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Midgut pH Profile and Energy Differences in Lipid, Protein and Glycogen Metabolism of *Bacillus thuringiensis* Cry1Ac Toxin and Cypovirus-infected *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae)

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ABSTRACT

In this research, the effect and interaction of Cry1Ac and *Helicoverpa armigera* Cytoplasmic Polyhedrosis Virus, HaCPV (Chinese strain) on body weight, midgut pH, total lipid, glycogen and soluble protein contents of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) larvae were studied. Cry1Ac is a toxic protein produced by *Bacillus thuringiensis* (Berliner). When larvae were separately exposed to HaCPV (3×10^7 PIB ml^{-1}) or Cry1Ac (0.9 µg g^{-1}), body weight, glycogen, soluble protein, and total lipid content of them, compared with the control, were significantly reduced. The effects of their combination at the same concentrations on the above mentioned parameters were more pronounced, which indicated their synergistic or additive influence. The midgut pH in larvae was significantly declined from 10.5 (in control) to 7.5 when larvae fed on diets containing HaCPV or Cry1Ac, and it was reduced to 8 when they were fed with their combination.

Keywords: Metabolism, Midgut pH profile, *Bacillus thuringiensis* Cry1Ac toxin, Cytoplasmic Polyhedrosis Virus, *Helicoverpa armigera*

INTRODUCTION

Since the discovery of Cytoplasmic polyhedrosis virus (CPV) by Ishimori in the silkworm (*Bombyx mori* (L.)) larvae in 1934 (Aruga 1971), it has been recognized as an important entomopathogen, especially among lepidopteran insects (Martignoni and Iwai, 1981). The site of CPV infection is usually limited to the gut wall, but can also spread to the fat body (Payne and Mertens, 1983). The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is one of the most important insect pest in the Old World due to its mobility, high polyphagy, short generation time and high reproductive rate (Fitt 1989; Sharma 2005). The use of genetic modified crops expressing *Bt* protein, which has been applied in cotton plant to protect from damage of pests provides a powerful way to control lepidopteran pests (Shelton *et al*. 2002). However, resistance monitoring of *H. armigera* in the field populations has suggested that there has been some decline in the susceptibility to Cry1Ac (Shen *et al*. 1998; Li *et al*. 2004; Gunning *et al*. 2005). Therefore, alternative control measures, such

as biological control agents, are required (Liu *et al*. 2005a; Liu *et al*. 2005b; Ma *et al*. 2008). Some of the studies indicated that combination of CPV and *B. thuringiensis* produced higher mortality than either CPV or *B. thuringiensis* in alone (Marzban *et al*. 2009; Katagiri *et al*. 1977). Bong and Sikorowski (1991a) reported that CPV acted synergistically in combination with bacterial contaminant (*B. subtilis*, *Escherichia coli*, *Staphylococcus epidermidis*, and *pseudomonas maltophilia*). May be Bt toxins proliferate at HaCPV infected larvae having lower gut pH, and synergistically induce the deleterious effects on the cotton bollworm. Our previous studies have shown that exposure of *H. armigera* to Cry1Ac or HaCPV or their combination had negative impact on growth and development of *H. armigera*, and resulted in reduction of pupation and pupal weight as well as delaying the developmental period (Marzban *et al*. 2009). It could have been due to the diversion of host energy to combat the pathogen (Wiygul and Sikorowski, 1991), or the disrupted midgut epithelium would have been unable to absorb nutrient. There is, however, little information on the effects of viruses and *B. thuringiensis* toxins on physiology and biochemistry of the host insect. Therefore, the present study was conducted to establish the relationship of time-concentration of lipid, glycogen, and protein when third instar *H. armigera* larvae were exposed to Cry1Ac-HaCPV in combination and alone. Furthermore, we measured pH value in the midgut of the cotton bollworm, in all treatments. The results clarified that *B. thuringiensis* Cry1Ac toxin and HaCPV combination affected *H. armigera* more when in alone, which added our information about interaction between virus and *B. thuringiensis* toxins against *H. armigera*.

