Isolation and functional characterisation of antibacterial peptide from the mosquito, *Aedes* (*Ochlerotatus*) *caspius* (Diptera: Culicidae)

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One antimicrobial peptide was isolated and purified to 5.50-fold from the crude lysate of immunised *Aedes caspius* larvae by $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography, and reverse-phase high performance liquid chromatography (HPLC). The peptide, named ACAm, was found to be heat stable below 80 °C and exhibited strong antimicrobial activities against Gram-positive and Gram-negative bacteria as well as against peptidoglycans and lipopolysaccharides of bacterial cell walls. The estimated molecular weight of ACAm by sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE), under non-reducing conditions, was 4 kDa. Using isoelectric focusing (IEF), ACAm exhibited pI in basic pH (8.4) belonging to cationic peptides.

Key words: antimicrobial peptides, antibacterial activity, *Aedes caspius*, mosquitoes, ion-exchange chromatography, RP-HPLC.

INTRODUCTION

Once an insect's innate immune system is activated, it produces many effector molecules including antimicrobial peptides (AMPs) (Bulet *et al*. 2004). AMPs have the potential as tools for new anti-infective drugs since they proved themselves as exciting antibacterial agents against multi-resistant bacteria (Zhou *et al*. 2014). They have been isolated from a wide range of organisms including different orders of insects (Mulder *et al*. 2013; Yi *et al*. 2014). Moreover, the sequence diversity and biological activity of invertebrate AMPs were reviewed lately (Tassanakajon *et al*. 2015) to provide a better understanding of the evolution pattern of these peptides. Although there are more than one million described insect species, only few antimicrobial peptides/polypeptides from various insect species have been characterised and addressed (Cytryñska *et al*. 2007).

Aedes caspius (Pallas, 1771) subgenus *Ochlerotatus* is a potential vector of the human pathogens *Spiroplasma sabaudiensc* and *Crisiulo sporaaedis* (Khodzhaeva & Issi 1989; LeGoff *et al*. 1990). It is known as the most important vector of the *Tahyna* (TAH) virus in Europe (Pilaski 1987) and in the Mediterranean region (Mitchell 1995) and also a potential reservoir of 'Rift Valley Fever Virus' (RVF) during inter-epizootic periods (Gad *et al*. 1999; Balenghien *et al. 2006)*. To date, AMP production in *Ae. caspius* remains elusive except for purification of a lectin from females (Ayaad 2008). Therefore, the present study mainly focused on the isolation of antimicrobial peptide/s from *Ae. caspius* third instar larvae and its *in vitro* antimicrobial properties.

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MATERIAL AND METHODS

Insects

Mosquitoes have been reared in our laboratories, Entomology Department, Faculty of Science, Cairo University, since 2012. Species identification was based on the third instar using the keys of Kirkpatrick (1925) and Harbach (1985, 1988) for the Egyptian mosquitoes. Mosquitoes were reared at 28 ± 1 °C and 80 ± 5 % RH under LD 16:8 photoperiod. Adults were supplied with a pad of cotton wool soaked with 10 % sucrose solution and the females were allowed to feed on the common quail, *Coturnix coturnix* for ovary maturation, twice a week. Third instar larvae were used in all experiments to avoid the prepupa conflict in fourth instar larvae and according to Carron *et al*. (2008).

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Insect immunisation and collection of haemocyte-free supernatant

Before injection, the bacterial cells were pelleted by centrifugation at 2000 *g* for 10 min at 4 °C, extensively washed with sterile Ringer's solution (pH 7.2) to the concentration of 1×10^8 cells/ml of log phase bacteria and then immediately used for the experiments. All bacterial strains were obtained from the Microbiology Division, Microanalytical Centre, Cairo University, Egypt, and Cairo Microbiological Resources Centre (Cairo MIRCEN), Ain Shams University, Egypt.

Insects (naïve *Ae. caspius*) were individually pricked with a fine needle dipped into combined bacteria (Gram-positive (G+ve) *Bacillus thuringiensis* var. *israelensis* and *Staphylococcus aureus,* and Gram-negative (G–ve) *Escherichia coli* and *Pseudomonas aeruginosa*) or with 20 μg of bacterial cell wall components like lipopolysaccharide (LPS) from *Salmonella enterica* (Sigma), soluble peptidoglycan (PGN) from *S. aureus* (Sigma) or the algal carbohydrate laminarin from *Laminaria digitata* (Sigma).

