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ISOLATION AND PARTIAL PURIFICATION OF ANTIMICROBIAL PEPTIDES/PROTEINS FROM DUNG BEETLE, *ONTHOPHAGUS TAURUS* IMMUNE HEMOLYMPH

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ABSTRACT

Antimicrobial peptides are important in the first line of the host defense system of all insect species. In the present study antimicrobial peptide(s) were isolated from the hemolymph of the dung beetle *Onthophagus taurus.* Both non induced and immune induced hemolymphs were tested for their antimicrobial activity against different bacterial strains and *C. albicans.* Induction was done by injecting *E. coli* into the abdominal cavity of the *O. taurus.* The non induced hemolymph did not show activity against any of the tested fungal and bacterial strains where as induced hemolymph showed activity against all tested bacterial strains but no activity against *C. albicans*. The induced hemolymph was subjected to non reducing SDS-PAGE and UV wavelength scan was performed to detect the presence of peptides. The immune induced hemolymph was purified by gel filtration chromatography to separate the proteins responsible for the antibacterial activity. The fractions within the peak were tested against those bacteria which previously showed sensitivity to the crude immune induced hemolymph. All fractions were found to be active against all tested bacteria with difference in zone of inhibition. The peptides are active against prokaryotes & not against eukaryotes. These properties reveal its unique characteristics and therapeutic application.

Keywords: Antimicrobial peptides/protein, immune induced hemolymph, SDS-PAGE, Gel filtration chromatography (GFC), UV wavelength scan

INTRODUCTION

Insects are probably the first successful scholars of combinatorial chemistry. They demonstrate a remarkable evolutionary success that can be attributed to a variety of reasons **(Labandeira and Sepkoski, 1993)**, among which their potent antibacterial defense reactions play a major role **(Cociancich *et al.*, 1994)**. These creatures are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites, but only a few encounters result in infection **(Gillespie *et al.*, 1997)** Particularly, in insects which lack an adaptive immune system, antimicrobial peptides play a crucial role in fighting against invading pathogens. They are synthesized in response to microbial infection or septic body injury mainly in insect fat body (functional equivalent of mammalian liver) and in certain blood cells, and then rapidly released into hemolymph where they act synergistically against microorganisms **(Lopez*et al.*, 2003; Irving*et al.*, 2004; Tzou*et al.*, 2002)**.

Insects are amazingly resistant to bacterial infections. To combat pathogens, insects rely on cellular and humoral mechanisms, innate immunity being dominant in the latter category. Upon detection of bacteria, a complex genetic cascade is activated, which ultimately results in the synthesis of a battery of antibacterial peptides and their release into the hemolymph **(Laszlo otvos, 2000)**. Peptides exhibiting antimicrobial activity are mainly small (5 kDa), amphipathic, cationic molecules **(Bulet *et al.*, 1999)**.

Most known antimicrobial peptides act toward microbial cell membrane causing permeability perturbations or even membrane disintegration due to pore forming or carpet-like mechanisms of action **(Bulet*et al.*, 1999; Boman, 2003; Yeaman and Yount, 2003)**. However, the proline-rich peptides seem to have a protein target and are not membrane-active **(Korner *et al.*, 2004; Otvos, 2002)**, while, on the other hand, the rare anionic antibacterial peptides acts by causing cytoplasmic protein precipitation and intracellular content flocculation **(Brogden*et al.*, 2003; Brogden, 1996; Lai*et al.*, 2002)**. Some peptides affects DNA and protein synthesis or proper folding of newly synthesized proteins **(Bulet*et al.*, 1999; Otvos, 2002; Casteels*et al.*, 1998)**. There are also known peptides affecting important intracellular processes, e.g. DNA and protein synthesis or proper folding of newly synthesized proteins **(Bulet*et al.*, 1999; Otvos, 2002; Casteels*et al.*, 1998)**. Certain antimicrobial peptides demonstrate anticancer activity, e.g. insect cecropins **(Casteels*et al.*, 1998; Małgorzata*et al.*, 2007)**.) and magainins from frog skin **(Moore *et al.*, 1994; Cruciani*et al.*, 1991)**. Generally, antimicrobial peptides are assumed in the near future as an alternative for the nowadays classical antibiotics. It was found that only 2.87µg of the protein could inhibit bacterial growth where as approx. 10 µg of conventional antibiotics was required to obtain similar result **(Seraj *et al.*, 2003)**. The advantages of antimicrobial peptides are: selectivity, fast killing, broad antimicrobial spectra and no resistance development **(Boman, 2003; Matsuzaki, 1999)**.

