Saudi Journal of Biological Sciences xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

First report of detection of the putative receptor of *Bacillus thuringiensis* toxin Vip3Aa from black cutworm (*Agrotis ipsilon*)

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ARTICLE INFO

Article history:
Received 5 November 2017
Revised 17 December 2017
Accepted 15 January 2018
Available online xxxx

Keywords:
Receptor
vip3Aa
Bacillus thuringiensis
BBMV

ABSTRACT

Black cutworm (BCW) *Agrotis ipsilon*, an economically important lepidopteran insect, has attracted a great attention. *Bacillus thuringiensis* (Bt) is spore forming soil bacteria and is an excellent environment-friendly approach for the control of phytophagous and disease-transmitting insects. In fact, bio-pesticide formulations and insect resistant transgenic plants based on the bacterium Bt delta-endotoxin have attracted worldwide attention as a safer alternative to harmful chemical pesticides. The major objective of the current study was to understand the mechanism of interaction of Bt toxin with its receptor molecule (s). The investigation involved the isolation, identification, and characterization of a putative receptor – vip3Aa. In addition, the kinetics of vip toxin binding to its receptor molecule was also studied. The present data suggest that Vip3Aa toxin bound specifically with high affinity to a 48-kDa protein present at the brush border membrane vesicles (BBMV) prepared from the midgut epithelial cells of BCW larvae.

1. Introduction

Bacillus thuringiensis (Bt) technology has been proved universally as a valuable alternative to the conventional synthetic insecticides. The use of Bt as a microbial insecticide offers several advantages over the harmful chemical insecticides. The species-specific action of the insecticidal crystal (Cry) proteins (ICPs) or vegetative insecticidal proteins (Vips) makes them safe to non-target or beneficial insects, vertebrates, environment, and the users (El-Menofy et al., 2014; Osman et al., 2015). The Bt toxins become active only after ingestion by insects. The alkaline pH and proteolytic enzymes present in the midgut solubilize the endotoxin protein and convert it to an active toxic form. These toxic compounds disrupt the midgut epithelial cells resulting in cessation of feeding and consequent insect mortality. The first step in the insecticidal action of Bt toxin is the recognition of target molecules inside the midgut of the insect host (Assaeedi et al., 2011; Abulreesh et al., 2012). Existing reports indicate that the target receptor molecules are located within or on the membrane surface of epithelial cells of the midgut of a susceptible insect. Although, the specific binding of these toxins to the brush border membrane vesicles (BBMV) of midgut has been reported for Cry toxin proteins (Hofmann et al., 1988; Osman, 2012), little is known about the molecular attributes of the toxin binding proteins of Vip 3Aa toxin proteins.

1.1. Insect receptors for cry toxins

The first step in the action mechanism of Bt toxins is the recognition and binding to the high-affinity site of specific receptors on the brush border surface of the midgut of susceptible insect host. Several immunological methods have been used to identify Bt

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2018.01.006
1319-562X/

Please cite this article in press as: Osman, G.H., et al. First report of detection of the putative receptor of *Bacillus thuringiensis* toxin Vip3Aa from black cutworm (*Agrotis ipsilon*). Saudi Journal of Biological Sciences (2018), https://doi.org/10.1016/j.sjbs.2018.01.006

toxin targets, which apparently reside in the luminal brush border of the larval midgut (Bravo et al., 2011; El-Ghareeb et al., 2012). The *B. thuringiensis* toxin receptor, BT-R_1, present in the midgut brush border membrane was purified and respective cDNA was cloned from the larvae of lepidopteran *Manduca sexta* (Vadlamudi et al., 1993, 1995). As determined by SDS-PAGE, the molecular mass of BT-R_1 is approximately 210 kDa with an isoelectric point of ~5.5. The BT-R_1 cDNA encodes a polypeptide with a predicted molecular mass of 192 kDa. The BT-R_1 is a cadherin-like glycoprotein. Recently, two other cadherin-like proteins of 175 and 180 kDa, showing amino acid sequence homology to BT-R_1 have also been identified, purified, and cloned from the lepidopteran *Bombyx mori* (silkworm) (Nagamatsu et al., 1998; Ihara et al., 1998). Moreover, a 210-kDa protein from the lepidopteran *Lymantria dispar* (Gypsy moth) was reported to bind Cry1Aa and Cry1Ab in ligand blot studies Valaitis et al., 1997. A 175-kDa protein from *B. mori* binds specifically to Cry1Aa with high affinity. Anti-BT-R antibodies were capable of reducing toxicity *in vivo* as well as blocking binding to the membrane vesicles *in vitro*. The study showed that the 175-kDa BT-R receptor was found only in the gut. Similarly, Midboe et al., (2001) also detected BT-R_1 exclusively in the midgut of *M. sexta*. Conserved features shared between protocadherins and the cadherin-like proteins along with the amino acid sequence homology between the toxin-binding proteins suggest that these integral midgut membrane proteins represent a novel class of invertebrate protocadherins and therefore were labeled as epithelial invertebrate protocadherins (E-IVPs). Recently, the midgut epithelial cells of *M. sexta* were shown to possess a putative Cry 1Ac toxin receptor, which is a 120-kDa glycoprotein (Yaoi et al., 1997).

