Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 35-44

INDUCTION OF CECROPIN-LIKE AND ATTACIN-LIKE ANTIBACTERIAL BUT NOT ANTIVIRAL ACTIVITY IN HELIOTHIS VIRESCENS LARVAE

Donald D. Ourth* [1], Timothy D. Lockey[1], and Harold E. Renis[2]

[1]Department of Biology, University of Memphis, Memphis, TN 38152

[2]The Upjohn Company Laboratories, Kalamazoo, MI 49001

Received February 19, 1994

SUMMARY: Inducible cecropin-like and attacin-like proteins were isolated from immune hemolymph obtained from vaccinated Heliothis virescens larvae. The attacin-like protein had a molecular weight of approximately 25,000 daltons and was not dialyzable. The cecropin-like peptide had an estimated molecular weight of 6,000-7,000 daltons and was dialyzable, heat-stable and sensitive to trypsin digestion. The cecropin-like peptide showed bactericidal activity against Escherichia coli and Enterobacter cloacae, and the attacin-like protein showed bactericidal activity against E. coli. The immune hemolymph was bactericidal against E. coli, E. cloacae and Pseudomonas aeruginosa. Ultrastructural cell envelope damage to E. coli, produced by the immune hemolymph, was observed by scanning electron microscopy. No antiviral activity by the inducible cecropin-like and attacin-like proteins was detected against herpes simplex virus-1 and the vesicular stomatitis virus.

Lepidopteran insects are known to produce antibacterial factors in the hemolymph in response to injection of bacteria (2,9,13). The best-defined antibacterial factors have been inducible proteins isolated from Hyalophora cecropia pupae called cecropins (M.W. 4,000-7,000) (3,4,10) which are bactericidal and attacins (M.W. 20,000-23,000) (4,10,11) which have limited antibacterial activity. In contrast with antibacterial immunity, little has been understood regarding insect antiviral immunity (5,6,7) until recently (17,18), even though insects can be infected with DNA and RNA viruses (1,14).

The purpose of this investigation was to demonstrate an inducible antibacterial humoral response in tobacco budworm (TBW) larvae, Heliothis virescens. Although the inducible antibacterial peptides of insects are known to have bactericidal activity against many bacteria, it has not been known if these inducible immune proteins also have antiviral activity. To accomplish the objectives of this study, cecropin-like and attacin-like proteins were isolated by gel filtration chromatography from immune

*To whom correspondence should be addressed. FAX: 901-678-4457.

0006-291X/94 $5.00

hemolymph obtained from larvae vaccinated with bacteria. The isolated larval immune proteins were then characterized and tested in vitro for antibacterial and antiviral activities against selected Gram-negative bacteria and enveloped viruses.

MATERIALS AND METHODS

Maintenance of Larvae: Tobacco budworm eggs were obtained from the Bioenvironmental Insect Control Laboratory, U.S. Department of Agriculture, Stoneville, MS. The eggs hatched after 3-4 days. The larvae were then reared individually in 30 ml plastic cups with lids; each cup contained 10 ml of diet (16,20). The cups were held at 24-25°C in a natural photoperiod.

Vaccination of Larvae: Tobacco budworm larvae were injected with live bacteria to induce an antimicrobial response. The vaccination period was for 24 hrs. An Enterobacter cloacae (ATCC 23355) vaccine was prepared by growth in tryptic soy broth for 24 hrs. at 30°C. Each TBW larva was inoculated with 10^6 viable bacteria at the base of a proleg; a 28-gauge needle with 0.5 ml syringe was used (16). The larvae were 14 days old (5th instar) at the time of vaccination and 15 days old at the time of bleeding (16). Two hundred larvae were inoculated with the vaccine. Two hundred twenty-four larvae were not injected with the vaccine and served as a normal hemolymph group.

Collection of Hemolymph: Two pools of normal and immune hemolymph were collected, respectively, from the unvaccinated and vaccinated TBW larvae with capillary tubes under ice-chilling conditions (16). Cell-free hemolymph was obtained by centrifugation at 10,000 rpm for 10 min. at 4°C (16). The hemolymph was stored at -80°C.

Protein Determination: Hemolymph protein was determined by the Lowry method (15) using a standard curve. Bovine serum albumin was used as the protein standard.

