Research Article

Partial Purification and Characterization of *Conus textile* Venom Phosphodiesterase I

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Phosphodiesterase I (5'-exonuclease, E.C. 3.1.4.1) was isolated and purified to apparent homogeneity from the venom of Conus textile *by a series of differential extractions and chromatographies using Sepharose 4B, DEAE-Cellulose 4B, and DEAE-Sephadex column. The M_r of the enzyme, obtained through Sepharose 4B chromatography, was estimated at 1,200,000. SDS polyacrylamide gel electrophoresis set the main subunit M_r at 60,000. The optimum conditions for the assay, with para-nitrophenyl thymidine-5'-phosphate as substrate, were pH 8.8, temperature at 40°C, and a 40 mM magnesium ion concentration in a buffer of high ionic strength. The pI, determined by isoelectric focusing, was about 7.8-7.9. The Eadie-Hofstee diagnostic plot gave values of $K_m = 3.7 \times 10^{-3}$ M and $V_{max} = 608$ µmoles p-nitrophenol liberated/mg protein/min. EDTA in a 0.01 M concentration was found to moderately inhibit the enzyme reaction.*

INTRODUCTION

Marine shells of the genus *Conus* have a highly developed venom apparatus composed of a long duct leading to the pharynx. It has been suggested that the venom apparatus is used primarily for catching, paralyzing, and possibly digesting prey. It may also function as a defensive weapon (1). Some *Conus* stings have been found to be lethal to man (2,3).

The venom has a high protein content which may be associated with enzymatic activity. Other venom components are carbohydrates, quarternary ammonium salts, and some high molecular weight compounds.

Fish, other molluscs, and marine worms are *Conus* prey. Such variety leads one to expect that the *Conus* venom has several digestive enzymes. Proteases, acetylcholinesterases, phosphodiesterases, some nucleotidases and phospholipases have been reported. Proteases were detected in the venom of several species of vermivorous cones (4). More specifically, *C. textile* was found to possess at least two proteolytic and three hemolytic components (5). Acetylcholinesterase was partially purified from *C. geographus* venom and found to be separable from the toxic components (6). Higher activities of protease, acetylcholinesterase, and phosphodiesterase were noted particularly in the granular fractions of *C. striatus* venom, suggesting that these granules serve as storage packets for digestive enzymes or zymogens as well as precursors of the toxic elements of *Conus* venom (7).

Although much has been published on *Conus* venom pharmacology, many aspects of *Conus* venom enzymology are still open for further investigation.

An earlier survey of several *Conus* species (*C. striatus, C. geographus, C. magus, C.*

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marmoreus, and *C. textile*) indicated significant phosphodiesterase I activity in *C. textile*.

It is within this framework that this study on phosphodiesterase I (5'-exonuclease E.C. 3.1.4.1) from *C. textile* was conceived. This enzyme digests nucleic acids, specifically removing successive mononucleotide units from the polynucleotide chain starting from the end bearing a free 3'-hydroxyl group regardless of the location of the monophosphoryl group (8).

The objective of this study is to isolate and partially purify phosphodiesterase I from *C. textile* venom and to do a preliminary investigation of some physical and chemical properties of the enzyme.

MATERIALS AND METHODS

C. textile specimens were collected off the coast of Marinduque Island in the Philippines and maintained alive at room temperature in well-aerated marine aquaria in the laboratory.

Live specimens were buried in ice for 30 min, after which the animals were removed from their respective shells. The venom apparatus was dissected out of the body cavity, freed of extraneous tissue and washed with cold distilled water. The venom was gently squeezed onto an ice cold stainless steel spatula, collected in small vials, lyophilized, weighed, and stored at -20°C until processed.

Crude venom suspensions were prepared by weighing out lyophilized venom and reconstituting with 0.05 M NH_4Ac, pH 7 to make a 10% suspension, allowing to stand in ice for 30 min with occasional stirring, and centrifuging at 22,000 x g for 30 min. The supernate was saved into a test tube and labelled 10% soluble fraction.

To the pellet was added 0.05 M NH_4Ac, pH 7/0.1% Triton X-100 to make a 20% suspension. This suspension was sonicated for 1 min and centrifuged for 10 min at 3,000 x g, the supernate decanted and set aside. With the resulting pellet, another 20% suspension in 0.05 M NH_4Ac, pH 7/0.1% Triton X-100 was prepared, then sonicated and centrifuged as described above. The supernates were collected, combined, and labelled 10% granular fraction.

Assays for protein, phosphodiesterase I, and protease were performed on these extracts.

