# A recombinant snake neurotoxin generated by chemical cleavage of a hybrid protein recovers full biological properties

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We previously reported the production of a fused snake neurotoxin composed of protein A and erabutoxin *a* in *E. coli* [1]. The hybrid had much lower toxicity and affinity for the acetylcholine nicotinic receptor than natural erabutoxin. By treating the hybrid with cyanogen bromide we generated a toxin which was purified in a single step by RP-HPLC. This compound, produced in a good yield, recovered all properties of native erabutoxin *a*, implying that the lower toxic activities of the hybrid were due to the bulky protein A and not to an incorrect folding of the toxin. This work serves as a basis for future studies of toxin-receptor interactions using engineered toxin mutants.

Snake neurotoxin; Recombinant protein; Cyanogen bromide cleavage; Acetylcholine receptor

## 1. INTRODUCTION

Previously, we reported on the direct expression in *E. coli* of a heterologous protein composed of protein A coupled to a snake neurotoxin. The fused toxin was capable of binding to the acetylcholine nicotinic receptor (AcChoR) but with a much lower affinity than native toxin [1], making the hybrid inappropriate for detailed studies of toxin-receptor interactions. The hybrid was also less toxic in vivo [1]. Possibly, the non-native biological properties of the fused toxin were due to an incorrect folding of the toxin and/or to a steric hindrance by the bulky protein A. This unclear situation prompted us to delete the protein A moiety from the toxin. Appropriate mutations in the plasmid [1] had located a methionine at position −1 of the toxin sequence. In the present paper we describe (i) the cleavage of the hybrid with cyanogen bromide; (ii) the purification of the generated toxin and (iii) the physicochemical and biological properties of this first recombinant snake toxin.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Amino acid composition analysis was performed using an automatic model 130 A amino acid analyzer following total acid hydrolysis (6 N HCl, 110°C, 24 h) and subsequent precolumn phenylisothiocyanate derivatization using the on-line model 420 A derivatizer; sequencing was performed on a pulsed liquid 477 A pro-

tein sequencer, and the resulting phenylthiohydantoin derivatives of amino acids were analysed by HPLC using the on-line model 120 A analyzer (Applied Biosystems, Foster City, CA, USA). Dichroic spectra were recorded at 22°C, using a CD III Jobin-Yvon dichrograph.

### 2.2. Expression and purification of the fusion protein

The fusion protein expressed in *E. coli* was purified using a one-step affinity chromatography on immobilized neurotoxin-specific monoclonal antibody [1].

### 2.3. Cyanogen bromide cleavage

In a typical experiment, 3 mg of fusion protein were incubated with 1 ml of a 90 mM CNBr solution in 70% formic acid for 24 h, at room temperature. The solution was then diluted 10-fold with water and freeze-dried.

### 2.4. Purification of the recombinant protein

The CNBr treated material was applied to a reverse phase HPLC column (Vydac C4, 4.6 × 250 mm) equilibrated in 0.1 M triethylamine adjusted to pH 3.2 with pure formic acid. A 60 min gradient of 0–40% acetonitrile at a flow rate of 1 ml/min was then applied. After freeze-drying, the purified protein was incubated in 100 $\mu$l of pure ethanolamine for 30 min at room temperature for deformylation [2] and then freeze-dried again. A desalting step was finally performed on a reverse phase HPLC column (Aquapore RP-300, 2.1 × 30 mm) using a TFA/CH$_3$CN/H$_2$O gradient system: 5 min elution with 0.1% TFA (A) followed by a 10 min gradient of 0–100% B in A (B: 70% CH$_3$CN in A) at a flow rate of 0.5 ml/min.

### 2.5. Biological assays

Radioimmunoassays were carried out as previously described [3] using $^3$H-labelled toxin $\alpha$ from *Naja nigricollis* [4] and M$\alpha$2-3 monoclonal antibody [5]. Specific binding and affinity of fused and cleaved erabutoxins for acetylcholine receptor-rich membranes were determined from competition experiments [6] using $^3$H-labelled toxin $\alpha$. Native erabutoxin a was purified from *Laticauda semifasciata* [7]. The lethal dose (LD$_{50}$) was determined by i.p. injections of purified cleaved protein in 20 g BALB/c mice.

