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ORIGINAL ARTICLE

Characterization of the immune induced antimicrobial peptide in *Drosophila melanogaster* and *Drosophila ananassae*

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Abstract. Insects can recognize invading pathogens and initiate an immune response. Among them, *Drosophila* has emerged as an invertebrate model for investigating innate immune responses in which antimicrobial peptides play a crucial role. In the present study, immune-induced antimicrobial peptides were characterized in *D. melanogaster* and *D. ananassae* using the agar well diffusion method, HPLC, SDS-PAGE and LC-MS/MS after infection with either *S. aureus* or *E. coli*. The HPLC revealed two and three differentially induced components, respectively, in *D. melanogaster* and *D. ananassae* flies infected with *S. aureus* and *E. coli*. The tricine SDS-PAGE analysis also revealed two and five differentially induced proteins, respectively, in *D. melanogaster* and *D. ananassae* infected with *E. coli*. In *E. coli* infected flies, the ~6 kDa band was produced at higher level. Based on LCMS/MS and Mascot analysis, the peptide was identified as a putative cecropin A-like peptide, and the data suggested that both species of *Drosophila* have exhibited a clear immune response. The flies were also able to discriminate between bacteria, as this putative cecropin A-like peptide was produced in flies infected with *E. coli* but not *S. aureus*.

INTRODUCTION

The fruit fly, *Drosophila melanogaster* has innate immunity against invading microbes. This includes both cellular and humoral immune responses (Lye, 2018; Meghashree & Nagaraj, 2020). Antimicrobial peptides (AMPs) are an important component in the first line of defence (Yuchen et al., 2019). AMPs are endogenous peptides with a molecular weight (MW) of ~2–22 kDa and they are released by the fat body (analogue of the liver) into haemolymph to clear off the microbial infections (Troha et al., 2019). The interactions of AMPs with Gram-positive and Gram-negative bacteria differ. The positively charged AMPs selectively interact with prokaryotes having a negatively charged bacterial cell-wall, including lipopolysaccharides (LPS) and phospholipids. Based on the available data (FlyBase), nine distinct classes of AMPs (23 members) are identified in *Drosophila* (Thurmond et al., 2019). Among them, attacin, diptericin, cecropin and drosocin are produced in response to Gram-negative bacterial infections (Imd pathway), metchnikowin and defensin in response to a Gram-positive bacterial infection (Toll pathway) and drosomycin only in response to fungal infection (Sheehan et al., 2018).

As fruit flies are genetically similar in the way they combat diseases as humans, they can be used to evaluate microbial infections and their associated immune responses (Baenas & Wagner, 2019). *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) are two commonly used pathogens in studies on the immune response in *Drosophila* and are recognized by two distinct immune pathways. Like *D. melanogaster*, *D. ananassae* is another cosmopolitan and domestic species with high mutable property (Singh & Yadav, 2015). Most of the studies on this species are on its behaviour, genetics and other evolutionary aspects (Kumar & Singh, 2017; Kaladchibachi et al., 2019; Singh et al., 2020) in which there are no reports of infection-induced antimicrobial peptides. Understanding the immune response in this species and how it compares with that of *D. melanogaster* will provide a better knowledge of whether these two species differ in their microbe-induced immunity.

Cecropin A was first isolated from the moth, *Hyalophora cecropia*. Later on, cecropin-like peptides were identified in different insects belonging to Diptera, Hymenoptera, Coleoptera, Lepidoptera and Isoptera, and have different names, including sarcotoxin-I (Buonocore et al., 2021), hinnavin (Wu et al., 2018) and papiliocin (Kim et al., 2010), etc. In *Drosophila*, there are four cecropin genes on chromosome 3R, which give rise to cecropin A1, cecropin A2, cecropin B and cecropin C peptides (Brian & Clark,

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2003), which are highly conserved in insects. There are also many research studies focused on identifying novel cecropin-like peptides in insects (Wu et al., 2015; Park & Yoe, 2017; Manniello et al., 2021).

Hence, the objective of this study was to determine whether the induction of immune-induced AMP in *D. melanogaster* and *D. ananassae* flies infected with either *E. coli* or *S. aureus* differed. To authenticate its antimicrobial efficacy, homogenates of flies were tested for antibacterial activity in vitro. In addition, the phylogenetic relationships of the identified peptide are also discussed.

MATERIALS AND METHODS

Fly stocks

D. melanogaster (1.002) and *D. ananassae* (11.001) were reared on an instant *Drosophila* diet supplemented with yeast and kept at room temperature (RT) under 12L : 12D conditions. The flies were obtained from the *Drosophila* Stock Center, University of Mysore, Mysore, Karnataka, India. For all experiments, 4–5 day old male and female adult flies (1 : 1) were used.

Bacterial species

Escherichia coli (MTCC 723) and *Staphylococcus aureus* (MTCC 7443) were obtained from MTCC, Chandigarh. All bacterial cultures were maintained on a nutrient agar medium. For liquid culture, bacteria were grown in sterile tubes containing 5 mL of nutrient broth (beef extract – 3g/L; peptone – 0.5g/L; NaCl – 0.5g/L), which was incubated for 24 h at 37°C before use. An optical density of 0.5 (OD_{600}) having 1×10^7 CFU/mL was used as an infectious dose and was obtained using a spectrophotometer (Multiskan Sky, Thermo scientific).

Bacterial infection

Flies were anesthetized and infected by inserting a tungsten needle into the lateral side of the thorax that had been dipped into either *S. aureus* or *E. coli* suspended in phosphate buffer saline (PBS). The treated flies were kept at RT by placing each of them in a fresh vial, laying the vial on its side until all flies recovered from the anesthesia in order to avoid the flies from becoming stuck in the food (Khalil et al., 2015). For the control group, flies were pricked with PBS dipped needle to create a non-septic injury.