Protein in crude venom, soluble fractions, and granular fractions was estimated by the method of Lowry *et al.* with bovine serum albumin as standard (9). In column effluents, eluted proteins were monitored either by the Lowry method or by their absorbance at 280 nm.

Phosphodiesterase I was measured by the sensitive assay method of Razzall and Khorana (10). Specific activity was expressed as units of phosphodiesterase I activity per mg protein (1 unit is equivalent to 1 mole p-nitrophenol liberated per min). The assay was standardized with respect to the following parameters: pH, temperature, buffer system, substrate concentration, and Mg^{++} requirements.

Non-specific alkaline phosphatase (phosphomonoesterase) was determined by the method of Laskowski (11). Specific activity was expressed as units of alkaline phosphatase per mg protein (a unit of activity is equal to 1 µmole of p-nitrophenol liberated per min).

Activity of 5'-nucleotidase was determined according to the method of Koerner and Sinsheimer (12). Specific activity was expressed as units of 5'-nucleotidase per mg protein, where 1 unit is equivalent to 1 µmole inorganic phosphate liberated per min.

Protease was assayed by the azocasein method essentially as described by Tomarelli *et al.* (13). Specific activity was expressed as units of protease activity per mg protein, where 1 unit is equivalent to 1 µg azocasein digested per min.

The Lineweaver-Burke and Eadie-Hofstee plots were constructed using the phosphodiesterase I assay on the partially purified fraction at various concentrations of substrate. Approximate values for the Michaeli's constant (K_m) and maximum reaction velocity (V_{max}) were calculated.

Using the partially purified enzyme preparation, the phosphodiesterase I assay was standardized in terms of optimum values of pH, temperature, and cofactor require-

ment.

Distribution of phosphodiesterase I activity after autolysis was determined. An aliquot of each of the 10% soluble and granular fractions was acidified to pH 3.6 with 0.2 M HAc. The mixtures were incubated at 38°C for 3 h to inactivate 5'-nucleotidase, then centrifuged at 12,000 x g for 10 min and decanted. Both the pellet and supernate from the 10% soluble fraction were lyophilized and reconstituted with 0.2 M TrisAc, pH 8.8. The supernate from the treated granular fraction was set aside, while the pellet was dissolved in a minimum volume of 0.2 M TrisAc, pH 6/0.1% Triton. Both supernate and reconstituted pellet were assayed for protein and phosphodiesterase I.

Phosphodiesterase I was purified by loading exactly 2.6 mL of the autolysis product onto a column of Sepharose 4B (1.3 x 100 cm) previously equilibrated with 0.2 M NH_4Ac, pH 6. Elutions were carried out in the same buffer at a flow rate of 0.5 mL/min. Fractions of 5.0 mL were collected and assayed for protein and phosphodiesterase I.

The active fractions from Sepharose 4B chromatography were pooled, lyophilized and reconstituted with 0.2 M NH_4Ac, pH 8.5 and applied to the DEAE-Sephadex column (2.0 x 20 cm) and eluted with a linear gradient of 0.2 -1.2 M NH_4Ac, pH 8.5 (2 x 75/mL) at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected and assayed for protein and phosphodiesterase activity.

The subunit molecular weight of phosphodiesterase I was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis using the method of Weber and Osborn (14). The subunit molecular weights were estimated from a graph of log M_r vs. electrophoretic mobility.

The M_r of phosphodiesterase I from *C. textile* was estimated by using a Sepharose 4B column (0.8 x 27 cm) previously equilibrated with 0.2 M NH_4Ac, pH 8.5 at 4°C. This column was standardized using blue dextran catalase, and hexokinase. Fractions were collected and assayed for proteins.

The DEAE active fraction (containing phosphodiesterase I) was lyophilized and reconstituted in 0.1% glycine. This glycine preparation was electrofocused in a 5% polyacrylamide slab gel containing ampholytes ranging from pH 3.5 to 9.5 and was pre-run at 3°C for 1.5 h at a constant power of 25 watts. The gel was fixed in 12% TCA for 15 min, stained in 0.1% Coomasie Blue for 30 min and subsequently destained in frequent changes of 10% methanol-10% acetic acid at 50°C until protein bands could be visualized.

RESULTS AND DISCUSSION

Preliminary studies were performed to determine the optimum assay conditions for the phoshodiesterase of the soluble fraction. Appreciable activity was observed over a broad pH range of 8.0 to 9.6 with a maximum at pH 9.2. Optimum temperature was between 40° and 45°C. Enzyme activity was not affected by changes in the Mg^{++} concentration of the assay mixtures which were incubated for 5 h. This could mean that the Mg^{++} requirement was met by amounts already in the crude venom suspension. Within the concentration range used, the reaction rate was still proportional with enzyme concentration. The assay was linear for the first 40 min but began to level off after that. All assays were therefore performed 15-20 min after the reaction was initiated.

