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Characterization and Chemical Modifications of Toxins Isolated from the Venoms of the Sea Snake, *Laticauda semifasciata*, from Philippines*

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ABSTRACT: The venom of *Laticauda semifasciata* from the Philippines, contains two toxins. These toxins were isolated and purified by means of Sephadex gel filtration and CM-cellulose column chromatography. The purity of the toxins was established using isoelectric focusing, electrophoresis, sedimentation velocity, and sedimentation equilibrium. Both toxins could be isolated in crystalline form. The lethality of the purified toxins increased five- to sixfold when compared to the original venom. The molecular weight as determined by amino acid composition, gel filtration, and by sedimentation equilibrium was approximately 6800 for both toxins. The amino acid compositions of the two toxins are quite similar.

Toxin a contains 62 amino acid residues and toxin b contains 61 amino acid residues. No free sulfhydryl groups were detectable. End-group analysis showed both toxins to be a single polypeptide chain with arginine at the N terminal and aspartic acid (asparagine) at the C terminal. The isoelectric points were determined by isoelectric focusing to be 9.15 for toxin a and 9.34 for toxin b. The toxicity of the toxins was unaltered by heating to 100° for 30 min or by exposure to pH extremes from 1 to 11. The toxicity was completely lost when the tryptophan residue in the purified toxins was modified with *N*-bromosuccinimide. However, no change in antigenicity was observed for the *N*-bromosuccinimide-modified toxins. No significant change in the toxicity was observed when the lysine and arginine residues of the purified toxins were modified.

The venoms of all sea snakes (*Hydrophiidae*) are extremely toxic (Tu, 1961; Tu and Ganthavorn, 1969; Homma *et al.*, 1964). These venoms are believed to act upon the neuromuscular junction of the victims (Rogers, 1902, 1903; Fraser and Elliott, 1905; Carrey and Wright, 1960a,b, 1961). The neurotoxic factors of sea snake venoms were first identified as protein by Arai *et al.* (1964). The isolation and preliminary characterization of some neurotoxic proteins from the venom of the sea snake, *Laticauda semifasciata* (*Erabu unagi*), from Japan have been described by Uwatoko *et al.* (1966a,b) and Tamiya *et al.* (1966). Enzymes, such as phospholipase A (Carrey and Wright, 1960a; Barme, 1958; Tu *et al.*, 1970; Setognchi and Ohbo, 1969), anti-coagulase (Barme, 1958; Cesaru and Boquet, 1936), L-leucyl-β-naphthylamide-hydrolyzing enzyme (Tu and Toom, 1967), and hyaluronidase (Barme and Detrait, 1959) have also been observed. In this paper, the isolation, purification, and the physicochemical and chemical characterization of two neurotoxic proteins from *Laticauda semifasciata* captured in the Philippines in the South China Sea are presented. In addition, we present some data concerning selective chemical modification of the tryptophan residue, lysine residues, and arginine residues. The antigenicity of *N*-bromosuccinimide-modified toxins is compared to that of the original toxins.

Materials

4-Mercuribenzoic acid, was purchased from Aldrich Chemical Co. Glutathione was obtained from the Nutritional Biochemicals Corp. Bacitracin, cytochrome *c* (horse heart), myoglobin (crystallized salt free, sperm whale), chymotrypsinogen A (beef pancreas, six-times crystallized, salt free), ovalbumin (two-times crystallized), trypsin were purchased as molecular weight markers (kit 8109) from Mann Research Laboratories. Blue Dextran, Sephadex G-10, and G-50 were obtained from Pharmacia Fine Chemicals, Inc; Cellex-CM from Bio-Rad; and Cellulose casing from the Union Carbide Corp. *N*-Bromosuccinimide, *O*-methylisourea, urea, guanidium chloride, and 1,2-cyclohexandione were obtained from the Aldrich Chemical Co. *N*-Bromosuccinimide was

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recrystallized from water before use. Agar plated for immunodiffusion were made of Difco bactoagar from the Difco Laboratories. Ampholine-carrier ampholytes and the electrofocusing column were the products of LKB Instruments, Inc. All other chemicals were of analytical grade. All solutions were prepared with deionized quartz-redistilled water.

Sea snakes, *L. semifasciata*, were captured in the caves of Gato Island, Philippines, by skin diving during the summer of 1967 by the senior author. To extract the venom, we decapitated the sea snakes and removed the venom glands by dissection. The dried glands of 600 sea snakes were thoroughly pulverized with an electric grinder, and the venom was extracted with distilled water. Insoluble tissue debris was removed by centrifugation, and the supernatant liquid was lyophilized.

Methods

Isolation Procedure. Fine-grade Sephadex G-50 was equilibrated in 0.1 M NaCl and 0.01 M phosphate at pH 6.5. Lyophilized venom (1 g) was dissolved in 2 ml of 0.01 M phosphate buffer at pH 6.5. This sample was applied to a 2 × 75 cm column and eluted with the equilibration buffer at a flow rate of 9 ml/hr in a cold room at 4°. The eluate was monitored at 280 mμ with ISCO Model UA-2 dual-beam ultraviolet analyzer equipped with a recorder and collected in 3-ml volumes. The tubes representing each peak were pooled and lyophilized. Each sample was desalted by passing through a 1.5 cm × 85 cm column of Sephadex G-10. Each fraction was tested for toxicity.

Carboxymethylcellulose powder was washed with 0.5 M HCl followed by deionized water, then 0.5 M NaOH containing 0.5 M NaCl, and finally with deionized water until no chloride could be detected using silver nitrate. A 2 × 40 cm column of CM-cellulose was equilibrated with 0.01 M phosphate buffer at pH 6.5. Desalted, lyophilized fraction (S-II) (400 mg) was dissolved in 2 ml of 0.01 M phosphate buffer (pH 6.5), and applied to the column. Elution was carried out with stepwise increase in the ionic strength by increasing sodium chloride concentration. The flow rate was 9 ml/hr.