Preparation of crude extract and isolation of haemolymph

Infected flies (n = 50) were homogenized in 120 µL 0.1% trifluoroacetic acid (TFA) at an ice-cold condition (Bhagavathula et al., 2017). The disrupted homogenate was further sonicated (QSonica 125, Thermo Scientific) at 20% amplitude for 5 cycles at an interval of 5 s. A 100 µL supernatant of each extract was collected after centrifugation at 10000 × g for 15 min at 4°C, freeze-dried using a lyophilizer (FreeZone, Labconco) for 10 h and stored at –80°C until further use. These lyophilized samples were used for HPLC analysis and antibacterial activity.

The haemolymph was collected by means of centrifugation (Dhar & Mishra, 2020) in which each fly after 24 h of bacterial infection was pricked with a needle to release the haemolymph. A 0.5 mL vial was punched with 4–5 tiny holes using a 24G syringe needle and all the pricked flies (n = 50) were added to it. This vial was put inside a 1.5 mL microcentrifuge tube from which haemolymph was collected after centrifugation for 10 min at 2000 × g and stored at –80°C (Damrau et al., 2015). From each vial, 2 µL of haemolymph was extracted and freshly isolated samples were used for protein quantification and SDS-PAGE analysis.

Protein quantification

The protein concentration in the haemolymph from control and infected (24 h) flies was quantified in five independent experiments (n = 50) by direct concentration measurement in which the haemolymph was diluted 1 : 5 with double distilled water. A 2 µL of diluted haemolymph was placed on a µDrop™ plate and absorbance was measured at 280 nm using Multiskan Sky spectrophotometer (Thermo Scientific™, USA). Protein concentration was determined based on this instrument's built-in protocol for the extinction coefficient of BSA.

In vitro antibacterial activity determined using the agar well diffusion method

The freeze-dried homogenate from *Drosophila* spp. obtained 24 h after injection with either *S. aureus* or *E. coli* or PBS (Control) were examined for antibacterial activity against the respective bacteria using the agar well diffusion method (Sewify et al., 2017). In brief, 5-mm diameter wells were made with a sterile cork borer (6 mm diameter) in nutrient agar plates spread with *S. aureus* or *E. coli*. The lyophilized homogenate (5 mg) was suspended in 50 µL PBS. Ciprofloxacin (100 µg/mL) was used as a standard. After incubation for 24 h at 37°C, the zone of inhibition was measured in terms of its diameter in mm.

HPLC analysis

The haemolymph from control and infected flies (24 h) was subjected to HPLC analysis. The lyophilized sample was dissolved in 0.1% TFA solution at a concentration of 25 mg/mL. A 20 µL of the crude sample was injected using a glass syringe into a C_{18} reverse-phase analytical column (5 µm particle size; 250 × 4.6 mm column) placed over an HPLC (Shimadzu). The solvent system included 0.1% TFA in Milli Q water (Solvent-A) and 80% aqueous Acetonitrile (ACN) with 0.1% TFA (Solvent-B) and the flow rate for the mobile phase was set at 1 mL/min. The elution was carried out with a linear gradient of 5–95% of solvent-B over a 60 min period. The eluted peaks were detected at 214 nm using an UV-DAD detector (SPD-M20A).

SDS-PAGE analysis

The profile of proteins in haemolymph was assessed by both Tris glycine (Laemmli, 1970) and tricine SDS-PAGE techniques (Schägger, 2006). A 30 µg of haemolymph from control and infected flies (24 h) were placed in wells. For Tris-glycine, 4% stacking and 12% resolving gel were used and ran at 150 V for 75 min. For tris-tricine SDS-PAGE, 4% stacking, 10% spacer and 16% resolving gel were used and ran at 150 V for 195 min. To determine the MW, high (11–245 kDa; Himedia) and low range protein markers (3–45 kDa; SRL) were used. After electrophoresis, separated protein bands were detected by silver staining method (Gromova & Celis, 2006). The density and number of bands were determined using Gelanalyzer software, version 19.1. The gel band of interest was cut out and placed in 1% acetic acid solution until required.

Protein identification

In-gel digestion

The piece of gel band was washed with 500 µL of wash solution (50% acetonitrile, 50 mM ammonium bicarbonate) and vortexed for 15 min at RT until it became opaque and stuck together. Then the gel band was spun down and the supernatant removed. A 3 mL of the Dithiothreitol solution was added to completely cover the piece of agar and incubated for 30 min at 56°C in an air thermostat. A 5 mL of Acetonitrile (ACN) was added, incubated for 10 min at RT and then the liquid was removed. A 3 mL of the iodoacetamide solution was added and incubated for 20 min at

RT in the dark. The piece of agar was placed in acetonitrile and then centrifuged to remove all the liquid. Trypsin buffer (13 ng/μL) was added until the gel band was covered and then kept in an ice bucket for about 90 min. A 1 μL of ammonium bicarbonate buffer (100 mM) was added to cover the gel band and keep them wet during enzymatic cleavage. 5% formic acid/ACN (1 : 2) was added and incubated for 15 min. After centrifugation, the supernatant was transferred to a new vial. Then the samples were lyophilized and dissolved in 20 μL of 2% acetonitrile/0.1% formic acid solution.

