J. Plant Prot. and Path., Mansoura Univ., Vol. 6 (1): 65 -72 , 2015

SYNERGISTIC EFFECT OF CADHERIN RECEPTOR OF *Helicoverpa armigera* (HÚBNER) TO NON-SPECIFIC *Bacillus thuringiensis* CRY1AC TOXIN AGAINST COTTON LEAFWORM; *Spodoptera littoralis* (BIOSDUVAL)

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ABSTRACT

Bacillus thruingiensis (Bt) strains are common bacteria in insect control field. Cadherin like protein receptor is consider as a key factor for binding Cry1A toxin to the Burch Border Membrane Vesicles (BBMV) in insect midgut. Our previous investigations have shown the importance of cadherin like protein as a synergistic factor to increase the toxicity of different types of specific Bt toxin against Lepidopterous insects. Small fragment of cadherin receptor (CadF3) of BtCry1Ac toxin of *Helicoverpa armigera* (Húbner) pest was isolated, cloned and expressed. The recombinant bacterial strain of BtCry1Ac toxin is inactive to cotton leafworm; *Spodoptera littoralis* (Biosduval). In the current study, the ability of CadF3 fragment to enhance the toxicity of BtCry1Ac toxin against cotton leafworm; *S. littoralis* was examined. The results revealed that CadF3 has a potential effect to BtCry1Ac to become active toxin on cotton leafworm. Thus, this data showed the capability of CadF3 fragment to play very essential role to activate BtCry1Ac in cotton leafworm midgut.

Keywords: *Bacillus thuringiensis*, cotton leafworm, Bt Cry1Ac toxin, Cadherin like protein, CadF3 fragment.

INTRODUCTION

Cotton leafworm; *Spodoptera littoralis* (Biosd.) is a major polyphagous pest that attack cotton and several vegetable crops in Egypt and over the globe. It causes severe damage in quantity and quality of crop yield (Hosny et al., 1986). Several Egyptian million pounds are paid yearly in cotton leafworm control. The chemical insecticides usually applied on the foliage of the plant. Since 1960, several chemical insecticides have been used in order to control this insect but as a result of repetitive use of chemical pesticides; several health problems have been generated. Therefore, the biopesticides formulations based on *B. thruingiensis* were used as an alternative measures that proved more safe to the nontarget organisms.

Bacillus thruingiensis (Bt) is a Gram positive soil bacterium that had been extensively used in insect pest control against several Lepidopteran pests. In the gut of susceptible insect, the insecticidal crystal protein (Protoxin) is solubilized into activated toxin form. The activated toxin crosses the peritrophic membrane and binds to specific receptors such cadherin and aminopeptidase-N (Abdullah et al., 2009). Eventfully, the cell gets lysis and pore formation produced that resulting insect death.

BtCry1Ac toxin is one of the most specific toxins to *H. armigera* but not to cotton leafworm (Moussa, 2009). It is very much essential to bind to cadherin like protein receptor that located at the BBMVs to complete it toxicity process. Though a specific Bt toxin has a narrow spectrum of activity, several types of Bt toxin have been characterized that have selective toxicity to different orders of insects (Schnepf et al., 1998). Our previous data showed that the cadherin fragment (Ectodomain region; 10-12) of *Manduca sexta* cadherin receptor enhances the toxicity of BtCry1Ac to beet armyworm; *Spodoptera exigua* (Hübner) (Abdullah et al., 2009). Development of Bt synergists is a continuous process to improve the inadequacy of Bt pesticides. Adopting synergists technology might lead to a reduced quantity of Bt needed to obtain good control, a wider spectrum of activity and lengthening of residual activity (Dubois et al. 1989 and Liu and Tabashnik, 1997). Later on, cadherin like protein; the receptor of BtCry1Ac in *H. armigera* pest was isolated, fragmented, cloned and the expressed protein of each fragment was tested for its synergism effect (data under publication). CadF3 fragment found to be a very effective one to enhance BtCry1Ac and BtCry1Ab against *H. armigera*. Therefore, the current study aims to examine cadherin fragment (CadF3) as a synergism factor to Cry1Ac against cotton leafworm; *S. littoralis* insect pest.

