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ELECTROPHORETIC SEPARATION OF BIOLOGICALLY ACTIVE CONSTITUENTS OF SCORPION VENOMS

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SUMMARY

Venoms of two species of scorpions *viz. Buthus tamulus* and *Palamneous gravimanus* were examined for their biologically active constituents after starch-gel electrophoresis.

Protease was present in both venoms, 5'-nucleotidase in *Palamneous gravimanus* venom and phosphodiesterase in *Buthus tamulus* venom only. The guinea-pig ileum-contracting factors and toxins were also identified.

Phospholipase A, cholinesterase and L-amino acid oxidase, the enzymes usually found in snake venoms, could not be detected in these venoms.

Buthus tamulus venom had two toxins one of which produced increased breathing and the other caused stiffening of the hind portion of the injected mice.

The *Palamneous gravimanus* venom had a guinea-pig ileum-contracting factor, but none of the electrophoretic fractions had a lethal effect on mice.

INTRODUCTION

The pharmacological action of venoms of a few species of scorpions have been tested[1-3]. DEL POZO[1] believed that the constituents of all scorpion venoms are basically similar and that the variations in toxicity and differences in specific pharmacological actions are due to the quantitative differences of several active chemical fractions. DINIZ AND GONÇALVES[2] have separated the active components of Brazilian scorpion venoms using filter paper electrophoresis and zone electrophoresis[5]. They found that only one component which affected the rabbit gut was toxic when injected into mice. MIRANDA AND LISSITZKY[4] have recently carried out a purification of toxins from two species of North African scorpions. The main characteristics of the toxins (scorpamins), as found by them were their pure neurotoxic nature, low molecular weight and stability on storage and to denaturing agents.

In a previous communication from this laboratory[6], we had reported the separation of enzymes and toxins from two species of snake venoms and described micro-methods for the detection of some of the enzymes in those venoms after starch-gel electrophoresis. We have extended those studies to venoms of two species of scorpions commonly found in India *viz. Buthus tamulus* and *Palamneous gravimanus*.

MATERIALS AND METHODS

Most of the methods followed in this investigation have been described in the previous communication[6], hence only a brief mention will be made of those which have been modified or of the new ones.

The venoms were supplied by the Entomology Department of the Haffkine Institute and had been obtained by electrical stimulation of the distal coil of the tail of each scorpion. Venom samples from a large number of each of the two species were pooled separately and lyophilized. A 20 % solution of the lyophilized venom of each species was made in distilled water and the solution was centrifuged at 3000 rev./min for 15 min. The supernatant was used for the electrophoretic experiments.

Some batches of the venom, especially those from *Palamneous gravimanus*, gave a highly viscous jelly-like mass though the electrophoretic pattern of the viscous venom was practically the same as the normal venom, under the same conditions of the experiment. Toxicity of the two venoms, as determined in this laboratory, on white mice weighing about 19–20 g by injecting 0.5 ml of the venom dilutions subcutaneously was found to be 1.0 mg for the *Buthus tamulus* venom and 5 mg for *P. gravimanus* venom. The toxicity of *B. tamulus* venom was 0.1 mg and that of *P. gravimanus* was 1.6 mg for suckling mice (15–16 days old and weighing about 5 g) as tested by injecting 0.5 ml of the diluted venoms intraperitoneally.

Starch-gel electrophoresis

The electrophoretic procedure was essentially the same as described previously[6]. Among the methods tried, the discontinuous system of buffers described by POULIK[7] was found to give the most satisfactory separation of the components. Different pH values were tried and pH 8.6 was found to be the most suitable for obtaining a good separation of the constituents of both venoms. Normal human serum was subjected to starch-gel electrophoresis on the same batch of hydrolysed potato starch used for the separation of the constituents of the venom samples; the separation obtained is represented in Fig. 1 for purposes of comparison. When the components were to be

