Egyptian Journal of Biological Pest Control, 15(1), 2005, 41-44

Changes in Lipid Contents due to Infection with *Bacillus thuringiensis kurstaki* in Larvae of the Greater Wax Moth, *Galleria mellonella* L. (Lepidoptera: Galleridae)

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(Received, April 15, 2005; Accepted, June 1, 2005)

ABSTRACT

Total Lipid contents decreased in *B.t. kurstaki* treated larvae especially on the 4th and 5th days post treatment since their respective amounts were about 1/3 and 1/2 of those in the control larvae. Concerning the saponifiable lipids, caproic acid appeared only in treated larvae on 2nd and 5th days post treatment. Meanwhile, linolenic acid appeared only in the untreated larvae on the 2nd day of the test. Thus, these two saponifiable fatty acid are characteristic to the diseased and healthy larvae of *G. mellonella*. On the other hand, 11 other fatty acids (caprylic, capric, lauric, myristic, myristolic, penta decylic, palmitic, margaric, stearic oleic, and linoleic acid) were detected in both larval groups at different intervals with variable amounts. Generally, their amounts were less in the treated than in the control larvae, with the exception of capric, palmitic, oleic and linoleic acids which were higher in the treated larvae than in those of the control larvae. For the Unsaponifiable lipids, cholesterol and stigmasterol were detected in the control larvae, but they were absent in the treated larvae. The cholestane was detected in both larval groups, but in irregular quantitative levels.

Key Words: *Bacillus thuringiensis kurstaki, Galleria mellonella,* physiopathology, lipids

INTRODUCTION

Lipids have assumed considerable functional significance during the evolutionary history of the class Insecta. They are essential structural components of the cell membrane and cuticle. They provide a rich source of metabolic energy for periods of sustained energy demand. Lipids facilitate water conservation both by the formation of an impermeable cuticular barrier (epicuticle) and by yielding metabolic water upon oxidation. In addition, they include important hormones and pheromones (Downer, 1978).

Studying the available literature showed that lipids were not studied before in *Galleria.mellonella* neither in healthy nor in *Bacillus thuringiensis* treated larvae. Meanwhile, they were studied in some other insects, *e.g. Popillia japonica* (Bennet and Shotwell, 1972), *Locusta megratoria* (Hoffmann, 1980), *Spodoptera littoralis* (Boctor and Salama, 1983), *Aedes aegypti* (Vivares *et al.*, 1989), and *Plutella xylostella* (Kumaraswami *et al.*, 2001).

The present study deals with the quantitative changes of total lipid content in larvae of *G. mellonella* post ingestion of spores and endotoxin crystals of *Bacillus thuringiensis* var. *kurstaki* of the commercial formulation Dipel 2X, as well as those of saponifiable and non-saponifiable lipids of the whole body content.

MATERIALS AND METHODS

Larval treatment

G. mellonella was reared in the laboratory on the diet described by Ibrahim *et al.* (1984), but bee honey was excluded from diet prepared for present experiments because of its known antimicrobial effect (Omar, 2004). The LC_{50} of the *B.t. kurstaki* formulation Dipel 2X (4.784 g/100g diet) was used for infecting the larvae *per os.* to allow obtaining about 50% survived diseased larvae among a period of at least 5 days post treatment (Omar, 2004). About 1500 larvae (L_3) were fed on the treated diet, while another 700 ones were fed on untreated diet to serve as control. Daily samples each of 100 larvae were collected from the treatment as well as from the control and processed for lipid determination.

Determination of lipids

The total lipid contents, saponifiable and unsaponifieble fatty acids were separated and fractioned according to the methods of Frings *et al.* (1972), Farag *et al.* (1986) and Elkattan (1995).

Separation of fatty acids and unsaponifiables

Lipid materials were saponified with methanolic KOH (20%w/v) for 24hrs at room temperature. The unsaponifiables were extracted three times with diethyl-ether (peroxide free). The aqueous layer was acidified by hydrochloric acid (20%v/v) and the liberated fatty acids were extracted three times with peroxide free diethyl ether. The combined extracts of unsaponifiables and fatty acid were washed several times with distilled water until the washing was neutral, then dried with anhydrous sodium sulfate.

