Mechanisms for Bt toxin resistance and increased chemical pesticide susceptibility in Cry1Ac10-resistant cultured insect cells

Kaiyu Liu, Jin Zheng, Huazhu Hong*, Jianxin Peng, Hong Yang and Rong Peng
Institute of Entomology, Central China Normal University, Wuhan, 430079, P. R. China; *Author for correspondence (e-mail: hzhong@mail.ccnu.edu.cn; phone: +86-27-67861485; fax: +86-27-67861485)

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Abstract

Cabbage looper moth (*Trichoplusia ni*) cell line BTI-Tn-5B1-4 (TnH5) has developed high-level resistance (>1000 fold) by the selection of Bt Cry1Ac10 toxin. In order to examine mechanisms of resistance to Cry1Ac10 toxin (biological pesticide), both general esterase activities and cell tolerance to osmotic lysis were compared between non-selected Cry1Ac10-susceptible *Trichoplusia ni* cell line TnH5-S and Cry1Ac10-resistant *Trichoplusia ni* cell line TnH5-R selected by Bt Cry1Ac10. The Cry1Ac10-resistant TnH5-R cells had lower general esterase activity than the non-selected TnH5-S cells, and the esterase isozyme bands for the Cry1Ac10-resistant TnH5-R cells were much weaker than that for the non-selected TnH5-S cells. Both activated Cry1Ac10 toxin and multi-toxin from *Bacillus thuringiensis* subsp. *aizawai* GC-91 (an engineering bacterium) could not inhibit the esterase activity both in the Cry1Ac10-susceptible and Cry1Ac10-resistant cells, but two chemical pesticides, chlopyrifos and methomyl, could greatly inhibit the esterase activities both in the TnH5-R and TnH5-S cells. On the other hand, cell tolerance to osmotic lysis caused by hypotonic solution for the Cry1Ac10-resistant TnH5-R cells was higher than that for the non-selected TnH5-S cells (2.5×). Based on these results, we made the following conclusions. The general esterase activities in the Cry1Ac10-resistant TnH5-R cells was not related to Bt Cry1Ac10 resistance, but the susceptibility to the two tested chemical pesticides increased in TnH5-R cells because of their lower esterase activity. The increase of cell tolerance to osmotic lysis for the Cry1Ac10-resistant TnH5-R cells may be one of the mechanisms for Bt toxin resistance because midgut cells of insects are also disrupted by an osmotic lysis caused by Bt toxin.

Abbreviations: Bt – *Bacillus thuringiensis*; DTT – DL-dithiothreitol; ELISA – enzyme-linked immunosorbent assay; MTT – tetrazolium salt; α -NA – α -naphthyl acetate; β -NA – β -naphthyl acetate; SDS – sodium dodecyl sulphate; TnH5-S – Cry1Ac10-susceptible TnH5 cell; TnH5-R – Cry1Ac10-resistant TnH5 cell

Introduction

Microbial insecticides based on *Bacillus thuringiensis* (Bt) have been used widely, and many transgenic plants expressing Bt toxin protein have

been grown for pest control. Many insects have developed high-level resistance to Bt toxins both in fields and in laboratories with the development of Bt pesticides and Bt-transgenic plants (Tabashnik et al. 1990; Zhao et al. 2000). Many possible

mechanisms of resistance to Bt crystal toxin have been proposed, but only a few have been proved (Liu et al. 2000).

