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Isolation and Characterization of *Pelamis platurus* (Yellow-bellied Sea Snake) Postsynaptic Isoneurotoxin

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Abstract—*Pelamis platurus* (yellow-bellied sea snake) venom contains several neurotoxins, the major toxin, which is most toxic, and two other isotoxins. The second most toxic neurotoxin (Pelamis toxin *b*) was isolated and characterized. It contains 60 amino acid residues with only one residue difference from the major toxin, Pelamis toxin *a*. The difference is at the tenth amino acid residue from the acid terminal. The isoelectric point of toxin *b* is 8·7. Raman spectroscopic examination of toxin *b* indicates that the toxin contains a considerable amount of antiparallel β-structure, β-turn, and random coil without α-helix as the amide I band appears at 1673 cm^{-1} and the amide III band at 1246 cm^{-1}. Circular dichroic studies also indicate a typical β-sheet structure. The Pelamis toxin *b* is a typical postsynaptic neurotoxin as it binds to the acetylcholine receptor competitively with a well known toxin, α-bungarotoxin. The LD50 of toxin *b* is 0·185 $\mu g\ g^{-1}$ in mice by intravenous injection, indicating high toxicity of a postsynaptic neurotoxin.

Snake venoms of Elapidae and Hydrophiidae are extremely toxic because they contain potent pre- and/or postsynaptic neurotoxins (Tu 1973; Dufton & Hider 1983).

A snake venom contains several isotoxins, but in most cases only the major toxin is isolated and studied in detail. Because of the small quantity and difficulty to isolate a minor toxin, most investigators have ignored them. *Pelamis platurus* venom contains several isotoxins. Only the major toxin, Pelamis toxin *a*, has been isolated and its properties characterized (Tu et al 1975; Wang et al 1976; Ishizaki et al 1984). However, it is of interest and importance to find how each toxin differs in chemical structure, property, and pharmacological property in order to understand the mechanism of toxic action.

Materials and Methods

Isolation

Sea snakes, *Pelamis platurus*, were captured on the Pacific coast of Costa Rica, Central America, in 1980. The neurotoxin was isolated from *P. platurus* venom by the method of Tu et al (1975) using a two-step gel chromatography procedure performed at 4°C.

The homogeneity of the toxin was checked using polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was done using the β-alanine acetate system previously described (Tu et al 1975).

Toxicity

Toxicity was assayed with white Swiss Webster mice between 18 to 22 g. Toxin in 100 μL of 0·9% NaCl (saline) was injected by intravenous route. The LD50 was determined with 5 to 10 mice per dose level, allowing a 24 h interval according to the method of Litchfield & Wilcoxon (1949).

Amino acid sequence

The purified major toxin was reduced and alkylated to make carboxymethylated toxin by the method of Fox et al (1977).

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The toxin fragments were made by incubating toxin with endopeptidase lysine-c (Boehringer Mannheim) for 24 h using an enzyme to substrate ratio of 1:20 (w/w). The mixture was then separated to peptide fragments by reverse phase HPLC (Beckman ultrasphere-ODS, 4·6 mm × 25 cm). The sample was eluted with water from zero concentration to the gradient of 50% acetonitrile–0·1% trifluoroacetic acid. Endopeptidase Arginine-C (Sigma) was also used to make different fragments, which were also separated by reverse phase HPLC.

Amino acid sequence was determined directly on an Applied Biosystems 470 A protein sequencer and PTH analyzer, on-line PTH amino acid separation system 120 A.

Spectroscopic assays

CD spectra were measured with a Jasco Model J-41G spectropolarimeter at 25°C. Protein samples were dissolved in distilled water (pH 6·8) at a concentration of 0·1 mM.

Raman spectra were obtained by excitation with the 514·5-nm line (Spectra Physics, Model SP-164 argon ion laser) with a green interference filter. The spectra represent an average of 10 scans and were recorded using a Spex Ramalog 5 Raman spectrophotometer and a Spex SCAMP data acquisition processor. All spectra were done with solid protein samples and were recorded from 400 to 1700 cm^{-1} with a 4 cm^{-1} spectral width resolution.