MATERIALS AND METHODS

Insect culture

The stock culture of cotton leafworm, *Spodoptera litoralis* (Biosduval) pest was maintained under laboratory conditions for several generations as described by El-Defrawi et al. (1964) with little modifications. The mother colony of cotton leafworm, *S. litoralis*, was collected from cotton field of Kafer Elsheikh Governorate. The larvae were fed on clean and dry castor bean leaves (*Ricinus communis*) in four litter glass jars. The castor bean leaves were changed daily. After pupation, the pupae were collected and kept in glass containers until adult emergence. The Adult were allowed to mate and feed on 10% sugar solution on cotton pad and lay eggs in the same jars. The eggs were collected on tissue paper and kept in small jars along with wetted cotton piece as a source of moist for hatching. The eggs were collected and kept in plastic cups for hatching. The laboratory condition was fixed at 26 ±1 °C, 70% RH and 16:8 (L: D) photoperiod.

Characterization and purification of *Bacillus thuringiensis* Cry1Ac

The Cry1Ac protoxin was partially purified according to methods previously described by Moussa and Gujar (2005). The recombinant bacterial cells of Bt Cry1Ac strain was inoculated in 5 ml Loria broth (LB) culture tube and incubated overnight at 150 rpm shaking with 37ºC. An amount of 1ml overnight culture was sub-cultured in one litter LB media with 1ml of 10% ampicillin stock solution and kept to grow for further 3 – 4 days under the previous conditions until the cells were lysised and crystals with the spores produced. The cells were pelleted down using 5200 rpm centrifuge at 4ºC for

10 min. The pellet was resuspended in lysis buffer (50mM Tris; PH 8.0; 50mM EDTA; 15% sucrose; 10µg/ml of lysozyme).

Sodim Dodsyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The partially purified toxin concentration was determined using the Bio-Rad protein assay based on the Bradford method using Bovine Serum Albumin (BSA) as standard (Bradfrod, 1976). The molecular weight of BtCry1Ac and CadF3 fragment was determined according to method described by Laemmli (1970). The sample of stock solution was resolved in 10% SDS-PAGE for separation and BtCry1Ac and CadF3 bands were visualized using Comassie Blue R-250. The partially purified toxin was quantified, pooled, aliquoted and stored at -20°C for further use.

Expression and purification of cadherin fragment (CadF3) of Helicoverpa armigera

The recombinant *E. coli* bacterial strain that carry CadF3 gene was overexpressed to get their inclusion bodies by cultured it in 5ml Loria broth (LB) overnight at150 rpm shaking and 37ºC. An amount of 1ml of the overnight culture was added to another flask that has one litter LB media and kept to grow for further 3 - 5 days under the same mentioned conditions. The cells were pelleted down using 5200 rpm centrifuge at 4ºC for 10 min. The cells were pelleted down using 5200 rpm centrifuge at 4ºC for 10 min. The cells were sonicated in Buffer B (50 mMNaPO4, pH 8.0, 300mMNaCl and 20mMImidazole) then centrifuged using 10000 rpm for 10min at 4°C. The supernatant was collected and passed through column (Anti Ni column) ten times and the purified CadF3 protein was eluted using elution buffer (50mM NaPO4, 300mM NaCl, and 250mM Imidazole).The concentration of the passed through CadF3 protein was determined using Bradford method as mentioned above and checked on SDS-PAGE gel. The sample was aliquoted and kept at – 20^{0}C for further assay.

Insect bioassay

To determine the LC_{50} of Cry1Ac toxin against cotton leafworm, a complete bioassay was performed using four different concentrations viz., 1.0, 2.0, 4.0 and 8 µg/g semi artificial diet (Moussa and Gujar , 2005). In order to investigate the synergistic effect of CadF3 protein on toxicity enhancement of BtCry1Ac toxin; the inclusion bodies of both CadF3 protein and Cry1Ac toxin were combined together at various mass ratios *viz.*, 1: 0.5, 1:1, 1:10, 1:50, 1:100 of Cry1Ac and CadF3 respectively. Subsequently, these doses were applied and sprayed on castor leaves. The castor leaves were brought freshly, washed with cleaned water then left on the bench at room temperature to dry before conducting the experiment. A number of 50 first neonates were released on the surface of the treated leaves and were considered as one replicate. Three different replicates were used per concentration. The leaves were then kept on glass jars (25 x 15 cm diameter). The larval mortality was estimated four days after treatment. Three different stages of cotton leafworm were tested to examine their sensitivity to BtCry1Ac toxin when it combined with CadF3 protein. The first neonates