Larval tissues (5 g) of both groups (naïve and immunised) were homogenised with 0.01 M of sodium acetate buffer (SAB) pH 5.4. Buffer was supplemented with protease inhibitors (final concentration 20 μl/ml) as recommended by the manufacturer (Complete-Protease Inhibitor Cocktail Tablets, Roche) in an ice-cold water bath. The homogenates were centrifuged at 12000 *g* for 15 min at 4 °C to remove haemocytes and chitin precipitates. The supernatant of the immunised body lysate (BL) was kept at –20 °C until needed to apply one of the following tests using disc diffusion assay (as described below): the antimicrobial activity of BL against different bacterial species and cell wall components; the assay of lysozyme (dose-dependent) activity against different bacterial concentrations of *E. coli*; or the time course activity where BL was directly collected at different intervals (6, 12, 24, 48 and 72 h) post-injection and assayed against *E. coli*. Controls in previous experiments were no spiking larvae (naïve control) and/or sterile saline.

Effect of heating

Aliquots (25 μl), of the naïve and extracted BL samples (0.50 mg protein/ml) were diluted (1/2) in 0.1 M SAB buffer (pH 5.4) and were assessed against *E. coli* using disc diffusion assay. BL samples were heated, in a water bath, for 5 min at 50, 80, or 100 °C and were assayed. The protein concentration was determined by Bradford (1976) method using bovine serum albumin (BSA) as a standard.

Microbial cultures

The following test microorganisms were employed for investigating their sensitivities toward the isolated samples: the G+ve *Bacillus subtilis* (ATCC 6051), *B. thuringiensis* var. *israelensis* (AM 6552), *S. aureus* (ATCC 12600), *Clostridium perfringens* (ATCC 3624), and *Streptococcus faecalis* (ATCC 19433); the G–ve *E. coli* (ATCC 11775), *P. aeruginosa* (ATCC 10145), *Serratia marcescens* (EMCC 1247), and *Stenotrophomonas maltophilia* (ATCC 17666); the yeasts *Candida albicans* (ATCC 26555), and *Saccharomyces cerevisiae* SAF® (Baker's yeast); the filamentous fungi *Beauveria bassiana* (ATCC 74040), and *Aspergillus flavus* (IMI 111023).

Antimicrobial activity assays (sensitivity tests)

Bacterial lysis assay of $(NH_4)_2SO_4$ fractions

Cultures of *E. coli* and *S. aureus* (mid-logarithmic phase) were diluted with fresh Luria Broth (LB) medium to a final cell density of 1×10^8 CFU/ml then mixed with a 1:1 v/v (100 μl) of two-fold serial concentration dilution of the test sample ($(NH_4)_2SO_4$ fractions) in fresh LB medium. The latter mixture was incubated overnight at 37 °C and then transferred into a 96-well microplate (100 μl per well) for absorbance measurement. Bacterial growth was determined by monitoring the increase in OD_{600}. Readings were taken after zero (to determine zero growth base line), 24 and 48 h incubation. The plates were incubated at 37 °C between readings.

Antimicrobial activity assay by Kirby-Bauer disc diffusion method

Antimicrobial activity of the crude BL or HPLC fraction was determined using a modified Kirby-Bauer disc diffusion method (Bauer *et al.* 1966). Standard methods M7-A3, M38-A, and M44-P approved by the National Committee for Clinical Laboratory Standards (NCCLS) were used for evaluating the susceptibilities of bacteria, filamentous fungi and yeasts (NCCLS, 1993, 1997, 2002, 2003). Briefly, 100 μl of the test bacteria/fungi were grown in 10 ml of nutrient broth solution until they reached a count of approximately 1×10^8 cells/ml for bacteria or 1×10^5 cells/ml for fungi (Pfaller *et al*. 1988). The microbial suspension (100 μl) was spread onto Mueller-Hinton agar

(BDH Laboratory Supplies, England) plates corresponding to the broth in which they were maintained. Blank paper disks (Schleicher & Schuell BioScience GmbH) with a diameter of 5.0 mm were impregnated with 10 μl of the tested samples concentration. Plates were incubated at 37 °C for 24 h for bacteria, at 25 °C for 72 h for filamentous fungi, and at 33 °C for 48 h for yeasts. The diameters of the inhibition zones were measured in millimetres (Bauer *et al.* 1966). Standard discs of tetracycline (antibacterial agent), amphotericin B (antifungal agent) served as positive controls for antimicrobial activity. Filter discs impregnated with 10 μl of ddH_2O were used as a negative control. The standard zones of inhibition have been determined for susceptible and resistant values.