Bactericidal Assay: To conduct the bactericidal assay, the same E. cloacae culture as used for vaccination was grown in tryptic soy broth for 24 hrs. at 30°C. The bactericidal assay was performed as previously described (16) using 25 µl of a culture dilution of E. cloacae in saline and 25 µl of hemolymph or 25 µl of a column purified protein fraction or 25 µl of saline. The incubation time of the mixture was 90 min. at 27°C. The tryptic soy agar plates were incubated for 48 hrs. at 25°C and the bacterial colonies then counted. An equation was used to calculate the percent bactericidal activity of the hemolymph and hemolymph fractions (19). Besides E. cloacae, cultures of Escherichia coli (ATCC 11229), E. coli (K-12, D31 strain) and Pseudomonas aeruginosa (ATCC 27853) were tested for their sensitivity to the immune and normal hemolymphs. Escherichia coli (ATCC 11229) and E. cloacae were tested for their sensitivity to the antibacterial activity of the cecropin-like and attacin-like hemolymph factors.

Antiviral Assay in Tissue Culture: The TBW normal and immune hemolymph protein fractions were tested for their antiviral activity in vitro in tissue culture against the herpes simplex virus (HSV) type 1, a DNA virus, and against the vesicular stomatitis virus (VSV), an RNA virus. Antiviral activities were determined as previously described (18) using a quantitative ($TCID_{50}$) cytotoxicity/virus inhibition test done in Vero cell tissue cultures that demonstrates viral cytopathic effects (CPE) and plaque formation (18,21,22). Cytotoxicity testing using uninfected Vero cells was also done.

Purification of Antimicrobial Factors from Hemolymph: Hemolymph from the inoculated TBW larvae was purified by gel filtration chromatography as follows. Hemolymph (0.5 ml) was applied to a Sephadex G-75-50 gel column (Pharmacia, Piscataway, NJ) in phosphate buffered saline (PBS), pH 7. After an initial large void volume peak, one very small peak and then two poorly-separated larger peaks of low molecular weight were seen. The latter two peaks were combined and concentrated,

and then 1 ml was applied to a Sephadex G-50-50 column (Pharmacia, Piscataway, NJ) in PBS, pH 7. Two ml of eluate were collected per tube from a column (1.5 cm x 30 cm) containing 50 ml of packed gel. Two well-separated peaks were obtained. The second peak was recycled by Sephadex G-50-50 gel filtration. These two protein peaks, isolated by gel filtration chromatography, were then tested for antibacterial and antiviral activities.

Three mg each of α-chymotrypsin (M.W. 21,000), lysozyme (M.W. 14,300) and insulin (M.W. 5,800) were used as molecular weight protein standards (Sigma, St. Louis, MO). Two ml fractions were collected from the Sephadex G-50-50 column, and the peak tube number was plotted against the log of the molecular weight standards to construct a standard curve. The molecular weights of the two isolated peaks were then determined.

Low mol. wt. hemolymph fractions of ≤10,000 and ≤3,000 were obtained by ultrafiltration and centrifugation using Ultrafree-MC filter units (Millipore Corp., Bedford, MA). These two protein fractions were also tested for antiviral activity.

High Pressure Liquid Chromatography (HPLC): An HPLC gel filtration column (Bio-Sil TSK-250, BioRad, Richmond, CA) and Beckman HPLC instruments Model 332 (Beckman Instruments, Fullerton, CA) were used to determine the purity and molecular weight of the cecropin-like immune hemolymph peptide. The column was eluted with 0.2 M Na_2HPO_4, pH 7 and had a flow rate of one ml/min. The molecular weight of the cecropin protein factor isolated by Sephadex G-50-50 gel filtration was determined by comparing its HPLC retention time with Bio-Rad gel filtration molecular weight standards (Bio-Rad, Richmond, CA) of thyroglobulin, 670,000; IgG,158,000; ovalbumin, 44,000; myoglobin, 17,000 and vitamin B-12, 1350.

Characterization of Hemolymph Cecropin Factor: The following experiments were done to obtain data on the chemical nature of the hemolymph antibacterial cecropin-like factor (8,10).

(a) The hemolymph cecropin factor was tested to determine if it was dialyzable (12,000 M.W. cut-off dialysis membrane).

(b) The hemolymph cecropin factor was tested to determine if it was heat-stable (heated at 60°C for 30 min.).

