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Cardiotoxin of the Indian cobra (*Naja naja*) is a pyrophosphatase

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Abstract. An inorganic pyrophosphatase has been purified to apparent homogeniety from Indian cobra (*Naja naja*) venom, with a ten-fold increase in specific activity. The enzyme activity is intrinsic to a protein fraction in the venom which is normally termed cardiotoxin, cobramine, cytotoxin and so on. The enzyme shows a low K_m (70 μM) and high heat stability. The enzyme was active against sodium pyrophosphate; it also hydrolyses a few mononucletides and sugar phosphates at much lower rates. The physiological significance of inorganic pyrophosphatase in venom is discussed.

Keywords. Cobra venom; *Naja naja;* cardiotoxin; pyrophosphatase.

Introduction

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, E.C.3.6.1.1) is not a hitherto acknowledged constituent of Indian cobra (*Naja naja*) venom, which is known to contain many other enzymes (Jimenez-Porras, 1970). Amongst the known enzymes, are two which have the ability to hydrolyze the pyrophosphate linkage in ATP, NAD^+ and coenzyme A (Kaye, 1960; Jimenez-Porras, 1970). We had earlier noted the presence of inorganic pyrophosphatase activity in both crude venom and in several basic protein fractions obtained from the venom (Achyuthan and Ramachandran, 1980). In this paper, we report the pyrophosphatase nature of one of those proteins (Fraction X), which is none other than cardiotoxin, a well-known non-neurotoxic protein of cobra venom.

Materials and methods

Crude venom of Indian cobra (*Naja naja*) (Batch No. 195-210) was obtained from Haffkine Institute, Bombay. Cobramine B (twelve year-old sample) was a gift from Dr. J. Wolff of the National Institute of Health, Bethesda, Maryland, USA. All other chemicals used were of analytical grade. All solutions were made in glass-distilled water.

Isolation and purity of Fraction X

Cobra venom was fractionated on columns of CM-Sephadex C-25 as described previously (Achyuthan *et al.,* 1980). Fraction X, the protein under study, is highly basic and eluted as the penultimate peak under the chromatographic conditions used. It had been found to be homogeneous on rechromatography and disc gel electrophoresis under conditions earlier described (Achyuthan *et al.,* 1980).

Enzyme assay

Inorganic pyrophosphatase activity was assayed according to Heppel (1955), using sodium pyrophosphate as substrate. The reaction mixture contained 0.5 mg enzyme protein, 1 μmol of pyrophosphate and 1.1 μmol of $MgCI_2$ in a final volume of 1.0 ml of 0.05 M Tris-HCl buffer, pH 7.2. At the end of 15 min of incubation (at 37° ±1°C), the inorganic phosphate liberated was estimated by the method of Fiske and Subbarow (1923). In all assays, appropriate corrections for controls were made. The specific activity of pyrophosphatase is defined as micromol of phosphate liberated per minute per mg of protein.

As traces of 5'-nucleotidase activity had been earlier found (Achyuthan and Ramachandran, 1980) in Fraction X, assays for nucleotidase activity at optimal substrate concentration (ten-fold higher, relative to pyrophosphate) were done. Phosphatase assays also were done at a ten-fold higher substrate concentration.

K_m and V_{max}

The Michaelis constants were calculated according to the direct linear plot of Eisenthal and Cornish-Bowden (1974).

Results

Purification and properties

Fraction X with the pyrophosphatase activity is obtained in an yield of 15.4% of the weight of crude venom. The specific activity (*V*max under standard conditions) corresponded to 0.12 I.U. indicating that a ten-fold purification had been achieved by fractionation of the crude venom. The enzyme activity in solution is stable for upto one month if kept in the frozen state. A twelve year-old Cobramine B sample (Larsen and Wolff, 1967) stored in the lyophilized state still displayed 50% of the activity shown by Fraction X. The enzyme has a broad pH optimum (pH 6.5-7.5). About 50% of this activity was evident even at the lower pH of 5.5 and the higher pH of 9.5. The reaction was zero order upto 50% hydrolysis of substrate.

