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ISOLATION AND BIOLOGICAL PROPERTIES OF SCORPION VENOM PROTEINS: IV-COMPARATIVE STUDY ON FOUR PROTEINASES ISOLATED FROM THE VENOM OF *ISOMETRUS VITTATUS*

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ABSTRACT:

1) Four proteinases were isolated from the venom of a local scorpion species Isometrus vittatus collected from Sindh region, Pakistan and named PRO,^{IA} PRO^{IB}, PRO^{IVA} and PRO^{IVB}
2) Successive chromatography on Sephadex G-50, CM-Cellulose and Sephadex G-100 yield 25.5 mg, 20.5 mg, 24.0 mg and 20.0 mg of PRO,^{IA} PRO^{IB}, PRO^{IVA}, and PRO^{IVB} respectively.
3) The purified toxins were homogenous by gel filtration chromatography and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) giving single peak and band with an apparent molecular weight of 80.0 kda, 75.5 kda, 19.0 kda and 17.0 kda by gel filtration and 81.0 kda, 74.7 kda, 18.25 kda and 17.2 kda by ¸SDS-PAGE.
4) PRO,^{IA} PRO,^{IVA} and PRO^{IVB} have LD_{50s} of 1.27 mg/kg, 1.91 mg/kg, 1.68 and 1.70 mg/kg respectively. The former two also caused marked alteration of serum enzyme and chemical constituent levels at sublethal dose whereas PRO^{IVA} and PRO^{IVB} were found to be less effective.
5) Enzymatic and biological activities of all four were inhibited by heating at 62°C. Both activities were also inhibited by 1.8 mM EDTA and 1.2mM PMSF. Activities were enhanced by Ca^{2+} but retarded and inhibited by Cu^{4+} and $Mg^{2+,}$ respectively.

Introduction

Isolation and purification of several toxins with enzymtic and non-enzymatic activities from scorpion species have been reported during past three decades (Minton, 1974; Tato *et al.,* 1978). But most of the components isolated were small molecular weight neurotoxins (Wright *et al*, 1977). The lethality and toxicity of scorpion venom is reported to be due to these neurotoxins in general and some enzymatic components in

particular. But the isolation of enzymatic and enzyme-like components have not been carried out in detail. However, non-enzymatic but extremely neuromxic components have been isolated from several medically important scorpion species including *Andro*c*tonus australis, Butlus occitanus tunetanus, Leiurus quinquestriatus quintquestriatus* and *Centruroides lmpidus limpidus* (Miranda *et al*., 1971; Tato *et al.,* 1978).

During past three decades, extensive enzymological studies have been carried out on *Palamens gravimanus* (Wright *et al,* 1977), *Tityus serrulatus* (Alagon *et al,* 1978). *Buthus tamulus, Vejovis spinigerus, Scorpio maurus, Buthacus arenicola* and *Hadrurus arizonensis* (Minton, 1974). Their venom were reported to contain alkaline phosphates, phosphodiesterase, 5-nucleotidase, protease, chloinesterase, acetylchloineaterase and lecithinasc (Minton, 1974). The present paper reports the isolation and biological activities of four proteinases from the venom of *Isometmv vittatus.*

Materials and Methods

Materials:

Whole venom was obtained by electrical milking of 60 specimen of *Isometmv vittatus* collected from interior of Sindh, Pakistan and lyophilized Sephadex G-50, Sephadex G-100, CM-Cellulose, phenyl methyl sulfonyl floride (PMSF), egg-yolk lecithin, human umbical cord hyaluronic acid, calibration kits for molecular weight determination were obtained from Sigma Chemical Company (St. Louis, M.O.), diagnostic kits of serum enzyme substrate were products of Boerhinger Chemicals (Germany), 45 pm membranes filter was purchased from Millipore Inc. (USA). All other reagents used were of analytical grade.