(c) The hemolymph cecropin factor was tested for its resistance to trypsin digestion (1 mg/ml trypsin treatment for 30 min. at 30°C) and the reaction then stopped by adding 1 mg soybean-trypsin inhibitor.

After each of the above experiments (a-c), the hemolymph cecropin factors were tested by the bactericidal assay to determine if they retained their antibacterial activity to E. coli versus untreated hemolymph.

Electron Microscopy: After incubation for 90 min. at 27°C with hemolymph, the E. coli (ATCC 11229) whole cells were pelleted, resuspended in PBS and filtered onto 0.22 micron-pore Nucleopore PC membranes (Millipore Corp., Bedford, MA). Twenty-four hr. cultures of E. coli were used. Bacteria on the membrane filters were fixed with 2.5% glutaraldehyde in cacodylate buffer, pH 7, dehydrated in ethanol, critical-point dried and sputter-coated with 15 nm gold (12). The E coli were imaged using a JEOL 840 scanning electron microscope (SEM). Hemolymphs from vaccinated and unvaccinated larvae were used. Saline treatment of bacteria was also done. The SEM study was done in the UM Electron Microscopy Center.

RESULTS

Immune hemolymph from the TBW larvae was purified by gel filtration chromatography using Sephadex G-75-50 and Sephadex G-50-50 columns (Pharmacia, Piscataway, NJ) in PBS, pH 7. Two well-separated peaks were obtained by Sephadex G-50-50 column chromatography (Fig. 1). Using α-chymotrypsin, lysozyme and insulin as molecular weight standards to construct a standard curve,

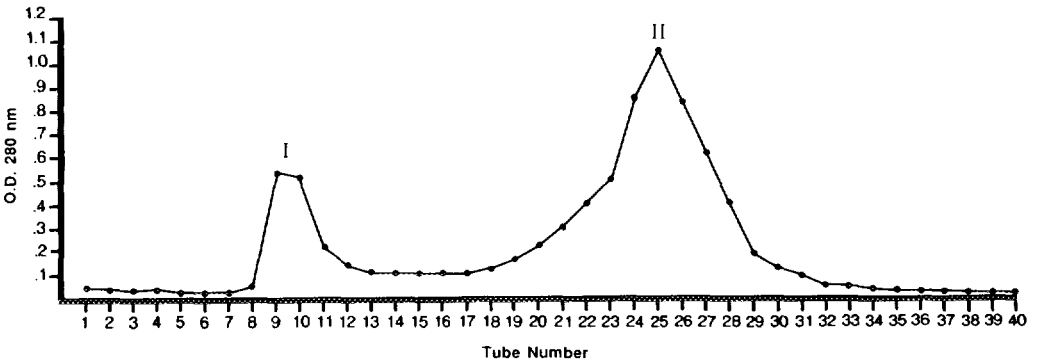

Figure 1. Sephadex G-50-50 gel filtration in PBS, pH 7, of the last two peaks obtained from Sephadex G-75-50 gel filtration. Two ml of eluate was collected per tube. Peak I contained an attacin-like (25,000 daltons) antibacterial factor, and Peak II contained a cecropin-like (6,000-7,000 daltons) antibacterial factor.

one peak was estimated to have a molecular weight of 25,000 daltons (attacin-like) and was not dialyzable. The recycled second peak had an approximate molecular weight of 7,000 daltons (cecropin-like) and was dialyzable. The 25,000 mol. wt. attacin-like peak, after dialysis, showed bactericidal activity (30%) against E. coli (Table 1). The 7,000 mol. wt. cecropin-like peak showed bactericidal activity against E. coli (44%) and E. cloacae (23%) (Table 1). After dialysis, the second cecropin-like peak showed 2% bactericidal activity against E. coli. Analysis of the second peak by HPLC showed it to be 91% pure with a mol. wt. of 6,142 daltons (Fig. 2). After column purification and concentration, the yield of both the attacin and cecropin purified

Table 1. Percent Bactericidal Activity of Hemolymph and Hemolymph Fractions from Heliothis virescens Larvae

Bacteria	Normal Hemolymph	Immune Hemolymph	Cecropin Fraction	Attacin Fraction
Escherichia coli K-12	0%	95%	ND	ND
Escherichia coli	0%	72%	44%	30%
Pseudomonas aeruginosa	2%	46%	ND	ND
Enterobacter cloacae	3%	41%	23%	ND

