CHROM. 6192

CHROMATOGRAPHIC SEPARATION OF THE VENOM OF *BUNGARUS MULTICINCTUS* AND CHARACTERIZATION OF ITS COMPONENTS

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(Received May 15th, 1972)

SUMMARY

The venom of *Bungarus multicinctus* has been fractionated on a CM-Sephadex C-50 column into eight major fractions by gradient elution with 0.05–1.0 *M* ammonium acetate buffer at pH 5.0–6.8. Fraction I is identified as guanosine by its spectral data at various pH values. Fractions II and III contain postsynaptic (α-type) neurotoxins, which produce an antidepolarizing neuromuscular block by combining irreversibly with the acetylcholine receptor of the motor endplate, but they are contaminated with acetylcholinesterase, phospholipase A, NADase and phosphomonoesterase. After twice re-chromatographing Fraction II on a CM-cellulose column, a homogeneous toxin (Fraction II_2), corresponding to α-bungarotoxin, is obtained. It is free from any enzymic activities so far tested. Fractions IV–VIII contain presynaptic (β-type) neurotoxins, which act on the motor nerve-endings, first enhancing and then depressing the transmitter release. By re-chromatography of Fraction V on a CM-cellulose column, a neurotoxin corresponding to β-bungarotoxin is obtained, which is free from hyaluronidase and other enzyme activities but still contains a small amount of impurity. α-Bungarotoxin consists of a single polypeptide chain of seventy-four amino acid residues cross-linked by five disulphide bridges, whereas β-bungarotoxin is composed of about hundred-eighty amino acid residues, probably with ten disulphide bonds.

INTRODUCTION

The venom of *Bungarus multicinctus* has been separated by zone electrophoresis on starch into four fractions[1]. One fraction is non-toxic but contains cholinesterase. One, called α-bungarotoxin, produces an antidepolarizing neuromuscular block by combining irreversibly with the acetylcholine receptor of the motor endplate. The two most electropositive fractions, called β- and γ-bungarotoxin, respectively, both produce a neuromuscular block by acting presynaptically on the motor nerve endings, leaving the sensitivity of the endplate to acetylcholine unaffected. Thus, it is evident that this venom contains two distinctly different types of neurotoxins, *viz.*, postsynaptic (α-type) and presynaptic (β-type) toxins.

Besides acetylcholinesterase[2], this venom has been shown to contain some other enzymic activities, such as NADase (formerly DPNase)[3], phospholipase A, L-amino acid oxidase and hyaluronidase[4]. Protease and other phosphatases, such as

phosphodiesterase, 5'-nucleotidase and ATPase, in this venom have been shown to be so feeble that their activities in each fraction cannot be determined[4].

Although it was demonstrated that the above neurotoxins did not contain any cholinesterase activity[1], it has not been shown whether or not they are free from other enzymic activities. The present work was undertaken to obtain homogeneous neurotoxins that are devoid of any known enzymic activities present in the crude venom.

MATERIALS AND METHODS

Venom

The venom of *Bungarus multicinctus* was freshly collected and diluted with an equal volume of distilled water. The insoluble residue was removed by centrifugation and the supernatant was lyophilized.

Column chromatography on CM-Sephadex

The procedure described by LEE *et al.*[5] was followed, with minor modifications. CM-Sephadex C-50 (Pharmacia, Uppsala) was equilibrated with 0.05 *M* ammonium acetate buffer, pH 5.0, and then packed into a column of 2.5 × 75 cm. The venom (0.9–1.0 g) dissolved in 6 ml of the same buffer was applied to the column in a cold cabinet (4–5°). A linear gradient of 0.05 *M* ammonium acetate buffer, pH 5.0 to 1.0 *M*, pH 6.8, with a flow-rate of 15–18 ml/h was used. Fractions of 3 ml were collected and their absorbancy was determined at 280 mμ. The fractions belonging to the same peaks were pooled, lyophilized and desalted on a Sephadex G-25 (coarse) column.

Re-chromatography on CM-cellulose column

Carboxymethyl-cellulose (Serva, Heidelberg) was purified and prepared according to the manufacturer's suggestions. The preswollen CM-cellulose was equilibrated with the starting buffer (0.05 *M* ammonium acetate, pH 5.0, unless otherwise indicated) and packed into a column of 1.2 × 50 cm. Either a linear gradient or a stepwise elution method was used for the fractions of different peaks separated by CM-Sephadex column chromatography.

Identification of nucleoside

The UV absorption spectra of Fraction I at various pH values were obtained with a Beckman DU spectrophotometer. The spectral data were calculated according to VOLKIN AND COHN[6].

