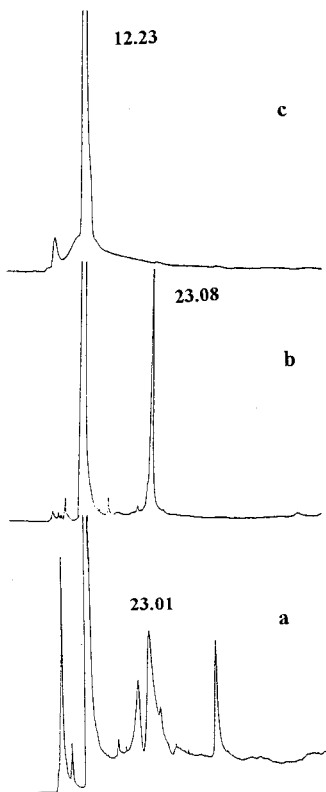


**FIG. 1.** Sequence of the *ctxC*-containing PCR-amplified DNA product. The sequence of the coding strand of the PCR-amplified product is shown. The initiation and stop codons for *ctxC* are shown in boldface, and the encoded amino acid sequence is illustrated in the single-letter codes underneath the nucleotide sequence. The DNA fragment shown between the two arrows was inserted into pET21b at the *NdeI* and *EcoRI* sites to obtain pCT3.



**FIG. 2.** SDS-PAGE of lane M- molecular weight marker proteins; lane 1- inclusion bodies dissolved in 0.1 M Tris-HCl (pH 8.7) containing 8 M urea and 2%  $\beta$ -mercaptoethanol; lane 2- expressed and refolded CTX III (rCTX III) and lane 3- native CTX III (nCTX III). The proteins composing the molecular weight marker kit are, myosin (204 Kda),  $\beta$ -galactosidase (121 Kda), bovine serum albumin (82 Kda), ovalbumin (56.2 Kda), carbonic anhydrase (34.2 Kda), soyabean trypsin inhibitor (28.1 Kda), lysozyme (19.4 Kda) and aprotinin (7.3 Kda).

pH 8.7, containing 8 M urea and 2%  $\beta$ -mercaptoethanol). SDS-PAGE of the dissolved inclusion bodies (Fig. 2, lane 1) revealed an intense band in the molecular weight range of 6000–7000. CTX III is expressed in large amounts with an yield of about 40 mg/L. The lane (Fig. 2, lane 1) in the SDS PAGE gel corresponding to the dissolved CTX III inclusion bodies revealed little impurity in the inclusion bodies due to other proteins. In comparison with the buffer control (Fig. 3c), reverse phase HPLC run of the native CTX III (nCTX III) isolated from the crude venom and dissolved in a denaturant buffer (0.1 M Tris-HCl, pH 8.7, containing 8 M urea and 2%  $\beta$ -mercaptoethanol) showed a major peak with a retention time of 23.08 min (Fig. 3b). HPLC profile of the inclusion



**FIG. 3.** HPLC profile of, **a** - inclusion bodies dissolved in a buffer consisting of 0.1 M Tris-HCl (pH 8.7), 8 M urea and 2%  $\beta$ -mercaptoethanol; **b** - nCTX III dissolved in the same buffer; and **c** - the buffer alone.

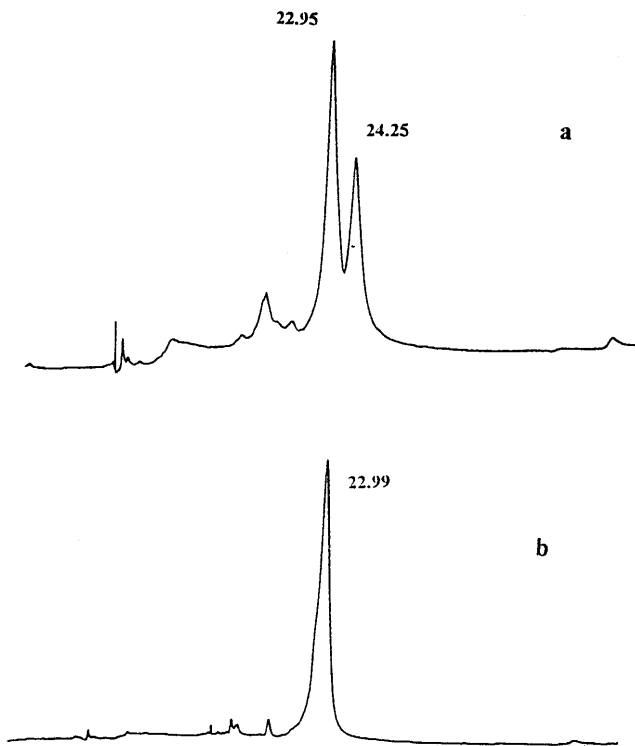


FIG. 4. Reverse phase HPLC profile, **a** - the refolded protein(s) mixture, **b** - rCTX III.

