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Proteomic identification of *Bacillus thuringiensis* subsp. *israelensis* toxin Cry4Ba binding proteins in midgut membranes from *Aedes* (*Stegomyia*) *aegypti* Linnaeus (Diptera, Culicidae) larvae

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ABSTRACT

Novel *Bacillus thuringiensis* subsp. *israelensis* (Bti) Cry4Ba toxin-binding proteins have been identified in gut brush border membranes of the *Aedes* (*Stegomyia*) *aegypti* mosquito larvae by combining 2-dimensional gel electrophoresis (2DE) and ligand blotting followed by protein identification using mass spectrometry and database searching. Three alkaline phosphatase isoforms and aminopeptidase were identified. Other Cry4Ba binding proteins identified include the putative lipid raft proteins flotillin and prohibitin, V-ATPase B subunit and actin. These identified proteins might play important roles in mediating the toxicity of Cry4Ba due to their location in the gut brush border membrane. Cadherin-type protein was not identified, although previously, we identified a midgut cadherin AgCad1 as a putative Cry4Ba receptor in *Anopheles gambiae* mosquito larvae [Hua, G., Zhang, R., Abdullah, M.A., Adang, M.J., 2008. *Anopheles gambiae* cadherin AgCad1 binds the Cry4Ba toxin of *Bacillus thuringiensis israelensis* and a fragment of AgCad1 synergizes toxicity. Biochemistry 47, 5101–5110]. Other identified proteins in this study that might have lesser roles include mitochondrial proteins such as ATP synthase subunits, mitochondrial processing peptidase and porin; which are likely contaminants from mitochondria and are not brush border membrane components. Trypsin-like serine protease was also identified as a protein that binds Cry4Ba. Identification of these toxin-binding proteins will lead to a better understanding of the mode of action of this toxin in mosquito.

1. Introduction

Aedes (*Stegomyia*) species vector dengue, yellow fever and chikungunya viral diseases. The challenge of controlling this medically important vector is compounded by emerging resistance to chemical pesticides. Controlling the larval stage is critical to mosquito control programs and biopesticides based on *Bacillus thuringiensis* subsp. *israelensis* (Bti) are widely used for this purpose. Bti carries a plasmid that encodes the insecticidal proteins Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa and Cyt2Ba (Berry et al., 2002). Each of these insecticidal proteins is deposited in inclusions that become part of the parasporal crystal of Bti.

The action of Cry toxins is best studied for the lepidopteran-active Cry1A toxins. The intoxication process is a complex event involving Cry1A binding to receptors, pre-pore formation, membrane insertion, activation of biochemical pathways culminating in midgut cell lysis and insect mortality. According to the Bravo et al. (Bravo et al., 2004) model, Cry1A toxin-binding to cadherin induces an internal protease cleavage of toxin, toxin pre-pore formation and subsequent binding to glycosylphosphatidyl inositol (GPI) anchored aminopeptidases (APN) and alkaline phosphatase (ALP). These events probably take toxin to the membrane surface where glycolipids function as Cry1 toxin receptors (Griffitts et al., 2005). The role of receptors in Cry1A toxin action was recently reviewed (Pigott and Ellar, 2007).

Relative to Cry1A toxin interaction with midgut tissue, less is known about the identities and roles of Cry toxin receptors in dipteran larvae. A 65-kDa protein that bound Cry4B and Cry11A toxin was identified in the midgut of *Aedes* larvae (Buzdin et al., 2002). This protein was identified as a GPI-anchored alkaline phosphatase (Fernandez et al., 2006) and determined to be

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a functional receptor for Cry11Aa toxin. A second GPI-anchored protein, a 100-kDa aminopeptidase in *Anopheles* species was determined to specifically bind Cry11Ba and is considered a potential toxin receptor (Abdullah et al., 2006; Zhang et al., 2008). The Cyt toxin component of Bti crystals has limited toxicity itself, yet serves as a synergist enhancing Cry protein toxicity to mosquito larvae. This synergism occurs via a mechanism whereby Cyt toxin itself binds to brush border membrane and functions as a receptor for Cry11Aa (Perez et al., 2005). Evidence suggests that some of the same types of proteins which function as receptors in Cry1A toxins in lepidopteran larvae may be involved in Cry toxin action against mosquitoes.

Novel Cry1 binding proteins have been identified in brush border membranes of lepidopteran larvae by combining 2-dimensional gel electrophoresis (2DE) and ligand blotting followed by protein identification using mass spectrometry. Using this approach ALP was identified as a Cry1Ac binding protein in brush border of *Manduca sexta* and *Heliothis virescens* (Krishnamoorthy et al., 2007; McNall and Adang, 2003). This identification was validated in *H. virescens* when ALP was demonstrated as a functional receptor molecule and loss of the enzyme correlated with Bt resistance to Cry1Ac (Jurat-Fuentes and Adang, 2004). Additional Cry1Ac binding proteins in lepidopteran brush border preparations detected by 2DE ligand blots approach includes actin, aminopeptidase, vacuolar-ATPase subunit A and a desmocollin-like protein (Krishnamoorthy et al., 2007; McNall and Adang, 2003). A proteomics-based approach using differential-in-gel electrophoretic (DIGE) analysis quantified altered levels of specific proteins in Bt susceptible and resistant larvae of *Plodia interpunctella* (Candas et al., 2003). Those authors detected changes in the levels of APN, V-ATPase and an F_1F_0-ATPase in resistant larvae.