were transferred by fine brush on the surface of the treated leaves. The negative and positive control treatments were also considered for comparison. The LC_{50} was determined and larval mortality was calculated and LC50 value was determined. Each experiment was performed several times in order to get accurate results.

RESULTS AND DISCUSSIONS

BtCry1Ac toxin band was detected at 130kDa on 10% SDS-PAGE (Fig. 1). The measuring of Cry1Ac toxin and CadF3 protein concentration absorbance at 595nm wave length showed that their concentrations were 1.0 µg/ µl and 6.7 µg/µl respectively, (Fig. 1 and 2). The toxicity data analysis of Cry1Ac showed its insensitivity to cotton leafowm and the LC_{50} of Cry1Ac was 39.30ug/g diet. The current result and earlier studies revealed that the Cry1Ac toxin is ineffective toxin to cotton leafworm (Van Frankenhuyzen, 1993). On contrast Moussa (2009) reported that Cry1Ac is very effective toxin against *H. armigera* and digested in its midgut causing high mortality compared with Cry2Ab which was remain unprocessed in insect gut 20 min after treatment.

In order to enhance Cry1Ac toxin, the cadherin fragment (CadF3) was expressed in Loria broth media and was mixed with Cry1Ac toxin at different mass ratio as mentioned above. The result showed that the mortality % of Cry1Ac toxin was increased from 16.6 % to 23.3 % when CadF3 protein was added with 1:0.5 mass ratio for Cry1Ac and CadF3, respectively. Also, it has been found that the mortality % of Cry1Ac was enhanced with 4.4 fold more when CadF3 protein added at mass ratio of 1: 50 where mortality was increased from 16.6 to 73.3% (Fig. 3). On the other hand, the mortality % of Cry1Ac was significantly increased (From 10 to 66.6 %) when the CadF3 cadherin fragment was added with 1: 50 mass ratio for Cry1Ac and CadF3 against the second instar larva of cotton leafworm (Fig. 4). But in case of the third instar larvae the mortality % was still increased (From 10 to 46.66%) with 1: 50 mass ratio (Fig. 5). When the ratio of CadF3 fragment was increased from 50 to 100 times than Cry1Ac toxin, the mortality % started decline with the first, second and third instar larvae assay. Overall, this result is not indicated that CadF3 fragment has the ability to enhance Cry1Ac toxin only but also it increases its potentiation to lighten its effect with the second and third instar larvae of cotton leafworm. Several of the anti-Lepidoptera Insecticidal Crystal Proteins (ICPs) have shown a very high toxic activity against the early-instar larvae of several agriculture major pests such as *Heliothis* and *Helicoverpa* spp. ,*Spodoptera* spp. , *Ostrinia nubilalis* (Hubner), and *Plutella xylostella* (Linnaeus), (Van Frankenhuyzen, 1993). However, some other species belong to Noctuidae family such as *Spodoptera frugiperda* and *Agrotis* spp are insensitive to ICPs. Chen et al., (2007) reported that the synergistic mechanisms to Bt toxins can be classified into three different groups: (i) enhancement of toxin docking and membrane insertion, (ii) damage of midgut bruch border membrane vesicles, which leads

to increased toxin permeability, (iii) disruption the bacterial community present in the insect midgut, which is vital for insect growth and development. However, the protease inhibitors responsible for digestion and conversion of Bt protoxin into toxin (The active form) have shown significant potentiation for Bt toxicity process (Liu and Tabashnik, 1997).

