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Purification and Chemical Characterization of the Major Neurotoxin from the Venom of *Pelamis platurus*†

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ABSTRACT: A major toxin was isolated from the venom of the sea snake *Pelamis platurus* (yellow-bellied sea snake) by Sephadex G-50 and carboxymethylcellulose column chromatography. The LD_{50} of the pure toxin (*Pelamis* toxin a) was 0.044 μg/g in mice representing a tenfold increase in toxicity after purification. The toxin was homogeneous in acrylamide disc gel electrophoresis and eluted as a single peak after isoelectric focusing in a sucrose density gradient column. The isoelectric point was 9.69; thus it is a highly basic protein. The toxin contained 55 amino acid residues with four disulfide linkages. When all disulfide linkages were reduced and alkylated, the toxic action of the pure toxin disappeared leading to the conclusion that the disulfide bonds of the neurotoxin were essential for toxic action.

Venoms from the sea snake family (Hydrophiidae) contain potent neurotoxic proteins which bind strongly to the acetylcholine receptor sites of the neuromuscular junction (Tu, 1974). Toxins from a number of the sea snake species which inhabit the coastal waters of Asia have been studied in detail. However, the venom of *Pelamis platurus* (yellow-bellied sea snake), a truly pelagic sea snake found in the Indian and Pacific Oceans and the only species known to inhabit the Pacific Coast of Central and South America, has not been examined in detail. The isolation and characterization of the major toxin from the venom of *P. platurus* is described in this report.

Materials and Methods

Venoms. Sea snakes, *Pelamis platurus*, were captured on the Pacific coast of Costa Rica, Central America, in 1973. Venom was extracted from the venom glands of 3069 snakes. The extraction was done by the method described previously (Tu and Hong, 1971).

Isolation Procedure. Crude venom (1.5 g) dissolved in buffer was loaded on a Sephadex G-50 column (3.5 × 110 cm) previously equilibrated with 0.01 *M* potassium phosphate buffer (pH 6.5) containing 0.1 *M* NaCl. The toxin was eluted with the same buffer at a flow rate of 18 ml/hr. The eluate, collected in 3-ml aliquots, was monitored at 280 nm with an ISCO Model UA-2 dual beam ultraviolet analyzer equipped with a recorder. For the accurate measurement of absorbance, each tube was determined again at 280 nm using a Beckman DB-G spectrophotometer. The tubes from each protein peak were pooled and lyophilized. The samples were then desalted by passage through a Sephadex G-10 column (2.5 × 40 cm) and each fraction was tested for toxicity.

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The desalted, lyophilized, toxic fraction (S-IV) was dissolved in 2 ml of 0.01 *M* phosphate buffer (pH 7.8), and applied to a CM-cellulose column (2.5 × 60 cm) which was equilibrated with the same buffer. Elution was carried out by stepwise increase in NaCl concentration, the flow rate being 9 ml/hr. Individual fractions in each peak were combined. Each combined fraction was tested for toxicity after desalting by a Sephadex G-10 column.

All purification procedures were performed at 4°.

Toxicity Test. (A) LD_{50} Test. The toxicity tests were performed by intravenous injection of 0.2 ml of the fraction to be tested into Swiss white mice weighing 20 g. Five to eight concentrations (four mice in each dose) were used for toxicity tests. The number of mice that survived for a 24-hr period was recorded. The toxicity was determined statistically by the method of Litchfield and Wilcoxon (1949) and expressed as the lethal dosage 50% (LD_{50}).

(B) Stability Test. To test the stability of the pure toxin at various temperatures, the toxin at concentrations equivalent to 1 LD_{50}, 2 LD_{50}, and 3 LD_{50} in 1 ml of isotonic saline solution was prepared and incubated at the selected temperature for 30 min. At the end of the incubation period, the sample was cooled to 22° and the toxicity test was done immediately. To determine the stability of the toxin at different pH values, 10 μl of the sample was mixed with 0.1 ml of 0.01 *M* buffer solution and incubated for 30 min at 22°. At the end of the incubation, the samples were diluted with saline to the appropriate concentrations and used immediately for toxicity tests. The following buffers were used for incubation: 0.01 *M* glycine-HCl for pH 2, 0.01 *M* acetate buffer for pH 4, 0.01 *M* succinate buffer for pH 6, 0.01 *M* Tris buffer for pH 8, 0.01 *M* glycine-NaOH buffer for pH 10.

