BIOCHEMICAL CHARACTERIZATION OF SOME TOXINS FROM *CONUS STRIATUS* VENOM

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ABSTRACT

Crude venom from *C. striatus* was found to have an LD_{50} of 8.5 μg protein per gram mouse injected intraperitoneally and an LD_{50} of 0.8 μg protein per gram carp injected intramuscularly. The minimum paralysis and death times for mice were estimated to be 1.9 and 4.8 min and for carps 0.5 and 3.5 min, respectively.

Using fish for bioassay, three toxins were resolved from the venom extract by phosphocellulose chromatography. Lethal peaks eluting at 0.01M, 0.2M and 0.27M of N-ethylmorpholine acetate buffer which accounted for 7%, 41% and 52% of total activity recovered from the column were designated as Toxins I, II and III respectively. Toxin I appears to be a small acidic peptide ($M_r < 1500$). On the other hand, Toxins II and III behaved as basic polypeptides with molecular weights of around 10,000-14,000 daltons. All toxins were found to be sensitive to heat and β-mercaptoethanol.

INTRODUCTION

Human injuries and fatalities caused by the marine snail *Conus* have been attributed mainly to fish eating species particularly *C. geographus* (1, 2). *Conus striatus* being piscivorous is also potentially dangerous to man and other vertebrates. In mice intraperitoneal injection of *C. striatus* venom has been observed by Kohn (3) to cause tonic spasm, ataxia, dyspnea hyper-excitability, violent scratching movements and partial paralysis prior to death. In fish the symptoms observed preceding death are color change, ataxia, convulsions or quivering and partial paralysis.

Pharmacologic studies on the action of *C. striatus* venom on muscle and nerve preparations from vertebrates have been done by Endean et al. (4, 5, 6), Freeman et al. (7), Kobayashi et al. (8) and Hanin et al. (9). Two toxins from the venom have been described by Strichartz et al. (10); one peptide toxin (M_r 7,000) produces a slow-decaying plateau after an action potential, and another component (which seems to have a higher molecular weight) produces repetitive firing in response to a single stimulus applied to

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frog sciatic nerve. Recently, Kobayashi et al. (11) isolated a cardiotonic glycoprotein (M_r 25,000) from the venom but failed to detect the 7,000-dalton peptide with their assay system (measurement of inotropic effect on guinea pig atria).

In this report, we describe the detection of three different toxins using fish as assay animal. Although two of the toxins may be similar at least in size to those previously described (10, 11), the low molecular weight toxin we found is certainly a novel one.

MATERIALS AND METHODS

Materials

Specimens of *C. striatus* were collected from around Marinduque Island. They were kept alive in well-aerated salt water aquaria and fed with anchovies twice a week. The mollusc was first immobilized by burying it in ice for 30 minutes before it was pulled out of the shell. The venom apparatus was dissected out and venom was extracted from the duct as previously described (12). The crude venom obtained was either used fresh or lyophilized before storage in a freezer. Animals used for bioassay of venom extracts and fractions were ten-gram mice of Japanese sDDy strain and two-gram fresh water carps, *Cyprinus carpio.*

Ion exchangers, molecular weight standards and enzymes were obtained from Sigma Chemical Company. N-ethylmorpholine (NEM) came from Pierce Chemical Company. All other chemicals were of reagent grade.

Protein Determination

The protein content of venom samples and fractions were determined according to the method of Lowry et al. (13) using bovine plasma albumin as standard.

Bioassay

The method described by Cruz (14) for testing biological activity of venom extracts of *C. geographus* on mice was adapted for *C. striatus.* Mice were starved for six hours prior to injection.

For bioassay of toxins using carps, the fish (about 2 g) was first taken out of water then laid flat on a watch glass or petri dish. Venom suspension or fractions resuspended in NSS were injected intramuscularly on the dorsal side taking care not to insert the needle beyond the 2-mm mark from the tip so as not to hit the main nerve or blood vessel. Each fish was placed in individual jars filled with aerated tap water. Symptoms after injection were observed. The time of onset of partial paralysis and the time of death were noted. Death time was taken when movement of the operculum was no

longer evident and when the fish did not respond to stimulation by a rod.

The standard dose-response curves were plotted as described by Cruz (14). For the determination of LD_{50}, the procedure of Miller and Tainter (15) was used.

