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Midgut aminopeptidase N isoforms from *Ostrinia nubilalis*: Activity characterization and differential binding to Cry1Ab and Cry1Fa proteins from *Bacillus thuringiensis*

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ABSTRACT

Aminopeptidase N (APN) isoforms from Lepidoptera are known for their involvement in the mode of action of insecticidal Cry proteins from *Bacillus thuringiensis*. These enzymes belong to a protein family with at least eight different members that are expressed simultaneously in the midgut of lepidopteran larvae. Here, we focus on the characterization of the APNs from *Ostrinia nubilalis* (*On*APNs) to identify potential Cry receptors. We expressed *On*APNs in insect cells using a baculovirus system and analyzed their enzymatic activity by probing substrate specificity and inhibitor susceptibility. The interaction with Cry1Ab and Cry1Fa proteins (both found in transgenic insect-resistant maize) was evaluated by ligand blot assays and immunocytochemistry. Ligand blots of brush border membrane proteins showed that both Cry proteins bound mainly to a 150 kDa-band, in which *On*APNs were greatly represented. Binding analysis of Cry proteins to the cell-expressed *On*APNs showed that *On*APN1 interacted with both Cry1Ab and Cry1Fa, whereas *On*APN3a and *On*APN8 only bound to Cry1Fa. Two isoforms, *On*APN2 and *On*APN3b, did not interact with any of these two proteins. This work provides the first evidence of a differential role of *On*APN isoforms in the mode of action of Cry proteins in *O. nubilalis*.

1. Introduction

Aminopeptidase N (APN) isoforms are metalloenzymes widely present in the insect midgut that cleave neutral amino acids from the N-terminus of peptides (Terra and Ferreira, 2012). They belong to the M1 metalloprotease family and their activity is dependent of zinc cations (Hooper, 1994). The main role of APNs in insect midgut is protein digestion: they hydrolyze, in conjunction with carboxypeptidases, oligopeptides resulting from the action of endoproteinases (Terra and Ferreira, 2012). In lepidopteran species, several isoforms of APNs have been described to be expressed simultaneously in the larval midgut (Angelucci et al., 2008; Crava et al., 2010; Khajuria et al., 2011; Simpson et al., 2008; Wang et al., 2005). Genes coding for these isoforms have been found organized in a single cluster in several lepidopteran genomes (Baxter et al., 2008; Crava et al., 2010; d'Alençon et al., 2010). All the lepidopteran APNs share the following characteristics: they have a molecular mass of about 100–150 kDa, the signal peptide is always located on the C-terminal end of the protein and a glycosylphosphatidylinositol (GPI)-anchor signal is present on the N-terminal end (Pigott and Ellar, 2007). In fact, the main feature of the lepidopteran APNs is that they are linked to the apical membrane of the midgut epithelium cells by a GPI-anchor (Lu and Adang, 1996; Agrawal et al., 2002). Due to this anchor, APNs are located in membrane regions called lipid rafts, rich in cholesterol and sphingolipids (Paulick and Bertozzi, 2008; Zhuang et al., 2002).

In addition to their role in digestion, APNs have been extensively studied for their interactions with the insecticidal Cry proteins produced during the sporulation of *Bacillus thuringiensis* (Bt) (Likitvivatanavong et al., 2011; Pigott and Ellar, 2007). Cry proteins are widely used in the control of insect pests, either as Bt-based formulates or expressed in transgenic plants resistant to insects (Bt crops) (Ferré et al., 2008). These toxins act by ingestion, leading to the insect death through a not yet fully understood mode of action (Vachon et al., 2012). It is commonly accepted that, in

Abbreviations: Ala-*p*-NA, alanine-*p*-nitroanilide; ALP, alkaline phosphatase; APN, aminopeptidase N; BSA, bovine serum albumin; BBMV, brush border membrane vesicles; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; hpi, hours post infection; O/N, overnight; LC, liquid chromatography; leu-*p*-NA, leucine-*p*-nitroanilide; lys-*p*-NA, lysine-*p*-nitroanilide; MOI, multiplicity of infection; MS, mass spectrometry; RT, room temperature.

