AD-754 455

FINAL REPORT, NOVEMBER 1, 1966 TO OCTOBER 31, 1970

Anthony T. Tu

Colorado State University

Prepared for:

Office of Naval Research

31 October 1970

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

AD754455

FINAL REPORT

ONR CONTRACT: N00014-67A-0299-0005

PERIOD: November 1, 1966 to October 31, 1970

PRINCIPAL INVESTIGATOR: Anthony T. Tu
Professor of Biochemistry
Colorado State University
Fort Collins, Colorado 80521

16

I. OBJECTIVE

1. To collect sea snake venoms.

2. To investigate chemical properties of sea snake venoms and pure toxins from these venoms.

II. ACCOMPLISHMENTS

A. Sea Snake Collecting

In 1967, sea snakes were collected in Japan, Formosa, Hong Kong, Thailand, Malaysia, and the Philippines, and a total of 2,600 sea snakes were captured In 1969, we captured over 7,000 additional sea snakes in Thailand, Malaysia, and the Philippines.

1. Thailand

The 1967 collecting period was July 1 to July 10. The number of snakes captured were: 68 Lapemis hardwickii, 11 Enhydrina schistosa, 146 Aipysurus eydouxi, 1 Pelamis platurus, Hydrophis cyanocinctus (data missing), Hydrophis spiralis (data missing), H. klossi (data missing), Kerilia jerdoni (data missing), Microcephelophis gracilis (data missing), and 50 unknown sea snakes. Some false sea snake, Acrochordus granulatus were captured. Acrochordus granulatus is a non-poisonous snake and, therefore, lacks any fangs. It is reported that they are found in rivers and estuaries. However, we found many of A. granulatus in the open sea in the Gulf of Thailand, both in 1967 and 1969. Aipsysurus eydouxi was the predominant species during the 10 day period. Lapemis hardwickii was the second most abundant sea snake in the 1967 collection.

Enhydrina schistosa is commonly found in the estuary in central Thailand and the snake moves upstream with the tidal influx of sea water. In the vicinity of the Bangkok area, E. schistosa is most abundant during the dry season (December to April), when sea water penetration is greatest due to the low water level of the rivers. In the Bangkok area, sea water can reach to Phra Pra Dang, which is 3 miles south of Bangkok, or about 10 miles from the river mouth. Fishermen there, have reported catches of about 1000 sea snakes per night during the dry season. An attempt was made to collect the sea snakes in the same spot in June 1967, but only two small E. schistosa and three A. granulatus were caught in one day.

In 1969, the collecting was made about 10 miles off the east coast of the Kra Isthmus with trawling nets attached to trawlers. Species, and number of sea snakes captured are summarized in Table 1. The data obtained from the 1969 collecting are statistically more significant than that of 1967, as a longer period of time was spent for the collection, which was from July 3 to August 13, 1969. Since a large number of sea snakes were captured, the distribution of each species can be fairly accurately projected for the summer period. Of the 5,306 sea snakes captured, 4,365 were Lapemis hardwickii, which accounted for 82 per cent of all the snakes. The next common species was Aipysurus eydouxi, which accounted for 4.5 per cent. Again large numbers of Acrochordus granulatus, (186) were captured in the open sea. This is the only nonpoisonous snake captured in the sea. Contrary to the reported record, A. granulatus can live in salt water. It may be that the snake possesses a salt gland so that it can survive in a high tonic environment.

Pelamis platurus is very rare in the Gulf of Thailand. Out of a total of nearly 6,000 sea snakes captured in 1967 and 1969 in Thailand, only one was P. platurus. Contrary to the report of the Royal Thai Navy, no genus of Laticauda was found in Thailand. Sea Snakes reported to be Found in Thailand and by the Royal Thai Navy are: Lapemis hardwickii, L. curtus, Enhydrina schistosa, Thalass-sphis viperina, Astrotia stokesii, Microcephalophis gracilis, Kerilia jerdonii, Pelamis platurus, Laticauda laticaudata affinis, L. colubrina, L. semifasciata, Emydocephalus imimae, Hydrophis spiralis, H. cyanocinctus, H. ornatus, and H. melanocephalus. The sea snakes in the Gulf of Thailand reported by Taylor are: Laticauda colubrina, L. laticaudata, Aipysurus eydouxi, Kerilia jerdoni, K. jerdoni siamensis, Astrotia stokesii, Kolpophis annandalei, Thalassophis viperina, Enhydrina schistosa, Pelamis platurus, Lapemis hardwickii, Hydrophis cyanocinctus, H. ornatus, H. caerulescens, H. torquatus, H. torquatus diadema, H. kosii, H. fasciatus, H. brooki, and H. mamillaris.