The recognition of pathogens and parasites by the invertebrate immune system may involve soluble proteins present in the hemolymph as well as proteins (receptors) localized at the surface of the hemocyte or other cells. The initial recognition may bring about communication to other population of cells through molecules that act as signals to stimulate a response **(Rameshkumar *et al.*, 2009)**

O. taurus spends most of its time in dung, where pathogens are abundant. Their larvae feed on the fungi, decaying organic matter, dung, and other organic materials found in dung. Therefore, it is likely defends itself against invading pathogens by means of antimicrobial compounds.

The purpose/objective of the present study was to isolate and purify antimicrobial peptides from dung beetle. In this study we isolated and purified antimicrobial peptides in immune induced hemolymph of *O. taurus* which may open up new avenue of research in a search to combat against pathogenic microbes.

MATERIAL AND METHODS

Bacterial & fungal strains

The bacterial strains that were used for screening antimicrobial peptides include *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Streptococcus sp., Staphylococcus aureus* and the pathogenic fungus *Candida albicans* were obtained from Dept. of Molecular Biology, University of Mysore and J.S.S Medical College, Mysore.

Collection of insects

O. taurus (Dung beetles) are collected freshly in and around Mysore and Nanjangud, Mysore District, Karnataka State, India where they were grown in natural environment during the month of December& January, and were brought to the laboratory regularly in a plastic container containing few amount of dung or wheat bran with sufficient amount of moisture.

Immunization of the insects

The broth containing 16 hour culture of *E. coli* which was cultured at 37°c and cells were pelleted by centrifugation at 10k. rpm at 4°C for 20 minutes. Immunization was done according to the method described by **Małgorzata *et al.*, (2007)**. Peptides expression was detected

Hemolymph collection

The *O. taurus* insects (non induced & immune induced) are surface sterilized with 70% ethanol and fixed on to the dissection plate. Hemolymph was collected from insect as described earlier **(Seraj *et al.*, 2003)**, and it is transferred in to a clean & chilled 2ml eppendorf tubes containing few crystals of ascorbic acid (vit-c) or phenyl thiourea to prevent melanization.

Preparation of hemocyte free hemolymph

The hemocyte-free hemolymph was obtained by centrifugation at 200g for 5 mins and subsequently the supernatant was spun down at 20,000g for 15 mins at 4°C to pellet cell debris **(Matsuzaki, 1999; Małgorzata*et al.*, 2007)**. The obtained hemocyte-free hemolymph is then stored at 4°C until it is used.

Spectrophotometry

The protein concentration of both non induced and immune induced hemolymph was measured in spectrophotometer (HITACHI U-2900) as described earlier **(Lowry *et al.*, 1951)**. U.V Wavelength scan for peptides were performed as described earlier **(Ping Fu *et al.*, 2009)**.

SDS-PAGE

Non reducing SDS-PAGE for both non induced and immune induced hemolymph was performed according to the method of **Laemmli U K. (1970)**.

Antimicrobial activity

Bacteria and fungus

The antimicrobial assays were done by well diffusion assay. Where all bacterial strains were cultured on nutrient agar pH 7.0 and *C. albicans* on Sabouraud agar medium pH 5.5. The agar surface was spreaded with 0.1ml of the microbial inoculum (24hrs old) & the wells were loaded with 20 µl of hemolymph. The plates were incubated over night at 37°c. The diameter of the clear zone was recorded.

Gel filtration chromatography

The immune induced hemolymph was applied to a sephadex G-50 gel filtration column (1×50cm) equilibrated with 0.1M ammonium acetate buffer, pH 6.4. Gel column was eluted with the same buffer at 15ml/hr flow rate i.e. 1.5ml /6min of eluent is collected in each tube. Fifty fractions were collected **(Kyung and Ourth, 2000)**. Fifty fractions were collected and measured at A280 nm and are again tested for antimicrobial activity.

