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LOCALIZATION OF PROTEINS AND ENZYMES IN THE GRANULES OF *Conus* VENOM

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ABSTRACT

The toxicity and enzyme activities of granules and soluble fraction extracted from various segments of the venom duct of *Conus striatus* were studied. Examinations reveal the granules to contain 60 to 67% of the total proteins. Using fish as assay animal, the soluble fraction was found to possess greater toxicity accounting for 63 to 73% of the total paralytic and lethal activities. Higher enzyme activities are associated with the granules which contain 74 to 85% of the total proteolytic activity, 80 to 84% of the total acetylcholinesterase activity and 90 to 94% of the total phosphodiesterase activity. Total proteins in the granules were noted to increase towards the posterior duct region except in the segment closest to the venom bulb while those in the soluble fraction increase from the anterior to the posterior region. Total paralytic and lethal activities were observed to increase towards the posterior duct region. Total enzyme activities increase from the anterior to the posterior region except in the segment adjacent to the venom bulb.

Treatment of the granules extracted from the entire venom duct with carboxypeptidase A led to the enhancement of paralytic activity. Electrophoretic patterns in polyacrylamide gels of aliquots withdrawn before and after incubation of the granules with carboxypeptidase A have shown difference in mobility of a major protein.

The results support the suggested role of granules as storage packets for enzymes and for inactive precursors of the toxic components of *Conus* venoms.

INTRODUCTION

Venom elaborated by marine snails of the genus *Conus* consists of microscopic granules suspended in a viscous fluid. Examination of the material inside granules of *C. magus* venom showed their physiologic activity to be different from that of the fluid vehicle (1). In *C. geographus*,

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toxicity of the granules was shown to be negligible compared with that of the soluble fraction (2).

Differences in sizes and shapes of granules from anterior and posterior regions of the venom duct of *C. magus* were noted by Endean and Duchemin (3). Small spherical bodies were found at the anterior regions while large sausage-shaped bodies were found at the posterior regions. Circular and oval granules were also observed by Kohn *et al.* (4) in the venom of *C. striatus.* Similarly, histologic examination of various segments of the venom duct of *C. geographus* showed two types of granules (2). Small ones which are eosin-staining were found at the anterior region while larger granules which are hematoxylin-staining were found at the middle and posterior regions.

Toxicity assays on venoms from various *Conus* species have revealed that the venom from posterior regions of the duct is much more lethal than that from anterior regions (5, 6). The lethal and paralytic activities are mainly due to small peptide toxins (7, 8, 9). In addition to the conotoxins, venoms contain enzymes like proteases, acetylcholinesterase and phosphodiesterase (10, 11, 12, 13). Both granules and soluble fraction from the anterior duct region of *C. geographus* were found to contain relatively high proteolytic activity whereas the middle and posterior regions showed very little activity. To date, no work has yet been published on the distribution of the two other enzymes.

Based on some biochemical and histologic data on *C. geographus,* Pali *et al.* proposed the possible role of venom granules as storage packets for digestive enzymes and precursors of conotoxins. This hypothesis is supported by the present report on the localization of enzymes and proteins in the granules of *C. striatus* venom.

MATERIALS AND METHODS

Specimens

The specimens of *Conus striatus* were collected from sea waters around Marinduque, Philippines. These were maintained alive in the laboratory in salt-water aquaria.

Reagents and Equipment

Azocasein was obtained from Calbiochem, San Diego, California. Carboxypeptidase A and α-chymotrypsin were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. All other biochemicals were from Sigma Chemical Co., St. Louis, Missouri.

A Sorvall centrifuge, Model RC2-B, equipped with SS-34 rotor and an Eppendorf centrifuge, Model 3200, were employed for sedimentation. Sonication was done using Sonifier Cell Disruptor, Model W185D from Branson

Sonic Power Co.

Preparation of Venom Samples

The venom apparatus was dissected as described by earlier workers (14, 15). Crude venom from the entire duct (CV) was extracted from the whole venom duct starting from the anterior region proximal to the pharynx down to the posterior region adjacent to the venom bulb. Crude venom from segment (CVS) was extracted after cutting the venom duct into five segments of equal length. The segment closest to the pharynx was labeled as segment 1 and that proximal to the venom bulb as segment 5.