Cadherin fragment of CR12-MPED is the first Cry synergist derived from an insect midgut protein (Chen et al., 2007). Cadherin-based synergists are not limited to lepidopteran cadherin that interacts with Cry1A toxin. Earlier studies revealed that cadherin receptor from other target insects may have potential as synergistic factor for other classes of Bt Cry toxins (Hua et al., 2008).This data is in accordance with those reported that a small toxin-binding fragment of *Manduca sexta* (Joh.) cadherin acts as a synergist of Bt toxins to *M. sexta*, *Heliothis virescens* F. and *Helicoverpa zea* (Boddie). Furthermore, Abdullah et al. (2009) reported that a BtCry1A toxin was significantly enhanced against *Agrotis ipsilon* (Hufnagel) and *S. exigua* when cadherin fragment (Ectodomain 10-12 region) of *M. sexta* (Linnaeus) was added. Generally, it can be concluded that enhancing of Cry toxicity process particular amount of cadherin receptor may play a key factor for protease inhibitors that may inhibit specific gut proteases that would deactivate Bt toxins, or it may reduce proteolysis or degradation of membrane bound receptors, thus increasing their half-life and their ability to bind Cry1A toxin (Pardo et al., 2009). However, this theory is not yet explainable and may need to be investigated. The current study is therefore intensifying the use of Bt synergists as new technology in order to improve the quality of biopesticdes based on Bt toxins. Also, adopting such technology might reduce the quantity of Bt needed to obtain worthy pest control and widen Bt activity.

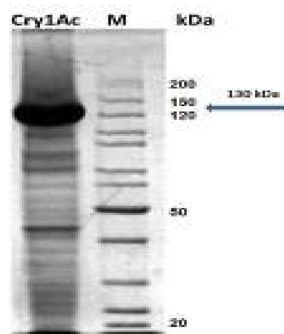

Fig.1: SDS-PAGE electrophoresis of Cry1Ac partially purified toxin, M: Represents PageRoler marker, Lanes 1, 2 and 3: Represent 10, 10 and 20ul of Cry1Ac loaded

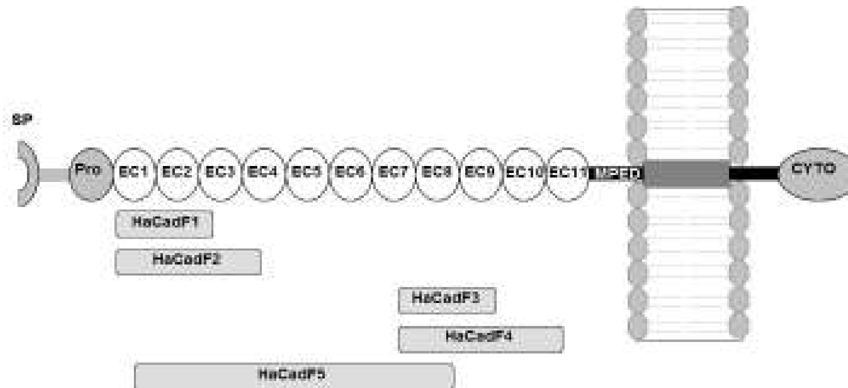

Fig. (2): Diagram of *H. armigera* cadherin receptor and its various domains. SP: Represents signal peptide; Pro: Represents proprotion region; EC: Represents ectodomain regions; MPED: Represents membrane-proximal extracellular domain; CYTO: Represents cytoplasmic domain; HaCadF: Represents recombinant proteins of cadherin fragments (CadF1, CadF2, CadF3, CadF4, and CadF5)

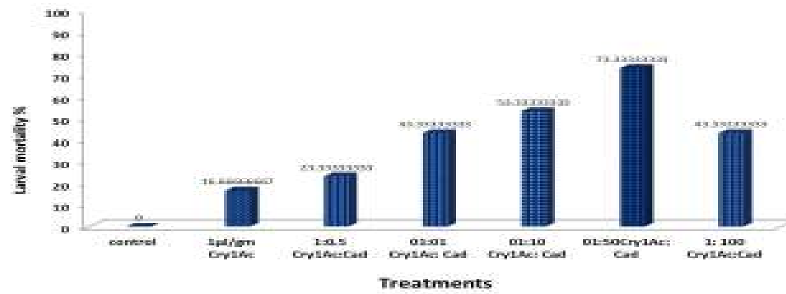

Fig. (3): Synergistic effect of CadF3 fragment to BtCry1Ac toxin against the first larval instar of cotton leafworm.