LC-MS/MS analysis

The sample was analysed using an ultra-high-performance LC with mass selective detection and an Ultimate 3000 series LC (Dionex, USA) coupled with ESI tandem mass spectrometer (micrOTOF-Q II) (Bruker, Germany). A 3 μL of sample was injected into LC precolumn (Pep map TM 100; 75 μm × 2 cm; Nanoviper C18, 3 μm; 100Å) and LC analytical column (EASY SPRAY PEPMAP RSLC C18 3 μm; 50 cm × 75 μm; 100Å) of an EASY-nLC 1200 LC instrument. Mobile phase A of 0.1% Formic acid in HPLC water and mobile phase B of 0.1% formic acid in acetonitrile was used. A linear gradient starting from 5% to 95% in 60 min with an 0.2 mL/min flow rate was recorded. The MS scan was carried within the 200–1800 m/z range and the data acquired in MS/MS (auto) scanning mode.

Mascot Search

The data analysis was carried out using MASCOT search engine. In MS/MS ions search, the SwissProt database was used with all entries option for Taxonomy. The other parameters used included trypsin as a proteolytic enzyme, Cysteine carbamidomethylation as fixed modification, methionine oxidation as variable modification, the error window for peptide mass was 10 ppm and fragment ion mass 0.6 Da. The decoy database was selected to calculate the false discovery rate. Only top rank peptide hits for given precursors were used for further protein identifications.

Phylogenetic analysis

The phylogenetic tree was constructed for seven cecropins: cecropin A (64 aa; P01507), A1 (63 aa; C0HKQ7), A2 (63 aa; C0HKQ8), B (63 aa; P14956) and C (63 aa; O16829), cecropin-2 (63 aa; XP_001955554.1) and sarcotoxin-1C (63 aa; XP_001955556.1) using the Maximum Likelihood method and JTT matrix-based model (Jones et al., 1992). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the cecropins analysed. The percentage of replicate trees in which the associated cecropins clustered together in the bootstrap test was shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model and then selecting the topology with the highest log-likelihood value. The evolutionary analyses were conducted in MEGA X v10.1.8 (Kumar et al., 2018).

Statistical analysis

The data were analysed using a two-way ANOVA for the protein concentration and antimicrobial activity with Bonferroni posthoc test using Graph pad Prism software 5.0. All the values are means ± SEM. Values were considered significant when $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

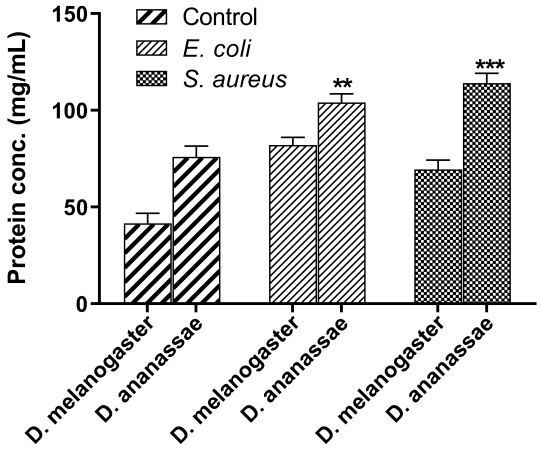

Fig. 1. Protein concentration in haemolymph. Mean protein concentration after infection of *D. melanogaster* (n = 50) and *D. ananassae* (n = 50) with either *S. aureus* or *E. coli*. Data presented as means ± SEM. Significance compared to control. $**P < 0.05$ and $***P < 0.01$.

RESULTS

The concentration of protein in haemolymph was greater in infected flies

The average total concentration of protein in haemolymph based on five independent experiments was found to be significantly higher in both *D. melanogaster* and *D. ananassae* 24 h after infection with *E. coli* or *S. aureus* (Fig. 1). In *D. melanogaster*, infection with *E. coli* resulted in a significantly greater protein production (82.1 mg/mL; $P < 0.01$) than infection with *S. aureus* (69.4 mg/mL). However, in *D. ananassae* protein concentration was higher after infection with *S. aureus* (114 mg/mL) than *E. coli* (104 mg/mL). In addition, there is a very significant difference in total protein concentration in the two species *Drosophila* following bacterial infections.

The *Drosophila* homogenate inhibits the growth of bacteria

The freeze-dried homogenate of bacteria-infected flies inhibited the growth of bacteria differently as seen in the zone of inhibition against both *S. aureus* and *E. coli* compared with standard ciprofloxacin. An inhibition zone was not observed in PBS control flies. However, the degree of inhibition of bacteria is different. The *E. coli* infected *D. melanogaster* and *D. ananassae* homogenates resulted in a larger zone of inhibition than that of the *S. aureus* infected flies (Table 1 and Fig. S1).

HPLC profile showed differential expression of immune induced molecules

The HPLC profile of haemolymph showed that *D. melanogaster* and *D. ananassae* infected with either *S. aureus* or *E. coli* had two and three differentially produced molecules, respectively. This shows that there is a clear quantitative difference in the expression of AMPs. In addition, the elution time indicated (within 30 min) that these molecules are possibly polar (Fig. 2).

Table 1. The zones of inhibition for in vitro antibacterial activity. Zone of inhibition measured against *S. aureus* and *E. coli* for whole-body homogenate samples of *D. melanogaster* and *D. ananassae* injected, respectively, with the two bacteria. Ciprofloxacin antibiotic was used as a standard. Data are represented as means ± SEM. Significance is shown compared to control. ***$P < 0.001$.