Cellulose Phosphate Chromatography

All sample preparations and chromatographic operations were done over ice or at 4° to $10^{o}C$ in a cold room. Crude venom weighing 0.5 to 0.7 g was extracted 3 times by dispersing and soaking for 5 min either in 0.01M NH_4Ac, pH 8.5 or 0.01M NEMAc, pH 8.5 followed by centrifugation at 10,000 rpm in a Sorvall RC2-B centrifuge (SS-34 rotor). A sonicator (Branson Cell Disruptor, Model W 185D) was used for the second and third dispersions. The supernates were combined and applied on a phosphocellulose column (2.2 x 17 to 22 cm) preequilibrated with the extraction buffer. This was eluted with 150 ml of the same buffer, followed by a linear concentration gradient with 0.01M and 1.5M NH_4Ac or NEMAc, pH 8.5 as limiting buffers.

Sephadex G-50 Chromatography

Toxic fractions corresponding to a peak from the phosphocellulose column were pooled, concentrated down to 5 ml by freeze-drying, applied on a 1.3 x 36 cm column of Sephadex G-50 then eluted with 2 bed volumes of 0.1M NEMAc, pH 8.5 at a flow rate of 1.0 ml per 5 min under a hydrostatic pressure of 38 cm at $10^{o}C$. The toxicity and protein content of fractions were determined.

Digestion of Toxins with Pronase

One-ml aliquots of the eluates exhibiting the highest toxicity from fractions A and B were lyophilized in several Eppendorf tubes then taken up in 15 μl of pronase solution (15 mg/ml in 0.01M phosphate buffer, pH 7.4) or 15 μl of the buffer as shown in Table 1. All samples were incubated at $37^{o}C$ for 2 hours then 15 μl more of the enzyme solution or buffer were added and the mixtures were incubated for 2 more hours. The samples were diluted with 10 μl distilled water and 10-μl aliquots were used for bioassay.

Reduction of Toxins with β-Mercaptoethanol

Aliquots (0.2 ml) from 5 tubes of fractions A and B were pooled in Eppendorf tubes then lyophilized and taken up in 20 μl of 0.1M. Tris buffer, pH 8.5 or 20 μl of 1.9M β-mercaptoethanol solution in the same buffer. The mixtures were diluted with 20 μl of water then incubated either at room temperature or $50^{o}C$ as indicated in Table 2 for 4 hours. For bioassay, 10 μl aliquots of the incubated mixture were injected to 2-g carps.

Disc Gel Electrophoresis

Analytical disc gel electrophoresis was done according to the method described in the Canalco Instruction Manual (16) for Research Disc Standard (RDS) gels.

RESULTS

Toxicity of *C. striatus* Venom

An LD_{50} of 8.5 μg protein per gram mouse was obtained when *C. striatus* venom was injected intraperitoneally. This agrees very well with the LD_{50} reported by Kohn et al. (3) for the same species. Comparison with reported toxicities for other cones reveal that *C. geographus,* the most dangerous species, is six times more toxic than *C. striatus.* Using fish as assay animal, a much smaller dose of *C. striatus* venom is required for lethality. The LD_{50} for carp is 0.8 μg protein per gram body weight, which is ten times less than the LD_{50} for mice.

Figure 1 shows the linear relationship between the reciprocal of dose and time of onset of partial paralysis and death. The minimum paralysis and death times obtained for mice were 1.8 and 4.8 min and for carps 0.5 and 3.5 respectively, thus confirming the greater sensitivity of fish to *C. striatus* venom. For this reason, fish instead of mice were used for locating active fractions from a chromatographic run. A toxicity unit was arbitrarily defined as the amount of toxin injected per gram carp which will cause partial paralysis in 2.2 min and death in 37 min. The times of onset of symptoms of paralysis and death were replotted versus dose expressed in terms of units of toxicity. All subsequent determinations of toxic activity were based on this standard curve.

Partial Purification of Toxins

The degree of separation and the relative amounts of toxins varied from one preparation to another when chromatographed on phosphocellulose. The best resolution was obtained under conditions indicated in Figure 2. Three major toxic peaks designated as I, II and III (which eluted at 0.01M, 0.2M and 0.27M NEMAc) accounted for 7%, 41% and 52% respectively of the total activity recovered from the column. For this reason Toxins II and III were taken together and designated as fraction B in all characterization experiments. The first active component, Toxin I was labeled as fraction A.