RESULTS

Spectrophotometry [Protein Estimation]

After successful isolation of hemolymph containing peptides from the insect. Total protein estimation was carried out for both immune induced and non induced hemolymph. The protein concentration of the crude non induced hemolymph was found to be 9 mg /ml. The protein concentration of the crude immune induced hemolymph was found to be 15 mg /ml (Figure 1).

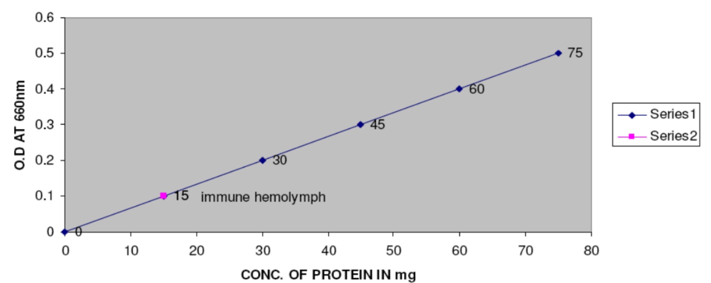

Figure 1 Estimation of protein in non induced hemolymph (Series 2 – 15mg/ml of protein in immune non induced hemolymph).

Spectrophotometry [Wave length scan]

In order to check the presence of active peptides both the immune induced and non induced hemolymph samples are subjected to U.V.Wavelength scan where the peptides shows maximum absorption at 220nm. The non induced hemolymph shows increase in the concentration of aromatic side chains than peptides. [Absorption at 260-280nm is contributed by aromatic side chains **(Ping Fu etal., 2009)**]. In Immune induced hemolymph the peptides were produced in higher concentration. Hence a positive peak at 210-220nm and it had the characteristic absorption peak of protein (peptides) (Figure 2).

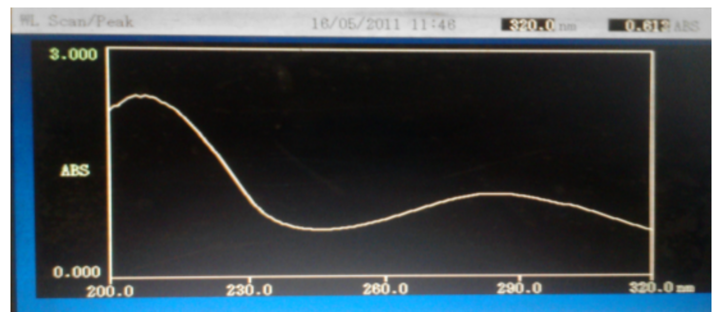

Figure 2 U.V. wavelengths scan of immune induced hemolymph

SDS-PAGE

After total protein estimation and wavelength scan both the crude hemolymph types are subjected to nonreducing SDS-PAGE in order to identify different protein bands. The results of non reducing SDS-PAGE of crude immune induced hemolymph were shown in figure 3. It is very much clear that the immune induced hemolymph is having more protein contents than the non induced hemolymph.

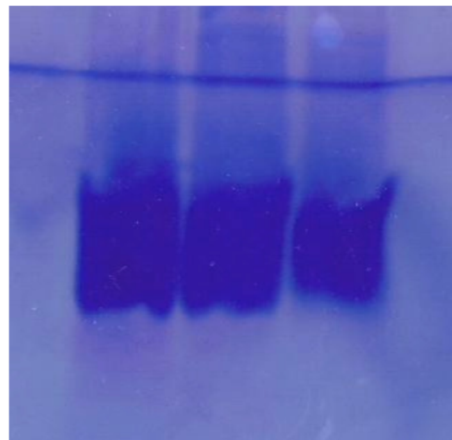

Figure 3 SDS PAGE (non reducing) profile of immune induced hemolymph.

Antimicrobial activity of the non induced and immune induced hemolymph

As the main objective of the present research is to isolate peptides active against microbes, we tested the activity of isolated peptides on microorganisms in culture plates by well diffusion assay. The non induced hemolymph did not show inhibition against any of the bacterial strains & *C. albicans*. The immune induced hemolymph was shown to be active against all bacterial strains with difference in zone of inhibition but no activity against *C. albicans* (Table 1).