Toxicity Test. The toxicity tests were done by injecting 0.2 ml of venom of varying concentrations intravenously into mice. Each chromatographic fraction was tested by the same procedure. Swiss white mice weighing 20 g each were used for the test. The number of mice that survived for a 24-hr period was observed. Six dosage levels of five mice each were used and the toxicity was determined statistically using the method of Litchfield and Wilcoxon (1949) and expressed as the lethal dosage 50%, the LD_{50} values (micrograms of toxin per gram body weight of mouse).

Polyacetate Electrophoresis. The electrophoresis on Gelman cellulose polyacetate strips was carried out in barbital buffer at pH 8.5, with a voltage of 250 V for 1.5 hr. Proteins bands were stained with 0.2% Amido Black in methanol, and washed with anhydrous methanol.

Isoelectrofocusing. The isoelectrofocusing was carried out by the method of Vesterberg and Svensson (1966) and Toom *et al.* (1970) after a sucrose gradient had been formed by means of a gradient mixer (LKB 8121). Purified, salt-free toxin (10 mg) was applied to a 110-ml isoelectrofocusing column which was cooled with circulating water in the cold room. The ampholyte concentration was 2% w/v. The pH ranged from 7 to 10. The potential was maintained at 500 V for 60 hr. At the end of the experiment, the column was drained and 2.0-ml fractions were collected. Absorption at 280 mμ was monitored on an ISCO Model UA-2 dual-beam ultraviolet analyzer equipped with an automatic recorder. The pH of each 2-ml fraction was measured by Radiometer pH meter type PHS 630 T.

Sedimentation Analyses. A Spinco Model E analytical ultracentrifuge equipped with a temperature control unit (RTIC) and a schlieren-interference optical system was used for the sedimentation velocity and sedimentation equilibrium experiments. The schlieren and interference patterns were recorded on Eastman Kodak metalographic and spectrographic II-G plates, respectively. The plates were read using a Nikon Model 6C microcomparator equipped with a rotational stage.

The sedimentation velocity experiments were performed at 20° and 59,780 rpm using a double-sector schlieren cell with an aluminum-filled Epon capillary-type synthetic boundary centerpiece. The sedimentation coefficient was calculated from the rate of movement of the maximum ordinate of the schlieren peak. The samples were prepared for ultracentrifugation by dissolving 10 mg of lyophilized toxin in 1 ml of 0.1 M glycine–NaOH buffer at pH 9.3 containing 0.15 M NaCl. Solutions were dialyzed against the same buffer prior to centrifugation. Three concentrations for each toxin were used to evaluate the sedimentation coefficient.

The sedimentation equilibrium experiments were performed at 20° and a rotor speed of 24,630 rpm using the interference optical system. A double-sector cell with an aluminum-filled Epon centerpiece was used, with a 3-mm column of solution prepared as above in the sample sector. FC-43 (0.02 ml) was used to provide a liquid bottom for the column of solution. Initial concentrations were determined for separative runs using a double-sector capillary-type synthetic boundary cell. Apparent equilibrium was established within 24 hr. Fringe patterns after 24 hr were used in calculations of the molecular weight according to procedures summarized by Chervenka (1969).

Molecular Weight by Gel Filtration. Estimations of the molecular weights of the toxins were made by the method of Andrews (1964). The Sephadex G-50 was hydrated for 48 hr with decantation to remove fine particles before packing in a 1.5 × 85 cm column. The buffer was 0.05 M Tris-HCl–0.1 M NaCl (pH 7.5). Blue Dextran was used to estimate void volume (V_0), and several proteins of known molecular weights were used to calibrate the column. The molecular weights of toxins a and b were estimated directly from the linear portion of the line describing the relationship between $(V_e - V_0)$ and the molecular weight.

Crystallization of Toxins. A solution containing 10 mg of toxin was precipitated at 2° by the stepwise addition of appropriate amounts of ammonium sulfate. The toxins precipitated at approximately 40% saturation. The resultant suspension was centrifuged for 15 min at 10,000*g* and 2°. The precipitate was suspended at 2° in 1 ml of 30% saturated ammonium sulfate solution containing 0.01 M phosphate buffer (pH 8.0). Crystallization occurred on standing undisturbed overnight at 2°. The toxin crystals were collected by centrifugation at 10,000*g* for 10 min.

Amino Acid Analysis. The amino acid composition of toxins were determined after acid hydrolysis at 110° according to the procedure of Moore and Stein (1957). Analyses were performed using a Beckman Model 120B amino acid analyzer.

The total tryptophan content of toxin was estimated by titration of the toxin with *N*-bromosuccinimide in 0.05 M acetate buffer at pH 4.0 containing 6 M urea as described by

Spande and Witkop (1967). The tryptophan content was also analyzed by the spectrophotometric method of Edelhoch (1967). Cystine was determined after performic oxidation by the method of Hirs (1967).

SH Group of Toxins. The sulfhydryl groups of the toxins were determined by titration with *p*-mercuribenzoate in 0.01 M phosphate buffer (pH 7.0) and in 6 M urea following the method of Riordan and Vallee (1967), and Benesch and Benesch (1962).

End-Group Analysis. The N-terminal amino acid was determined by dansyl method of Gray (1967), modified by Bustin and Cole (1969); 10 mμmoles of each toxin was used. The dansyl derivatives of the amino acids were separated and identified by thin-layer chromatography on polyamide sheets by the method of Woods and Wang (1967).

The C-terminal residue was determined by the titration method of Matsuo *et al.* (1965, 1966) as modified by Holcomb *et al.* (1968), in which, pyridine and acetic anhydride were used. The tritiated amino acids were identified by spraying the thin-layer plates with 0.2% ninhydrin reagent and measuring the radioactivity with a Packard Model 3310 liquid scintillation spectrometer.

Ultraviolet Absorption Spectra. The absorption spectra were obtained with a recording Beckman DBG spectrophotometer. Purified, lyophilized toxin was dissolved in 0.05 M acetate at pH 4.0. The absorption spectra were determined at a concentration of 1 mg/ml.