The presence of contaminating enzymes such as 5'-nucleotidase and non-specific phosphatases in venom preparations has been noted in several reviews (11, 15, 16). Dolapchiev *et al.* described an incubation step at 37°C for 3 h to inactivate 5'-nucleotidase (17). The stability of phosphodiesterase I to similar pre-treatment was studied by incubating crude venom extract in buffers of differing pHs at 37°C for 2 h. Phosphodiesterase I assays after incubation indicated that the enzyme was unaffected by this treatment.

The distribution of protein, phosphodiesterase I, 5'-nucleotidase, phosphomonoesterase, and protease between the granular and soluble extracts, and between the pellet and supernate of the incubated 10% granular fraction is summarized in Table 1. The bulk of protease activity was located in the soluble fraction while 5'-nucleotidase and phosphodiesterase activity were found largely in the granular fraction.

Most of the proteins had molecular weights in the region of 100,000 to 130,000. Fig. 1 shows also the elution volumes of

Table 1. Distribution of enzyme activities in *C. textile* venom.

Fraction	Phosphodiesterase I (units x 10^{-2})	5'-nucleotidase (units)	Phosphomonoesterase (units x 10^{-2})	Protease (units x 10^{-2})
10% soluble	5.6	0.73	14.8	14.0
10% granular	17.8	2.32	28.5	3.6
Total	23.4	3.05	43.3	17.6
Incubated 10% Granular Fraction				
Supernate	5.0	0.75	1.3	0
Pellet	11.6	0.12	10.9	0
Total	16.6	0.87	12.2	0

molecular weight markers as indicated by arrows: blue dextran, 2,000,000; catalase, 250,000; and hexokinase, 100,000.

The granular fraction showed three protein peaks labelled g_1, g_2, and g_3 (Fig. 1) with M_r of 5,000,000, 1,200,000, and 540,000. Maximum phosphodiesterase activity was found over peak g_2. Peaks g_1 and g_3 exhibit high specific activity for phosphomonoesterase as shown in Fig. 1A.

Active fractions from Sepharose 4B chromatography were pooled, lyophilized, reconstituted, and assayed for protein and phosphodiesterase I before application to a DEAE-Cellulose column. Upon passing a 0.2-1.2 M NH_4Ac, pH 8.5 gradient through the column, several protein peaks were detected (Fig. 2). The active peak was eluted at a concentration of 0.60-0.65 M NH_4Ac, pH 8.5. These results suggest that the enzyme was tightly bound to the exchanger because a high ionic strength was required to elute it from the column.

The DEAE-Cellulose active fractions were rechromatographed on a DEAE-Sephadex column run at a gradient of 0.2-1.2 M NH_4Ac, pH 8.5. This resulted in a major protein peak eluted at a concentration of 0.60 M NH_4Ac, coincident with maximum phosphodiesterase activity (Fig. 3).

Table 2 summarizes the effectiveness of the various steps of purification and the corresponding yields. After the DEAE-Cellulose step, only 2% of the protein remained, the rest having been removed by the various