For the determination of the distribution of toxicity and enzyme activities, CV extracted from ten venom ducts and CVS extracted from twenty venom ducts were pooled. CV and CVS were made up to about 5% or 10% (w/v) solution with H_2O. CVS was sedimented at 12,000 rpm for 10 minutes to separate the granules (GS) from the soluble fraction (SS). The SS was saved; the GS was washed twice with H_2O, centrifuged each time, and resuspended in H_2O; the washings were combined with the SS. The GS was disrupted by sonication over ice at 50 watts for 20 seconds, three times at 20-second intervals.

Protein Analysis

The protein content of each sample was determined according to the method of Lowry *et al.* (16) using bovine serum albumin as the standard. Absorbance readings were taken using a Spectronic 20 equipped with a microcell adapter.

Toxicity Assay

The fish used for toxicity assays were fresh water carps *(Cyprinus carpio)* weighing 1.0 to 1.5 g. The bioassay was based on the linear dependence of paralysis and death times on the reciprocal of dose as originally devised by Cruz (17) for mice and modified by Pali (4) for fish. One unit of activity (U) of *C. striatus* toxin was arbitrarily defined for this study as the amount of toxin injected per gram of fish which can cause complete paralysis in 16 min and death in 33 min. Five fish were used for all samples assayed.

Assays for Enzyme Activities

The optimum pH and temperature for each of the enzymes were determined using pooled crude venom from the whole duct of *C. striatus.* These conditions were then used for assaying the enzyme in the granular (GS) and soluble fractions (SS) of the segments.

Proteolytic activity was assayed according to the method of Charney and Tomarelli (18) using 10-μl aliquots of GS or SS. With azocasein as the

substrate, one unit of activity (U) is arbitrarily defined as the amount of enzyme which yields an A_{440} of 1.0 after 10 min of incubation at pH 9 and 40°C.

The acetylcholinesterase activity of 5-μl aliquot of GS or SS was examined employing the method of Hestrin (19). One unit of activity (U) is equivalent to the disappearance of one μmole of acetylcholine per 30 minutes at pH 7 and 35°C.

The phosphodiesterase activity of 20-μl aliquot of GS or SS was determined according to the method of Koerner and Sinsheimer (20) using bis(p-nitrophenyl) phosphate as substrate. One unit of activity (U) is equivalent to the liberation of one nmole of p-nitrophenol per 14 hours at pH 8 and 40°C.

Activation by Carboxypeptidase A

Crude venom(CV) extracted from twenty five venom ducts were pooled and made up to about 5% solution with 0.1 M ammonium acetate buffer, pH 8.5. CV was sedimented using Eppendorf centrifuge at maximum speed for two minutes to separate the granules (G) from the soluble fraction (S). G was washed twice, centrifuging each time and resuspended in 0.1 M ammonium acetate, pH 8.5. The suspension was sonicated over ice three times at 50 watts for 20 seconds, at 20-second intervals. An aliquot of G was taken and centrifuged at maximum speed for two minutes to separate the granule debris (GD) from the granule extracts (GE). GD was washed twice, centrifuging each time, then resuspended in the buffer.

An aliquot of each sample was separately incubated with different proteases (α-chymotrypsin, elastase, trypsin and carboxypeptidase A) at a ratio of 25 μg of protease per mg of proteins. Incubation was carried out at 37°C for 6 hours. Toxicity assays using five fish per mixture were done before and after incubation. Of the various proteases used, carboxypeptidase A was found to enhance the paralytic activity of G.

A mixture of G and carboxypeptidase A in 0.1 M NH_4Ac, pH 8.5 at a ratio of 25 μg of carboxypeptidase A per mg of G proteins was prepared and incubated at 37°C for 6 hours. Samples were withdrawn at various times of incubation, immersed in ice and tested for toxicity, using five fish per sample. Controls prepared using 0.1 M ammonium acetate, pH 8.5 and carboxypeptidase A were similarly tested.

