IMMUNE REACTIONS IN THE *BACILLUS THURINGIENSIS* RESISTANT INSECT

REAKSI IMUN PADA SERANGGA YANG TAHAN TERHADAP BACILLUS THURINGIENSIS

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ABSTRACT

Insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* (Bt) in sprays and transgenic crops are extremely useful for environmentally sound pest management. However, some important agricultural insect pests show increasing resistance to Bt-toxin. This resistance problem has prompted intensive investigation on the mechanisms of resistance to Bt including genetic and molecular aspects of the mode of asction. This study demonstrates a possible resistant mechanism by using an immunological approach. Based on the assumption that Bt-toxin is a tetramertic lectin, a model of *Drosophila* immune receptor has been used to explore *B*t- resistance as a result of glycosilation modification. Findings in this study suggest that increase of soluble *Bt*-binding molecules in the gut lumen of resistant insects correlates with increased resistance against other pathogens. This is compatible with the assumption that the Bt-binding protein in resistant insects is an immune complex.

ABSTRAK

Crystal protein dari Bacillus thuringiensis (Bt) dalam penyemprotan dan tanaman transgenik adalah sesuatu yang sangat bermanfaat dari aspek linmgkungan dalam program pengelolaan hama. Namun beberapa hama penting pertanian telah menunjukkan peningkatan ketahanan terhadap Bt-toxin. Masalah ketahanan tersebut mendorong penelitian yang intensif tentang mekanisme ketahanan termasuk dari aspek genetika dan molekuler . Penelitian ini menunjukkan kemungkinan mekanisme ketahanan dengan menggunakan pendekatan imunologi. Berdasarkan asumsi bahwa Bt-toxin merupakan lectin tetramerik, suatu model reseptor imun Drosophila telah digunakan untuk eksplorasi ketahanan serangga terhadap Bt sebagai akibat terjadinya modifikasi glykosilasi. Hasil penelitian ini menunjukkan bahwa meningkatnya molekul pengikat Bt yang terlarut pada cairan usus serangga tahan berkaitan dengan meningkatnya ketahanan terhadap patogen lainnya. Hal ini sesuai dengan asumsi bahwa protein yang mengikat Bt pada serangga tahan merupakan suatu komplek imun.

Key words: Immune , *Bacillus thuringiensis*, Resistant
Kata kunci: Imun, Bacillus thuringiensis*, Ketahanan*

INTRODUCTION

Bacillus thuringiensis is a gram positive bacterium that produces insecticidal crystal proteins (ICPs) during sporulation, which are toxic to certain insect larvae. The target of *B. thuringiensis* crystal delta-endotoxins is the midgut (Hofte and Whiteley, 1989; Gill *et al*, 1992; Knowles and Ellar, 1986). This is one of the most successful pathogenic microorganisms that has been used as biological control agent against a large variety of insect pests. However, some lepidoptera, which are of economic importance, show increasing resistance to chemical insecticides (Bryan, 1991), such as the diamond back moth (*Plutella xylostella)* and cotton bollworm (*Helicoperva armigera*). Currently these pests are the only field populations known with resistance to Bt toxin (Tabasnik, 1990; Schnepf *et al*, 1998). This resistance problem initiated intensive investigations on the mechanisms of resistance to *B. thuringiensis*. Findings suggest that the mechanisms of resistance are complicated, involving multiple gene aspects (Aronson *et al*, 1999; Gahan *et al*, 2001).

Genetically, laboratory development of resistance is more likely to involve polygenes (multiple-genes, each having a small impact on the selected trait). In contrast, development of resistance in the field is more likely to involve a single major genes (Gahan *et al*, 2001). Whereas different mechanisms have been observed in resistant population selected under laboratory conditions, only one major mechanism has been reported so far for resistance developed under field conditions. Instead of genetics of resistance in insect population, there are several biochemical basis of resistance such as altering proteolytic processing and binding site modification (Schnepf *et al*, 1998). A subsequent study demonstrated a genetic linkage between decreased susceptibility to CryIAc and the absence of a major gut protease , and CryIAc resistance in *P.xylostella* was demonstrated to be due to dramatically reduced binding (Tabashnik *et al*, 1997).

Since p85 is found in hemolymph and produced by fat body cells, the question is whether the protein is part of the defense system. Although p85 is naturally occurring attempts to stimulate the production of new proteins or to enhance existing levels have met with varying degree of success. The question is: Is Bt-toxin capable of stimulating the insect to defend itself through the accumulation of defense molecules?

