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STARCH-GEL ELECTROPHORESIS OF VENOMS OF INDIAN KRAIT AND SAW-SCALED VIPER AND IDENTIFICATION OF ENZYMES AND TOXINS

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SUMMARY

Venoms of krait (*Bungarus caeruleus*) and saw-scaled viper (*Echis carinatus*) were subjected to starch-gel electrophoresis and the different enzymic and toxic principles in the separated components were identified. Krait venom was found to possess 4 toxic components which might account for its highly toxic nature whereas the saw-scaled-viper venom was found to have one toxin which was identified as a proteolytic enzyme having a strong coagulant action.

INTRODUCTION

Electrophoretic techniques such as free electrophoresis, filter-paper electrophoresis and electrophoresis on potato starch have been widely used in the analysis of snake venoms[1-5].

We have previously reported[6] the identification of enzymes and toxins in venoms of Indian cobra and Russell's viper after electrophoretic separation on starch gel.

We have extended these studies to two more venoms of Indian snakes *viz.* krait (*Bungarus caeruleus*) and saw-scaled viper (*Echis carinatus*). The present communication incorporates the results obtained.

MATERIALS AND METHODS

The venoms from a large number of kraits and saw-scaled vipers which had been pooled separately for the two species and lyophilized, were obtained from the Entomology Department of the Haffkine Institute.

Starch-gel electrophoresis was carried out according to the procedure described in detail in a previous communication from this laboratory[6]. Normal human serum was subjected to starch-gel electrophoresis using the batch of hydrolysed potato starch employed for the separation of the constituents of venom; the separation of the constituents of venom; the separation obtained is represented in Fig. 1 for purpose of comparison. Twenty percent aqueous solution of lyophilized krait and saw-scaled-viper venom was used for electrophoresis. It was first centrifuged to remove the suspended material.

0.025 ml of the venom solution, absorbed on a thin strip (20 × 2 mm) of Whatman filter paper No. 3 MM was applied to the gel. After electrophoresis, the starch blocks were cut into 10-mm wide segments and marked as +1, +2, +3 etc. starting from the point of application towards the anode and −1, −2, −3 etc, from the point towards the cathode.

These segments were kept frozen in covered petri dishes at −20° overnight and on the following day, after thawing, were pressed in a 20-ml syringe which had a side nozzle. About 0.4 ml of fluid was obtained from each segment. The fluid from each segment was tested for enzyme activity and toxicity.

The methods used for testing for coagulase, phosphodiesterase, posphomonoesterase, 5'-nucleotidase, cholinesterase, phospholipase A, L-amino acid oxidase, nucleases, and toxins have been described in detail in a previous communication[6].

For proteases a slightly modified procedure based on that described by KOCHWA *et al.*[7] was used. 0.1 ml of the test solutions was placed in separate drops on X-ray-film strip which had been exposed and fixed. The film strip was incubated in a closed petri dish in a humid incubator at 37° for 5–6 h. After incubation, the film was washed gently with water and dried. The spots on which the test fluids had been applied were stained with Amido Black 10 B and the strip was washed with 1 % acetic acid. The areas showing proteolytic activity did not stain with the dye.

For detection of nucleases the procedure was the same as described previously[6] except that 0.9 % sodium chloride and 0.01 % magnesium chloride were included in the nucleic acid–agar. For cholinesterase an additional substrate was employed besides the esters of 2,6-dichlorophenolindophenol[8]. A 0.1 % solution of indoxyl butyrate in acetone was added dropwise with shaking to 2.0 ml of Tris–HCl buffer (pH 8.8) until white flakes began to appear in the solution. The mixture was layered thinly over the starch block and the block left at 25° in a water-saturated cabinet. After about 15 min a bluish-green band due to the deposition of indigo appeared at the site of the esterase activity. After about 90 min the block could be washed with water to remove the excess of the substrate. A reference block was stained for proteins to locate the position of the component having the cholinesterase activity.

The toxic components were detected by injecting 0.5 ml of the diluted fluid (1:3) from each segment intravenously into white mice weighing about 18–20 g. The criterion of toxicity was death of the injected mice within 24 h. The time of death and the symptoms produced after injecting different components were also noted.

RESULTS

Fig. 2 shows the results of the starch-gel electrophoretic blocks stained for proteins. The different active principles that were identified are given in the legend.

