Isolation, evaluation and purification of antibacterial peptides from rhinoceros beetle, *Oryctes rhinoceros* (L.)

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Abstract

Antibacterial peptides were isolated from immune haemolymph of Rhinoceros beetle (*Oryctes rhinoceros* L.) larvae and evaluated for antibacterial activity against bacterial pathogens, viz., *Klebsiella pneumoniae*, *Micrococcus luteus*, *Bacillus subtilis*, *Paenbacillus macerans* and *Pseudomonas* sp. The peptides were found to be stable upto 37°C and antibacterial activity observed at pH 7.0. The peptide were active against all the pathogens with minimum inhibitory concentrations varying between 1.56 to 6.25 µM. Protein profiling on 16% Tricine sodium dodecyl sulphate polyacrylamide gel revealed that the molecular size of two antimicrobial peptides of 17 and 9 kDa, which was also observed as two peak fractions from reverse phase high pressure liquid chromatography (RP-HPLC) exhibiting antibacterial activity. These peptides were purified by Gel filtration chromatography and RP-HPLC upto 15 and 37 fold purifications, respectively.

Keywords: Antibacterial peptide, *Oryctes rhinoceros*, Tricine SDS-PAGE, Antibacterial activity, MIC, Gel filtration, RP-HPLC

Introduction

Antimicrobial peptides represent a unique and diverse group of molecules, classified into several subgroups based on the amino acid composition and structure. These are short proteins generally composed of 12 to 100 amino acids and are typically small cationic and amphipilic moieties exhibiting a wide spectrum of antimicrobial activity (1-4). These peptides include two or more positively charged residues provided by arginine, lysine, in acidic environments, histidine, and a large proportion (generally >50%) of hydrophobic residues (5-7). They are referred as host defense peptides which are found in many species of fungi, insects, frogs and mammals including human (8-10), although the first report was from bacterial origin (11).

The expression levels of antimicrobial peptides could be constitutive or inducible upon infectious or inflammatory stimuli (3,4,12). These short moieties serve as the basic form of innate immunity accumulating abundantly in epithelial and immune cells. Firstly, they represent a naturally occurring means of combating pathogenic challenge by rapid microbicidal activity (13). They are reported to have a direct effect on bacteria (14-18), fungi, (3,19,20) or viruses (21,22), by damaging or destabilizing them and suggested to be involved in the orchestration of the innate immune and antimicrobial immune-modulatory responses (23,24).
Some antibacterial peptides have been isolated and purified from insects of different orders, such as dipterans, isopterans, lepidopterans and coleopterans (12,25-28). A few well-characterized antimicrobial peptides in insects include; mastoparan, poneratoxin, cecropin, moricin and melittin (8,27,29-31). This paper reports the antimicrobial peptides isolated and purified from Rhinoceros beetle, *Oryctes rhinoceros*, a coleopteran pest of coconut palm and confirmation of antibacterial activity against five bacterial strains (*K. pneumoniae, M. luteus, B. subtilis, P. macerans* and *Pseudomonas* sp.).

**Materials and methods**

**Microbial cultures**

The bacterial cultures, *viz.*, *K. pneumoniae, M. luteus, B. subtilis, P. macerans* and *Pseudomonas* sp., which were isolated and identified by morphological and biochemical tests in our previous studies were used for the present study. The microbial cultures were grown and maintained in Nutrient agar medium.

**Insect culture**

The larvae of rhinoceros beetle, *O. rhinoceros* was collected from the farmyard, reared in laboratory condition using composts of cow dung, plant debris as source of food and allowed to grow until the pre-pupal stage.

**Immunization and Haemolymph collection**

The pre-pupal larva of *O. rhinoceros* (average body weight 16.5 g/larvae) was injected with $10^6$ cfu viable log phase culture of *M. luteus* and haemolymph was collected as per the methodology described by Cytrynska et al. (2007), (32).

**Protein and Protease assay**

Protein quantification was done by Lowry’s method (33) using Bovine Serum Albumin as standard. Protease assay was performed using pepsin (Sigma) as standard (25).

**Antibacterial activity assay**

Antibacterial activity of the peptide was assayed by the method as described by Faber et al. (2005) (9). Plate growth inhibition assay was performed with ~ $2 \times 10^6$ logarithmic-phases of bacterial strains spread plated in Luria Bertani (LB) agar (pH 7.2) plate and incubated at 37°C overnight in an incubator (Remi) and zone of inhibition was recorded.

**Effect of different temperature and pH on antibacterial activity**

The lyophilized crude peptide sample from the larvae induced by *M. luteus* was dissolved in PBS (150 mM, pH 7.0) to make a final concentration of 10 µg/ml. The effect of temperature and pH on antibacterial activity was assessed by varying the temperature at -20, 4, 15, 25, 37, 50, 70, 90 and 100°C at constant pH 7.0 and varying the pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, at constant temperature 37°C for 24 h, respectively. Microbial growth was monitored by multiwell reader (BioRad) and absorbance read at 600nm. Bacterial culture without sample was used as control.

