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Study on Humoral Defense Factor Involved in the Innate Immune Responses of Periplanetaamericana

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Abstract—*Antibacterial peptides are important for nonspecific defense in many animals. They have been characterized from mammals, amphibians and chelicerates, but only in few species of insects ie. Drosophila, Bombyx, honey bee and housefly. The insect haemolymph has a range of defense substance which includes agglutinins, lysosomes, bacteriocidins and non-lysozyme factors. The production of antibacterial proteins represents the first line of defense mechanism in insects. The experiment was carried out to identify and partially characterize the antibacterial protein produced in the haemolymph of American cockroach Periplanetaamericana in response to Bovine serum albumin and Escherichia coli injection. Agglutination assay, Inhibition zone assay, Immunodiffusion assay was done to identify the antibacterial substance and it was characterized based on it's trypsin susceptibility, SDS-PAGE and by bactericidal activity. From the results, it was concluded that the antibacterial substance produced in the cockroach is a cationic proteins which has bactericidal and agglutination activity.*

1. INTRODUCTION

The extensive use of antibiotics for the human welfare resulted in the emergence of many drug resistant bacteria, which is a major drawback. Besides, many of the antibiotics are not cost effective and have various side effects. So the antibacterial peptides of invertebrates and plants have drawn attention of the scientists which may be the possible, better alternative for the antibiotics.

Current estimates are that insect account for 90% of all extant animal species. Insect colonize almost all ecological niches except sea. Insects have been remarkably successful in evolution. One of the most important reasons is that they are resistant to microbial infection build up a rapid, large spectrum, host defense that shares some fundamental characteristics with the innate immune response of vertebrates.The body fluids of insects lack immunoglobulins and immunological memory, but they contain a range of naturally occurring and inducible humoral defense factors. Natural substances include agglutinins, lysozyme, other lysins, non-lysozyme bacteriocidins, and immobilization factor. The production of antimicrobial molecule is central to the protection against diseases[13].

Numerous antimicrobial peptides have been characterized from insects. Cecropins, drosocin, abaecin, apidaecin, hymenoptericin, coleoptericin, defensing, scorpianin, diptericin, bombninin, mytilins and myticins are some of the purified peptides from insects.All these inducible antimicrobial peptides share the following common characters; all are heat stable, trypsin sensitive, low molecular weight basic proteins induced by variety of stimuli like injury, biological antigenic substances from proteins to live bacteria. They are synthesized and secreted in fat body and later released in to haemolymph on getting the signal.

In the present study we attempted to identify theantibacterial protein using immune diffusion assay,bacterial agglutination assay, haemagglutination assay, inhibition zoneassay and SDS-PAGE. It is also tested for its susceptibility to trypsin.

2. MATERIALS AND METHODS

2.1. Experimental animal

The adult American cockroach is reddish brown with a pale brown or yellow band around the edge of the pronotum. They were procured and maintained in a plastic container at room temperature with the supply of sterile water and biscuits.

2.2. Immunization

Adult cockroaches were anesthetized with ether and they were immunized with saline, 0.1ml of Bovine serum albumin (BSA 1mg/ml) and log-phase bacterial culture in bacterial saline (0.8%) with 1 ml syringe between the 4^{th} and 5^{th} abdominal sternitesand then were kept undisturbed for 24hrs and fed normally.During this procedure the needle was inserted horizontally and anteriorly in a slow fashion. If the haemolymoph leaked out upon withdrawl of the syringe, the

animal was discarded. They recovered from the anesthetic state within 20 min with no ill effects. Another unimmunized group was also maintained as control. After 24hrs of immunization the haemolymph was collected by cutting the leg segments between the trochanter and the femur in an eppendorf tube containing few crystals of Phnyl Thio Urea (PTC). It prevents the melanization of haemolymph. The collected haemolymph from each group was pooled and stored in ice-cold eppendorf tubes at -4^{o}C and used whenever there is need. The haemolymph collected form nonimmunized insect served as control serum. The haemolymph should be kept in ice throughout the experiment to avoid coagulation and to ensure the antibacterial activity.

2.3. Bacterial agglutination assay

The term agglutination is concerned with the reaction of antibody with particulate antigen. Agglutination activity of the haemolymph was detected by serial two fold dilution of 20µl of bacterial agglutintation sample with 20µl of Tris-HCl buffer or bacterial saline. The last well was considered as the control and from the first well the number was countedas, 1, 2, 3……10, 20µl of bacterial saline was added to each well. 10µl of the haemplymph was introduced in the first well and dilution was made up in 2-fold steps by adding 10µl of the haemplymph from the first well to second well, from the second to third well and so on. 20µl of 2.5% *E.coli* was added and mixed well. The microtitre plate was incubated for one hour at 37^{0}C. The degree of aggregartion of *E.coli* was noted by comparing with the control. The result of agglutination test is expressed as titre, which is the recioprocal of the highest dilution at which agglutination occurs.