This study demonstrates a possible resistant mechanism by using an immunological approach. Based on the assumption that Bt-toxin is a tetramertic lectin, a model of *Drosophila* immune receptor have been used to explore *B*t-resistance as a result of glycosilation modification.

MATERIALS AND METHODS

Genetic resistance to low levels of Bt-endotoxins has been selected in several species in the laboratory, but is common in field populations of diamondback moth (Ferre and Van Rie, 2001). A *Helicoverpa armigera* strain was used, which has been selected in the laboratory for resistance to low levels of *Bt*-toxin. Resistant insect populations were subsequently back-crossed four times with a susceptible population to generate nearly isogenic lines of resistant (ISOC4) and susceptible (ANGR) insects.

In order to be able to detect the existence of the predominant protein in the tested caterpillars, Western blots technique was used**.** In addition, the induction of defense reaction assay was used to analyses the correlation between Bt-binding and the immune status of insect. Melanisation assays using individual resistant and susceptible caterpillars were conducted to examine the different status of immune reaction between resistant and susceptible strain. To test the assumption that Bt-resistance may protect against other pathogens, the Baculovirus assays were conducted. The methods used in this investigation are described as follows:

Western blots. Proteins were separated by SDS-PAGE. The transfer of separated proteins onto nitrocellulose filter was carried out in a Mini Trans-Blot cell (Bio-Rad) applying 200 mA/70 V for about 1 h using transfer buffer containing 192 mM glycine, 25 mM Tris-base and 20% (v/v) methanol (Towbin *et al.*, 1979). After preincubation of the filters in first blocking solution (8% (w/v) non-fat milk powder and 0.02% sodium azide in PBS) for 1 h at room temperature with gentle agitation, the primary antiserum was added to a specified final concentration, and the incubation was continued for another 2 h. Then, the filters were washed three times, 10 min each, with gentle agitation: twice in PBS and once in 150 mM NaCl, 50 mM Tris-HCl pH 7.5. If not stated otherwise, alkaline phosphatase-conjugated secondary anti-rabbit antibodies (Pierce) diluted 1:10000 in second blocking solution (5% (w/v) non-fat milk powder, 0.02% sodium azide, 150 mM NaCl, 50

mM Tris-HCl pH 7.5) was added to the blot. The blots were incubated for 2 h at room temperature. After the incubation with secondary antibodies, usually alkaline phosphatase-conjugated anti-rabbit IgG (Pierce) in a 1:10000 dilution, the filters were washed twice for 10 min each in 150 mM NaCl, 50 mM Tris-HCl pH 7.5 with moderate shaking. Finally, for the visualisation of the protein bands, the blots were stained with NBT and BCIP as described by Sambrook *et al.* (1989).

Blots developed with *Helix .pomatia.(H.p)* lectin conjugated with peroxidase were washed three times after immunoblotting in TBST for 15 min each with agitation. *H.p.* lectin was added to the last wash to a final dilution of 1:10000. The filters were then incubated for at least 2 h. Blots were washed four times in TBST for 10 min each and eventually immersed into staining solution (a tip of a spatula of DAB and 40 µl of H_2O_2 in 20 ml of 10 mM Tris-HCl pH 7.5). Rinsing the blots in DDW stopped the staining reaction, and the blots were dried on air.

Induction assay: Fourth instar larvae from diamond back moth (*Plutella xylostella*) were fed with leaf dip assays bioassay method. Leaf disc of 10 cm diameter were cut with a metal puch hole from the fully expanded leaves of Chinese cabbage (Pak choi) plants. Leaf discs were dipped for five seconds in freshly prepared distilled water suspensions of various inducers such as Bt-toxin (Delfin WG) (1 ppm), Killed Bt (1 ppm), E.coli (1 ppm), Bt-toxin (0.1 ppm), Killed Bt (0.1 ppm), E.coli (0.1 ppm), combination of all inducers and control (ditilled water) in 100 ml baker. Each leaf disc was placed on a corrugated sheet of aluminium foil for drying up at room temperature. The leaf disc were placed into petri dishes of 14 cm diameter containing a single 70 mm filter paper (Whatman No.1) moistered with distilled water.10 larvae of fourth instar were placed in each petri dish. After 48 hours the survivors from each treatment were dissected for induction of p85 analyses.

Melanisation assays: Plasma from five or single caterpillar (resistant and susceptible *H.armigera*) was collected in the absence of PTU and light absorbance measure at a wavelength of 490nm.Gut extracts were obtained by homogenizing gut, including gut contain, in 1 ml PBS. Debris was removed by centrifugation (5,000 g, 5 min) and light absorbance of the supernatant was measured in the presence of 10 mM DOPA.

