Identification and purification of a novel high-affinity receptor BTR_{250} from Pink Bollworm (*Pectinophora gossypiella*) for *Bacillus thuringiensis* Cry1A toxins

(Received: 10.05.2004; Accepted: 30.05.2004)

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ABSTRACT

The pink bollworm Pectinophora gossypiella Saunders (PBW) is a devastating cotton pest worldwide. Bacillus thuringiensis Cry1A toxins are lethal to pink bollworm (P. gossypiella) larvae. Cry1Ac binds to ~250, 200, 120, and 115 kDa proteins in the brush border membrane vesicles. The toxin binds specifically and with high affinity (K_d ~10 nM) to the receptors binding sites. A competition ligand blot using unlabeled Cry1Ac to compete with ^{125}I-Cry1Ac shows that binding is abolished by Cry1Ac. Consequently, Cry1Ac binding to the mentioned proteins is specific and more likely to be of a mediating toxicity in P. gossypiella. The Cry1Ac immuno-precipitation gives rise to three major bands of ~200 and 120/115 kDa. Interestingly, a novel 250 kDa band was observed to bind with 125I-Cry1Ac. The work was focused on the purification of the 250 kDa protein using ion exchange chromatography and 2D gel electrophoresis. Collectively, these results demonstrate that BTR250 in junction with the 200 kDa Cadherin-like receptor may play an important role in Cry1A toxicity to P. gossypiella. Protein sequencing and DNA cloning of this new receptor will help in identification of the novel receptor and deciphering its nature. Understanding the molecules involved in toxin binding will help in developing strategies for insect resistance management and development of better bio-pesticides.

***Keywords**: Pectinophora gossypiella, Bacillus thuringiensis, Cry toxin, PBW-BTR_{250}.*

INTRODUCTION

Control of the PBW using *Bacillus thuringiensis* (Bt) formulations has shown very promising results during the last few decades (Perlak *et al.*, 1990). *Bacillus thuringiensis* is a gram-positive, spore-forming bacterium that forms a parasporal crystal which contains insecticidal toxins (Bulla *et al.*, 1980; Höfte and Whiteley, 1989). *Bacillus thuringiensis* (Bt), as a biopesticide, is a viable alternative for the control of insect pests in agriculture. Bt use is also compatible with sustainable and environmentally friendly agricultural practices. Bt produces insecticidal proteins (Cry toxins) during sporulation as parasporal crystals. These crystals are predominantly composed of one or more proteins, called endotoxins. These toxins are highly specific to their target insect; are safe to humans, vertebrates and plants; and are completely biodegradable. The mode of action of Cry toxins is a multistage process. Crystal toxins ingested by susceptible larvae

dissolve in the alkaline environment of the larval midgut, thereby releasing soluble proteins. The inactive protoxins are then cleaved at specific sites by midgut proteases, yielding 60-kDa protease-resistant active fragments. The active toxin then binds to specific membrane receptors on the apical brush border of the midgut epithelium columnar cells (Bravo *et al.*, 1992; Hofmann *et al.*, 1988). Therefore, receptors on the brush border membrane are a key factor in determining the specificity of Cry toxins. After binding, the toxin apparently undergoes a large conformational change leading to its insertion into the cell membrane (Li *et al.*, 1991). The Cry toxin molecules then aggregate through toxin-toxin interactions, leading to the formation of lytic pores (Sobero´n *et al.*, 2000), which disrupt midgut ion gradients and the trans-epithelial potential difference. This disruption is accompanied by an inflow of water that leads to cell swelling and eventual lysis, resulting in paralysis of the midgut and subsequent larval death (Schnepf *et al.*, 1998). A number of putative receptor molecules for lepidopteran specific Cry1A toxins have been identified. In *Manduca sexta,* Cry1Aa, Cry1Ab, and Cry1Ac proteins bind to a 120-kDa aminopeptidase N (APN) (Denolf *et al.*, 1997) and to a 210-kDa cadherin-like protein ($Bt\text{-}R_1$) (Belfiore *et al.*, 1994, Vadlamudi *et al.*, 1995). In *Bombyx mori*, Cry1Aa binds to a 175-kDa cadherin-like protein (Bt-R175) (Nagamatsu *et al.*, 1998) and to a 120-kDa APN (Yaoi *et al.*, 1997). In *Heliothis virescens,* Cry1Ac binds to two proteins of 120 and 170 kDa, both identified as APN (Gill *et al.*, 1995). In *Plutella xylostella* and *Lymantria dispar* APNs were identified as Cry1Ac receptors (Denolf *et al.*, 1997, Luo *et al.*, 1997, Valaitis *et al.*, 1995). In *Ostrinia nubilalis*, Cry1Ab binds to 205 kDa cadherin-like and 170 kDa APN (Mostafa *et al.*, 2003). In *P. gossypiella* Cry1Ab toxin binds to 200-kDa cadherin receptor ($PBW\text{-}BTR_2$) which was cloned, sequenced and characterized (Maaty, 1999). The toxins have high binding affinity (Kd =12.8 nM) to PBW $BT\text{-}R_2$ and mediate toxicity to mammalian cells expressing $BT\text{-}R_2$ cDNA. In the present study, we report the Cry1Ac toxin binding to a novel 250 kDa protein in *P. gossypiella*. This study is focused on the identification and purification of the novel 250 kDa protein.