2.4. Passive agglutination assay

This is done to study the antibacterial activity of the induced peptide against soluble antigen (BSA), by coupling the antigen to an insoluble indicator cells such as sheep red blood celss. Chromic chloride is an effective coupling agent for linking proteins to red blood cells. Sheep RBC in Alsevers solution are wahsed thrice with physiological saline (0.15N) by centrifuging at 3000rpm for 10mins. To one volume of packed SRBC equal volumeof BSA at concentration of 5mg/ml saline and equal volume of chromic chloride solution at concentration of 1mg/ml saline was added. The mixture was agitated by hand for 10mins and kept in a water bath at 37^{0}C for 15mins with intermittent agitation. The reaction is abruptly stopped by diluting with saline. The BSA coupled cells were washed thrice in saline and resuspended to a required concentration in saline.

10µl of the haemolymph sample was diluted with 10µl of physiological saline in the microtitre palte till the 11th well and 12th well was kept as negative control. Then 10µl of 1% BSA coupled SRBC in saline was added to each well. The plate was hand shaken for effective mixing of reagents and incubated for an hour at 37^{0}C and for more hours at 10^{0}C. The highest dilution of the sample which shows detectable agglutination is recorded.

2.5. Immunodiffusion assay

To study the immunological identity existed between the immune serum obtained from insect and the soulble bacterial proteins, conventional immunodiffusion assy was carried out in agar plates.The antigen solution was prepared by shaking a thick suspensionof bacteria in 1% SDS solution containg 50mM EDTA. The haemolymph obtained from insect served as antiserum.1% agarose was poured on to a glass slide withour air bubbles and kept on a leveled surface. After the solidification of agarose, using the template, wells were punched. The centere well was filled with antigen. In one perpheral well, control haemolymph was added and in another well haemolymph from immunized insect was added. The agar plates were left in humid champers at 37^{0}C observed over a period of 48hrs.

2.6. Assesment of antibacterial activity - inhition zone assay (IZA)

The whole haemplymph cannot be used as such, since it contains bacterial pathogens. They were removed by heat treatment in acidic medium. The haemolymph was mixed with equal volume of acetate buffer pH 3.6 (40mM ammonium acetate and 1M acetic acid (2:1 v/v) and was left in a boiling water bnath for 5minutes. The contents were shaken continuously, this procedure results in the precipitation of all other proteins except the basic catioinc proteins. The precipitate was removed by centrifugation at 12,000g for 20min inrefrigerated centrifuge and its supernatant was used for assessing the antibacterial ativity.

In this procedure the antibacteial activity of the haemplymph of insect was assessed by following the method of Hoffmann *et al*., 1981[10]. The agar plates were prepared by pouring 10ml of Luria broth to sterile petriplates and the plates were inoculated with log phase *E. coli*. On these agar plates 2mm diameter wells were punched using a sterile gel punch. The wells were filled with 8µl of hemolymph and the petridishes are incubated at 37^{0}C for about 15-20h. The bacterial multiplication was accompanied by a slow diffusion of the serum in to the agar. This procedure was a modification fo the paper disc method of Kirby and Bauer used to determine the suceptibility of bacterial isolates growth occurred was regarded as zone of inhibition of bacteial growth. The diameter of the zone was measure and recorded.

2.7. SDS-PAGE

The electrophoresis was performed on 12% separating gel which was stained with Coomassie blue R-250 [12]. 15 µl of ammonium acetate buffer treated samples were loaded in a predetermined order into the bottom of the wells by

micropipette. Once the bromophenol dye reached the bottom, the gel was then transferred to a staining tray for staining for a minimum of 4 hrs at room temperature. The gel was then destained by soaking it in the methanol: acetic acid solution without the dye on a slowly rocking platform for 4-8Hours, changing the destaining solution three or four times.Once the bands are visible, it was photographed for future use.

