Purification and Characterization of Two Antimicrobial Peptides from Bacterial-challenged Haemolymph of *Bombyx mori* Larva.

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ABSTRACT

Defense peptides and proteins constitute key factors in insect humoral immune response against invading microorganisms. In this study, biochemical approach was designed to purify and characterize two peptides which appeared in larval haemolymph of *B. mori* after bacterial challenge. The results showed a significant increase of total protein of the bacterial-challenged haemolymph and then declined over time. This suggested that the AMPs are upregulated and released in haemolymph as "acute phase response" of the insect. Full antimicrobial activity was observed for the immune haemolymph at 24 h p.i. Fractionation of the immune haemolymph extract on a reversed phase C-18 column allowed effective separation of 5 fractions containing mainly proteins and peptides of molecular masses below 20 kDa. After fractionation, one out of three fractions (fraction# 5) exhibited the strongest antimicrobial activity. Finally, two peptides (5.8 and 4.3 KDa) were purified and one of them (4.3 KDa) showed full antimicrobial activity and very weak hemolytic activity up to concentration of 100 µg/ ml. These results were consistent to the results of quantitative protein analysis. Conclusively, this study demonstrated that the antimicrobial activity of the immune haemplymph is related to the presence of two antimicrobial peptides.

Keywords: *Bombyx mori*, antimicrobial peptides, HPLC, hemolytic activity.

INTRODUCTION

The high rate of emerging multiresistant bacterial and viral strains resulted in increased demands for new effective antibiotics. In such cases, insufficient antibiotic research and use of less effective antibiotics could have bad impacts on human and/ or animal health and productivity. The first case of bacterial resistance to several classes of antibiotics was documented in 1977 (Jacobs *et al.*, 1978) and it can resist a whole host of drugs, 26 years later (Reinert *et al.*, 2005). In addition, several new bacterial diseases have been discovered in the past decades. In this context, there is an urgent need for a new generation of antibiotics to complement the panel of drugs that are available to the clinicians and to provide new tools for multitherapy treatment (Bulet and Stöcklin, 2005). For decades, one major area of interest for the discovery and study of new antibiotics was the investigation of antimicrobial peptides (AMPs) derived from insect immune defense reactions. Defense peptides are key factors in innate immunity against bacteria and fungi in vertebrates as well as invertebrates. Particularly, in insects which lack an adaptive immune system, antimicrobial peptides play a crucial role in fighting against invading pathogens (Hertu *et al.*, 1998, Irving *et al.*, 2004, Tzou *et al.*, 2004). This issue has demonstrated alternative antimicrobial strategies, due to insect immune defense relies solely on innate immunity (Hoffman *et al.*, 1998). One component of the defense weapons developed by insects to rapidly

eliminate invading pathogens is the fast and massive production of potent AMPs (Hertu *et al.*, 1998, Bulet *et al.*, 2003). AMPs are rapidly and transiently synthesized mainly in insect fat body (functional equivalent of mammalian liver) and in certain blood cells, and then rapidly released into haemolymph, where they act synergistically against microorganisms. They are synthesized in response to microbial infection or septic body injury (Bulet *et al.*, 2003). From a large number of about 890 AMPs of eukaryotic origin identified to date, more than 180 were described in insects. Peptides exhibiting antimicrobial activity are mainly small ($\approx$5 kDa), amphipathic, cationic molecules. On the basis of amino acid sequence and structural characteristics they are divided into three broad classes: (i) linear a-helical peptides without cysteine residues, *e.g.* cecropins; (ii) peptides whose structure is stabilized by disulfide bridges (cysteine-stabilized peptides), *e.g.* defensins; (iii) peptides with an overrepresentation of proline and/or glycine residues (Bulet *et al.*, 1999). Generally, AMPs are assumed in the near future as an alternative for the nowadays classical antibiotics. The advantages of AMPs are: selectivity, fast killing, broad antimicrobial spectra and no resistance development (Boman, 2003, Matsuzaki, 1999).

In this paper, we report on purification, partial characterization, antimicrobial and hemolytic activity spectra of two peptides present simultaneously in the immune haemolymph of *B. mori* larvae. Such peptides could be useful in the control of plant, human and veterinary microbial infection.

MATERIALS AND METHODS

Insects and bacterial strains

Disease free laying (DFL) of a silkworm breed was brushed and reared separately as per the standard rearing techniques described by Dandin *et al.* (2003) on fresh leaves of mulberry. The 3rd to 5th instar larvae were kept under controlled conditions (25 °C, 65-70% RH and 14L: 10D photoperiod cycle) at the insectary of the Department of Entomology, Faculty of Science, Cairo University, Giza, Egypt.

The experiments were conducted on the 5th instar larvae and repeated thrice at three different times.

Three gram (+) bacteria, *Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus sanguinis* and four gram (-) bacteria, *Escherichia coli* (D$_{31}$), *Psuedomonas aeruginosa*, *Proteus mirabilis* and *Klebsiella pneumoniae* were obtained from the Unit for Genetic Engineering and Agricultural Biotechnology, Faculty of Agriculture, Ain Shams University and used for insect immunization and antibacterial activity assay. Bacteria were grown in a peptone medium (1%), supplemented with 1% meat extract and 0.5% NaCl, at 37 °C in a rotary shaker.