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## 3. RESULTS AND DISCUSSION

### 3.1. Purification and characterization

The hybrid possesses four methionine residues that are all located before the erabutoxin sequence and one of them was purposely located at position $-1$ of erabutoxin a by site directed mutagenesis [1]. Treatment with CNBr was therefore expected to release the toxin moiety. Chromatography on the RP-column of the cleaved hybrid led to three main resolved components which were investigated for their ability to compete with a radioactive toxin in binding with a toxin-specific monoclonal antibody named $M\alpha$2-3 [5]. Components 1 and 3 were recognized by the antibody and coeluted respectively with native erabutoxin and the hybrid. Component 1 was treated with ethanolamine [2], desalted and rechromatographed on RP-HPLC using a highly resolutive TEAF system [6]. Fig. 1 shows that recombinant erabutoxin ($Ea_r$) coeluted on RP-HPLC and coelectrophoresed on polyacrylamide gel with native erabutoxin a ($Ea_n$). In both cases, $Ea_r$ appeared homogeneous. Approximately 0.25 mg of $Ea_r$ was obtained from 3 mg hybrid.

The amino acid compositions of the recombinant and native toxins were identical within the experimental error (Table I). In both cases however, the Ile content was lower than expected from sequence data (Table II).
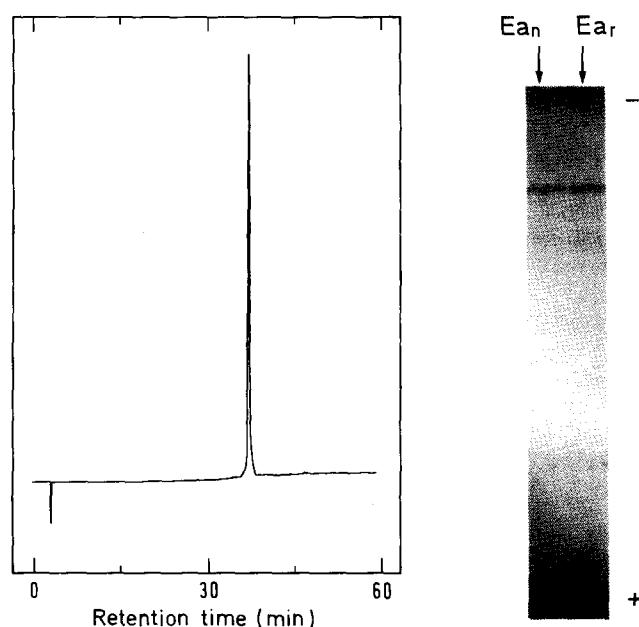


Fig. 1. Analysis of recombinant erabutoxin a by RP-HPLC and isoelectric focusing. Left, approximately 5 μg of native erabutoxin a and 5 μg of recombinant erabutoxin a were mixed and loaded onto a Vydac C4 column (250 × 4.6 mm) and eluted with a 60 min gradient of 0–40% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The detection was monitored at 210 nm. Right, native ($Ea_n$) and recombinant ($Ea_r$) erabutoxin a were submitted to isoelectric focusing in a 8% polyacrylamide gel using a pH 3.5–10 Ampholine gradient as previously described [10]. About 20 μg of protein were loaded in each lane. Gels were stained with Coomassie blue.

Table I

Amino acid compositions of native or recombinant erabutoxin a

| Amino acid | From sequence analysis | Native erabutoxin | Recombinant erabutoxin |
|---|---|---|---|
| Asx | 5 | 5.29 | 5.26 |
| Glx | 8 | 8.69 | 8.72 |
| Ser | 8 | 8.07 | 8.08 |
| Gly | 5 | 5.25 | 5.34 |
| His | 1 | 0.99 | 1.15 |
| Arg | 3 | 2.81 | 2.77 |
| Thr | 5 | 5.58 | 5.48 |
| Ala | 0 | 0.07 | 0.15 |
| Pro | 4 | 4.11 | 4.09 |
| Tyr | 1 | 1.04 | 0.93 |
| Val | 2 | 1.57 | 1.56 |
| Met | 0 | 0.04 | 0.00 |
| Ile | 4 | 2.42 | 2.40 |
| Leu | 1 | 0.97 | 1.13 |
| Phe | 2 | 2.06 | 2.13 |
| Lys | 4 | 4.03 | 3.95 |
| Cys | 8 | ND | ND |
| Trp | 1 | ND | ND |