Many articles have reported the role of esterase in resistance of insects to chemical insecticides (Devonshire 1991; Ishaaya 1993). The susceptibility of insects to some chemical pesticides changes with the esterase activity because the esterase can bind to, sequester and detoxify them. Zhu et al. (2004) reported that esterase activity was compared *in vitro* between malathion susceptible and resistant (selected) strains which had 11-fold higher resistance to malathion, and more than 6-, 3- and 10-fold higher activity were obtained with the resistant strain using α -naphthyl acetate (α -NA), β -naphthyl acetate (β -NA), and *p*-nitrophenyl acetate, respectively. Esterase also seems to be related to Bt resistance in insects. A recent article reported that a colony of Cry1Ac-resistant *Helicoverpa armigera* larvae had much higher esterase activity than Cry1Ac-susceptible larvae, the increased esterase in Cry1Ac-resistant larvae could bind to and sequester Bt Cry1Ac toxin, but the esterase of Cry1Ac-susceptibility larvae could not. So a new mechanism for Bt toxin resistance has been proposed, in which esterase sequesters Bt Cry1Ac toxin (Gunning et al. 2005). But we do not know whether this mechanism is common in Bt resistant insects or cells.

In addition, One of Bt resistance possible mechanisms in which the ionic compensation or balance increases has been proposed because midgut cells of insects are disrupted by the osmotic lysis after Bt crystal toxin treatment (Heckel 1994; Akhurst et al. 2000). However, this mechanism still has not been proved. Some articles have reported that one of the important proteins for the ionic compensation or balance was pICln (protein current chloride nucleotide sensitive). It is a chloride channel protein and related to cell tolerance to hypotonic stress (Paulmichl et al. 1992; Krapivinsky et al. 1994). The increase of ionic compensation or balance was related to the increased cell tolerance to hypotonic solution. So we can prove this Bt toxin resistance mechanism by the assay of the change of cell tolerance to osmotic stress.

In this work, we tried to examine the above mentioned mechanisms for Bt toxin resistance using Cry1Ac10-resistant (selected) TnH5-R and the susceptible (non-selected) TnH5-S cells (Liu et al. 2004). The employed methods were comparison

of esterase activity and cell tolerance to osmotic lysis between the Cry1Ac10-resistant TnH5-R and Cry1Ac-susceptible TnH5-S cells. Some interesting results have been obtained.

Materials and methods

Insect cell lines

Tested cell lines were *Trichoplusia ni* BTI-TN-5B1-4 cell (TnH5) (Granados et al. 1991), *Helicoverpa zea* IMC-HZ-1 (Hz) cell (Hink and Ignoffo 1970), *Spodoptera litura* ZSU-SL-1 (Sl) cell (Xie et al. 1988), and *Spodoptera frugiperda* Sf9 cell (Summers and Smith 1987). *Trichoplusia ni* TnH5 cell line was cloned from *T. ni* embryonic cell line BTI-TN-5B1-28 (Granados et al. 1991). All the cell lines were cultured at 28 °C in 25-ml flask containing 2.5 ml Grace's insect medium (Gibco-BRL) supplemented with 10% FBS, (Gibco-BRL), and subcultures were performed every 2–4 days.

In a previous article, we have reported that *T. ni* cell line TnH5 was the most susceptible to Cry1Ac10 among the tested eight insect cell lines, which was named TnH5-S cell by us. It has developed high-level resistance (>1000-fold) by the selection of Cry1Ac10 toxin, and has middle to high cross-resistance to a few of Bt crystal toxins (Liu et al. 2004). TnH5 cells selected by Cry1Ac10 toxin for 104 and 107 times were named TnH5-R104 and TnH5-R107 cells, respectively.

Bt crystal toxins

The preparation of parasporal crystals of *B. thuringiensis* subsp. *aizawai* GC-91 (Bt formula, Ciba co.) and Cry1Ac10 protoxin were described elsewhere (Liu et al. 2004). Cry1Ac10 protoxin or crystal protoxin from *B. thuringiensis* subsp. *aizawai* GC-91 was dissolved in 50 mM carbonate buffer (pH 10.5) containing 15.4 mg/ml DTT at 37 °C for 2 h. After that, protoxin was activated at 37 °C for 2 h by 0.5 mg/ml trypsin solution prepared by adding trypsin directly into the carbonate buffer.