Methylation of fatty acids

Fatty acids of standards and samples were converted to methyl esters using ethereal solution of diazomethane. Fatty acids were dissolved in 0.5ml of anhydrous diethyl ether and methylated by drop wise addition of diazomethane solution until the yellow color (Vogel, 1975). The mixture was then left at room temperature for 15min and the solvent was evaporated on a water bath maintained at 60°C. Finally the methyl ester of fatty acids, were dissolved in pure chloroform and aliquots of this solution was subjected to GLC analysis.

The methyl esters of the fatty acids, the unsaponifiables and standard compounds were

analyzed by using a GC Pye unicam gas chromatograph equipped with dual flame ionization detector.

GLC of fatty acid methyl esters and unsaponifiables

The fractionation of fatty acid methyl esters was conducted by using coiled glass column (1.5mx4mm) packed with Diatomite-C (100-120 mesh) and coated with 10% polyethylene glycol adipate (PEGA). The column oven temperature was programmed at 8°C/min from 70°C to 190°C, then isothermally at 190°C for 45min with nitrogen flow rate of 30ml/min.

The unsaponifiables were also fractioned on a coiled glass column (2.8m x 4mm) packed with Diatomite-C (100-120 mesh) and coated with 1% ov-17. The oven temperature was programmed at 10°C/ min from 70°C to 270°C, then isothermally at 270°C for 25 min and nitrogen flow rate was 30ml/min. In both fatty acid methyl esters and unsaponifiables detector, injector temperatures were generally 300°C and 280°C.

RESULTS AND DISCISSION

1. Total lipids

As sown in Table (1), the total lipids estimated in healthy and *B.t. kurstaki* treated larvae of *G. mellonella* varied along the 5 successive days post treatment. The pathogen caused a remarkable decrease in their amounts in the treated larvae. The decrease reached its maximum value after 4 days (13.04%) compared to 37.33% in the healthy larvae. There was one exception at the 3rd day, since lipid contents of untreated larvae was less than of the treated ones. Lipid reduction in *B. thuringiensis* treated larvae may be due to the consumption of these lipids by the developing vegetative cells of the bacterium and/or preventing lipid synthesis as a phsiopathological effect in the host larvae.

Table (1): Total lipids % in *B.t. kurstaki* treated (T) and control (C) larvae of *G. mellonella* among 5 days post treatment.

Days after treatment	Total lipids%	
	Treatment	Control
1	33.30	27.30
2	45.00	50.00
3	17.86	3.40
4	13.04	37.33
5	26.68	50.88

2. Saponifiable lipids

Using a standard contains 15 fatty acids that differ in number of carbon atoms and in their retention time by running in the GLC (Table 2), the control and the *B.t. kurstaki* diseased larvae of *G. mellonella* sampled one day post feeding on the contaminated diet showed the absence of lauric acid where it was present by 0.138% of the total saponifiable fatty acids in the control (Table 3). On the other hand, treated larvae showed 3 fatty acids, *i.e.* pentadecylic (0.945%), oleic (16.832%) and linoleic acid (15.782%) which were absent in larvae of the control. Meanwhile, 5 other fatty acids were presented in both treated and control larvae but differ quantitatively in their concentrations (Table3).Two days post treatment, data resulted the absence of caprylic acid from larvae of the control, while it was detected in the treated larvae by 0.070%. On the contrary, linolenic acid appeared in the control (0.139%), but was absent in the treatment. Another 6 fatty acids were present in both the treated and control larvae. Concentrations of these fatty acids were 0.924 and 0.135% for capric acid, 30.177 and 29.189% for myristoleic acid, 9.429 and 9.734% for palmitic acid, 31.424 and 54.661% for margaric acid, 11.704 and 3.974% for oleic acid, and 14.742 and 1.764% for linoleic acid in larvae of the treatment and control, respectively.