**Determination of the minimal inhibitory concentration (MIC)**

The minimal inhibitory concentration was done according to the method followed by Cytrynska et al. (2007). (32). Minimal inhibitory concentration (MIC) was recorded based on the lowest concentration that completely inhibited the growth of the bacteria.

**Tricine SDS-PAGE**

16% Tricine –SDS-PAGE was performed to resolve the peptide by the method of Schagger (2006), (34) using low molecular weight (3000-43,400 Dalton) protein markers (Genei, Bangalore) and stained with Coomassie brilliant blue stain.

**Purification of the antimicrobial peptides**

**Gel filtration chromatography**

Gel filtration purification using sephadex G-50 column equilibrated with ammonium acetate buffer (100 mM, pH 7.0) was done as per the protocol of Peng et al. (2008), (12). The freeze-dried (lyophilized) sample was reconstituted in known volume of distilled water and 1.5ml heat-treated sample was passed through Sephadex G-50 Column (Hidex Himedia; 60 x 1.0 cm column). Two ml fractions were collected from the column at the flow rate of 15 ml/h, up to 72 fractions and the protein content was monitored at 280 nm by an UV-double beam spectrophotometer (Systronics; 2101). The fractions with antibacterial activity were pooled and used for further purification.

**Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)**

The active pooled fractions of gel filtration step were
passed through reverse-phase HPLC (HP–1100 series) RP-18 Merck column (50mm×4.6μm) column as per the procedure described by Imamura et al. (1999). (30). The protein fractions were eluted in an elution range of 0 to 70% acetonitrile in water for 40 min at a flow rate of 1ml/min. The protein was monitored as done with gel filtration chromatography.

**Results and Discussion**

Antimicrobial peptides often called as host defense peptides (HDPs) are short cationic molecules produced by the immune systems of many multi-cellular organisms and play a central role as effector molecules of innate immunity. In humans, both resident and infiltrating cells on the skin synthesize and secrete small peptides, viz., cathelicidins and defensins that demonstrate broad-spectrum antimicrobial activity against bacteria, fungi, and enveloped viruses (13). Few of the peptides that are well characterized in insects include mastoparan, poneratoxin, cecropin, moricin and melittin (27,29-31,35). The present study establishes that larvae of the coconut rhinoceros beetle, *O. rhinoceros*, family: Scarabaeidae and order: Coleoptera respond to a bacterial challenge by the rapid appearance of antibacterial peptides in the haemolymph. This result correlates early investigation in *Heliothis virescens* (3). Earlier, antibacterial peptides have been isolated and purified from insects of different orders, viz., dipteran (26,36,37), isopteran (3), lepidopteran (12,32,35,38) and coleopteran (25,27,28,30,31).

**Antibacterial activity**

Haemolymph was collected from the *M. luteus* immunized beetle, *O. rhinoceros* at the third instar non-feeding stage with bacteria. The appearances of anti bacterial activity in the haemolymph, monitored by the plate growth inhibition assay against *K. pneumoniae, M. luteus, B. subtilis, P. macerans* and *Pseudomonas* sp. revealed that after 48 h there was prominent clear zone formation of sizes, 0.62 mm, 0.7 mm, 0.42 mm, 0.45 mm and 0.54 mm, respectively. Peng et al. (2008) have reported that lepidopteran antibacterial peptides had strong inhibition against *Staphylococcus aureus, Escherichia coli K12-D31*, and *Salmonella derby* (12). In the present study, the zones of inhibition remained prominent even after two weeks of incubation but subsequently, there was decrease in zone size. Untreated larvae (Control) were found to be devoid of significant zone of inhibition. Similarly, antibacterial activity of antimicrobial peptides has earlier been reported against pathogenic bacteria (14-18,30). Three structurally related antibacterial peptides (acalooleptins A1, A2, and A3) from the haemolymph of immunized larvae of *Acalolepta luxuriosa* (Coleoptera) shared the same 6 N-terminal amino acid residues and demonstrated antibacterial activity against few Gram-negative bacteria (30).