Substrate and other effects

Above a final concentration of 6 mM sodium pyrophosphate the activity decreases and is about 40% at a pyrophosphate concentration of 16 mM (figure 1). Otherwise, the activity is constant over the.range of substrate concentration, 0.2-6 mM.

The effect of metal ions, tris, chelators, thiols and some anions is shown in table 1. High concentrations of many metal ions and anions inhibit the enzyme activity to varying extents. The activity is stable over a broad range of buffer (tris) concentrations, full activity being retained even upto 0.8 M tris. Chelating agents, thiols and reagents for thiol groups have no effect on enzymatic activity (table 1). The protein contains no thiol groups as can be ascertained by reaction with *p*-hydroxymercuribenzoate by the method of Boyer (1954).

The enzyme is optimally active between 40^0C and 60^0C, below and above which the activity declines. The enzyme activity is remarkably stable, withstanding

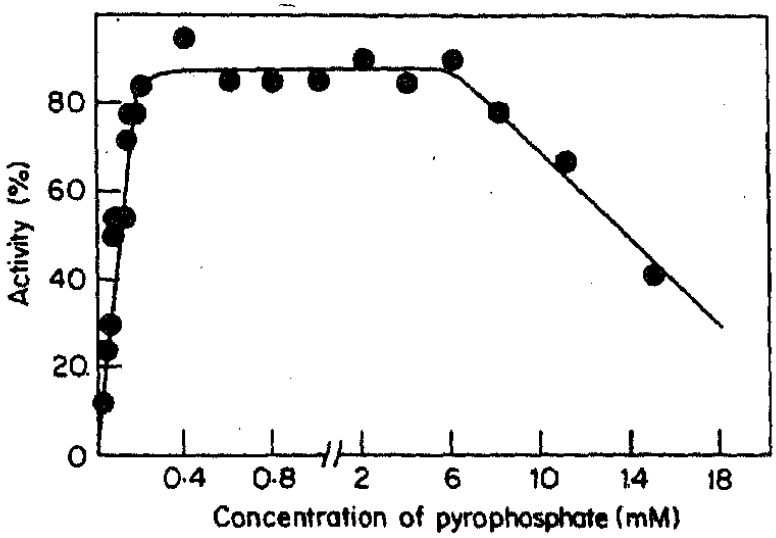

Figure 1. Effect of substrate concentration on enzyme activity. The enzyme (0.5 mg) was incubated with varying concentrations of sodium pyrophosphatase (upto 15 μmol/ml). Other conditions of incubation are described in the text. At the end of 15 min the inorganic phosphate liberated was estimated.

Table 1. Effect of metal ions, anions, tris, chelators, thiols and thiol reagents on *Naja naja* pyrophosphatase

Ingredient	Concentration (mM)	Inhibition* (%)
Metal ions:		
Cu^{2+} and Ag^{+}	0.5	65-73
	10	70-88
Ca^{2+}	0.5	68
	10	83
Zn^{2+}, Cd^{2+} and Hg^{2+}	0.5	70-75
	10	77-90
Mn^{2+}	0.5	57
	10	69
Ni^{2+} and Co^{2+}	0.5	24-73
Anions:	10	57-77
WoO_4^{2-}	10	17
MoO_4^{2-}	10	24
Oxalate	10	10
F^{-}	10	82
CN^{-}	10	50

Thiols (cysteine, reduced glutathione, and dithiothreitol) at 10 mM have no effect on activity at pH 7.0. N-Ethylmaleimide and iodoacetate (0.01 to 10 mM) have no effect.

Tris-HCl (pH 7.5), upto 0.8 M and the metal chelators, 8-hydroxyquinoline, and α, α′-bipyridyl and ethylene-diaminetetraacetate, at concentrations upto 10 mM have no effect on enzyme activity.