ND: Not Done

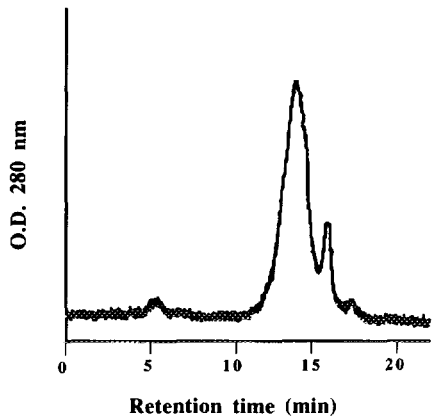

Figure 2. High pressure liquid chromatography of the cecropin-like peptide isolated by Sephadex G-50-50 gel filtration. An approximate molecular weight of 6,142 daltons was obtained.

fractions was 2 mg/ml. The two fractions were tested for antiviral activity against HSV-1 and VSV, but no antiviral activity was found (Table 2).

The bactericidal activity of the cecropin-like peptide was heat-stable, resisting heating at 60°C for 30 min. However, the cecropin-like peptide was sensitive to

Table 2. Antiviral (AV) Activity and Cytotoxicity (Tox) of Hemolymph Fractions from Heliothis virescens Larvae

	HSV-1 (mg protein/ml)*			VSV (mg protein/ml)*		
Hemolymph	Tox	AV	R**	Tox	AV	R**
Attacin Fraction (25,000 M.W.)						
Immune	0.55	>0.55	1	>0.55	>0.55	1
Cecropin Fraction (6,000 M.W.)						
Immune	0.5	>0.5	1	>2.0	>2.0	1
Protein Fraction (≤10,000 M.W.)						
Immune	>1.2	>1.2	1	>1.1	>1.1	1
Normal	>0.72	0.16	4.5	>0.72	>0.72	1
Protein Fraction (≤3,000 M.W.)						
Normal	1.5	0.37	4.1	>1.5	>1.5	1

*mg protein/ml which protects 50% of Vero cells from viral
**cytopathology or is cytotoxic for 50% of noninfected Vero cells.
R is cytotoxicity divided by antiviral activity.
HSV-1: Herpes Simplex Virus-1.
VSV: Vesicular Stomatitis Virus.

trypsin-treatment at 30°C for 30 min. which resulted in loss of bactericidal activity (0%) to E. coli.

Immune hemolymph from the vaccinated TBW larvae showed bactericidal activity against E. coli K-12 (95%), E. coli (72%), P. aeruginosa (46%) and E. cloacae (41%) (Table 1). Normal hemolymph showed little or no bactericidal activity (Table 1).

Ultrastructural evidence of bacterial cell envelope damage against the E. coli whole cells by the immune hemolymph proteins, produced by the vaccinated larvae, was readily seen by SEM (Fig. 3a). The morphological damage to E. coli was extensive. No structural damage was seen with hemolymph from unvaccinated larvae (Fig. 3b). Saline treatment of bacteria also showed no structural damage by SEM (Fig. 3c).

Low mol. wt. hemolymph fractions of ≤10,000 and ≤3,000 were obtained by ultrafiltration and centrifugation using Ultrafree-MC filter units. They were tested for antiviral activity against HSV-1 and VSV but no antiviral activity was detected (Table