Acrylamide Gel Electrophoresis. Discontinuous polyacrylamide gel electrophoresis was carried out to check the homogeneity of the toxin. The resolving gel was prepared by addition of 6 ml of acetate–tetramethylethylenediamine buffer solution at pH 4.3 to the mixture of 7.5 ml of 28% acrylamide and 10.5 ml of 0.25% ammonium persulfate solution. The stacking gel was prepared by mixing 1 ml of acetate–tetramethylethylenediamine (pH 6.65) buffer solution with 2 ml of 5% acrylamide and 1 ml of 0.4% riboflavine. The tubes were placed in the apparatus containing the lower chamber buffer solution (mixture of 3.6 ml of glacial acetic acid and 50 ml in 1 *N* NaOH diluted to 1 l., pH 5.1). The sample was dissolved in diluted chamber buffer (mixture 3.56 g of β-alanine and 2.23 ml of glacial acetic acid diluted to 1 l., pH 4.15) containing 10% glycerol and 50 μl was applied by layering under the upper chamber buffer. At 4°, the gels were run at 1 mA/tube for 30 min and at 2 mA/tube for 2 hr at which time the Methyl Green dye had approached the bottom of the gel. The gels were removed from the tubes and immersed in 12.5% trichloroacetic acid for 1 hr, stained by 0.025% Coomassie Blue in Cl_3CCOOH solution for 2 hr, and then destained by 7% acetic acid (Chrambach et al., 1967).

Isoelectric Focusing. The isoelectric focusing experiment was performed according to the method of Vesterberg and Svensson (1966). A sucrose gradient, formed by means of a gradient mixer (LKB 8121) with 2 mg of salt-free, purified toxin dissolved in the light solution, was loaded into the 110-ml column. The pH range of the ampholine was from 9 to 11, the initial voltage was 400 V, and the electric current was 3.8 mA. After 60 hr, the current had dropped and stabilized at 0.5 mA and the experiment was terminated. The contents of the column was collected in 70-drop fractions.

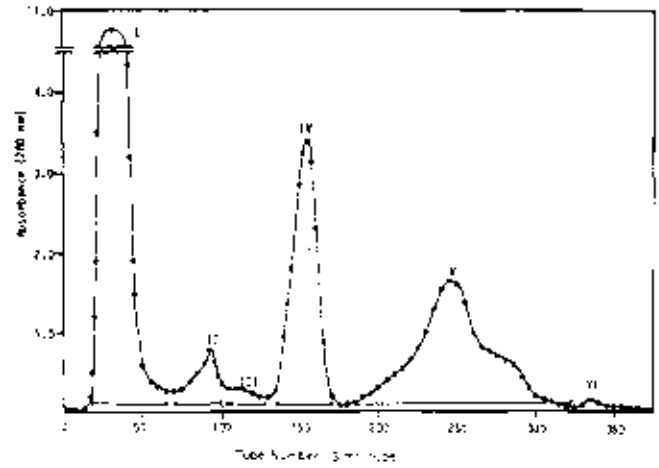

FIGURE 1: First step fractionation of toxin from *Pelamis platurus* venom. The Sephadex G-50 column was equilibrated with 0.01 *M* NaCl. A volume of 220 ml of buffer solution was collected from the column before starting to record. Major toxic fraction is indicated by the shaded area.

Absorbance at 280 nm was determined on a Beckman DB-G grating spectrophotometer. The pH of each fraction was measured on a Radiometer pH meter, Type pHS 630 T.

Amino Acid Analysis. The amino acid composition of the pure toxin was determined according to the procedure of Moore and Stein (1948). Hydrolysis was carried out at 110° for 24, 48, and 72 hr in constant boiling HCl. The analyses were done on a JEOL amino acid analyzer, Model JLC-6AH. The tryptophan content was analyzed by the spectrophotometric method of Edelhoch (1967) and by alkaline hydrolysis according to the method of Hugli and Moore (1972). Cystine was determined as half-cystine by the method of Crestfield et al. (1963); 2 mg of pure toxin was reduced with mercaptoethanol in 8 *M* urea and subsequently alkylated with iodoacetic acid after alkylation. Excess reagents were separated from protein by a Sephadex G-50 column. The sample was collected, lyophilized, and then hydrolyzed in HCl. The free sulfhydryl groups of the toxin were determined by direct alkylation with iodoacetate in 8 *M* urea. The sample was then hydrolyzed with constant boiling point HCl and the amino acid composition was determined as described before.