Aliquots were withdrawn before and after incubation and saved in the freezer. These were lyophilized using an Eyela freeze drier (FD-1) and resuspended in H_2O. An aliquot containing about 100 ug of proteins analyzed by polyacrylamide gel electrophoresis according to the method described in Canalco Instruction Manual for Model 1200 (21). The staining procedure and calculation of mobilities were based on the method of Weber and Osborn (22).

RESULTS AND DISCUSSION

Protein and Toxicity Distribution

In agreement with the findings of Balagtas (9) 60 to 67% of proteins in the crude venom of *C. striatus* was found associated with the granular fraction. Figure 1A shows that the soluble fractions consistently have lower levels of protein which slowly increase as one goes from the anterior to the posterior region of the venom duct.

Granules from segments 1 and 2 were found to contain much lower amounts of protein than those from segments 3, 4 and 5. The highest protein content was found in the granular fraction of the fourth segment. Histochemical tests by Endean and Duchemin (3) have also indicated that protein content of the cores of venom bodies (granules) increases towards the posterior duct region.

On the otherhand Figures 1B and 1C indicate much higher specific paralytic and lethal activities in the soluble than in the granular fraction. Toxicity is lowest near the pharynx or anterior region and steadily increases from segments 1 to 5. The findings of Endean and Rudkin (6), and Pali *et al.* (2) have shown similar increases in toxicity towards the posterior region. Pali, however, found venom from a very short portion of the duct (that closest to the venom bulb) to have very little material, protein and toxic activity. In the above experiment this portion is included in segment 5.

Hydrolytic Enzymes

Three hydrolytic enzymes (proteases, acetylcholinesterase and phoshodiesterase) were assayed in the venom of *C. striatus.* Optimum conditions for activity were determined and found to be as follows:

	Optimum pH	Optimum Temperature
Proteolytic activity	9.0	40°C
Acetylcholinesterase	7.0	35
Phosphodiesterase	8.0	40

These conditions were then used in determining the distribution of enzymes along the venom duct.

Figure 2 shows much higher specific activities of the hydrolytic enzymes of *C. striatus* in the granular fraction as compared to the soluble fraction. Both the proteolytic activity (Figure 2A) and phosphodiesterase level (Fig. 2B) were found to be highest in the fourth segment. The level of acetylcholinesterase (Fig. 2C) was essentially uniform throughout the venom duct.