Microzone electrophoresis

Electrophoresis on a cellulose acetate membrane strip was carried out in a Beckman Model R-101 microzone electrophoresis cell. It was performed in 0.1 *M* phosphate buffer at pH 7.4 with a potential of 300 V for 30 min. Protein bands were stained with Ponceau-S and rinsed with 5 % acetic acid.

Sedimentation studies

A Hitachi analytical ultracentrifuge was used with a Schlieren optical system for the sedimentation velocity experiment. The sedimentation coefficients were

determined at a protein concentration of 5 mg/ml in 0.1 *M* acetate buffer at pH 5.7. Photographs were taken at various time intervals after the rotor had attained full speed.

The molecular weight was determined on a Spinco Model E analytical ultracentrifuge with an interference optical system by the sedimentation equilibrium method[7], dissolving the protein in 0.005 *M* Tris + 0.1 *M* NaCl at pH 7.0. The measurements were carried out after centrifugation for 24 h at 20° and a rotor speed of 12590 r.p.m. The molecular weight of the toxin was extrapolated to zero protein concentration. The partial specific volume was calculated from the amino acid composition[8].

Amino acid analysis

The purified toxins were hydrolyzed with twice-distilled hydrochloric acid in evacuated sealed tubes at 110° for 40 h. The evaporated hydrolysates were then analyzed for amino acid composition in a Beckman Spinco automatic analyzer[9]. Cystine was determined as cysteic acid after oxidation with performic acid. Tryptophan residue was determined by the UV absorption method[10].

Assay of toxicity

The lethality of each fraction was assayed in mice (NIH strain) weighing 15–20 g by intraperitoneal injection, unless otherwise indicated. The LD_{50} was computed according to the method of LITCHFIELD AND WILCOXON[11].

Assay of neuromuscular blocking action

Isolated biventer cervicis nerve-muscle preparation of the chick[12] was suspended in 20 ml of Krebs' solution, which was aerated with 95 % oxygen and 5 % carbon dioxide at 37°. Indirect stimulation was applied through the tendon at a rate of 6 pulses per minute with supramaximal rectangular pulses of 0.1 msec. For differentiation between α- and β-types of bungarotoxins, 10 μg/ml of acetylcholine was applied for 60 sec after the muscle ceased to respond to indirect stimulation.

Measurement of enzyme activities

Acetylcholinesterase (E.C. 3.1.1.7) activity was measured by the same method as described by CHANG AND LEE[2], except that the enzyme solution was pre-incubated with 0.08 *M* magnesium chloride solution at 37° for 1 min. Phospholipase A (E.C. 3.1.1.4) activity was measured by the indirect haemolytic method of COLLIER[13], modified by YANG *et al.*[14]. Cat blood was used instead of human blood. Phosphomonoesterase (E.C. 3.1.3.1) activity was measured according to the method of GULLAND AND JACKSON[15], modified by SUZUKI AND IWANAGA[16]. NADase (nicotinamide dinucleotide phosphohydrolase, E.C. 3.6.1.9) activity was measured according to the method of SUZUKI *et al.*[3]. Hyaluronidase (E.C. 4.2.99.1) was measured by the turbidimetric method of DIFERRANTE[17].

Reagents

Lecithin (egg lecithin), *p*-nitrophenyl phosphate, NAD (diphosphopyridine nucleotide), hyaluronic acid and cetyltrimethylammonium bromide were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

RESULTS

Chromatographic separation on a CM-Sephadex column

A typical chromatographic pattern is illustrated in Fig. 1. The venom was separated into eight major fractions. The recovery and yield of each fraction together