Sample	Zone of inhibition (diameter in mm)			
	D. melanogaster		*D. ananassae*	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
PBS (Control)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Infected (*E. coli* and *S. aureus*)	11.1 ± 0.15***	10.0 ± 0.10	11.5 ± 4.50	9.0 ± 5.20
Ciprofloxacin (Standard)	18.0 ± 0.0	21.0 ± 2.00	18.0 ± 0.10	17.0 ± 1.50

Detection of a cecropin A-like peptide in *E. coli* infected flies using SDS-PAGE and LC-MS/MS analysis

The SDS-PAGE analysis of haemolymph protein using the Tris-glycine method revealed several electrophoretic bands with MW ranging from ~11–242 kDa, based on a densitometry analysis. Several proteins are up and down-regulated during infection with either *E. coli* or *S. aureus*. Among them, three (58, 34, and 13 kDa) and five proteins (45, 33, 27, 14, and 11 kDa) were markedly produced in *D. melanogaster* and *D. ananassae*, respectively (Fig. 3).

As the Tris-glycine method doesn't resolve the low MW peptides, haemolymph samples from *D. melanogaster* and *D. ananassae* infected with *S. aureus* or *E. coli* were separated using the tris-tricine method. The data showed that a single protein band of ~6 kDa (based on the retention factor calculated using gelanalyzer software) was differentially produced in both *D. melanogaster* and *D. ananassae* injected with *E. coli*, but not with *S. aureus* or PBS (Fig. 4A). In addition, one protein band in *D. melanogaster* and four protein bands within 14 kDa, were highly produced in *D. ananassae* infected with *E. coli* (Fig. 4B) and these protein bands were not recorded in the two species of flies infected with *S. aureus*. The data also showed that the control groups of these two flies have distinct protein profiles. The higher molecular weight protein bands were also differentially produced in the *E. coli* infected flies. Here,

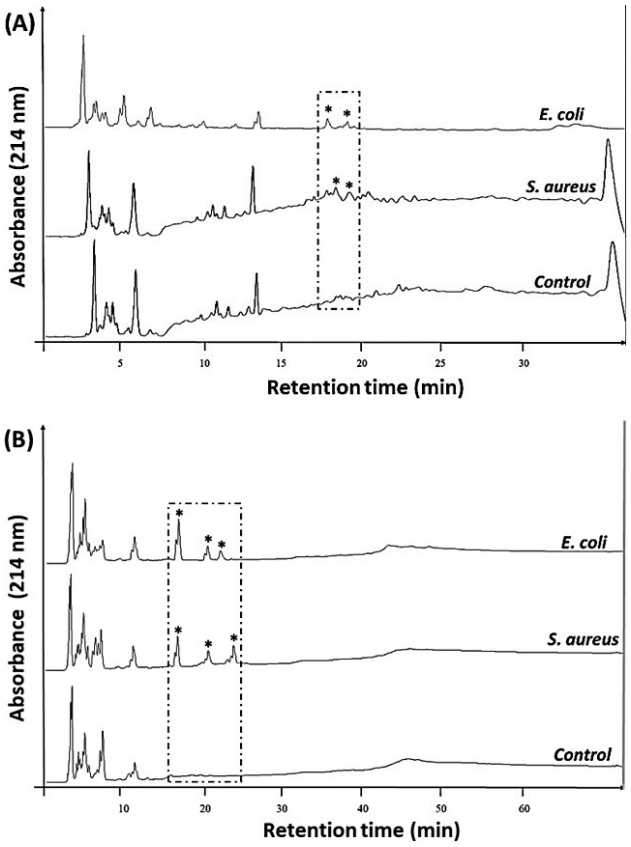

Fig. 2. HPLC chromatogram of *Drosophila* haemolymph after infection. The HPLC profile of haemolymph obtained from (A) *D. melanogaster* and (B) *D. ananassae*, 24 h post-injection with PBS (control), *E. coli* or *S. aureus* show two and three differently expressed molecules, respectively. *Peaks of immune-induced components.

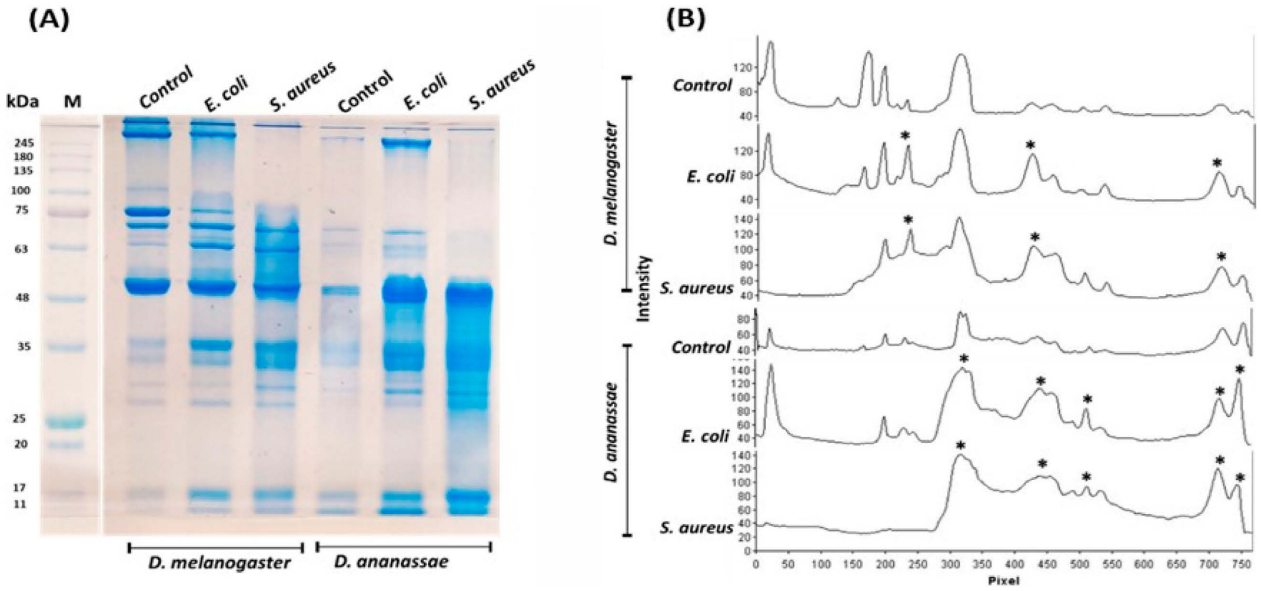

Fig. 3. Tris-glycine SDS PAGE and densitometry analysis. (A) The Tris-glycine SDS-PAGE analysis of the haemolymph and (B) its densitometry analysis. *Differentially induced proteins. M – Marker.