When the cell-free haemolymph of challenged larvae was treated with protease, no antibacterial activity was observed. The antibacterial activity seen in the *O. rhinoceros* is due to a family of these particular peptides as protease assay confirmed that the molecules responsible for this antibacterial activity are peptides and not due to any salt or proteins. Earlier, in an antibacterial assay, protaetins were reported to show antibacterial activities against a group of Gram-positive and Gram-negative bacteria (25). Faber et al. (2005) have reported high level of antibacterial activity of human lactoferrin 1-11 and gentamicin peptides against chronic methicillin-resistant *S. aureus* osteomyelitis (9). The antibacterial activity of the induced BTI-Tn-5B1 cell line from Lepidoptera with heat inactivated *Escherichia coli* DH5α was found to be the highest, and the antibacterial activity was found to be increasing gradually to the highest level at 16 h after stimulation (12). *Nicrophorus* secretions were demonstrated to attenuate the growth of naturally occurring microorganisms likely to be found colonizing the carrion resource, and that the active antimicrobial components of the secretions are small antimicrobial peptides (AMPs) similar to those produced by other insects (28). It has been explained that antibacterial peptides are evolutionarily conserved and are usually positively charged and have both a hydrophobic and hydrophilic sides that enable the molecules to be soluble in aqueous environments yet also enter lipid-rich membranes (6). These peptides kill target cells through diverse mechanisms once they reach the target microbial membrane. They possess a wide array of biological mechanisms from direct killing of invading pathogens to modulation of immunity and other biological responses of the host (24).

**Effect of temperature and pH on activity of the peptide**

All the test organisms were found to thrive well at same optimum temperature and pH. Hence, the present experiment on effect of temperature and pH on antibacterial property of the peptide was assayed with only one pathogen, *M. luteus*. Among the various temperatures tested, the peptide was found to be stable up to 37°C and retained its activity at temperature 37°C or below in buffer strength of 150 mM (Fig.1). The peptides from *Musca domestica* larvae were very thermo-stable and had antibacterial activity against Gram-positive bacteria such as *B. subtilis* (39). Besides maintaining the stability of bioactive peptides by removing the exotic proteases, the heat-chromatography could also extract non-peptides with antibacterial activity (39). Similarly, the effect of pH varied from 2.0 to 9.0 at buffer strength of 150 mM. It

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revealed that the peptide retained its antibacterial activity only at neutral pH (7.0) condition. These peptides were found to be highly pH sensitive; as loss in its antibacterial activity at pH ranges above or below neutral was observed (Fig. 2). In other words, in acidic and alkaline pH, no significant activity was observed. This narrow pH range for a good activity of the peptide can be a disadvantageous for use as a commercial anti microbial drug. But the stable activity at room temperature and below at neutral pH gives room for stabilizing the peptide by chemical means that could be used as an antibacterial drug.

Minimal inhibitory concentration (MIC)

Minimal inhibitory concentration (MIC) serves as a critical minimum level of concentration required for confirming antibacterial activity. MIC levels of *O. rhinoceros* antimicrobial peptides determined at various peptide concentrations (0.781 to 100 µg/ml) in a microtitre plate assay against five bacterial pathogens is reflected in Table-1. The assay revealed that the peptide was active against *Micrococcus luteus* even at a lower concentration of 1.56 µM and a higher concentration of 6.25 µM was found to be effective against *P. macerans* and *B. subtilis*. These values were comparable with antimicrobial activity reported by termcin (3) and moricin (35). *Galleria mellonella* immune haemolymph defensin like peptide inhibited fungal and sensitive bacteria growth in a concentration of 2.9 and 1.9 µM, respectively (32). Metalnikowins were reported to inhibit Gram-negative bacteria growth at a concentration range from 50 to 200 µM depending on the isoforms, (40). Contrarily, higher side (211.1 µM) of minimal inhibitory concentration of lebocin3 was effective against *E. coli* in nutrient broth (38). It was also suggested that lebocins act synergistically in reducing the minimum inhibitory concentration of other antimicrobial peptides.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Minimal inhibitory concentration (MIC) µM</th>
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</thead>
<tbody>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>3.12</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>6.25</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>1.56</td>
</tr>
<tr>
<td><em>Paenibacillus macerans</em></td>
<td>6.25</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>3.12</td>
</tr>
</tbody>
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Tricine-SDS-PAGE

16% Tricine-SDS-PAGE was done for each purification step to check the changes in the peptides molecular profile on the basis of sizes. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 KDa (34). In the present study, there were four prominent bands observed in the gel with the molecular weight ranging from 9 kDa to 43kDa Dalton in the crude protein from immunized larvae of *O. rhinoceros*. The higher range bands were eliminated as they are not falling within the range as reported for antimicrobial peptides. Antimicrobial peptides (AMPs) are reported to be small molecular weight proteins with the molecular sizes in the range of 7 to 12 kDa (6,32,35,38). For instance, protaetins 1 and 3 purified and characterized from larval haemolymph of *Protaetia brevitarsis*, a fruit tree pest in Korea was reported to have molecular masses of 7.5 and 12 kDa, respectively on Tricine SDS-PAGE while protaetin 2 was determined to be 9kDa size by MALDI-TOF mass spectrometry (25).