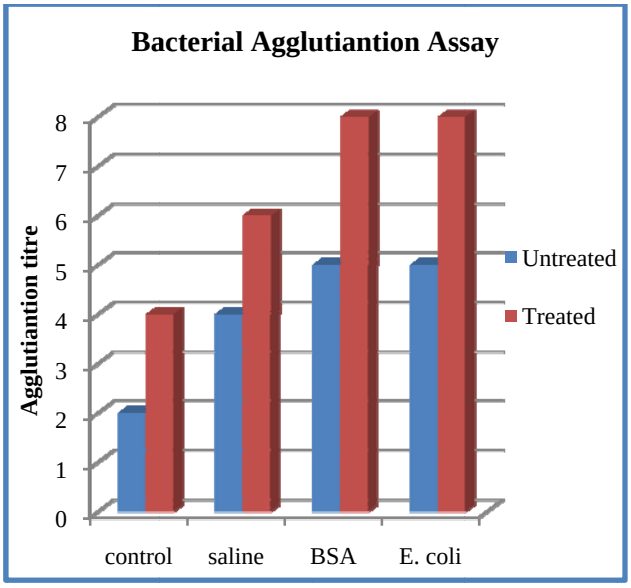
2.8. Susceptibility to trypsin

The antibacterial substance was subjected to proteolytic activity by incubating 5mg of protein with trypsin (9mg/ml) in phosphate buffer saling (PBS) at pH 7.0 for 30mins and 1hr at 37^{o}C. At the end of the incubation the activity was tested by IZA.

3. RESULTS

3.1. Bacterial agglutination assay

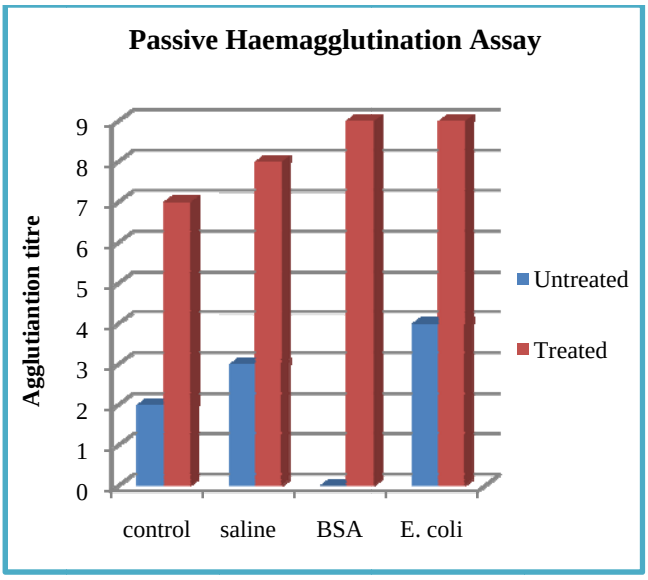
The haemolymph of control and induced inseect was analysed for bacterioagglutinin. It is summarized in Graph I. The agglutiantion titre is very high in the treated haemolymph irrespective of the induction. The highest activity wasobserved in the haemolymph injected with BSA and bacteria.



Graph 1: The bacterial agglutination of all the samples

3.2. Passive haemagglutination assay

The haem agglutination patterns of whole haemolymph and treated haemolymph from cockroaches against sheep erythrocytes were determined (Fig. 2). The treated haemolymph showed strong haem agglutination property than whole haemolymph. The highest titre is observed in the treated haemolymph collected from the bacteria injected cockroaches (Graph 2).



Graph 2: The passive haemagglutination of all the samples

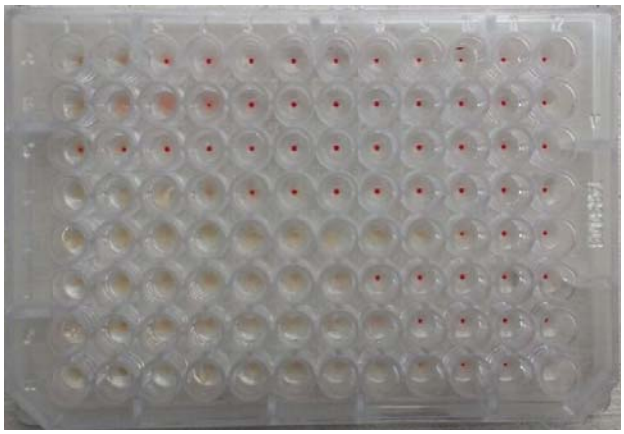


Fig. 1: Result of passive agglutination assay – first 4 wells are whole haemolymph in the order of control, saline, BSA and *E. coli*. The last 4 wells are treatedhaemolymph in the order of *E. coli*, control, saline and BSA

3.3. Immunodiffusion assay

It was done to identify whether the antibacterial protein has any precipitation ability against BSA and *E. coli*. Absence of precipitation line in control and immunized haemolymph indicates the absence of natural and induced precipitating antibodies.

3.4. Antibacterial activity

The activity of control and immunized haemolymph was confirmed by inhibition zone assay (Fig. 2) against highly resistant strain of *E. coli*. The results shows that there is a bacteria free zone surrounding the well in which the haemolymph from bacteria injected group was placed. The

bacterial growth was inhibited to the extent of 8mm diameter. No zone was observed in any other well where control and treated haemolymph were placed.