Enzyme activities:

Hyaluronidase, alkaline phosphomonoestorase, phospholipase, acetyl-cholinesterase and proteinase activities of whole venom and isolated fractions were determined by the methods described earlier (Aird and Da Silva, 1991; De Hass et al, 1968; Ellman *et al*, 1961) using human umbical cord hyaluronic acid, σ-nitrophenyl phosphate, egg-yolk lecithine, acetylthiocholine and casein respectively, as substrate.

Protein estimation:

Total protein content of crude as well as isolated fractions were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Isolation procedure:

All fractionation steps were performed at 10°C.

Gel filtration on Sephadex G-50:

Lyophilized crude venom (600 mg) was dissolved in 3ml 0.2N1 ammonium acetate buffer (pH 8.2) and insoluble material was removed by centrifugation and filtration through micropore membrance filters. Supernatant was applied to a column of Sephadex G-50 (2.5 x 85.0 cm) equilibrated with 0.2M ammonium acetate buffer (pH 8.2). The elution was carried out with same buffer at a flow rate of 20ml/hour. Fractions of 4ml were collected and all fractions corresponding to each peak (or the portion of highest enzymatic activity) were pooled and lyophilized. Standard gel filtration markers were also run on same column under similar condition for calibration.

Ion-exchange chromatography:

CM-Cellulose column (3.0 x 25.0 cm) was equilibrated with 0.2M ammonium acetate buffer (pH 8.2). 94.0 mg and 90.0 mg of lyophilized fractions of peaks I and IV with proteinase activity from Sephadex G-S0 (previous step) was dissolved in 2.0 ml of 0.2M ammonium acetate buffer (pH 8.2) and applied to the column. Elution was carried out with a linear gradient from O.OM to 0.3M sodium chloride in buffer for peak I while from O.oM to 0.4M NaCl in case of peak IV. Two fractions each from I (I_A and I_B) and IV (IV_A and IV_B) were obtained. All four were separately pooled lyophilized, redissolved and again fractioned on same column at a flow rate of 30 ml/hr and 4 ml fractions were collected.

Gel filtration on Sephadex G-100:

Isolated fractions of I_A, I_B, IV_A and IV_B, giving single peaks on CM-Cellulose column in previous step was loaded on to a column of Sephadex G-100 (2.5 x 90.0 cm) equilibrated and eluted with 0.2M ammonium acetate buffer (pH 8.2). The column was calibrated with standard gel filtration markers under similar conditions.

Molecular weight determination:

Gel filtration

Approximate molecular weights were determined by calibrating gel filtration column of Scphadex C-50 and Sephadex G-100 with-bovine serum albumin (66,000), carbonic anhydrase (29,400) and cytochrome C (12,200). Calculations was done by plotting Ve/Vo of known proteins against their molecular weights.

SDS-polyacrylamide gel electrophoresis:

Molecular weight determination was also carried out with SDS-PAGE according to the method of Laemmli (1970) on 10.0% gel. Bovine serum albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphotale dehydrogenase (36,800), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100) and α-lactalbumin (14,200) were used to form the standard curve.

Biological activity:

Lethality

Lethality was determined by administration (i.p.) of a 2.0 ml/kg dose containing 1.0 mg/ml of either crude venom or purified fraction in albino rats of either sex (150-200 gm). Each experiment was repeated twice with six rats at each dose.

Effects on blood components:

Sublethal (1/3 LD_{50}) of purified fractions was administered intraperitoneally in albino rats (150-200 gm) of either sex. Blood was collected after 24 hours and scrum was obtained. Serum levels of creatine phosphokinase, lactate dehydrogcnase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphotase, β-amylase, cholesterol, triglyceride, total lipid, direct bilirubin, total bilirubin, total protein, albumin and creatinine were analysed by Hitachi 705 biochemical autoanalyzer using standard kits of enzyme substrate from Boehringer (Germany) according to the instructions of manufacturer. Experiment was repented thrice and results were expressed as means + S.E.M.

Chemical and physical stability:

Crude venom as well as purified proteinases were examined for their chemical and physical stability after heating at temperature from 40°C to 90°C in disodium hydrogen phosphate/sodium dihydrogen phosphate buffer (pH 6.4) for 10-30 min. The effect of ethylene diamine tetraacetic acid (EDTA), PMSF and some divalent ions on the activity of proteinases were also examined.