Stability of Toxins. The stability of the toxins at various temperatures was tested by heating the saline toxin solution to a particular temperature for 30 min with immediate subsequent cooling water. The toxicity was determined as described above. To determine the stability of the toxins at different pH's the following solutions were used: 0.01 M HCl–KCl for pH 1 and 2, 0.01 M acetate buffer for pH 4, 0.01 M phosphate buffer for pH 6 and 8, 0.01 M glycine–NaOH buffer for pH 10 and 11. The toxicity after each treatment was determined by the method used for the temperature stability tests.

Chemical Modifications

Modification of Tryptophan with N-Bromosuccinimide. The oxidation of toxins by *N*-bromosuccinimide was carried out by the method of Freisheim and Huennekens (1969). Successive 10-μl aliquots of 5 mM aqueous *N*-bromosuccinimide solutions were added to 2 ml of 1.48×10^{-4} M of toxin a or 1.47×10^{-4} M toxin b in 0.05 M acetate buffer (pH 4.0). The oxidation of tryptophan residues was followed by the decrease in the absorbance of 278 mμ using a Beckman DB-G spectrophotometer. Lethality of the toxins at different stages of modification were measured by injecting 0.2-ml solution of modified toxin intravenously into five Swiss white mice (20-g body weight) for each dose. Six doses were used for the LD_{50} measurement. The control contained only *N*-bromosuccinimide. The LD_{50} was calculated according to the method of Litchfield and Wilcoxon (1949).

Modification of Arginine with 1,2-Cyclohexanedione. The arginyl residues of the toxins were chemically modified using the reagent 1,2-cyclohexanedione (Toi *et al.*, 1967). Five mg of the toxin was dissolved in 1.5 ml of 0.1 M triethylamine buffer (pH 11.0) and 0.5 ml of 1,2-cyclohexanedione solution (8.9 M) was added. After standing for 24 hr at room temperature the reaction mixtures were dialyzed in the cold against deionized water for 24 hr using cellulose casing. The lyophilized toxins were assayed for toxicity as described above. The extent of modification was estimated by determining the arginyl residues using a modified Sakaguchi reaction (Izumi, 1965a,b). The decrease in arginine was also measured by amino acid analysis after the hydrolysis of modified toxin.

Modification of Lysine with O-Methylisourea. Purified toxin (5 mg) was dissolved in 2 ml of 0.6 M *O*-methylisourea (pH 10.5). The solution was incubated at 25° for 72 hr, then desalted with cellulose casing and dialyzed against deionized water for 24 hr and lyophilized. The lyophilized toxins were assayed for their toxicities by the methods described above. The extent of guanidination was estimated by determining the homoarginine content after acid hydrolysis by the Sakaguchi reaction as modified by Izumi (1965a,b). The amino acid analyzer was used to determine the decrease of lysine and appearance of homoarginine.

Immunodiffusion. Immunodiffusion was carried out at 0° for 12 hr in 1% Difco agar made in 0.025 M barbital buffer at pH 8. Native and *N*-bromosuccinimide-modified toxins were used at a concentration of 20 mg/ml.

TABLE I: Isolation of Toxic Principles from *Laticauda semifasciata* Venom from Philippines by Sephadex G-50 and CM-cellulose Chromatographies.

Fraction	Protein (mg)	Recov of Protein (%)	Toxicity LD_{50} (μg/g)
Venom	1000		0.28
First fractionation[a]			
S-I	171	17	Nontoxic[b]
S-II	392	39	0.13
S-III	286	29	Nontoxic
S-IV	93	9	Nontoxic
S-V	52	5	Nontoxic
Recovery	994	99	
Second fractionation[c]			
CM-I	122	12	1.60
CM-II	12	1	1.60
CM-III	62	6	0.39
CM-IV	84	8	0.07
CM-V	82	8	0.05
CM-VI	23	2	1.40
Recovery	385	37	

[a] The Sephadex G-50 column was loaded with 1 g of venom. [b] All the mice survived an intravenous injection of 20 μg of lyophilized fraction per g of body weight. [c] The CM-cellulose column was loaded with 400 mg of S-II fraction.

Results

Isolation of the Toxins. Five fractions were obtained by passing the venom through a Sephadex G-50 column (Figure 1A). The recovery of protein and the toxicity of each fraction is presented in Table I. Only fraction S-II was found to be toxic, and the LD_{50} was 0.13 μg/g. The LD_{50} value of the original venom was 0.28 μg/g. Thus, the fractionation resulted

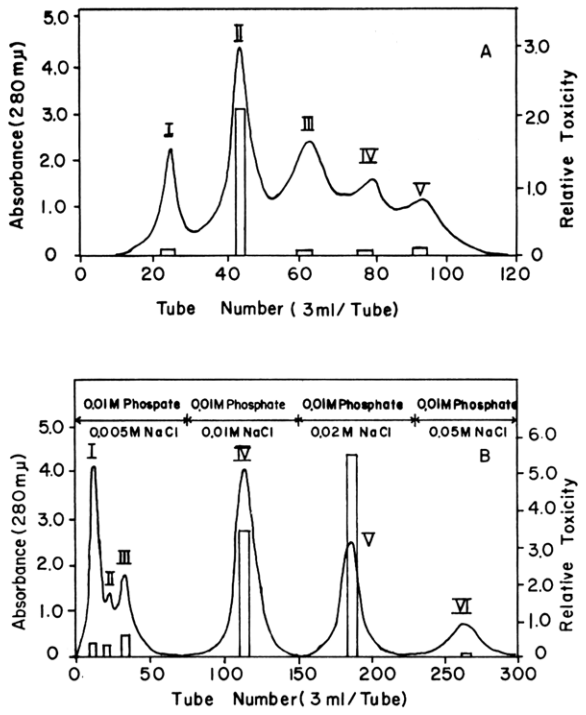

FIGURE 1: Isolation of toxins from *Laticauda semifasciata* venom. (A) Absorbance at 280 mμ and toxicity profiles of the crude venom after passage through a column of Sephadex G-50. The absorbance at 280 mμ is shown by the solid line. The toxicity of each fraction is shown by the rectangular histogram under each peak. (B) Absorbance at 280 mμ and toxicity profiles of fraction S-II after passage through a column of CM-cellulose. The rectangular histograms indicate the toxicity of each fraction.

in approximately a twofold increase in toxicity. Fraction S-II of Sephadex G-50 filtration was desalted with a Sephadex G-10 column by eluting with deionized water. The desalted S-II fraction was applied to a CM-cellulose column. Two

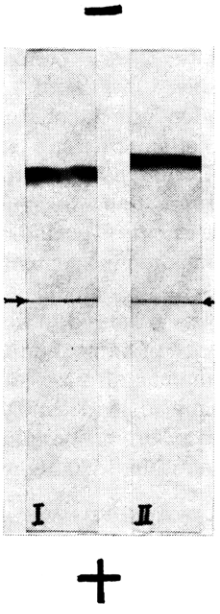

FIGURE 2: Polyacetate electrophoresis employing a barbital buffer (pH 8.5). The points of application of a 50-mg/ml sample are indicated by the arrows. The samples were electrophoresced for 90 min. The applied voltage was 250 V. Strip I is for toxin a, strip II for toxin b.