MATERIALS AND METHODS

Bacterial strains and toxin purification

Recombinant Cry1Ac protoxins (*Bacillus* Genetic Stock Center, The Ohio State University) were prepared from *E. coli* JM-103 and trypsinized essentially as described by Lee *et al.* (1992). In addition, the soluble trypsinized 60-kDa toxins were subjected to FPLC NaCl salt gradient purification over an HR-5/5 Mono-Q anion exchange column (Pharmacia) prior to quantitation, radio-iodination, and use in bioassays. All toxin protein quantitations were performed using the bicinchoninic acid method (Pierce Chemical Co.) with bovine serum albumin (BSA, Fraction V) as a standard.

Preparation of brush border membrane vesicles (BBMV)

Early fourth-instar larvae were kept on ice for 1 hr and midguts were surgically removed from the larvae. BBMV were prepared from midgut tissues by the differential magnesium precipitation method of Wolfersberger *et al.* (1987) in the presence of protease inhibitors (5 mg/ml pepstatin, antipain, aprotonin, leupeptin, 1 mM PMSF, and 5 mM benzamidine). The final pellet was resuspended in buffer A (300 mM mannitol, 5 mM EGTA, and 17 mM Tris-HCl, pH 7.5) containing the protease inhibitors, flash frozen

in liquid nitrogen, and stored at –85°C.

Protein iodination

All Cry toxins used in this work were radioiodinated using the chloramine-T method (Hunter and Greenwood, 1962) with ^{125}I-Na (NEN Dupont). Ten µg of toxin were mixed with 5 µl of ^{125}I-Na (0.5 mCi) in 100 µl of NaHPO4 buffer (0.5 M, pH 7.4) with 25 µl of Chloramine T (4 mg/ml). The reaction mixture was agitated for 20-25 sec at 23°C and the reaction was stopped by adding 50 µl of $Na_2S_2O_5$ (4.4 mg/ml). Free iodine was removed by gel filtration on an excellulose desalting column (Pierce), equilibrated with PBS containing 10 mg/ml BSA.

Toxin binding assays

Homologous competition inhibition binding assays were performed as described by Keeton *et al.* (1998). A total of 25 µg of BBMV were incubated with 1.2 nM ^{125}I-Cry1Ac toxin in the presence of increasing concentrations (0 to 1000 nM) of the appropriate unlabeled homologous toxin (Cry1Ac). Incubations were performed in 100 µl of binding buffer (PBS/0.2% BSA) at 25°C for 30 min. Radiolabeled and unlabeled toxins were mixed together before adding them to the BBMV. Unbound toxins were separated from BBMV-bound toxin by centrifugation at 14,000-x g for 10 min. The pellet containing bound toxin was washed three times in ice cold binding buffer by gentle mixing and radioactivity in the final pellet was measured using a Beckman Gamma 5500 counter. Binding data were analyzed by the PRISM program (GraphPad Software Inc., San Diego). The dissociation constant (K_d) of labeled toxins was calculated from three separate experiments. The equilibrium binding parameters were estimated by analyzing the data with the PRISM computer program.