Reagents

Fast blue B salt (*o*-dianisidine, tetrazotized), Fast blue RR salt, α -naphthol, α -NA, β -NA and trypsin

were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) and coomassie brilliant blue G250 were purchased from Bio-Rad laboratory (Hercules, CA). Chlopyrifos and methomyl were provided by the institute of pesticides, Central China Normal University, P.R. China.

Total-esterase determinations

The cells of six cell lines were collected and then were suspended in 0.15 M NaCl solution containing 1 mM EDTA (pH 7.0), respectively. Then the cells of the six cell lines were sonicated in order to disrupt cell membrane at 8 W for 3 min (6×30 sec with 30 sec pauses) in an ice-water bath, respectively. After that, they were centrifuged at $15,000 \times g$ for 20 min at 4 °C. Then the supernatants were transferred to fresh microtubes and used as enzyme sources for the analysis of esterase. Total esterase activities were determined using 1-NA (α -NA) as the substrate in 0.04 M PBS (phosphate buffer solution, pH 7.0) according to the modified method that was briefly described below (Asperen 1962). The prepared enzyme solution was diluted in 0.04 M PBS, and 10 μ l of diluted enzyme solution was incubated in the 2.92 ml reaction solution at 30 °C for 30 min. The reaction solution was 0.04 M phosphate buffer containing 0.3 mM 1-NA (α -NA). Enzymatic reactions were stopped by adding 80 μ l of stop solution, which was prepared by a mixture of 2 volumes of 1% (w/v) fast blue B solution and 5 volumes of 5% (w/v) SDS solution. After 15 min, the absorbance was determined at 600 nm with a blank in which the volume of the supernatant (10 μ l of diluted enzyme solution) was replaced by the same volume of 0.04 M PBS (pH 7.0). The concentration of substrate hydrolysed was determined from a standard curve of α -naphthol.

Analysis of esterase isozyme

The prepared total esterase samples from six cell lines were loaded onto a non-denaturing polyacrylamide gel. Then gels were run at 200 V for 3 h or so. After the electrophoresis had been finished, the gels were stained for esterase activity in a staining solution, which consisted of 0.2% α -NA (w/v), 0.2% β -NA (w/v) and 0.2% Fast Blue RR

salt (w/v) in 0.04 M phosphate buffer (pH 7.0), in the dark at 30 °C for 30 min or so. Finally, the stained gel was fixed in the fixing solution, which consisted of 5% (v/v) acetic acid, 30% (v/v) ethanol and 15% (v/v) glycerin.

Inhibition of total esterase activity

Two chemical insecticides, activated Cry1Ac10 toxin and the multi-toxins from Bt GC-91 were used to study the inhibition of total esterase activities using α -NA as substrate. Ten μ l of the enzyme preparations was incubated with 2.92 ml of 0.3 mM α -NA solution, which contained different concentration of insecticides (chlopyrifos, methomyl, Bt multi-toxins or Cry1Ac10) at 30 °C for 30 min. The residual activities of the total esterases on α -NA were measured with the method of Asperen (1962).

All protein determinations were made by the Bradford protein assay using BSA fraction V (Sigma) as a standard.

Comparison of cell tolerance to osmotic lysis

The method was described briefly as following (Mosmann 1983). The Cry1Ac10-resistant cells were added into 42 wells in the left part of a 96-well cell culture plate with $2-3 \times 10^5$ cells/well, the non-selected Cry1Ac10-susceptible cells were added into 42 wells in the right part of a 96-well cell culture plate with $2-3 \times 10^5$ cells/well, and the other 12 wells at the last row of the cell culture plate were kept without cells and were used as blanks in the MTT assay. These cells were cultured overnight for the adherence. Non-diluted Puck's solution (pH 6.8) was isotonic for insect cells, which contained 8 g/l NaCl, 0.4 g/l KCl, 1 g/l glucose and 0.35 g/l NaHCO₃ (pH 6.8). Before the assay, a dilution series of Puck's solution were prepared by adding sterile distilled water.