Carbohydrate Test of Toxin. Carbohydrate was determined by the method of Winzler (1955); 0.1 ml of toxin (1 mg/ml) was added to 1 ml of 0.1% orcinol in 70% H_2SO_4, then heated at 100° for 20 min.

Results

Purification of Toxin. Isolation of pure toxin was achieved in two steps using Sephadex G-50 and CM-cellulose chromatography (Figures 1 and 2). Three toxic fractions were obtained from Sephadex G-50 chromatography. The most toxic fraction (S-IV) exhibited toxicity 4.7 times higher than the original venom (Table I). The most toxic fraction was used for further purification. The seventh fraction (CM-VII) from the CM-cellulose fractionation was much more toxic than any other fraction as indicated by the low value of LD_{50} (Table I). Moreover, fraction CM-VII accounted for most of the protein in the second fractionation step. The LD_{50} value of the pure toxin was 0.044 μg/g indicating that the toxicity increased ten times after the

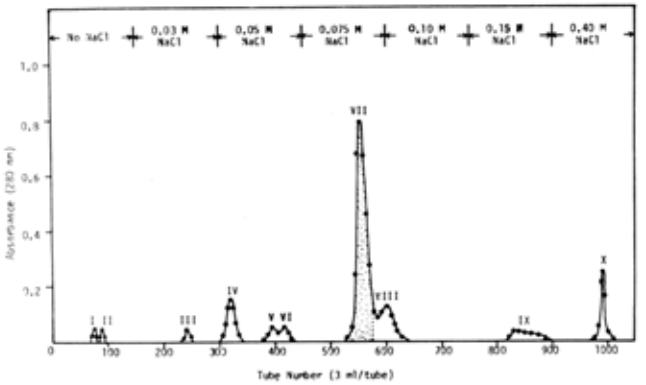

FIGURE 2: Second step fractionation of toxin *Pelamis platurus* venom. The most toxic fraction from the first step fractionation (S-IV) was further purified by using a CM-cellulose column 2.5 × 60 cm. The fractions were eluted with 0.01 *M* potassium phosphate buffer at pH 7.8 with increasing NaCl concentration at a flow rate of 9 ml/hr.

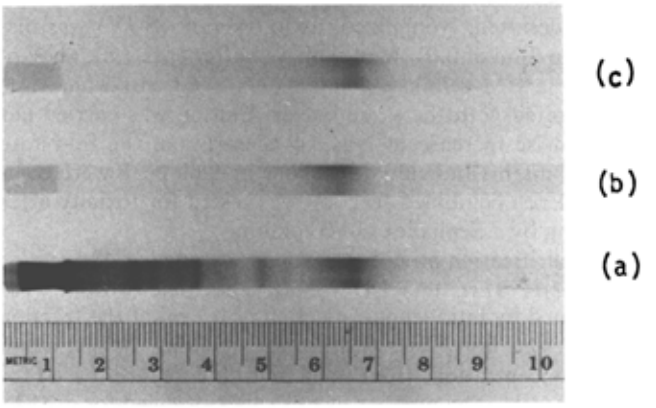

FIGURE 3: Polyacrylamide gel electrophoresis: (a) crude venom; (b) fraction S-IV; (c) fraction CM-VII.

Table I: Isolation of Toxic Principle from *P. platurus* Venom by Sephadex G-50 and CM-Cellulose Column Chromatography.