Immunoprecipitation of Cry1Ab binding protein

Immunoprecipitation was carried out according to Vadlamudi *et al.* (1993). Twenty five µl of Cry1Ac antiserum were added to 1 ml of protein A-Sepharose CL-4B equilibrated in washing buffer (1% Nonidet P-40, 6 mM EDTA, 50 mM Tris-HCl, and 250 mM NaCl) and mixed for 1 hr at 4°C. After washing the blot three times with washing buffer, 700 µg of Cry1Ac toxin were added and the mixtures were incubated for an additional 1 hr at 4°C and washed again three times with washing buffer. Pink bollworm BBMV proteins (6 mg) were solubilized in 1 ml of washing buffer containing 1% NP-40 and protease inhibitors (10 µg/ml pepstatin, antipain, aprotonin and leupeptin; 5 mM iodoacetamide; and 1 mM PMSF). Unsolubilized proteins were removed by centrifugation. Solubilized proteins were filtered through a 0.45 µm filter, added to 1 ml of Sepharose-protein A beads linked to Cry1Ab toxins, and the sample was stirred gently for 1 hr at 4°C. Sepharose beads were centrifuged and washed four times with washing buffer containing 0.25% NP-40 and 0.02% SDS. The toxin-binding protein complex was dissociated from the beads by heating in sample buffer as described by Laemmli (1970) and the binding proteins were Coomassie stained and detected by blotting with ^{125}I-Cry1Ac.

Radioligand blotting

The immunpoprecipitated binding proteins were solubilized, separated by 7.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane as described by Keeton *et al.* (1998). Blots were blocked with TBS (10 mM Tris-HCl and 0.9 % NaCl) containing 5% non-fat dry milk powder, 5% glycerol, 0.5% Tween-20, and 0.025% sodium azide for 2 hr at 25°C. Blocking buffer was removed and membranes were incubated for 2

hr at 25°C in an equal volume of fresh blocking buffer containing 2 x 10^5 cpm/ml (1-1.25 nM) of ^{125}I-Cry1Ac toxins. Finally, membranes were washed three times with fresh blocking buffer for 10 min each, rinsed once with TBS, dried, and exposed to Kodak X-ray film at –80°C.

Two-dimensional gel electrophoresis and immunodetection of pink bollworm Cry1A receptor

The precipitated protein was run into two separate two-dimensional gel electrophoresis. After electrophoresis, one gel was stained with Coomassie blue and the other was transferred to a PVDF membrane, blocked with 5% nonfat dry milk in PBS buffer and incubated at 4°C overnight in the same blocking buffer containing 10 µg/ml of Cry1Ac. Unbound toxin was washed with PBS. Antibodies raised in rabbits against the 60-kDa Cry1Ac toxin were diluted 1:1000 and hybridized to the membrane for 2 hr at 25°C then the blot was washed with PBS. Peroxidase-conjugated goat anti-rabbit IgG was diluted 1:3000 in TBS blocking buffer and hybridized to the membrane for 2hr, then membrane was washed extensively with PBS. Visualization of the bound toxin was accomplished using the ECL Western blotting detection method (Amersham).

Mono-P ion-exchange chromatography purification for BTR250 binding protein

Solubilized PBW BBMV proteins were applied to a Mono-P ion-exchange column (0.5 X 5 cm) attached to an FPLC system (Pharmacia) equilibrated in buffer A [1% CHAPS, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM Mg_2SO_4, 10% glycerol and protease inhibitor cocktail] at 4°C. Bound material was eluted from the column with buffer A containing an increasing concentration of KCl, collected in 1 ml fractions and monitored at 280 nm.