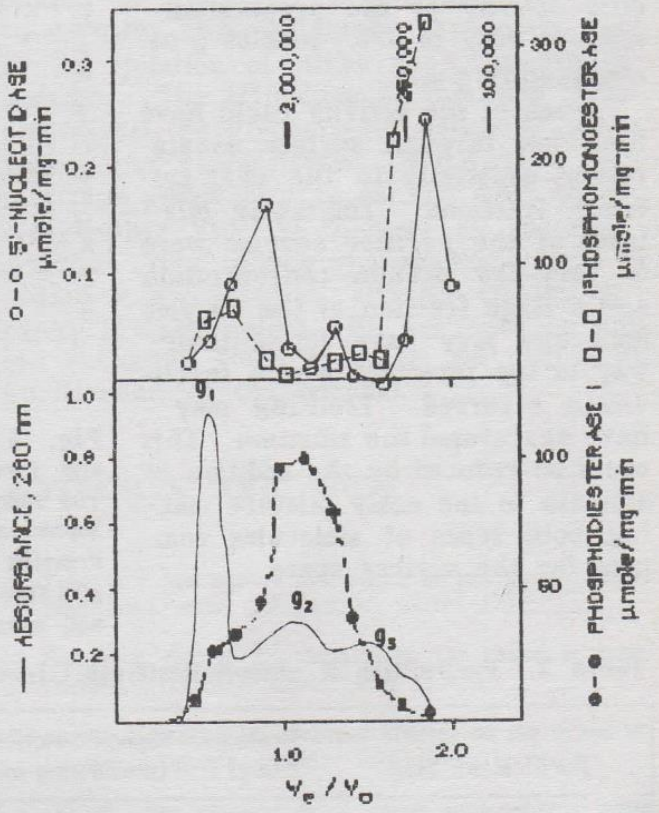

Fig. 1. Sepharose 4B gel filtration of incubated 10% granular fraction. The sample (34.3 mg protein) was applied to a 1.3 x 100 cm Sepharose 4B column equilibrated with 0.2M TrisAc, pH 6 and eluted with the same buffer. The flow rate was 0.5 mL/min at a constant hydrostatic pressure of 90 cm. Fractions of 5 mL were collected and assayed for enzyme activity.

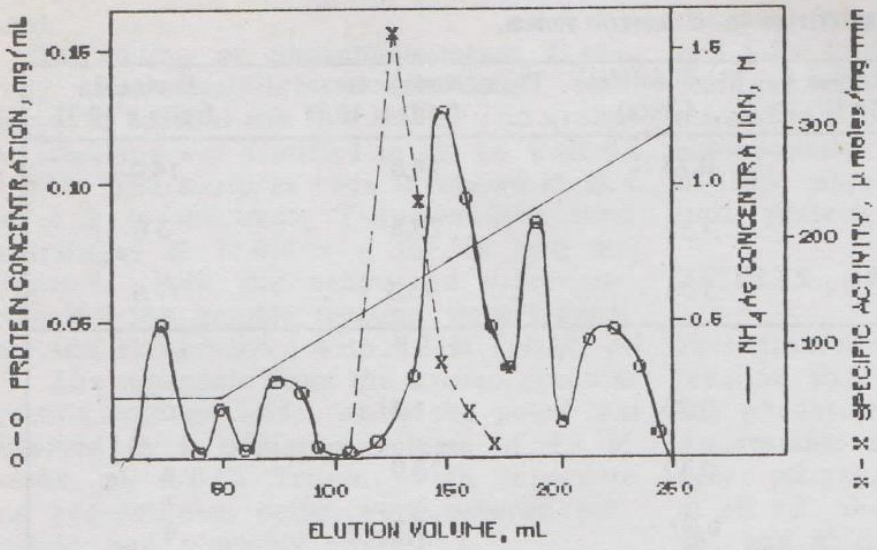

Fig. 2. Chromatography on DEAE-Cellulose of lyophilized and reconstituted phosphodiesterase I fractions from Sepharose 4B. The sample (7.36 mg protein) was loaded onto a 2.0 x 17 cm column and charged with 0.2 M NH_4Ac, pH 8.5. Elution was continued with a gradient of 0.2-1.2 M NH_4Ac, pH 8.5 (2 x 100 mL).

fractionations. While all the purification steps resulted in 18% recovery of enzyme activity, the specific activity of the preparation resulted only in 176.5 μmoles p-nitrophenol/mg-min.

Much of the activity could have been lost through surface denaturation, especially in the DEAE-Cellulose fractions. The assay mixtures of the purified enzyme were of very low protein concentration and a large fraction of the enzyme molecules may have found their way to the interface where inactivation occurred. Frothing may have aggravated the situation. This could be reduced by the addition of albumin to the assay mixture making both types of molecules compete for the surface space.