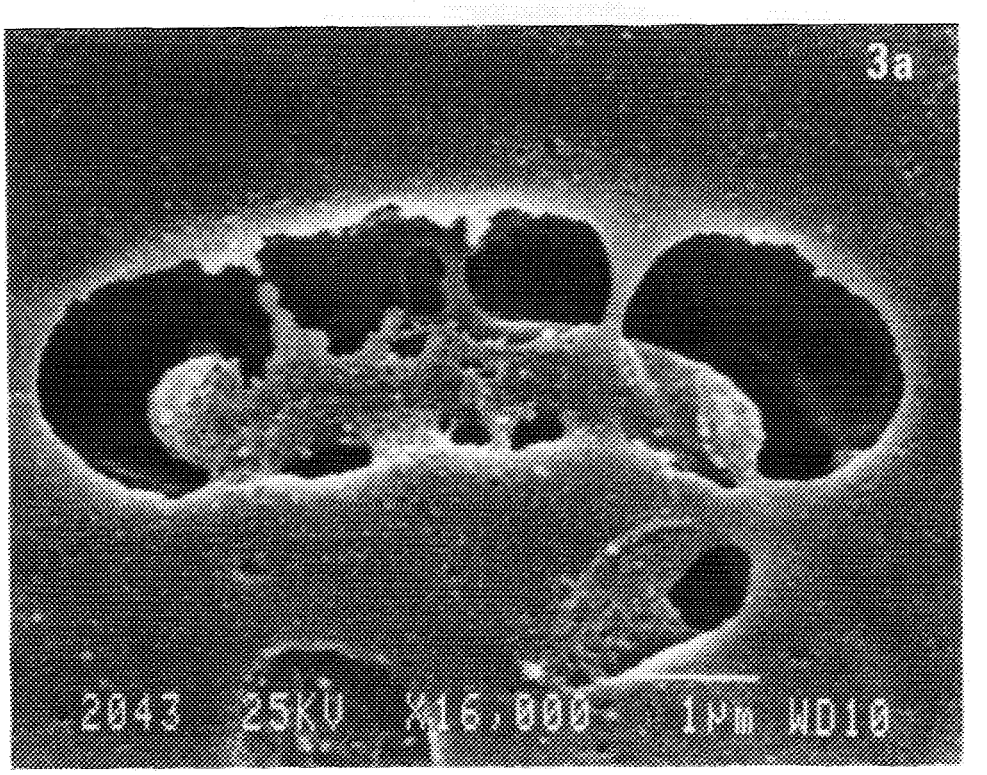

Figure 3. Scanning electron microscopy of effect of Heliothis virescens hemolymph on Escherichia coli bacteria. (a) Immune hemolymph from vaccinated larvae showing bacterial cell envelope damage (x16,000). (b) Normal hemolymph from unvaccinated larvae showing no structural damage to bacteria (x16,000). (c) Saline treatment of bacteria showing no structural damage (x20,000).