Isolation and purification of antibacterial peptide

$(NH_4)_2SO_4$ fractionation

The cell-free supernatant was mixed with 10 % ammonium sulphate. The supernatant was dialysed against dH_2O and phosphate buffered saline (PBS, pH 7.2) and kept in 4 °C for 24 h. After storage it was centrifuged at 4700 *g* for 15 min at 4 °C. The fractionated protein samples were concentrated by lyophilisation, then reconstituted in approximately one-twentieth the original volume of supernatants with sterile SAB. The antibacterial activity was monitored throughout the purification process using bacterial lysis assay (Lehrer *et al*. 1991). Fraction showing the highest positive antibacterial activity (from the 40 % precipitate) was pooled and stored at –70 °C till use in the subsequent steps.

Ion-exchange chromatography

An aliquot of 10 ml of the 40 % precipitate was lyophilised, reconstituted in 1 ml of 0.1 M SAB and subjected to ion-exchange chromatography using a CM Sepharose® Fast Flow (Sigma) that had been pre-equilibrated with 1.0 M SAB and packed into 1.5 × 20 cm column. The column was then re-equilibrated with 0.1 M SAB and washed with 250 ml of the same buffer. The protein sample was applied onto the column and then eluted by a linear gradient of 0.1–1.0 M SAB at a flow rate of 1.0 ml/min. The eluate was monitored at 280 nm (Bio-Rad EM-1 Econo UV Monitor, Berkeley, CA, U.S.A.). Fractions, 5 ml each, corresponding to each peak were lyophilised and reconstituted with 50 μl SAB. The antibacterial activities of the eluted protein fractions were estimated against *E. coli* using disc diffusion assay. Fractions with positive antibacterial activity were pooled, lyophilised and stored at –70 °C.

Reverse-phase high performance liquid chromatography (RP-HPLC)

The active fraction from one CM-Sepharose run was reconstituted in 100 μl PBS, brought up to 2 ml in 0.02 M SAB and was further purified by HPLC on a C18 (RP18, ODS, octadecyl) reverse-phase column (Delta-Pak, 15 μm, 100 Å, 3.9 × 300 mm) connected to a Agilent 1100 Series HPLC G1314A VWD complete chromatography system. A 45-min linear gradient from 0.1 % (v/v) trifluoroacetic acid (TFA) up to 70 % (v/v) acetonitrile in 0.01 M SAB (acidified with 0.1 % (v/v) TFA) at a flow rate of 0.5 ml/min at 25 °C was adapted. The elution pattern was monitored at 220 nm. One millilitre fractions were collected, lyophilised, reconstituted in 10 μl of SAB, and tested for antibacterial activity using the disc diffusion assay. To attain and assess purity, the collected active fractions after the initial HPLC were rechromatographed on C18 RP-HPLC and eluted with 70 % ethanol, 30 % water, at a flow rate of 0.5 ml/min, and detected at 220 nm.

Molecular characterisation of isolated ACAm

Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE)

Active fraction was subjected to electrophoresis by Tris–glycine buffer system method of SDS-PAGE running on a vertical slab Mini-PROTEAN® II cell according to the protocol of Schägger & von Jagow (1987). After separation, the gels were fixed by gentle shaking for 30-min in 10 % acetic acid, 50 % methanol (v/v) and stained with Coomassie Brilliant Blue R-250. The relative molecular weight of the purified ACAm was determined by running a low molecular weight marker (Sigma).

Isoelectric focusing (IEF)

IEF was performed under non-denaturing conditions (*i.e.* in the absence of urea) with the Bio-Rad Mini Protein II system (Bio-Rad Laboratories). A 12-ml solution of 5 % polyacrylamide (24.25 % acrylamide: 0.75 % piperazinediacrylamide [Bio-Rad Laboratories]), 5 % glycerol, and 2 % ampholytes, Biolyte 3-10 (Pharmacia Biotechnology Inc.), was degassed and polymerisa-

tion was initiated by adding 20 μl each of 10 % ammonium persulphate and TEMED. After polymerisation, the anode buffer, 0.02 M phosphoric acid, and cathode buffer, 0.04 M sodium hydroxide, were added, and the sample wells were filled with overlay buffer (0.87 ml of water, 0.1 ml of 50 % glycerol, 15 μl of Biolyte 5-7 (Bio-Rad Laboratories), 5 μl of Biolyte 3-10 (Bio-Rad Laboratories), and 20 μl of 1 % bromophenol blue. Samples and standard (isoelectric focusing standard [Bio-Rad Laboratories]) were dissolved in an equal volume of 50 % glycerol and layered under the overlay buffer. Samples were electrophoresed by stepped increases in voltage (100 V for 15 min, 200 V for 30 min, 300 V for 45 min, and 400 V for 30 min). The outer buffer chamber was stirred with a magnetic stirrer throughout electrophoresis to dissipate heat. After electrophoresis, the gels were carefully removed and fixed in a solution of 12 % trichloroacetic acid, 4.5 % sulfosalicylic acid, and 40 % methanol for a period of 2 to 4 h with one change in the fixing solution. The gels were then washed for 2 to 8 h with water and stained with Coomassie Brilliant Blue R-250. The pH gradient was measured directly by slicing an unused portion of the gel in 3-mm sections and placing the sections in 1 ml of water under vacuum for 2 h.