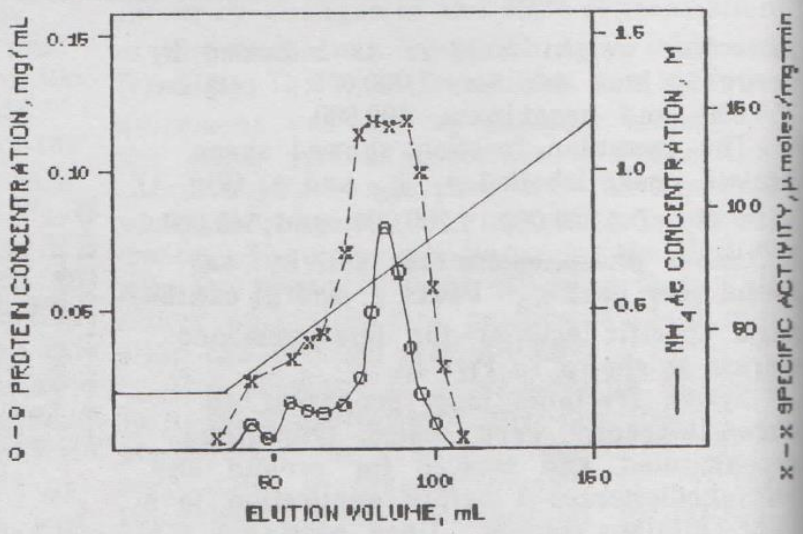

Fig. 3. Chromatography on DEAE-Sephadex of lyophilized and reconstituted phosphodiesterase from DEAE-Cellulose. The sample (1.76 mg protein) was loaded on a 2.0 x 20 cm Sephadex column equilibrated with 0.2 M NH_4Ac, pH 8.5. Proteins were eluted with a 0.2-1.2 M NH_4Ac, pH 8.5 gradient at a flow rate of 0.5 mL/min. Fractions of 5 mL were collected and assayed for enzyme activity.

Table 2. Purification of phosphodiesterase I from *C. textile* venom.

Purification Step	Protein (mg)	Specific Activity (μmoles/mg-min)	Purification-fold	Total Activity (units)	Recovery (%)
10% granular extract	79.20	22.5	1.0	1780	100
pellet, incubated extract	34.30	30.3	1.4	1040	58
Sepharose 4B	7.35	84.8	3.8	624	35
DEAE Cellulose*	1.76	176.5	7.9	311	18

Some activity may also have been lost during lyophilization. Dialysis as a concentration step ought to be considered as an alternative. If phosphodiesterase I activity is proven to be lost through dialysis, this may mean that some activators or cofactors associated with the crude enzyme are lost through the walls of the membrane during dialysis.

The active fractions from the DEAE-Sephadex column were used in characterizing the enzyme. SDS polyacrylamide gel electrophoresis showed a single peak with a subunit M_r of 60,000. Rechromatography on a Sepharose 4B column of a fraction of the active DEAE-Sephadex fraction gave a protein with an approximate M_r of 1,200,000. This suggests a conglomeration of twenty different subunits to form the 1,200,000 dalton molecule. Isoelectric focusing yielded a sharp band of proteins at pH 7.8- 7.9. K_m and V_{max} values were calculated from Eadie-Hofstee (Fig. 4) and Lineweaver-Burke plots. The values obtained by the method of least

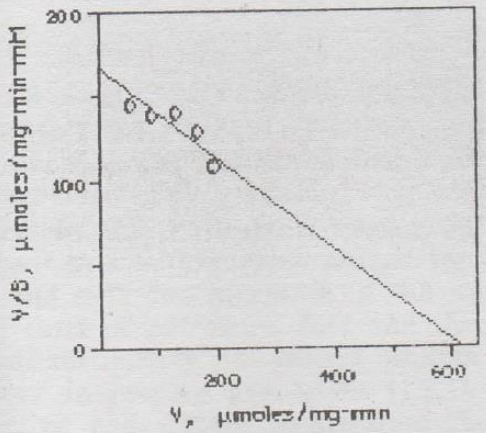

Fig. 4. Eadie-Hofstee diagnostic plot for C. textile phosphodiesterase I. Specific activity of the purified enzyme was measured at varying concentrations of p-nitrophenyl thymidine-5'-phosphate. The values were calculated to be 3.7 x 10^{-3} M for K_m and 608 μmoles p-nitrophenol liberated/mg protein-min for V_{max}. Each point represents the mean value of four determinations.

squares were comparable with an average K_m value of 3.85 x 10^{-3} M. The V_{max} from both methods gave an average of 629 μmoles p-nitrophenol liberated/mg protein-min.

The optimum values of pH, temperature, and Mg^{++} concentration for the assay of the partially pure enzyme were determined to be : optimum pH , 8.8 ; and optimum temperature, 40°C. These values are slightly lower than the values obtained with the crude extract. This is probably so because all the Mg^{++} initially present were washed out by the sequence of column elutions. Dependence of enzyme activity on Mg^{++} concentration became apparent in the pure enzyme.

5'-Exonuclease of snake venoms is a glycoprotein. If the same could be said of the *Conus* enzyme, it is conceivable that a significant amount of interaction exists between the carbohydrate residues of the enzyme and the galactose moieties constituting the agarose matrix. Also, charged carboxyl groups and sulfate groups (from agaropectin contaminants) may act as weak cation exchangers further retarding the elution of glycoprotein molecules.

Affinity chromatography has been used in the isolation of snake venom phosphodiesterase. The efficiency of this step is derived from the glycoprotein nature of snake venom phosphodiesterase. Purification of this type yields as high as 35% with an accompanying 350-fold increase in specific activity. If *C. textile* phosphodiesterase I is indeed a glycoprotein, affinity chromatography in combination with gel filtration and ion exchange could enhance the degree of purification of the enzyme. ※

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