MATERIALS AND METHODS

1. Cytoplasmic *Polyhedrosis* Virus propagation and purification

Cytoplasmic Polyhedrosis virus of *H. armigera* **(**HaCPV) was produced in vivo in the second instar larvae that were infected with HaCPV by spraying a suspension of 3×10^7 PIB ml^{-1} on the artificial diet. Six days after infection, the larval midgut was homogenized in deionized water and strained through 35-µm -pore-size mesh nylon cloth to remove large debris. The filtrate was layered on the top of a HS-40 Ludox continue gradient and centrifuged at 16000g for 45 min (Green *et al*. 2006; Shapiro *et al*. 2005). The resulting band containing purified virus was recovered, washed by sterile distilled water three times, and held in 0.1 mM NaOH at 5ºC.

2. Cry1Ac toxin preparation

Cry1Ac toxin was purified from the HD-73 strain of *B. thuringiensis* subsp. *kurstaki*, provided by the Institute of Microbiology, Chinese Academy of Science. The strain was grown in LB medium (tryptone 1%, yeast extract 0.5%, NaCl 1%, pH 7-7.2) at 28ºC for 3-4 days at which time the separation of crystals from spores was confirmed by microscopic observation. The spores and crystalline parasporal bodies mixture were centrifuged at 8000g for 10 min at 4ºC, and the pellet was washed five times with distilled water. Then, it was kept in an alkaline buffer (CHAPS 0.1 M, β-mercaptoethanol

10 mM, pH 10.5) at 28ºC for 1 hr until the crystal protein had been dissolved. The solution was further centrifuged at 8000g for 15 min. The pH of supernatant was adjusted to 5-6 with 1 M HCl and stored overnight at 4ºC to precipitate the toxin. Finally the precipitate was collected by centrifugation and washed three times with distilled water. The Cry1Ac toxin was lyophilized and dissolved in double distilled water.

3. Insect rearing and infection

The eggs of *H. armigera* were surface-sterilized by immersing in 2% formaldehyde for 15 min at room temperature. Then, they were washed several times with tap water and finally rinsed with sterile distilled water. The eggs were allowed to air-dry on paper towels and left to hatch in 10 cm × 6 cm plastic bags at 26ºC. The larvae were individually fed by artificial diet (Bot 1966) at 26ºC and 65% RH with a 14:10 hr photoperiod, and adults were fed in a diet with 10% honey solution. For treating experiments, the newly moulted third-instar (6 days old) larvae were forcedly fed diets that sprayed with HaCPV (3×10^7 PIB ml^{-1}) or/and containing 0.9 µg g^{-1} Cry1Ac toxin in alone and combination, and the untreated larvae were used as control. 45 larvae were used for each treatment in three replicates and in successive days, at day 2, day 4, and day 6 post treatments.

4. Measurement of pH inside the gut

Vital pH indicators, bromothymol blue (pKa 7), cresol red (pKa 8.0), phenolphthalein (pKa 9.1) and alizarine yellow R (pKa 11.0), were separately mixed into the artificial diet at a concentration of 0.4% (W/W). The four treatments repeated three times, including HaCPV (3×10^7 PIB ml^{-1}), Cry1Ac (0.9 µg g^{-1}), their combination, and a control were independently applied by mixing them in diets separately, with 20 larval individuals at each treatment. The treated larvae were transferred to tubes, and after 24 hr, the larval bodies dissected under a stereoscopic microscope. Color of each region of the digestive tube was compared to the standard pH values covering intervals at 0.5 pH units (Gontijo *et al*. 1998).