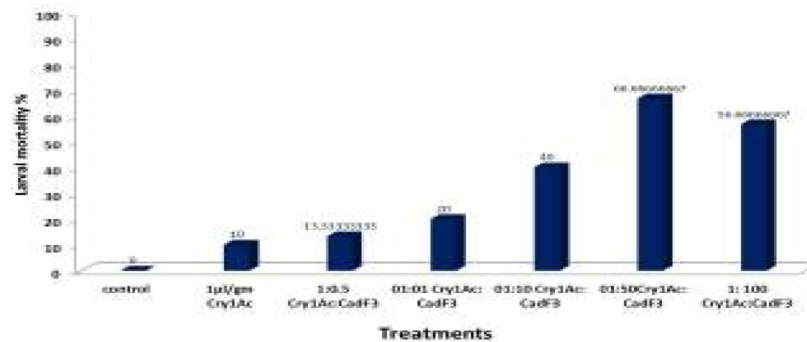

Fig. (4): Synergistic effect of CadF3 fragment to BtCry1Ac toxin against the second larval instar of cotton leafworm.

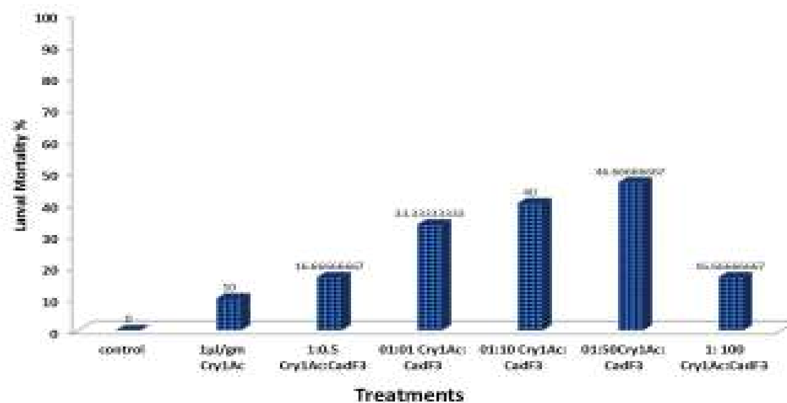

Fig. (5): Synergism effect of CadF3 fragment to BtCry1Ac toxin against the third larval instar of cotton leafworm.

Acknowledgment

The author is gratefully acknowledged the Agriculture Research Development for Fund (ARDF) for funding this work through the project number 244. Also, I would like to thank the board of ARDF organization for their support, encourages and caring throughout the project.

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التأثير التحفيزى لمستقبل الكادهيرين فى دودة اللوز الامريكية للمركب البكتيرى الــ Cry1Ac الغير متخصص على حشرة دودة ورق القطن

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بكتريا الباسيليس من الانواع الشائعة الاستخدام فى مجال مكافحة الآفات الحشرية. يلعب المستقبل البروتينى الكادهيرينى دورا هاما فى عملية ارتباط التوكسين البكتيرى بالغشاء المبطن لجدار معدة الحشرة. وقد أوضحت الدراسات السابقة أن المستقبل يعمل كعامل محفز لزيادة فعالية السمية ضد عدد كبير من الحشرات التابعة لرتبة حرشفية الاجنحة. وفى هذه الدراسة تم اختبار القطعة رقم ثلاثة من المستقبل البروتينى للتوكسين البكتيرى الـــ Cry1Ac كعامل محفز لنفس التوكسين البكتيرى (Cry1Ac) الغير متخصص على حشرة دودة ورق القطن عند نسب مختلفة. وقد أوضحت النتائج قدرة هذه القطعة البروتينية ثلاثة من المستقبل البروتينى الكادهيرينى على زيادة فعالية التوكسين البكتيرى (Cry1Ac) وتحويلة من توكسين غير نشط الى توكسين نشط وفعال.