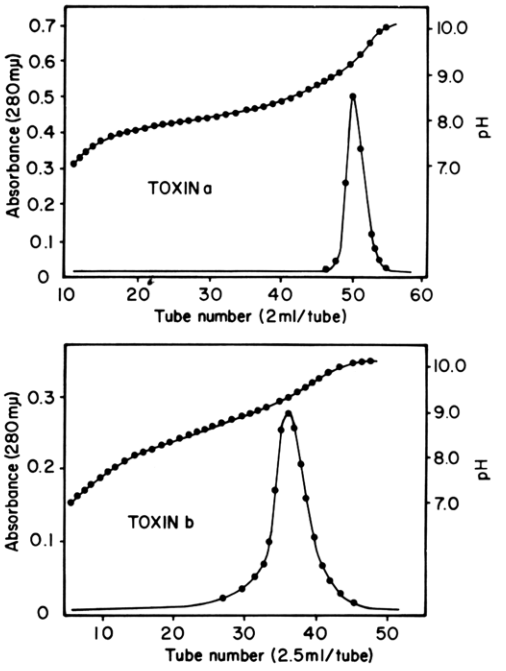

FIGURE 3: Isoelectric focusing profiles of purified toxins. Details of the experimental procedure are given in the text.

toxic fractions were isolated. They were designated as toxins a and b (Figure 1B, CM-IV, CM-V). LD_{50} was found to be 0.07 and 0.05 μg per g for toxins a and b, respectively. The CM-cellulose fractionation resulted in a greater than twofold increase in toxicity. Comparison with the original venom LD_{50} of 0.28 μg/g demonstrates a fourfold increase in toxicity for toxin a and a 5.6-fold increase in toxicity for toxin b. Both toxins a and b gave the typical absorption spectrum of

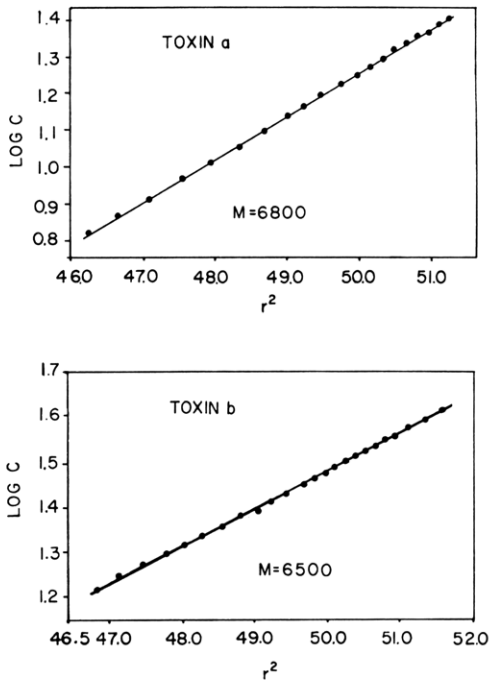

FIGURE 4: Plot of log C *vs.* r^2 for a sedimentation equilibrium experiment, where C refers to concentration in terms of interference fringes. The rotor speed was 24,630 rpm and the temperature was 20°. A 3-mm column of solution was used.

TABLE II: Amino Acid Composition of Toxins a and b from *Lacticauda semifasciata* Venom.

		Molar Ratio of Toxins at Different Durations of Hydrolysis Based on Leucine = 1.0								Nearest Integer	
		24 hr		48 hr		72 hr		Cor[a]			
Amino Acid	(Toxin)	a	b	a	b	a	b	a	b	a	b
Lysine		4.13	4.52	3.82	4.58	4.48	4.81	4.14	4.64	4	5
Histidine		1.05	1.80	1.00	1.01	1.09	1.00	1.04	1.26	1	1
Arginine		3.05	2.78	2.72	1.79	2.71	2.21	2.82	2.26	3	2
Aspartic acid		4.56	4.17	4.59	4.21	4.77	4.29	4.64	4.22	5	4
Threonine		4.92	4.23	3.53	3.42	3.34	2.36	5.60	5.36	6	5
Serine		6.33	4.69	3.98	4.06	3.46	1.54	7.20	6.40	7	6
Glutamic acid		8.22	8.47	7.84	7.72	7.79	7.54	7.95	7.76	8	8
Proline		3.98	3.99	3.77	3.73	3.89	4.01	3.85	3.91	4	4
Glycine		5.33	5.69	5.10	5.58	5.26	5.51	5.23	5.59	5	6
Alanine		0	0	0	0	0	0	0	0	0	0
Valine		2.42	2.67	2.32	2.45	2.40	2.57	2.38	2.56	2	3
Methionine		0	0	0	0	0	0	0	0	0	0
Isoleucine		4.05	3.31	3.63	4.18	3.84	3.63	3.84	3.71	4	4
Leucine		1.00	1.12	1.00	1.15	1.04	1.44	1.01	1.23	1	1
Tyrosine		1.10	1.15	1.09	0.99	1.08	0.98	1.09	1.37	1	1
Phenylalanine		1.60	2.07	1.89	2.00	2.07	1.95	1.85	2.01	2	2
Half-cystine[b]								7.89	7.56	8	8
Tryptophan[c]								0.95	0.82	1	1
Total residue number										62	61
Minimal molecular weight										6840	8677

[a] Results from three different hydrolysis times were extrapolated to zero time to obtain the value for threonine, and serine. Average values for the remaining amino acids are reported. [b] These values were determined after oxidation of the toxins with performic acid followed by 24-hr hydrolysis in HCl. [c] Tryptophan was determined by spectrophotometric methods.

a protein with a maximum extinction at approximately 280 mμ. The recovery of protein and the toxicities of other fractions were also determined. The results are summarized in Table I.