Fraction	Protein (mg)	Recovery of Protein (%)	Percent Composition Based on Venom (%)	Toxicity Lethal Dosage 50% (μg/g)
Venom	1500			0.435
First fractionation (G-50)[a]				
S-I	690	46.0	46.0	Nontoxic[b]
S-II	139	9.3	9.3	Nontoxic
S-III	14	0.9	0.9	0.75
S-IV	168	11.2	11.2	0.093
S-V	388	25.9	25.9	Nontoxic
S-VI	6	0.4	0.4	0.425
Recovery	1405	93.7		
Second fractionation (CM-cellulose)[c]				
CM-I	2.8	1.7		Nontoxic
CM-II	1.9	1.1		Nontoxic
CM-III	4.0	2.4		Nontoxic
CM-IV (*Pelamis* toxin b)	14.1	8.4	1.0	0.15
CM-V	8.6	5.2		8.4
CM-VI	7.4	4.4		7.4
CM-VII (*Pelamis* toxin a)	68.6	41.1	4.6	0.044
CM-VIII (*Pelamis* toxin c)	24.0	14.4	1.6	0.31
CM-IX	9.6	5.7		0.93
CM-X	14.5	8.7		1.00
Recovery	155.5	93.1		

[a] The Sephadex G-50 column was loaded with 1.5 g of venom. [b] All mice survived after intravenous injection at the concentration of 10 μg/g of body weight of the mouse. [c] The carboxymethylcellulose column was loaded with 167 mg of S-IV fraction from the first fractionation.

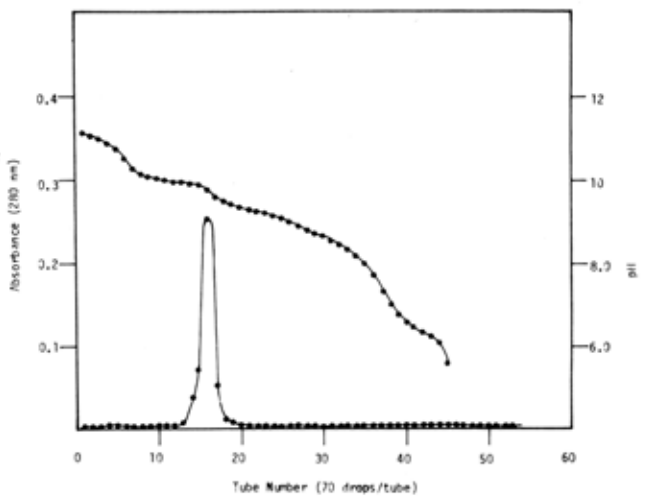

FIGURE 4: Isoelectric focusing profile of purified toxin, *Pelamis* toxin a (fraction, CM-VII).

two-step purification. This major toxic fraction was called *Pelamis* toxin a.

Establishment of Homogeneity. Purity of the toxin was established by acrylamide disc gel electrophoresis and isoelectric focusing in a sucrose gradient. Only one band was observed after electrophoresis of the toxin. Due to the low molecular weight of the toxin, the band was relatively diffuse because the toxin has such a low molecular weight (Figure 3). The isoelectric profile obtained from the isoelectric focusing column indicated one distinct peak with an isoelectric point of 9.69 indicating that the toxin is a highly basic protein (Figure 4).

Amino Acid Composition. Data from amino acid analyses after HCl hydrolysis for 24, 48, and 72 hr are listed in Table II. The values for serine and threonine were obtained by extrapolation to zero time. The toxin contains only 1 mol of tryptophan when determined by the spectrophotometric method or by amino acid analysis after alkaline hydrolysis (Table II). Eight cysteine residues were found in the toxin (Table III). In order to confirm that the toxin did not contain free sulfhydryl groups, the toxin was alkylated without prior reduction and then hydrolyzed in HCl. So *S*-carboxymethylcysteine was observed (Table III). This indicates that all the cysteine residues are in the form of disulfide bridges.

Absence of Carbohydrate. The test for carbohydrate gave negative results indicating that there was no orcinol sensitive carbohydrate in the purified toxin. Thus, it was concluded that the toxin was not a glycoprotein.

Molecular weight of the major toxin was determined by two different methods: amino acid composition and gel filtration. The minimum molecular weight calculated from the amino acid composition (Table II) is 6223, while gel filtration gave a value of approximately 6600.

Stability of Toxin. After incubation at 50° for 30 min,

Table II: Amino Acid Composition of the Major Neurotoxin from *P. platurus* Venom.