Sensitivity of Toxins to Proteases

Fractions A and B from the phosphocellulose column were treated with pronase or incubated with buffer as shown in Table 1. Carps injected

Table 1. Sensitivity of Toxins to Pronase

Sample	Toxin*	Pronase soln µl	Phosphate buffer µl	Observation
Pronase control	–	15 + 15	–	all normal
Fraction A pronase digest	+	15 + 15	–	all normal
Fraction A control	+	–	15 + 15	Symptoms observed were angular swimming, swirling and lateral repose. Average death time = 41.9 min
Fraction B pronase digest	+	15 + 15	–	all normal
Fraction B control	+	–	15 + 15	Symptoms observed were similar to those listed above. Average death time = 108.3 min

**Aliquots of Fractions A and B were lyophilized in the reaction tubes and then taken up either in 0.01 M Na phosphate buffer, pH 7.4 or in a solution of pronase (15 mg/ml) in the same buffer. Reaction mixtures were treated as described under Materials and Methods.*

with pronase-digested fraction A behaved normally within 8 hours of observation while those injected with the fraction incubated only with the buffer died after an average of 41.9 min. With fraction B the control caused death after an average time of 108 min whereas the pronase-digested sample did not even produce any sign of paralysis. These results suggest Toxins I, II and III to be polypeptides.

Reduction of Toxins with β-Mercaptoethanol

The sensitivity of the toxins to β-mercaptoethanol was tested as shown in Table 2. Incubation was done both at room temperature and 50°C. In both cases fractions A and B were inactivated by β-mercaptoethanol which reduces disulfide bonds.

Heat Sensitivity

The controls for the β-mercaptoethanol experiment demonstrated that the toxins are stable at 50°C even after 14 hours incubation. However, toxins heated at 100°C for 15 minutes were inactivated. The activity of fraction A was almost completely destroyed, retaining only 5.7% of its original activity. The toxicity of fraction B was completely lost as indicated by the apparently normal behavior over an 8-hour observation period of carps injected with the heated fraction.

Table 2. Reduction of *C. striatus* Toxins with β-Mercaptoethanol (β-ME).

Sample	Toxin*	Temp. °C	β-ME μl	Buffer μl	Observation
1. β-ME control	–	25	20	–	all normal
2. Fraction A (reduced)	+	25	20	–	all normal
3. Fraction A (control	+	25	–	20	Angular swimming, spinning, swirling and lateral repose. Average paralysis time = 3.9 min; ave. death time = 45.0 min
4. Fraction A (reduced)	+	50	20	–	all normal
5. Fraction A (control)	+	50	–	20	Same symptoms as for #3. Ave. paralysis time = 4.67 min; Ave. death time = 39.9 min
6. Fraction B (reduced)	+	25	20	–	all normal
7. Fraction B (control	+	25	–	20	Angular swimming, spinning and lateral repose. Ave. paralysis time = 4.6 min; Ave. death time = 152.2 min
8. Fraction B (reduced)	+	50	20	–	all normal
9. Fraction B (control)	+	50	–	20	Same symptoms as for # 7. Ave. paralysis time = 8.23 min; Ave. death time = 115.5 min

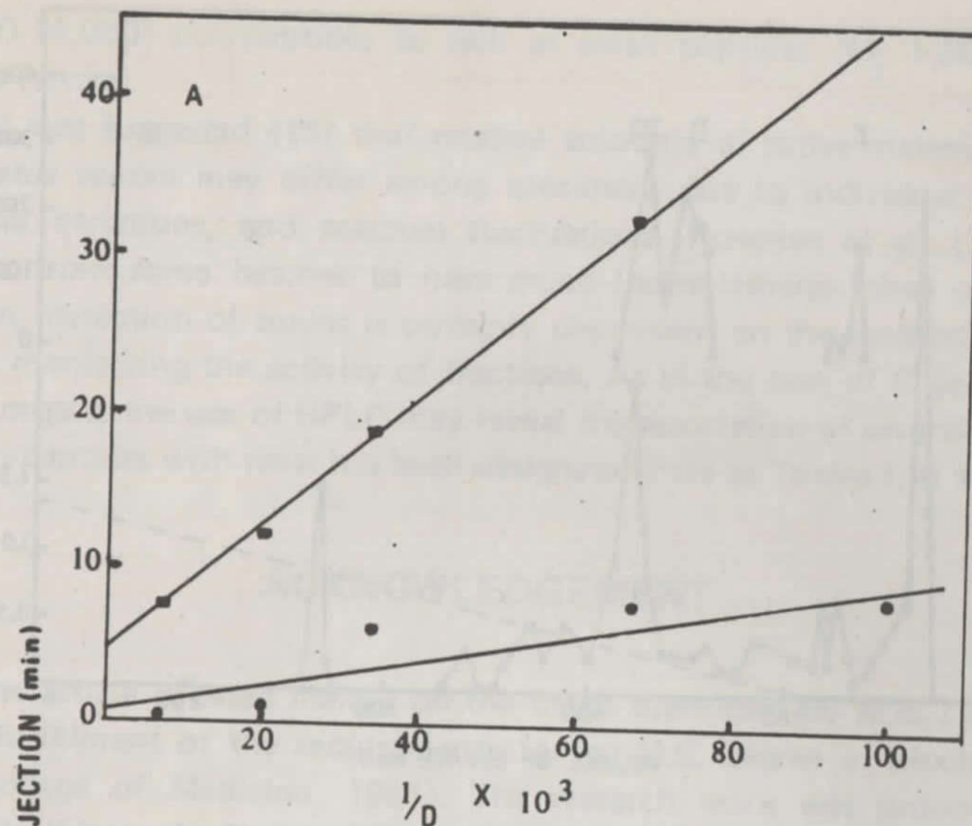
**Toxin samples were lyophilized in Eppendorf tubes then taken up either in 0.1M Tris buffer, pH 8.5 or 1.9M β-ME solution in the same buffer. Reaction mixtures were treated as described under Materials and Methods.*