Insect immunization and haemolymph collection

Insect immunization was performed by injecting 20 newly moulted 5th instar larvae with 10 μl of approximately 1×10^6 (cells/ ml) viable, log phase bacteria dissolved in membrane-filtered saline using a thin-needled microsyringe. Bacterial strains were used for immunization separately or in combinations (Table 1). Haemolymph was sampled at 24, 48 and 72 h p.i. at 4 °C (500 μl/ each), containing few crystals of phenylthiourea to prevent melanization. Usually, 10 ml of haemolymph was collected, from about 15 to 20 larvae (irrespective of sex), by piercing a proleg with a fine, sterile needle. Haemolymph was centrifuged for 15 min at 1,800 rpm, then the cell-free haemolymph was aliquoted (100 μl to 1 ml each), and stored at -80 °C for a weak until investigated. The same procedures were applied to the control group except it was injected with saline without bacteria.

Quantitative protein analysis

Total protein concentration was quantified spectrophotometrically in both the control and the bacterial-challenged samples using Bio-Rad protein assay kit (Bio-Rad, USA) following the manufacturer's protocol. The difference between the control and the treated samples was considered accumulated AMP in the haemolymph (subtraction method). Standard curve was constructed by using Bovine Gamma Globulin (BGG). Haemolymph volumes were corrected for total protein concentration all over the antibacterial experiments.

Preparation of haemolymph extracts

The cell-free haemolymph was used for extraction of peptides. Acidic/ methanolic extracts of cell-free haemolymph were obtained by the method adapted from Schoofs *et al.* (1990). The haemolymph was diluted 10 times with the extraction solution consisting of methanol: glacial acetic acid: water (90: 1: 9, v/ v/ v) and mixed thoroughly. Precipitated proteins were pelleted by cooling centrifugation at 14000 rpm for 30 min. The obtained supernatant was collected, freeze-dried and the pellet was dissolved in 0.1% trifluoroacetic acid (TFA). For lipid removal from the extract, the same volume of *n*-hexane was added, the sample was vortexed and centrifuged at 14000 rpm for 10 min at 4 °C. The upper fraction containing lipids was removed and an equal volume of ethyl acetate was added to the water-soluble fraction (containing peptides). After vortexing and centrifugation the water-soluble fraction was freeze-dried and stored at -80 °C until needed.

Purification of immune haemolymph peptides

The freeze-dried immune haemolymph, deprived of lipids, was dissolved in 0.1% TFA and subjected to the first step of purification using a Supelcosil LC-18-DB 4.6 mm x 250 mm column, two buffer sets (A: 0.1% TFA (v/ v), B: 0.07% TFA, 80% acetonitrile (v/v)), and a linear gradient from 20 to 70% of buffer B over 30 min and 1 ml/ min flow rate. This step and all next chromatographic steps were performed on a Dionex P680 HPLC system (Dionex, Sunnyvale, CA, USA). The resulting fractions were subjected to freeze-drying, redissolved in water and visualized by staining with Coomassie Brilliant Blue after gel electrophoresis. Antimicrobial activity of the obtained fractions was determined as described below. Identified peptide-containing fractions exhibiting antibacterial activity were then subjected to the second step of purification using gel filtration chromatography on a Superose 12 HR 10/ 30 column (Pharmacia Biotech, Uppsala, Sweden), 50 mM ammonium acetate buffer pH 7.5 supplemented with 30% (v/ v) acetonitrile and 0.4 ml/ min flow rate. The collected peptide-containing fractions were finally purified to homogeneity using the previously described Supelcosil LC-18-DB column and a TFA/ water/ acetonitrile buffer set as experienced (many gradients were tried). The purified peptides were freeze-dried and stored at -80 °C until needed. Before use for antimicrobial activity tests, they were dissolved in apyrogenic water.

Polyacrylamide gel electrophoresis (PAGE) and localization of antibacterial activity

Native-PAGE of the collected samples of haemolymph at 24, 48 and 72 h p.i. was carried out to assess the induction of antibacterial peptides in the different haemolymph samples. It was carried out in 15% polyacrylamide gels pH 4, using a discontinous buffer system (Gabriel, 1971), but omitting the stacking gel. The acrylamide/ bisacrylamide ratio was 60: 0.8. The gels were run at 200 V until the tracker dye (methyl green) was running off the gel (approximately 2.5 h).

To localize bands with antibacterial activity the gels were incubated for one hour in a rich bacterial medium which contained 0.2 M sodium phosphate pH 7.4, and

streptomycin, 100 µg/ ml. The gel was then overlaid with 5 ml of melted agar, 0.6% in the same medium, containing about 2 x 10^5 viable *E. coli* D_{31} cells. On top of this was poured another layer of agar without bacteria, and the gel was incubated at 37 °C overnight. After incubation overnight at 37 °C, antibacterial activity in the bands was visualized as areas without bacterial growth.

Sodium dodecylsulfate (SDS)-PAGE of denatured proteins was carried out in 20% polyacrylamide gels pH 8.8, in a discontinous buffer (Maizel, 1971), but omitting the stacking gel. The acrylamide/ bisacrylamide ratio was 50: 1. The gels contained no SDS before electrophoresis. The protein samples were pretreated with 1% SDS and 1% β-mercaptoethanol for 5-10 minutes at 100 °C. The gels were run at 100 V until the tracker dye (bromphenol blue) was leaving the gel (approximately 6 h). All gels were fixed in 20% 5-sulfosalicylic acid, stained with Coomassie Brilliant Blue R_{250} in 7%, acetic acid, and destained in 7% acetic acid.