Following the same rationale above to identify novel toxin-binding proteins, in this study Cry4Ba binding proteins in the midgut proteome of *Aedes aegypti* were detected on blots of 2DE gels. Proteins that bound toxin on blots were identified from the corresponding protein in stained 2DE gels by mass spectrometry. The tryptic peptide patterns of 12 groups of toxin-binding proteins matched with high-confidence scores to 12 proteins in *A. aegypti* protein databases. ALP isoforms were predominant among the identified Cry4Ba binding proteins.

2. Materials and methods

2.1. Bacterial strain, toxin purification and ^{125}I-labeling

Escherichia coli DH5α harboring the *cry4Ba* gene was cultured to produce Cry4Ba inclusion bodies and activated toxin produced as previously described (Abdullah et al., 2003). Protein was quantified using the Bradford protein assay (Bradford, 1976) with BSA as standard. Purified Cry4Ba toxin (10 μg) was labeled with 5 μCi of $Na^{125}I$ (GE Healthcare) using the chloramine-T reagent according to Garczynski et al. (Garczynski et al., 1991). Labeled toxin was separated from free iodine by gel filtration on sephadex G-50 (Sigma) resulting in ^{125}I-Cry4Ba with a specific activity of 6 μCi/μg input toxin. Labeled toxin was stored at 4 °C for further use.

2.2. Mosquitoes

A. aegypti (UGAL strain) was maintained at 27 °C, 70–80% humidity with a photoperiod of 14L: 10D. Larvae were fed ground brewers yeast, lactalbumin and rat food-chow (1:1:1 ratio) daily for 6 days. Fourth instar larvae were collected on a nylon mesh and then washed thoroughly with deionized water to remove food particles and molted skin. Larvae were dried briefly on filter paper with gentle suction and stored at −80 °C until needed for brush border membrane fraction (BBMF) preparation.

2.3. BBMF preparation

Whole body homogenate was prepared by adding 4 g of frozen larvae to 16 ml ice cold homogenization buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5) containing 1 mM PMSF. Larvae were homogenized with 40 strokes of a glass–teflon homogenizer and BBMF isolated using the magnesium precipitation method according to Silva-Filha et al. (Silva-Filha et al., 1997). The final BBMF protein concentration was determined as above. Enrichment of the brush border marker enzymes ALP and APN was determined using leucine-*ρ*-nitroanalide and *ρ*-nitrophenyl phosphate as substrates, respectively (Terra and Ferreira, 1994). Enrichment in the final BBMF preparation relative to the initial homogenate ranged from 5- to 7-fold for APN activity and 8- to 10-fold for ALP activity.

2.4. Preparation of protein samples for 2DE

Proteins were precipitated from BBMF (100 μg protein) using Plus-One cleanup kit (GE Healthcare) according to the manufacturer's instruction. The final precipitant in a microfuge tube was dissolved in 100 μl solubilization buffer [7 M urea, 2 M thiourea, 2% CHAPS, 2% caprylyl sulfobetaine, 18 mM DTT, 2% carrier ampholytes (pH 3–10 or 4–7, Plus-one; GE Healthcare). To increase solubilization, the microfuge tube containing the BBMF protein sample was floated in a sonicating water bath containing cold water, replacing the water every 5 min to avoid potential artifacts created by urea in warm water. Protein samples were then centrifuged at 13,000 g for 10 min at room temperature; the supernatant was collected and protein amount determined with a 2D quantification kit (GE Healthcare).

2.5. IEF

For IEF, solubilized BBMF (60–80 μg protein) in 150 μl rehydration solution (solubilization buffer plus 0.002%(w/v) of bromophenol blue) were loaded onto a 13 cm immobilized pH gradient (IPG) strip (pH 4–7 or pH 3–10, nonlinear, GE Healthcare) and overlaid with 2 ml of plus-one IPG strip cover fluid (GE Healthcare). After 15–17 h passive rehydration, IEF was performed on a Multiphor-II flatbed system according to the manufacturer's guidelines (2D Electrophoresis Principles and Methods, GE Healthcare) with an additional initial step of 30 min low voltage (150 V) step to facilitate improved entry of high molecular-sized proteins into the IPG strip. Strips were stored at −80 °C or used directly in the equilibration step. Strips were first equilibrated for 15 min in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS and 0.002%(w/v) of bromophenol blue) containing 1% DTT (w/v). The strips were then equilibrated in equilibration buffer containing 4% iodoacetamide (w/v).

2.6. Second-dimensional electrophoresis

SDS-PAGE was performed on an Ettan DALT*six* large vertical electrophoresis system (GE Healthcare). The equilibrated IPG strip was transferred onto a 12% SDS-PAGE gel. Electrophoresis was carried out at 20 °C using a three-phase program: 5 mA for 30 min, 10 mA for 60 min, and 15 mA until the dye front was near the bottom of the gel. Proteins were stained with Deep Purple stain (GE Healthcare) or transferred overnight onto a PVDF (polyvinylidene difluoride; Immobilon P, Millipore) membrane. Stained gels and gels for ligand blotting were run in parallel.