Baculovirus assay: Similar sized third instar larvgae from resistant and susceptible *H.armigera* strains were fed with suspensions of 10^7/ml of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). Since *H. armigera* is semipermissive to AcMNPV lethality was determined by counting the dead larvae over several days. Each treatment was performed with at least 20 caterpillars.

RESULTS AND DISCUSSION

A Soluble Bt-binding Protein in the Gut Lumen

It is widely accepted that there is more than one *Bt*-binding protein in the insect midgut. The proteins that bind *B*t toxin in lepidopteran insects described so far are 120 kDa and 220 kDa proteins which are integral part of the brush border membrane vesicle (BBMV) fraction (Martinez-Ramirez *et al*, 1994; Knight *et al*, 1994; Sangadala *et al*, 1994).

However, when proteins were extracted under denaturing conditions directly from the gut, additional *Bt*-binding proteins were isolated. For example, in the diamondback moth (*Plutella xylostella*), a 85 kDa protein as found as the major band labelled with both the Bt toxin and HPL (Figure 1). This suggests that under denaturing conditions (SDS-PAGE and Western blots), Bt toxin and HPL bind predominantly to p85. The fact that p85 was not found in BBMVs suggests that the flycoprotein was probably lost during the BBMV fractionation process. Therefore, it is probably not attached to the brush border membrane microenvironment, but is prevalent in the gut lumen of dissected larvae and in the whole insect. This glycoprotein showed weak Coomassie staining in several tissues compared to the strongly stained triple bands at 78, 80 , 82 kDa, which correspond to arylphorin, phenoloxidase (PO) and apolipophorin II (Figure 2a). With HPL staining the p85 protein was the predominantly labelled glycoprotein (Figure 2b). p 85 glycoprotein was detected in several tissues of DBM, such as gut, fat body, hemolymph and gut content (Figure.3). This observation suggests that p85 was probably produced in the fat body and released into the gut lumen (Figure 2A). The observation that isolated peritrophic membrane contains little amounts of p85 (Figure 2B) suggests that the protein is associated with, but not a structural part of the peritrophic membrane.

To analyse the possible function of p85 as a toxin-binding protein further, resistant and susceptible DBM larvae were examined. In the resistant DBM strain, HPL binding to p85 was stronger than those in the susceptible strain

(Figure 5). A possible explanation for this is that the amount of p85 in the gut lumen is increased in the resistant strain. Recently, the identification of glycoproteins involved in coagulation revealed a similar variation (Li *et al*, 2002). For example, proteins of immune-induced larvae formed non-soluble complexes, whrereas proteins from non-induced larvae remained soluble (D.Li, unpubl. data). Given its presence in hemolymph, one possible role of p85 would be an involvement in coagulation reactions.

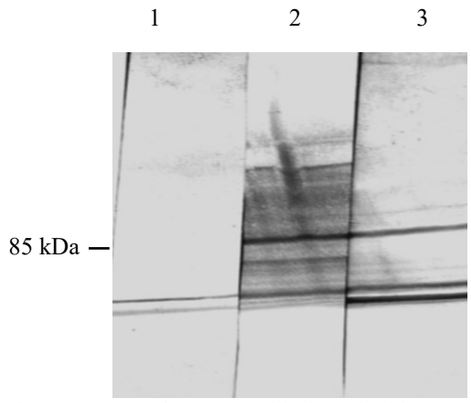


Figure 1. Bt-toxin CryIAc binds to lepidopteran glycoproteins. Western blot containing protein extracts from fourth inster caterpillars were probed with 1) preserum of anti-CryIAc-antibodies using peroxidase-conjugated secondary antibodies, 2) Peroxidase-conjugated HPL, 3) antibodies against CryIAc, using peroxidase-conjugated secondary antibodies.

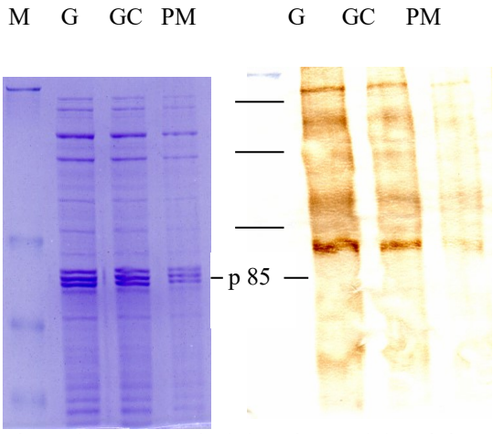


Figure 2. Coomassie (A) and HPL (B) staining of different tissues of diamondback moth caterpillars. HPL only stain p85 and high molecular weight proteins extracted fron the gut (G), gut content (GC) and peritrophic memberane (PM)