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Lepidoptera, Cry proteins are solubilized in the larval midgut and then proteolytically converted into activated toxins which cross the peritrophic membrane. Subsequently, Cry activated proteins interact with one or more receptors located on the brush border membrane of the epithelial cells. According to the model proposed by Bravo's group (Pardo-López et al., 2012), these interactions allow oligomerization of Cry proteins with further binding to a second membrane receptor that facilitates the insertion of the oligomer into the membrane and the formation of poorly selective pores which lead to the insect death by cell colloid-osmotic lysis. In the model proposed by Bulla's group (Ibrahim et al., 2010), specific binding of Cry proteins to the midgut receptor causes death by activation of an intracellular cascade signal. A lot of efforts have been done to identify the functional receptors of Cry proteins in insects and two main categories of proteins have been described: a cadherin-like protein, and GPI-anchored proteins such as APNs and alkaline phosphatases (ALPs) (Likitvivatanavong et al., 2011; Pigott and Ellar, 2007). The Bravo's group model claims that both cadherin and GPI-anchored proteins are involved in the insertion of the toxin oligomer in the membrane (Pardo-López et al., 2012) whereas Bulla' group's experiments indicated that only cadherin is essential for the Cry toxic action (Ibrahim et al., 2010). Recently, a third molecule that has never been considered in the proposed models has been described as a functional receptor for Cry proteins, the ABCC2 transporter (Tanaka et al., 2013).

An APN protein was first proposed as a Cry receptor in *Manduca sexta* (Knight et al., 1994). Since then, many other APNs from different lepidopteran species have been isolated (Masson et al., 1995; Lee et al., 1996; Shitomi et al., 2006), or cloned and expressed, to show their ability to bind Cry proteins (Garner et al., 1999; Agrawal et al., 2002; Banks et al., 2003; Sivakumar et al., 2007; Pigott and Ellar, 2007; Chen et al., 2009). To date, at least eight different classes of APNs have been defined in Lepidoptera (Crava et al., 2010), and binding to Cry proteins was demonstrated for at least one member of six of these classes (Budatha et al., 2007; Pigott and Ellar, 2007). All these binding studies have been carried out with different lepidopteran species and with different techniques and, in most cases, they have focused just on one or two APN isoforms. Although the overall results confirm the role of APNs as Cry binding proteins, they do not shed light on which isoforms are the ones involved in the interaction with Cry proteins within each lepidopteran species. Further evidences of the involvement of the APNs in the mode of action of Cry proteins come from studies aimed to characterize lepidopteran strains resistant to different Cry proteins. In some cases, reductions of APN expression or mutations in APN coding sequences have been proposed as responsible of resistance (Herrero et al., 2005; Tiewsiri and Wang, 2011; Yang et al., 2010; Zhang et al., 2009).

Ostrinia nubilalis Hübner, commonly known as European corn borer, is one of the most damaging lepidopteran pests of corn in temperate climates. Nowadays, it is efficiently controlled by transgenic corn expressing the insecticidal Bt protein Cry1Ab or a combination of Cry1Ab and Cry1Fa (Siegfried and Hellmich, 2012). The aim of the present study was to determine the APN isoforms responsible for binding of Cry1Ab and Cry1Fa proteins in the *O. nubilalis* midgut. To achieve this goal, five *O. nubilalis* APN isoforms (*On*APNs) have been expressed in Sf21 cells, first to characterize them by substrate specificity and inhibitor susceptibility, and second to assess their ability to bind Cry1Ab and Cry1Fa proteins.