5. Extraction, separation and colorimetric determination of lipid, protein and glycogen

The age, instar and weight of infected *H. armigera* larvae with Cry1Ac or/and HaCPV were recorded. To allow excreting the residues of gut, the larvae were starved for 6 hr after 2, 4 or 6 days infecting. Each larva transferred into a non-disposable glass culture tube and dried at 90 ºC in a heat block for 12 hr. Then, their dry weight was separately recorded and stored at -80 ºC for further use. Extraction and separation of lipid, protein and glycogen from the bodies of *H. armigera* larvae was carried out as described by Zhou *et al*. (2004). Lipid was quantified using vanillin assay (van Handel, 1985a). Protein was measured by the BCA Protein Assay Kit (Biosynthesis Biotechnology Company, Beijing, China) and according to the manufacturer's instructions bovine serum albumin was used as a standard. Glycogen was measured by anthrone assay (van Handel, 1985b). The glycogen standard was purchased from Sigma Chemical Co. All biochemical analyses were carried out at 3-5 replicates.

6. Data analysis and statistics

Statistical analysis of the data was performed by one way analysis of variance (ANOVA); all tests were run using the program Statistical Package for the Social Sciences (SPSS 1998). All of the treatments were separated and compared using a one-way Duncan test, and they were conducted with P = 0.05.

RESULTS

1. Midgut pH in *H. armigera* larvae

The use of pH indicator dyes (bromothymol blue, cresol red, phenolphthalein and alizarine yellow R) allowed the first direct measurement of pH in the larvae's guts of *H. armigera*, it was successfully used to obtain the pH range inside the digestive tube of larvae that infected with Cry1Ac-HaCPV. The pH was higher in the control versus Cry1Ac-HaCPV treatments. The results showed that the pH of the midgut in the uninfected control larvae was constantly kept at 10.5 after treatments of 24 hr, 3 days and 5 days. However the pHs of the hindgut and foregut were lower than 10.5. Moreover the pH of the midgut in the Cry1Ac-infected larvae was 7.5, approximately same to HaCPV-infected larvae. The pH of the midgut in the Cry1Ac-HaCPV combination infected larvae was approximately 8. In all of Cry1Ac-HaCPV treatments, the midgut pH values at day 5 after treatment increased to pH 8.8. Based on these results, it is evident that the pH values of the midgut reduced from 10.5 to 7-8 after 24 h treatment with Cry1Ac-HaCPV, and increased to 8.8 after 5 days.

2. Body mass and water content

The wet mass, dry mass and water content of *H. armigera* larvae were assayed during critical development stages of the larval stadium, and significant differences detected in larval wet and dry masses when the third instar of *H. armigera* were forcedly fed diets containing Cry1Ac-HaCPV, wet mass at day 2 after treatment: $F_{[3, 16]} = 359.96$; $P < 0.000$; wet mass at day 4 after treatment: $F_{[3, 16]} = 524.5$; $P < 0.000$; wet mass at day 6 after treatment: $F_{[3, 10]} = 329$; $P < 0.000$; dry mass at day 2 after treatment: $F_{[3, 16]} = 185.53$; $P < 0.000$; dry mass at day 4 after treatment: $F_{[3, 16]} = 298.16$; $P < 0.000$; and dry mass at day 6 after treatment: $F_{[3, 10]} = 170.38$; $P < 0.000$: (Table 1). The wet and dry masses in control were significantly higher than other treatments and in combination of Cry1Ac-HaCPV were the lowest. Significant differences were detected in total water contents of bodies in all post treatments. Total water content in all of treatments decreased in larval development period but it was constantly the highest in combination of Cry1Ac-HaCPV.

3. Glycogen, Protein and Lipid

Changes in the profile of glycogen contents third instar of *H. armigera* when forcedly fed diets containing Cry1Ac-HaCPV are shown in Fig. 1. Significant differences were detected in level of glycogen in Cry1Ac-HaCPV infected and uninfected larvae in three successive times, glycogen at day 2 after treatment: $F_{[3, 16]} = 686.29$; $P < 0.000$;

glycogen at day 4 after treatment: $F_{[3, 16]} = 195.6$; $P < 0.000$; and glycogen at day 6 after treatment: $F_{[3, 10]} = 116.23$; $P < 0.000$.