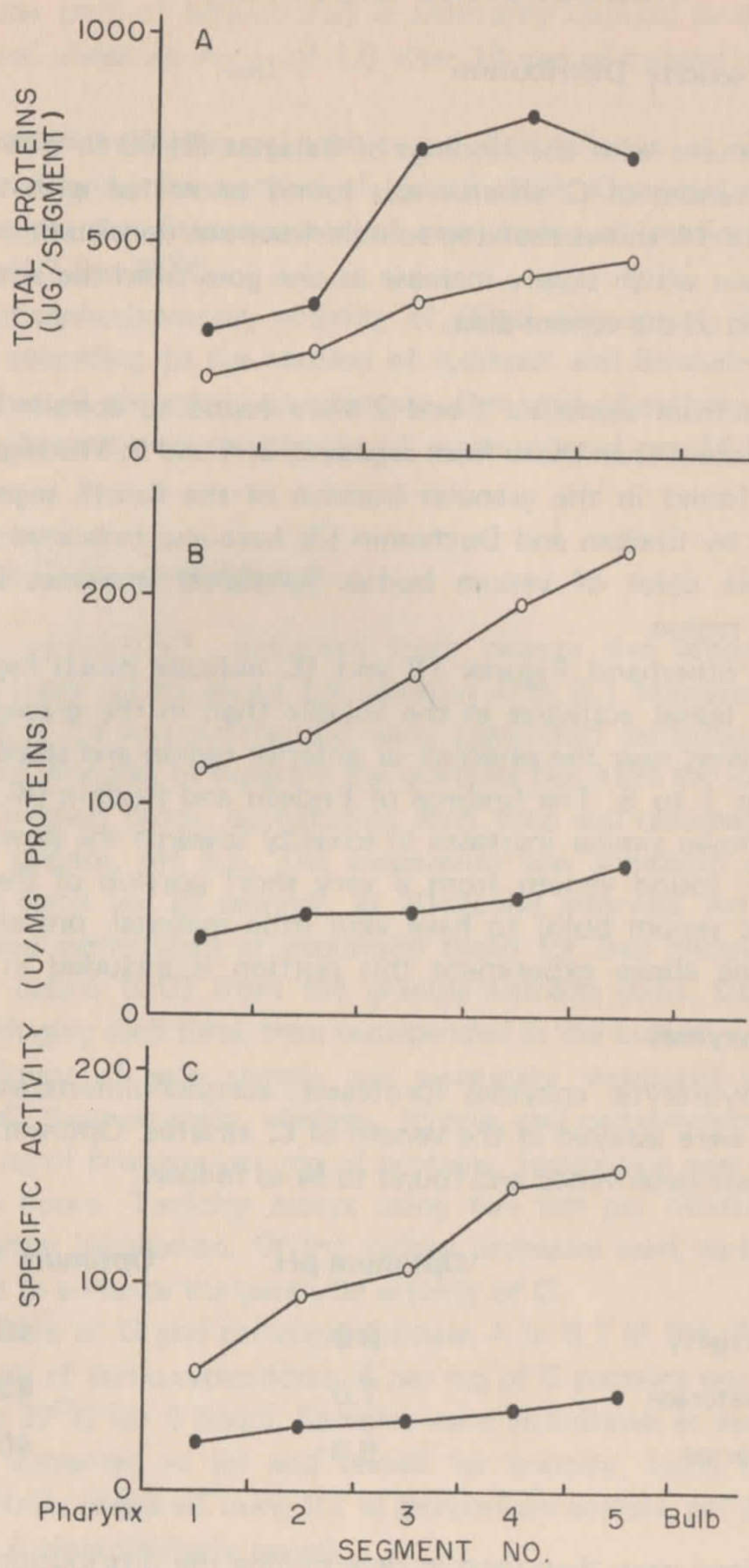

Figure 1. Comparison of protein and toxicity distribution along the venom duct. The protein content (A), paralytic activity (B) and lethal activity (C) of the soluble (o—o) and granular (●—●— fractions of successive segments of venom ducts were determined as described in the text.