Amino Acid	Amino Acid Residues[a]				Nearest Integer
	24 hr[b]		48 hr[b]	72 hr[b]	Corrected
Lysine	5.42	(5.23)[c]	5.47	5.41	5.43	5
Histidine	1.89	(1.99)	1.92	1.86	1.89	2
Arginine	3.05	(3.08)	2.97	3.02	3.01	3
Aspartic acid	5.70	(5.74)	5.93	5.51	5.70	6
Threonine	5.94	(6.06)	5.32	4.63	6.64	7
Serine	4.11	(4.18)	3.52	2.90	4.81	5
Glutamic acid	7.28	(6.80)	6.97	6.54	6.93	7
Proline	1.35	(1.24)	1.37	1.17	1.29	1
Glycine	3.54	(3.29)	3.48	3.33	3.45	3
Alanine	0.97	(0.99)	0.97	0.95	0.96	1
Half-cystine	8.17	(7.87)	8.37	7.63	8.05	8
Valine	1.03	(0.96)	1.03	1.01	1.02	1
Methionine	1.00	(1.03)	0.98	1.04	1.01	1
Isoleucine	1.72	(1.78)	1.77	1.85	1.78	2
Leucine	1.02	(1.03)	1.03	1.02	1.02	1
Tyrosine	0.98	(0.98)	0.99	0.96	0.98	1
Phenylalanine	0.00	(0.00)	0.00	0.00	0.00	0
Tryptophan					0.92[d]	1
					0.97[e]	1
S-Carboxymethylcysteine					8.34[f]	8
Percentage of recovery	99.26		94.20	90.32	94.59	
Total residue number						55

[a] Results obtained after hydrolysis for three different time periods were extrapolated to zero time to obtain values for threonine and serine. Average values for the remaining amino acids are reported. [b] These values were normalized to average molar ratio to alanine, valine, methionine, leucine, and tyrosine. [c] Values in parentheses were obtained from the pure toxin in the second purification. [d] Tryptophan was determined by alkaline hydrolysis for 24 hr. [e] Tryptophan was determined by spectrophotometric methods. [f] These values were determined for cysteine after reduction and alkylation of the toxin followed by Crestfield et al. (1963) method, and by hydrolysis for 24 hr in HCl.

Table III: Amino Acid Compositions of Carboxymethylated Toxin and Reduced, Carboxymethylated Toxin from the Venom of Sea Snake, *P. platurus*.

Amino Acid	Carboxymethylated Toxin	Reduced, Carboxymethylated Toxin
Lysine	5.23	5.33
Histidine	1.87	1.80
Arginine	3.04	3.39
S-Carboxymethylcysteine	0.00	8.34
Aspartic acid	5.79	6.01
Threonine	5.95	5.67
Serine	4.17	4.20
Glutamic acid	7.15	7.11
Proline	1.33	1.36
Glycine	3.44	3.03
Alanine	0.97	0.98
Half-cystine	8.17	0.00
Valine	1.01	1.03
Methionine	1.02	1.07
Isoleucine	1.81	1.82
Leucine	1.04	1.07
Tyrosine	0.96	0.98
Phenylalanine	0.00	0.00

Table IV: Stability of Toxin at Different Temperatures.

Temp (°C)	Dose		
	0.044 μg/g (1 LD_{50})[a]	0.088 μg/g (2 LD_{50})[a]	0.132 μg/g (3 LD_{50})[a]
Room temp	2/4[b]	4/4	4/4
50	0/4	4/4	4/4
75	0/4	0/4	0/4
100	0/4	0/4	0/4

[a] Toxin was incubated at different temperatures for 30 min. Mortality of mice was determined after intravenous injection at each lethal dosage of 20 g body weight of mouse. [b] Values shown are death per 4 mice injected.

the toxin still retained toxicity. All experimental animals died (Table IV) at a dose of toxin which was two times the LD_{50} value when the incubation was carried out at 50°. However, at 75°, the toxin was detoxified.

The toxicities as expressed by the LD_{50} values were 0.083, 0.080, 0.078, 0.063, and 0.080, respectively, at pH 2, 4, 6, 8, and 10. Thus, the toxin was stable in both acidic and basic solutions.

Importance of Disulfide Bonds for Toxic Action. From amino acid composition of the toxin (Table II), it is clear that all eight sulfur atoms in the toxin are used for four disulfide bonds. In order to determine the importance of the disulfide bonds, the toxin was reduced with mercaptoethanol and subsequently alkylated. The amino acid composition of the reduced and alkylated toxin indicated all cystines were converted to *S*-carboxymethylcysteine (Table III). Carboxymethylated toxin, even at very high doses, was nontoxic. Five mice each received a 10 μg/g (230 LD_{50}) dose of toxin intravenously. Since none of the mice died, the experiment indicates that disulfide bonds are essential for toxic action.

