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 peptides could inhibit bacterial growth 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; Matsuoka, 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

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.

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

<table>
<thead>
<tr>
<th>Name of the organism</th>
<th>Activity</th>
<th>Zone of inhibition (diameter in millimeter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. coli</td>
<td>+</td>
<td>12</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>+</td>
<td>14</td>
</tr>
<tr>
<td>P. aerogena</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>Streptococcus sp.</td>
<td>+</td>
<td>12</td>
</tr>
<tr>
<td>S. aureus</td>
<td>+</td>
<td>10</td>
</tr>
</tbody>
</table>

Legend: ‘-’ means no activity; ‘+’ means active.

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. aerogenosa.

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

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Immune hemolymph (1mg/ml) Zone of inhibition (diameter in millimeter)</th>
<th>Gentamicin (1mg/ml) Zone of inhibition (diameter in millimeter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>P. aerogena</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Streptococcus sp.</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>S. aureus</td>
<td>8</td>
<td>20</td>
</tr>
</tbody>
</table>

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). Lebocins peptides inhibit growth of Gram negative and Gram positive bacteria. It is known that prolinrich peptides like P. prasina metalkinwos and B. mori lebocins, are similar to genetically modified proline-rich peptides which are active against sensitive microorganisms in relatively high concentrations. Metalkinwos inhibit Gram-negative bacteria growth at a concentration range from 50 to 200 mM depending on the isofrom (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 (300 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 (Harao et al., 1995; Furukawa et al., 1997; Yamakawa et al., 1999).

It has been observed in various insect species that bacteria injected into the haemoocoel 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 si-
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 staining 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 to 37 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. Figure 6 shows the results of the fractions of the tested bacteria with different 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 Momori & Natori, (1990) from P. americana. Fraction-29 did not showed activity against E.coli but showed activity against other Gram positive bacteria except Strepoccus sp. (Figure 7). Fraction-30 was active except against S. aureus, fraction-31 is not active against S. aureus and P. aeruginosa, 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. aeruginosa 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 now resistant to general synthetic antibiotic like Gentamicin, penicillin, etc. At this point it also important to note that resistance development in bacteria, they may be involved in signaling mechanism. Therefore, they are used to prevent resistance development in pathogens.

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 Omphalophagus 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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REFERENCES