Table 1 Antimicrobial activity of the immune induced hemolymph. (The Antimicrobial activity of the immune induced hemolymph was done by well diffusion assay against *C. albicans* & bacterial strains)

Name of the organism	Activity	Zone of inhibition (diameter) in millimeter
C. albicans	-	-
E. coli	+	12
B. subtilis	+	14
P. aerugenosa	+	6
Streptococcus sp.,	+	12
S. aureus	+	10

Legend: '–' means no activity; '+' means active.

Gel filtration chromatography

Since the non induced hemolymph did not show activity against any of the tested microorganisms, and only the immune induced hemolymph showed activity against many of the bacteria hence it was subjected to GFC and purified as mentioned above **(Kyung and Ourth, 2000)**. All the 50 fractions were measured at 280 nm to detect the presence of active peptides.

Antimicrobial activity in gel filtration fractions

Since the crude immune induced hemolymph showed activity against test bacterial strains, it is purified by GFC and the fractions within the peak i.e., between fraction 28 and 37 are again used to test the presence of active peptides. The result of the action of peptides in invitro studies are shown in the figure 4 to 8.

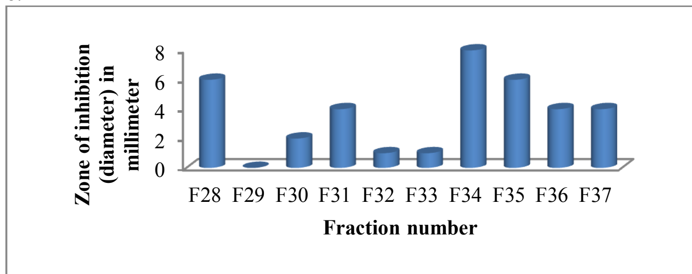

Figure 4 Antimicrobial activity of gel filtration fractions against *E.coli*.

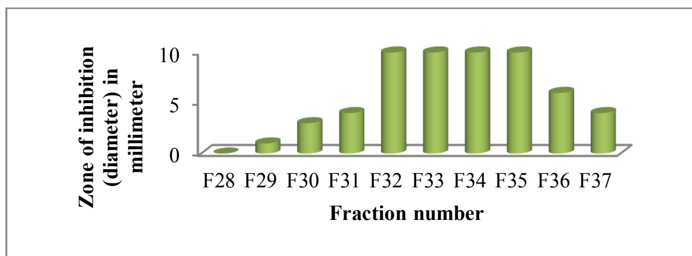

Figure 5 Antimicrobial activity of gel filtration fractions against *B. subtilis*.

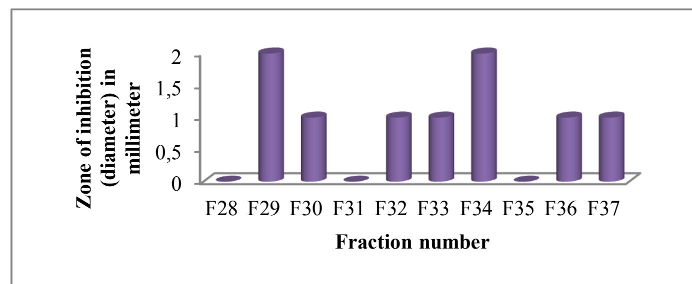

Figure 6 Antimicrobial activity of gel filtration fractions against *P.aerugenosa*.

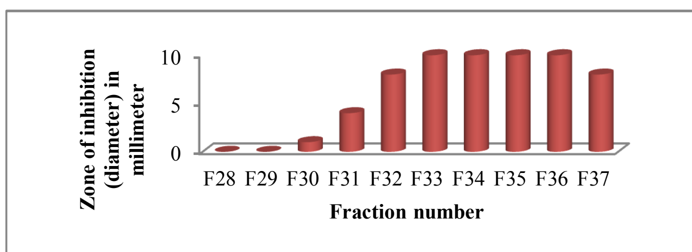

Figure 7 Antimicrobial activity of gel filtration fractions against *Streptococcus sp.,*.