Discussion

From Figure 2 and Table I, it is clear that *P. platurus* venom contains more than one toxic fraction. The most toxic fraction (*Pelamis* toxin a) was ten times more toxic than the original venom. The venom also contained other toxins with higher toxicity than the original venom. Toxin b was three times more toxic and toxin c was 1.4 times more toxic than crude venom even though they may have been impure. The major toxin, *Pelamis* toxin a, comprised 4.5% of the venom. The percentages for toxin b and c were 0.95

Table V: Comparison of Amino Acid Composition of *Pelamis* Toxin a to Those of Other Sea Snake (Family: Hydrophiidae) Toxins.

Subfamily	Hydrophiinae	Hydrophiinae	Laticaudinae	
Genus and species	*Pelamis platurus*	*Lapemis hardwickii*	*Laticauda semifasciata*	
Geographical origin	Costa Rica	Thailand	Philippines	
			Toxin a	Toxin b
Lysine	5	5	4	5
Histidine	2	2	1	1
Arginine	3	3	3	5
Aspartic acid	6	6	5	4
Threonine	7	8	6	5
Serine	5	6	7	6
Glutamic acid	7	8	8	8
Proline	1	3	4	4
Glycine	5	4	5	6
Alanine	1	1	1	1
Half-cystine	8	8	8	8
Valine	1	1	2	3
Methionine	1	1	1	1
Isoleucine	2	2	4	4
Leucine	1	1	1	1
Tyrosine	1	1	1	1
Phenylalanine	0	0	2	2
Tryptophan	1	1	0	1
Total residue	55	61	62	61

and 1.6%, respectively. Therefore, *Pelamis* toxin a was not only the most toxic but also the major toxin from the view point of composition. It is an interesting observation that all pure toxins have comparable LD_{50} values, ranging from 0.04 to 0.085 for the subfamily of Hydrophiinae (Tu, 1974). The low LD_{50} values indicate that all sea snake toxins are extremely toxic.

The isoelectric point of *Pelamis* toxin was 9.69 which is comparable to those reported of other sea snake toxins. The following values have been reported for pure toxins from different sea snake venoms: 9.2 for the major toxin of *Enhydrina schistosa* (Tu and Toom, 1971), 9.9 for *Lapemis hardwickii* major toxin (Tu and Hong, 1971), and 9.2 and 9.3 for two toxins obtained from *Laticauda semifasciata* (Tu et al., 1971).

The total of 55 amino acid residues for the *Pelamis* toxin a is a low value when compared to most Type I neurotoxins (Tu, 1973). However, the amino acid composition is similar to that of *Lapemis hardwickii* (Table V) even though the *L. hardwickii* toxin has 61 amino acids. There are two subfamilies (Hydrophiinae and Laticaudinae) in the family of sea snakes (family, Hydrophiidae). This similarity of *Pelamis* toxin a to the *Lapemis hardwickii* toxin is reasonable since they belong to the same subfamily of Hydrophiinae. Yet, it is fascinating that two snakes, separated geographically by such a distance, have major toxins with a very similar amino acid composition, molecular weight, and isoelectric point. *Pelamis platurus* was captured on the Central American coast, while *Lapemis hardwickii* was obtained in the Gulf of Thailand. The amino acid composition of *P. platurus* major toxin differs somewhat from the toxins of *Laticauda semifasciata* which belongs to the different subfamily of Laticaudinae (Table V).

Structure function relationships in sea snake toxins have been studied extensively by a number of workers. The importance of tryptophan and tyrosine residues in sea snake toxins has been demonstrated. The absence of toxicity in the alkylated *Pelamis* toxin a indicated that the native compact structure with four disulfide bonds was essential for toxicity. Similar results were obtained with the venom of *Naja naja atra* (Formosan cobra). Yang (1965, 1967) reduced the toxin with β-mercaptoethanol or oxidized it with performic acid and observed that toxicity was lost.