Criteria of Purity. The single protein band shown by electrophoretic experiments on polyacetate strips (Figure 2) suggests that toxins a and b are each a single component.

Isoelectric focusing (pH 7–10) profiles of toxins a and b also suggest a single component in each case (Figure 3). The isoelectric points were determined to be 9.15 and 9.34 for toxins a and b, respectively.

Within the limits of our measurements both the sedimentation velocity and the sedimentation equilibrium results suggest that both toxin a and toxin b are homogeneous. The log C *vs.* r^2 plots for an equilibrium sedimentation experiment are shown in Figure 4.

Both toxins a and b were crystallized in rectangular form as shown in Figure 5A,B.

Amino Acid Analysis. The amino acid compositions of the two toxins are summarized in Table II. The results represent the average of three determinations after 24-, 48-, and 72-hr hydrolysis in HCl. The values for threonine and serine were obtained by extrapolating to zero time. Cystine was determined as cysteic acid after oxidation with performic acid. Eight moles of cysteic acid was found. Since no detectable sulfhydryl groups were found by titration of the toxins in 6 M urea with *p*-mercuribenzoate, all cysteic acids must be derived from the disulfide linkages in the toxins. The tryptophan content was determined by a *N*-bromosuccinimide titration according to the methods of Spande and Witkop (1967). A typical *N*-bromosuccinimide titration is shown in Figure 6. The alternative spectrophotometer method of Edelhoch (1967) was also used to determine the tryptophan content of the toxin. In each case only one tryptophan residue was found in each toxin. The percent recovery of amino acid residues was 93% for toxin a and 95% for toxin b. The results of amino acid analysis indicate that toxin a is composed of 62 amino acid residues, whereas, toxin b is composed of 61. The minimum molecular weight of each toxin estimated from amino acid composition is 6840 for toxin a and 6677 for toxin b, respectively.

N- and C-Terminal Amino Acids. Amino-terminal analyses were performed using approximately 10 mμmoles of each purified toxin. The predominent spot of dansyl-arginine on polyamide thin-layer sheets indicated that arginine is the N-terminal amino acid in both toxins a and b. The carboxyl-terminal amino acid was determined by the selective tritiation method of Holcomb *et al.* (1968). Only aspartic acid showed significant radioactivity. This indicated that aspartic acid or asparagine was the carboxy-terminal amino acid in both toxins a and b.

Molecular Weights and Sedimentation Coefficients. The average molecular weights of toxin a and b were estimated from the results of sedimentation equilibrium studies using $M = (RT/(1 - \bar{v}\rho)\omega^2)((\mathrm{d}\ln c)/\mathrm{d}r^2)$.

Plots of log concentration *vs.* r^2 are presented in Figure 4. Molecular weights estimated from these plots were 6800 for toxin a, and 6500 for toxin b. The partial specific volume

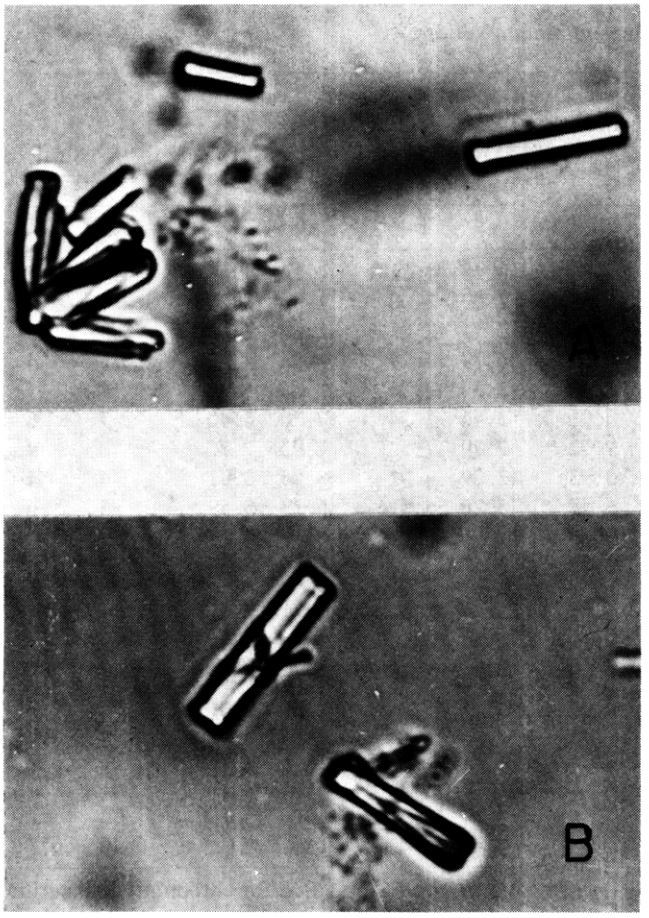

FIGURE 5: Crystals of toxins a (A) and b (B). Both are shown at 450 times magnification.

was estimated from the amino acid composition, by the method of Cohn and Edsall (1943) and found to be 0.71. Thus, within the limits of the methods used we conclude that the molecular weights of the toxins are equal.

Sedimentation velocity studies of the purified toxins revealed a single peak in the Schlieren photographs at concentrations of 3.4, 5.0, and 6.7 mg per ml for toxin a; 3.5, 4.5, and 6.0 mg per ml for toxin b. The sedimentation coefficients were determined to be 1.45 ± 0.03 S for toxin a and 1.32 ± 0.03 S for toxin b. No significant concentration dependence was shown over the range studied.