One-way ANOVAs indicated that the effects of Cry1Ac-HaCPV on soluble protein content of *H. armigera* larvae differed significantly between control and Cry1Ac-HaCPV treatments in combination and alone, soluble protein at day 2 after treatment: $F_{[3, 16]} = 188.19$; $P < 0.000$; soluble protein at day 4 after treatment: $F_{[3, 16]} = 73.66$; $P <0.000$; and soluble protein at day 6 after treatment: $F_{[3, 10]} = 9.96$; $P < 0.004$. Soluble protein in control was significantly higher compared with treatments. However, it was the lowest amount measured in Cry1Ac-HaCPV combination. Soluble protein content, especially in control, dropped to the lowest at prepupa stage, and was not observed in Cry1Ac-HaCPV combination (Fig. 2).

Significant differences detected in total lipid between control and Cry1Ac-HaCPV infected third instar *H. armigera* larvae. Total lipid at day 2 after treatment: $F_{[3, 16]} = 813.54$; $P < 0.000$; total lipid at day 4 after treatment: $F_{[3, 16]} = 569.18$; $P < 0.000$; and total lipid at day 6 after treatment: $F_{[3, 10]} = 1814.99$; $P < 0.000$. Total lipid in control was significantly higher compared with treatments. However it was the lowest in combination of Cry1Ac--HaCPV treatment. There was a significant difference in total lipid among treatments throughout the 6 days experiment. The fat body is the principal store of lipid in the insect's body. The amount stored varies with the stages of development and state of feeding of the insect. Lipid stores normally increase during periods of active feeding and its accumulation was the highest in control at prepupa time (Fig. 3).

Table 1. Fresh and dry weight and water content body of *Helicoverpa armigera* larvae fed by artificial diets containing Cry1Ac/HaCPV in combination and alone for 48 hr and measured at 2, 4, and 6 days after treatments [a]

Treatment	Wet mass (mg) [b]			Dry mass (mg)			Water content (%)		
	2 day	4 day	6 day	2 day	4 day	6 day	2 day	4 day	6 day
Control	49 ± 1.8 d	157.2 ± 5.6 c	329 ± 7.7 d	8.5 ± 0.5 c	31.9 ±1.6 c	89.9 ± 2.3 c	82.7 ± 0.6 a	79.7 ± 0.6 a	72.7 ± 0.4 a
HaCPV	27.3 ± 0.3 c	90.3 ± 2.6 b	226 ± 8.8 c	4.8 ± 0.1 b	16.7 ± 0.6 b	47.5 ± 3.9 b	82.3 ± 0.2 a	81.5 ± 0.7 a	79.1 ± 1.1 b
Cry1Ac	11.6 ± 0.7 b	39.8 ± 0.7 a	105 ± 3.9 b	1.6 ± 0.1 a	6.9 ± 0.1 a	18.5 ± 1.6 a	86.1 ± 0.4 b	86.3 ± 0.4 b	82.5 ± 0.9 c
HaCPV+ Cry1Ac	8 ± 0.2 a	28.4 ± 0.3 a	71.9 ± 1.5 a	1.1± 0.7 a	4.8 ± 0.1 a	12 ± 0.6 a	85.9 ± 0.6 b	89.3 ± 0.7 c	83.4 ± 0.6 c

[a] The third instar larvae of *H. armigera* larvae were infected by of HaCPV and Cry1Ac toxin in combination and alone for 48 h, then fed a normal artificial diet..

[b] The data in the table are means (±SE). Means within the same column followed by a different letter are significant at P < 0.05, Duncan test.

DISCUSSION

In current research, in treated *H. armigera* with Cry1Ac, HaCPV or their combination, the amount of glycogen, total lipid and soluble protein were reduced, in which this reduction was noticeable at 2nd day and more pronounce at 4th day after treatment. Wiygul and Sikorowski (1978) reported that oxygen respiration in infected larvae was highest at day 5, there after, gradual decline was begun. In this research, combination of Cry1Ac and HaCPV showed the highest effect on biochemical constituents when

followed by Cry1Ac or HaCPV alone. Depletion of glycogen may be due to direct utilization of this compound for energy generation. Decrease in total lipid and soluble protein under Cry1Ac-HaCPV stress could be due to several mechanisms. These include: 1. formation of lipoproteins which are utilized for repairing of damaged cell and tissue organelles, 2. direct utilization by cells to cellular immune response for energy requirements, 3. Lipid and protein attributing to the energy requirement of the virus, 4. the infection of midgut epithelial cells reduce the rate or efficiency of food absorption. However, further studies are required to understand which of the above mentioned mechanisms are induced in the present system.