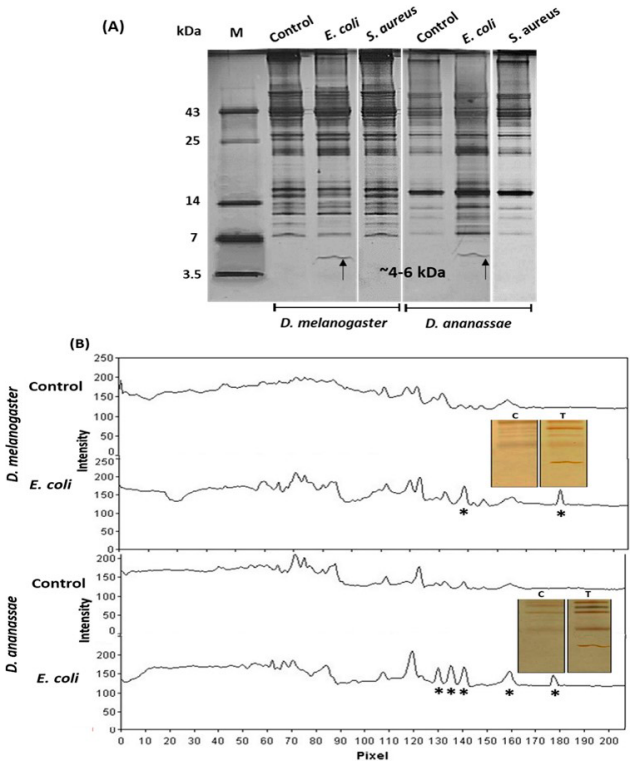

Fig. 4. Tris-tricine SDS PAGE and densitometry analysis. (A) The Tris-tricine SDS-PAGE analysis of the haemolymph from *Drosophila* infected with *S. aureus* or *E. coli* showed one differentially expressed peptide of ~6 kDa against *E. coli* infection. (B) The densitometry analysis showed two and five immune induced proteins in *E. coli* infected *D. melanogaster* and *D. ananassae* flies, respectively. *Differentially induced proteins. M – Marker; C – Control haemolymph and T – treated haemolymph of *E. coli*.

D. melanogaster and *D. ananassae* infected with *E. coli* showed three and two highly produced proteins, respectively, compared to PBS injected flies (Fig. 5).

The Mascot search of the LC-MS/MS analysis of a protein band (~6 kDa) from both *E. coli* infected *D. melanogaster* and *D. ananassae* gave a fragment ion with sequence AGPAVAVVGQATQIAK, which is similar to cecropin A of 6952 Da with a protein sequence coverage of 26% (Table 2 and Fig. 6). The mass spectrometry proteomics data are deposited in the 'ProteomeXchange Consortium' via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD025417 with DOI: 10.6019/PXD025417 for *D. melanogaster* and PXD025419 with DOI: 10.6019/PXD025419 for *D. ananassae*.

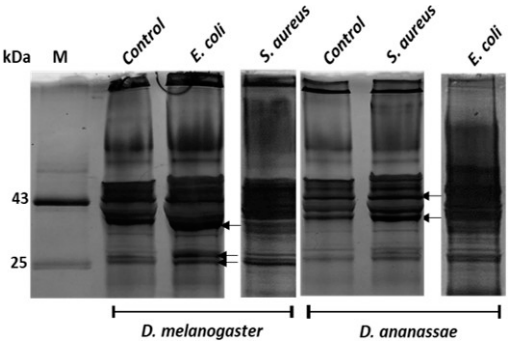

Fig. 5. Tris-tricine SDS PAGE for high-range proteins. The Tris-tricine SDS-PAGE analysis for high-range proteins of the haemolymph from *D. melanogaster* and *D. ananassae* infected with *E. coli* showed three and one differentially expressed proteins, respectively (Arrow); M – Marker.

Phylogenetic analysis of the putative cecropin A-like peptide sequence

Cecropins occurred in insects before the divergence of Diptera, Lepidoptera and Coleoptera. The phylogenetic tree (Fig. 7A) indicate that *Drosophila* cecropins are present in one branch and *Hyalophora*'s cecropin in another branch. The result of a multiple sequence alignment and phylogenetic analysis indicate that the putative cecropin A-like peptide has an approximately 11.7% sequence similarity with other *Drosophila* cecropins (Fig. 7B). The result also support the independent evolution of the cecropin peptide family in these insects (Tassanakajon et al., 2015).

DISCUSSION

Studies on the immune system of insects help in understanding the complexity of the immune system and the vital role of AMPs in insects' innate immunity. *Drosophila* has been a suitable model for studying the role of antimicrobial peptides in neutralizing circulating pathogens. The measurement of protein concentration in complex mixtures other than cell lysates can be better assessed at 280 nm. The increased total protein level recorded could be due to bacterial infection resulting in a higher metabolic rate in these flies. The type of signal generated by *E. coli* could be stronger and have resulted in a higher metabolic rate and hence increased total protein level in *E. coli* infected flies than in *S. aureus* infected flies.