Antimicrobial assay

In vitro antimicrobial studies were carried out on the crude immunized haemolymph, the fractions containing peptides of low molecular weight and the purified peptides, from the HPLC experiment. Antimicrobial activity was estimated by the agar disk diffusion method with minor modifications (Favel *et al.*, 1994, NCCLS, 1997). Five milliliters of 0.6 % melted LB agar (52 °C) were mixed with 100 µl of viable bacterial suspension (1.6×10^9 cells/ ml), and poured into a 15 cm plastic dish. Five microliters of each haemolymph and protein samples were applied to a 6 mm diameter paper disk and incubated at 37 °C. Haemolymph volumes were corrected for total protein concentration all over the experiment. Penicillin (10 mg/disc; obtained from Sigma) and normal saline solution were used as positive and negative controls, respectively. *S. aureus*, *B. subtilis*, *S. sanguinis*, *E. coli* (D_{31}), *P. aeruginosa*, *P. mirabilis*, and *K. pneumoniae* bacteria were used for testing the antimicrobial activity. Inhibition zone diameters of five replicates were measured after 24-48 h. The degree of growth inhibition was quantitatively evaluated after 16 h by comparison with the growth inhibition resulting from the positive control.

Hemolytic assay

Hemolytic assay was performed using human, cow and rabbit erythrocytes as previously described with slight modification (Ryge and Hansen, 2005). Briefly, the erythrocytes were washed three times (3000 rpm) with phosphate buffered saline (0.15 M, pH 7.2) and diluted to a final concentration of 0.5% in the same buffer (PBS). To each well of a polypropylene microtiter plate 75 µl of the diluted erythrocytes and 75 µl of peptide solution at 100, 75, 50, 25 and 12.5 µg/ ml were added. After incubated for 1 h at 37 °C, samples were centrifuged at 4000 rpm for 10 min and 100 µl of the supernatants were transferred to a new 96-well microtiter plates. The optical density of supernatant was determined at 405 nm with an ELISA plate reader. Negative control (zero hemolysis) and positive control (100% hemolysis) were achieved by suspending the erythrocytes in 10 mM PBS and Triton X-100, respectively. The hemolysis percentage was calculated as follows: $[(A_{peptide}-A_{PBS})/(A_{Triton}-A_{PBS})] \times 100$. All hemolysis determinations were performed in triplicate and are the average of three independent determinations using the same stock solution.

RESULTS

Quantitative protein analysis

Quantitative protein analysis of the crude haemolymph of control and bacterial-challenged *B. mori* was determined at 24, 48 and 72 h p.i. (Table 1). Statistical

analysis of the data revealed that the increase of total protein concentration in all cases of bacterial-challenged insects were significant, when compared to control, at 24 and 48 h p.i. *Df, F* and *P* values were illustrated in Table (1). Meanwhile, no significant difference was observed between control and bacterial-challenged insects, at 72 h p.i. (*df*= 9, *F*= 0.4 and *P*= 0.9). At 24 h p.i, the total protein concentration of the haemolymph immunized with a gram (-) bacterium was significantly higher (*P*< 0.01) than that immunized with a gram (+) bacterium or with a combination of both, as well (Table 1). It was observed that the increase in total protein concentration of bacterial-challenged insects was significantly higher (*P*< 0.001) than control at 48 h p.i. However, no significant difference (*P*> 0.05) was observed between gram (-) and gram (+) bacterial challenge at 48 h p.i. (Table 1). In addition, no significant difference (*P*> 0.05) was observed between control and bacterial-challenged insects at 72 h p.i. (Table 1).

Generally, the total protein concentration decreases over time from 24 to 72 h p.i. within the same treatment. This decrease was statistically significant (*P*< 0.05) in all cases except the decrease between 24 and 48 h p.i. in the case of combination$_2$, combination$_3$, and *S. sanguinis* bacteria (*P*> 0.05). No significant difference (*P*> 0.05) was observed in the case of control (Table 1).

Table 1: Quantitative protein analysis of the control and the immune haemolymph of *S. littoralis* at different times post-immunization by different bacterial srains.

Immunization by	Protein concentration at different hours post-infection (µg/ ml) Mean ± S.E.		
	24 h p.i.	48 h p.i.	72 h p.i.
Cont-H*	672.0±7.9^{a**}	649.4± 6.9^a	676.4±6.2^a
E. coli (-ve)	2008.8±11.2^b	974.8± 3.3^b	712.4± 2.0^a
S. aureus (+ve)	1106.8±41.7^{bc}	859.6± 29.9^{ab}	708.6±9.7^a
Combination	1097.6±46.5^{bc}	920.2± 24.7^b	716.4± 9.9^a
P. vulgaris (-ve)	2087.4±49.8^b	960.8± 28.7^b	702.6±14.4^a
B. subtilis (+ve)	1186.6±47.2^{bc}	933.8± 49.9^b	709.2± 9.5^a
Combination	1074.8±52.2^{bc}	932.2± 21.2^b	696.4±48.5^a
K. pneumoniae (-ve)	2097.4±38.0^b	977.0± 46.5^b	722.6± 28.0^a
S. sanguinis (+ve)	1140.0±60.9^{bc}	982.4± 78.2^b	719.4±13.1^a
Combination	1012.8±15.6^{bc}	953.8± 20.1^b	731.2± 42.6^a
Df, F, P	9, 156.4, 0.0	9, 7.1, 0.0	9, 0.4, 0.9

*Cont-H: Untreated crude haemolymph.
**Means with different letters are significant at 95% confidence limits.