RESULTS AND DISCUSSION

Controls from the two groups, naïve larvae and SAB saline extracts, and samples of immunised body lysate challenged by different kinds of inducers were directly subjected to the growth inhibition zone assay to detect responses (Fig. 1). All inducers (bacteria or cell wall components) increased the antimicrobial activity. The immunised body lysate of *Ae. caspius* larvae showed its highest activity against G–ve *E. coli* and G+ve *B. thuringiensis* and *S. aureus* bacteria, respectively; and the lowest activity against the algal carbohydrate laminarin. These observations are an instance for supporting the potent broad spectrum activity of *Aedes* AMPs against bacteria and some of the

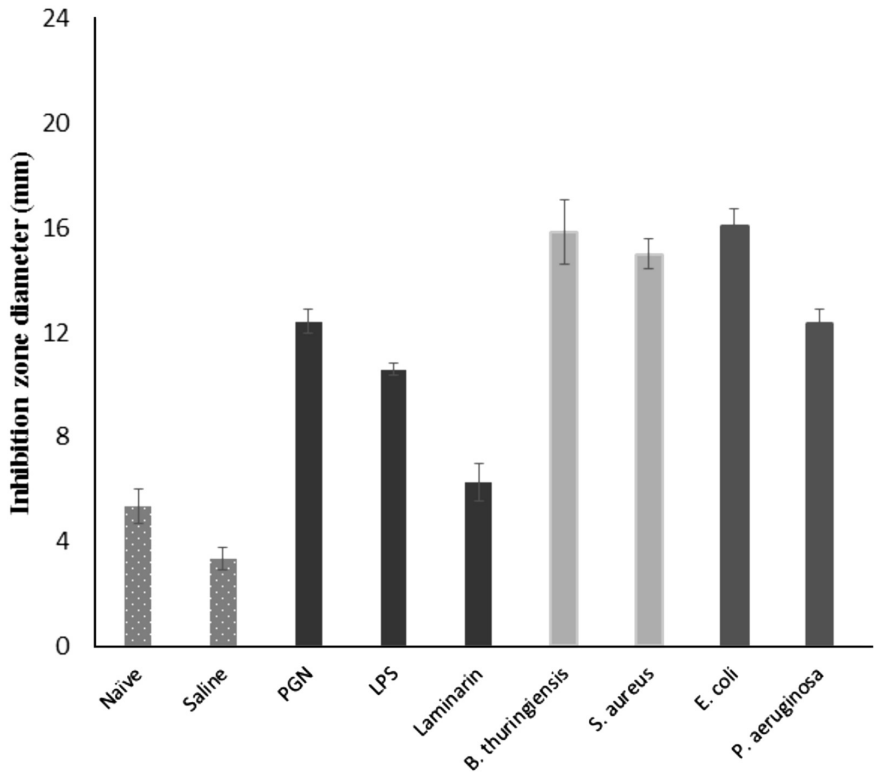

Fig. 1. Induction of antimicrobial activity in third instar larvae of *Aedes caspius* by different bacterial species and bacterial cell wall components. Each point represents the mean ± S.E. from three replicates. Means with different letters are significantly different at $P < 0.05$.

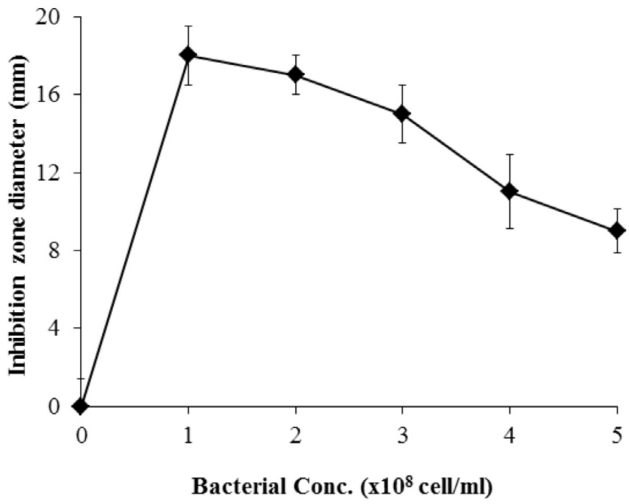

Fig. 2. Lysozyme activity of the immunised body lysate of *Aedes caspius* third instar larvae against *Escherichia coli*. Each point represents the mean ± S.E. from three replicates.