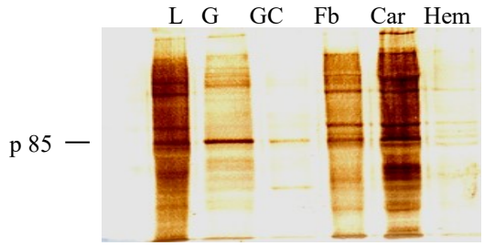


Figure 3. Western blot of protein extracts from individual different tissue of dbm larvae stained with hpl lectin. p85 kda glycoprotein exist in different tissues of dbm such as whole larvae (L), gut (G), gut content (GC), fat body (FB), car-cases (CAR) and hemolymph (HEM)

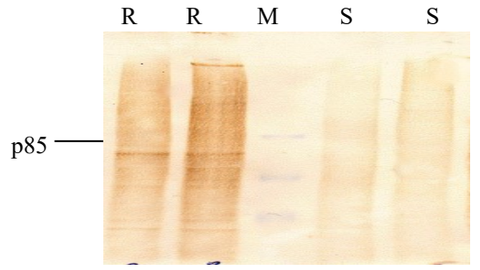


Figure 4. Western blot of total protein ex-tracts from individual resistant (R) and susceptible (S) DBM cater-pillars stained with peroxidase-conjugated hpl, showing a some-what stronger staining of p85 in resistant strain compared to p85 staining in susceptible strain.

Immune Elicitors in the Gut

In this study an analysis of the soluble midgut proteins for their ability to react to microbial infection is of interest. Therefore, attempts to correlates Bt-binding with the immune status are necessary and induction of defense reaction experiment in the diamond back moth by feeding of insect pathogenic bacteria was carried out. This is to analyse possible immune induction by gut derived pathogen or immune elicitor. Lethal and sub lethal concentrations of Bt-toxin were treated to the fourth instar larvae of DBM and monitored the p85 amount after 48 hrs treatment with different inducers. Lethal concentration of Bt caused damage in the gut which appear to prevent the induction of p85 (Figure 1. In sub lethal concentrations), Bt was able to induce p85 similar to other inducers, such as LPS, E.coli (Figure 5; 1, 3, 7). However, when inducers were mixed with lethal concentration of Bt, their ability to induce p85 was reduced (Figure 5; 2,4,6)

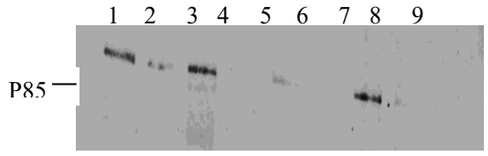
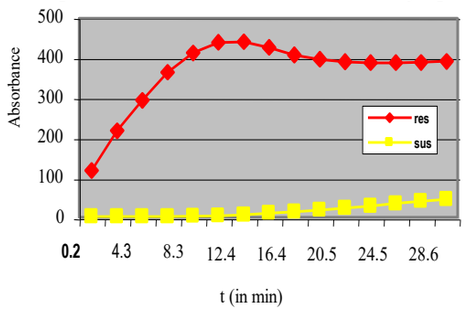


Figure 5. Induction of the p85 from midgut extract of dbm 48 hrs after feeding caterpillar with several immune-elicitors. 1. LPS, 2.LPS +toxin (0.1 ppm), 3. *E.coli,* 4. *E.coli* + toxin (0.1 ppm) , 5.killed bt, 6. killed bt + toxin (0.1 ppm), 7. toxin 0.01 ppm, 8. toxin 0.1 ppm, 9. control (sterile water). note that apithelial tissue structures were intact in all cases, except when toxin was used in lethal dosis (0.1 ppm). under these conditions total protein patterns are altered.

Melanisation Reactions

The enzyme phenoloxidase (PO) is responsible for the melanisation-reactions in caterpillars and plays a role in defence reactions. Since spontaneous melanisation of hemolymph extract, which is posed to air, is generally used as a reliable indicator for the immune-status of an insect, melanisation assays were performed with individual resistant and susceptible caterpillars. Cell-free hemolymph from Bt-resistant caterpillars differed from susceptible caterpillars in that the melanisation-reaction started from a higher level and increased to a higher level compared to hemolymph from susceptible insects, where the start of the reaction was delayed for several minutes and produced less melanised reaction products (Figure. 6.1).