Purification of the immune haemolymph peptides

The fractionation of the immune haemolymph extract on a reversed phase C-18 column allowed effective separation of 5 fractions containing mainly proteins and peptides of molecular masses below 20 kDa (Fig. 1E). The obtained fractions were tested for antimicrobial activity and relative high level of antibacterial activity against gram (+) and gram (-) was detected in fractions# 2, 3 and 5, containing the most abundant low-molecular weight peptides (Table 3). For further purification, the fractions# 2, 3 and 5, containing the most abundant low-molecular weight peptides (below 7 kDa) and exhibiting high antibacterial activity, were chosen. The second step embraced gel filtration chromatography and allowed isolation of single peptide

components from the chosen fractions (Fig. 1E). Although each fraction resolved during gel filtration into two separate peaks (Fig. 1C and D), only two low-molecular weight peptides were chosen to be tested for antimicrobial activity (peptide# A and B). These peptides were then desalted and purified to homogeneity by a reversed phase chromatography step on a C-18 column. The final peptide preparation gave single bands on SDS-PAGE gels and single peaks on a C-18 column.

Antimicrobial assay

Tables (2-4) show a summary of the antimicrobial screening of the crude immunized haemolymph, three active fractions and two purified peptides, based on microbial growth inhibition zone (in mm). Based on the quantitative protein analysis, antimicrobial activity of the crude *Klebsiella*-immunized haemolymph (the highest protein concentration) was investigated at 24, 48 and 72 h p.i. (Table 2). Significant increase in antimicrobial activity of the immunized haemolymph (inhibition zone more than positive control) was found against gram (-) and gram (+) bacteria at 24 h p.i.

Table 2: Antimicrobial activity of the immunized haemolymph (*K. pneumoniae*) collected at different times post-immunization, against gram (-) and gram (+) bacteria.

Microorganism (gram stain)	Antimicrobial activity of the immunized haemolymph collected at					
	24 h p.i.*		48 h p.i.		72 h p.i.	
	24 h**	48 h	24 h	48 h	24 h	48 h
E. coli (-ve)	++	+++	++	++	(+)	+
K. pneumoniae (-ve)	+++	+++	++	++	(+)	+
P. vulgaris (-ve)	++	+++	++	++	(+)	+
B. subtilis (+ve)	+++	+++	++	+++	+	+
S. aureus (+ve)	++	+++	+	++	(+)	+
S. sanguinis (+ve)	+++	+++	++	+++	+	+

*: Time of sampling post-immunization.
**: Time of taking reading.
(+): Inhibition zone less than 1 mm surrounding the 6 mm paper disk
+: Inhibition less than positive control.
++: Inhibition comparable to positive control.
+++: Inhibition more than 10mg penicillin; inhibition zones of references = 13±1 mm diam.

Table (3): Antimicrobial activity of the fractions containing low molecular weight peptides, against gram (-) and gram (+) bacteria.

Microorganism (gram stain)	Antimicrobial activity of the immune haemolymph collected at					
	Fraction# 2		Fraction# 3		Fraction# 5	
	24 h*	48 h*	24 h	48 h	24 h	48 h
E. coli (-ve)	+	++	++	+++	+++	+++
K. pneumoniae (-ve)	(+)	+	+	++	+++	+++
P. vulgaris (-ve)	+	++	++	+++	+++	+++
B. subtilis (+ve)	++	+++	+++	+++	++	+++
S. aureus (+ve)	++	++	+++	+++	+++	+++
S. sanguinis (+ve)	+	++	++	+++	+++	+++

*: Reading taken after this time.
(+): Inhibition zone less than 1 mm surrounding the 6 mm paper disk
+: Inhibition less than positive control.
++: Inhibition comparable to positive control.
+++: Inhibition more than 10mg penicillin; inhibition zones of references = 13±1 mm diam.

Table 4: Antimicrobial activity of the purified antimicrobial peptides on gram (-) and gram (+) bacteria.

Microorganism (gram stain)	Antimicrobial activity of the immunized haemolymph collected at			
	Peptide# A		Peptide# B	
	24 h*	48 h*	24 h	48 h
E. coli (-ve)	++	+++	+++	+++
K. pneumoniae (-ve)	++	++	+++	+++
P. vulgaris (-ve)	+	++	++	+++
B. subtilis (+ve)	+++	+++	+++	+++
S. aureus (+ve)	++	++	++	+++
S. sanguinis (+ve)	+++	+++	+++	+++

*: Reading taken after this time.
(+): Inhibition zone less than 1 mm surrounding the 6 mm paper disk
+: Inhibition less than positive control.
++: Inhibition comparable to positive control.
+++: Inhibition more than 10mg penicillin; inhibition zones of references = 13±1 mm diam.

However, it was comparable to or more than the positive control at 48 h p.i. The least activity of the immunized haemolymph was observed at 72 h p.i. Generally, antimicrobial activity was comparable to or less than the positive control in the case of *E. coli*, *P. vulgaris* and *S. aureus* bacteria (Table 1).

The crude *Klebsiella*-immunized haemolymph was subjected the first step of purification and five fractions were obtained from this step of purification. These fractions were analyzed by SDS-PAGE (Fig. 1E), and three of them were found to contain abundant low-molecular weight peptides (fractions# 2, 3 and 5). The antimicrobial activity of these three fractions was investigated (Table 3). Notably, fraction# 5 exhibited the strongest antimicrobial activity (more than the positive control) among the three tested fractions. The activity was comparable to or more than the positive control in the case of fraction# 3. However, the least activity was observed in the case of fraction# 2. *K. pneumoniae* showed more resistance to fractions# 2 and 3, followed by *E. coli*, *P. vulgaris* and *S. sanguinis*. It is worthy mentioned that the fraction# 5 displayed its full antimicrobial activity against all tested bacteria at 48 h post-treatment (Table 3).