Neurotoxin-acetylcholine receptor binding

Acetylcholine receptor was isolated from *T. californica* electroplax tissue using the method of Saitoh & Changeux (1980).

Toxin binding to receptor was determined using the method of Schmidt & Raftery (1973).

The binding of Pelamis toxins *a* and *b* was measured by determining the decrease in [^{125}I]α-bungarotoxin (Bgtx) binding to receptor following preincubation of the receptor with varying amounts of toxins *a* and *b* for 1 h. In other words, [^{125}I]α-Bgtx was used as a non-reversible back-titrant to measure specific binding of a ligand competing for the same receptor (Fulpius et al 1980; Juillerat et al 1982).

Following preincubation of receptor with varying amounts of toxins *a* and *b*, a saturating concentration of [^{125}I]α-Bgtx was added. This mixture was then incubated for 1 h, and the counts min^{-1} determined as above.

Results

Isolation

Pelamis platurus venom was fractionated according to the method of Tu et al (1975). A reproducible fractionation pattern was obtained. The yield of toxin *b* was only 1·2% compared with toxin *a* of 4·1%. Homogeneity of the purified toxin *b* was established by two independent methods, polyacrylamide gel electrophoresis and by an analytical isotachophoresis. The single band of acrylamide electrophoresis is shown by two methods.

Sequence

Amino acid sequence of toxin *b* was established by the Edman method using three methods. They are:

a) carboxymethylated toxin *b*; b) fragments of carboxymethylated toxin *b* after endopeptidase lysine c treatment; c) fragments of carboxymethylated toxin *b* after endopeptidase arginine c treatment.

The fragments were separated by the reverse phase HPLC. The amino acid sequence for carboxymethylated toxin *b* was established directly up to residue 42. Amino acid sequences of two fragments obtained after endopeptidase lysine-c are shown in Fig. 1. Only one fragment was used for the sequence study for the peptide fragments obtained after endopeptidase arginine-c treatment. The sequence from this fragment corresponds to No. 32 to No. 60 residues.

Isoelectric points of toxins *a* and *b* were found to be 9·4 and 8·7, respectively.

Spectroscopic analysis

The Raman frequencies of the amide I and III are most frequently used for the determination of peptide backbone conformation. The amide I band arises from the coupled C=O stretching vibrations of the peptide bond. The α-conformation normally appears in the region of 1650–1658 cm^{-1}, random coil at 1664–1666 cm^{-1}, and β-sheet conformation in the region of 1665–1680 cm^{-1} (Tu 1986). The amide III band originates from "in-plane" vibration of the peptide bond. The α-conformation usually gives very high amide III bands of 1260–1298 cm^{-1}; random coil 1242–1252 cm^{-1}; and β-sheet at 1235–1240 cm^{-1}. The β-reverse turn conformation using oxytocin as a model compound shows the amide I band at 1663–1666 cm^{-1} and the amide III band at 1260–1266 cm^{-1} (Tu 1986). The β-reverse turn conformation shows the amide I band at 1656–1668 cm^{-1} and the amide III band at 1255 cm^{-1} (Tu 1986). Judging from the amide I band at 1673 cm^{-1} this clearly indicates the presence of anti-parallel β-sheet structure (Fig. 2). The 1246 cm^{-1} band of the amide III region is at a lower frequency end for random coil but at a higher frequency for β-sheet structure. Therefore, the band at 1246 cm^{-1} is best explained as a mixture of β-sheet and random coil. The presence of random coil also can be seen in the amide I band. There is a shoulder at 1665 cm^{-1} that is a clear indication of random coil. The absence of α-helix is very clear from the Raman spectrum.