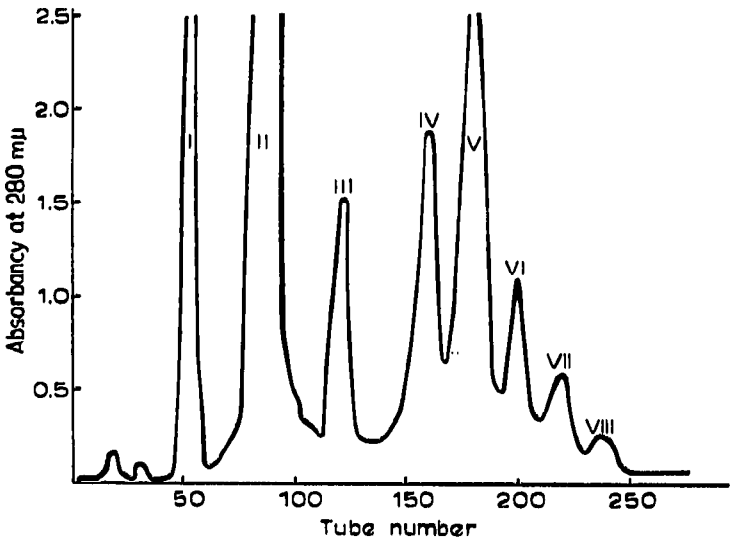

Fig. 1. Chromatography of *Bungarus multicinctus* venom (904 mg) on CM-Sephadex C-50 column (2.5 × 75 cm) by gradient elution with ammonium acetate buffer, from 0.05 *M*, pH 5.0, to 1.0 *M*, pH 6.8. Eluates of 3 ml each were collected with a flow-rate of 15–18 ml/h.

with the toxicity in mice and the neuromuscular blocking activity in the chick biventer cervicis muscle are given in Table I. From UV spectra and the Folin reaction, Fraction I was found to be a non-protein component, whereas Fractions II–VIII were protein in nature. As shown in Table II, comparison of the spectral data of Fraction I with those of authentic guanosine at various pH values confirms the finding of WEI AND LEE[18] that the non-protein component of this venom is guanosine. All of the seven

TABLE I

SEPARATED FRACTIONS OF *Bungarus multicinctus* VENOM BY CM-SEPHADEX CHROMATOGRAPHY AND THEIR PROPERTIES

Fraction No.	*Yield*		*Approximate LD_{50} (i.p.) in mice (μg/g)*	*Chick biventer cervicis muscle*		*Type of neurotoxin*
	mg	%		*N-M block (10^{-6} g/ml) (min. ± S.E.)*	*ACh (10 μg/ml) response after N-M block*	
I	9.63	1.06	> 15	No N-M block		(guanosin
II	277.90	30.74	0.15–0.3	13.5 ± 0.87 (ref. 4)	—	α
III	71.95	7.96	0.4 –0.5	28.0 ± 2.79 (ref. 5)	—	α
IV	137.95	15.26	0.5 –0.6	79.3 ± 7.08 (ref. 3)	+	β
V	158.95	17.58	0.02–0.03	21.0 ± 0.89 (ref. 5)	+	β
VI	51.55	5.70	0.04–0.06	34.0 ± 2.03 (ref. 4)	+	β
VII	23.10	2.56	0.02–0.04	23.0 ± 0.29 (ref. 4)	+	β
VIII	19.20	2.12	0.04–0.05	29.3 ± 1.30 (ref. 4)	+	β
Total	740.60	81.92				

TABLE II

COMPARISON OF THE SPECTRAL DATA BETWEEN FRACTION I AND AUTHENTIC GUANOSINE

Sample	*pH*	$\lambda_{max.}$ (mμ)	*250/260*	*280/260*	*290/260*
Fraction I	0.08	257	0.94	0.70	0.49
	6.6	253	1.17	0.66	0.27
	12.2	257–266	0.89	0.63	0.15
Guanosine (authentic)	0.08	257	0.93	0.68	0.48
	6.6	253	1.14	0.69	0.28
	12.2	257–266	0.88	0.61	0.12

other fractions contain neurotoxins, judging from the gross symptoms produced in mice and their neuromuscular blocking action on the chick biventer cervicis muscle preparation. Among them, the neurotoxins in Fractions II and III belong to the α-type, whereas those in Fractions VI–VIII belong to the β-type, as judged from the acetylcholine response of the chick muscle after neuromuscular blockade.

Re-chromatography of Fractions II–VII on a CM-cellulose column

Further purification of the neurotoxic fractions was carried out by re-chromatography on a CM-cellulose column. As shown in Fig. 2, each of Fractions II–IV was