Results

Isolation of proteinases (I_A, I_B, IV_A, IV_B):

Crude venom (600 mg) from *I. vittatus* was applied to a Sephadex G-50 column and

eluted with 0.2M ammonium acetate butler (pH 8.2) which give seven peaks (Fig. 1). Proteinase activity was found in peaks 1 and IV while alkaline phosphomonoesterase in peak VII and VIII. Peak I (93.0 mg) with proteinase activity of 2.57 units/mg was subjected to chromatography on CM-Cellulose (ion-exchange) equilibrated with 0.2M ammonium acetate buffer (pH 8.2) with a linear gradient of 0.0M to 0.3M sodium chloride (Fig. 2, Setp 1). Two peaks I_A and I_B were obtained. The former have 3.0 units/mg and the later 2.75 units/mg of proteinase activity and lethality of 1.90 mg/kg and 1.25 mg/kg respectively.

PRO I_A (22.0 mg) was again loaded on to CM-Cellulose column and eluted under similar conditions (Fig. 2, Step 2) thus giving single peak with no change in enzyme activity. For final purification PRO IA (19.0 mg) was rechromatographed on Sephadex G-100 and eluted with 0.2M ammonium acetate buffer (pH 8.2) (Fig. 2, Step 3). No increase in specific activity was observed but a 1.5-fold increase in lethality was noted (LD_{50} = 1.28 mg/kg). Similarly PRO In (28.0 mg) from step I (Fig. 2) was fractionated on CM-Cellulose column with a linear gradient of TOM to 0.3M sodium chloride in 0.2M ammonium acetate buffer (pH 82) (Fig. 2, Step 4). A single peak was obtained. Final purification was achieved on Sephadcx G-100 (Fig. 2, Step 5) with 0.2M ammonium acetate buffer (pH 8.2), with an increase in enzyme activity of 3.25 units/mg.

PRO IV (90.0 mg) was also subjected to CM-Cellulose column (Fig. 3, Step 1) under same conditions as PRO I_A. Rechromatography of the fractions (85.0 mg) on same column with a gradient of 0.0M to 0.4M NaCl gives two peaks IV_A and IV_B with former having 4.4 units/mg and the later 4.3 units/mg of proteinase activity (Step 2, Fig.3). Pro IV_A (33.0 mg) was chromatographed on CM-Cellulose column and eluted with, 0.2M ammonium acetate buffer (pH 8.2) with 0.0M to 0.4M NaCl in buffer. A single peak was noted (Step 3, Fig.3), 34.0 mg of PRO IV_B was also treated in same way and a single peak was noted with no increase in enzyme activity (Step 4, Fig. 3). For final purification both PRO IVA and IV_B were rechromatographed on Sephadex G-100 (Step 5, 6, Fig. 3).

Molecular weights:

The molecular weight of peak I and IV from step 1 (Fig. 1) were approximately 8L.0 kda and 18.0 kda by calibration on Sephadex GSO column. Calibration was carried out with a series of standard proteins with known molecular weights. Molecular weights of PRo I_A, Ip, IV_A and IVu were found to be 80.0 kda, 75.5 kda, 19.0 kda and 17.0 kda after final purification on Sephadex G-100 (Fig.4). Their molecular weights coincide with those determined by SDS-PACE on 10.0% gel which were 81.0 kda, 74.7 kda, 18.25 kda and 17.2 kda respectively (Figs).

Biological properties:

LD_{50} of crude venom was found to be 2.8 mg/kg while that of purified PRO I_A, I_B, IV_A and IV_B, 1.27 mg/kg, 1.91 mg/kg, 1.68mg/kg and 1.70mg/kg respectively. Purification on Sephadex G- 100 leads to 2.24-4.0 increase in lethality as compared to crude venom.

All four proteinase also caused marked alteration in serum enzyme and biochemical component level. Serum enzyme levels were increased owing to administration of sublethal dose (1/3 LD50) of both toxins. The animals survived only upto 24 hr in cases of PRO IV_B thus exhibiting an active lethal potency but a low ability to induce tissue damage.