* Values for Zn^{2+}, Cd^{2+}, Cu^{2+} and Co^{2+} and for WoO_4^{2-} and MoO_4^{2-} are corrected for protein precipitation. Mg^{2+} (1-10 mM) has no effect on activity.

heating at 60°C upto 30 min without any loss in activity (figure 2). It also retains 80% of its activity after heating for 10 min at 90°C, after which time however, the enzyme is rapidly inactivated (figure 2). The Q_{10} values obtained are 1.8 (20°C-30°C) and 1.1 (30°C-40°C). The energy of activation calculated from the Arrhenius plot is 10.12 Kcal/mol.

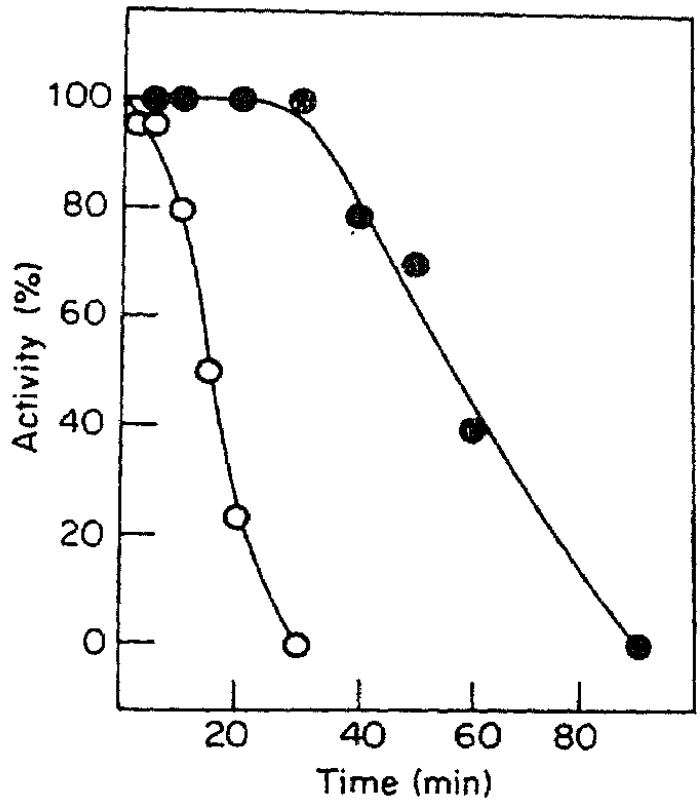

Figure 2. Effect of heat treatment at 60°C (●) and 90°C (O) on enzyme activity. The enzyme solution was heated in a water bath at the desired temperature (±1°C) for different durations. The solutions were taken out, cooled rapidly and assayed for pyrophosphatase activity as described in the text.

Substrate specificity

Besides pyrophosphate, the enzyme also hydrolyzes a few nucleotides and sugar phosphates, though at markedly reduced rates (table 2). The K_m value for hydrolysis of UMP (uridylic acid) was 100-fold higher than that for pyrophosphate and the corresponding V_{max} was only a third.

Discussion

No studies of inorganic pyrophosphatases in cobra venoms exist. Johnson *et al.* (1953) had observed the existence of very weak inorganic pyrophosphatase activity in cobra venom. They had observed that one mg of venom will hydrolyze 1.5 µmol of pyrophosphate in *six days.* The prolonged incubation period would make one wonder whether the observed activity was intrinsic to venom. The present study demonstrates beyond doubt the existence of inorganic pyrophosphatase in cobra venom. The enzyme activity is associated with a basic protein which is also a toxin.