The three fractions were subjected to the second step of purification and two promising antimicrobial peptides (peptide# A and B) were selected to be tested for antimicrobial activity (Table 4 and Fig. 1E). The full antimicrobial activity of the peptide# B (more than the positive control) was observed at 48 h post-treatment. However, the antimicrobial activity of the peptide# A was comparable to or more than the positive control, except in the case of *P. vulgaris* (Table 4). Generally, the purified peptides exhibited a broad antimicrobial activity against all tested gram (+), gram (-) bacteria but notably was relatively less active against *S. aureus* and *P. vulgaris* at 24 h post-treatment (Table 4).

Hemolytic activity analysis

The purified peptides (A and B) evidenced no or very weak hemolytic activity against human, cow and rabbit red blood cells, even up to 50 µg/ ml concentration (Table 5). Peptide# B exhibited weaker hemolytic activity than peptide# A. Acceptable percentages of hemolysis (2.8-5%) were observed at higher concentrations (75 and 100 µg). Hemolytic activity of the peptide# A toward human RBCs did not exceed a maximum point of 4.4% at 100 µg concentration. However, the slight increase (4.8 and 5%) of hemolytic activity toward cow and rabbit RBCs still standing to the acceptable level. Statistical analysis of the data revealed that the increases in hemolytic activity of the purified peptides toward human, cow and rabbit RBCs are significant at the concentrations of 75 and 100 µg/ ml. *Df*, *F* and *P* values are illustrated in Table (5). In such cases, the significant increase could be neglected (*i.e.*

considered to be standing to the acceptable level at all tested concentrations up to 100 µg/ ml), because the increase in hemolytic activity was compared to zero value (for concentration up to 50 µg/ ml) (Table 5).

Table 5: Hemolytic activity of the purified peptides against human, cow and rabbit red blood cells.

Treatment	% Hemolysis (µg/ml)					
	Peptide# A			Peptide# B		
	Human RBCs	Cow RBCs	Rabbit RBCs	Human RBCs	Cow RBCs	Rabbit RBCs
Control	0^a	0^a	0^a	0^a	0^a	0^a
12.5 µg	0^a	0^a	0^a	0^a	0^a	0^a
25 µg	0^a	0^a	0^a	0^a	0^a	0^a
50 µg	1.40 ± 0.24^a	1.60 ± 0.25^a	1.80 ± 0.37^b	0^a	0^a	0^a
75 µg	3.60 ± 0.51^b	3.40 ± 0.51^{ab}	3.20 ± 0.37^b	2.80 ± 0.37^b	3.00 ± 0.32^b	2.60 ± 0.40^b
100 µg	4.40 ± 0.51^b	4.80 ± 0.66^b	5.00 ± 0.63^{bc}	2.80 ± 0.37^b	2.80 ± 0.20^b	3.40 ± 0.25^b
df, F, P	5, 40.46, 0.00	5, 33.40, 0.00	5, 38.49, 0.00	5, 44.80, 0.00	5, 96.29, 0.00	5, 67.20, 0.00

*Means with different letters are significant at 95% confidence limits.

Native and SDS-PAGE, and localization of the antimicrobial activity

The native forms of the purified proteins were analyzed by electrophoresis system for basic proteins. Fig. (1A) shows that the isolated peptides (A and B) are essentially pure. However, peptide B contained a single impurity which moved faster than the main spot, causing irregular shape of the band.

The immunized haemolymph was subjected to native-PAGE at intervals after bacterial injection, and the antibacterial activities were detected by overlaying bacteria seeded agar. Fig. (1B) showed that that seeding of *E. coli* on top of a gel provided a means to demonstrate the antibacterial activity of proteins. A localized antibacterial activity of two protein bands with distinct electrophoretic mobility was demonstrated using bacteria seeded agar method. Meanwhile, no antibacterial activity was detected in the case of non-immunized haemolymph (Fig. 1B).

Fig. (1E) showed the two peptides with low molecular weight (A and B), and exhibiting relative higher antimicrobial activity. The molecular weights of the two peptides as revealed by Tricine SDS-PAGE were 5.8 and 4.3 KDa, respectively (Fig. 1E).

DISCUSSION AND CONCLUSION

Defense peptides and proteins constitute key factors in insect humoral immune response against invading microorganisms. It is generally assumed that each insect species possesses an individual set of antimicrobial peptides synthesized in response to non-self recognition. In this study, we purified and partially characterized two *B. mori* peptides which appeared in larval haemolymph after bacterial challenge. They probably comprise a part of the defense peptide repertoire of *B. mori*. The significant increase of the bacterial-challenged haemolymphs suggested that the AMPs are upregulated and released in haemolymph as "acute phase response" of the insect and then declined over time. Agreeable results were reported by Brogden *et al.* (2003) who stated that after immunization, the level of antimicrobial activity in the haemolymph increases significantly. Also, it was clear that this response differ in correspondence to inducer (bacterial strain). The observation that a gram (-) inducer was more efficient than a combination needs more investigation, especially at molecular level. Consequently, full antimicrobial activity of the immune haemolymph was observed at 24 h p.i. These results are consistent to the results of quantitative protein analysis. The immune haemolymph is then fractionated and one of the tested