2. Philippines

In 1967 and 1969, we collected sea snakes inside the caves of Gato Island. Inside the caves, there was a large number of sea snakes on the surface of the water in rock crevices in the water, and on the rock. Sea snakes were captured by skin diving inside the caves. There were two species in this area. One was Laticauda semifasciata and the other was Laticauda colubrina. 800 L. semifasciata were captured in 1967 and 600 in 1969.

Sea snakes present in the sea of Philippines reported by Taylor are: Aipysurus eydouxi, Laticauda laticaudata, Laticauda colubrina, L. semifasciata, Hydrophis fasciatus, H. ornatus, H. cyanocinctus, H. ornatus, Lapemis hardwickii, and Pelamis platurus. Specimens in the National Museum, Manila, observed by the author, were Hydrophis fasciatus, H. spiralis, Pelamis platurus, and Lapemis hardwickii. Sea snakes identified from the specimens collected by Alaban Serum and Vaccine Laboratories were Lapemis hardwickii, Hydrophis fasciatus atriceps.

3. Malaysia

In Malaysia, sea snakes were captured in the Strait of Malacca, near Penang Island by setting fish traps, which were immersed in the water for 12 hours. Usually, at least one snake was caught in each trap. The variety of sea snakes captured was very similar to the ones we obtained in Thailand.

Sea snakes present in the coastal water of Sarawak (in Borneo) are: Laticauda laticaudatus, L. colubrina, Aipysurus eydouxi, Kerilia jerdoni, Enhydrina schistosa, Hydrophis cyanocinctus, H. spiralis, H. melanosoma, H. caerulescens, H. torquatus, H. brookei, H. fasciatus, Thalassophis anomalus, Lapemis hardwickii, Microcephalophis gracillis, Pelamis platurus, and Praescutata viperina.

4. Other Areas

The most common sea snake in the vicinity of Hong Kong is *Hydrophis cyanocinctus*, which accounts for 70% of all the sea snakes captured by fishermen in Hong Kong. Other sea snakes frequently captured are: *Hydrophis ornatus ornatus*, *Microcephalophis gracilis*, *Pelamis platurus* and *Praescutata viperina*.

In the summer of 1967 collection, *Hydrophis cyanocinctus*, *H. ornatus*, *Pelamis platurus*, *Microcephalophis gracilis*, *Lapemis hardwickii*, and one unknown species were captured.

In 1967, we collected 600 Pelamis platurus from the northern coast of Formosa. In southwestern Formosa, we obtained a large number of genus Hydrophis, however, no venom was extracted as there were too many different species and subspecies within this genus, which made proper identification very difficult.

In Amami Island, Japan, we obtained 500 *Laticauda semifasciata*. Comparison to those of Philippine origin is made later in this manuscript.

B. Toxicology

In general, venoms of sea snakes are more toxic than those of land snakes. The LD_{50} in mice by I.V. is listed in Table 2.

The quantity of venom that can be obtained from sea snakes is much smaller than the amount that can be obtained from land snakes. The yield of venom from sea snakes collected was from 0.6 to 19 mg per snake.

C. Chemistry

1. Comparison of Venom from Sea Snake and Land Snake

Recent studies have shown that venom from the snakes of the family Hydrophiidae are much simpler in composition than the venom of the land snake.

Isoelectric points of sea snake venom toxins are very basic; they are all around or above 9, while that of a land snake, *A. rhodostoma*, has an isoelectric point of about 7.0.

2. Isolation

Since all snake venoms contain a rather large number of proteins, purification is achieved by more than two step column chromatography. Toxins were isolated either using the combination of Sephadex G-50 and CM-cellulose Chromatography or repeating use of CM-cellulose with a different buffer. Venoms used for isolation were *Lapemis hardwickii* from Thailand, *Laticauda semifasciata* from the Philippines, and *Enhydrina schistosa* from Malaysia. Phospholipase A was isolated from venom of *L. semifasciata* from Amami Island, Japan, by a two step purification utilizing CM-cellulose and DEAE column chromatography.

3. Criteria of Purity

In each preparation, 3 or 4 of the following methods were used to confirm the purity of isolated toxins or the enzyme.

a. Sedimentation pattern in analytical ultracentrifuge.

b. Polyacetate electrophoresis at different pH values.

c. Isoelectric focusing.

d. Rechromatography in column.

e. Straight line in the plot of log C against r^2 in sedimentation equilibrium.

f. Crystallization

4. Physical and Chemical Properties

Physical and chemical properties of isolated toxins and phospholipase A are summarized in Table 3.

The toxins contain a total amino acid residue of either 61 or 62. The phospholipase A consists of 108 amino acid residues.

Molecular weights of toxins and phospholipase A were determined by a combination of s and D, data from sedimentation equilibrium, amino acid composition, or quantitative Sephadex elution method. These results are summarized in Table 4.