The pyrophosphatase described here is "neutral" and displays a broad pH optimum of 6.5-7.5. Inorganic pyrophosphatases with acidic, neutral and alkaline

Table 2. Organic phosphates and pyrophosphate as substrates for *Naja naja* pyrophosphatase

Compound	Effectiveness*
Pyrophosphate	100
Cytidylic acid (CMP –3′)	25
Adenylic acid (AMP –5′)	25
Uridylic acid (UMP –5′)	22
Guanylic acid (GMP –5′)	25
Glucose–1–phosphate	13
Glucose–6–phosphate	13
Others**	0

* The effectiveness of the enzyme on sodium pyrophosphate under standard assay conditions is taken as 100. Under standard assay conditions, other substrates are scarcely attacked. The values entered in the table are obtained at ten-fold higher substrate concentration (see text).

** Several other organic phosphates (viz. thymidylic acid (TMP –5′), cyclic–AMP, adenylic acid (ATP -5′), cytidylic acid (CTP -5′), inosinic acid (ITP -5′), uridylic acid (UTP -5′ and UDP -5′), cofactors (FMN, FAD, NADP, TPP), glycerophosphates (α– and β–), fructose –1, 6 -diphosphate and p-nitrophenyl phosphate) which were tested were not hydrolyzed.

pH optima are known (Butler, 1971; Josse and Wong, 1971). The Michaelis constant (70 μM) is quite low and indicates high affinity of the anionic substrate for the cationic enzyme protein. Fraction X (Cardiotoxin II) is a highly basic protein. It is possible that basic amino acid residues in the protein are involved in substrate binding. A similar high affinity for substrate has been observed for several other pyrophosphatases (Bailey and Webb, 1944; Naganna *et al.,* 1955). Unlike pyrophosphatases of yeast, brain and rat liver which are inactive at pyrophosphate concentrations of 0.5-3 mM, the venom enzyme remains unaffected at concentrations upto 6 mM.

Most pyrophosphatases have an absolute requirement for divalent metal ions, usually Mg^{2+}, for activity (Butler, 1971; Josse and Wong, 1971). Cobra venom pyrophosphatase (Fraction X) is neither activated by added Mg^{2+} nor inhibited by ethylenediaminetetraacetate (EDTA) (table 1). Varying the ratio of the concentrations of Mg^{2+} to pyrophosphate widely in an effort to enhance enzyme activity turned out to be of no consequence. High concentrations (1-10 mM) of two other chelating agents (table 1) have no inhibitory effect. Fluoride (10 mM) causes 82% inhibition of the enzyme. Instances are known where inhibition by fluoride is related to removal of essential cations such as Ca^{2+} and Mg^{2+}, or to formation by F^- of complexes with substrates or products which then inhibit the enzyme.

The inhibition by other metal ions and anions (table 1) follows set patterns reported by earlier workers (Naganna *et al.,* 1955; Butler, 1971; Josse and Wong,

1971) and in part may be accounted for by protein precipitation, complexing with pyrophosphate and other ill-defined interactions. Inhibition by cyanide amounts to 50%, and this may have to do with the stability of four disulfide bonds which are known to be present in the protein of Fraction X (Srinivasa, Achyuthan and Ramachandran, unpublished data) which lacks thiol groups. Thiols and reagents for thiol groups have generally no effect on enzyme activity (table 1). Thus, cobra venom pyrophosphatase is not an – SH enzyme, unlike some known pyrophosphatases (Butler, 1971).

Cobra venom pyrophosphatase (Fraction X) is remarkably stable to heat (figure 2). Cardiotoxin II (Cobramine B) activity also is known not to suffer attrition in toxicity on heating for moderate durations (Larsen and Wolff, 1967). In so far as heat stability is concerned, Fraction X shares this property with the pyrophosphatases of *E. coli* studied by Josse & Wong (1971) and of *Proteus vulgaris* studied by Swartz *et al.* (1958).

Pyrophosphatases studied hitherto show varying degrees of substrate specificity (Butler, 1971; Josse and Wong, 1971). The venom enzyme also attacks at slow rates some mononucleotides and two sugar phosphates (table 2).