fractions exhibited higher antimicrobial activity. Finally, two peptides (5.8 and 4.3 KDa) were purified and one of them (4.3 KDa) showed full antimicrobial activity and very weak hemolytic activity up to concentration of 100 μg/ ml. Early immunological studies conducted at the beginning of the 20th century, were focused on the role of the morphologic elements of the haemolymph. At that time, a strong, heat-stable haemolymph antibacterial activity was observed after insect immunization. Late 1970s brought a series of biochemical investigations on immunized insects that resulted in isolation of novel classes of antimicrobial peptides called cecropins and attacins (Chadwick *et al.*, 1991). Presently, the insect haemolymph is one of the richest sources of novel antimicrobial agents; the number of novel inducible antibacterial peptides of insects exceeds now 180. Additionally, antimicrobial peptides are assumed as an alternative for the classical antibiotics. They are advantageous because their selectivity, fast killing, broad antimicrobial spectra and no resistance development (Matsuzaki, 1999, Boman, 2003). As it was stated earlier, many antimicrobial peptides have been discovered in insects. In *Drosophila melanogaster*, more than 20 antimicrobial peptide genes were identified and their peptide products were grouped into seven families: attacins, cecropins, defensins, diptericins, drosomycins, drosocin and metchnikowin (Irving *et al.*, 2004). Although *D. melanogaster* is the best characterized organism concerning insect innate immunity, the first insect inducible antibacterial peptides, cecropins, were isolated and characterized from bacteria-challenged pupae of the lepidopteran insect, *Hyalophora cecropia* (Steiner *et al.*, 1981). Since then peptides with antimicrobial activity have been purified and described in many other insect species belonging to different orders: Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Trichoptera and Odonata (Hetru *et al.*, 1981). Recently, the lepidopteran insect, *Galleria mellonella*, has been developed as a model organism for studying innate immunity mechanisms (Cotter *et al.*, 2000, Leger, *et al.*, 2000, Brennan *et al.*, 2002, Dunphy *et al.*, 2003, Reeves *et al.*, 2004, Kavanagh and Reeves, 2004, Mylonakis *et al.*, 2005). So far, more than 13 inducible *G. mellonella* peptides with antimicrobial activity have been characterized (Schuhmann *et al.*, 2003, Kim *et al.*, 2004, Lee *et al.*, 2004, Cytryńska *et al.*, 2007). Antimicrobial peptide homologous to *B. mori* cecD and a proline-rich peptide of unique amino acid sequence were purified by Mak *et al.* (2001) however, the antimicrobial activity spectrum of both peptides was not determined.

Conclusively, we demonstrated that the antimicrobial activity of the immune haemplymph is related to the presence of two peptides that are able to kill bacteria. The present work describes purification and partial characterization of these two antimicrobial peptides. Molecular studies on such peptides will be very helpful in understanding their mode of action and structure-function relationships.

REFERENCES

Boman, H.G. (2003): Antibacterial peptides: basic facts and emerging concepts. *J. Intern. Med.*, 254: 197–215.

Brennan, M.; Thomas, D.Y.; Whiteway, M. and Kavanagh, K. (2002): Correlation between virulence of Candida albicans mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol. Med. Microbiol.*, 34: 153–7.

Brogden, K.A.; Ackermann, M.; McCray Jr, P.B. and Tack, B.F. (2003): Antimicrobial peptides in animals and their role in host defences. *Int J. Antimicrob. Agents.*, 22: 465–78.

Bulet, P. and Stöcklin R. (2005): Insect Antimicrobial Peptides: Structures, Properties and Gene Regulation. Protein and Peptide Letters J. , 12: 13-11.

Bulet, P.; Charlet, M. and Hetru, C. (2003): In *Innate Immunity,* (Ezekowitz, R.A.B. and Hoffmann, J.A., Eds.) pp. 89-107. *Humana Press, Totowa, N.J.*

Bulet, P.; Hetru, C.; Dimarcq, J-L. and Hoffmann, D. (1999): Antimicrobial peptides in insects: structure and function. *Dev. Comp. Immunol.,* 23: 329–344.

Chadwick, J.S.; Aston, W.P. and Gupta, A.P. (1991): Immunology of insects and other arthropods. pp. 347–370. *Boca Raton: CRC Press.*

Cotter, G.; Doyle, S. and Kavanagh, K. (2000): Development of an insect model for the *in vivo* pathogenicity testing of yeasts. *FEMS Immunol .Med. Microbiol.,* 27: 163–169.

Cytryńska, M.; Mak, P.; Zdybicka-Barabas, A.; Suder, P. and Jakubowicz, T. (2007): Purification and characterization of eight peptides from *Galleria mellonella* immune haemolymph. *Peptides,* 28: 533–546.

Dandin, S.B.; Jayant J. and Giridhar, K. (2003): Hand Book of Sericulture Technologies. *Central Silk Board, Bangalore, India,* 53.

Dunphy, G.B.; Oberholzer, U.; Whiteway, M.; Zakarian, R.J. and Boomer, I. (2003): Virulence of Candida albicans mutants toward larval *Galleria mellonella* (Insecta, Lepidoptera, Galleridae).*Can. J. Microbiol.,* 49: 514–24.