bacterial cell wall components. Extracts from immunised and naïve insects all demonstrated basal activity against the bacteria, but the immunised insect showed much stronger antibacterial activity than the native insect, suggesting that antibacterial peptide genes could be up-regulated by immunisation. A similar result has been obtained by Yamauchi (2001) who found a trace of antibacterial activity in the haemolymph of *Anomala cuprea* (Coleoptera) naïve larvae. Also, Lowenberger *et al*. (1999) detected an activation of the defensin gene expression during metamorphosis of the naïve *Aedes aegypti*. Once insect is infected or injured, AMPs are rapidly synthesised in the insect fat body and in certain blood cells, and then secreted into the haemolymph where they act synergistically against microorganisms (Hoffmann & Reichhart 2002; Irving *et al*. 2004). The expression levels of antimicrobial peptides could be constitutive or inducible upon infectious or inflammatory stimuli (Bulet *et al*. 1999).

The BL of *Ae. caspius* larvae immunised by combined four bacteria species, achieved higher inhibition activities against low bacterial concentrations to the limit of 3×10^8 of *E. coli* (Fig. 2); then the lysozyme activity gradually declined showing concentration-dependent killing of organism. Moreover, this study showed that the BL activity, as inhibition zone diameter, was ~2.5-fold higher (17.50 ± 0.68) than naïve control (7.25 ± 0.58) against *E. coli* after 12 h of immunisation; then the activity declined to record 8.25 ± 0.49 after 72 h (Fig. 3). This is in accordance with Okada & Natori (1983) for *Sarcophaga peregrine* and in contrast to Yamauchi (2001) who recorded long duration of activity in *A. cuprea* against *Micrococcus luteus* reached three weeks after induction. These may be due to the differences between mode of action of AMPs towards Gram+ve and Gram–ve bacteria or may be correlated with the long persistence of *A. cuprea* developmental stages and their habitat in the soil that is rich in microorganisms.

The lyophilised BL was thermo-stable below 80 °C, and then the antibacterial assay revealed weaker anti-*E. coli* when the extract was heated for 5 min at 80 °C or 100 °C (Fig. 4). Dang *et al*. (2006) and Lu *et al*. (2007) found that the antibacterial peptide/s from *Bactrocera dorsalis* and *Musca domestica* (Diptera) were very thermo-stable. Whilst, the antibacterial property of *Oryctes rhinoceros* (Coleoptera) peptide was found to be stable only up to 37 °C against *M. luteus* (Rabeeth *et al*. 2012).

Bacterial lysis assay performed on the fractionated extract of *Ae. caspius*, using $(NH_4)_2SO_4$ precipitation at OD_{600}, showed antimicrobial activity in all fractions with the 40 % fraction exhibiting the highest level of activity against both *S. aureus* and *E. coli*. (Table 1). The antimicrobial activity increased after 48 h of bacterial incubation with 100 μl tested fractions. The 40 % $(NH_4)_2SO_4$ precipitation was fractionated by CM-Sepharose Fast Flow column (Fig. 5). We have identified five peaks (I, II, III, IV and V), which was eluted with

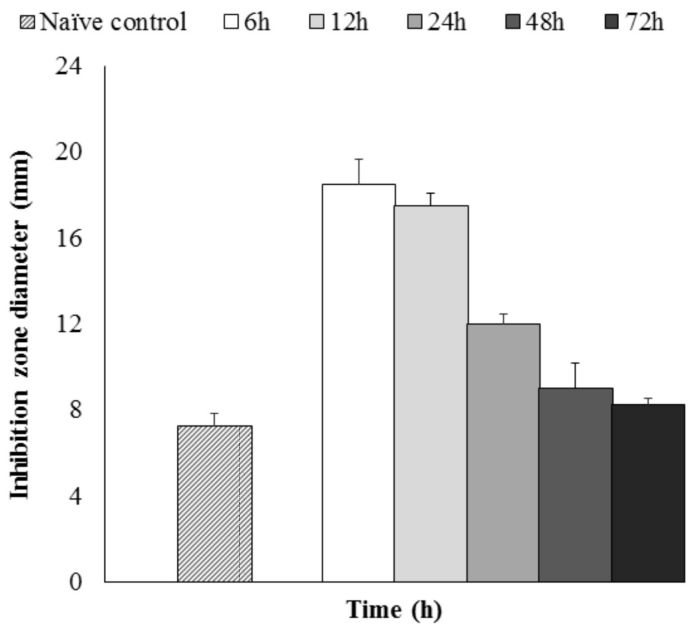

Fig. 3. Time-dependent activity of the immunised body lysate of *Aedes caspius* third instar larvae against *Escherichia coli*. Each point represents the mean ± S.E. from three replicates.