1.2. Insect resistance mechanisms

In order to understand the mechanism of development of resistance, it is imperative to examine the fate of crystal proteins within the midgut of insects. In the first step, the crystal protein is solubilized and proteolytically degraded by proteinases in the gut of susceptible insect host. Consequently, a 60–70 kDa core proteinase-resistant toxin is produced. The toxin eventually interacts with a specific receptor(s) presumably creating pores in the midgut cell membranes and causing an ionic imbalance. This cascade of events may lead to septicemia in the insect. Given the multiple steps involved in the processing of the Cry protein to form an active toxin, it is not very surprising that the insect populations might develop various means of resistance against the toxin. Any alteration in insect gut physiology or biochemical system could disrupt the consecutive process and may result in toxin resistance and, therefore, several mechanisms of insect resistance to Bt toxins have been proposed (Osman et al., 2013). The current study was aimed at the identification and molecular characterization of Bt toxin Vip3Aa and its putative receptor in black cutworm, *Agrotis ipsilon*, a lepidopteran corn pest.

2. Materials and methods

2.1. Preparation of Brush Border Membrane Vesicles (BBMV)

The early 4th instar larvae of black cutworm were placed on ice for 1 h and were dissected to remove the midguts. Then, BBMV were prepared from the midgut tissues by following differential magnesium precipitation method (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 midgut tissue was homogenized in nine volume of buffer "A" (300 mM Mannitol, 5 mM EGTA and 17 mM Tris–HCl, pH 7.5) using a glass Teflon homogenizer (9 strokes up and down at 3000 rpm). An equal volume of 24 mM $MgCl_2$ solution was added to the homogenate and further re-homogenized. The homogenate was incubated on ice for 15 min and centrifuged at 4500 rpm for 15 min at 4 °C. The supernatant was transferred to a fresh tube and again centrifuged at 31,000 *g* for 30 min at 4 °C. The supernatant was discarded and the pellet was re-suspended in half the volume of buffer "A" and this step was repeated. The final pellet was re-suspended in buffer "A" containing protease inhibitors, flash frozen in liquid nitrogen, and stored at −85 °C.

2.2. Protein analysis of BBMV by SDS-PAGE

The integument or the extracted fluid was dissolved in sample buffer (0.06 M Tris HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and bromophenol blue). The samples were heated for 5 min in boiling water bath and separated on 10% polyacrylamide gel as described by Laemmli (1970). The gels were stained with 0.1% Coomassie brilliant blue R–250 and de-stained in 7% (v/v) acetic acid containing 50% methanol.

2.3. Protein iodination

Purified Vip3Aa toxin used in this work was radioiodinated using the chloramine-T method (Hunter and Greenwood, 1962) with ^{125}I-Na (NEN Dupont, Billerica MA) Carrier free. About 10 μg of toxin was mixed with 5 μL of ^{125}I-Na (approximately 0.5 mCi) in 100 μL of $NaHPO_4$ buffer (0.5 M, pH 7.4) with 25 μL of Chloramine-T (4 mg/mL). The reaction mixture was vortexed for 20–25 s 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 a Sephadex G–50 column equilibrated with PBS containing 10 mg/mL BSA.

2.4. Toxin binding assays

Homologous competition inhibition binding assays were performed as described by Keeton and Bulla (1998). About 25 μg of BBMV were incubated with 1.2 nM^{125}I-Vip3Aa toxin in the presence of different concentrations (0–1000 nM) of appropriate unlabeled homologous toxin (Vip3Aa) in 100 μL of binding buffer (PBS/0.2% BSA) at 25 °C for 30 min. The radiolabeled and unlabeled toxins were mixed together before adding to the BBMV. The unbound toxins were separated from BBMV-bound toxin by centrifugation at 14,000*g* for 10 min. The pellet containing bound toxin was washed three times with ice-cold binding buffer by gentle vortexing and radioactivity in the resultant pellet was measured using a Beckman Gamma 5500 counter. The binding data were analyzed by using the PRISM program (GraphPad Software Inc., San Diego, CA, USA).

2.5. Radioligand blotting

About 200 μg of BBMV protein was solubilized, separated by 7.5% SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane as described by Francis and Bulla (1997). The 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 h at 25 °C. Blocking buffer was removed and the membranes were incubated for 2 h at 25 °C in an equal volume of fresh blocking buffer containing 2×10^5 cpm/mL (1–1.25 nM) of ^{125}I-Vip3Aa toxin either in the presence or absence of unlabeled toxins. Finally, the membranes were washed thrice with fresh blocking buffer for 10 min each, rinsed once with TBS, dried, and exposed to Kodak X-ray film at 80 °C.

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