The agar well diffusion method is commonly used for screening the antibacterial activity of AMPs. Here, a putative cecropin A-like peptide produced due to bacterial infection could be the reason for the antimicrobial activity recorded against bacterial pathogens. In a recent study (Park & Yoe, 2017), the minimum inhibitory and bactericidal

Table 2. Mascot search results. Protein identification obtained using Mascot software search of corresponding gel bands from *D. melanogaster* and *D. ananassae*.

Source of protein	LC-MS/MS detected sequence	Observed mass (Da)	Mascot score	Protein identified	Uniprot acession no.	Mass (Da)	Sequence coverage (%)
Drosophila melanogaster	AGPAVAV VGQATQI AK	740.9282	111	Cecropin-A (similar to *Hyalophora cecropia*)	P01507	6952	26
Drosophila ananassae		740.9328	58				

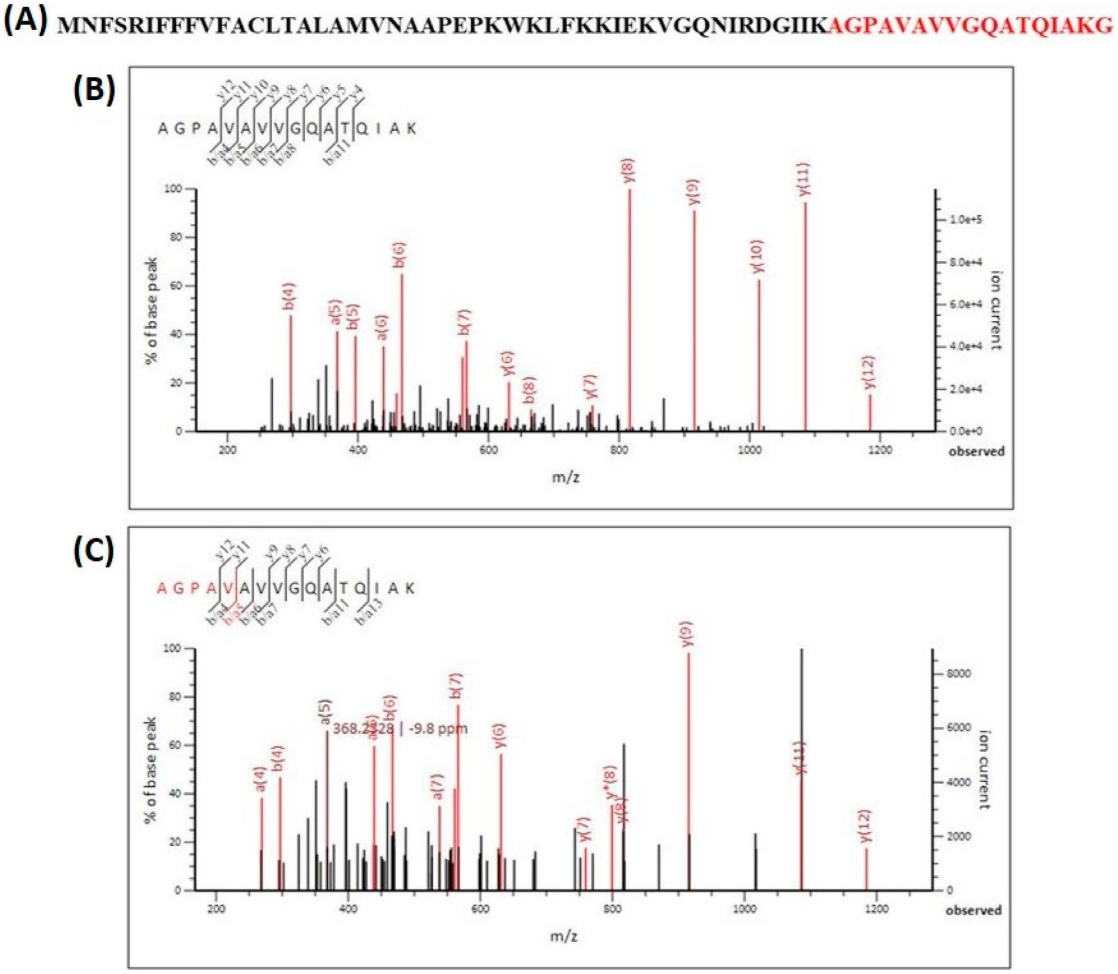

Fig. 6. Amino acid sequence and fragmentation spectra of the tryptic peptide. (A) The full sequence of cecropin-A in which the red coloured sequence is the LC-MS/MS identified tryptic peptide and its spectra sourced from the haemolymph of (B) *D. melanogaster* and (C) *D. ananassae*.

concentrations evaluated for a cecropin-like peptide has higher antibacterial effects against Gram-negative bacteria. In the *S. aureus* infection, though a cecropin A-like peptide was not recorded, there were other immune-induced proteins with higher MWs, which could also account for the in vitro antimicrobial activity recorded in these infections. In addition, the HPLC profile of haemolymph also confirms the presence of immune-induced components in flies infected with *S. aureus*.