RESULTS AND DISCUSSION

Cotton insects reduce yield by almost 10% worldwide. One group, in particular, the bollworm/budworm complex is the most damaging, causing a 2.7% loss. Insect damage reduced the overall cotton yield by more than 1.7 million bales and produces a great financial loss (Gouge *et al.*, 1997). Biological control of the cotton pests using *B. thuringiensis* formulations and transgenic plants has been in use for a number of years and is growing rapidly. Concerns that BT formulations or transgenic plants expressing the cry1A genes of *B. thuringiensis* may evoke emergence of either resistant or tolerant strains of cotton insects such as *P. gossypiella* (pink bollworm), as has occurred with some other insects, have prompted the search for a better understanding of the interaction between the Bt toxin proteins and their respective insect receptors. A better understanding of the receptors involved in toxicity and mode of action of Cry proteins is needed to develop strategies that may prevent or slow the occurrence of resistance.

The research described herein was targeted towards the identification and characterization of the Cry toxin receptor(s) for the PBW. Ligand blot experiments showed that proteins of 250, 200, 120 and 115 kDa bind Cry1Ac toxin (Fig. 3, lane 1). Competition assays for Cry1Ac binding to *P. gossypiella* BBMV show that binding of labeled Cry1Ac is almost completely inhibited by unlabeled Cry1Ac at 1 µM which is an indication of specific binding of the toxin. In many insects, the high-affinity receptor appears to be a cadherin-like protein with a large molecular mass, e.g., tobacco horn worm THW-BTR_1, (Vadlumudi *et al.*, 1995), pink bollworm PBW-BTR_2 (Maaty, 1999) and silk

worm BTR_{175} (Nagamatsu *et al.*, 1998). At this time, we can only speculate on the relation between the novel 250 kDa binding protein and these Cadherin-like receptors. The functions of these cadherin-like proteins in the mode of action of *B. thuringiensis* toxins are not known yet. It is generally thought to disrupt ionic balance in the midgut epithelium (Knowles, 1994). However, at least one type of cadherin has been shown to be the crucial receptor for the binding of the gram-positive intracellular pathogen *Listeria monocytogenes* to the plasma membrane of nonphagocytotic epithelial cells (Finlay and Cossart, 1997; Mengaud *et al.*, 1996). It is conceivable that in acting as a receptor for the Cry1A toxins, BT-R is responsible, either directly or indirectly, for mediating the intercalation of the lepidopteran-specific toxins into the brush border membranes of intoxicated larvae (Dorsch *et al.*, 2002).

Furthermore, the detailed mechanisms of the Cry1A toxin interactions with the midgut BBMV of the pink bollworm also have been presented. The purified Cry1Ac was activated by trypsin digestion followed by column purification (Keeton *et al.* 1998). The activated 60 kDa Cry1Ac toxin (Fig. 1) was used in a homologous competition binding assay. The assay was performed with 1 μM iodinated Cry1A toxin and various concentrations of the (0- 1000 nM) unlabeled toxin. Cry1Ac showed high binding affinity to BBMV proteins (Fig. 2). Fifty-percent inhibition of Cry1A binding was observed at concentration 12.8 nM of the corresponding unlabeled toxin. These data indicate that the toxin binds specifically with high affinity. The binding site concentration, B_{max}, and the equilibrium dissociation constant, K_d, was calculated from the three separate homologous competition inhibition experiments by analyzing the data with the GraphPad computer program. The K_d value was in the low nM range ~12.8 nM whereas the B_{max} for Cry1Ac was 8.6 pmole/mg tissue. The Hill coefficient for Cry1Ac was 0.77 indicating a negative binding cooperativity for the toxins against the BBMV proteins. Significantly, for Cry1Ac, the data were best accommodated by a two binding site model with high and low affinity binding sites. These results indicate high affinity binding of Cry1A toxins to the BBMV receptors. This high affinity binding may be a determinant of the specificity of lepidopteran insects for Cry1A toxins rather than Cry3 (Colepoteran specific) and Cry11 (Dipteran specific) toxins. Binding of the Cry1A toxins to BBMV proteins was specific and saturable (Fig. 2).