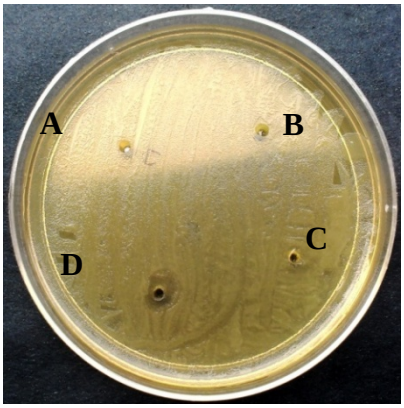


Fig. 2: Result of inhibition zone assay. The haemolymph was added in the following order, A. Control, B. Saline, C. BSA, D. *E.coli*

3.5. SDS-PAGE

The crude and treated haemolymph was subjected to SDS-PAGE (Fig. 3). Four proteins were identified with molecular weights 243kDa, 120, 75 and 63kDa which was measured against the standards used. In the 3rd lane, there is one particular band which is very prominent and is absent in control lane(1 and 2). This particular band is slightly present in the haemolymph of cockroaches injected with *E. coli*.

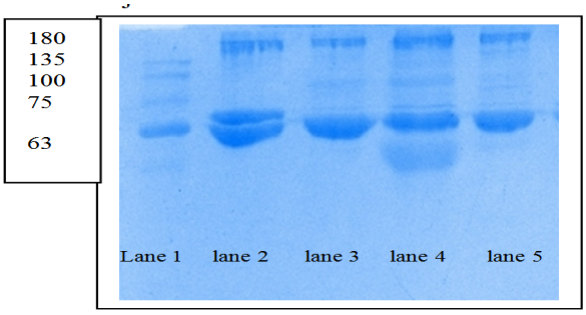


Fig. 3: SDS-PAGE gel showing the different protein bands induced in the haemolymph of cockroaches.Lane1. MarkerLane 2. Control, Lane 3. Saline, Lane 4. BSA injected group, Lane 5. *E.coli* injected group

3.6. Susceptibility to trypsin

To analyze the nature of the antibacterial substance, it was subjected to trypsin. The trypsin treated immunized haemolymph shows no antibacterial activity indicating that the active principle of the immunized haemolymph is a protein. It was evidence by the non-inhibition of growth of bacterial cultures when trypsin digested antibacterial substance was used for IZA.

4. DISCUSSION

Our study clearly shows the production of antibacterial protein against soluble and bacterial antigens which has agglutinating activity in the haemolymph of induced cockroaches. Biosynthesis of inducible antimicrobial peptide to bacterial challenge have been reported in insects belonging to the orders Lepidoptera [2, 10, 11], Diptera [4,7,18], Coleoptera [5, 14, 15, 17], in exopterygote Hemiptera [8, 16] and also in Hymenoptera [6]. These proteins are reported to possess pronounced antibacterial defense reaction.

In our study the agglutination titre was increased by the injection of both cellular and soluble antigen. The nonspecific activity of haemolymph was also observed in agglutination assay where both BSA and bacterial induced haemolymph showed the aggluination activity against both soluble and particulate antigen. When compared to the control the titre was increased even in the slaine injected group. This may be due to the production of the proteins not only to the infection but also to any stress like handling or any other environmental stress in natural conditions.This shows the importance of innate immune system for survival in these animals. Biosysnthesis of these proteins to injury in insects is also observed by Boman *et al*., 1981[3]. Injection of *Streptomyces griseus* in to American cockroach resulted in an increase in agglutination titre of haemolymph was also studied by Basseri *et al*., 2008[1]. They also suggested that these proteins may be lectins involved in the immunorecognition and block bacterial infection in insects. Haemagglutinating activity in the haemolymph of uninduced Ameican cockraoches was also observed by Scott [13].

In our study 6 bands were observed in the induced haemolymph of the cockroaches after subjecting it to SDS-PAGE. In a similar study seven bands were observed in the gel after injection with *E. coli*. Seraj *et al*., 2003 [18] have isolated 67 kDa and 61kDa proteins which has potential antibacterial activity from the haemolymph of American cockroach after injection with *E. coli*. They also observed the appearance of these proteins starting from half an hour and reaches its peak between 9 and 12 hrs.

We didn't observe any precipitation line in the immunodiffusion assay. In contrast, *Periplaneta americana* challenged by honey bee venom produced a humoral factor that behaved like an antibody molecule forming preciptin line with homologous antigens on agar gel plates [19]. Another interesting obsevation was the formation of specific preciptin band on immunodiffusion plates using soluble proteins of bacteria with immune seum of *Musa domestica*[9]. There are no other reports available on the occurrence of insects humoral factors capable of precipitating antigens on Ouchterlony gels.

We also observed the antibacterial activity in the haemolymph of cockroaches injected with *E. coli*. The proteins can be

further isolated, purified and characterized for the better understanding of the insect innate immune system and to explore the potential of this for therapeutic applications.

5. ACKNOWLEDGEMENT

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