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Protease Digestion and Role of N-acetyl Galactosamine in the Binding Characteristics of *Bacillus thuringiensis* Delta-endotoxin (Cry 1Ac) to Purified Receptor of *Helicoverpa armigera*

Kausar Malik and S. Riazuddin
National Center of Excellence in Molecular Biology, University of the Punjab Lahore, Pakistan

Abstract: Proteins synthesized by the bacterium *Bacillus thuringiensis* are potent insecticides. When ingested by susceptible larvae they rapidly lyse epithelial cell lining of the midgut. The receptor protein in *Helicoverpa armigera* midgut appeared as single band on Non-SDS-PAGE but on SDS-PAGE. It resolved as two subunits (120kDa, 70kDa). We observed that the sugar N-acetyl galactosamine (GalNAc) showed no effect on binding of CryIAc toxin to receptor protein or in other words, toxin binding to receptor was not inhibited by GalNAc. This finding suggest that GalNAc might be not a component of a Cry1Ac toxin receptor Proteolysis of receptor proteins with trypsin and gut juice of *Helicoverpa armigera* showed that ~120Kda was digested while, ~70 kDa was trypsin and gut juice resistant and showed binding to CryIAc in ligand blots Proteolysis of receptor protein with pronase and proteinase-K showed digestion of ~120 kDa , ~70kDa and less than 40 kDa bands were appeared.

Key words: N-acetyl galactosamine, proteinase-K, *Helicoverpa armigera, Bacillus thuringiensis,* ligand blots

Introduction

The Gram-positive spore-forming bacterium *Bacillus thuringiensis*. produces insecticidal crystal protein known as δ-endotoxin or ICPs(Oddou, *et al*. 1991).
ICPs are expressed during sporulation and are accumulated as parasporal crystals. Depending on toxin specificity, a wide range of insect larvae (e.g. Lepidoptera, diptera and coleoptera) is susceptible to different ICPs (Jaquet *et al*., 1987, Hofte *at al*., 1989, Lereclus *et al*., 1989).The crystal protein is composed of inactive protein which are proteolytically cleaved to yield active toxin sub-units of approx. 60kDa, following solubilization of the protoxin in the insect midgut (Luthy *et al*., 1980). The toxic domain is located in the N-terminal half of the protoxin (Schnepf *et al*., 1985, Hofte *et al*., 1986. Geiser *et al*., 1986) . *In vivo* biological studies of susceptible insects which have ingested toxin, show a swelling of midgut epithelial cells, resulting in cell lysis and disruption of gut wall (Heimpel *et al*., 1960, Percy *et al*., 1983). *In vitro* experiments with cell lines have confirmed these observation Murphy *et al*. (1976), Nishiitsuji *et al*. (1979), Ebersold *et al*. (1980), McCarthy *et al*. (1988), Mathavan *et al*. (1989), Knowles *et al*. (1987). The binding of *Bacillus thuringiensis* toxin to brush border membrane vesicles (BBMVs) from different lepidopteran species, has been reported (Hofmann *et al*., 1988, Van Rie *et al*., 1989,1990). The activated toxin bind with high affinity to receptors on the apical membrane, and this is followed by insertion of the toxin into the epithelial membrane and is followed by toxin oligomerization and the formation of a pore, which is resulted in osmotic imbalance (Gill *et al*., 1992; Chow *et al*., 1989; Walter *et al*., 1994, Slatin *et al*., 1990; Tabashnik *et al*., 1994) Insect mortality occures several hours to days after ingestion of the toxin. The noctuid *Helicoverpa armigera*, an important agricultural pest, is susceptible to Bt. toxins, such as Cry 1Ac.
In this report we have compared the role of GalNAc in the binding of a pure CryIAc δ-endotoxin to receptor from Lepidopteran larvae *Helicoverpa armigera* and whether Gal NAc was involved in the interaction of the toxin with its natural target tisssue, the brush border membrane of the gut of susceptible insect we have employed a ligand blotting technique to visualize the effect of GalNAc on the binding properties of CryIAc proteins to Receptor in brush border membranes (Hofte & Whiteley, 1989) . In this paper the term receptor refers to a toxin binding molecule in an insect membrane.It does not imply a function, as the role of receptors in the mode of action of *Bacillus thuringiensis* toxin is not yet understood.
Proteolysis of receptor protein to check the stability of ~120kDa and ~70kDa subunits of CryIAc receptor by the addition of proteases was also done.