In order to further examine the specificity of binding of the toxin to the BBMV proteins, BBMV proteins were solubilized in 1% Nonidet P-40 and immuno-precipitated with anti-toxin-protein A-Sepharose beads. Immunoprecipitation of BBMV proteins with anti-Cry1Ac antiserum were performed using Cry1Ac, which has high binding affinity to PBW BBMV. The mixture of bound material was solubilized in SDS sample buffer containing 2-mercaptoethanol. Electrophoresis and staining of the gel with Coomassie blue (Fig. 3, lane 2) revealed a major band of ~250 kDa, demonstrating selective precipitation of the 250-kDa toxin-binding protein. Radioligand blotting with ^{125}I-Cry1Ac showed bands of ~250, 200, 120 and 115 kDa (Fig. 3, lane 1), indicating precipitation of the same binding protein as that identified in stained lane (Fig. 3 lane 2). The low-molecular weight bands at ~60 and 52 kDa correspond to the Cry1Ab toxin and the heavy chain of IgG, respectively. The amount of ^{125}I-Cry1Ac toxin bound to the 120/115-kDa bands is ~15-times more toxin than the 250-kDa bands. The 120/115-kDa proteins proven to be heat shock proteins which act as nonspecific binding sites (sink) for the toxin (Maaty, 1999). Therefore, BTR_{250} is

potentially the specific binding site on the BBMV which mediates toxicity.

In order to purify these binding proteins we ran 2D gels for the BBMV proteins. The 2D pattern shows wide range of protein in molecular weight and isoelectric focusing point (pI) values (Fig 4A). Western blot analysis for the 2D separated BBMV proteins using Cry1Ac antisera shows a one spot in the middle of the blot in the range of pH 7 and molecular weight of ~100 kDa which probably represents the 115/120 kDa band (Fig. 4B). On the other side, the 250 kDa protein could not be seen which may be due to the hard nature of the 2D gels to resolve the high molecular weight proteins. Membrane proteins such as BTR_{250} represent a challenge for urea buffer used in the 2DE. To overcome this problem, we switched to ion exchange chromatography to purify the protein. The protein was successfully separated onto mono-P columns and the band appeared in fractions 15 and 16 well defined and clears from any associated proteins (Fig. 5). The purified band will serve as useful material for protein sequencing or mass spectrometry analysis to identify the novel protein in the future work.

The protein sequencing and the identification of this novel BT binding protein will pave the road for cloning and studying the role of the receptor in insect toxicity. The information presented in this study is the first necessary step leading to an understanding of the molecular biology of the toxin receptor in the pink bollworm. This step will be crucial in gaining a better control over the use of these bio-pesticides by allowing the engineering of more effective toxins in terms of longer persistence in the field, higher toxicity, and, ultimately, the preclusion of resistance development. For these reasons, it is imperative to gain a complete understanding of the mode of action of these toxins and the significance of receptors in this mechanism. An understanding of the Cry1A toxin receptor interactions in this cotton pest will facilitate our understanding of other economically important insect crop pests.

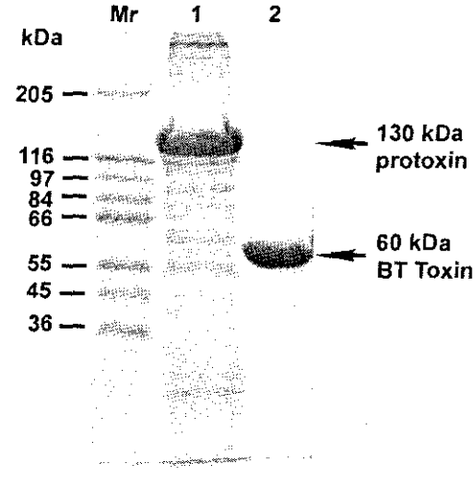

Fig. (1): Bt toxin activation. SDS-PAGE for Mono-Q anion exchange purified Cry1Ac toxin. The 130-kDa protoxin in lane 1 and the purified trypsin activated 60-kDa toxin in lane 2, (Mr = marker proteins).