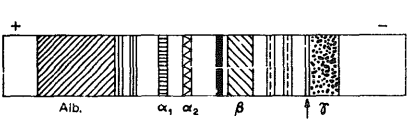

Fig. 1. Diagrammatic representation of the separated components of normal human serum.

tested on the surface of the starch gel after electrophoresis of the venom, 0.025 ml of the venom solution was applied to the starch block by a strip of Whatman No. 3MM filter paper (2 × 20 mm) and when the activity was to be tested on the eluates, 0.05 ml of the venom was applied by first adsorbing it on starch grains which were then inserted into a narrow furrow in the centre of the starch gel according to the procedure of SMITHIES[8]. Electrophoresis was carried out at about 200 V and 19–20 mA for 6 h. After the electrophoresis, a reference strip was stained with Amido Black 10 B and the remaining strips were tested for different biologically active components. The starch

blocks were cut into 10-mm segments and marked as +1, +2, +3 etc. from the point of application towards the anode side and −1, −2, −3 etc. from the same point towards the cathode side. They were left overnight in closed petri dishes at −20° and next day after thawing were pressed in a 20-ml syringe with a side nozzle. The fluid so expressed, which measured 0.3–0.4 ml, was then used for testing different enzyme activities and toxicity.

Enzyme activities

In order to obtain a good comparison with the snake venoms already reported from this laboratory[6] all the enzymes detailed in the snake venoms were looked for in both scorpion venoms.

Proteases were detected as follows. A slightly modified procedure based on that described by KOCHWA *et al.*[9] was used. X ray film (Kodak) was fixed with sodium thiosulphate and after washing it was thoroughly dried. The fluids to be tested (0.1 ml) were placed on this film and the film was kept in a closed petri dish and incubated for 5–6 h in a humid incubator at 37°. After this period, it was gently washed with tap water and dried at room temperature. After drying, a drop of Amido Black 10 B solution was applied to the spots on which the test samples had been placed and kept at room temperature for 5 min. The film strip was then washed with 1 % acetic acid and dried. The proteolytic activity was indicated by the appearance of a clear transparent zone whereas a dark blue spot of stained protein was left in the absence of proteolytic activity.

Nucleases were tested by overlaying the starch blocks after electrophoresis with 1 % agar containing 3 % yeast RNA (sodium salt) and 0.9 % sodium chloride and 0.01 % magnesium chloride. After incubation at 37° in a humid incubator overnight, the blocks were flooded with 0.5 M HCl.

Phosphodiesterase was detected by treatment of the starch blocks with 0.1 % *p*-nitrophenyl UMP. Phosphomoesterase was tested by incubation of the test fluids with sodium *p*-nitrophenylphosphate. 5'-Nucleotidase activity was tested by incubating the fluid from each segment with AMP at 37° and estimating the inorganic phosphate released[6]. The presence of coagulase, phospholipase A, cholinesterase and L-amino acid oxidase was tested as before[6].

The toxic components were located by injecting 0.3 ml of the eluate from each segment intraperitoneally into suckling white mice 15–16 days old and weighing about 5 g. The component which caused the death of mice in 24 h was taken as containing the toxin.

The toxicity tests were also repeated by injecting the eluates intravenously into white mice weighing 18–20 g. The symptoms produced were also noted.

The guinea-pig ileum-contracting factor was tested as follows: A guinea-pig weighing about 350 g was starved for 24 h after which it was sacrificed and the ileum removed and washed with 0.9 % sodium chloride. One end was fixed to the hook at the bottom of a bath containing 6 ml of Ringer solution at 37° and the other end was fixed to a light metal lever. Oxygen was bubbled through the solution at a slow rate. The contractions were recorded on a smoked rotating drum. The fluid from each electrophoretic segment was added to the Ringer solution and allowed to remain in contact with the ileum preparation for 90 sec. It was washed and after it had relaxed to the normal state, the fluid from the adjacent segment was tested. Standard

histamine solution was used to test the sensitivity of the gut before and after the experiments.