Drosophila is considered to be good a model for understanding the variability in conserved genes expressed in closely related species (Hodgins-Davis et al., 2009). The tricine SDS-PAGE method has been less used for identifying immune-induced AMPs in *Drosophila*, but as shown here, can clearly be used to identify low MW peptides. The production of a specific AMP against *E. coli* by both flies indicates that inducible immune genes may have been conserved in these two flies (Hanson et al., 2016). AMPs are not produced in uninfected or PBS-pricked flies (Li et al., 2019; Feng et al., 2021; Kapila et al., 2021). They are not constitutively expressed in *Drosophila* haemolymph. Further, the level of induced immunity was stronger against *E. coli* than *S. aureus*. This is in accordance with earlier reports in which several AMPs are elicited against Gram-negative bacterial infections, but not non-flagellated Gram-positive bacterial infections (Lemaitre et al., 1997). In another report, cecropin is strongly expressed in *Drosophila* cell lines by bacterial lipopolysaccharide and flagellin, but weakly by peptidoglycan (Samakovlis et al., 1992). This confirms that *E. coli* can elicit a stronger immune response than *S. aureus* in *Drosophila* spp. Thus, as in the mammalian immune system, where different pattern recognition receptors are involved in the identification of lipopolysaccharide (TLR-4) and peptidoglycan (TLR-2), *Drosophila* might be able to discriminate the different compositions of the membranes of Gram-positive and Gram-negative bacteria (Takeuchi et al., 1999).

Among several antimicrobial peptides of *Drosophila* origin, cecropin A, a 4.3 kDa peptide (active form), which was first isolated from the haemolymph of the Lepidopteran *H. cecropia* (Mylonakis et al., 2016) is mainly expressed during Gram-negative bacterial infections (Wen et al., 2019), as it is an α-helical antimicrobial peptide that mainly kills Gram-negative bacteria (Fu et al., 2004). Diptericin (9 kDa) and drosocin (2.19 kDa) are also produced in response to Gram-negative bacterial infections. In this study, the putative cecropin A-like peptide expression was

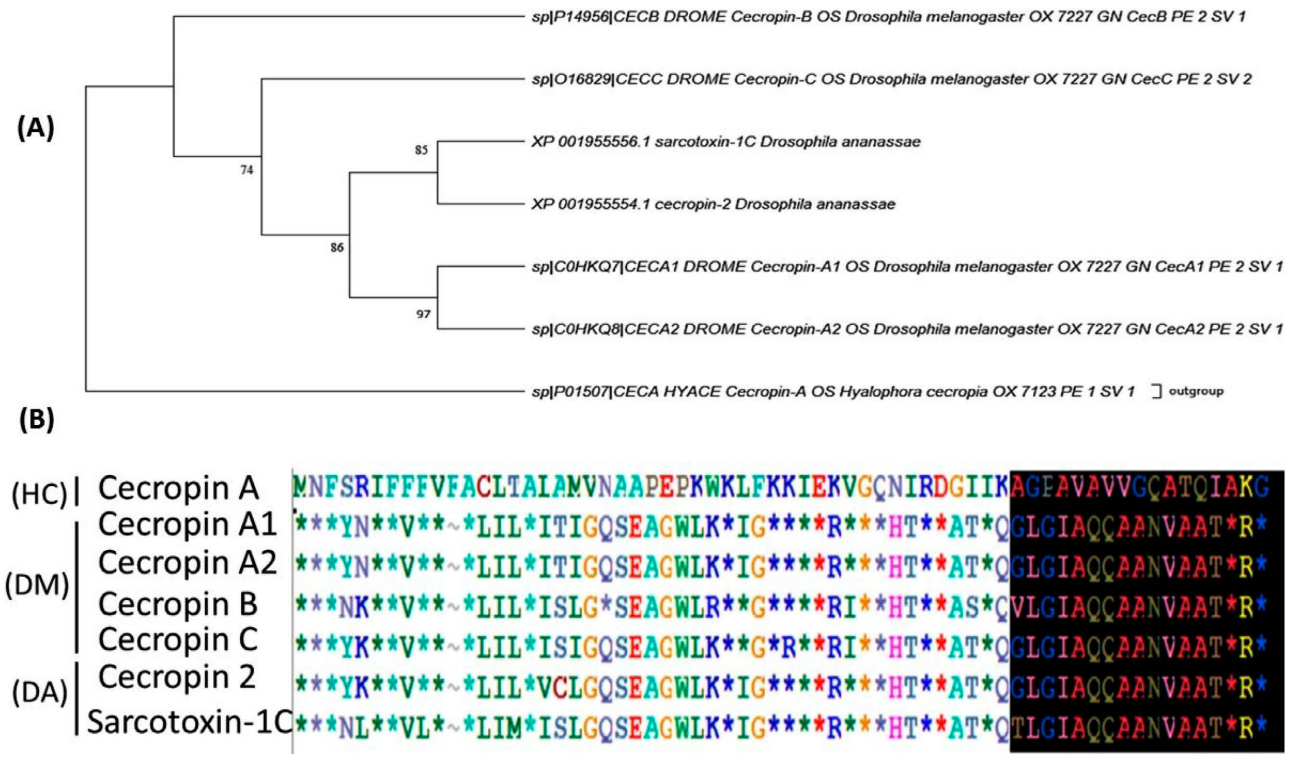

Fig. 7. (A) Phylogenetic analysis of seven cecropins. The phylogram of the amino acid sequence of cercopins from *Drosophila melanogaster* (4), *D. ananassae* (2) and *H. cecropia* (1) was generated in MEGA X v10.1 using Maximum Likelihood method with 1000 replications of the bootstrapping analysis. Outgroup and the scientific names are labelled with Uniprot accession no. (B) Multiple sequence alignment of cecropins. Sequence alignment of full amino acid sequence of cecropin from *H. cecropia* (HA) with that of *D. melanogaster* (DM) and *D. ananassae* (DA). The amino acid sequences (AGPAVAVVGQATQIAKG) identified from LC-MS/MS and Mascot analysis on the black background indicate other cecropins. Identical residues are indicated by *.

recorded only in haemolymph from *Drosophila* infected with *E. coli*.