From the data of amino acid composition, molecular weight, isoelectric point, and chemical modification, it is apparent that *P. platurus* major toxin is not only very similar to toxins of other sea snakes, but also to those of cobra venom toxins. The Type I neurotoxins have molecular weights of 6700 to 7000, while those of Type II are about 7800 (Tu, 1973). Most of the cobra (genus, *Naja*) venoms and krait (genus, *Bungarus*) venoms contain both Type I and Type II toxins. However, at this point in time all reports indicate sea snake venoms contain only Type I toxins. Since Type I toxins are of somewhat simpler molecules than Type II toxins, it has been proposed that the former are more primitive molecules from the viewpoint of molecular evolution (Tu, 1973). Based on this assumption, sea snakes are more primitive forms of snakes than terrestrial venomous snakes.

Addendum

After this manuscript was submitted, Liu et al. (1975) reported the isolation of a toxin from the venom of *Pelamis platurus* captured in Formosan waters. The amino acid composition of the toxin of this manuscript and that reported by Liu et al. are similar.

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Hydrogen–Tritium Exchange Kinetics of Soybean Trypsin Inhibitor (Kunitz). Solvent Accessibility in the Folded Conformation†

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ABSTRACT: The hydrogen exchange kinetics of Kunitz soybean trypsin inhibitor (STI) has been studied at pH 2, 3, and 6.5. From the temperature dependence of proton exchange at low pH, the contribution of major, reversible protein unfolding to the hydrogen exchange kinetics has been determined. Exchange directly from the folded conformation is characterized by an apparent activation energy (E^*_{app}) of approximately 25 kcal/mol, close to that of the chemical exchange step. At pH 6.5 the protein is more temperature stable than at low pH, and exchange of all but $\simeq$8 protons can be observed to exchange with $E^*_{app} \simeq 27$ kcal/mol. This implies that all but $\simeq$8 protons are accessible to exchange with solvent in the solution structure of folded STI. Estimates can be made of the average number of water molecules per molecule of STI consistent with a solvent accessibility model of hydrogen exchange kinetics. These estimates indicate that very few water molecules within the protein matrix are necessary to explain the exchange data. Calculations are done for the STI hydrogen exchange kinetics at pH 3, 30°, approximating STI structure by a sphere of radius = 18 Å. These calculations indicate an average of $\simeq$4 water molecules in the shell from 13 to 16 Å from the center of the molecule, while <1 water molecule is indicated in the innermost 13 Å. These calculations also suggest that there are $\simeq$190 water molecules associated with the outermost 1.5–2 Å of the sphere. While these values are consistent with a hydrophobic region in the central protein matrix, they indicate more solvent accessibility in the outer 1/3 of the molecule than the static accessibility estimates made from X-ray coordinates. Our results suggest that any protein movements or fluctuations responsible for solvent accessibility in proton exchange processes are localized in the outer regions of the globular structure.

Solvent accessibility of buried groups in folded proteins is fundamentally related to the dynamic behavior of native proteins in solution and to the solvent contribution to the driving force of protein folding.

In protein tritium–hydrogen exchange experiments, the back exchange kinetics of tritium-labeled peptide amide protons with solvent is measured. It has long been recognized that proton exchange in native proteins is many orders of magnitude slower than that in the unfolded conformation (Linderstrøm-Lang, 1955). In analyzing the processes responsible for the slowed exchange in the folded conformation, we have determined the contribution of major unfolding transitions to the overall exchange rates of native proteins (Woodward and Rosenberg, 1971a,b; Woodward et al., 1975a). From this, conditions for exchange only from the folded conformation have been characterized (Woodward et al., 1975a).

We have interpreted hydrogen exchange kinetics of proteins in the folded conformation in terms of solvent accessibility, rather than in terms of hydrogen bond breakage, per se (Woodward et al., 1975a). That is, the number of slowly exchanging protons in a folded protein is equal to the number with restricted solvent accessibility which may, or may not, be intramolecularly hydrogen bonded. The mechanism of solvent exposure may, and probably does, involve fluctuations of the protein, but these fluctuations need not involve H-bond breakage. It appears to us unlikely that the mechanism of protein fluctuations which accounts for solvent accessibility is a "breathing" process involving reversible, localized denaturations, accompanied by the breakage of several hydrogen bonds, because denaturants such as ethanol and urea do not affect the exchange rates (see below, and Woodward et al., 1975a,b).

We find that under conditions in which exchange occurs only from folded soybean trypsin inhibitor (STI),[1] all but

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[1] Abbreviation used is: STI, soybean trypsin inhibitor.