End group analysis indicated that there is a histidine at the amino-terminal and aspartic acid or asparagine at the carboxy-terminal for the toxin of Lapemis hardwickii venom. For toxins a and b of Laticauda semifasciata venom from the Philippines, the amino-terminal is arginine and the c-terminal is aspartic acid (asparagine).

Amino acid compositions of purified toxins and phospholipase A are summarized in Table 5. All toxins contain 8 moles cysteine, 8 moles glutamic acid, 1 mole each of tryptophan, leucine, and tyrosine. It is remarkable that there is a common number of residues for certain amino acids regardless of geographical origin. Other amono acid compositions are also remarkably similar. Only the toxin from Lapemis hardwickii contains methionine. Amino acid composition of phospholipase A is quite different from those of other toxins.

5. Chemical Modification

The trypiophan residue was chemically modified using a specific reagent, N-bromosuccinimide, on toxins isolated from the venoms of Laticauda semifasciata, Enhydrina schistosa, and Lapemis hardwickii. These toxins contain only one mole of tryptophan residue. After the modification, the toxicity disappeared completely. Two other reagents, 2-nitrophenylsulfenyl chloride and 2-hydroxyl-5-nitrobenzylbromide, were used for the modification of tryptophan residue of the toxin isolated from the venom of Lapemis hardwickii. Toxicity of the toxin disappeared agian on modification. It is thus concluded that the tryptophan residue is important for toxic action.

In contrast to tryptophan, the modification of the majority of raginine and lysine residues did not alter the toxicities of toxin a and b of Laticauda semifasciata venom. The result of chemical modification of sea snake venom toxins is summarized in Table 6.

6. Absence of Enzyme in Purified Toxins

The venom of E. schistosa contains a number of enzymes. Sixteen substrates were used to test various enzyme activities. The venom shows the following enzyme activities: clotting activity, hyaluronidase, alkaline phosphatase, phosphodiesterase, deoxyribonuclease, acetylcholinersterase, and leucine aminopeptidase. However, the venom does not contain such enzyme activities as ribonuclease, acid phosphatase, amino acid esterase (with N-benzoyl-L-arginine ethyl ester, N-benzoyl-L-tyrosine ethyl ester, p-toluenesulfonly-L-arginine methyl ester, and acetyl-L-tyrosine ethyl ester as substrates), and proteases (with casein and hemoglobin as substrates). NOne of the above enzymes are found in purified toxins.

7. Properties of Phospholipase A

By using ovolecithin of known composition in the 1 and 2 positions, the enzyme is shown to be specific for the 2 position liberating mainly unsaturated fatty acids.

Of the substrates tested, only phosphatidycholine was hydrolyzed. Of the two phosphatidycholines tested, ovolecthin was hydrolyzed at a much more rapid rate than the synthetic lecithin containing only the saturated fatty acid, palmitic acid. All other substrates tested, namely phosphatidyl ethanolamine, phosphatidyl-L-serine, phospharidyl inositide, phosphatidic acid, lysolecithin, sphingomyelin, cerebromide, and cardiolipin were not hydrolyzed. The enzyme was most active at pH 8.0, and at temperatures between 35 and 40°. The activation energy calculated from Arrhenius plot is 6,900 cal per mole.

The enzyme exhibited hemolytic activity which was greatly intensified by the addition of lecithin. The purified phospholipase A was nontoxic, nonhemorrhagic, and exhibited only slight myolytic activity. It was found that phospholipase A ev en in presence of ovolecithin had very lettle effect on the mouse embryo cells in tissue cultures.

D. Immunology

Neutralization capacity of commerical antivenin (Commonwealth Serum Laboratories, Melbourne, Australia) *in vitro* was tested against homologous and heterologous venoms. The antivenin was not only effective for homologous venom, but it also evfectively neutralized 3 heterologous venoms tested. One ml of serum neutralized 176 times the LD_{50} value for its own venom, 160 LD_{50} value for *Pelamis platurus* venom from Formosa, 120 LD_{50} value for the venoms of *Hydrophis cyanocinctus* from Malaya and *Lapemis hardwickii* from Thailand.

Table 1

Record of Sea Snake Collecting in Thailand

From July 3 to August 13, 1969

Snake	Number
Lapemis hardwickii	4305
Aipysurus eydouxi	146
Hydrophis cyanocinctus	92
Hydrophis ornatus	73
Enhydrina schistosa	73
Kerilia jerdonii siamensis	55
Praescutata viperina	99
Microcephalophis gracilis	16
Thalassophis anomalus	4
Unknown	165
Acrochordus granulatus (non-poisonous)	186
TOTAL	5306

