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Partial biochemical characterization of Indian spider venom

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ABSTRACT

Spiders belong to the group of arthropods, they are found in the terrestrial habitat. Spider venoms are an incredible source of biologically active substances which selectively target a variety of vital physiological functions in both insects and mammals. Venom of spiders are heterogeneous not only between the species but also within the species. They are made up of complex mixtures of biologically active enzymatic and non enzymatic components. Many toxins isolated from spider venom have been valuable in helping to determine the role diversity of neuronal ion channels and the process of exocytosis.

Introduction

Spider belongs to

Phylum: Arthropoda

Class: Arachnida

Order: Araneida

Family: Lycosidae

Species: Hiposa (Funnel Web Spider)

Spiders are the ancient group of arthropods. They are the most successful invertebrates on the land with nearly 40,000 species,have been described world wide (15) Spiders are found in every terrestrial habitat. Spiders are not generally harmless to humans.However several species of spiders are responsible for harmful and even fatal bites.When spider bites occurthey are usually accidental and/or defensive.

Most spiders feed predominantly on insects, after the prey is captured, pider inject the venom. Spider egin digestion externally regurgitataing digestive fluid on to the prey before swallowing the liquid meal(16).

Among arthropods,spider venoms have been explored less than scorpions, owing to difficulty in obtaining biological material ,although the diversity of spider species and ecological niche is far greater than that of scorpion(3).

Venom Components:

Like the venoms of other animals such as snakes, scorpion and lizards, venoms of spiders are heterogenous not only between the species. They are made up of complex mixtures of biologically active enzymatic and non-enzymatic components. The major constituent of spider venom are proteins, polypeptides, free amino acids, monoamines andinoganic salts. Spider produces a variety of toxins which effect the nervous system of the prey. (4) toxins that stimulate transmitter release and toxins blocking post synaptic cholinergic receptor. Most if not all, spider venoms characterized to date have been found to be either proteins, peptides or acyl polyamines and have a variety of action through out the nervous system(5).Neurotoxins isolated from spider venom can be classified according to their mode of action.

Toxins affecting glutametergic transmission, calcium (Ca^{2+}), sodium (Na^{2+}),potassium (K^{+}) and chloride (Cl^{-}) channel toxins. Enzymes are important and common components of venom of many animals including bees, wasps, arachnids, snakes and lizards with several possible functions. In this respect the venom of spiders is not different ,containing awide variey of enzymatic activity. Hyaluronidase enzyme has been found in venom of many spiders in mygalomorphs and labidognaths spider species documented to date were reported to have considerable amount of hyaluronidase activity .The venom hyluronidase is frequently referred to as the “spreading factor” as it promotes the spreading of toxins from the site of injection or bite. This process is involved in distorting the integrity of extracellular matrix by breaking down hyaluronic acid present in it(4).

Proteases are also the major constituents of spider venom(6) reported that the spider venom proteases are homologous tometalloproteinases which induce systemic effect like platelet aggregation and intravascular dissemated coagulation which depend on the extracellular molecules such as fibronetin and fibrinogen. Venom also shows the phosphodiesterases (7), alkaline phosphatases (8,9) esterases(10) ATPases(11) kininases (endopeptidase) (12)and phospholipase A (13) activities.

Spider venom represents a incredible source of biologically active substances whch selectively target a variety of vital physiological function both in insects and mammals. Many toxins isolated from spider venom have been valuable in helping to determine the role diversity of neuronal ion channel and process of exocytosis.

Tillnow no work has been done in India on any species of spider found here. The present work is proposed with the objective of studying the biochemical and pharmacological effect of Indian spider venom.

Materials:

Spider venom from species HIPOSA (Funnel web Spider)

Hyaluronic acid,N-acetyl glucosamine, phosphotidyl choline, hyaluronidase, bovine serum albumin, para-dimethyl amino benzaldehyde,gelatin ,fat free casein were from BDH chemicals and all the chemicals used inthis study were of analytical grade

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Methods:

Spider collection and venom preparation

Spiders were collected from Manasagangotri Mysore (India).Spiders were anaesthized,venom glands were dissected out and ground in 0.9%Nacl in an eppendorf homogenizer. The resulting suspension was centrifuged (2000rpm for 2 mins).The supernatents were pooled and aliquots were frozen at -20^{0}C until use.

Protein Estimation

The protein concentration was determined by the method of Lowry et.al.(09)using BSA standard(0-75µg) 0-1ml aliquots of BSA standard solution was taken in clean and dry test tubes,5ml of Lowry's reagent(98ml of 4% sodium carbonate +1ml of 2% sodium potassium tartarate+1ml of 2%copper sulphate)was added.After 15 mins 0.5ml of FC reagent(1:1 diluted with water) was added and left for 30 mins. The optical density was measured at 660nm.