On the 3rd day, the absence of caproic and myristic acids was recorded in the control larvae, while they were found in diseased larvae by concentrations of 0.054 and 6.034%, respectively. On the other hand, linoleic acid was absent in the treatment, but presented in the control larvae by a concentration of 2.011%. Nine fatty acids were recorded in both the treated and control larvae differing in their concentrations, where some were higher in the treatment than in the control or *vice versa*.

Four days post treatment, 4 fatty acids became absent in the diseased larvae ,but still present in the healthy ones by the concentrations of 0.095% for capric, 1.276% for lauric, 15.727% for margaric and 7.026% for linoleic acid. Meanwhile, 6 fatty acids were found presented in both larvae of the treatment and the control. These fatty acids were caprylic, myristolic, pentadecyclic, searic and oleic acids.

Concerning larvae of *G. mellonella* processed for fatty acid analysis 5 days post treatment, data presented in Table (3) showed that caproic acid was absent in the healthy larvae of the control, while it was found by a concentration of 0.259% among fatty acids of the *B.t. kurstaki* treated larvae. On the other hand, 4 fatty acids were found in the control, but they were absent in the treatment, *i.e.*, capric (0.143%), myristoleic (6.737%), oleic (26.360%), and linoleic acid (14.269%). Meanwhile, 7 other fatty acids were found in both larvae of the treated and untreated control.

In general, the three long chain fatty acids lenolenic, archidic and behinic were nearly absent in both treated and untreated larvae except in the first group two days post treatment. It could also be stated that the pathogen enhanced the synthesis of the two fatty acids oleic and lenoleic in the host especially in the first two days post treatment. The lenoleic acid disappeared completely in the treated larvae after 72 hours post treatment. This fatty acid is an essential fatty acid and its absence causes a decrease in the oxidative phosphorylation process.

Table (2): Carbon atoms and retention time (min.) for the standard saponifiable fatty acids used in measuring fatty acid content of *B.t. kurstaki* treated and control larvae of *G. mellonella*.

	No. of carbon atoms	RT/min.	Fatty acid	No. of carbon atoms	RT/min.
Caproic	6 : 0	3.137	Margaric	17 : 0	20.050
Caprylic	8 : 0	6.100	Stearic	18 : 0	21.600
Capric	10 : 0	9.050	Oleic	18 : 1*	22.167
Lauric	12 : 0	11.883	Linoleic	18 : 2*	23.800
Myristic	14 : 0	14.500	Linolenic	18 : 3*	26.083
Myristoleic	14 : 1*	15.200	Arashidic	20 : 0	29.000
Pentadecylic	15 : 0	16.380	Behinic	22 : 0	44.400
Palmitic	16 : 0	17.183			

*Number of double bonds

Table (3): Percentages of recovered saponifiable lipids in *B.t. kurstaki* treated (T) and control (C) larvae of *G. mellonella*.

Name of F.A.		Days after treatment				
		1	2	3	4	5
Caproic	T	-	-	0.054	-	0.259
	C	-	-	-	-	-
Caprylic	T	0.022	0.070	9.076	0.643	5.680
	C	1.093	-	8.013	4.410	2.563
Capric	T	0.444	0.924	0.309	-	-
	C	0.323	0.135	0.137	0.095	0.143
Lauric	T	-	-	2.233	-	1.511
	C	0.138	-	0.389	1.276	0.505
Myristic	T	-	-	6.034	-	28.072
	C	-	-	-	-	1.948
Myristoleic	T	21.708	30.177	19.830	34.909	-
	C	28.543	29.189	36.436	28.436	6.737
Pentadecylic	T	0.945	-	4.569	3.428	4.335
	C	-	-	4.894	3.504	1.053
Palmitic	T	14.024	9.429	9.494	4.463	4.335
	C	12.399	9.734	9.866	3.504	1.053
Margaric	T	27.943	31.424	19.239	-	14.714
	C	28.339	54.661	20.240	15.727	2.741
Stearic	T	-	-	5.312	1.431	10.482
	C	-	-	7.389	1.550	7.881
Oleic	T	16.832	11.704	5.919	1.787	-
	C	-	3.974	4.571	10.845	26.360
Linoleic	T	15.782	14.742	-	-	-
	C	-	1.764	2.011	7.026	14.269
Linolenic	T	-	-	-	-	-
	C	-	0.139	-	-	-
Arashidic	T	-	-	-	-	-
	C	-	-	-	-	-
Behinic	T	-	-	-	-	-
	C	-	-	-	-	-

Table (4): Standard unsaponifiable lipids and their retention time (RT).