By combining both amide I and III bands, it seems that Pelamis toxin *b* contains a fair amount of antiparallel β-sheet structure and random coil without much α-helix. A broad 1246 cm^{-1} band also suggests the presence of β-turn structure. A quantitative analysis based on the equation of Lippert et al (1976) suggests that toxin *b* contains a high amount of antiparallel β-sheet structure and random coil without α-helix (Table 1). Lippert et al's equation is well accepted, however, it does not include the β-turn term.

When toxin *b* was subjected to heat treatment at 94°C for 15 min, the 1673 cm^{-1} amide I band shifts to 1667 cm^{-1} and the amide III band at 1246 cm^{-1} shifts to 1249 cm^{-1}, suggesting the random content is slightly increased. However, there is no significant change in S-S stretching vibration at 508–510 cm^{-1}. The biggest change is a change in tyrosine doublet intensity at 850 and 830 cm^{-1}.

Pezolet et al (1976) devised the method to determine the content of β-sheet only. Using this method, the β-sheet contents of toxin *a* and toxin *b* were found to be 38·2 and 28·4%, respectively. These values are close to the values calculated from Lippert et al's method.

There is one mole of tyrosine in toxin *b*. The Raman lines at 850 and 830 cm^{-1} are related to the microenvironment of the tyrosine side chain (Yu et al 1973). The origin of the doublet is attributed to Fermi resonance between the ring-breathing and out-of-plane vibrations of the *p*-substituted benzene (Siamwiza et al 1975). Upon heating, there is a reversal of intensity ratio for the 850/830 cm^{-1} doublet. This

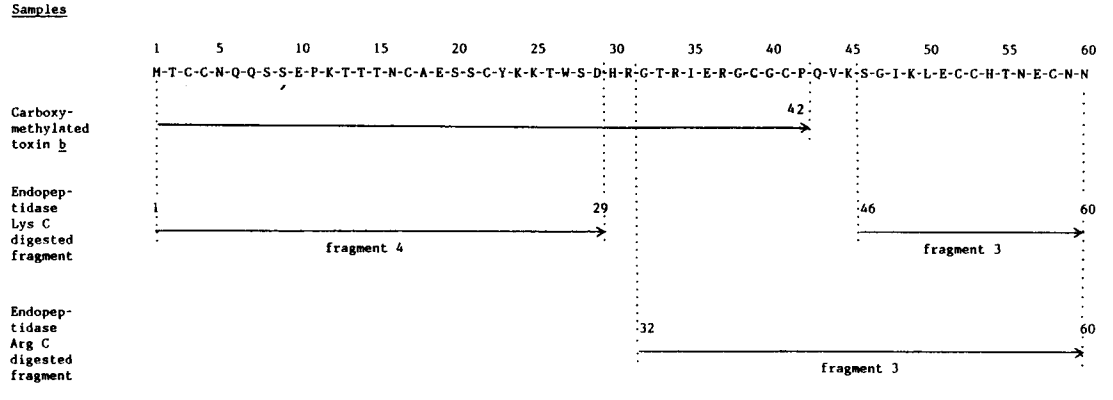

FIG. 1. Amino acid sequence of Pelamis toxin *b*. Arrows under the amino acid indicate the length of each Edman degradation experiment.

