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Primary structure of α-bungarotoxin

Six amino acid residues differ from the previously reported sequence

Mitsuhiro Ohta, Kiyoe Ohta, Hiroshi Nishitani and Kyozo Hayashi*

Department of Biochemistry, Clinical Research Center, Utano National Hospital, Ondoyamacho 8, Ukyoku, Kyoto 616 and **Department of Biology, Gifu Pharmaceutical University, Gifu 502, Japan*

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α-Bungarotoxin (α-BuTx) was isolated from the venom of the Formosan banded krait (*Bungarus multicinctus*). The amino acid sequence was determined by a combination of conventional methods. In contrast to the sequence of α-BuTx reported by Mebs et al. [(1971) Biochem. Biophys. Res. Commun. 44, 711–716], our results revealed the presence of Ser-Pro-Ile, Pro-His and Gln-Arg at positions 9–11, 67–68 and 71–72 from the amino-terminal, respectively, and not Ile-Pro-Ser, His-Pro and Arg-Gln as reported previously.

α-Bungarotoxin; Amino acid sequence; Neurotoxin; Acetylcholine receptor; (*Bungarus*)

1. INTRODUCTION

Venoms from snakes belonging to the families Elapidae (cobras, mumbas, tiger snakes, black snakes, taipan, etc.) and Hydrophidae (sea snakes) are highly toxic and produce flaccid paralysis and respiratory failure [2]. These proteins have been designated as neurotoxins, which are subdivided into two groups with respect to the mode of the inhibitory action at the neuromuscular junction. Postsynaptic neurotoxins bind selectively to the nicotinic acetylcholine receptors (AChR) on the postsynaptic membrane at the neuromuscular junction. The primary structures of a number of postsynaptic neurotoxins [3,4] and the tertiary structures of some of them have been elucidated [5–8].

In particular, α-bungarotoxin (α-BuTx), a principal protein isolated from the crude venom of the elapid snake (*Bungarus multicinctus*), has been most widely used in pharmacological and biochemical studies on the nicotinic AChR and as a diagnostic tool in the evaluation of anti-AChR antibody in myasthenia gravis [9]. The primary structure of this toxin was determined by Mebs et al. [1]. In this report we determined the amino acid sequence of α-BuTx, and compared it with the sequence reported previously.

Correspondence address: K. Hayashi, Department of Biology, Gifu Pharmaceutical University, Gifu 502, Japan

Abbreviations: HPLC, high-performance liquid chromatography; PTH-, phenylthiohydantoin derivatives

2. MATERIALS AND METHODS

2.1. *Isolation of α-BuTx from crude venom*

The lyophilized venom of *B. multicinctus* was obtained from the Miami Serpentarium (USA). α-BuTx was purified by one-step column chromatography on CM-Sephadex C-25 using a linear gradient from 0.05 M ammonium acetate (pH 5.8) to 1 M ammonium acetate buffer (pH 7.0) as in [10]. Each fraction obtained was desalted on a Sephadex G-25 column and lyophilized. Final purification of α-BuTx was achieved by reversed-phase HPLC on a Cosmosil 5C18 column (Nakarai, Japan) with 0.1% trifluoroacetic acid containing a linear gradient from 10 to 50%

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acetonitrile. *S*-carboxymethylated (Cm) derivatives of α-BuTx was prepared by the method of Crestfield et al. [11].

2.2. *Fragmentation of Cm-α-BuTx*

A 20 nmol sample of Cm-α-BuTx was digested in 0.1 M Tris-acetate buffer (pH 8.5) at 37°C for 2 h with 1% (w/w) lysyl endopeptidase [12], which was produced by *Achromobacter lyticus* (EC 3.4.21.50) (Wako, Japan). The peptides thus obtained were separated by reversed-phase HPLC on a Cosmosil 5C18 column with a linear gradient from 0 to 60% acetonitrile containing 0.1% (v/v) trifluoroacetic acid (fig.1).

2.3. *Amino acid sequence analysis*

Protein and peptides were automatically sequenced by means of an Applied Biosystems sequencer (model 470A) equipped with an on-line PTH-analyzer (model 120A). Polybrene was used as a carrier [13].

2.4. *Amino acid analysis*

Protein and peptide samples were hydrolyzed with 6 N HCl at 110°C for 24 h in evacuated sealed tubes. The amino acid compositions were determined with an amino acid analyzer (Hitachi model L-8500).