10–40 % 1 mol/l ammonium acetate, showing one peak (V) with significant antibacterial activity exhibited 18–22 mm diameter clear zone in the disc diffusion assay against *E. coli* (Fig. 5); however, other fractions did not reveal any antibacterial properties. The antibacterial fraction (active fraction) was thus pooled and lyophilised, then re-dissolved in SAB solvent and was further purified by RP-HPLC where only one fraction (black arrow in Fig. 6) was detected as positive to antibacterial activity assay. This peptide was then further checked on the C18 RP-HPLC. As shown in Fig. 7, only one peak (marked with black arrow) has been determined and this single peak was named ACAm fraction. Earlier, three structurally related antibacterial peptides with molecular mass of 8 kDa were purified by RP-HPLC and characterised from the haemolymph of immunised *Acalolepta luxuriosa* larvae (Imamura *et al*. 1999). Also, Liu *et al*. (2009) reported that only two antimicrobial peptides from *Tenebrio molitor* larvae were purified through RP-HPLC. The fat body of an insect species can respond to bacterial infection by the synthesis of either a large number of antimicrobial substances (*e.g*. *Drosophila* adults) or relatively few molecules (*e.g*. a single peptide in the dragonfly *Asechna cynanea*) (Bulet *et al*. 1999).

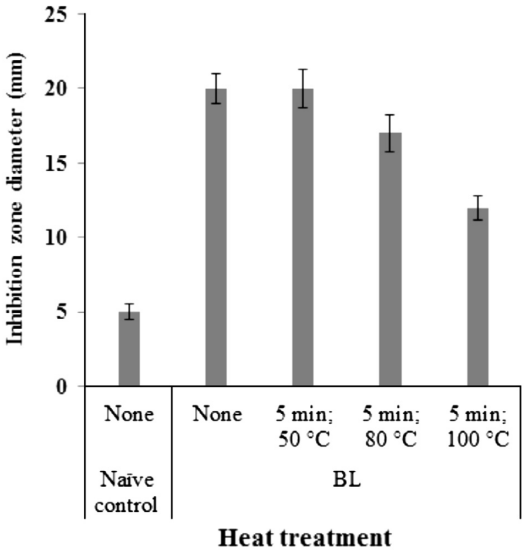

Fig. 4. Heat stability of the body lysate of *Aedes caspius* third instar larvae diluted (1/2) in SAB (pH 5.4, 0.1M). BL = supernatant of immunised body lysate (0.50 mg protein/ml). The data shown are mean ± S.E. from three replicates.

The ACAm fraction was tested against several bacteria, yeasts and fungi (Table 2). It indicated strong antimicrobial activity against G+ve and G–ve bacteria, poor activity against filamentous

Table 1. Bacterial lysis assay of ammonium sulphate fractions of BL of *Aedes caspius* third instar larvae.

Bacteria Gram +/–	Time (h)	$(NH_4)_2SO_4$ fractions (%)					
		Starting material	20	40	60	80	100
		OD_{600} (mean ± S.D.)					
E. coli (–)							
	24	0.17 ± 0.05	0.14 ± 0.03	0.41 ± 0.03	0.13 ± 0.03	0.25 ± 0.07	0.27 ± 0.04
	48	0.21 ± 0.03	0.23 ± 0.05	0.54 ± 0.01	0.33 ± 0.05	0.35 ± 0.07	0.46 ± 0.03
S. aureus (+)	24	0.21 ± 0.05	0.24 ± 0.02	0.58 ± 0.02	0.25 ± 0.03	0.27 ± 0.04	0.28 ± 0.06
	48	0.26 ± 0.04	0.28 ± 0.04	0.72 ± 0.04	0.38 ± 0.05	0.40 ± 0.08	0.49 ± 0.05