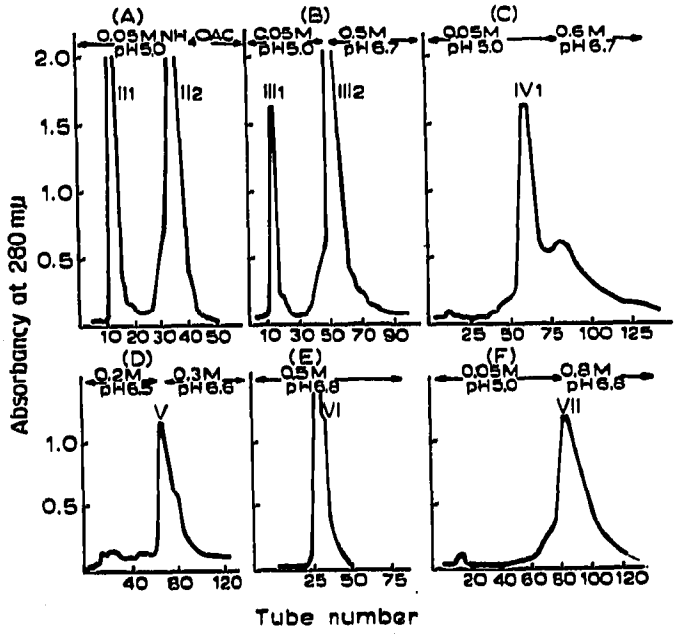

Fig. 2. Re-chromatography of Fractions II–VII on CM-cellulose column (1.2 × 50 cm). A, Fraction II (115.6 mg); B, Fraction III (133.1 mg); C, Fraction IV (76 mg); D, Fraction V (99 mg); E, Fraction VI (51.6 mg); F, Fraction VII (39.7 mg). The concentration and pH of ammonium acetate buffer used for elution are indicated on the top of each figure.

separated into two peaks, whereas only one peak was obtained from each of Fractions V–VII after re-chromatography. The protein recovery of each fraction together with the LD_{50} in mice and the neuromuscular blocking activity in the chick biventer cervicis muscle is given in Table III. Among the four fractions (II_1, II_2, III_1 and III_2)

TABLE III

SEPARATED FRACTIONS AFTER RE-CHROMATOGRAPHY ON CM-CELLULOSE COLUMN AND THEIR PROPERTIES

Fraction No.	*Recovery* (%)	*LD_{50} in mice* ($\mu g/g$)		*Neuromuscular block* (10^{-6} *g/ml*) (*min.* ± *S.E.*)	*Remarks*
		I.p.	*S.c.*		
II					Various enzymic activities
II_1	24.3	1.10 (0.84–1.43)			
II_2	48.4	0.15 (0.10–0.22)	0.21 (0.18–0.25)	13.5 ± 1.2 (ref. 4)	α-Bungarotoxin; free from enzymic activities
III					
III_1	23.1	0.25 (0.22–0.29)		35.2 ± 4.6 (ref. 5)	α-Type neurotoxin
III_2	71.0	0.74 (0.69–0.80)		42.6 ± 3.4 (ref. 5)	α-Type neurotoxin
IV					
IV_1	55.6	2.12 (1.40–3.20)			β-Type neurotoxin
IV_2	21.3	0.28 (0.21–0.38)			β-Type neurotoxin
V	44.8	0.014 (0.011–0.018)	0.04 (0.033–0.048)	17.8 ± 1.0 (ref. 4)	β-Bungarotoxin; free from enzymic activities
VI	79.2	0.053 (0.039–0.072)			β-Type neurotoxin
VII	39.7	0.016 (0.011–0.022)		28.0 ± 0.87 (ref. 5)	β-Type neurotoxin

that contain α-type neurotoxin, Fraction II_2 was found to be the most potent and free from enzymic activities so far tested (see Figs. 4–7). Among the six fractions (IV_1, IV_2, V, VI, VII and VIII) that contain β-type neurotoxin, Fraction V was found to be the most toxic and also free from enzymic activities so far tested. These twice re-chromatographed Fractions II_2 and V will be hereafter called as α- and β-bungarotoxin, respectively.

Distribution of enzymic activities

The distribution of enzymic activities of the venom after chromatographic separation on a CM-Sephadex column is shown in Fig. 3. Most enzymic activities except hyaluronidase distributed around Fractions II and III, but all of the peaks of enzymic activities did not coincide with any of the peaks of protein fractions.