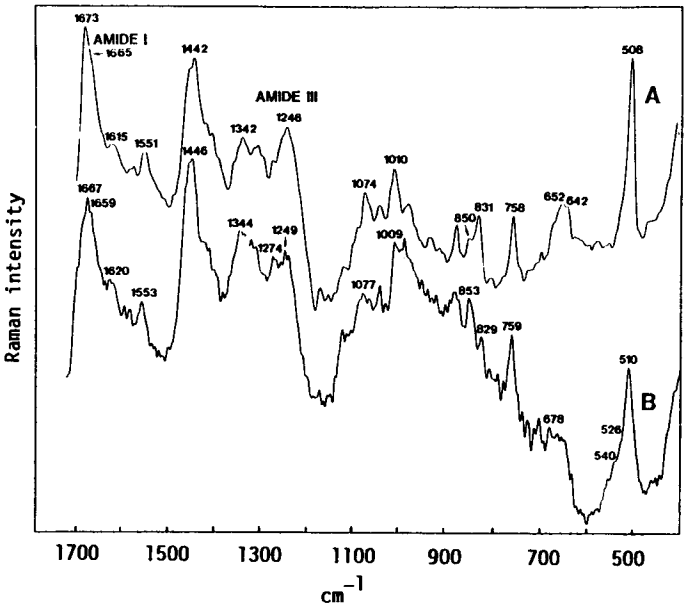

FIG. 2. Raman spectra of (A) native toxin *b* and (B) after treatment at 94°C and 15 min. Experimental condition: radiant power, 80 mW (A), 60 mW (B); integration time, 0·5 s; scale, 1000 pulse s^{-1}, slit width, 500 μm; scan increment, 0·5 cm^{-1}; number of scans, 10.

Table 1. Comparison of toxin *a* and toxin *b* isolated from *Pelamis platurus* venom.

	Toxin *a*	Toxin *b*
Yield (%)	4·09	1·23
LD50 ($\mu g\ g^{-1}$)	0·13	0·19
pI	9·4	8·7
Amino acid residues	60	60
Amino acid residue at position 10	glutamine	glutamic acid
N-Terminal	methionine	methionine
C-Terminal	asparagine	asparagine
Secondary structure by Lippert's et al's method		
α-helix	0	0
β-sheet	39·3	39·8
random coil	61·4	58·5
by Pezolet's method		
β-sheet	38·2	38·4

is interpreted as that the single tyrosine residue is exposed upon heating.

A sharp S-S stretching vibrational band was obtained (Fig. 2), suggesting that the four disulphide bonds have a similar geometry of *gauche-gauche-gauche* conformation for C-C-S-S-C-C network (Nash et al 1985). After heat treatment, the major peak remained at 510 cm^{-1} (untreated one at 508 cm^{-1}), but new shoulders appeared at 526 and 540 cm^{-1}. The frequencies of new shoulders are almost exactly at frequencies for *gauche-gauche-trans* and *trans-gauche-trans* conformation. This means that heat treatment does not affect the overall conformation of four disulphide bonds significantly, but a small fraction of disulphide bonds forms new conformations of *gauche-gauche-trans* and *trans-gauche-trans*. This finding agrees with the result of peptide backbone conformation detected from the amide I and III bands. From the Raman spectroscopic study, it can be concluded that the major conformation including peptide backbone structure and disulphide bonds remain unchanged. But it is observed that there is a microenvironmental change for the tyrosine side chain as well as a small portion of disulphide bonds which change the conformation.

Conformation of pelamis toxin *b* was also analysed by circular dichroism measurements. Native toxin *b* was recorded at pH 7·0 from 200 to 250 nm (Fig. 3). The occurrence of the 214 nm minimum in CD can be attributed to the presence of anti-parallel β-chain that was also clearly shown from Raman spectrum. The presence of β-reverse turn is also possible but not entirely clear from the CD spectrum. Oxytocin which contains one β-reverse turn shows a similar extreme at 208 nm, which is fairly close to 214 nm of β-sheet structure (Hruby et al 1982).

ACR binding

Neurotoxins usually show high toxicity. The LD50 of toxin *b* was 0·185 $\mu g\ g^{-1}$ in mice by intravenous injection. The LD50

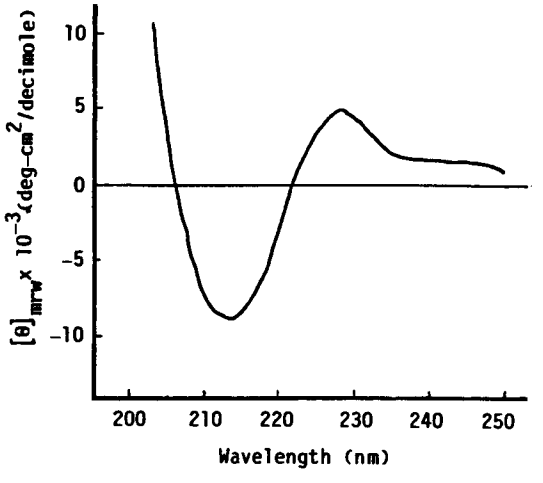

FIG. 3. The CD spectrum of toxin *b* in the peptide region. Mean residue ellipticities (θ mrw) are based on mean residue weight of 100 for toxin *b*.