3. RESULTS AND DISCUSSION

3.1. *N-terminal sequence of α-BuTx*

More than 20 protein fractions containing a major postsynaptic neurotoxin (α-BuTx) and presynaptic neurotoxins (β-BuTxs) were obtained by CM-Sephadex ion-exchange column chroma-

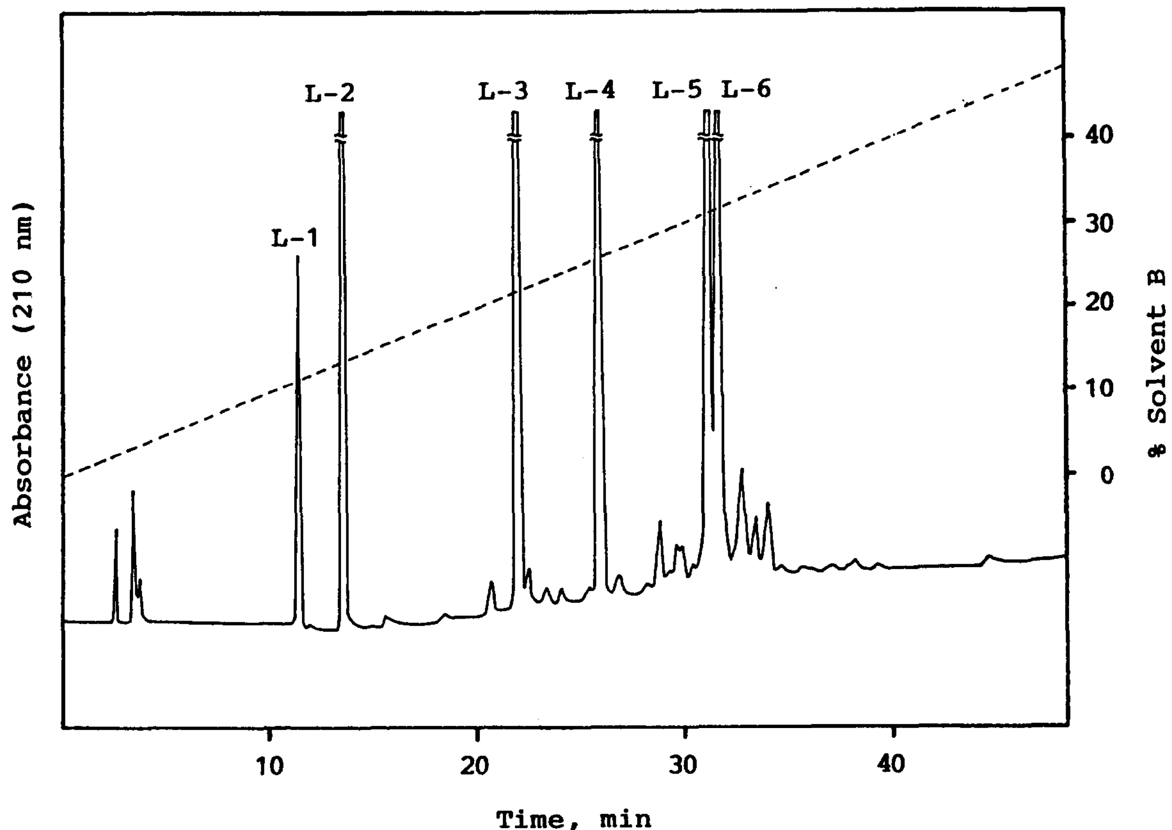

Fig.1. Chromatographic separation of the six peptides. The digest was applied to a Cosmosil 5C18 column, and the peptides were eluted with a linear gradient from 100% (v/v) solvent A to 40% solvent A/60% solvent B in 60 min. Solvent A: 0.1% trifluoroacetic acid in distilled water; solvent B: 0.1% trifluoroacetic acid in acetonitrile. (——) Absorbance (210 nm), (---) % solvent B.

Table 1

Amino acid compositions of the peptides digested with lysyl endopeptidase

Amino acid	Cm-α-BuTx	L-1	L-2	L-3	L-4	L-5	L-6
Cm-cysteine	9.14 (10)		1.05 (1)	1.98 (2)	2.15 (2)	2.14 (2)	3.06 (3)
Aspartic acid	4.19 (4)		1.10 (1)	1.07 (1)		1.04 (1)	1.03 (1)
Threonine	6.61 (7)			1.84 (2)	0.94 (1)		3.69 (4)
Serine	5.53 (6)			1.00 (1)	0.93 (1)	1.82 (2)	1.85 (2)
Glutamic acid	5.20 (5)	1.09 (1)		2.11 (2)	1.00 (1)		1.00 (1)
Proline	7.75 (8)	0.99 (1)	1.78 (2)	0.89 (2)	0.85 (1)		2.80 (3)
Glycine	3.99 (4)	1.12 (1)			1.01 (1)	1.18 (1)	1.05 (1)
Alanine	4.94 (5)				1.81 (2)	1.06 (1)	1.79 (2)
Valine	3.83 (5)			0.87 (1)	1.21 (2)		1.23 (2)
Methionine	1.00 (1)					0.72 (1)	
Isoleucine	1.41 (2)						1.12 (2)
Leucine	1.82 (2)				0.97 (1)		0.88 (1)
Tyrosine	1.90 (2)			0.64 (1)			0.75 (1)
Phenylalanine	0.98 (1)					0.86 (1)	
Lysine	5.72 (6)		1.00 (1)	2.00 (2)	1.00 (1)	1.00 (1)	1.00 (1)
Histidine	2.11 (2)		0.95 (1)				0.98 (1)
Arginine	2.98 (3)	1.00 (1)				0.91 (1)	0.92 (1)
Tryptophan	+ (1)					+ (1)	
Yield (%)		74	72	69	77	57	72
Position		71–74	65–70	52–64	39–51	27–38	1–26