Molecular Weight and Purity

Molecular weights of the toxins were estimated by gel filtration using a Sephadex G-50 column. Toxin I eluted beyond the fractionation range of G-50, after bacitracin; it appears to be a small peptide (M_r<1500). Fraction B which is a mixture of Toxins II and III showed only a single toxin peak suggesting the two toxins to have similar molecular weights (10,000 – 14,000 daltons). The elution profiles on Sephadex G-50 also indicated fractions A and B to be contaminated with proteins and peptides not toxic to fish. Disc gel electrophoresis patterns confirmed the presence of these impurities.

DISCUSSION

The data presented in this report indicate Toxin I to be a small acidic peptide whereas Toxins II and III appear to be basic and much bigger polypeptides. All toxins are sensitive to the sulfhydryl reagent, β-mercaptoethanol, suggesting the importance of cystine residues for activity, i.e., in maintaining the proper conformation for toxicity.



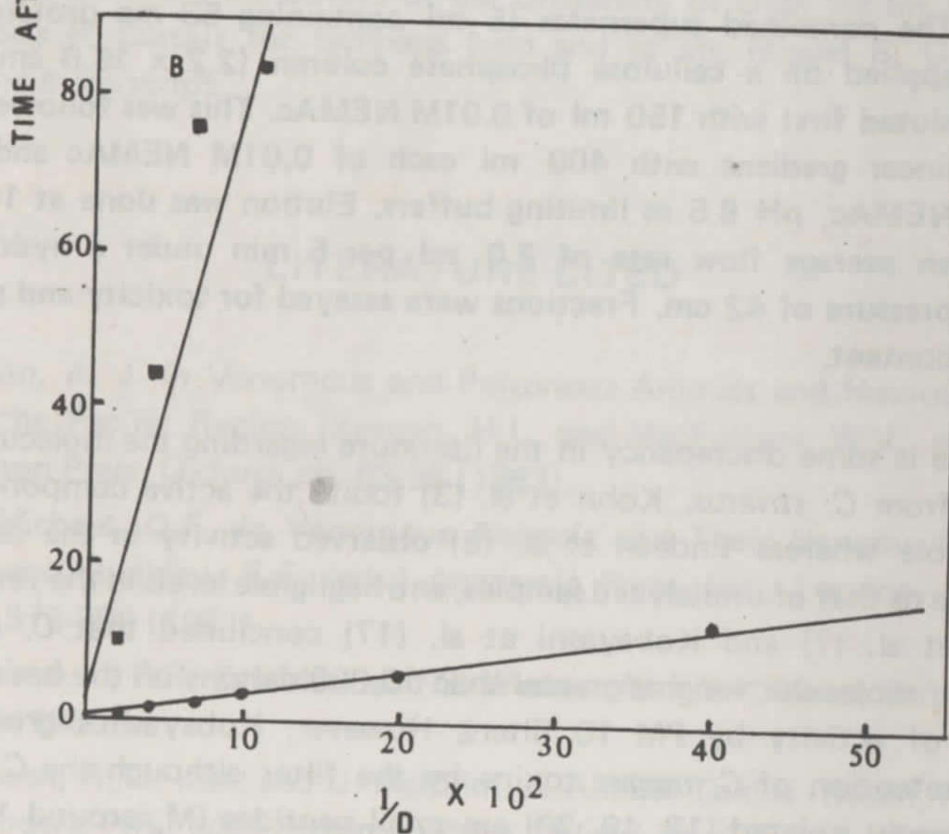


Figure 1. Dose response curve for mice and carps injected with *C. striatus* venom. Crude venom suspension in 0.9% NaCl was injected to ten-gram mice (A) and two-gram carps (B). The dose is expressed in μg venom protein per gram animal. Each point represents the average paralysis (●) or death (■) time for ten animals.

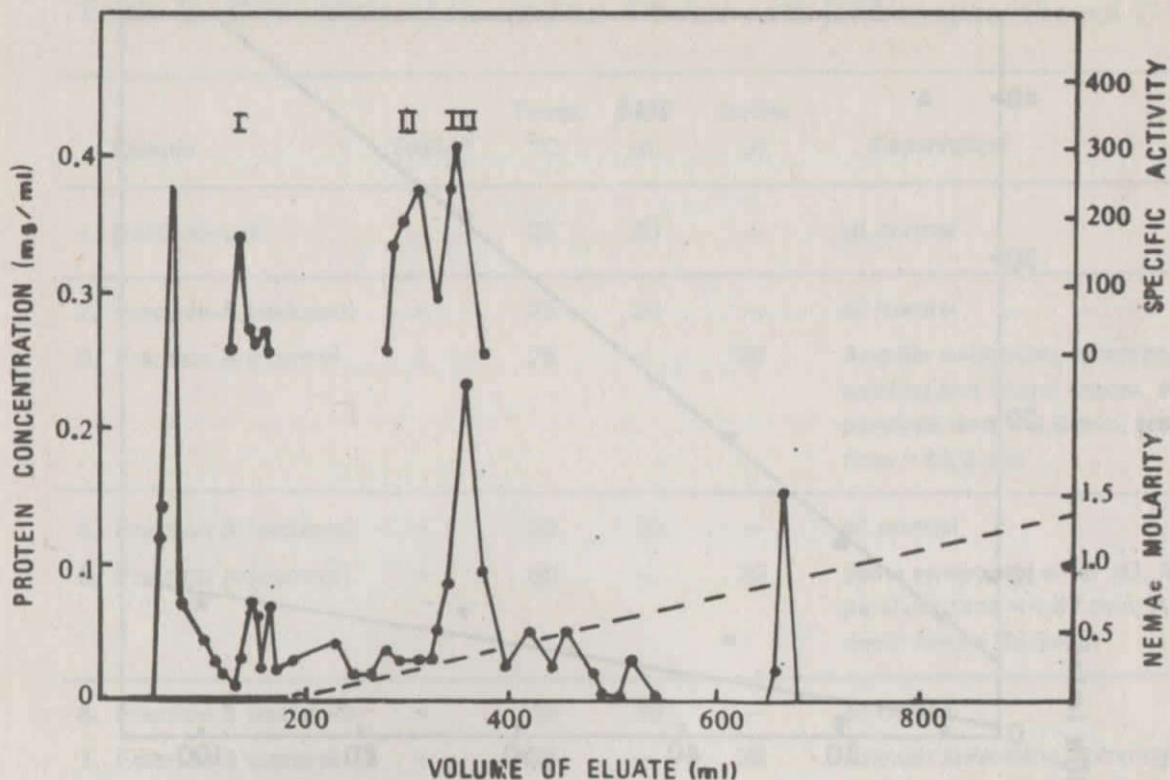


Figure 2. Phosphocellulose fractionation of *C. striatus* venom. Crude venom (0.66g) was extracted three times with 0.01M NEMAc, pH 8.5. The combined supernates (5 ml containing 53 mg protein) was applied on a cellulose phosphate column (2.2 x 16.6 cm) then eluted first with 150 ml of 0.01M NEMAc. This was followed by a linear gradient with 400 ml each of 0.01M NEMAc and 1.5M NEMAc, pH 8.5 as limiting buffers. Elution was done at 10°C at an average flow rate of 2.0 ml per 5 min under a hydrostatic pressure of 42 cm. Fractions were assayed for toxicity and protein content.