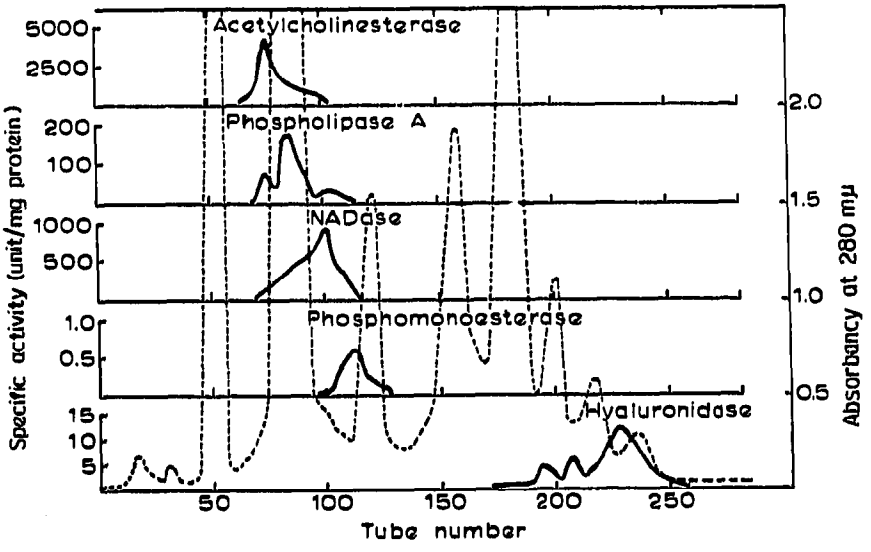

Fig. 3. Distribution of enzyme activities of *Bungarus multicinctus* venom in the eluates from CM-Sephadex column chromatography. Each enzyme activity is expressed as unit per milligram of protein. The dotted line indicates protein content estimated by the absorbancy at 280 mμ.

Acetylcholinesterase (*E.C. 3.1.1.7*). Acetylcholinesterase activity was found to distribute around Fraction II in a single peak (Fig. 3). After re-chromatography of Fraction II on a CM-cellulose column, the cholinesterase activity was concentrated in a high narrow peak around the starting point of Fraction II_1, and α-bungarotoxin (Fraction II_2) was found to be free from this enzyme activity (Fig. 4).

Phospholipase A (*E.C. 3.1.1.4*). Three peaks of phospholipase A activity were detected around Fraction II. The main peak was located in Fraction II with two minor peaks on its two sides (Fig. 3). After re-chromatography of Fraction II on a CM-cellulose column, three peaks of phospholipase A activity around Fraction II_1 still appeared with the main peak in the middle (Fig. 5), suggesting that there are at least three different molecular species of phospholipase A in this venom. No phospholipase A activity was detected in α-bungarotoxin (Fraction II_2).

NADase (*E.C. 3.6.1.9*). The peak activity of NADase was found between Fractions II and III (Fig. 3). After re-chromatography of Fraction II on a CM-cellulose column, NADase activity was concentrated in a narrow peak between Fractions II_1