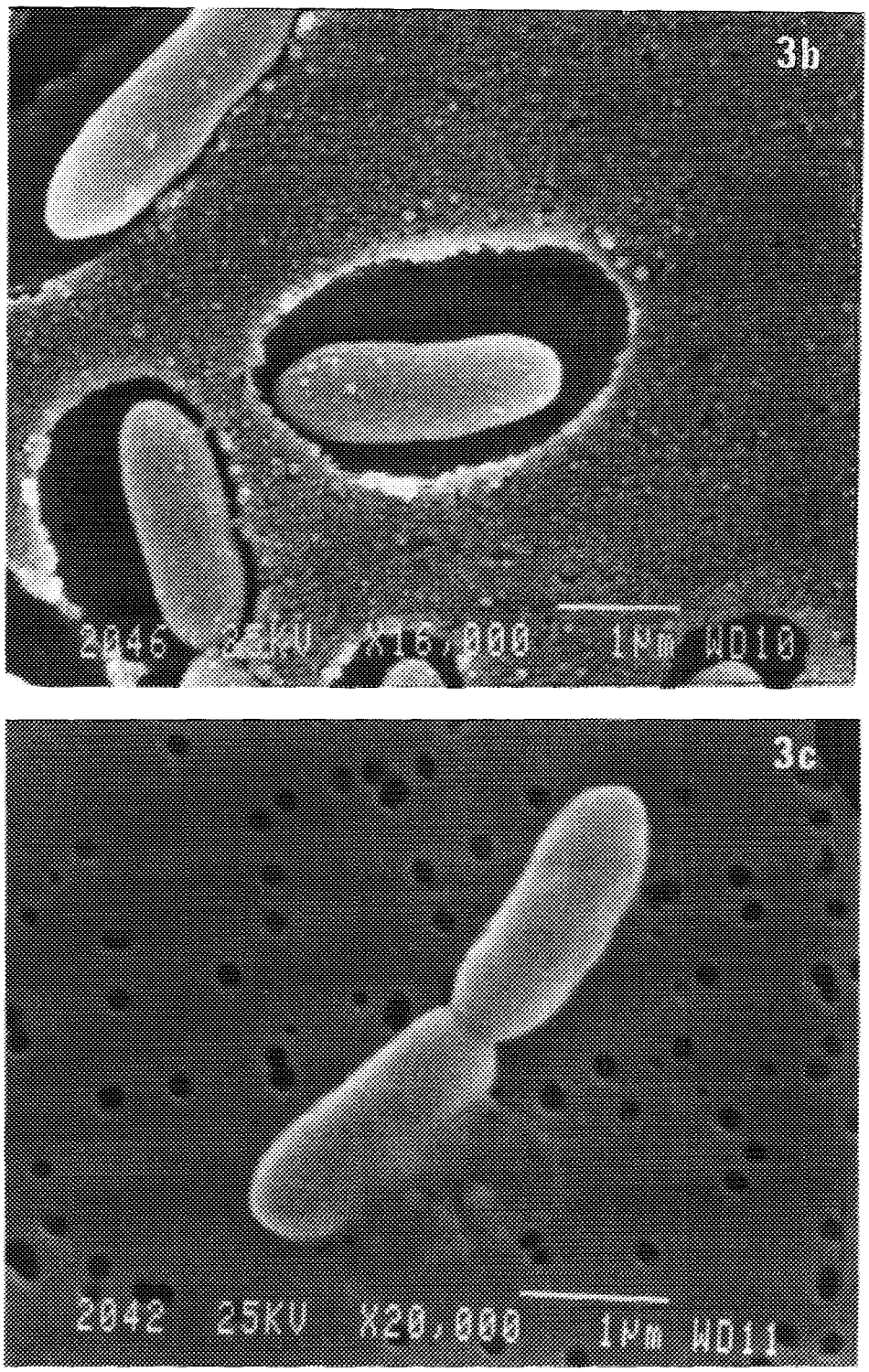

Figure 3 - Continued

2). A major low mol. wt. cecropin peak of 6,142 daltons that was 91% pure by HPLC was isolated that showed bactericidal activity against E. coli and E. cloacae (Table 1; Fig. 2). The cecropin-like protein was further tested for antiviral activity but none was found (Table 2). Both the cecropin and attacin inducible responses, although antibacterial (Table 1), were not antiviral (Table 2). Antiviral activity was therefore not inducible by vaccination.

DISCUSSION

Heliothis virescens is an important agricultural pest in the U.S.A. and is highly resistant to nearly all chemical insecticides. Research studies on the development of insect resistance to bacteria and viruses are needed to better understand the insect response to microorganisms. Insect immunity should be studied in detail since biological and chemical agents might be used to inhibit the insect defensive response for the control of insect pests.

Induced humoral antibacterial proteins have been described before in lepidoptera (4,9,10). This investigation, however, describes for the first time the presence of inducible antibacterial activity in H. virescens larvae against E. coli, E. cloacae and P. aeruginosa (Table 1). Characterization of the hemolymph cecropin-like antibacterial factor was done. The cecropin-like protein was found to be dializable as it passed through a 12,000 mol. wt. cutoff dialysis membrane. It was determined to be trypsin-sensitive and was heat-stable. The cecropin-like peptide had an estimated mol. wt. of 7,000 daltons by Selphadex G-50-50 gel filtration chromatography and a mol. wt. of 6,142 daltons by HPLC (Fig. 1 and 2). The attacin-like protein had an estimated mol. wt. of 25,000 daltons by Sephadex G-50-50 gel filtration chromatography (Fig. 1).

Antiviral activity of the TBW hemolymph was determined by calculating the therapeutic index (R value) or the measure of cytotoxicity (mg/ml) divided by the antiviral activity (mg/ml). An R value of 10 is needed in order for antiviral activity to be effective in animals (22). The cecropin and attacin fractions both had R values of 1 against the DNA and RNA viruses tested here (Table 2). From the R values obtained, the data indicate that the inducible cecropin-like and attacin-like proteins were not antiviral against HSV-1 and VSV (Table 2). We have recently found that the Heliothis phenoloxidase-activated melanization reaction is antiviral and can inactivate DNA and RNA viruses (17,18).