Favel, A.; Steinmetz, M.D.; Regli, P.; Olivier, E.V.; Elias, R. and Balansard, G. (1994): *In vitro* antifungal activity of triterpenoid saponins. *Planta Med.,* 60: 50–53.

Gabriel, O. (1971): Analytical disc gel electrophoresis. *Methods Enzymol*., 22: 565-578.

Hetru, C.; Hoffmann, D.; Bulet, P.; Brey, P.T. and Hultmark, D. (1981): Molecular mechanisms of immune responses in insects. London: Chapman & Hall., 40–66.

Hoffmann, J.A.; Kafatos, F.C.; Janeway, C.A. and Ezekowitz, R.A. (1999): Phylogenetic perspectives in innate immunity. *Science,* 284: 1313-1318.

Irving, P.; Troxler, L. and Hetru, C. (2004): Is innate enough? The innate immune response in *Drosophila*. *C.R. Biol.,* 27: 557–570.

Jacobs, M.R.; Koornhof, H.J.; Robins-Browne, R.M.; Stevenson, C.M.; Vermaak, Z.A.; Freiman, I.; Miller, G.B.; Witcomb, M.A.; Isáacson, M.; Ward, J.I. and Austrian, R. (1978): Emergence ofmultiply resistant pneumococci. *New England J. of Med.,* 299: 735-740.

Kavanagh, K. and Reeves, E.P. (2004): Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. *FEMS Microbiol. Rev.,* 28: 101-112.

Kim, C.H.; Lee, J.H.; Kim, I.; Seo, S.J.; Son, S.M. and Lee, K.Y. (2004): Purification and cDNA cloning of a cecropin-like peptide from the great wax moth, *Galleria mellonella. Mol. Cells,* 17: 262–266.

Lee, Y.S.; Yun, E.K.; Jang, W.S; Kim, I.; Lee, J.H. and Park, S.Y. (2004): Purification, cDNA cloning and expression of an insect defensin from the great wax moth, *Galleria mellonella. Insect Mol. Biol.,* 13: 65–72.

Leger, R.; Screen, S.E. and Shams-Pirzadeh, B. (2000): Lack of host specialization in *Aspergillus flavus. Appl. Environ. Microbiol.,* 66: 320–324.

Maizel, J.V. and Jr, P.B. (1971): Methods in Virology (Maramorosch, K. & Koprowski, H., eds) *Academic Press, New York and London*, 5: 179-246.

Mak, P.; Chmiel, D. and Gacek, G.J. (2001): Antibacterial peptides of the moth *Galleria mellonella* . *Acta Biochim. Pol.,* 48: 1191–1195.

Matsuzaki, K. (1999): Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. *Biochim. Biophys. Acta.,*1462: 1–10.

Mylonakis, E.; Moreno, R.; El Khoury, J.B.; Idnurm, A.; Heitman, J. and Calderwood, S.B. (2005): *Galleria mellonella* as a model system to study *Cryptococcus neoformans* pathogenesis. *Infect. Immun.,* 73: 3842–3850.

NCCLS (1997): Performance standards for antimicrobial disc suspectibility tests. *Approved Standard NCCLS PublicationM2-A5. Villanova, PA, USA.*

Reeves, E.P.; Messina, C.G.; Doyle, S. and Kavanagh, K. (2004): Correlation between gliotoxin production and virulence of *Aspergillus fumigatus* in *Galleria mellonella. Mycopathologia,* 158: 73-79.

Reinert, R.; Ringelstein, A.; Van der Linden, M.; Yu, M.; Al-Lahham A. and Schmitz, F. (2005): Molecular epidemiology of macrolide-resistant *Streptococcus pneumoniae* isolates in Europe. *J. Clinical Microbiol.*, 43: 31294–31300.

Ryge, T.S. and Hansen, P.R. (2005): Novel lysine-peptoid hybrids with antibacterial properties. *J. Peptide Sci.,* 11: 727–734.

Schoofs, L.; Holman, G.M.; Hayes, T.K.; Nachman, R.J. and DeLoof, A. (1990): Locust atachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. *FEBS Lett.,* 261: 397–401.

Schuhmann, B.; Seitz, V.; Vilcinskas, A. and Podsiadlowski, L. (2003): Cloning and expression of gallerimycin, an antifungal peptide expressed in immune response of greater wax moth larvae, *Galleria mellonella. Arch. Insect Biochem. Physiol.,* 53: 125–133.

Steiner, H.; Hultmark, D.; Engström, A.; Bennich, H. and Boman, H.G. (1981): Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*, 292: 246–248.