For the Cry1Ac10-resistant cell wells, after the medium was carefully aspirated by a micropipette, a series of diluted Puck's solution was added into the wells at 100 μ l/well in the treatment group in the 1st to the 5th line of wells, and one dilution was for five wells. Non-diluted Puck's solution was added into the Cry1Ac10-resistant cell wells at 100 μ l/well in the control group in the 6th line of wells of the cell

culture plate, and the number of control wells was 7. The same procedure was performed for the Cry1Ac10-susceptible cells. After incubation of the Cry1Ac10-resistant and susceptible cells at 28 °C for 12 h, a microtiter plate assay which used the tetrazolium salt MTT was used to quantify viable cells. The solution in each well was replaced by 100 μ l of 0.5 mg/ml MTT solution. During subsequent incubation at 37 °C for 4 h, the dehydrogenase in viable cells converted partial tetrazolium salt into purple water-insoluble formazan, but the dead cells did not. Then the solution in each well was again replaced by 100 μ l of SDS-saturated isopropanol. The 96-well plate had been shaken slightly for 30 min in the dark until the purple formazan crystals were completely dissolved. The formazan formed could be quantitated in an ELISA plate reader at 570 nm. The OD₅₇₀ values positively correlated with the number of the viable cells in the well, and the results were calculated according to the following formula.

Cell mortality = 1 – the mean of OD₅₇₀ values for treatment wells/the mean of OD₅₇₀ values for control wells. Then the lethal dilution for 50% mortality was calculated according to common regression analysis (Probit assay). Tolerance Ratio = the lethal dilution for 50% mortality of Cry1Ac-resistant cells/that of Cry1Ac-susceptible cells.

Results

Decrease of the total esterase activity in the Cry1Ac-resistant TnH5-R cells

The Cry1Ac-resistant TnH5-R107 cells had the lowest total esterase activity among the six cell

lines, and the total esterase activity in Cry1Ac-susceptible TnH5-S cells was 4.3-fold and 3.9-fold higher than that in the TnH5-R107 and TnH5-R104 cells, respectively (Table 1).

Assay of the esterase isozyme phenotype in the Cry1Ac10-resistant TnH5-R cells

Esterase isozyme phenotypes of six cell lines were shown in Figure 1, and they had distinct difference. The esterase bands in the Cry1Ac-resistant TnH5-R cells were much weaker than that in the Cry1Ac-susceptible TnH5-S cells.

Inhibition of the esterase activity

α -NA esterase activity in both the Cry1Ac10-susceptible TnH5-S and the Cry1Ac10-resistant TnH5-R107 cells was inhibited greatly by the two chemical pesticides, chlopyrifos and methomyl (Data were shown in Figures 2 and 3), but both the activated Cry1Ac10 and the multi-toxin from Bt GC-91 had no influence on the esterase activity in both cells (Data were not shown). The results indicated that chlopyrifos and methomyl could bind to esterase in Cry1Ac10-susceptible TnH5-S and the Cry1Ac10-resistant TnH5-R cells, but the activated Bt toxins seemed not to bind to esterase in them, so the decrease of the general esterase activity in the Cry1Ac10-resistant TnH5-R cells was not related to the Bt Cry1Ac10 toxin resistance. However, the esterase in the Cry1Ac10-resistant TnH5-R cells seemed to bind to and sequester less chemical pesticides than that in the Cry1Ac-susceptible TnH5-S cells because TnH5-R

Table 1. General esterase activity of six cell lines.

	Cell type					
	TnH5-S ^b	TnH5-R104 ^c	TnH5-R107 ^d	Hz	Sl	Sf9
Esterase Activities ^a	0.331 $\pm$ 0.041	0.067 $\pm$ 0.005	0.062 $\pm$ 0.011	0.09 $\pm$ 0.008	0.075 $\pm$ 0.008	0.067 $\pm$ 0.006
Ratio ^e	5.3	1.1	1	1.5	1.1	1.1

^aActivity unit is μ mol.mg⁻¹.min⁻¹.

^bTnH5-S, Cry1Ac10-susceptible TnH5 cells.