Dissection of gut tissues from resistant caterpillars frequently revealed a blackening of the peritrophic membrane and gut content due to melanisation reactions (Figure 8). This could indicate the presence of hemolymph-derived immune molecules in the gut lumen confirming the observation from confocal microscopy. To examine melanisation at a quantitative level we performed melanisation assays in gut extracts, which showed an increased melanisation in the gut of Bt-resistant caterpillars (Figure. 6.2)



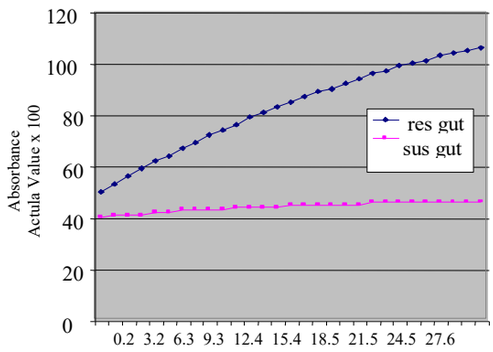


Figure. 6. Melanisation reactions in *H. armigera* strains that are resistant and susceptible to bt-toxin. 1) cell-free hemolymph from 3rd instar caterpillars was diluted in pbs-solution and relative absorbance measured over 30 min.note a slight reduction of relative absorbance in hemolymph from the resistant strain due to coagulation reactions. 2) gut extracts without gut contents were measured in the presence of 100mm 3,4-dihydroxyphenylalanine (DOPA). although the shape of absorbance curves varies slightly between hemolymph from individual caterpillars, the difference in absorbance between the two strains always exceeded 200 units, with hemolymph from susceptible caterpillars showing variability due to possible immune-induction by wounding or food derived elicitors.

The *B. Thuringiensis*–resistant Strain May be Resistant to Other Pathogens

If the Bt-resistant strain is constitutively immune-induced producing immune-reactive components in the gut lumen, Bt-resistance may protect against other pathogens as well. We tested this assumption by feeding caterpillars with baculoviruses. Although it is not known, whether baculoviruses use GalNAc-containing glycoproteins to gain access to gut cells, the prevalence of these glyco-determinants in the gut (Figure 7) could indicate a virus-glycoprotein interaction. When *Autographa californica* M Nucleopolyhedrovirus (AcMNPV) was applied to caterpillars from both strains, Bt-

resistant caterpillars were less vulnerable to the virus than susceptible caterpillars (Figure. 7). Similar results were obtained when virions were injected into the hemocoel (not shown).

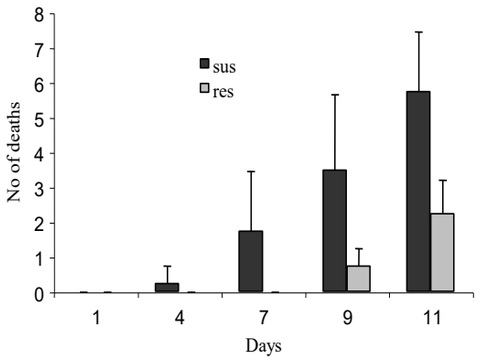
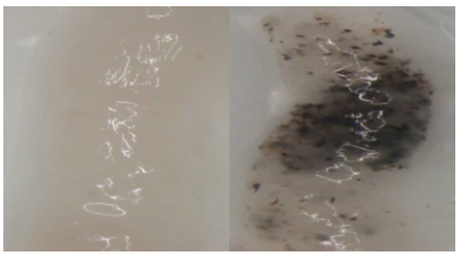


Figure. 7. Mortality rate in baculoviruz-treated caterpillars from bt-resistant and susceptible strains. *h. armigera* 3rd instar larvae were fed on artificial food mixed with a suspension of 10^7/ml of *Autographa californica* multiple nuclear poly-hedrosis virus (A*c*MNPV). *H. armi-gera* is semiper-missive to *ac*mnpv. the virus titre, resulting in low mortality rates, was chosen to mimic low bt-toxin levels. each treatment was repeated three times with at least 20 caterpillars each. The difference in mortality rates was highly significant for each time point. no mortality was observed in non-treated insects.



A B

Figure 8. Dissected gut tissues from a sus-ceptible caterpillar of *H. armigera* (A) and a resistant caterpillar (B) shows blackening of the peritrophic membrane and pieces of gut content in resistant caterpillar.

CONCLUSIONS

Increase of soluble *Bt*-binding molecules in the gut lumen of resistant insects correlates with increased resistance against other pathogens. This is compatible with the assumption that the Bt-binding protein in resistant insects is an immune complex.

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