Indirect haemolytic activity

5ml of freshly citrated blood was centrifuged for 10 mins at 1,500 rpm, the supernatant is discarded and the cells obtained washed repeatedly with ice cold PBS 1ml of packed cells +1ml of egg yolk+8mlof this suspension is taken in each tube with the enzyme sample .This is incubated for 30 mins,or 1 hr.The reaction and the reaction is stopped by adding 9ml of ice cold PBS, this is mixed and centrifuged at 2000rpm for 10 mins and the supernatant is taken and read at 540nm.

Phospholipase Assay

Phospholipase assay was done as described by Bhat and Gowda(2)This method involves the measurement of fatty acid liberated by the enzyme catalysed hydrolysis of phosphotidyl choline (PC). 1ml of the reaction mixture contains 1000nmoles of PC ,0.2ml of solvent ether ,0.1ml of Tris HCL buffer (Ph 4) ,0.02ml of $CaCl_2$. The reaction is initiated by adding the venom sample.The reaction mixture was incubated at 37^{0}C for 1hour. The reaction was terminated by adding 0.5ml of Doles mixture (isopropyl alcohol+petroleum ether+1NHCL,40:10:1)and the contents of the tubes were mixed for one minute and centrifuged at 1000 g for 10minat room temperature.All the lipids present were extracted in the upper organic phase which was transferred to another test tube containing 0.5ml of chloroform petroleum ether (5:1v/v)mixture followed by the addition of 0.5ml of cobalt reagent .The contents were mixed thoroughly for 1min and centrifuged for 10mins at 1000rpmat room temperature. Cobalt selectively form asoap with fatty acids which was extracted in the upper phase. The upper phase 0.5ml was carefully transferred to a tube containing 0.75ml of 1 nitro 2 naphthol indicator solution. Cobalt forms a coloured product with the indicator.The intensity of the coloured product is directly proportional to the amount of free fatty acid present.After 30mins the contents were diluted by adding 2ml of methanol and the absorbance was read at 540nm using a colorimeter.Phospholipase activity was expressed in nano molesof fatty acid released/min/mg of protein.

NAG Estimation by hyaluronidase activity

Hyaluroniase activity was assayed by estimating the amount of N-acetyl glucosamine released according to method Reisig et.al(18).The activity was expressed as moles of N-acetyl glucosamine released/min/mgg of protein.

Substrate Gel Assay:

This was carried out by (5,12).Hyaluronic acid was incorporated at final concentration of 0.17mg/ml into polysaccharide gel matrix (10%) 50µgms of each venom samples were prepared under non reducing condition. Clear zone against the blue background of undigested hyaluronic acid indicates the corresponding lane was carefully sectioned and stained with commassie brilliant blue R-250.Since the included hyaluronic acid does not react with dye.

Caseinolytic Assay

Caseinolytic activity was assayed according to the method of Muruta et.al(13) using 4% of fat free casein in Tris HCl (0.2 M PH 8.5)buffer as substrate.The enzyme was incubated with the substrate at 37^{0}C and at the end of the incubation the undigested casein was precipitated with TCA and centrifuged ,proteolysis product in the supernatant was determined with FC reagent.After 30mins the blue colour developed was read at 660nm. 1 unit of activity is defined as the amount of enzyme required to cause the increase in OD by 0.01 at 660nm per min enzyme activity is expressed in terms of specific activity.

Gelatinolytic Assay:

Gelatin was copolymerized in to 10% w/v SDS polyacrylamide gel at final concentration of 0.5mg/ml gelatin ,the reconstituted spider venom (75µg) were prepared under non reducing conditions. Following electrophoresis consequently the gel was washed 5% and 2.5% Triton X 100 for 1hr and the washed 3 times in distilled water to rmove SDS. After which it was stained with commassie blue R-200 and subsequently destained in methanol:acetic acid: water,gelaitnase activity in the spider venom sample was evident as a transparent zone due to gelatin degradation.

Alkaline phosphor mono esterase activity

Alkaline phosphor mono esterase activity was assayed by a modification of the method by Lo et.al.(10) 0.1ml of the venom sample was added to an assay mixture containing 0.5ml of 0.5M glycine buffer ph 8.5 ,0.5ml of 0.01 M para nitro phenyl phosphate and 0.3 ml of 0.01M $MgSO_4$. The mixture was incubated at 37^{0}C for 30mins. At the end of the incubation period 2ml of 0.2N sodium hydroxide was added and allowed to stand for 20 mins the absorbance at 440nm was measured.One unit of alkaline phosphor mono esterase activity was defined as the increase in 0.001 absobance unit/min at 440nm.