^cCry1Ac-resistant TnH5 cells selected by Cry1Ac10 toxin for 104 times.

^dCry1Ac10-resistant TnH5 cells selected by Cry1Ac10 for 107 times.

^eRatio = Esterase activity in each cell line/Esterase activity in TnH5-R107 cell line. There was statistically significant difference for general esterase activity between the TnH5-S and TnH5-R (TnH5-R104 or TnH5-R107) cells ($p < 0.05$).

Figure 1. Polyacrylamide gel showing esterase activities in six cell lines. There were Sl, Hz, Sf9, TnH5-R104, TnH5-R107 and TnH5-S cell lines from the left to the right. Sl was *Spodoptera litura* ZSU-SL-1 cell line, Hz was *Helicoverpa zea* IMC-HZ-1 cell line, Sf was *Spodoptera frugiperda* cell line, and TnH5-S was non-selected Cry1Ac10-susceptible TnH5 cell line, TnH5-R107 was the Cry1Ac10-resistant TnH5 cell line selected by Cry1Ac10 for 107 times, and TnH5-R104 was the Cry1Ac-resistant TnH5 cell line selected by Cry1Ac10 toxin for 104 times. Each track contained 35 μ g protein.

Figure 2. Esterase inhibition by methomyl in the Cry1Ac10-susceptible TnH5-S cells (B) and the Cry1Ac10-resistant TnH5-R107 cells (C). Total esterase activity of the preparations without chemical insecticides was 100%. The error bars indicate standard errors from three independent experiments. TnH5-S cells were non-selected Cry1Ac10-susceptible TnH5 cells. TnH5-R107 cells were Cry1Ac10-resistant TnH5 cells selected by Cry1Ac10 for 107 times.

Figure 3. Esterase inhibition by chlopyrifos in the Cry1Ac10-susceptible TnH5-S cells (B) and the Cry1Ac10-resistant TnH5-R107 cells (C). Total esterase activity of the preparations without chemical insecticides was 100%. The error bars indicate standard errors from three independent experiments. TnH5-S cells were non-selected Cry1Ac10-susceptible TnH5 cells. TnH5-R107 cells were Cry1Ac10-resistant TnH5 cells selected by Cry1Ac10 for 107 times.

cells had lower esterase activity. So the Cry1Ac-resistant TnH5-R cells may be more susceptible to both tested chemical pesticides.

during 12 h, but that for TnH5-S was 1.89-fold ($0.53 \times$ Puck's) (Table 2). So the Cry1Ac10-resistant TnH5-R cells were more tolerant to dilution than the TnH5-S cells.

Cry1Ac10-resistant TnH5-R cells was more tolerant to osmotic stress

Discussion

The dilution of Puck's solution for 50% mortality of TnH5-R cells was 4.54-fold ($0.22 \times$ Puck's)

The total esterase activity in both TnH5-R104 and TnH5-R107 cells was close to that in Sl, Hz and

Table 2. Dilution of Puck's solution for 50% mortality between resistant TnH5-R and TnH5-S cells.

Cell	Line reg.	<i>r</i>	$\times$ Puck's	D_{50}^a	Mean $\pm$ SE ^b ($\times$ Puck's)	FL95 ($\times$ Puck's)
TnH5-S ^c	$y = -2.2 - 7.2x$	-0.97	0.49	2.04	0.53 ± 0.044	0.46-0.60
TnH5-S	$y = -3.3 - 14x$	-0.95	0.59	1.69		
TnH5-S	$y = -1.6 - 5.8x$	-0.94	0.52	1.92	0.22 ± 0.029	0.17-0.27
TnH5-S	$y = -3.0 - 10x$	-0.90	0.51	1.96		
TnH5-R ^d	$y = -2.0 - 3.1x$	-0.96	0.22	4.55		
TnH5-R	$y = -2.6 - 3.8x$	-0.94	0.21	4.76		
TnH5-R	$y = -3.9 - 6.6x$	-0.98	0.26	3.85		
TnH5-R	$y = -2.8 - 3.9x$	-0.95	0.19	5.26		

^aDilution for 50% mortality indicated the dilution of Puck's solution for 50% mortality of cells, and Puck's solution is an isotonic solution for insect cells.