ND, not determined

Table II

Automated sequence analysis of the 50 first residues of recombinant erabutoxin a

| Cycle number | Residue assigned | Amount (pmol) | Cycle number | Residue assigned | Amount (pmol) |
|---|---|---|---|---|---|
| 1 | R | 191 | 32 | F | 65 |
| 2 | I | 1486 | 33 | R | 110 |
| 3 | C | ND | 34 | G | 36 |
| 4 | F | 1089 | 35 | T | 19 |
| 5 | N | 551 | 36 | I | 71 |
| 6 | H | 219 | 37 | I | 37 |
| 7 | Q | 710 | 38 | E | 12 |
| 8 | S | 167 | 39 | R | 41 |
| 9 | S | 216 | 40 | G | 43 |
| 10 | Q | 524 | 41 | C | ND |
| 11 | P | 484 | 42 | G | 32 |
| 12 | Q | 416 | 43 | C | ND |
| 13 | T | 199 | 44 | P | 11 |
| 14 | T | 207 | 45 | T | 4 |
| 15 | K | 490 | 46 | V | 8 |
| 16 | T | 191 | 47 | K | 10 |
| 17 | C | ND | 48 | P | 8 |
| 18 | S | 62 | 49 | G | 9 |
| 19 | P | 266 | 50 | I | 10 |
| 20 | G | 270 | 51 | | |
| 21 | E | 126 | 52 | | |
| 22 | S | 55 | 53 | | |
| 23 | S | 31 | 54 | | |
| 24 | C | ND | 55 | | |
| 25 | Y | 96 | 56 | | |
| 26 | N | 96 | 57 | | |
| 27 | K | 148 | 58 | | |
| 28 | Q | 114 | 59 | | |
| 29 | W | 47 | 60 | | |
| 30 | S | 25 | 61 | | |
| 31 | D | 17 | 62 | | |

Analysis was carried out with about 1.5 nmol of reduced, $S$-pyridylethylated protein. ND, not quantitatively determined

This is probably due to a low yield on acid hydrolysis of the Ile-Ile bond [8], found at positions 36–37 in the toxin sequence. To assess that the four isoleucine residues were effectively present in $Ea_r$, we submitted its reduced, S-pyridylethylated form to microsequencing up to residue 50. As shown in Table II, the elucidated amino acid sequence of $Ea_r$ is identical to that of $Ea_n$.

### 3.2. Structure

We investigated the overall structure of $Ea_r$ by analysing its far-ultraviolet circular dichroism characteristics (Fig. 2). Within experimental error, the spectra of $Ea_n$ and $Ea_r$ superimposed, indicating that the dominant $\beta$ sheet structure of erabutoxin [9] is preserved also in $Ea_r$.