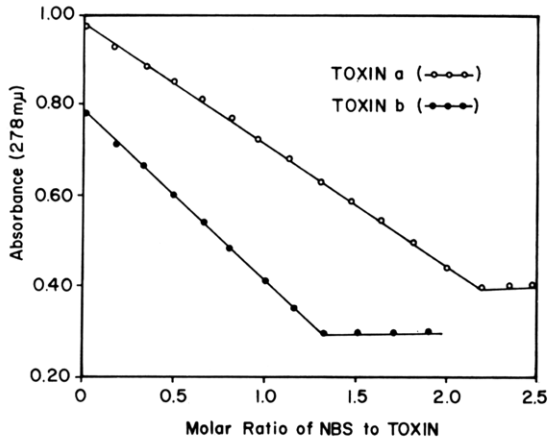

FIGURE 6: Determination of tryptophan residue of toxin a and toxin b with *N*-bromosuccinimide. The concentrations were 1.49×10^{-4} M for toxin a and 1.47×10^{-4} M for toxin b in 0.05 M acetate buffer (pH 4.0) containing 6 M urea.

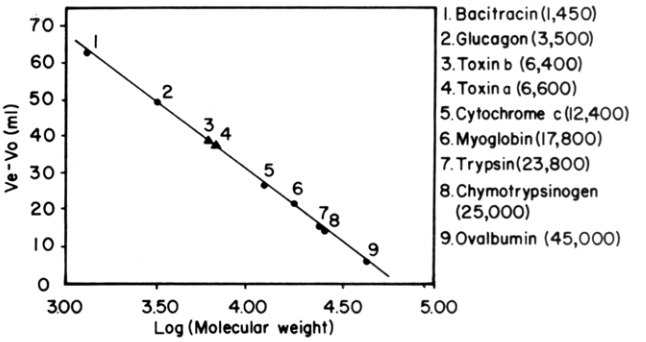

FIGURE 7: Molecular weights of purified toxins by gel filtration. A 1.5 × 85 cm column of Sephadex G-50, equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl was used. Standard proteins used for the calibration are listed on the graph. The molecular weights were estimated from the linear plot.

Molecular Weight by Gel Filtration. The molecular weights of the toxins were determined by measuring the elution volume on a column of Sephadex G-50 (Figure 7). Toxins a and b were found to have a molecular weight of 6600 and 6400, respectively. These values are in agreement with the minimum molecular weights calculated from amino acid composition and with the molecular weights from sedimentation equilibrium.

Effect of Temperature and pH. Table III summarizes the temperature and pH stability of the toxins. The results indicate that toxicity of the toxins is not significantly temperature dependent, even when heated at 100° for 30 min. No significant changes in toxicities were observed from pH 1 to 11 indicating that both toxins a and b are stable over wide range of pH.

Modification of Tryptophan. The oxidation of the tryptophan residue in the toxins was based on the principle that the indole chromophore of tryptophan is converted into oxindole

TABLE III: Stability of Toxins at Different Temperature and pH.

	Toxicity (LD_{50})[a]	
	Toxin a	Toxin b
Temperature (°C)		
25	0.06	0.05
60	0.07	0.05
80	0.06	0.06
100	0.06	0.05
pH		
1	0.06	0.04
2	0.07	0.04
4	0.07	0.04
6	0.07	0.05
8	0.07	0.04
10	0.05	0.04
11	0.05	0.04

[a] LD_{50} is expressed as micrograms of toxin per gram body weight of the mouse.

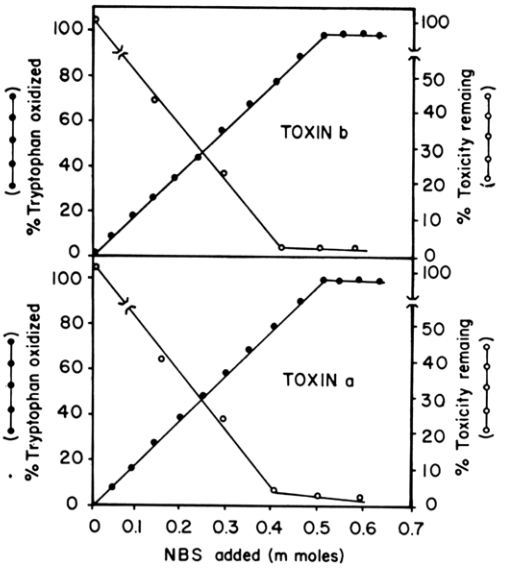

FIGURE 8: Modification of toxins with *N*-bromosuccinimide and its effect on toxicity. Aliquot of 10 μl of 5 mM *N*-bromosuccinimide were used for 1.48×10^{-4} M toxin a and 1.47×10^{-4} M toxin b step by step. All reactions were carried out in 0.05 M acetate buffer (pH 4.0). The toxicities of *N*-bromosuccinimide-modified toxins at different stages of oxidation were tested as described in the text.

on oxidation with *N*-bromosuccinimide (Spande and Witkop, 1967). The relationship between toxicities and the oxidation of tryptophan residue in the toxins are presented in Figure 8. The toxicity of each toxin progressively decreased as the tryptophan residue was modified. The toxicity was lost completely when all tryptophan in the sample was oxidized by *N*-bromosuccinimide.

It has been reported by Ramachamdran and Witkop (1959) that *N*-bromosuccinimide modification may cause cleavage of proteins at the peptide bond containing tryptophan. To check that the toxins were not cleaved under the conditions used, the modified toxins were applied to polyacetate electrophoresis. The single protein band of *N*-bromosuccinimide-modified toxin a or b, suggested no cleavage had occurred. Further the mobilities of the modified toxins were similar to those of the native toxins.

To determine whether or not amino acid other than tryptophan were modified by the *N*-bromosuccinimide treatment, we performed an amino acid analysis of the *N*-bromosuccinimide-modified toxin. No significant change in amino acid composition were noted for the *N*-bromosuccinimide-modified toxins. The data are summarized in Table IV.

Antigenicities of toxins a and b before and after *N*-bromosuccinimide modification were examined against the rabbit serum antibody for original venom by the Ouchterlony immunodiffusion technique. There was no change in antigenic activities for toxin a or b after modification. A single fused precipitin line was observed with neighboring antigens (Figure 9).