Materials and Methods

Chemicals were purchased from sigma. Acrylamide, N, N-methylene-Bis-acrylamide sodium dodecyle sulphate (SDS), glycine, Trition X-100. SDS molecular weight protein marker, polyvinylidene difluoride membrane and brad-ford protein assay reagents were from Bio-Rad. *H.armigera*. Larvae were obtained from CAMB insectory.

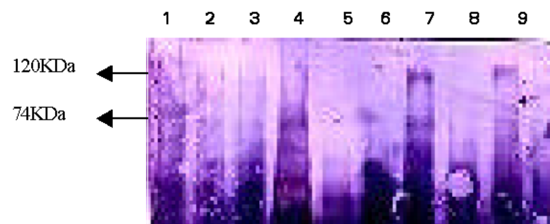

Fig. 1: Ligand Blot treated with GalNAc. Lane1. HMW marker, Lane 4. Receptor+trypsin, Lane5, Receptor+Proteinase-k, Lane6. Receptor+pronase, Lane7,9. Receptor

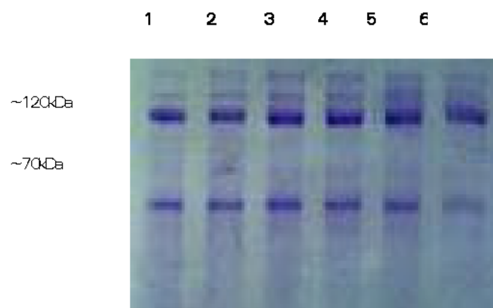

Fig. 2: Purified receptor protein solubilized in solutions containing different concentration of CHAPS, resolved on 8% SDS-PAGE. Lane1. 0.2%, Lane2. 0.4%, Lane3. 0.6% Lane4. 0.8% lane5. 1.0%, Lane6. 1.2%.

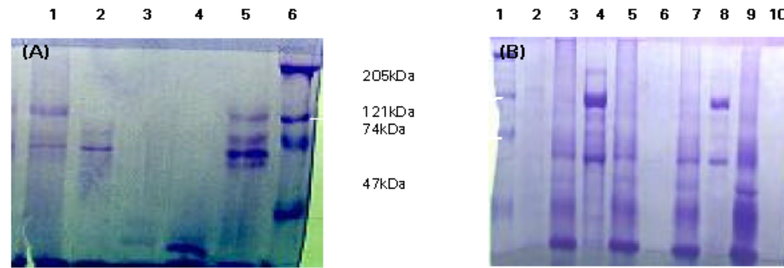

Fig. 3: a) Protease digestion of purified receptor of *H. armigera*, resoved on 8% SDS-PAGE. Lane1. Receptor, Lane2. Receptor+Trypsin, Lane3. Receptor+protenase-k, Lane4. Receptor+oribase, Lane6. HMW marker
b) Gut juice digestion of purified receptor of *H. armigera*, resoved on 8% SDS-PAGE. Lane1. HMW marker, Lane3,7. Receptor+Gut juice, Lane4,8. Untreated receptor, Lane9. Gut juice of *H. armigera*

Preparation of CryIAc: Bt. var kurstaki HD73 was grown in liquid medium (10g tryptone,5g yeast extract , 10g NaCl per liter) at 30°C until cell lysis. The spore/crystal mixture was harvested and washed twice with 1M NaCl, 0.1%. Triton X-100 crystals were solubilized at room temperature for 2hr in 50mM Na_2CO_3, pH 10.2, 10mM dithio-threitol. Solubilized crystal protein was treated with trypsin (20:1, w/w) at room temperature for 2h or overnight.

Preparation of brush border membrane vesicles: *H.armigera*. Larvae were reared on artificial diet, midguts were dissected from 5th instar larvae and either immediately used for preparing BBMV or stored at -70°C. BBMV, were made using the $MgCl_2$ precipitation method of Wolfersberger *et al*. (1987).

Solubilization and purification of BBMV protein: BBMVs, were solubilized in a buffer containing 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio)]-1-propane sulphonate, 20mM tris-HCl, pH 7.4, 150mM NaCl, 5 mM EDTA, 1 mM PMSF. Solubilized BBMVs were purified by column chromatography (Bio-gel, Anion exchange) and resolved on SDS-PAGE and non-SDS-PAGE.