Table 2

Yield and Toxicity of Sea Snake Venoms

Venom	Origin	Yield (mg/snake)	LD_{50} (µg/g)	Year Collected
Aipysurus eyedouxi	Thailand	0.6	> 4	1967
Enhydrina schistosa	Malaya	----	0.90	1967
			0.98	1969
	Thailand	8.1	0.14	1967
		14.0	0.21	1969
Hydrophis cyanocinctus	Hong Kong	2.1	-----	1967
	Malaya	----	0.35	1967
	Thailand	18.0	-- --	1969
H. ornatus	Thailand	19.0	2.2	1969
Lapemis hardwickii		5.2	0.71	1967
		2.4	1.40	1969
Laticauda semifasciata	Japan	7.10	0.28	1967
	Philippines	16.0	0.28	1967
		19:0	0.45	1969
Pelamis platurus	Formosa	2.0	0.18	1967
		9.3	0.28	1968

Table 3

Physicochemical Properties of Sea Snake

Venom Toxins and Phospholipase A

	Enhydrina schistosa[a] (Malaysia)	*Lapemis hardwickii*[b] (Thailand)	*Laticauda semifasciata*[a] (Philippines)		*Laticauda semifasciata*[a] (Japan)
			Toxin		
	Toxin	Toxin	a	b	Phospholipase A
Isoelectric Point	9.20	9.85	9.15	9.34	6.70
Sedimentation Coefficient ($s^{o}_{20,w}$)	1.4	1.13	1.52	1.43	1.93
Diffusion Coefficient ($D^{o}_{20,w}$ cm^2/sec)	15.5×10^{-7}	13.7×10^{-7}			14.1×10^{-7}
Parital specific volume	0.70	0.70	0.71	0.71	0.71
Amino terminal		His	Arg	Arg	
Carboxy terminal		Glu	Asp	Asp	
Amino Acid Residue	61	62	62	61	108

[a] venom was collected in 1967.
[b] venom was collected in 1969.

Table 4

Molecular Weight of Sea Snake Venom

Toxins and Phospholipase A

Sea Snake	Origin	amino acid composition	S and D	Sedimentation equilibrium	Gel filtration	Reference
Lapemis hardwickii toxin	Gulf of Thailand	6774	6800	6800		Tu and Hong, 1971
E. schistosa toxin	Strait of Malacca	6878	7300			Tu and Toom, 1971
Laticauda semifasciata	Gato Island Philippines					Tu, Hong and Solie, 1971
Toxin a		6840		6800	6600	
Toxin b		6677		6500	6400	
Laticauda semifasciata Phospholipase A	Amami Island, Japan		11000		10700 10400	Tu, Passey and Toom, 1970

Table 5

Amino Acid Composition of Sea Snake

Venom Toxins and Phospholipase A

	Lapemis hardwickii	*E. schistosa*	*Laticauda semifasciata* (Philippines)		*Laticauda semifasciata* (Japan)
	Toxin	Toxin	Toxin a	Toxin b	Phospholipase A
Lysine	5	5	4	5	7
Histidine	2	2	1	1	2
Arginine	3	3	3	2	4
Aspartic acid	6	6	5	4	11
Threonine	8	8	6	5	6
Serine	6	6	7	6	7
Glutamic acid	8	8	8	8	9
Proline	3	3	4	4	5
Glycine	4	5	5	6	10
Alanine	1	1	0	0	8
Valine	1	1	2	3	4
Methionine	1	0	0	0	1
Isoleucine	2	2	4	4	3
Leucine	1	1	1	1	5
Tyrosine	1	1	1	1	10
Phenylalanine	0	0	2	2	3
Half-cystine	8	9*	8	8	12
Tryptophan	1	1	1	1	1
Total Residue	61	62*	62	61	108

* Nine residues of cysteine and a total residue of 62 were found by a calculation based on the average molar ration to leucine, alanine, and valine. Eight residues for cysteine and 61 total 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.

Table 6

Summary of Chemical Modifications

Toxin	Amino Acid	Reagent	Toxicity	Amino Acid Residue Before Modification	After Modification	Number of Amino Acid Residue Modified
<u>Laticauda semifasciata</u> (Philippines)						
Toxin a	Tryptophan	N-bromosuccinimide	-	1	0	1
	Arginine	1,2-cyclohexanedione	+	3	2	1
	Lysine	O-methylisourea	+	4	1	3
Toxin b	Tryptophan	N-bromosuccinimide	-	1	0	1
	Arginine	1,2-cyclohexanedione	+	2	1	1
	Lysine	O-methylisourea	+	5	1	4
<u>Enhydrina schistosa</u> (Malaysia)	Tryptophan	N-bromosuccinimide	-	1	0	1
<u>Lapemis hardwickii</u> (Thailand)	Tryptophan	2-nitrophenylsulfenyl chloride	-	1	0	1
		2-hydroxy-5-nitrobenzylbromide	-	1	0	1
		N-bromosuccinimide	-	1	0	1

+ = Toxic
- = Non-toxic