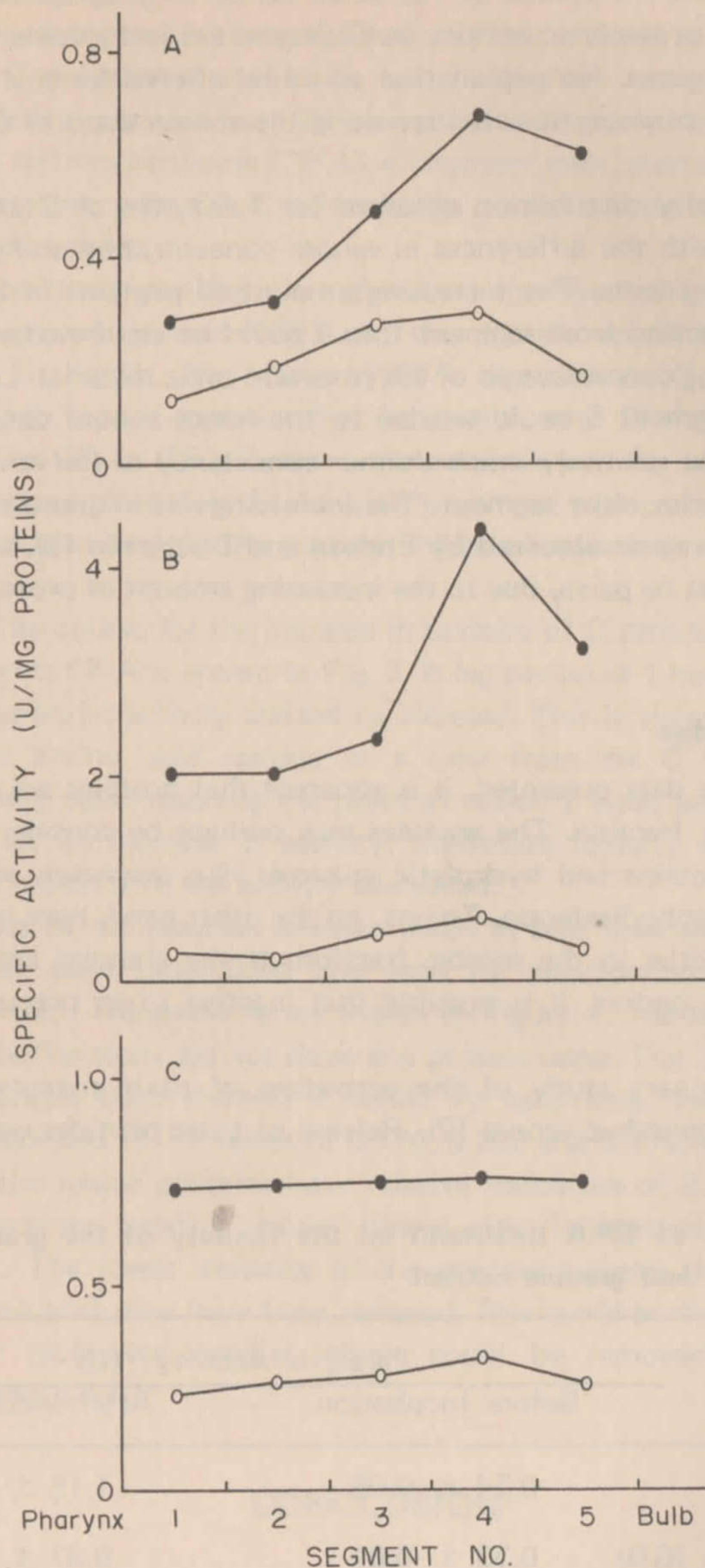

Figure 2. **Distribution of hydrolytic enzymes along the venom duct of *C. Striatus*. Proteolytic (A), phosphodiesterase (B) and acetylcholinesterase (C) activities of the granular (●–●) and soluble (o–o) fractions were determined for successive segments of the venom duct as described under Materials and Methods.**

In contrast the studies of Pali et al. (2) on *C. geographus* venom have shown highest proteolytic activity in both granules and soluble fraction from the anterior regions. No explanation could be offered for this difference in distribution of proteolytic activities along the venom ducts of *C. striatus* and *C. geographus.*

The activity distribution observed for 3 enzymes of *C. striatus* venom is consistent with the differences in venom concentration and protein levels in different segments. The increasing amount of proteins in both granules and soluble fraction from segment 1 to 4 could be attributed at least in part to the increasing concentration of enzymes and toxic material. Lower enzyme activities in segment 5 could be due to the lower venom concentration as indicated by the relatively much thinner consistency of the venom extracted from the posterior most segment. The increasing size of granules towards the posterior duct region observed by Endean and Duchemin (3), and Pali *et al.* (2) could at least be partly due to the increasing amount of proteins contained in the granules.

Activation Studies

From the data presented, it is apparent that proteins are concentrated in the granular fraction. The granules may perhaps be considered as storage packets for proteins and hydrolytic enzymes like proteases, acetylcholinesterase and phosphodiesterase. Toxins, on the other hand, have higher specific and total activities in the soluble fraction. If the granules are serving as a general storage packet, it is possible that inactive toxin precursors exist in the granules.

A preliminary study of the activation of toxin precursors has been done on *C. geographus* venom (2). Release of toxic peptides was effected by

Table 1. Effect of CP-A treatment on the toxicity of the granule, granule debris and granule extract.

Sample	Paralytic Activity (U) Before Incubation	Paralytic Activity (U) After Incubation
Granule (G)	0.34 ± 0.08	1.15 ± 0.37*
Granule Debris (GD)	0.33 ± 0.04	0.37 ± 0.08
Granule Extract (GE)	0.34 ± 0.08	0.36 ± 0.06

Five fish were used for the bioassay of each sample

Statistically significant at $\alpha = 0.05$.

treating the granules with elastase. These peptides elicited symptoms evinced by purified *C. geographus* toxins. To test if activation can also be done with *C. striatus* venom, granules from 25 cone shells were pooled and aliquots were treated with carboxypeptidase (CP-A), α-chymotrypsin, elastase and trypsin. Of these proteases, only CP-A increased the paralytic activity of the granules to a significant extent.