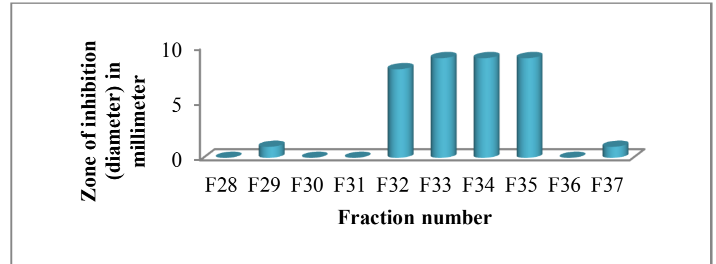

Figure 8 Antimicrobial activity of gel filtration fractions against *S.aureus*.

Comparison of insect peptides with standard antibiotic

The insect peptides are active against all bacteria's as mentioned above. So attempts have been made to compare the action of antibacterial peptides obtained from immune induce insects with the standard antibiotic Gentamicin by well diffusion assay. The results were shown in the Table 2. The fractions which showed maximum inhibition for each bacteria was loaded to one well with 1mg/ml concentration where the agar plate was previously spread with respective bacteria's and to another well Gentamicin with 1mg/ml concentration was loaded (Table 2).

Table 2 Comparison of insect peptides with standard antibiotic Gentamicin

Bacteria	Immune hemolymph (1mg/ml) Zone of inhibition (diameter) in millimeter	Gentamicin (1mg/ml) Zone of inhibition (diameter) in millimeter
E. coli	8	20
B. subtilis	12	20
P. aerugenosa	2	4
Streptococcus sp.,	10	20
S. aureus	8	20

DISCUSSION

Defense peptides and proteins constitute key factors in insect humoral immune response against invading microorganisms. It is generally assumed that each insect species possesses an individual set of antimicrobial peptides synthesized in response to non-self recognition **(Malgorzata *et al.*, 2007)**. In this study the non induced hemolymph & the induced hemolymph of the dung beetle were screened for the antimicrobial activity against different pathogenic bacterial strains and *C. albicans*.

The non induced hemolymph did not show inhibitory activity against any of the test bacterial strains & fungus. It does not indicate that peptides are absent but it may be present in lesser quantity so that no visible action in invitro studies is seen. But the immune induced hemolymph showed activity against all tested bacteria and no activity against fungus. Hence the peptide is active against prokaryotes and doesn't affect the fungus which is a eukaryote.

Similarly, *D. melanogaster* metchnikowins peptides have no activity against Gram-negative bacteria but they inhibit growth of *M. luteus* and filamentous fungus *N. crassa* **(Bulet *et al.*, 1999)**. Abaecins peptides inhibit growth of Gram negative and Gram positive bacteria. It is known that prolinerich peptides like *P. prasina* metalnikowins and *B. mori* lebocins, are similar to genetically modified proline-rich peptides which are active against sensitive microorganisms in relatively high concentrations. Metalnikowins inhibit Gram-negative bacteria growth at a concentration range from 50 to 200 mM depending on the isoform **(Chernysh *et al.*, 1996)**. Similarly, the minimal inhibitory concentration of lebocin 3 tested against *E. coli* in nutrient broth was determined for 211.1 mM (800 mg/ml) **(Hara *et al.*, 1995)**. It was suggested that lebocins can serve to reduce the minimum inhibitory concentration of other antimicrobial peptides acting synergistically **(Hara*et al.*, 1995; Furukawa*et al.*, 1997; Yamakawa*et al.*, 1999)**.

It has been observed in various insect species that bacteria injected into the haemocoel elicit the synthesis of number of peptides and proteins which are active singly or in concert against the invaders and are secreted into the hemolymph **(Gillespie *et al.*, 1997)**. Induction is a common process in many insect species. In the present study induction of such peptide(s) was done by injecting *E.coli* into the abdominal cavity of the dung beetle. The immune induced peptides were active against tested bacterial strains and this result suggests that peptides are produced to combat bacterial infection. Some proteins present in the hemolymph of invertebrates may be both constitutive & inducible such as p47 of *C. capitata* **(Charalambidis *et al.*, 1996)** & lysozyme **(Gillespie *et al.*, 1997)**. They are sometimes acts as signaling molecule (p47) and good antibacterial (lysozyme).