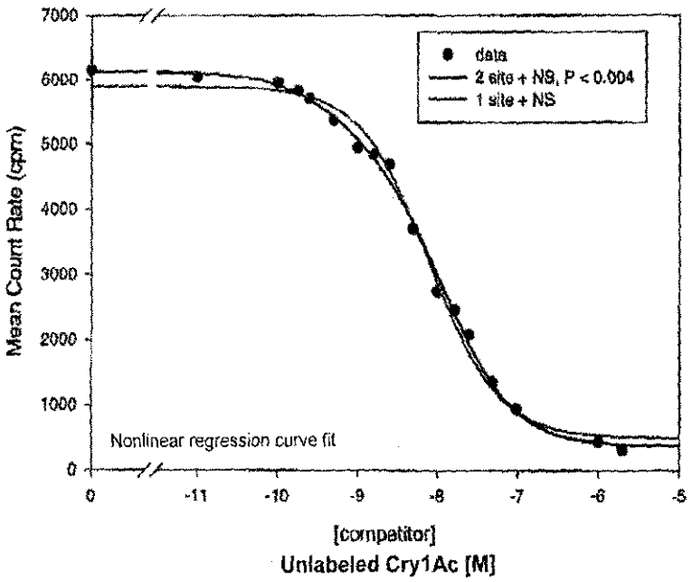

Fig. (2): Homologous competition inhibition binding assays. Binding of ^{125}I-labeled Cry1Ac toxin to BBMV proteins. BBMV (25 µg) were incubated with labeled toxin in the presence of increasing concentrations of the corresponding unlabeled toxin (0.01-1µM). Each point represents the mean of three independent experiments.

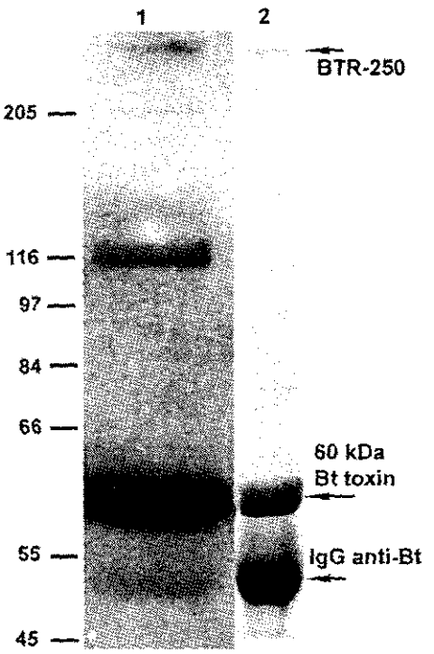

Fig. (3): Immunoprecipitation of the Cry1Ac toxin binding protein. The Cry1Ac binding protein was precipitated by adding anti-Cry1Ac serum to Sepharose protein-A. Cry1Ac was added followed by incubation with the solubilized P. gossypiella BBMV proteins. Bound proteins were separated and blotted onto PVDF membrane. Detection of the binding protein was carried out by Coomassie stain (lane 2), and ligand blotting with ^{125}I-Cry1Ac (lane 1).

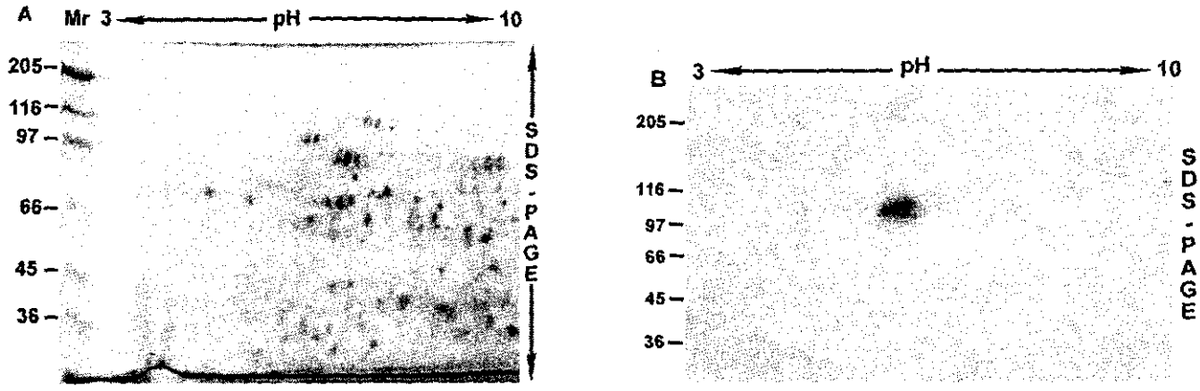

Fig. (4): 2-Dimentional Electrophoresis map of the PBW-BBMV proteins. 250 µg solubilized BBMV proteins were applied to 3-10NL pH gradient IPG strips. The second dimension is SDS-PAGE in 12 % acrylamide gel slabs. The gels were stained with Coomassie stain (A) or blotted to PVDF membrane and incubated with Cry1Ac then anti-Cry1Ac antibodies (B). Positions of molecular mass markers (in kilodaltons) are indicated.