2. Experimental

2.1. Purification of Cry1Ab and Cry1Fa proteins

Cry1Ab and CryFa proteins were obtained from recombinant *Escherichia coli* strains kindly provided by Dr. R. de Maagd (Wageningen University, The Netherlands). Protein expression, purification and trypsin activation were carried out as described by Herrero et al. (2004). The activated proteins were analyzed by 12% SDS-PAGE and quantified by densitometry using the software 1-D Manager 2.0 (TDI).

Activated Cry proteins were purified by anion-exchange chromatography using an ÄKTA Explorer system (GE Healthcare). To this purpose, they were dialyzed overnight (O/N) at 4 °C against 20 mM Tris/HCl buffer, pH 8.6. The dialyzed solution was centrifuged, filtered and loaded on a 5 ml HiTrap Q HP anion-exchange column (GE Healthcare) previously equilibrated with the above mentioned buffer. Cry proteins were eluted from the column by a NaCl gradient. Fractions were individually analyzed by 12% SDS-PAGE. Fractions containing Cry proteins were pooled, quantified by Bradford assay and stored at −20 °C until use.

2.2. Brush border membrane vesicles (BBMV) preparation

Midguts were dissected from fifth-instar *O. nubilalis* larvae, washed in ice-cold MET buffer (250 mM mannitol, 17 mM Tris−HCl, 5 mM EGTA, pH 7.5) containing 1 mM PMSF, then frozen in liquid nitrogen and kept at −80 °C until use. BBMV were prepared following the $MgCl_2$ differential precipitation method described by Wolfersberger et al. (1987) with minor modifications. Midguts were homogenized with a Potter homogenizer in ten volumes of MET buffer with 1 mM PMSF and the Roche Protease Inhibitor Cocktail (Roche). The homogenate was filtered through three layers of gauze and one volume of 24 mM $MgCl_2$ was added to the filtrate. The mixture was gently mixed, incubated for 15 min on ice and centrifuged at 2500 *g* for 10 min at 4 °C. The supernatant was transferred to a new centrifuge tube and centrifuged at 48,000 *g* for 20 min at 4 °C. The pellet was resuspended in half the volume of MET buffer initially used and homogenized again with the Potter homogeneizer. Incubation with $MgCl_2$ and subsequent centrifugations were repeated as described above. The final pellet was resuspended in MET buffer diluted 1:1 in water and the total protein concentration was quantified by Bradford assay. Freshly prepared BBMV were directly loaded on 10% SDS-PAGE for ligand blot assays and protein identification.

2.3. Ligand blot and protein identification by LC-MS/MS

Replicate samples of equal amounts of BBMV proteins (80 μg/well) were loaded on 10% SDS-PAGE. After electrophoresis, the replicated lanes containing separated BBMV proteins were excised from the gel. One of the lanes was stained with Comassie blue for protein identification while the others were employed for transferring proteins to a nitrocellulose membrane Amersham Hybond™ ECL (GE Healthcare) using a semi-dry transfer unit Amersham Biosciences TE70 (GE Healthcare) for ligand blot assays. To this purpose, membranes with transferred proteins were blocked O/N at 4 °C with PBS supplemented with 0.1% Tween 20 (PBS-T) and containing 3% of Membrane Blocking Agent (GE Healthcare). Then, membranes were probed with 75 nM activated Cry1Ab or Cry1Fa protein in PBS for 2.5 h at room temperature (RT) and subsequently washed three times for 5 min in PBS-T. Cry1Ab or Cry1Fa bound to BBMV proteins were detected by incubation of the membrane with anti-Cry1Ab/Cry1Ac or anti-Cry1Fa monoclonal mouse antibodies (Abraxis) (1:5000 in PBS-T) followed by washing with PBS-T and 1 h incubation with goat anti-mouse horseradish peroxidase conjugated antibody (Sigma) (1:5000 or 1:10000 in PBS-T for Cry1Ab or Cry1Fa detection, respectively). Detection was performed with the Amersham ECL Western Blotting Analysis System (GE Healthcare) following the manufacturer's instructions and using an ImageQuant LAS4000 imaging system (GE Healthcare).