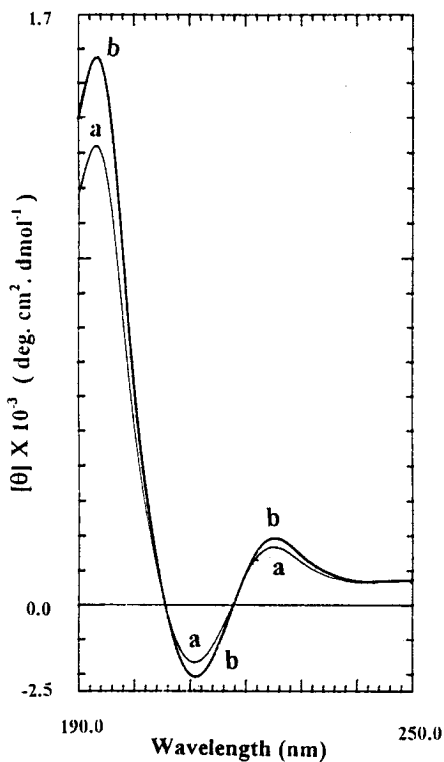


FIG. 5. Far UV CD spectra of, **a** - nCTX III and **b** - rCTX III.



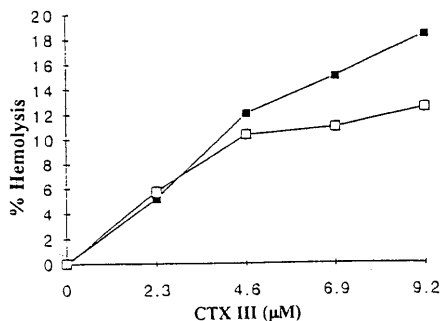


FIG. 7. The dosage dependent haemolytic assay of □-□, rCTX III and ■-■, nCTX III.

bodies dissolved in the same buffer (Fig. 3a) revealed a substantial peak (retention time 23.01 min) corresponding to that obtained from nCTX III, and a few additional peaks implying that there are very few proteins trapped along with CTX III in the inclusion body. This result is consistent with that obtained on SD-PAGE. Far UV CD spectra of this protein peak (expressed CTX III) showed a CD band centred at 203 nm, indicating that the expressed CTX III obtained after the HPLC run is disordered, as a random coil (data not shown).

The expressed and disordered CTX III sample was subjected to slow refolding (by dialysis) under oxidizing conditions. The refolded sample, on reverse HPLC showed two peaks with retention times of 22.95 and 24.25 min (Fig. 4a). The major peak with the retention time of 22.95 min, on rechromatography on the reverse phase column under identical conditions shows a single peak (Fig. 4b), implying that the protein sample is pure. SDS gel electrophoresis of this refolded protein (rCTX III) sample also gave a single band corresponding to a molecular weight of about 6500 (Fig. 2, lane 2). SDS PAGE of nCTX III sample, run as a control also shows a single band at the same position on the gel as that of the rCTX III (Fig. 2, lanes 2 and 3). These results clearly demonstrate that the major peak (Fig. 4a, retention time of 22.95 min) in the refolded sample is pure and it is the same as native CTX III.

It is important to establish that the expressed and rCTX III folds are similar to the nCTX III protein. Identical as the nCTX III, far UV CD of rCTX III revealed a 212 nm negative CD extrema (Fig. 5), characteristic of the  $\beta$ -sheet conformation. NOESY spectrum (data not shown) of rCTX III showed that the refolded protein possess both the double and the triple stranded antiparallel  $\beta$ -sheets (19,20). A comparison of the DQF-COSY spectra of the nCTX III and the rCTX III, (Figs. 6A and 6B), in the finger print region showed that almost all the intra residue cross peaks which are observed in nCTX III (Fig. 6A) could also be visualized in the spectrum of the rCTX III sample (Fig. 6B).

Another critical test for the correct folding of rCTX III is its biological activity. CTX III has been reported to possess haemolytic activity (1). We performed a dosage dependent haemolytic assay for nCTX III and rCTX III. It was found that the haemolytic activity observed for rCTX III was similar to the one obtained for nCTX III (Fig. 7). Thus, we are not only able to express CTX III in high yields (40 mg/L) but also are successful in purifying CTX III from the inclusion bodies and later refolding the protein into the native biologically active form.

Previously, there have been attempts to express other cardiotoxin isoforms as fusion proteins (21,22). However, the purification of the expressed protein was never achieved. To the best of our knowledge, this is the first report wherein the direct expression and purification of the cardiotoxins produced in *E. coli* are accomplished. In our opinion, the successful expression of CTX III in high yields would also facilitate the generation of site-directed mutants, which in turn would prove useful in understanding the structure-function relationship and protein folding aspects in this class of proteins.

## ACKNOWLEDGMENTS

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