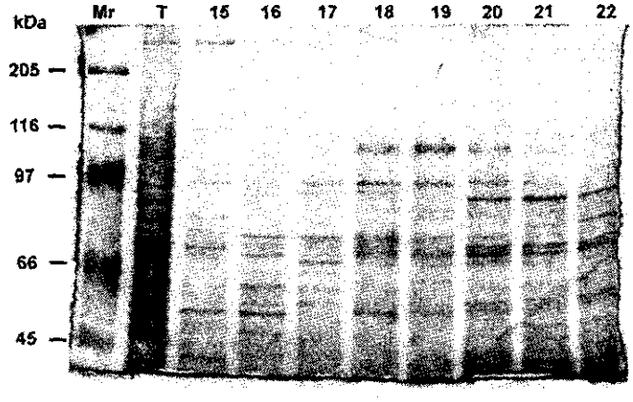

Fig. (5): Bt binding proteins column purification. Elution profile of CHAPS-solubilized BBMV on a Mono-P FPLC column. Five mg of CHAPS-solubilized BBMV were applied to an HR5/5 Mono-P column. Bound proteins were eluted and 25 (l of each 1-ml sample were separated by 7.5% SDS-PAGE. Solubilized total BBMV proteins are shown in lane T. Molecular weight markers are indicated on the left (Mr)

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الملخص العربي

تعريف وتنقية لمستقبل جديد BTR_{250} من حشرة لوز القطن القرنفلية (*Pectinophora gossypiella*) عالي الارتباط مع البروتين السام (Cry1A) من بكتريا باسيلس ثيرنجنسيس

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دودة لوز القطن القرنفلية تعتبر أحد اخطر آفات القطن على مستوى العالم والمكافحة الحيوية لهذه الحشرة باستخدام بكتريا الباسيليس ثورينجينسيس أظهرت كفاءة عالية على مدى العقود الماضية ومن المعروف أيضا أن البروتينات (Cry1A) شديدة السمية لدودة لوز القطن القرنفلية. وجدنا أن هذه السموم وهى Cry1Ac ترتبط مع بر وتينات موجودة فى الأغشية المبطنة لخملات المعى المتوسط ذوات أوزان جزيئية حوالى 250 120 ،200 و 115 كيلو دالتوان. أظهرت تجربة Competition ligand blot باستخدام بروتين Cry1Ac الموسوم باليود المشع 125 قدرة Cry1Ac الغير موسوم على التنافس وطرد البروتين الموسوم من مكانه على المستقبل ،والأكثر من ذلك تم حساب (k_d(toxin-receptor dissociation constant حيث كانت قيمته 12 نانومول من البروتين السام ،وهذا يظهر مدى تخصص الارتباط وشدته. من خلال النتائج السابقة يمكن القول أن البر وتينات السامةCry1Ac لهم مستقبل أو أكثر فى البروتين الغشائي لمعى الحشرة حيث يرتبطون به بدرجة عالية من التخصص. تركزت الدراسة على تنقية هذا البروتين بعدة وسائل منها أستخدام التحليل الكروما توجرافى وفصل البروتينات المعى المتوسط على الرحلان ثنائى الأبعاد (2DE) .فى النهاية يمكن القول أن التعرف على دور هذا البروتين فى عملية السمية للحشرة سوف يساعد على فهم الوظائف الحيوية لهذا المستقبل والمسار الحيوي لتفاعل هذه السموم مع المستقبلات وذلك سوف يمدنا بمعلومات مهمة وضرورية عن هذه المستقبلات ودورها فى قتل الحشرات وتفادى تكوين مقاومة لهذه السموم والمساعدة على تطوير أنواع جديدة من السموم البروتينية ذات المدى الأوسع لهذه الحشرات.