The value for the underlined amino acids was taken as 1.0 or 2.0. Numbers in parentheses represent the integers from sequence analysis. Tryptophan was determined spectrophotometrically

tography. The yield of α-BuTx was 15.8% of the crude venom. The amino acid composition of α-BuTx is listed in table 1, and agrees with that of previous reports. The N-terminal sequence of α-BuTx was determined to be the following:

H-Ile-Val-X-His-Thr-Thr-Ala-Thr-Ser-Pro-Ile-Ser-Ala-Val-Thr-X-Pro-Pro-Gly-Glu-Asn-Leu-X-Tyr-Arg-Lys-Met-Trp-X-Asp-Ala-Phe-X-Ser-Ser-Arg-Gly-Lys-Val-Val-Glu-Leu-Gly-X-Ala-Ala-Thr-X-Pro---.

We did not detect any PTH amino acid at position X and deduced X to be a Cys residue. The N-terminal sequence was identical with that of the previous report except for positions 9 and 11 (---Ile-Pro-Ser---).

3.2. *Amino acid compositions and sequences of the peptides from lysyl endopeptidase digest*

The lysyl endopeptidase digest of Cm-α-BuTx was separated by HPLC into 6 fragments, L-1–L-6 (fig.1), and their amino acid compositions were determined (table 1). The value of valine in peptide L-4 and those of valine and isoleucine in peptide L-6 were remarkably low because of insufficient

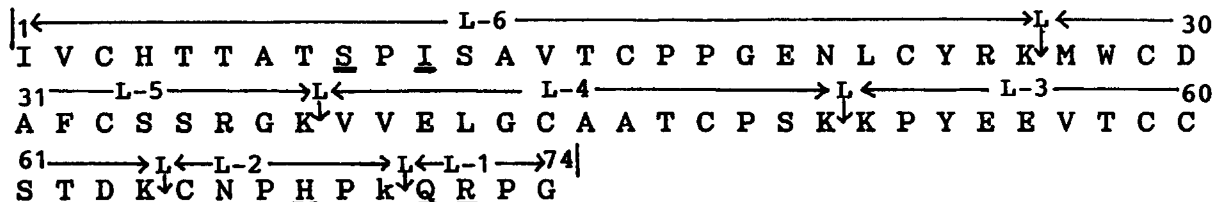

Fig.2. Amino acid sequence of α-bungarotoxin from Formosan krait venom (*B. multicinctus*). L refers to the lysyl endopeptidase peptides. Underlined residues differ from the sequence in the report of Mebs et al. [1].

Table 2

Amino acid sequence of Cm-α-BuTx

Peptide no.	Sequence
L-1	Gln-Arg-Pro-Gly
L-2	Cys-Asn-Pro-His-Pro-Lys
L-3	Lys-Pro-Tyr-Glu-Glu-Val-Thr-Cys-Cys-Ser-Thr-Asp-Lys
L-4	Val-Val-Glu-Leu-Gly-Cys-Ala-Ala-Thr-Cys-Pro-Ser-Lys
L-5	Met-Trp-Cys-Asp-Ala-Phe-Cys-Ser-Ser-Arg-Gly-Lys
L-6	Ile-Val-Cys-His-Thr-Thr-Ala-Thr-Ser-Pro-Ile-Ser-Ala- Val-Thr-Cys-Pro-Pro-Gly-Glu-Asn-Leu-Cys-Tyr-Arg-Lys

Right-handed arrows indicate that the sequence was elucidated by automated Edman degradation

hydrolysis of the peptide bond of Val-Val (L-4) and Ile-Val (L-6) under standard conditions. Their sequences on 6 peptides from L-1 to L-6 are summarized in table 2. Five peptides, except L-1, contained one or two lysyl residues. This means that the enzyme hydrolyzes specifically the carboxy-terminal peptide bond of lysine residues. When the amino acid compositions and sequences of these 6 peptides were compared with previous reports, different amino acid sequences were observed in the three peptides, L-1 (Gln-Arg-Pro-Gly), L-4 (Cys-Asn-Pro-His-Pro-Lys) and L-6 (Ile-Val----Ser-Pro-Ile----Arg-Lys) at positions 71–72, 67–68, and 9 and 11, respectively. In spite of the identical amino acid composition the reported sequence contained 'reversed sequences' within each peptide. The amino acid sequence of α-BuTx is shown in fig.2, in which the underlined residues differ from those in the previous report. This revised sequence of α-BuTx has a higher degree of homology of the C-terminal sequence of the *Naja naja oxiana* neurotoxin I [14] than the unrevised sequence.

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