of toxin *a* was 0·13 $\mu g\ g^{-1}$ (Ishizaki et al 1984). Therefore, both toxins *a* and *b* are extremely toxic and are in the range of other snake postsynaptic neurotoxins.

To determine that the pelamis toxins are postsynaptic type neurotoxins, their bindings to ACR were investigated. As can be seen from Fig. 4, as the concentrations of pelamis toxins *a* and *b* are increased, the binding of a well known postsynaptic toxin [^{125}I]α-Bgty decreased. This indicates that both toxins *a* and *b* competitively bind to the same site as α-Bgtx. This shows that both toxin *a* and toxin *b* are indeed postsynaptic type neurotoxins. Although the data shown in Fig. 4 are qualitative, toxin *b* binds slightly less than toxin *a*. While native toxin *b* is active in ACR binding, heat denatured toxin *b* lost binding activity as shown in the flat line in Fig. 4.

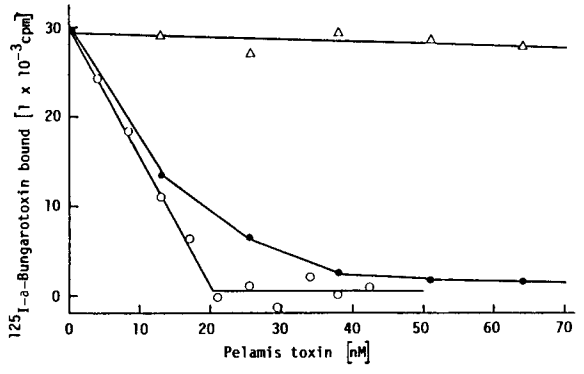

FIG. 4. Comparison between the binding of toxin *a* (●), toxin *b* (○), and heat treated toxin *b* (△) to acetylcholine receptor (ACR). For the assay, the acetylcholine receptor preparation was incubated with increasing amounts of toxins for 1 h. Then a saturating amount of [^{125}]α-Bgtx was added to the preparation and incubated for 1 h more. Each data point is a single experimental result.

Discussion

So far nearly a hundred or so postsynaptic neurotoxins have been analysed for their amino acid sequences. Pelamis toxin *b* interestingly has glutamic acid residue at position 10. The only other toxin containing glutamic acid residue at this position is toxin I of *Naja mossambica mossambica* venom (Gregoire & Rochat 1977). Compared with Pelamis toxin *a*, toxin *b* differs only at position 10. The replacement of glutamine to glutamic acid increases one negative charge in the toxin. This fact is immediately reflected in a lower isoelectric point of toxin *b* (Table 1). The isoelectric points of toxins *a* and *b* are 9·4 and 8·7, respectively. The replacement of one residue did not affect the toxicity of ACR binding ability significantly, although a slight decrease in toxicity (slight increase in LD50) and a slight decrease in ACR binding ability (Fig. 4) are noticed.

It is generally believed that the basicity of the neurotoxin is important for ACR binding. All neurotoxins isolated so far are basic proteins with pI of 9 or above. The pI of α-Bgtx is 9·5, while the ACR-α-Bgtx complex has a pI value of 5·2. The pI of ACR itself must be more acidic (Raftery et al 1971). It is reasonable to assume that the reduction of the isoelectric point from 9·4 to 8·7 due to the replacement of glutamic acid by glutamine is responsible for the slight reduction in toxicity and binding ability to ACR.

Acknowledgement

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