Bacteria were incubated at 37 °C for 24–48 h in a rotary shaker (250 rpm) in liquid LB medium containing 100 µl of the tested fractions. Absorbance of the cultures at 600 nm was determined. Each treatment was repeated three times. Each point represents the mean values of OD changes at the end of culture period, which were adjusted to remove background absorbance due to inoculated medium. Positive control constituted the bacteria + LB, while the negative control was LB alone. BL = the cell-free supernatant of total body lysate immunised by *B. thuringiensis, S. aureus, E. coli* and *P. aeruginosa.*

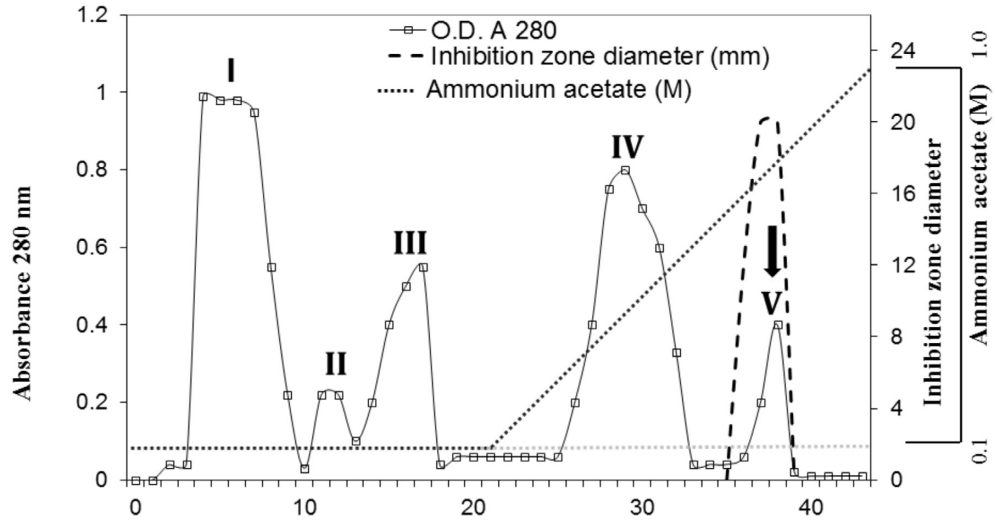

Fig. 5. Purification of 40 % $(NH_4)_2SO_4$ precipitation using ion-exchange chromatography on CM-Sepharose. Fraction V exhibiting antibacterial activity against *Escherichia coli* using disc diffusion assay.

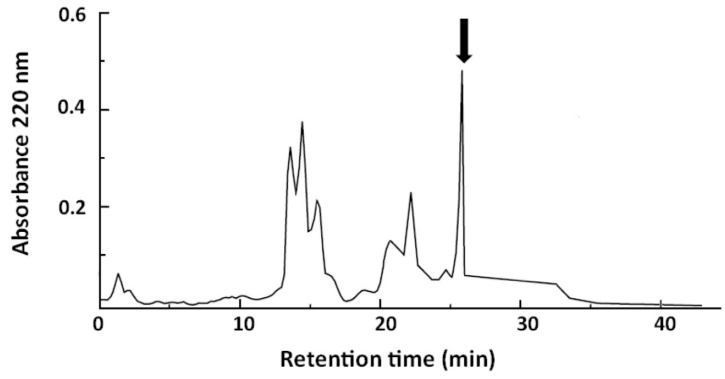

Fig. 6. Further purification of the antibacterial fraction using reverse-phase HPLC. Black arrow shows the peak with antibacterial activity against *Escherichia coli* using disc diffusion assay; the elution pattern was monitored at 220 nm.

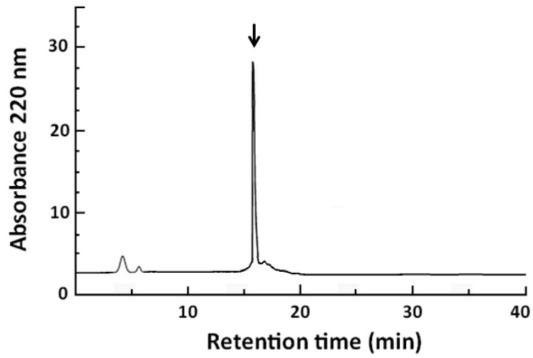

Fig. 7. Final purification of the antibacterial fraction on C18 RP-HPLC where it was eluted with 70 % ethanol, 30 % water, at a flow rate of 0.5 ml/min, and detected at 220 nm. Black arrow shows the peak with antibacterial activity against *Escherichia coli* using disc diffusion assay.

Table 2. Antimicrobial activities of isolated ACAm against different bacteria, yeasts and fungi.