Modification of Arginine Residue. Amino acid analyses (Table V) indicated that toxin a contained 3 moles of arginine and toxin b contained 2 moles. Only one arginine residue was modified in both toxins as the number of arginine residues were reduced to 2 and 1 for toxins a and b, respectively, after reaction with 1,2-cyclohexanedione. A second independent method was also employed to determine the number of arginine residues remaining by the Sakaguchi reaction as modified by Izumi (1965a,b). By the Sakaguchi method,

TABLE IV: Amino Acid Compositions of *N*-Bromosuccinimide–Toxins.

Amino Acid	Control[a]		After Modification	
	Toxin a	Toxin b	Toxin a	Toxin b
Lysine	4.3	4.7	4.1	4.6
Histidine	1.2	1.1	1.2	1.1
Arginine	3.1	2.3	3.1	2.2
Aspartic acid	4.8	4.3	4.9	4.2
Threonine	4.9	4.4	5.0	4.4
Serine	6.5	5.3	6.8	5.1
Glutamic acid	7.8	7.9	8.0	8.1
Proline	3.6	3.7	3.9	3.9
Glycine	5.2	5.6	5.1	5.8
Alanine	0	0	0	0
Half-cystine[b]	4.5	4.7	4.5	4.8
Valine	2.4	2.7	2.2	2.7
Methionine	0	0	0	0
Isoleucine	4.2	3.9	4.1	3.8
Leucine	1.0	1.0	1.0	1.0
Tyrosine	1.1	1.1	1.1	1.2
Phenylalanine	1.8	1.9	1.9	2.1

[a] Amino acid composition of toxins treated with 0.05 M acetate buffer (pH 4.0). [b] These values were obtained from samples which were not oxidized with performic acid.

2 moles of arginine remained in toxin a and 1 mole in toxin b after modification. Thereafter, this method also confirmed that only one arginine residue was modified for both toxins a and b. The results are summarized in Table VII.

No changes in toxicities were observed for toxin a or b after modification.

Modification of Lysine Residues. Amino acid analyses (Table VI) indicated that the lysine content was reduced to one when toxins a and b were modified with *O*-methylisourea. There were 4 moles of lysine in toxin a and 5 moles for toxin b before modification. Another method was used to determine the number of lysine residues modified. When the lysine residues were modified with *O*-methylisourea, the reactive lysine residues were converted into homoarginine which was then determined by the method of Izumi (1965a,b). By this

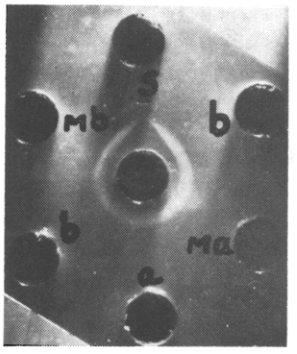

FIGURE 9: The immunodiffusion pattern of the native and tryptophan-modified toxins. Rabbit antiserum was applied in the center well. The sample in the side wells are a (toxin a), b (toxin b), Ma (modified toxin a), Mb (modified toxin b), and S (saline). The samples contained 20 mg of toxin/ml.

TABLE V: Amino Acid Composition of the Arginine-Modified Toxins.

Amino Acid	Control		After Modification	
	Toxin a	Toxin b	Toxin a	Toxin b
Lysine	3.8	4.7	4.4	4.5
Histidine	1.0	1.1	1.2	1.2
Arginine	2.7	2.2	1.5	1.1
Aspartic acid	4.6	4.3	4.8	4.2
Threonine	4.5	4.1	5.1	4.2
Serine	4.9	4.2	4.8	4.7
Glutamic acid	7.8	7.9	7.6	8.4
Proline	3.8	3.8	4.4	3.9
Glycine	5.1	5.5	4.6	5.7
Alanine	0	0	0	0
Half-cystine	7.6	7.6	7.5	7.8
Valine	2.3	2.5	1.9	2.7
Methionine	0	0	0	0
Isoleucine	3.6	3.7	3.9	3.8
Leucine	1.0	1.0	1.0	1.0
Tyrosine	1.0	0.9	1.1	1.1
Phenylalanine	1.9	1.9	1.7	2.1

method, 3 moles of homoarginine were found for toxin a and 4 moles for toxin b. Thus, two independent determinations gave identical results. These modifications produced no change in toxicity of the toxins. The results of the modification of the lysine residue are summarized in Table VII together with the results of the modification of the arginine residues.

Discussion

On the basis of the results presented in the previous sections it appears that the venom of the sea snake *L. semifasciata* from the Philippine Sea contains two types of toxin. We have labeled these toxin a and toxin b. The evidence for their independent nature is the clear resolution achieved by CM-cellulose chromatography. The homogeneity of the two toxins fraction was substantiated by sedimentation velocity, sedimentation equilibrium, Sephadex gel filtration, isoelectric focusing, electrophoresis, and crystal formations. From per cent recovery of proteins in fractionations, it was found that *L. semifasciata* venom contained 8.2% toxin a and 8.0% toxin b. By isolating toxins, toxicities increased four times for toxin a and 5.6 times for toxin b.

TABLE VI: Amino Acid Compositions of Lysine-Modified Toxins.

Amino Acid	Control		After Modification	
	Toxin a	Toxin b	Toxin a	Toxin b
Lysine	4.1	4.6	0.8	1.1
Histidine	1.1	1.0	1.1	1.4
Arginine	3.1	1.8	3.4	2.2
Homoarginine	0	0	3.5	4.0
Aspartic acid	4.5	4.2	4.8	3.9
Threonine	4.9	4.4	4.5	4.3
Serine	5.6	4.3	6.3	4.2
Glutamic acid	8.2	7.7	8.1	8.3
Proline	3.9	3.7	4.3	3.9
Glycine	5.3	5.6	5.1	5.6
Alanine	0	0	0	0
Half-cystine	7.7	7.6	7.8	7.5
Valine	2.2	2.6	2.4	2.8
Methionine	0	0	0	0
Isoleucine	4.1	4.2	4.4	3.8
Leucine	1.0	1.1	1.2	1.0
Tyrosine	1.0	1.2	1.0	0.9
Phenylalanine	1.6	2.1	2.2	1.9

TABLE VII: Summary of the Modification of Arginine and Lysine Residues.

