Partial purification and characterization of antimicrobial peptide from the hemolymph of cockroach *Periplaneta americana*

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**ABSTRACT**

The partial purification and characterization of antimicrobial peptide (AMP) from the hemolymph of cockroach, *Periplaneta americana*, was studied. Hemolymph was drawn from cockroach and the AMP was purified on a sephadex G-75 gel filtration column. The gel filtration showed two peaks, I and II, and only peak II showed five active fractions, namely, 12, 13, 14, 15, and 16, of antimicrobial activity. Fraction 13 showed the highest microbial Inhibition Concentration (MIC) and a single protein band on SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) with a molecular mass of 60.2 kDa. Purified antimicrobial protein exhibited the highest antimicrobial activity against *Escherichia coli* at 30°C, pH 6.0, and 5 mM calcium ion. The AMP showed higher activity of MIC against lipopolysaccharides and β-1,3 glucan during 5 hours of exposure. The study concludes that the AMP from hemolymph was effective against microbes or was able to recognize the molecular pattern of microorganisms.

1. **INTRODUCTION**

Antimicrobial peptides (AMPs) are evolutionarily highly conserved molecules and constitute an important component of insect immunity. It is observed in all organisms of the domain of life, from bacteria to humans. The major family of AMPs, which has been characterized, is the defensin [1]. During the past two decades, several AMPs were isolated and purified from a wide range of animals, including both vertebrates and invertebrates, and have been reviewed by Martin [2]. These peptides show a broad spectrum of activity against a range of microorganisms including Gram-negative and Gram-positive bacteria, protozoa, yeast, fungi, and viruses. They have the potential to overcome bacterial resistance, thus making them a promising candidate for therapeutic drugs [3].

The cockroaches are seen in habitats that are always exposed to potential pathogenic microorganisms and parasites, but only a few encounters resulted in infection [4], because insects, on recognizing the infections, often initiate a complex genetic cascade and produce AMPs into the hemolymph [5,6]. Some insects synthesize inducible AMPs, such as lysozyme, a constitutive molecule like lipopolysaccharide (LPS) binding protein, which have been isolated from the hemolymph of the American cockroach, *Periplaneta americana* [7,8]. This protein also acts as an opsonin [9–11].

The American cockroaches mostly live in sewage and sewer pipe, which harbor high density of bacteria or other microbes that are potentially pathogenic. Hence, it is most likely possible that these insects may defend foreign microbes invading its body by means of AMPs. The AMPs are usually synthesized in fat body or hemolymph to eliminate microorganisms [12–14]. This work was carried out to partially purify and characterize hemolymph AMP from *P. americana*.

2. **MATERIAL AND METHOD**

2.1. **Insects Rearing**

The cockroaches *P. americana* were collected from godowns and reared in the laboratory in dark containers at room temperature 27°C ± 1°C and 12:12 light:dark (L:D) cycle. All experiments were conducted at room temperature unless specified.

2.2. **Collection of Hemolymph From Non-Induced and Induced Microbes**

For collecting hemolymph, the cockroaches were divided into four groups of 30 cockroaches. Group I as control (non-induced
with *Escherichia coli*), group II with induced *E. coli*, group III as control (non-induced with β-1,3 glucan), and group IV with β-1,3 glucan induced.

### 2.2.1. Collection of hemolymph for AMP assay after treatment with β-1,3 glucan and LPS

Before collecting the hemolymph from the non-induced (control) group, adult cockroaches were topically sterilized with 70% ethanol. The control cockroach was injected with 10 µl of Carlson’s saline as described by Jomori and Natori [9] in the abdominal cavity, whereas, for induced, 10 µl of *E. coli* (0.01 µg/10 µl) (10⁴ cells/ml) or β-1,3 glucan (Sigma) (0.01 µg/10 µl) was injected in the abdominal cavity using 190 µl Carlson saline as a medium. The hemolymph of control and induced *E. coli* or β-1,3 glucan was collected after 1, 3, and 5 hours from the coxal membrane of legs, using a sterile syringe from all groups. The collected hemolymph was centrifuged at 1200× g for 15 minutes and the supernatant was stored at 4°C for further use. The resulting supernatant was used as a source of AMP and assayed. The protein content of all samples was determined by Lowry’s method [15] or by UV (ultra violet) spectrophotometer using BSA (Bovine Serum Albumin) as standard protein.

### 2.2.2. The bacterial and yeast strains

For screening, the AMP from hemolymph compounds was based on evidence as mentioned in earlier studies [16,17]. We used *E. coli* ATCC25972, which was susceptible to all antibiotics. The β-1,3 glucan used in the present study was obtained from Sigma.

### 2.3. Purification Procedure

The hemolymph collected was analyzed for AMP. The collected hemolymph was centrifuged at 700× g for 10 minutes. The supernatant was loaded on a gel filtration column of Sephadex G-75 (1.5 cm × 13 cm), which was previously equilibrated with 20 mM ammonium acetate buffer (pH 6.4). The aliquot was collected at a flow rate of 0.5 ml/min, a volume of 3 ml was collected as described by Kyung et al. [18] with minor modification. The protein content in aliquot was assayed at 280 nm absorbance, and the AMP activity of each fraction was measured according to the procedure described in the following section.