The sonicated granules were therefore fractionated by centrifugation to give the granule extract (GE) and the granule debris (GD) which was resuspended in buffer. The effect of CP-A digestion on G, GD and GE is shown in Table 1. It is apparent that incubation with CP-A has no effect on the toxicity of GD or GE. However, the paralytic activity of unfractionated granules increased more than 3-fold upon treatment with carboxypeptidase A. Somehow fractionation of the granules inhibited activation of the toxins by CP-A.

The time course for the increase in toxicity of *C. striatus* granules upon incubation with CP-A is shown in Fig. 3. A lag period of 1 hour was observed before the paralytic activity started to increase. This is expected since CP-A removes one amino acid residue at a time from the C terminal of the precursor. Only upon reaching the point at which a toxic peptide is released would one see an increase in activity. Maximum toxicity was observed at 5 hours after which time the activity decreased.

Aliquots of the reaction mixture taken at zero time and after 6 hours of incubation were lyophilized and used for polyacrylamide gel electrophoresis (PAGE). The patterns are shown in Figure 4. The blank containing enzyme and buffer alone did not show any protein band. The 2 gels containing granular fractions both showed 3 bands. At zero time, the major protein bands corresponded to Rf values of 0.170, 0.252 and 0.979. After 6 hours of incubation, the major proteins have relative mobilities of 0.171, 0.200 and 0.977. Apparently band B moves slower after incubation with carboxypeptidase A. The lower mobility of the protein suggests that a negatively charged amino acid may have been released. This could be due to cleavage of aspartate or glutamate residue which could be removed by prolonged incubation with CP-A.

CONCLUSION

Proteins and hydrolytic enzymes such as protease, acetylcholinesterase and phosphodiesterase were found to be associated with granules. There is an increasing trend in the granular contents as one goes from the anterior to the posterior region but with the peak coinciding with the second to the last segment. On the other hand, the soluble fraction contains much higher paralytic and lethal activities than the granules. Toxicity is lowest near the pharynx and steadily increases towards the posterior region.

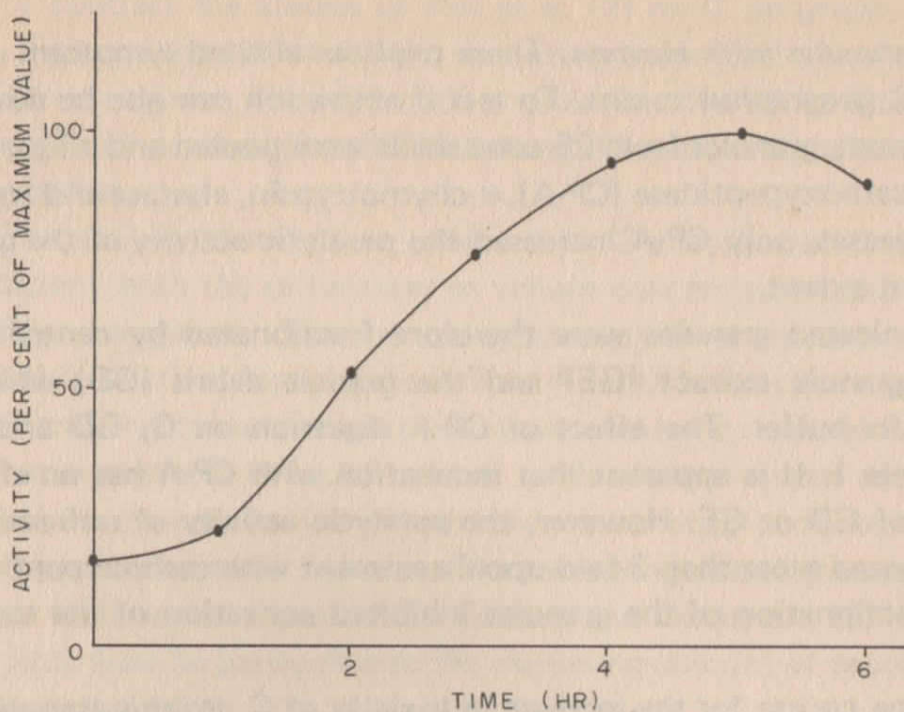

Figure 3. Time course for the release of toxins from granules by CP-A treatment.