It was found that krait venom separated into 10 components of which 3 migrated towards the anode, 6 migrated towards the cathode and one component remained at the point of application. Under the conditions of the experiment a 20 % solution of saw-scaled-viper venom did not give a very clear separation although the different components could easily be differentiated on the starch blocks after staining. More dilute solutions (5 or 10 %) of the venom gave clearer separations, although the number of components was the same. Saw-scaled-viper venom separated into 7 components, 5 moving towards the anode, one remaining at the point of application and

a very faint component moving towards the cathode (marked No. 7 in strip B of Fig. 2). In this respect it differed from other snake venoms examined in this laboratory[6] by the same procedure which had components moving both to the anode as well as the cathode at pH 8.6.

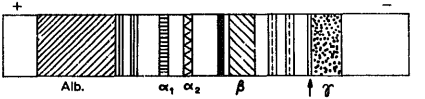

Fig. 1. Diagrammatic representation of the separated components of normal human serum.

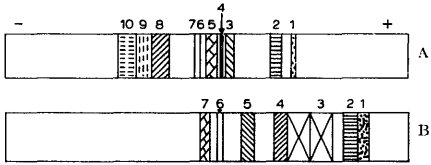

Fig. 2. Starch-gel electrophoresis of venoms of krait and saw-scaled viper. Stain: Amido Black 10 B. Arrow indicates the point of application of the venom sample. Strip A (krait venom) starting with the fastest component on the anode side: 1, protease; 2, phospholipase A; 3, L-amino acid oxidase, phosphodiesterase, nuclease; 4, point of application (arrow), cholinesterase; 5, L-amino acid oxidase, toxin with a late lethal action, also 5'-nucleotidase; 6, L-amino acid oxidase. The zone between Components 6 to 8 was highly toxic. Strip B (saw-scaled-viper venom): 1, not identified (protein); 2, 3, protease, strong coagulase and toxin; 4, L-amino acid oxidase; 5, phosphodiesterase. The zone between components 5 and 6 had the 5'-nucleotidase activity. 6, 7, not identified (protein).

The electrophoretic patterns of the two viperine venoms examined in this laboratory *viz.* Russell's-viper and saw-scaled-viper venoms are quite different. The former had protein components moving both towards the anode and the cathode, whereas in the case of saw-scaled-viper venom all the components except one moved to the anode.

The krait-venom cholinesterase was found on the point of application.

This cholinesterase hydrolysed the acetyl, butyryl and caproyl esters of 2,6-dichlorophenolindophenol but acted much more readily on indoxyl butyrate. The use of indoxyl butyrate had one distinct advantage: after the detection of the enzyme, the starch block could be washed and preserved for future reference. Cholinesterase could not be detected in saw-scaled-viper venom. This was true in the case of Russell's-viper venom also[6].

In krait venom, the phosphodiesterase occupied a position immediately on the anode side of the point of application. The nuclease was also located at the same position by the RNA–agar technique.

5'-Nucleotidase in the same venom migrated to the negative side, like those of the venoms of cobra and Russell's viper[6], but its activity was considerably less than that of the other venoms. The proteolytic activity in krait venom was located in the faint fastest band on the anode side.

Krait-venom L-amino acid oxidase was found in two distinct components, one moving to the anode the other to the cathode.

Toxicity experiments revealed that each of the krait-venom Components, 6, 7, 8 and 9 (Fig. 2) on the negative side killed mice on intravenous injection within less than 60 min. Component No. 5 (Fig. 2) had a late lethal action on mice. Krait venom has thus at least 4 toxic components, thus differing from the other 3 venoms examined in this laboratory[6].

The enzymes present in the toxic fractions of krait venom were 5'-nucleotidase, cholinesterase in component No. 5 (Fig. 2) and L-amino acid oxidase in Components 5 and 6 (Fig. 2).

The electrophoretic fractions of krait venom had practically no effect on the coagulation time of recalcified human plasma.

In the saw-scaled-viper venom the nuclease activity detected by the RNA–agar technique was very faint at the same position as the phosphodiesterase. Phospholipase A could not be detected. 5'-Nucleotidase activity was detected in the first fraction on the anode side whereas in all other venoms it moved to the cathode side under identical experimental conditions.

Saw-scaled-viper venom had a very prominent proteolytic enzyme moving a considerable distance on the anode side. A powerful coagulase was detected at the same position. Intravenous injection of the electrophoretic fractions revealed that saw-scaled viper venom had a toxic component moving the same distance as the proteolytic enzyme and the coagulase. Table I gives the relationship between the proteolytic and coagulase activities and the toxicity of the electrophoretic fractions.