### 2.4. Antibacterial Assay

The antibacterial assay was carried out by the agar well diffusion method as described by Perez et al. [19]. For control, paper soaked with 20 µl of non-induced hemolymph was used. The plates were incubated overnight at 37°C, and the diameter of the clear zone was measured.

#### 2.4.1. Effect of pH

The effect of pH was studied by varying pH ranges pH 3.0, 4.0, 5.0, 6.0, and 7, respectively. The reaction mixture contains 50 µl AMP aliquot added with 50 µl phosphate buffer of different pH, well mixed and incubated for 15 minutes at room temperature, and assayed for antibacterial assay.

#### 2.4.2. Effect of temperature

The effect of temperature was studied by incubating the aliquot to different temperatures, namely, 10°C, 20°C, 30°C, and 40°C in a heat block for 20 minutes. A 50 µl AMP aliquot was mixed with 50 µl 20 mM Phosphate Buffered Saline (pH 7.8) and kept for 5 minutes, followed by heating on a heating block at different temperatures for 10 minutes; 50 µl AMP aliquot from each incubated temperature was assayed for antibacterial assay.

#### 2.4.3. Effect of metal ion on AMP

50 µl of the purified AMP sample was mixed with 50 µl of different concentrations of calcium, namely, 3, 4, 5, and 6 mM were added and gently mixed well and incubated for 20 minutes. The sample was poured in petri dishes, and antimicrobial assay was carried out.

### 2.5. Determination of Microbial Inhibition Concentration (MIC)

MIC was determined by the broth dilution method [20]. About 0–120 µl/ml of AMP was incubated into the nutrient broth and incubated for 24 hours at 37°C. MIC was defined as “the lowest concentration of the peptides inhibiting visible growth.” After incubation, absorbance was measured at 620 nm.

### 2.6. SDS-PAGE

The SDS-PAGE of the isolated peptide was carried out on 12% separating gel according to the procedure described by Laemmli [21]. The electrophoresis was carried out at a constant voltage of 100 V for 3 hours. The molecular weight of the protein band was determined using standard medium-range molecular markers: 97.4 KDa phosphorylase-b, 66 KDa-Bovine serum albumin, 43 KDa-Ovalbumin, 29 KDa-Carbonic anhydrase, 20.1 soybean trypsin inhibitor, and 14.3 KDa-lysozyme.

### 3. RESULTS AND DISCUSSION

#### 3.1. Chromatographic Profile of Peptide

The purification of the peptide is shown in Table 1, and the elution pattern on gel filtration is shown in Figure 1. The elution fraction showed two peaks of which only fraction 2 showed antimicrobial activity against *E. coli*. The five fractions are 12, 13, 14, 15, (0.01 assayed for antibacterial assay. 5.0, 6.0, and 7, respectively. The reaction mixture contains 50 µl of the peptides inhibiting visible growth.” After incubation, absorbance was measured at 620 nm.

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#### 3.2. Effect of Different Temperatures, pH, and Calcium Ions

The effect of different temperatures on the purified peptides (peak II, F13) against *E. coli* is shown in Figure 2. The peptide showed the zone of inhibition (mm) as 3, 4, 6, and 4 mm for temperatures 10°C,
20°C, 30°C, and 40°C, respectively. A decline in the zone of inhibition was observed at 40°C. Dang et al. [23] and Lu et al. [24] reported thermostable antibacterial peptides in *Bactrocera dorsalis* and *Musca domestica* (Diptera). However, in *Oryctes rhinoceros* (Coleoptera), peptide was only stable up to 37°C against *Micrococcus luteus* [25].

The effect of different pH on the antimicrobial activity of purified peptides (peak II, F13) was determined against *E. coli* (Fig. 3). It was observed that with a change in pH, the antimicrobial activity of peptide also changed. The AMP activity was observed between pH 3–7, maximum activity was observed at pH 6, and thereafter a decrease was observed. The result showed that the activity of purified peptide is pH dependent and varies for *E. coli*. The study showed a broad pH range of 3–7, and no pH activity was observed below pH 3. It appears that the peptide possibly requires an acidic environment for exhibiting its optimum antimicrobial activity. Rabeeth et al. [25] reported antimicrobial activity at pH 7 in rhinoceros beetle, *Oryctes rhinoceros* (L.), at pH 6.2 in *Brevibacillus laterosporus* [26], at pH 6.8 in bovine Lactophoricin [27]. Some higher or lower pH values have also been reported: pH 5.2 for *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Enterobacter sakazakii* [28]; pH 5.0 for *Clitocybe sinopica* [29]; pH 7.0 for *Bacillus megaterium* [30]; and pH 7.0 for *Bacillus subtilis* [31]. The peptide was found to be highly sensitive to pH because at high or low pH resulted in the loss of antibacterial activity.