There is some discrepancy in the literature regarding the molecular size of toxins from *C. striatus*. Kohn et al. (3) found the active components to be dialyzable whereas Endean et al. (5) observed activity in the dialysate comparable to that of undialyzed samples, and negligible levels in the retentate. Freeman et al. (7) and Kobayashi et al. (17) concluded that *C. striatus* toxins have molecular weights greater than 10,000 daltons on the basis of the retention of activity by PM 10 filters. However, Kobayashi's group also observed retention of *C. magus* toxins by the filter although the *C. magus* toxins recently isolated (18, 19, 20) are small peptides (M_r around 1,500 – 3,000). Perhaps the membrane filters used are not readily permeable to charged peptides.

Strichartz et al. (10) found two toxins from *C. striatus* venom one of which is 7,000 daltons. The other toxin they detected could be identical to the glycoprotein (M_r 25,000) isolated by Kobayashi et al. (11). On the other hand our results show the presence of relatively high molecular weight

(10,000-14,000) polypeptides as well as small peptides (M_r 1,500) in *C. striatus* venom.

It was suggested (11) that relative amounts of active materials in the *C. striatus* venom may differ among specimens due to individual and geographical variations, and seasonal fluctuations. Freeman et al. (7) found extracts from some batches to have much lower lethality than others. In addition, detection of toxins is certainly dependent on the method of assay used in monitoring the activity of fractions. As in the case of *C. geographus* and *C. magus,* the use of HPLC may reveal the association of several peptides and polypeptides with what has been designated here as Toxins I, II and III.

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LITERATURE CITED

1. Kohn, A. J. *in* Venomous and Poisonous Animals and Noxious Plants of the Pacific Region (Keegan, H.L. and MacFarlane, W.V., eds.) Pergamon Press, Oxford, pp. 83-96 (1963).
2. McMichael, D.F. *in* Venomous Animals and Their Venoms (Bucherl, W. and Buckley, E.E., eds.) Academic Press, Inc., London, Vol. III, pp. 373-393 (1971).
3. Kohn A.J., P.R. Saunders and S. Wiener. *Ann N.Y. Acad. Sci.* **90:706-**725 (1960).
4. Endean, R., J. Izatt and D. McColm. *In* Animal Toxins (Rusell, F.E. and Saunders, P.R., eds.) Pergamon Press Oxford, pp. 137-144 (1967).
5. Endean, R., H. Williams, P. Gyr and J. Surridge. *Toxicon* 14:267-274 (1976).
6. Endean, R., P. Gyr and J. Surridge. *Toxicon* 15:327-337 (1977).
7. Freeman, S.E., R.J. Turner and S.R. Silver. *Toxicon* 12:587-592 (1974).

8. Kobayashi, J.H. Nakamura and Y. Ohizumi. *Br. J. Pharmac.* 73:583-585 (1981).
9. Hahin, R., G.K. Wang, G.R. Strichartz, J. Schmidt and B.I. Shapiro. Biophys. J. 33:124a (1981)
10. Strichartz, G.R., G.K. Wang, J. Schmidt, R. Hahin and B.I. Shapiro. *Fed. Proc.* 39:2065 (1980).
11. Kobayashi, J., H. Nakamura, Y. Hirata and Y. Ohizumi. *Biochem. Biophys. Res. Commun.* 105:1389-1395 (1982).
12. Cruz, L. J., G.P. Corpuz and B.M. Olivera. *Veliger* 18:302-308 (1976).
13. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. *J. Biol. Chem.* 193:265-274 (1951).
14. Cruz, L.J. *Acta Medica Philippina* 13:13-16 (1977).
15. Miller, L.C. and M. Tainter. *Proc. Soc. Exper. Biol. Med.* 57:261-263 (1944).
16. Canalco Research Disc Electrophoresis Instructions. Canalco Industrial Corporation, Rockville, Maryland (1969).
17. Kobayashi, J., H. Nakamura, Y. Hirata and Y. Ohizumi. *Toxicon* 20:823-839 (1982).
18. McIntosh, M., L.J. Cruz, M.W. Hunkapiller, W.R. Gray and B.M. Olivera. Arch. Biochem. Biophys. 218:329-334(1982).
19. McIntosh, J.M., B.M. Olivera, L.J. Cruz and W.R. Gray. J. Biol. Chem. 259:14343-14346 (1984).
20. Kobayashi, J., H. Nakamura and Y. Ohizumi. *Eur. J. Pharmacol.* 86: 283-286 (1983).