We have shown marked similarities and some notable differences in the immune responses to bacterial infection by *D. melanogaster* and *D. ananassae*. Though the protein profiles were different in these two species, the AMP was recorded in both species in response to infection with *E. coli*, but not *S. aureus*. Previous research also indicates that many of the differentially expressed genes in *D. melanogaster* during the parasitoid-specific immune response have similar transcriptional responses in other closely related species of *Drosophila*. The gene expression profiles in *D. melanogaster* and *D. simulans* are very similar (Salazar-Jaramillo et al., 2017). In addition, 83% of protein-coding genes in *D. ananassae* are homologous to those in *D. melanogaster* (Uniprot, 2021a), which could explain the similar protein patterns recorded in SDS gels of the two species studied.

In this study, the LC-MS/MS-based mascot analysis detected a peptide with 16 amino acid sequences (AGPAVAVVGQATQIAK), which is similar to the cecropin-A from *H. cecropia*, with a good Mascot score and sequence coverage. However, the identified putative cecropin A-like peptide has only 37–41% similarity with other *Drosophila* cecropins (Uniprot, 2021b). Hence, the present finding of a cecropin A-like peptide is an addition to the pool of already known cecropins, produced by Lepidoptera like *H. cecropia*, *Bombyx mori*, Dipteran *Musca domestica* and Coleopteran *Acalolepta luxuriosa*, etc. In addition, as a part of the evolutionary link, the Lepidopteran cecropin is similar to those reported for Diptera. For example, the cecropin from Brachycera (Diptera) is closely related to the Lepidopteran cecropin (Brady et al., 2019) and the mosquito cecropin (Diptera) is more similar to the *B. mori* cecropin-D (Lowenberger et al., 1999). Notwithstanding, that the remaining unidentified region of this putative cecropin A-like peptide may have a similar or different amino acid sequence to the *Hyalophora*'s cecropin. There are many other cecropin like peptides, such as sarcotoxin-I (Buonocore et al., 2021), papiliocin (Kim et al., 2010), stomoxyn (Boulanger et al., 2002b; Landon et al., 2006) and hinnavin (Yoe et al., 2006), etc.

Further genomic analysis could validate this peptide as either cecropin A or its ortholog in *Drosophila*. The detection of a particular cecropin in several species of Diptera, like *Drosophila*, is not uncommon. Cecropin B is produced by both *D. sechellia* and *D. simulans*, and cecropin C by *D. takahashii*, *D. simulans* and *D. sechellia* (Uniprot, 2021b). Similarly, in this study, a putative cecropin A-like peptide was detected in both *D. melanogaster* and *D. ananassae*. This data support the tree topology as the obtained sequence is most similar to *H. cecropia.* In other words, each sequence is more similar to its ortholog in another species than to other members in the same species. That cecropin-A1, A2, B, C are conserved in *D. melanogaster* indicates that both paralogs are from duplication of a cecropin-like

peptide ancestor. The homology of the sequences of cecropin A (*H. cecropia*) and cecropins (A_1, A_2, B, and C) from *D. melanogaster*, indicate that the detected peptide region of cecropin A has two amino acids similar to known cecropins of *D. melanogaster* and *D. ananassae*.

This study has shown how different bacterial infections generate distinct immune responses in different genetic backgrounds. This study is novel as there are no reports on the evaluation of immune responses in *D. ananassae* and this is the only report of the production of putative cecropin A-like peptide in *Drosophila* spp.

CONCLUSION

This study has shown there is an antibacterial immune response in *D. melanogaster* and *D. ananassae* against *S. aureus* and *E. coli*. The production of a putative cecropin A-like peptide in both species of *Drosophila* is reported here for the first time against infection with *E. coli*, but not against *S. aureus*. The role of this peptide in innate immunity with comparison to other paralogues needs to be investigated.

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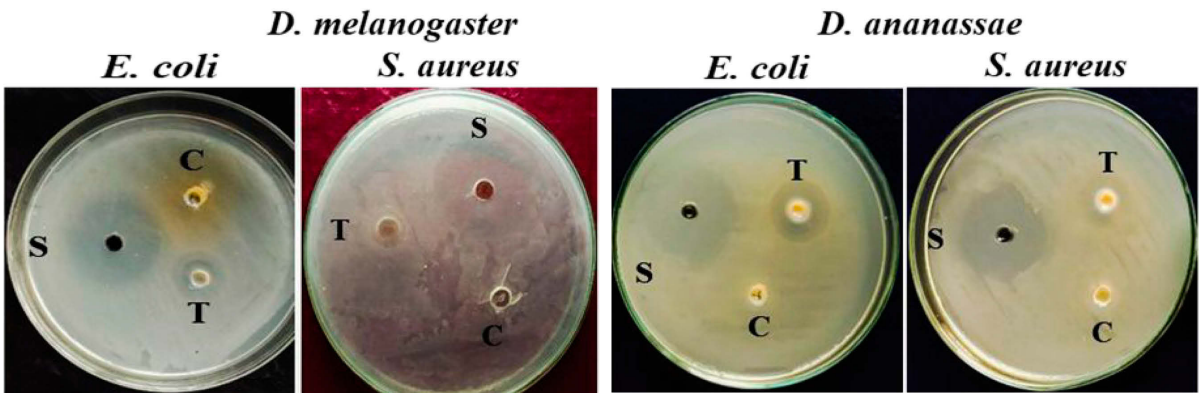

Fig. S1. Antibacterial activity of the *Drosophila* homogenate. The whole-body homogenates from both species of fly infected with either *S. aureus* or *E. coli* had shown the zone of inhibition. C – Control (PBS); T – Treated; S – Standard (ciprofloxacin).