These experiments were repeated several times. The correspondence of the proteolytic and coagulase activities with the toxicity was so marked that in some experiments the electrophoretic fractions which had been first tested for protease by the photographic film technique and found positive, were toxic to mice, whereas the remaining fractions had practically no effect on mice when injected intravenously.

The symptoms produced by the toxic fraction of saw-scaled-viper venom were quite different from those of krait venom. When the latter's fractions were injected the animals gradually lost consciousness and the respiration stopped within 30–90 min. With the saw-scaled-viper venom, about 5 min after the injection, the mice looked apparently highly uncomfortable and developed convulsions before finally collapsing within about 15 min.

TABLE I

CORRELATION BETWEEN PROTEASE, COAGULASE AND TOXICITY IN THE SAW-SCALED-VIPER VENOM

	Segment No. on the anode side									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Proteolytic acitivity	—	—	—	±	+	+	+	±	—	—
Coagulation time* (sec) of recalcified human plasma in presence of 0.1 ml of the same fractions	120	113	100	56	10	9	6	54	150	150
Effect of intravenous injection of the corresponding fractions on mice	—	—	—	—	D	D	D	—	—	—

+, present; ±, detectable; —, not detectable; D, injected animals died; *, the mean coagulation time with normal saline as the control was 173 sec.

There was only a faint L-amino acid oxidase activity in the saw-scaled-viper venom which corresponded to Component 4 in Fig. 2.

DISCUSSION

The electrophoretic patterns of the two species of venoms examined show a marked difference under identical experimental conditions.

The krait belongs to the family Elapidae and saw-scaled viper belongs to that of Viperidae. The electrophoretic pattern of krait venom had some apparent resemblance to that of the cobra venom[6]. However, when the enzyme activities of the different components were tested, krait venom showed marked differences from those of cobra venom. There were 4 toxins in krait venom whereas cobra venom had only one electrophoretic component lethal to mice.

The presence of more toxins accounts for the very high overall toxicity of krait venom compared with the other three venoms examined in this laboratory. The toxicity of krait venom is approximately five times as high as that of the other venoms[10].

The toxic component of cobra venom has no enzyme activity whereas in the krait venom cholinesterase, 5'-nucleotidase and L-amino acid oxidase are associated with the toxic components. Phosphodiesterase and 5'-nucleotidase have the same electrophoretic mobilities as the cobra and Russell's-viper enzymes[6]. The phosphodiesterase occurs in the non-toxic anodic zone and is also active on RNA giving a positive RNA–agar test. It is interesting to note that this type of test was very prominently given by cobra and krait venoms unlike Russell's-viper and saw-scaled-viper venom in which the test was not very distinct though both venoms had considerable phosphodiesterase activity. The anode fractions of krait venom are non-toxic when injected into mice, as in the case of cobra venom. It is interesting to note that in this venom also phospholipase A moves to the anode zone, containing the non-toxic components.

The presence of cholinesterase, 5'-nucleotidase and L-amino acid oxidase in the toxic fractions of krait venom does not necessarily imply that these enzymes contribute to the toxicity of the venom. Their electrophoretic mobilities may be the same as those of the toxins, since it is known that these enzymes are not very toxic[11]. All these three enzymes had no toxic action on mice in the venoms of cobra and Russell's viper as shown previously[6].

The cholinesterase in krait venom was found to be electrophoretically quite distinct from that of cobra venom as it remained at the point of application and also appeared in the first toxic fraction on the cathode side. However, it did not differ in specificity with the substrates used in the present investigation.

The 5'-nucleotidase in saw-scaled-viper venom was electrophoretically different from that in the other three venoms, in moving to the anode side. It was one of the prominent enzymes in this venom and quantitatively much more than that present in krait venom.

The toxic component in the saw-scaled-viper venom had a strong proteolytic activity and a powerful coagulant action, suggesting that the venom interferes with the circulation of blood in the envenomated animal. This is in good agreement with the findings of other workers[9] who observed a similar action with Russell's-viper

venom. However, unlike the saw-scaled-viper venom enzyme- the Russell's-viper coagulase activity could be electrophoretically separated from the toxic components[6]. The saw-scaled viper coagulase activity was much stronger and had a different electrophoretic mobility at pH 8.6 than the Russell's-viper enzyme. The electrophoretic fractions of Russell's-viper venom were all non-toxic and the anode fractions had to be combined in order to restore its toxicity.

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