Tzou, P.; De Gregorio, E. and Lemaitre, B. (2002): How *Drosophila* combats microbial infection: a model to study innate immunity and host–pathogen interactions. *Curr. Opin. Microbiol.,* 5: 102–110.
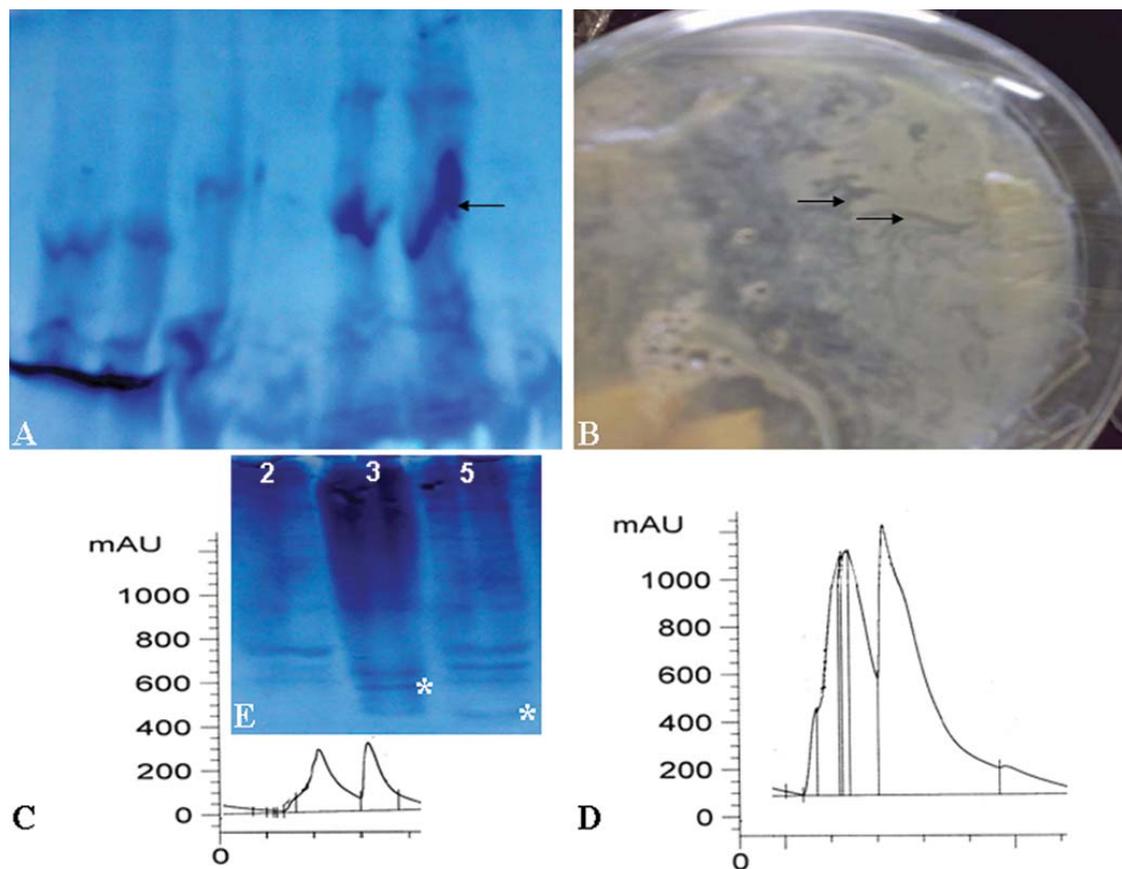
Fig. 1: (A): Native-PAGE showing the induced antimicrobial peptide. (B): localization of the antimicrobial activity on the gel using bacteria seeded agar method. (C&D): HPLC results indicating two peaks of the purified peptides. (E): SDS-PAGE showing the two antimicrobial peptides at 5.8 and 4.3 KDa molecular mass. Arrows and asterisks indicate the antimicrobial peptides.

RABIC SUMMARY

تنقية وتوصيف اثنين من الببتيدات المضادة للميكروبات من دم يرقات فراشة الحرير بومبيكس موراى المستحثة مناعيا بالبكتيريا

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تشكل الببتيدات والبروتينات الدفاعية عوامل رئيسية في الاستجابة المناعية للحشرات ضد غزو الكائنات الحية الدقيقة. وقد تم تصميم التجارب باستخدام تقنيات بيوكيميائية متقدمة لتوصيف اثنين من الببتيدات التي ظهرت في دم يرقات فراشة الحرير بومبيكس موراى المستحثة مناعيا بالبكتيريا. و أظهرت النتائج وجود زيادة معنوية فى كمية البروتين الكلي في دم اليرقات المستحثة بالبكتيريا ، ثم تراجعت هذه الزيادة بمرور الوقت. و أظهرت الدراسة أن تنظيم إفراز البروتينات المضادة للبكتيريا في دم اليرقات المستحثة بالبكتيريا ما هو إلا "استجابة المرحلة الحادة" للحشرة. وقد لوحظ نشاط مضاد للميكروبات كامل لالدموي اليرقات المعالجة بالبكتيريا بعد 24 ساعة من المعالجة. وعندما أجريت التجارب لتجزئة دم اليرقات المستحثة بالبكتيريا باستخدام عمود التجزئة C-18 ، وأظهر الفصل التجزيئي وجود خمسة أجزاء فعالة تحتوي على كمية وفيرة من البروتينات أقل من 20 كيلو دالتون. و أظهر الجزئ رقم 5 أقوى نشاط كمضاد للميكروبات. و أخيرا، تم تنقية اثنين من الببتيدات المضادة للبكتيريا (5.8 و 4.3 كيلو دالتون). وأظهر الببتيد (4.3 كيلو دالتون) وجود نشاط أعلى كمضاد للميكروبات ، بينما كان نشاطه الانحلالي تجاه كرات الدم المختلفة ضعيفا جدا حتى تركيز 100 ميكروجرام / مل. و كانت هذه النتائج متسقة مع نتائج التحليل الكمي للبروتينات. والخلاصة أن هذه الدراسة أظهرت أن النشاط المضاد للميكروبات فى دم اليرقات المستحثة بالبكتيريا يرتبط بوجود اثنين من الببتيدات المضادة للميكروبات التي لها القدرة على قتل البكتيريا. و من الممكن أن تكون هذه الببتيدات مفيدة فى مقاومة العدوى البكتيرية للنبات و الحيوان و الإنسان.