Heat and chemical stability:

All four protcinascs at a concentration of 50 mg/nil in disodium hydrogen phosphate/sodium dihydrogen phosphate buffer (pH 6.4) were monitored at various temperatures and then cooled Their enzymatic activity was then determined as described in methods. PRO IV_A and PRO IV_B were fully active unto 40°C.

The effects of PMSF, EDTA and some divalent ions were examined on protcinascs. 50 mg/ml of each proteinases were incubated with 1.0-2.0 mM EDTA and 1.0-2.0 mM PMSF. Their lethal as well as enzyme activity was inhibited by 1.8 mM EDTA and 1.2 mM PMSF. 50mg/ml of purified enzymes were incubated with 5 mM of divalent ions ($Ca,^{2+}$, Mg^{2+} and Cu^{2+}) in 10 mM acetic acid buffer containing 10 mM sodium chloride (pH 6.2) (Sugihara *et al*, 1985) for 15 min at 37°C and then analyzed for enzyme activity. Ca^{+} enhanced proteinase activity while one third of the activity was lost in the presence of Cu^{2+} and completely inhibited in the presence of Mg^{2+}

Discussion

The knowledge about the enzymes of scorpion venom is meager. Only few reports are available on the presence of specific enzymatic activities in scorpion venom (Min-ton, 1974). In this study we report the purification of four proteinases namely PRO IA, In, IV_A and IV_B from the venom of a local scorpion *I. vitatus*. When venom was subjected to Sephadex G-50 chromatography at pH 5.2, at least seven protein fractions with proteinase and alkaline phosphomonoestcrase activities were obtained. Previously Wright *et al.* (1977) reported the presence of hyaluroniduse and an alkaline phosphomonocsterase in the venom of *Palumneus gravimanus*. A high molecular weight hyaluronidase was also isolated from the venom of *Tityus serrulatus* (Alagon *et al,* 1978).

Only two reports are available on the presence of acetylcholinesterase in venom of scorpions *Vejovis spinigerus* (Russel *et al.*, 1968) and *Hadrurus arizonensis* (Saunders and Johnson, 1970). In addition, phosphodiestcrase, 5-nucleotidase and lecithinase activities were also reported for a number of scorpion species (Minton, 1974). A number of non-enzymatic toxins lethal to mammals were also isolated from G. *limpidus limpidus* (Tato *et al,* 1978). Recently six toxins with varying lethality were isolated from the venom of *A. mauretanicus mauretanicus* by HPLC, one from *T. serrulatus* and four from *T. discreoans* (Borges *et al,* 1990; Ceard *et al*., 1992; Zerrouk *et al,* 1991).

All four proteinases also induce marked alteration of serum enzyme and biochemical constituent levels suggesting a possible cytotoxie activity of these toxins. Presence of cytotoxic components in venom of snake and arthropod, including scorpion, is not uncommon and responsible for mild to severe tissue and organ damage. Acute pancreatitis in children and gastric injuries in ruts were reported for the venom of *T. trinitatis* and *L. quinquestriatus* (Soler *et al.,* 1991) and *T. serrutatus* (Cunha-Melt) *et al*, 1981). Apart from cytotoxicity, muscle paralysis, respiratory distress and convulsion was also noted 24 hr after administration of a sublethal doses of purified proteinases.