RESULTS

Comparison of the starch blocks stained for proteins and the ones tested for different biological activities was made in order to determine the positions of the different active components.

Fig. 2 gives the electrophoretic separation of the two venoms on starch gel.

The electrophoretic pattern of both venoms were fairly consistent with different batches of the venoms of the same species collected at different times though at times slight variations were observed. However, the positions of the biologically active components were the same in all experiments under identical conditions.

In the case of *B. tamulus* venom as many as nine well-defined zones could be distinguished after staining with Amido Black 10 B. Five were found to migrate to the anode side and four to the cathode side from the point of application. In the case of *P. gravimanus* venom, 5 components moved to the cathode side and 3 to the anode side. The identification of the active components is given in the legend to Fig. 2.

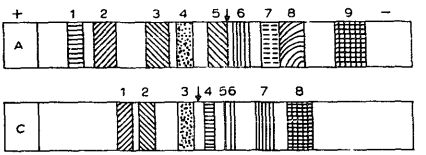

Fig. 2. Starch-gel electrophoresis of scorpion venoms. Stain: Amido Black 10 B. Arrow indicates the point of application of the venom sample. Strip A: *B. tamulus* venom. Bands No. 1, 2, 6, 9, unidentified protein components; 3, protease; 4, phosphodiesterase; 5, toxin causing increased rate of breathing in injected mice; 7, guinea-pig ileum-contracting factor (corresponding to 3 in Fig. 3); 8 guinea-pig ileum-contracting factor (corresponding to 6 of Fig. 3); 7,8 were toxic to mice. Strip C: *P. gravimanus*. 1,5'-nucleotidase; 2, guinea-pig ileum-contracting factor (segment No. 4 on anode side in Fig. 4); 5, 6, proteolytic activity; 3, 4, 7, 8, unidentified protein components.

Both the venoms had detectable proteolytic activity but their electrophoretic migration was found to be in different directions at pH 8.6.

The electrophoretic fractions of both venoms were found to have practically no effect on the coagulation time of recalcified normal human plasma. Phospholipase A could not be detected in any of the electrophoretic fractions. Similarly, cholinesterase and L-amino acid oxidase were not detected in either venom.

Phosphodiesterase activity was present only in *B. tamulus* venom in detectable amounts, though it did not degrade polymerized ribonucleic acid as tested by the RNA-agar technique.

P. gravimanus venom had a strong 5'-nucleotidase moving a considerable distance on the anode side at pH 8.6. *B. tamulus* venom had no action on AMP.

The guinea-pig ileum-contracting factor was found to move to the cathode side in the case of *B. tamulus* venom. It separated into two zones. The results are represented in Fig. 3. The toxicity experiments revealed that *B. tamulus* venom had two distinct toxins, one moving to the anode side and one moving to the cathode side. The first

component on the anode side, when injected intravenously into mice, induced vigorous breathing and restlessness in the experimental animals followed by death. It did not have a contracting effect on the guinea-pig ileum preparation.

The toxic component on the cathode side was found to coincide with the guinea-pig ileum-contracting factor. Intravenous injection of these fractions caused marked paralysis of the hind limbs up to the waist of the injected mice, followed by death.

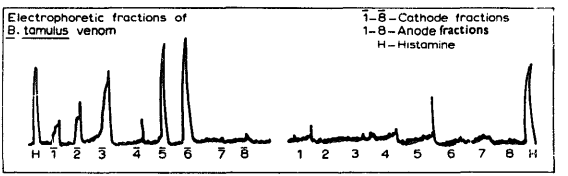

Fig. 3. Kymograph obtained by testing electrophoretic fractions of *B. tamulus* venom using guinea-pig ileum preparation. 1,2,3... etc., anode fractions; 1̄,2̄,3̄... etc., cathode fractions. H, 0.1 μg of histamine.