The large number of PIB within infected cells indicates a great expenditure of the insect's metabolic energy. Wigyul and Sikorowski (1978) found that CPV-infected tobacco budworm larvae had much higher oxygen uptake than healthy larvae. This is an indicative of extensive metabolism for synthesis of viral materials. Thereby, the diversion of energy for viral synthesis could adversely affect normal functions of the insect. CPV causes significant damage to the midgut epithelium and some of organelles such as mitochondria and rough endoplasmic reticulum infected columnar cells deteriorate (Bong and Sikorowski 1991b). Most of the digestive and adsorptive functions of the insect occur in the midgut. The damage caused by HaCPV or Cry1Ac would adversely affect the insect normal growth and development. Marzban *et al*. (2009) reported that *H. armigera* larvae were exposed to Cry1Ac/HaCPV in combinations and alone, the pupation rate was significantly decreased. They showed, larval and pupal periods longed, and pupal weight were significantly decreased. Simmons and Sikorowski (1973) found that CPV-infected larvae weighed 50% lower; lived 4 days longer and produced eggs 68% fewer compared to uninfected larvae.

Bouwer *et al*. (2009) believed that the cellular immune response was the most likely reason why inoculation survivors had high metabolic rates. The cellular immune response of insects has been implicated in defense system of Lepidoptera against baculoviruses for a long time. More recently, as some infected *H. zea* larvae were able to clear *Autographa californica* (Speyer) MNPV infection. Washburn *et al.* (1996) demonstrated that the insect's immune system was effective against viral pathogens. The immune system was suppressed and infection was more intensive in Lepidopteran larvae treated with biological agents, indicating that the cellular immune response was a significant factor in preventing the spread of infection (Washburn *et al*. 1996). The percentage of lipid on day 6 after treatment was significantly changed due to the accumulation of lipid in preparation for pupation in untreated larvae but the percentage of lipid in treated larvae decreased.

Our results may provide an overall picture of glycogen, protein, and lipid metabolism during larval period under Cry1Ac/HaCPV stress. The depletion of glycogen and lipid contents could be due to utilization of these reserves for energy generation as a result of Cry1Ac-HaCPV-induced stress. Decline of protein content in untreated larvae may indicate a physiological adaptability to compensate for pupation. Animals require high energy under stress conditions and the energy demand may have led to the stimulation of protein catabolism. The reduction of protein content in Cry1Ac-HaCPV

treatment might be due to a mechanism of lipoprotein formation, which is used to repairing damaged cell and tissue organelles (Sancho *et al*. 1998; Rambabu and Rao 1994). Protein depletion in tissues may be attributed to a physiological mechanism by retaining free amino acid content in haemolymph, to compensate for osmoregulatory problems encountered due to the leakage of ions and other essential molecules during the stress (Srivinas 1986).

The present results are in agreement with results of Marzban *et al*. (2009) who showed the most of Cry1Ac-HaCPV combinations had additive effect in the toxicity and in combinations of Cry1Ac at lowest and HaCPV at highest concentrations synergism. *H. armigera* exposed to Cry1Ac-HaCPV showed that toxicity of Cry1Ac was same as HaCPV and their effects could be additive or synergistic. In the present study, sublethal doses of Cry1Ac-HaCPV had a toxic impact on metabolic pathway in *H. armigera* larvae. Moreover, the decrease in the level of glycogen, protein, and lipid contents, which adversely affected the growth, development, and reproduction of *H. armigera*. Finally it can be concluded that HaCPV improves effectiveness of Bt crops (GM), which is important for successful IPM programs.

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