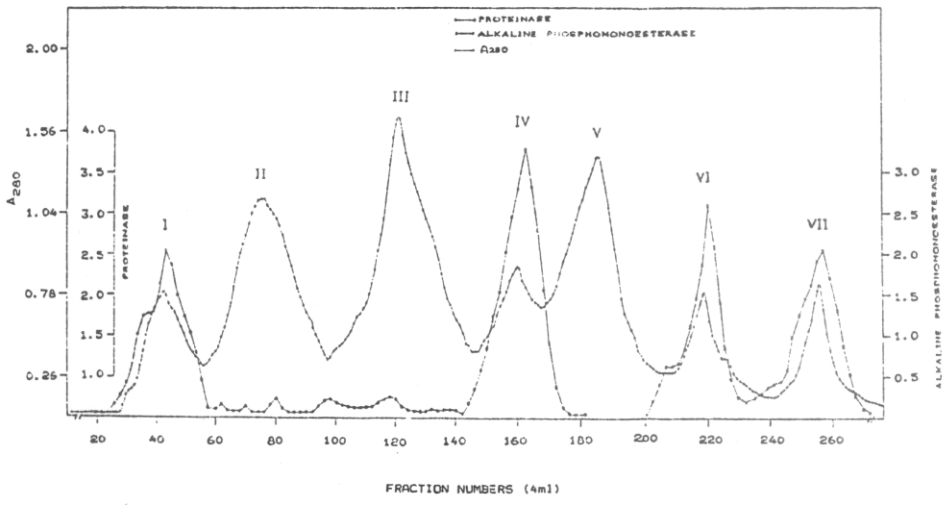

Fig. 1: Fractionation of scorpion *Isometrus vitatatus* venom; Gel filtration of 600 mg venom on a 2.5 x 85.0 cm column of sephadcx G-50 in a 07M ammonium acetate buffer (pH 8.2). Flow rate was 20m1/hour. Fractions of 4 ml were collected. Each fraction was tested for enzymatic activity as described in methods.

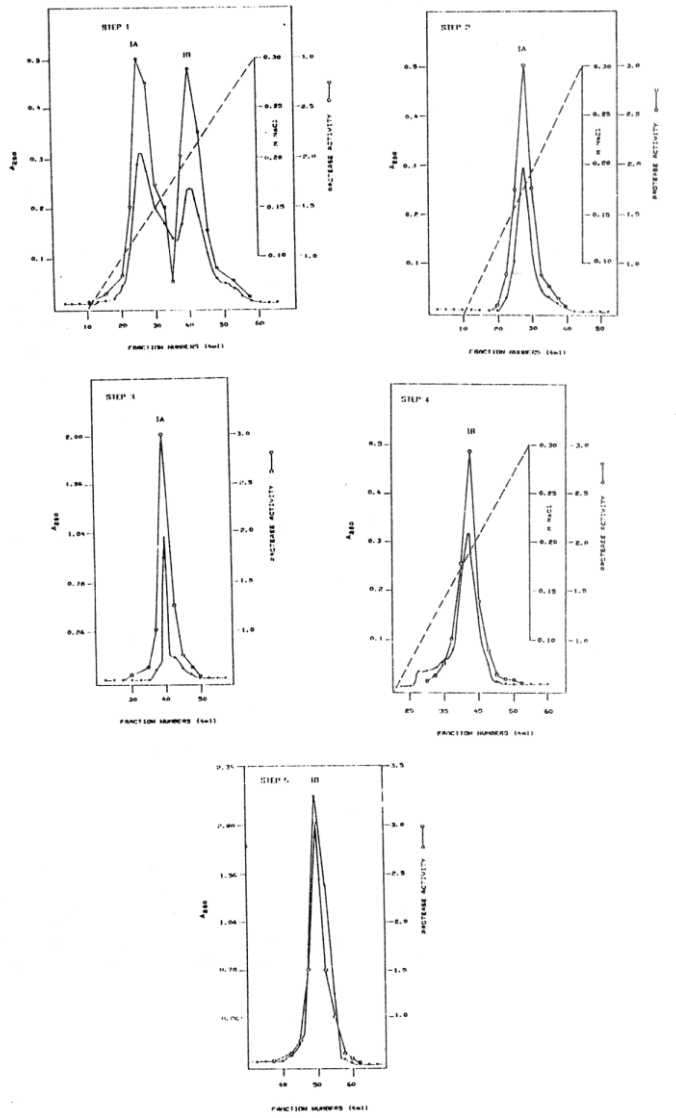

Fig. 2: Isolation scheme of proteinases I_A and I_B.