Bactericidal activity by insect cecropins is directed against bacterial infections. Immune hemolymph from the vaccinated TBW larvae was bactericidal against E. coli, E. cloacae and P. aeruginosa (Table 1). The cecropin-like and attacin-like proteins isolated from the immune TBW hemolymph demonstrated bactericidal activity against E. coli and E. cloacae (Table 1). Structural damage to E. coli was produced only by the immune hemolymph proteins obtained from the vaccinated larvae (Fig. 3a). The

bactericidal effect of the Heliothis cecropins and attacins against E. coli was readily observed by SEM. No antiviral activity was detected against HSV-1 and VSV (Table 2). The cecropin and attacin hemolymph column preparations and also the ≤10,000 M.W. and ≤3,000 M.W. protein fractions had R values of 1-4 indicating no antiviral activity (Table 2) (22).

This investigation describes for the first time the presence of an inducible antibacterial humoral response by Heliothis virescens lepidopteran larvae. Both cecropin-like and attacin-like antibacterial but not antiviral responses were seen. Characterization of the low molecular weight cecropin-like peptide was done. Bacterial cell damage by the immune hemolymph was observed by SEM.

ACKNOWLEDGMENT

The authors thank Dr. Hans Boman, University of Stockholm, Sweden for providing the Escherichia coli K-12, D31 strain.

REFERENCES

1. Adams, J. R. and Bonami, J. R. (Editors) (1991) Atlas of Invertebrate Viruses. CRC Press, Boca Raton, FL.
2. Boman, H. G. (1981) Microbial Control of Pests and Plant Diseases 1970-1980. H.D. Burges (Editor). pp. 769-784. Academic Press, New York, NY.
3. Boman, H. G. and Hultmark, D. (1981) TIBS 6, 306-309.
4. Boman, H. G. and Hultmark, D. (1987) Ann. Rev. Microbiol. 41, 103-126.
5. Briese, D. T. and Podgwaite, J. D. (1985) Viral Insecticides for Biological Control. K. Maramorosch and K. E. Sherman (Editors). pp. 361-398. Academic Press, New York, NY.
6. Chadwick, J. S. and Dunphy, G. B. (1986) Hemocytic and Humoral Immunity in Arthropods. A. P. Gupta (Editor). pp. 287-330. John Wiley and Sons, New York, NY.
7. Christensen, B. M. and Tracy, J. W. (1989) Amer. Zool. 29, 387-398.
8. DeAzambuja, P., Freitas, C.C. and Garcia, E.S. (1986) J. Insect Physiol. 32, 807-812.
9. Dunn, P. E. (1986) Ann. Rev. Entomol. 31, 321-339.
10. Gotz, P. and Boman, H. G. (1985) Comprehensive Insect Physiology, Biochemistry and Pharmacology. G. A. Kerkut and L. I. Gilbert (Editors). v. 3. pp. 453-485. Pergamon Press, New York, NY.
11. Hultmark, D., Engstrom, A., Andersson, K., Steiner, H., Bennich, H. and Boman, H. G. (1983) EMBO J. 2, 571-576.
12. Jenkins, J.A., Rosell, R., Ourth, D.D. and Coons, L.B. (1991) J. Aquatic Animal Health 3, 16-22.
13. Kanost, M. R., Kawooya, J. K., Law, J. H., Ryan, R. O., Van Heusden, M. C. and Ziegler, R. (1990) Advances in Insect Physiology. v. 22. pp. 299-396. Academic Press, New York, NY.
14. Kurstak, E. (Editor) (1991) Viruses of Invertebrates. Marcel Dekker, New York, NY.
15. Lowry, O. H, Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
16. Ourth, D. D. (1988) J.Econ. Entomol. 81, 148-151.

17. Ourth, D. D. and Renis, H. E. (1991) Amer. Zool. 31, 90A.
18. Ourth, D.D. and Renis, H.E. (1993) Comp. Biochem. Physiol. 105B, 719-723.
19. Ourth, D.D. and Wilson, E.A. (1981) Devel. Comp. Immunol. 5, 261-270.
20. Raulston, J. R. and King, E. G. (1984) Advances and Challenges in Insect Rearing. E. G.King and N. C. Leppla (Editors). pp. 167-175. Agricultural Research Service (Southern Region), U.S. Department of Agriculture, New Orleans, LA.
21. Rinehart, K. L., Gloer, J. B., Wilson, G. R., Hughes, R. G., Li, L. H., Renis, H. E. and McGovren, J. P. (1983) Federation Proc. 42, 87-90.
22. White, D. O. and Fenner, F. J. (1986) Medical Virology. 3rd ed. Academic Press, New York, NY.