Two prominent bands of 9 and 17 kDa sizes were present
only in the challenged samples which was absent in the unchallenged samples (Fig. 3). The pooled fractions from the gel filtration and RP-HPLC purifications showing antibacterial activity also revealed the presence of these two bands at the same position in 16% Tricine-SDS-PAGE. These results corroborates with earlier reports. The major antibacterial peptides extracted from Musca domestica larvae by heat-chromatography were determined with Mw 6.2-17.2 kDa in Tricine-SDS-PAGE and pl 5.59-5.91 with IEF-PAGE (39).

Fig 3: 16% Tricine SDS-Polyacrylamide gel profile of Oryctes rhinoceros antibacterial peptides. M- Low molecular weight protein marker, S1 & S2- Gel filtration peptide fraction of haemolymph of Micrococcus luteus immunized Oryctes rhinoceros.

Purification of the antibacterial peptides

The crude sample of the peptide (cell free haemolymph) was purified by using Sephadex G-50 column chromatography and RP-HPLC. By gel filtration chromatography and RP-HPLC purifications, 15 and 37 fold purifications of the peptides were achieved (Table-2). Earlier, Yoon et al. (2003) has purified antibacterial peptides named protaeitins by gel filtration, preparative acid-urea PAGE, and reversed-phase FPLC to such purification extents (25). However, Only 5.3 fold purification could be achieved by heat chromatography in case of Musca domestica larvae antimicrobial peptides (39).

In gel filtration, out of the 72 fractions, the fractions 27 to 41 showed inhibitory effects in plate inhibition assay confirming its antimicrobial activity. These fractions with high inhibitory effect were pooled, lyophilized and purified by RP-HPLC. RP-HPLC purification resulted in four peaks of which two peaks, one major and one minor peak, corresponding to 17 kDa and 9kDa bands, respectively in 16% Tricine/6M Urea polyacrylamide gel exhibited antibacterial activity. Earlier, three structurally related antibacterial peptides (acaloeloptins A1, A2, and A3) with a molecular mass of 8 kDa was purified by RP-HPLC and characterized from the haemolymph of immunized larvae of the Udo longicorn beetle, Acalolepta luxuriosa (30). Reverse phase HPLC analysis of the haemolymph of immunized and naive larvae showed that acaloeloptins A1, A2, and A3 were inducible and suggested that all three peptides were produced in a single insect.

Similarly, Liu et al. (2009) has reported that antimicrobial peptides from T. molitor L. larvae were purified through RP-HPLC, though produced several peaks, only 4th and 9th peaks could be identified as antimicrobial peptides. They further demonstrated that there are more than one antimicrobial peptides and anti-bacterial substances which have anti-tumour cell K562 activity (18). Peng et al. (2008) have identified a new antibacterial peptide with a molecular weight of about 8000 Da was preferentially induced in Trichoplusia ni BTI-Tn-5B1 cells in 16 h after stimulation (12). These results evinces that the there could be possibility of more than one type of antimicrobial peptides produced at a time in the same insect. Therefore, if these two peaks/bands represent same peptide but different isoforms or two different antimicrobial peptides, needs to be investigated. This study forms the basis for further work on characterization and chemical structural analysis which could facilitate a single novel drug development against these five tested bacterial pathogens.

Table 2: Purification profile of antibacterial peptides from haemolymph of Micrococcus luteus immunized Oryctes rhinoceros.

<table>
<thead>
<tr>
<th>Purification technique</th>
<th>Total protein (µg)</th>
<th>Antibacterial activity*</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude peptide</td>
<td>783</td>
<td>0.7 mm</td>
<td>-</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>545 µg</td>
<td>0.69 mm</td>
<td>15</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>282 µg</td>
<td>0.67 mm</td>
<td>37</td>
</tr>
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* Antibacterial activity was recorded as size of zones of inhibition

Conclusion

Antimicrobial peptides have been identified in several insects belonging to different orders, predominantly from coleopterans (25,27,28,30,31). The present study also has proved that antimicrobial peptides isolated from rhinoceros beetle, Oryctes rhinoceros, a coleopteran insect has broad spectrum antibacterial activity. It is also evident from several
studies recently that apart from showing antimicrobial activity, these peptides are involved in a remarkably broad range of host defense related functions including neutralization of some bacterial toxins and augmentation of both innate and adaptive immune mechanisms (7,28, 41). Since several of them have proved to be effective against antibiotic resistant bacteria, these peptides are being widely used as blueprints for the design of new antimicrobial agents, (42). Antimicrobial peptides based therapies are attractive candidates as alternative antibiotic treatments, since they offer several potential advantages over currently used classes of drugs.

Acknowledgement

The authors are thankful to Dr. M. Aruchami, Secretary, Kongu Nadu Arts and Science College, Coimbatore, for providing infrastructure facilities in the Department of Biotechnology and constant support for conducting this project.

References