Since immune induced hemolymph showed antibacterial activity UV wavelength was performed (Figure 4) which showed maximum absorption peak between 210-220nm indicating the presence of peptides compared to that of non induced hemolymph where it is rich in aromatic aminoacids than active peptides.

Then it was subjected to non reducing SDS-PAGE to determine the number & size of proteins present in the hemolymph. Since there is no breakage of disulfide bonds a thick overlapping bands (Figure 6) were detected in the gel but it was not possible to determine at this stage which protein (s) is responsible for the observed antibacterial activity. Wave length scan and non reducing SDS-PAGE was also performed for non induced hemolymph (Figure 3 and 5 respectively) to identify the presence of other proteins, figure 1 also showed the presence of proteins in non induced hemolymph, but they are not directly involved in killing the bacteria, they may be involved in signaling mechanism.

To determine the peptides/protein(s) responsible for the observed antibacterial activity the immune induced hemolymph was subjected to GFC. The figure 7 shows few peaks from which the fraction number 28 to37 has maximum absorption at 280nm hence they were selected for further studies, where they were again used to test the presence of antimicrobial peptides as mentioned above. From those 10 fractions most of the fractions showed activity against all tested bacteria with difference in zone of inhibition (Figure 4 to 8). The 28th fraction (in the peak) was active only against gram negative bacteria *E.coli* (Figure 4) and showed no activity against other tested gram positive bacteria. As its activity was observed only against *E.coli* the activity may be attributed to Lipopolysaccharide (LPS)-binding protein (LBP) in the hemolymph. Similar LPS binding protein (450kDa) was also purified and characterized by **Jomori & Natori, (1990)** from *P. americana*. Fraction-29 did not showed activity against *E.coli* but showed activity against other Gram positive bacteria except *Stretococcus sp.,* (Figure 7). Fraction-30 was active except against *S. aureus*, fraction-31 is not active against *S. aureus* and *P. aerugenosa,* fraction-32, 33, 34, 35 was active against all bacteria with difference in zone of inhibition (Figure 4 to 8). Among all tested bacteria *P. aerugenosa* was more resistant to the insect peptides (Figure 6). The purified proteins are predominantly active against the gram-positive bacteria; it suggests that the antibacterial activity of the peptides is related to the cell wall of the bacteria. It may be assumed that the proteins identified in this study might play an important role in the self-defense against bacterial infection in dung beetle singly or in concert. Even though the comparative study of the antibacterial peptides with the antibiotic Gentamicin showed a less activity (Table 2), it might be due to destruction of antibacterial peptides during isolation and storage by other proteases and also in *invivo* condition these peptides may work together/synergistically and combat against the infection. Further studies are needed to work out the combined effect of peptides. At this point it also important to remember the development of resistance to general synthetic antibiotic like Gentamicin, penicillin, etc by variety of infectious microorganisms. It is also believed that antimicrobial peptides are assumed in the near future as an alternative for the nowadays classical antibiotics (**Małgorzata *et al.*, 2007)**. The advantages of antimicrobial peptides are many *viz.*, selectivity, fast killing, broad antimicrobial spectra and no resistance development **(Matsuzaki, 1999; Papo N and Shai Y, 2005)**. However, the present results are preliminary and future studies will be done following the EUCAST and/or CLSI methods to confirm the antimicrobial property of peptides.

CONCLUSION

The main idea of this research is to isolate novel antimicrobial peptides from dung beetle and to study its action against different human pathogens. From this research we found that *Onthophagus taurus* (dung beetle) has the ability to produce peptides to combat against human pathogens when an immune challenge is done. Before immune challenge antimicrobial peptides were found in negligible amount. Whereas after induction peptide concentration increased tremendously in the hemolymph. Since the peptides isolated from the insect showed activity against different bacteria we can conclude that this insect species can produce antimicrobial peptides which has wide range of activity against microbes (Broad spectrum). Broad spectrum antimicrobials are beneficial than specifically targeting molecules, because there will be no resistance development by pathogens. Further work has to be done to improve purification step without damaging the peptides so that the peptides can act equally or higher than that of conventional antibiotics. And sequencing of the peptides would be advantageous.

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