Ligand Blotting: After resolvation of protein on 8% SDS-PAGE, Purified receptor protein transferred to PVDF membrane using a triglycine buffer as suggested by the manufacturer. After transfer, membrane were blocked by 5% dry skim Milk in TBST (20mM Tris-HCl, pH 7.4, 150mM NaCl) for 1 hr, three times washing with TBST buffer, then incubated one blot in 200mM N-acetyl galactonsamine (GalNAc) for 1 hr, other blot remained untreated, results shown in Fig. 1.
After washing with TBST buffer, incubated both blot in cryIAc toxin. Cry 1Ac toxin bound to the nitrocellulose filter was first detected by sequential incubation with primary (rabbit antitoxin) antibody, followed by secondary (peroxidase-Cojugated goat anti-rabbit) antibody as described by knowles *et al*. (1991). The peroxidase color reaction was developed as described by Hawkes *et al*. (1982).

Protease digestion: Gut juices of *H.armigera* were obtained by squeezing 5th instar Larvae Receptor protein was digested with trypsin and gut juice of *H.armigera*., proteinase-K, pronase in a buffer containing 0.1% SDS, 0.125M Tris-HCl, 1mM EDTA, 10% Glycerol, for the 1hr incubation at 25°C. The reactions were stopped by adding sample buffer and heating at 98 °C for 3 min and loaded in 8% SDS-PAGE (Fig. 3A,3B).

Results and Discussion

One of the first step in the mechanism of action of Bt. toxin involved the binding of the toxin to a specific binding protein on the surface of the midgut cells of the susceptible insects. In the previous study, we have identified the proteins, which can recognize and bind ICPs from *Bacillus thuringiensis*. These binding proteins are located on the brush border membrane of midgut cells from *H.armigera* larvae. After SDS-PAGE and transfer of the protein to a nylon membrane a multi-step incubation, involving the binding of specific toxins to the immobilized membrane proteins and subsequent visualization with toxin specific monoclonal antibodies was used to identify putative toxin binding proteins.

Identification of Cry 1Ac toxin binding receptor and effect of GalNAc on binding proteins: Brush border membrane vesicles for *H.armigera* was purified according to the method of Wolfersberger *et al.* (1987) and solubilized using the zwitterionic detergent CHAPS Column chromatography, purified proteins resolved on SDS-PAGE and non-SDS-PAGE. In case of non-SDS-PAGE, protein has single band while in case of SDS-PAGE, it resolved in two subunits of ~120kDa and 70kDa as in Fig. 2.
After Gel electrophoresis, the proteins were blotted onto a polyvinylidene difluoride membranes. In ligand blotting using Cry 1Ac toxin, we observed that there is no effect of GalNAc on the binding of Cry 1Ac and receptor, so it seems that sugar GalNAc is not the part of receptor protein. Thus the important conclusion is that carbohydrate moiety does not seems to be involved in the binding of toxin to the membrane protein. (Garczyinski *et al*., 1991). In other words, no effect of GalNAc on toxin-vesicle interaction was observed in binding assay. This might mean that in this insect the receptor lacks GalNAc, or simply that GalNAc in solution can not block the receptor binding as in *Pieris brassicae*

Protease treatment of *H.armigera* receptor: To obtain more information about the fragment of the receptor protein *H.armegera*, receptor was treated with different proteases prior to gel electrophoresis. Fig. 3A shows the pattern of bands seen with non treated receptor and trypsin treated. Gut juices and trypsin gave same patterns which consisted only of 70kDa and which appeared trypsin and gut juices resistant while ~120 kDa was digested with both as in Fig. 3A and 3B. In contrast, after proteinase-K or pronase digestion, the receptor protein degraded to a large extent, only <40kDa band appeared on gel (Fig 3A).
The corresponding ligand blot demonstrated that treatment with trypsin and gut jucies even at higher concentration, had no influence on the binding of the toxin Cry 1Ac to the 70kDa protein. However treatment with proteinase-K and pronase,

completely abolished the binding of toxin. This result was expected, since the membrane proteins were derived from the insect midgut and thus have to be resistant to digestive enzymes. The binding of the toxin completely disappeared after treatment of receptor protein with proteinase-K and pronase, due to the degradation of ~120Kda and ~70Kda receptor protein subunits. The precise mechanism of Bt. toxicity is not known (Daniela *et al.*, 1999). The generally accepted model is that following toxin binding to a receptor protein or macromolecule, the toxin undergoes a conformational change that facilitates the toxin insertion into apical cell membrane of insect midgut columnar cells. This initial binding is then followed by oligomerization of the bound toxin.

The cation selective pore formed by this oligomer caused a disruption of osmotic balance in the midgut epithelial layer. This disruption of midgut function ultimately lead to the insect death.

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