Microorganism	*Inhibition zone diameter (mm/sample)		
	Standard		Sample
	†Tetracycline	‡Amphotericin B	Isolated ACAm
Gram-positive bacteria			
B. subtilis	19	–	16
B. thuringiensis israelensis			12
S. aureus	24	–	18
C. perfringens	22	–	14
S. faecalis	26	–	16
Gram-negative bacteria			
E. coli	20	–	16
P. aeruginosa	22	–	18
S. marcescens	17	–	14–16
S. maltophilia	20	–	12
Yeasts			
C. albicans	–	38	2
S. cerevisiae	–	28	–
Filamentous fungi			
A. flavus	–	35	7
B. bassiana	–	38	5

The antimicrobial spectra of purified ACAm were tested by disc diffusion method. Positive control: †tetracycline (antibacterial agent) and ‡amphotericin B (antifungal agent), negative control: sterile double distilled water (DDW).

fungi and null activity against yeasts using disc diffusion assay. This may suggest the utility and adaptability of the ACAm fraction in binding with G+ve and G–ve bacterial membrane with respect to other microbes. AMPs from other biological sources were reported to have highly selective toxicity and broad antimicrobial spectra against bacteria, yeast, filamentous fungi, parasites and even enveloped viruses (Bahar & Ren 2013).

The analysis of ACAm using Tris–glycine SDS-PAGE yielded one band in the gel with Mw 4 kDa while using IEF-PAGE exhibited ACAm as a cationic peptide with pI 8.4 (Fig. 8). Many references found that AMPs were small molecular weight proteins with molecular sizes ranged from 4 to 17 kDa (Yoon *et al*. 2003; Cytryñska *et al*. 2007; *Yi et al*. 2014).

After using all previously mentioned purification steps, the crude sample of *Ae. caspius* larvae was purified to 5.5-fold purification (Table 3). Only

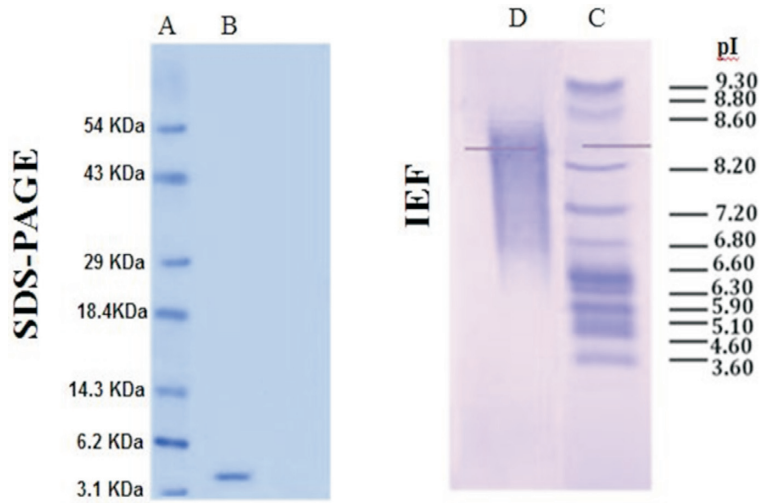

Fig. 8. SDS-PAGE and isoelectric focusing (IEF). Lanes: A, low MW marker; B, ACAm purified fraction; C, isoelectric focusing standard; and D, ACAm purified fraction.

Table 3. Purification of the antimicrobial peptide (ACAm) isolated from BL of *Aedes caspius* third instar larvae.

Step	Total activity (titre.vol)	Specific activity (IZD/mg)	Fold purification
6000 rpm supernatant	300	13.4	–
$(NH4)_2SO4$ fractionation	90	19.14	1.40
CM-sepharose IE chromatography	625	22.53	1.70
HPLC-reverse phase final step	85	73.92	5.50

Total activity was expressed as titre × volume.
Specific activity was expressed as titre/mg of protein.
BL = the cell-free supernatant of total body lysate immunised by *B. thuringiensis, S. aureus, E. coli* and *P. aeruginosa.*

5.3-fold purification could be achieved by heat chromatography in *M. domestica* larval antimicrobial peptides (Lu *et al*. 2007).

CONCLUSION

This study isolated an antimicrobial peptide from third larval instar of *Ae. caspius* which has broad spectrum antibacterial activity with heat resistance below 80 °C and forms the basis for further work on peptide sequencing and chemical structural analysis which may contribute considerably to both the understanding of the immune response in *Ae. caspius* and could facilitate a single novel drug development against several bacterial pathogens.

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