What is intriguing is that cobra venom pyrophosphatase (Fraction X) is also a toxin in its own right. (Achyuthan *et al*., 1980; Achyuthan and Ramachandran, 1980). It closely resemble the group of basic polypeptides severally named cardiotoxin, cytotoxin and cobramines (Jimenez-Porras, 1970). All these are related (similar, if not identical) polypeptides having low toxicity and strong cardiotoxic activity (Jimenez-Porras, 1970). Evidence based on a variety of chemical, physical, biological and enzymatic properties of Fraction X and Combramine B points to their close identity (Achyuthan *et al.,* 1980). The amino acid composition of Fraction X is also similar to that reported for the cardiotoxin group of polypeptides (Srinivasa, Achyuthan and Ramachandran, unpublished data). The pronounced inotropic effect of Fraction X on isolated ventricle muscle is similar (unpublished data) to that of cardiotoxins. Disc electrophoresis of Fraction X, Cobramine B and a mixture of the two proteins show identical mobilities at pH 4.2 and 6.0 (figure 3), and at pH 7.5 and 8.0 (not shown). Both Fraction X and a twelve year-old sample Cobramine B, isolated by Larsen and Wolff (1967) by a different procedure, and tested by us, display pyrophosphatase activity, with the latter retaining half as much specific activity (0.06 I.U./mg) as the former. The name cardiotoxin seems appropriate for protein fractions IX and X, in view of the well-known cardiovascular effects produced by such basic polypeptides of venom, and for future reference we would like to identify the later eluting protein fraction, X, as Cardiotoxin II.

Whether the pyrophosphatase activity is causally related to the toxicity of cardiotoxin is hard to say that the moment. Also, the physiological significance if any, of inorganic pyrophosphatase *per se* in venom is not quite clear. Fractions IX and X, which are active in this manner, account for nearly one third the weight of crude venom. Venom pyrophosphatase can indeed contribute to near complete hydrolysis of certain important cellular constituents like ATP and ADP by the respective hydrolytic enzymes present in venom, by further hydrolysis of pyrophos-

phate released as product by such enzymes. This would naturally deplete the cell of vital compounds.

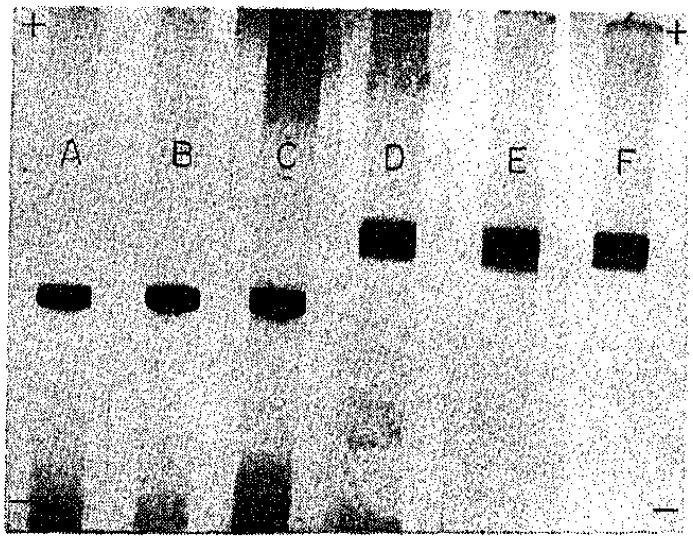

Figure 3. Disc electrophoresis of Fraction X, Cobramine B and a mixture of the two proteins at pH 4.2 (A, B and G) and at pH 6.0 (4, 5 and 6). Electrophoresis was carried out on 7.5% gels with tris-glycine (pH 8.5) as the matrix buffer. Current: 4 mA/tube.

Electrolyte: 0.2 M acetate buffer (pH 4.2) or 0.2 M citrate phosphate buffer (pH 6.0). Time of run: 60 min (for 1, 2 and 3 gels) and 90 min (for gels 4, 5 and 6). The gels were stained for 5 min in 1% Amido Black 10B in 7% acetic acid and destained with 7% acetic acid.

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