P. gravimanus venom also had a factor which caused contractions of the guinea-pig ileum and it moved to the anode side. However, this fraction was comparatively less active on the gut. The contractions produced by the two venoms therefore are different in nature. With the guinea-pig ileum-contracting factor of *P. gravimanus* venom few washings were required for the ileum to relax to the normal state whereas with *B. tamulus* venom fractions, several washings were required and it took more than 100 sec for the ileum to relax to its normal state. Another observation was that the contractions produced by the *B. tamulus* venom fractions were slightly delayed. The contractions produced by the electrophoretic fractions of *P. gravimanus* venom are given in Fig. 4.

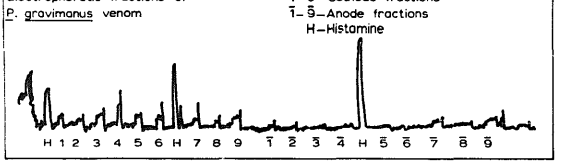

Fig. 4. Kymograph obtained by testing electrophoretic fraction of *P. gravimanus* venom, using guinea-pig ileum preparation. 1,2,3... etc., anode fractions; 1̄,2̄,3̄... etc., cathode fractions; H, 0.1 μg of histamine.

Almost all the anode fractions of *P. gravimanus* venom showed slight contraction on guinea-pig ileum preparation, fraction No. 4 corresponding to Component No. 2 in Fig. 2 showing the maximum contraction. This may be due to trailing of the active component because of the highly viscous nature of the solution of that venom.

None of the electrophoretic fractions of *P. gravimanus* venom had a lethal effect

by intravenous injection into mice weighing 18–20 g or intraperitoneal injection into suckling mice.

DISCUSSION

The results obtained clearly indicate that venoms of different species of scorpions do differ in their nature and composition. The major differences observed in the two venoms investigated are: (a) dissimilarity of their electrophoretic patterns obtained under identical conditions, (b) differences in electrophoretic mobilities of their proteolytic enzymes and (c) qualitative differences in the pharmacological activities in the guinea-pig ileum-contracting factors in the two venoms.

B. tamulus venom fractions on the cathode side produced slightly delayed contractions and were of much stronger type in both the separated factors, and resistant to several washings. The same fractions produced the lethal effect with paralysis of the posterior portion of the injected animals. The guinea-pig ileum-contracting factor was found to be separated into two zones by the electrophoretic technique. Both these zones had a lethal effect on mice. The presence of a phosphodiesterase hydrolysing *p*-nitrophenylUMP in *B. tamulus* venom is interesting. It was detected in all batches of the venom though in much smaller proportions than that in snake venoms. This enzyme could not be detected in *P. gravimanus* venom.

The presence of a strong 5′-nucleotidase in the venom of *P. gravimanus* was very striking. It moved a considerable distance on the anode side. This enzyme was present in all batches of the venom of this species and was found to occupy a position very nearly the same as the guinea-pig ileum-contracting factor. 5′-Nucleotidase was not detected in *B. tamulus* venom fractions even in highly concentrated solutions.

The most striking difference between the two venoms is their mode of toxicity. *B. tamulus* venom had two distinct toxins, one moving to the cathode side at pH 8.6 which also coincided with the guinea-pig ileum-contracting factor. This observation is in good agreement with that of DINIZ AND GONÇALVES[2,5], who also made similar observations on the venoms of two species of Brazilian scorpions. This factor produced paralysis in the injected animals which was followed by death. The other toxin in *B. tamulus* was found to move to the anode side at pH 8.6 and the intravenous injections of that fraction were followed by increased breathing and death. These symptoms were not produced by any of the fractions of *P. gravimanus* venom when injected intravenously into mice weighing 18–20 g or intraperitoneally into suckling mice.

The number of enzymes detected in both venoms is definitely smaller than that reported in most of the snake venoms. Whereas the snake venoms are believed to have a digestive role besides their killing action, the scorpion venoms primarily appear to have a defensive function.

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