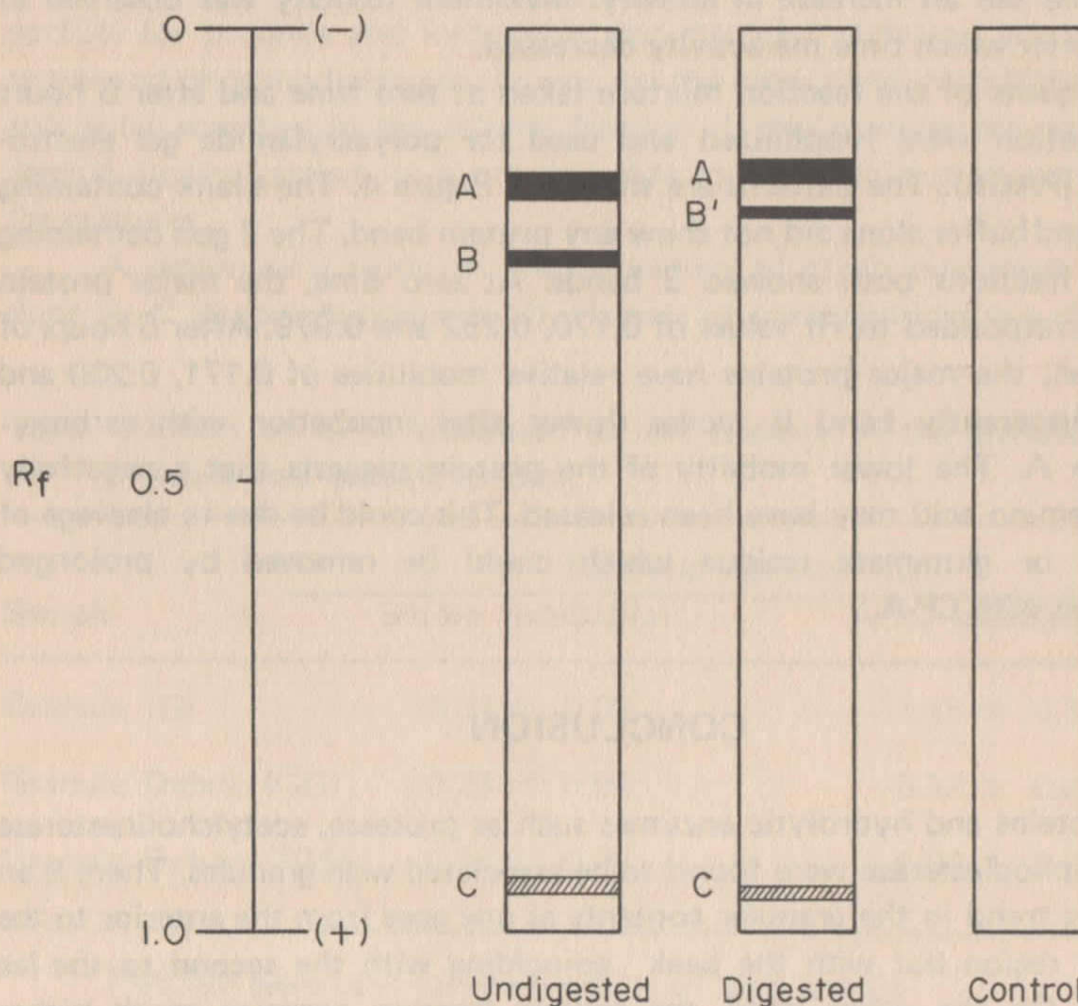

Figure 4. PAGE pattern of digested and undigested granule patterns.

Incubation of granules with carboxypeptidase A increased toxicity. This increase is accompanied by a change in mobility of one of the protein bands visualized after polyacrylamide gel electrophoresis. These data together with those of Pali *et al.* (2) suggest that granules contain toxin precursors. The granules may therefore be considered as storage packets not only for hydrolytic or digestive enzymes but also for the precursors of conotoxins.

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