We observed that at 5 mM calcium ion, the antibacterial activity showed the highest activity against *E. coli* (Fig. 4). Sakhivel and Palani [32] reported that Ca$^{2+}$ and Mn$^{2+}$ ions enhanced the antimicrobial activity from *Bauhinia purpurea*. Wen et al. [33] reported the presence of an anionic AMP in *Bombyx mori* and in the wax moth *Galleria mellonella* [34], which causes surface alterations in *E. coli* and the death of this bacterium along with lysozyme [34]. A higher concentration is reported to destroy the structure of the cell membrane through electrostatic interaction with LPS [35].

### 3.3. Effect of Purified Peptide on Apparent Proliferation of *E. coli*

The active purified peptide chromatographic fraction was used to determine MIC in culture media. The MIC increased with an increase in microbial peptide concentration (Fig. 5). The highest value of MIC was observed in F13 (Fig. 6). The *E. coli* growth

| Step                  | Volume (ml) | Protein A$_{280}$ (mg/ml) | Total Protein (mg/ml) | Total activity (AU) | Specific activity (U/mg) | Yield (%) | Purification (fold) |
|-----------------------|-------------|---------------------------|-----------------------|--------------------|--------------------------|-----------|--------------------|
| HLS                   | 7.2         | 0.538                     | 64.8                  | 336.96             | 5.2                      | 100       | 1                  |
| Gel Filtration (F-13) | 3.0         | 0.358                     | 43.2                  | 129.6              | 3.0                      | 38.46     | 0.57               |
reduced to 57.05% at 100 µg and to 93.86% by 200 µg. The percentage inhibitory activity of purified protein against *E. coli* showed that the peptide was active against *E. coli* at a lower concentration of 4.5 × 10^{-3} mM in F16 and a higher concentration of 1.115 × 10^{-3} mM in F13. In *G. mellonella*, immune hemolymph defensing-like peptide inhibited bacterial growth at a concentration of 2.9 and 1.9 µM.[36] Liu et al. [37] reported inhibition of bacterial growth at a concentration of 50–200 µM depending on the isoforms.

### 3.4. Effect of LPS and β-1,3 Glucan on MIC

The MIC for the control hemolymph before injection of β-1,3 glucan and LPS was 1.17 × 10^{-3} and 1.190 × 10^{-3} respectively. However, after 1, 3, and 5 hours after injection with β-1,3 glucan, the values observed were 1.585 × 10^{-3}, 1.68 × 10^{-3}, and 1.75 × 10^{-3}, and for LPS, the values were 1.64 × 10^{-3}, 1.79 × 10^{-3}, and 1.8 × 10^{-3}, respectively (Fig. 7). The inhibitory activity was measured against *E. coli*. A similar report for AMP was obtained for *B. mori* [38]. In sand fly, *Phlebotomus duboscqi*, bacteria injection resulted in the release of defensin family peptides with antibacterial activity in their hemolymph [39]. Andrejko and Mizerska-Dudka [40] reported that the antimicrobial cecropin proteins remain active even after 18 hours of infection by *E. coli* in *G. mellonella*, and the peak activity remains active till 48 hours in the immune system of the insect. In other insects, the maximum antimicrobial activity after injection was observed for 12 hours [41]. Erler et al. [42] reported that *E. coli* is an effective inducer of insect humoral immune responses. The insect hemocytes being a repository of AMPs, therefore, may involve in the activation of a complex innate immune system involving cellular and humoral responses [43,44]. We observed that apart from *E. coli*, β-1,3 glucan too showed a higher MIC, suggesting AMP may have both antibacterial and antifungal activity. However, how exactly the, β-1,3 glucan is influenced by AMP is not known.

### 3.5. SDS-PAGE

The pattern of separation of purified protein by gel filtration showed a single band of 60.2kDa molecular weight (Figure 8). Seraj et al. [17] reported a molecular weight of 61 kDa antibacterial protein from the American cockroach, *P. americana*. Similarly, Rethna Priya [45] reported a 77 kDa antibacterial protein molecule in *Clibanarius clibanarius*. Morishima et al. [46] reported a peptide of 6 1 kDa in *Bombyx mori*, and a 64 kDa in *Hyalophora cecropia* [47].

### 4. CONCLUSION

The AMP purified from *P. americana* exhibited a potential antimicrobial activity against *E. coli*. The purified protein was effective at an optimum pH 6.0 and at temperature 30°C. At 5 mM calcium ion, the antibacterial activity was higher. The activity of the purified protein was influenced by the changes in pH, temperature, and concentration of calcium ions. The molecular
weight of the purified protein was determined as 60.2 kDa by SDS-PAGE. The study showed that the MIC increased with an increase in microbial peptide concentration. The AMP showed an increased MIC activity against \( E. \ coli \) and \( \beta-1,3 \) glucan, till 5 hours. The study concludes that the AMP from hemolymph may be effective against microbes or will have a specific recognition pattern against microbial infection. It is not surprising that cockroaches carry harmful pathogenic microorganisms that are contagious between humans and animals. However, these microbes do not have a pathogenic effect on cockroaches; surely, the answer may lie in their complicated specific immune defense mechanisms, which are now being studied in detail to understand their mechanisms for the benefit of mankind.

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