Toxins	Residues of Arg	Residues of Lys	Toxicity LD_{50} (μg/g)
Toxin a			
Control	3	4	0.070
Arginine modified	2		0.063
Lysine modified		1	0.065
Number of amino acid residues modified	1	3	
Toxin b			
Control	2	5	0.050
Arginine modified	1		0.054
Lysine modified		1	0.053
Number of amino acid residues modified	1	4	

The sedimentation coefficients were 1.45 ± 0.03 and 1.32 ± 0.03 S for toxins a and b, respectively. From sedimentation equilibrium the molecular weight was determined to be 6800 ± 300 for toxin and 6500 ± 300 for toxin b. Sephadex gel filtration and amino acid composition gave molecular weights of 6600 and 6400, and 6840 and 6670 for toxins a and b, respectively. From these data we can reliably estimate the molecular weight to be 6800 ± 500.

The slight difference in amino acid compositions suggested in Table II cannot be totally justified by the composition data. Nevertheless, we are confident the compositions are different and that we can make the following number of positive statements. Neither toxin has a free SH group. Each toxin has four disulfide bonds. Neither toxin has methionine or alanine. The acid–base residue composition, the isoelectric point, and the migration rate in polyacetate electrophoresis are consistant.

Low molecular weights and high contents of disulfide bonds of toxins suggest that these toxin molecules have rather compact structure thus probably accounting for high stability of toxicities at high temperature and over a wide range of pH.

Comparison of the amino acid composition of toxins for the venoms of different sea snakes and cobras are summarized in Table VIII. It is well known that venoms of snakes from the family *Elapidae* such as cobras and kraits and the family *Hydrophiidae* (sea snakes), contain potent neurotoxins (Tu and Tu, 1970). The data in Table VIII, collected from the studies of numerous investigators, demonstrate the similarities of these toxins isolated from the venoms of snakes from widely varying geographical areas. For instance,

TABLE VIII: Amino Acid Compositions of Toxins from Snake Venoms.

Amino Acid (Toxin)	*Laticauda semifasciata* (Philippines) Toxin a	Toxin b	*Naja haje*[a] *haje*	*Naja naja*[b] *atra*	*Naja nigricollis*[c]	*Laticauda*[d] *laticaudata*	*Laticauda*[d] *colubrina*	*Laticauda semifasciata* (Reinwardt) Japan Erabu Toxin a	Erabu Toxin b	*Enhydrina*[e] *schistosa*
Lysine	4	5	6	3	6	4	4	4	4	5
Histidine	1	1	2	2	2	2	2	1	2	2
Arginine	3	2	4	6	3	5	5	3	3	3
Aspartic acid	5	4	7	8	7	9	9	5	4	6
Threonine	6	5	7	8	8	4	4	5	5	8
Serine	7	6	4	4	2	6	6	8	8	6
Glutamic acid	8	8	7	7	6	7	7	8	8	8
Proline	4	4	4	2	5	5	5	4	4	3
Glycine	5	6	5	7	5	5	5	5	5	5
Alanine	0	0	0	0	0	0	0	0	0	1
Valine	2	3	1	1	2	1	1	2	2	1
Methionine	0	0	0	0	0	0	0	0	0	0
Isoleucine	4	4	3	2	3	2	2	4	4	2
Leucine	1	1	1	1	2	1	1	1	1	1
Tyrosine	1	1	1	2	1	1	1	1	1	1
Phenylalanine	2	2	0	0	0	1	1	2	2	0
Half-cystine	8	8	8	8	8	8	8	8	8	9[f]
Tryptophan	1	1	1	1	1	1	1	1	1	1
Total Residue	62	61	61	62	61	62	62	62	62	62
Minimum molecular weight	6840	6677	6835	6952	6787	6970	6970	6850	6870	6981

[a] Botes and Strydom (1969). [b] Chang and Hayashi (1969). [c] Karlsson, Eaker, and Porath (1966). [d] Sato *et al.* (1969). [e] Tu and Toom (1971) [f] Nine residues of cystine were found by a calculation based on the average molar ratio to leucine, alanine, and valine. Eight residues were found if the calculation was based on the ratio to leucine alone. Leucine yielded the smallest number of moles in the amino acid analysis.

the venoms of the sea snakes *Laticauda semifasciata* from the Philippines; *Lauticauda ladicauda*, *Laticauda colubrina*, and *Laticauda semifasciata* (Reinwardt) from Japan; *Enhydrina schistosa* from the Strait of Malacca; and the venoms of cobras *Naja haje haje* from North Africa; *Naja naja atra* from Formosa, and *Naja nigricollis* from the southern portion of the African continent, all yield toxins with very similar molecular weights.

Chemical modification of particular amino acid residues has been used extensively to map out the active center of enzymes. This method was adapted to toxins in order to find which amino acid residue is essential for toxic action. It is shown in our study that chemical modification of a tryptophan residue in toxins leads to the complete loss of toxicity. Since there is only one tryptophan residue in both toxin a and b, it is clear that the tryptophan residue is essential for toxic action of toxin a or b. In contrast to tryptophan, the modification of the majority of arginine and lysine residues did not alter the toxicities of toxin a and b. Although three of four ϵ-amino groups of lysine residue in toxin a, and four of the five in toxin b can be converted into guanidinium group (Table VII), no marked alteration in toxicity was found. We may conclude that three of four lysine residues in toxin a and four of five in toxin b are not essential for toxic action.

Only one arginine residue in both toxin a and b can be modified with the reagent, 1,2-cyclohexanedione (Table VII). Therefore, we can conclude that at least one of the arginine residues in toxin a and b is not essential for toxic action.

It is of interest to note that while the tryptophan residue is essential for toxic action, it is not essential for antigenic action. In the immunodiffusion study, the modified and nonmodified toxins formed a single precipitin band. This strongly suggests that there is no change in antigenicity after modification.

In this investigation, we have shown that chemical modification technique which has been frequently used to study structure function relationships of enzyme molecules can be applied to toxins to study the toxicity–structure relationships.

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