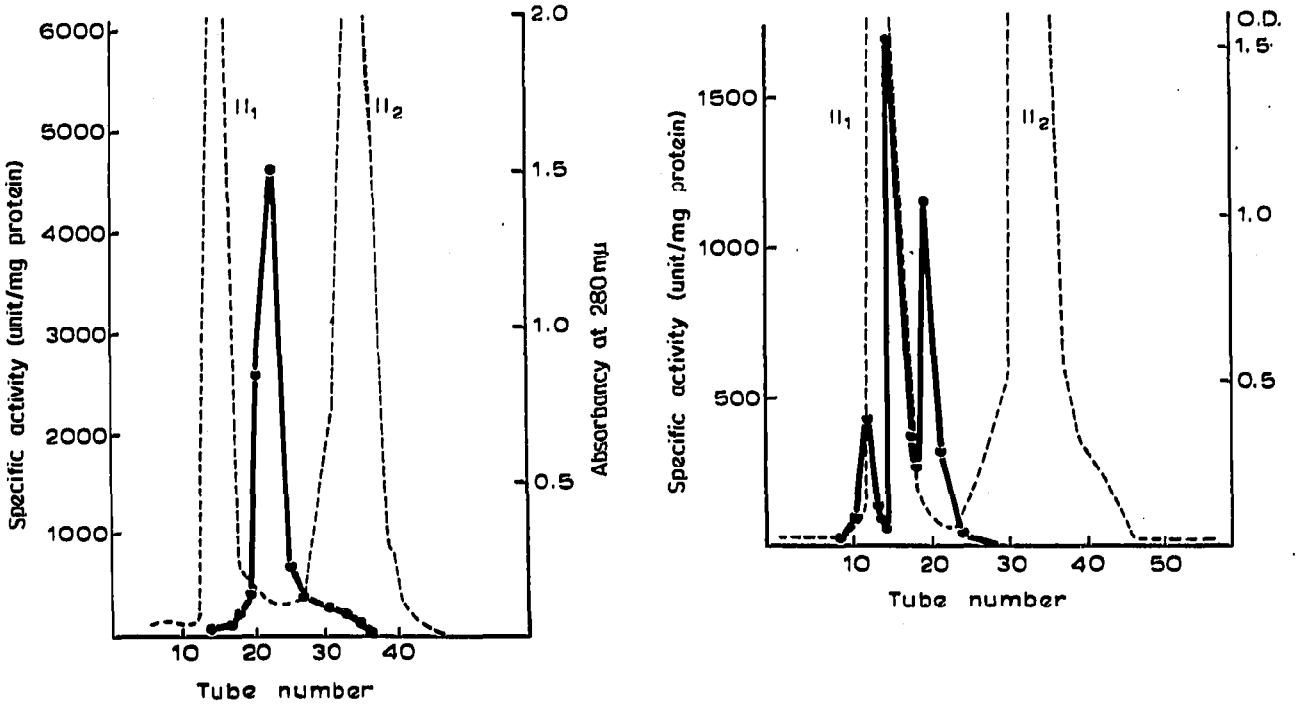

Fig. 4. Distribution of acetylcholinesterase activity (solid line) after re-chromatography of Fraction II on a CM-cellulose column. The dotted line indicates protein content.

Fig. 5. Distribution of phospholipase A activity (solid line) after re-chromatography of Fraction II on a CM-cellulose column. The dotted line indicates protein content.

and II_2 (Fig. 6). A trace of NADase activity found in Fraction II_2 disappeared after twice re-chromatographing Fraction II_2 on a CM-cellulose column.

Phosphomonoesterase (*E.C. 3.1.3.1*). Phosphomonoesterase activity was detected in a narrow range around the starting portion of Fraction III (Fig. 3). After re-

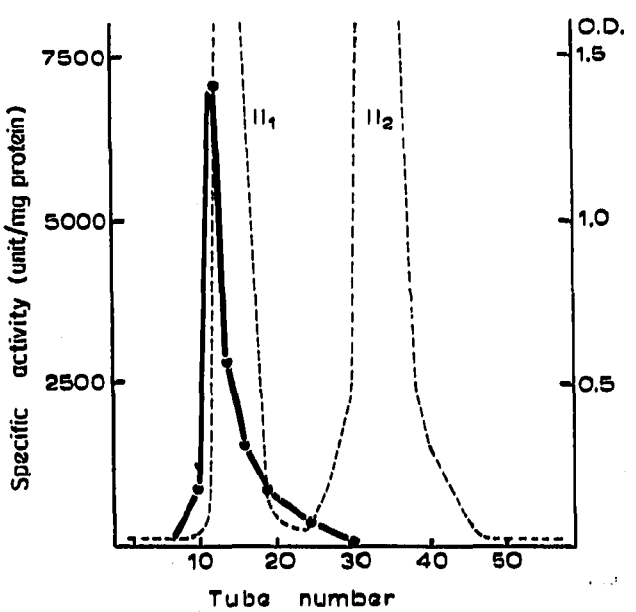

Fig. 6. Distribution of NADase activity (solid line) after re-chromatography of Fraction II on a CM-cellulose column. The dotted line indicates protein content.

chromatography of Fraction III on a CM-cellulose column, the enzyme activity was concentrated in a high peak at the starting portion of Fraction III_1 (Fig. 7).

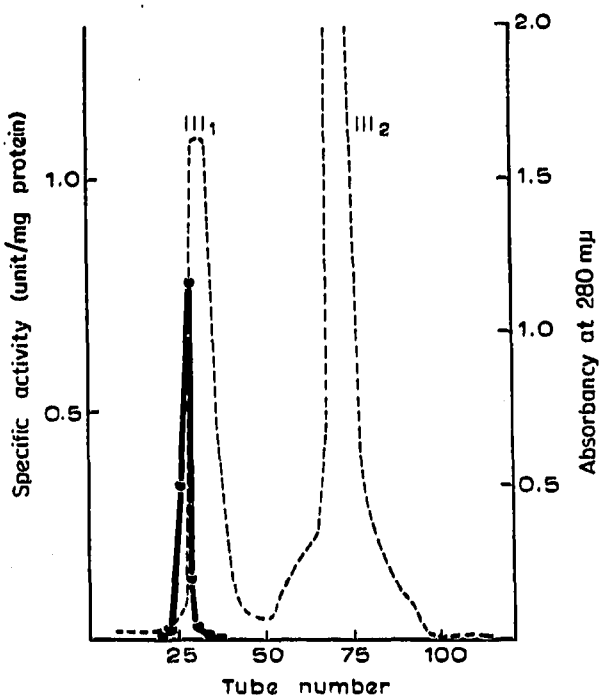

Fig. 7. Distribution of phosphomonoesterase activity (solid line) after re-chromatography of Fraction III on a CM-cellulose column. The dotted line indicates protein content.