We probed locally the structure of $Ea_r$ in two ways. Firstly, we examined the antigenicity of $Ea_r$ using $M\alpha2$-3, a monoclonal antibody which is highly specific for correctly folded neurotoxins and which recognizes a large domain encompassing the three adjacent loops
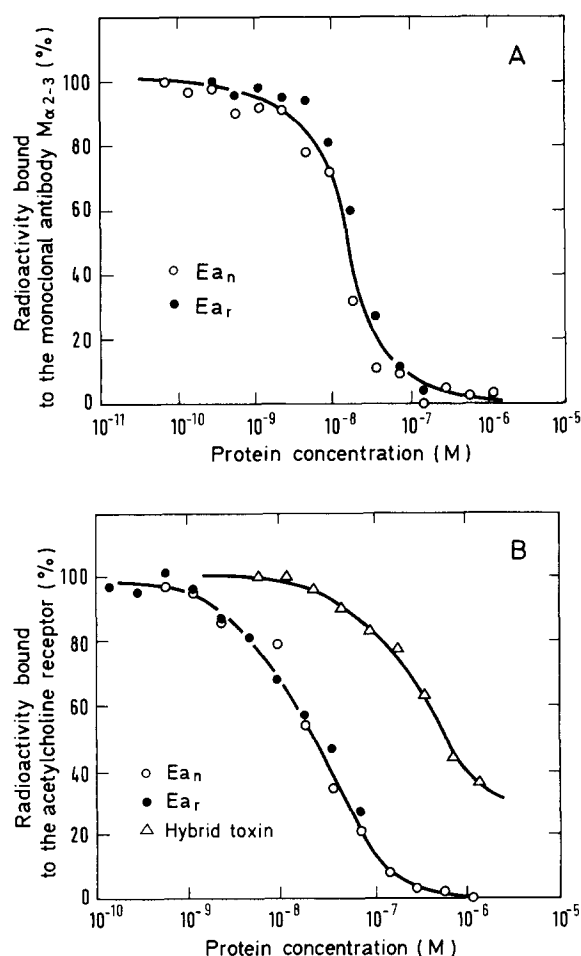
on the concave side of neurotoxins [5]. As shown on Fig. 3A, $Ea_r$ and $Ea_n$ are equally potent at competing with $^3$H-labelled toxin $\alpha$ for $M\alpha2$-3. The epitope is therefore highly similar in both toxins. Secondly, we probed the AChoR binding site of $Ea_r$ by determining in vitro its affinity for a suspension of membrane AChoR [6]. This experiment was of particular interest since the fused toxin had an affinity constant about 70-fold lower than $Ea_n$ for AChoR (Fig. 3B) and also was about 7-fold less toxic in vivo. As shown in Fig. 3B, $Ea_r$ and $Ea_n$ are strikingly equally potent at binding to AChoR. Furthermore, we found that they have the same toxicity to BALB/c mice ($LD_{50} = 2 \pm 0.5 \mu g/20$ g mouse). These results support our former proposal that the low biological activity of fused toxin was due to the presence of the bulky protein A moiety and not to an incorrect folding of the toxin [1].
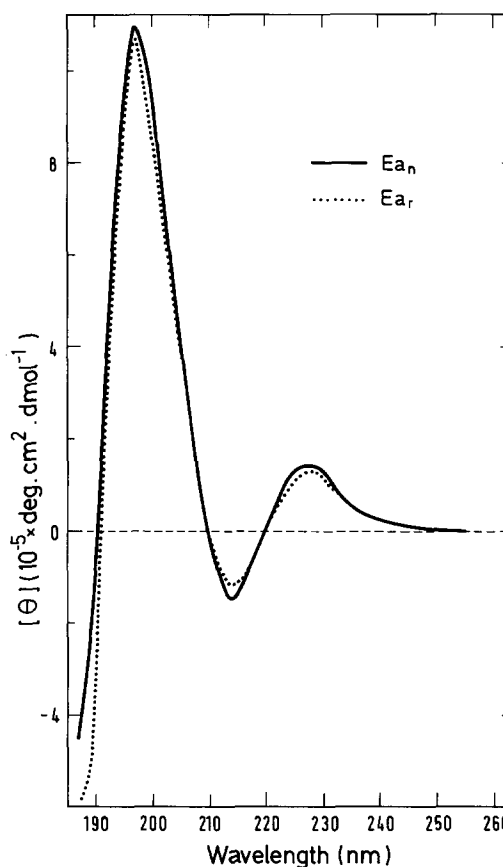


Fig. 3. Biological assays. (A) Binding of $^3$H-labelled toxin $\alpha$ (3.4 nM, 17 Ci/mmol) to the monoclonal antibody $M\alpha2$-3 (11.2 nM) in the presence of varying amounts of either native erabutoxin a (3 × 10$^{-7}$ to 3 × 10$^{-10}$ M) ($Ea_n$) or recombinant erabutoxin a (1.5 × 10$^{-7}$ to 5 × 10$^{-10}$ M) ($Ea_r$). Incubation time was 24 h at 4°C. Toxin-antibody complexes were precipitated by polyethylene glycol [4]. (B) Inhibition binding curve of $^3$H-labelled toxin $\alpha$ (6 nM) to the acetylcholine receptor (3.8 nM) in the presence of varying amounts of either protein A-erabutoxin a hybrid (10$^{-6}$ to 5 × 10$^{-9}$ M), native erabutoxin a (10$^{-6}$ to 10$^{-10}$ M) ($Ea_n$) or recombinant erabutoxin a (10$^{-7}$ to 10$^{-10}$ M) ($Ea_r$). Incubation time was 6 h at 20°C. The mixtures were filtered through Millipore filters as previously described [6].



Fig. 2. Far-ultraviolet circular dichroism analysis of recombinant erabutoxin. Circular-dichroic spectra of native erabutoxin (1.9 × 10$^{-5}$ M) ($Ea_n$) or recombinant erabutoxin (1.7 × 10$^{-5}$ M) ($Ea_r$) were recorded in water. The cell path length was 0.05 cm.

Altogether, our results indicate that the recombinant toxin is virtually identical with native erabutoxin a. This is the first report showing that a recombinant animal toxin has the same physicochemical and biological properties as the natural toxin. Since the recombinant toxin can be produced in the milligram range from the fused toxin, we now have in hand a well-suited system to prepare appropriate mutants for studying toxin-acetylcholine receptor interactions.

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