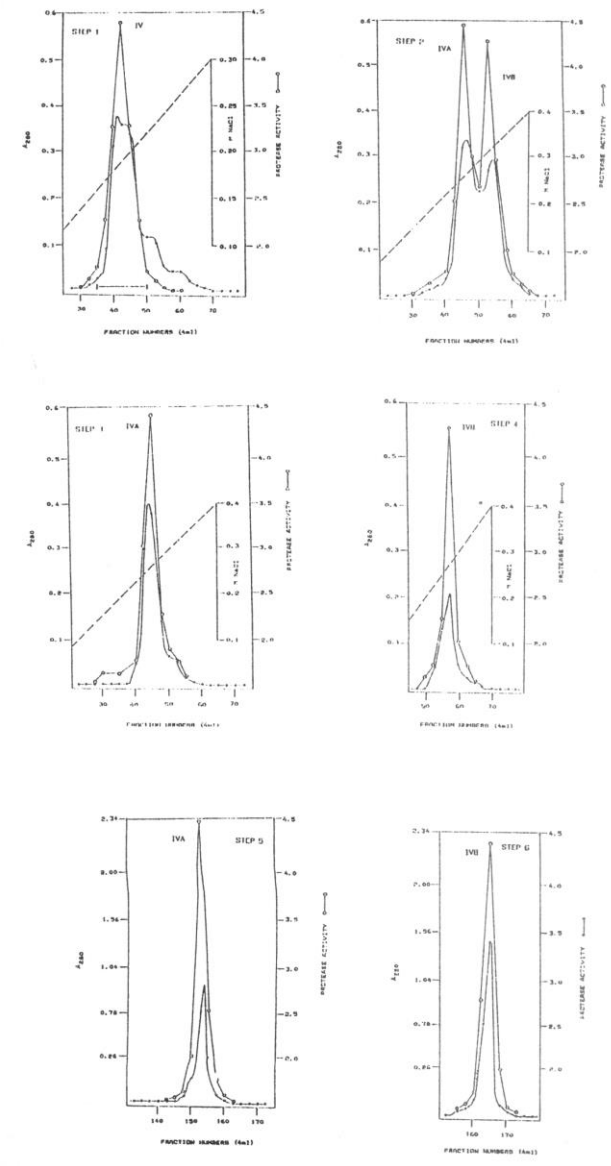

Fig. 3: Isolation scheme of proteinases IV_A and IV_B.

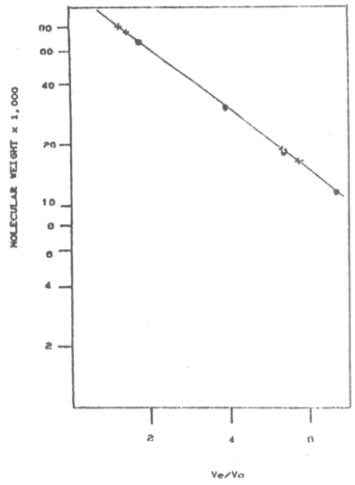

Fig. 4: Molecular weight determination of proteinases by Gel filtration on Sephadex G-100. Markers are bovine serum albumin (66 kda), carbonic anhvdrase (29.4 kda), cytochrome c (12.2 kda), in descending order. Cross indicate the point of four proteinases.

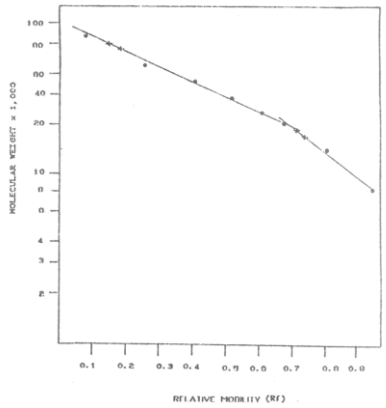

Fig.5: Molecular weight determination of four proteinases by SDS-PAGE on 10.0% gel. Standard protein markers are bovine serum albumin, Egg albumin, Glyceraldehyde-3-phosphate dehydrogenase. Carbonic anhydrase, Trypsinogen, Trypsin inhibitor and α-lactalbumin in descending order. Cross indicate the point of four proteinases.

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