Hyaluronidase (*E.C. 4.2.99.1*). Three peaks of hyaluronidase activity were found between Fractions V and VIII. The main peak was located between Fractions VII and VIII (Fig. 3). A trace of hyaluronidase activity was also detected in Fraction V, but after re-chromatography on a CM-cellulose column, Fraction V (β-bungarotoxin) was found to be free from this enzyme activity.

Criteria of homogeneity of bungarotoxins

After electrophoresis on cellulose acetate membrane at pH 7.4, α-bungarotoxin migrated towards the cathode as a single band, while β-bungarotoxin was found still not to be quite homogeneous (Fig. 8). The ultracentrifugal pattern obtained with α-bungarotoxin showed a symmetrical single peak with a sedimentation coefficient

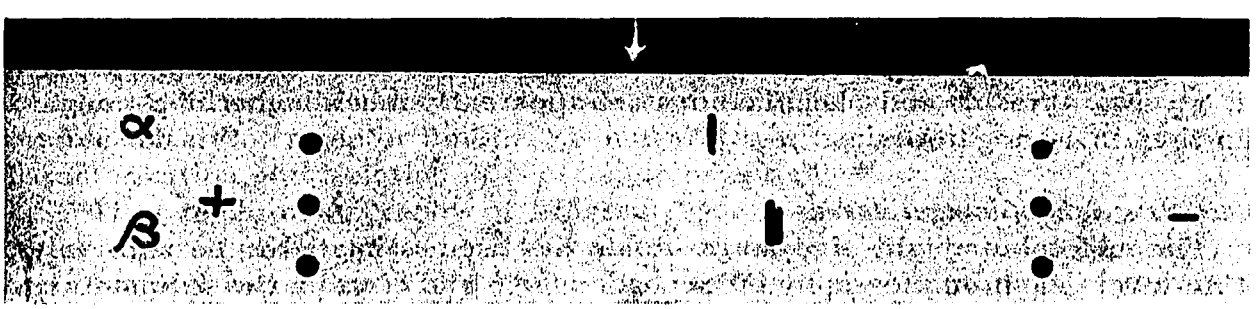

Fig. 8. Microzone electrophoresis of α- and β-bungarotoxin at pH 7.4 with a potential of 300 V for 30 min. The arrow indicates the place where the samples were applied.

($S_{20,w}$) of 1.45 S. Although β-bungarotoxin was also sedimented as a single peak with a sedimentation coefficient of 2.05 S, the pattern of the peak was not quite symmetrical, suggesting the presence of either impurity or aggregate.

Amino acid composition of bungarotoxins

The amino acid composition as well as the primary structure of α-bungarotoxin has recently been reported[19]. As shown in Table IV, the amino acid composition of

TABLE IV

AMINO ACID COMPOSITION OF BUNGAROTOXINS

Amino acid	*β-Bungarotoxin*			*α-Bungarotoxin*
	g/100 g protein[a]	*Residues per molecule*[b]	*Nearest integer*	*From sequence analysis*[c]
Lysine	7.11	12.61	13	6
Histidine	3.09	5.15	5	2
Arginine	9.39	13.84	14	3
Aspartic acid	11.24	22.02	22	4
Threonine	4.99	11.01	11–12	7
Serine	2.08	5.29	6	6
Glutamic acid	6.85	12.01	12	5
Proline	3.31	7.60	8	8
Glycine	4.44	16.33	16	4
Alanine	3.64	11.09	11	5
Half-cystine	8.65	18.84	19–20	10
Valine	1.82	4.09	4	5
Methionine	1.18	2.05	2	1
Isoleucine	4.03	8.04	8	2
Leucine	3.40	6.78	7	2
Tyrosine	9.23	13.08	13	2
Phenylalanine	3.78	5.91	6	1
Tryptophan[d]		2.61	3	1
Total	88.23		*ca.* 180	74

[a] Average values from duplicate analyses of 40-h hydrolysates.
[b] The values for residues per molecule were based on a molecular weight of 28,500.
[c] MEBS *et al.*[19].
[d] Estimated by the UV absorption method.

β-bungarotoxin is different from that of α-bungarotoxin. While α-bungarotoxin consists of seventy-four amino acid residues in a single chain cross-linked by five disulphide bridges[19], β-bungarotoxin is composed of about hundred-eighty residues, probably with ten disulphide bonds. No detectable free sulphydryl groups were found in both toxins by amperometric titration. The relative contents of arginine, aspartic acid, glycine, tyrosine and phenylalanine are apparently much higher in β-bungarotoxin than in α-bungarotoxin.