^bStandard error.

^cCry1Ac10-susceptible TnH5 cells.

^dCry1Ac-resistant cells selected by Cry1Ac toxin for 86 times. There was statistically significant difference for the dilution of 50% mortality between the resistant TnH5-R and susceptible TnH5-S cells ($p < 0.05$).

Sf9 cell lines (Table 1). Initially the Cry1Ac10-resistant TnH5-R cells were suspected to be the contaminated cells by SL, Hz or Sf9 cell lines, but this possibility was excluded because the isozyme phenotype of the Cry1Ac-resistant TnH5-R cells was different from that of the other cell lines (Figure 2).

There are a few of reports about the studies of the esterase in the Bt-resistant insects, but their results were different. The general esterase activity of the Cry1Ac10-resistant TnH5-R cells decreased in our experiment and seemed not to be related to Bt toxin resistance because the total esterase activity in both tested Cry1Ac10-resistant TnH5-R and Cry1Ac10-susceptible TnH5-S cells could not be inhibited by Bt toxin. However, the contrary results have been reported by other researchers. Gunning et al. (2005) reported the general esterase activity in a Cry1Ac-resistant *Helicoverpa armigera* strain increased greatly, and could be inhibited strongly by Cry1Ac. Gunning et al. (2005) inferred from these results that the esterase in the Bt resistant strain could sequester Bt toxin by binding to, and proposed a new mechanism for Bt toxin resistance, which was that the development of Bt resistance in some insects resulted from sequestering of Bt toxin by esterase. Above all, it is not that all esterases bind to and sequester Bt toxin, and the new mechanism for Bt toxin resistance proposed by Gunning et al. (2005) might not be common in Bt resistant insects or cultured insect cells. So the decrease of the general esterase activity in some Bt resistant insects or cultured insect cells was not curious.

The decrease of esterase activity and the strong inhibition by chemical insecticides in TnH5-R cells were good news for pest control. Now there are some articles about the increase of susceptibility to chemical insecticides in Bt resistant insects or Bt pretreated insects. Justin (1989) reported the increased insecticide susceptibility in *Heliothis armigera* (HBN) and *Spodoptera litura* F. larvae due to Bt Berlin treatment. *Helicoverpa armigera* larvae selected by Bt toxin had negative cross-resistance to chemical pesticides, deltamethrin, fenvalerate, phoxin and endosulfa, and the highest negative cross-resistance was 60-fold or so (Liang et al. 2000). We inferred from these results that the Cry1Ac10-resistant TnH5-R cells with the lower esterase activity might be more susceptible to some chemical pesticides.

The active Bt toxin interacts with the midgut epithelium of susceptible insects to elicit a change in osmotic balance which results in cell lysis, so Heckel (1994) proposed that there existed a mechanism of ionic balance or ionic compensation for Bt resistance. Some researchers thought that the rate-determining step in gaining volume control involved the activation of potassium and chloride conductance pathways (Ackerman et al. 1994). Insects or insect cells must be widely resistant to many different Bt toxins if there exists this Bt toxin resistance mechanism in them. In fact, some researchers have reported that some Bt-resistant insects had low to middle cross-resistance against other Bt toxins (Gould et al. 1992; Zhao et al. 2001; Liu et al. 2001; Jurat-Fuentes et al. 2003). The Cry1Ac10-resistant TnH5-R cells selected by Cry1Ac10 also had low to middle cross-resistance to Cry1C, which was not used for the selection (Liu et al. 2004). So we proposed strongly the increase in the cell tolerance to the osmotic lysis, which might result from the increased ionic balance or ionic compensation, would be one of Bt toxin resistance mechanisms.

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