Unsaponifiables	RT (min.)
Cholestane	21.233
Cholesterol	25.783
Stigmasterol	28.900
B-Sitosterol	30.150

Table (5): Percentages of recovered unsaponifiable lipids by GLC in *B.t. kurstaki* treated (T) and control (C) larvae of *G. mellonella*.

Unsaponifiables		Days after treatment				
		1	2	3	4	5
Cholestane	T	3.037	2.719	73.150	1.155	18.668
	C	10.711	3.635	3.481	-	1.009
Cholesterol	T	-	-	-	-	-
	C	-	6.535	-	4.118	-
Stigmasterol	T	-	-	-	-	-
	C	11.470	-	-	3.754	-
B-Sitosterol	T	-	-	-	-	-
	C	-	-	-	-	-

3. Unsaponifiable lipids

Table (4) show the standard four unsaponifiable lipids used for measuring the lipid contents in the experimental larvae of *G. mellonella*.

As shown in table (5), there was completely absence of the unsaponifiable B-sitosterol in both *B. t.* treated and untreated *Galleria* larvae along the time of experiment. On the other hand, the pathogen caused an absence of both cholesterol and stigmasterol in the treated larvae during the 5 days of the test. The two compounds were present in the control larvae at the second and fourth day for the first acid, and at the first and fourth day for the stigamasterol. The cholestene values increased by the time in the treated larvae while the opposite appeared in the control ones (Table 5). Meanwhile, cholesterol and B-sitosterol were absent in both larvae of the treatment and the control, but stigmasterol was found only in the control representing a rate of 11.470%.

The above mentioned results indicated that *B. thuringiensis* caused a remarkable decrease in the total lipids of *G. mellonella* larvae. This decrease may be due to the consumption of lipids by the pathogen or/and preventing lipid synthesis in the infected larvae as part of its pathogenic process in the host. Fast (1981) mentioned that the bacterium *B.t.* affect its lepidopterous hosts via endotoxins that acts at the surface of midgut epithelial cells causing a rapid loss of ATP from the cells, the gut micrivilli swell, during which the larval feeding is inhibited.

Regarding effect of the pathogen on the fatty acids in *Galleria* larvae, it was found that caproic acid appeared only in infected larvae, while linolenic acid appeared once only in the control larvae. Meanwhile, all other 11 fatty acids appeared in both treated and control larvae. Only margaric acid was present at higher rates in the control larvae than in treated ones, but its case was *vice versa* for most of the other 9 fatty acids. It could be also stated that the pathogen increased the synthesis of the two fatty acids oleic and lenoleic especially in the first two days after treatment, since they appeared in high concentrations in the infected larvae. Lenoleic acid is considered as an essential fatty acid since its absence causes a decrease in the oxidative phosphorylation process in the cells. The completely absence of cholesterol and stigmasterol in the treated larvae may be due to the inhibition of larval feeding known as feeding stop symptom.

Other changes in lipid contents of insects due to infection with *B. thuringiensis* were reported, *e.g.,* by Hoffmann (1980) through injection of *B. t.* vegetative cells into the locust haemolymph of *L. migratoria*; Boctor and Salama (1983) in *S. littoralis*; Vivares *et al.*, (1989) in *A. aegypti* treated with *B. t.* var. *israelensis*. Also, Bennet and Shotwell (1972) studied lipids in the Japanese beetle; *P. japonica* infected with *B. popillae* and found that neutral lipids account for 70% of total lipids in both normal and diseased larval haemolymph; while phospholipids account for 30% of the total lipids. There were also major differences in lipid contents between *B. t.*-resistant (PXR) and susceptible (PXS) larvae of *P. xylostella* reported by Kumaraswami *et al.* (2001), where certain lipid fractions in PXR accountedless than half the level found in PXS (glycolipids).

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