Molecular weight of bungarotoxins

The molecular weight of α-bungarotoxin has been estimated to be 7840 $\pm$ 580 by thin-layer gel chromatography on Sephadex G-50 and 8000 by the sedimentation equilibrium method[19]. A formula weight of 7983 was given from its amino acid composition.

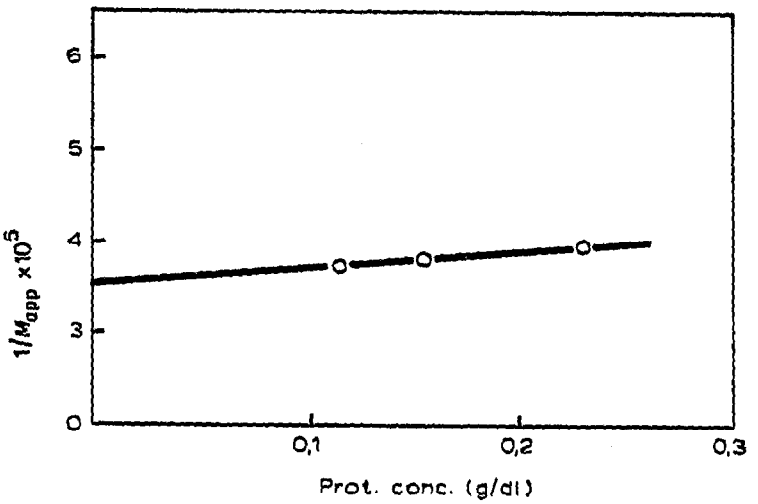

Fig. 9. Plot for $1/M_{app}$ with respect to the concentration of β-bungarotoxin in 0.005 *M* Tris + 0.1 *M* NaCl, pH 7.0, at 20°.

The molecular weight of β-bungarotoxin was also determined from the results of sedimentation equilibrium, assuming the partial specific volume of the toxin to be 0.72. As shown in Fig. 9, its apparent molecular weight (M_{app}) was estimated by extrapolating to zero protein concentration and found to be about 28,500.

DISCUSSION

Chromatography of *Bungarus multicinctus* venom on a CM-Sephadex column gave rise to eight major fractions, as indicated in Fig. 1. From the spectral data at various pH values, Fraction I was identified as guanosine, confirming our previous finding[18]. Fractions II–III contained neurotoxins of the α-type, whereas Fractions IV–VIII contained those of the β-type, as judged from the acetylcholine response of the chick biventer cervicis muscle after neuromuscular blockade. As Fractions II–III also contained various enzymes such as acetylcholinesterase, phospholipase A, NADase and phosphomonoesterase, they were re-chromatographed on a CM-cellulose column. After twice re-chromatographing Fraction II, an enzyme-free toxin was obtained that corresponds to α-bungarotoxin. This toxin showed a single homogeneous band on microzone electrophoresis at pH 7.4. Homogeneity has also been proved by polyacrylamide gel electrophoresis, analytical ultracentrifugation, amino acid analysis and end group analysis[19]. Its molecular weight as well as amino acid composition has been estimated, giving a formula weight of 7983. It consists of a single polypeptide chain of seventy-four amino acid residues cross-linked by five disulphide bridges. Its amino acid sequence has also been determined[19]. Because of its specific and irreversible nature of receptor binding, α-bungarotoxin has recently been used for the characterization and isolation of cholinergic receptor[20–26].

After re-chromatography of Fraction V on a CM-cellulose column, a trace of hyaluronidase activity present in this fraction could be eliminated. The neurotoxin thus obtained corresponds to β-bungarotoxin. The molecular weight of β-bungarotoxin was estimated to be 28,500. This toxin is composed of about hundred-eighty amino acid residues, probably with ten disulphide bonds. CHANG AND LEE[1] reported

LD_{50} (s.c.) values of 0.3 and 0.089 μg per gram of mouse for α- and β-bungarotoxin, respectively, while LD_{50} (s.c.) values of 0.21 and 0.04 μg per gram of mouse were found for the corresponding toxins, respectively, in the present work. In accordance with their different modes of action, α- and β-bungarotoxin have been found to differ from each other not only in their amino acid compositions but also in their ORD and CD patterns[27].

ACKNOWLEDGEMENTS

This work was supported in part by the National Science Council, Republic of China. The authors are indebted to Dr. K. NARITA, Institute for Protein Research, Osaka University, for amino acid analyses, to Dr. K. KAKIUCHI of the same Institute for molecular weight determinations, and to Mr. YUN-MING CHEN for assistance in testing the neuromuscular blocking action of fractionated toxins.

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