Results:

Spider venom was studied for various enzymatic activities such as phospholipase activity, haemolytc activity, hyaluronidase activity, protease activity, and alkaline phosphomono esterase activity. The venom released free fatty acids egg phosphotidyl choline with a specific activity 133nmoles/min/mg/protein. The venom also showed indirect haemolytic activity on human erythrocytes with a percentage lysis of 11% when 75µg of venom protein was used in the reaction mixture. The venom also showed hyaluronidase activity by releasing N-acetyl glucosamine when determined colorimetrically. The specific activity of hyaluronic acid hydrolysis was found to be 0.9X 10^{-2}units/min/mg of protein. This activity was further confirmed by zymogram assay in which the venom revealed an activity band corresponding to molecular mass of about 60KD. The transluscent band in the gel against a dark blue background of Stains All indicated the enzyme activity .

Spider venom when tested for proteolytic activity showed activity when casein as well as gelatin were used as substrates .The venom degraded casein with a specific activity of 8.94 units/min/mg of protein.

Further the proteolytic activity of the venom was confirmed on gelatin zymogram assay in which the venom revealed a clear activity band corresponding to a molecular mass of about 20 KD while rest of the gel was opaque due to coomassie brilliant R 250 stain.

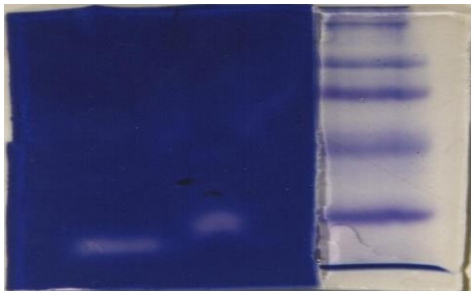

Figure : Substrate Gel Assay (Hyaluronidase)

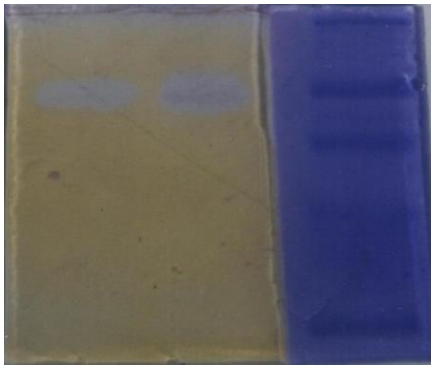

Figure 2: Gelatinolytic Assay

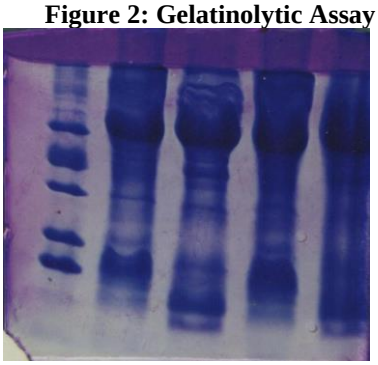

Figure: Electrophoretic pattern of spider venom

Spider venom also showed alkaline phosphatase activity. It degraded the para nitro phenyl phosphate with a specific activity of 8.94 units/min/mg protein.

Table I gives the summary of various ezyme activities determined. Figure2 gives the SDS PAGE pattern of the venom under both reduced and non reduced conditions. There was not much of a difference noticed in the banding pattern of the venom, except for few additional bands corresponding to low molecular forms of protein revealed in the reduced condition.

ENZYME	METHOD	ACTIVITY Units/min/mg
Hyaluronidase	NAG estimation	$0.9x10^{-2}$
Alkaline phosphomono esterase	PNPP METHOD	8.94
Protease	Caseinolytic	8.94

Discussion:

Spider venoms represent an incredible source of biologically active substances which selectively target a variety of vital physiological functions in both insects and mammals. Venom showed different enzyme activities such as protease, hyaluronidase, phospholipase and alkaline phosphomonoesterase activities.

The venom released free fatty acids when egg phosphatidyl choline was used as a substrate suggesting the presence of phospholipase enzyme. This was further confirmed by haemolytic activity which is semi quantitative determination of phospholipase activity.

The general gelatinolytic activity is usually found to be associated with hemorrhagic metallo proteinases, which are usually homologous to matrix metalloproteinases. Thus the gelatinolytic activity of the venom could be related to its possible hemorrhagic activity. This activity along with hyaluronidase activity degrade the major components of extra cellular matrix leading to local tissue damage during envenomation and this might have an influence on the systemic effect as well. In conclusion this study explores the different activities of spider venom and provides an insight into the nature of hyaluronidase as well as proteolytic enzymes.

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