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Proteolytic processing of *Bacillus thuringiensis* Vip3A proteins by two *Spodoptera* species

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ABSTRACT

Vip3 proteins have been described to be secreted by *Bacillus thuringiensis* during the vegetative growth phase and to display a broad insecticidal spectrum against lepidopteran larvae. Vip3Aa protoxin has been reported to be significantly more toxic to *Spodoptera frugiperda* than to *Spodoptera exigua* and differences in the midgut processing have been proposed to be responsible. In contrast, we have found that Vip3Ae is essentially equally toxic against these two species. Proteolysis experiments were performed to study the stability of Vip3A proteins to peptidase digestion and to see whether the differences found could explain differences in toxicity against these two *Spodoptera* species. It was found that activation of the protoxin form and degradation of the 62 kDa band took place at lower concentrations of trypsin when using Vip3Aa than when using Vip3Ae. The opposite effect was observed for chymotrypsin. Vip3Aa and Vip3Ae protoxins were effectively processed by midgut content extracts from the two *Spodoptera* species and the proteolytic activation did not produce a peptidase resistant core under these in *vitro* conditions. Digestion experiments performed with *S. frugiperda* chromatography-purified digestive serine peptidases showed that the degradation of the Vip3A toxins active core is mainly due to the action of cationic chymotrypsin-like peptidase. Although the digestion patterns of Vip3A proteins do not always correlate with toxicity, the peptidase stability of the 62 kDa core is in agreement with intraspecific differences of toxicity of the Vip3Aa protein.

1. Introduction

Vegetative insecticidal proteins (Vip) are produced by *Bacillus thuringiensis* (Bt) and secreted during the vegetative phase of growth. Vip1 and Vip2 proteins act as binary toxins and are toxic to coleopterans (Shi et al., 2004) and aphids (Sattar and Maiti, 2011), andVip3 proteins are toxic to lepidopterans (Estruch et al., 1996; Bhalla et al., 2005; Fang et al., 2007; Liu et al., 2007; Hernández-Rodríguez et al., 2009; Yu et al., 2010) (for a summary of toxicities, see van Frankenhuyzen and Nystrom, 2002 and Milne et al., 2008). Vip proteins are structurally different from *B. thuringiensis* Cry and Cyt δ-endotoxins produced during the late growth phase, and share no sequence homology with them. This is reflected in their different targets (Lee et al., 2003; Abdelkefi-Mesrati et al., 2009; Ben Hamadou-Carfi et al., 2013), which makes Vip proteins very promising to combat resistance in target pest insects, in combination or rotation with Cry proteins. Recombinant Bt strains transformed by *vip3A* genes in laboratory experiments showed more than 10-fold increase of the oral toxicity against *Spodoptera exigua* and *S. littoralis* (Sellami et al., 2011). Vip3A has already been incorporated into transgenic cotton and maize to confer additional resistance against a wide range of lepidopteran insect pests (Raybould and Quemada, 2010).

Most of the few studies carried out so far on the mode of action of Vip3 proteins have been done with Vip3Aa. Vip3Aa is synthesized by *B. thuringiensis* as a full length protein of approximately 90 kDa. Upon ingestion, the protoxin undergoes proteolytic processing by larvae midgut lumen peptidases, yielding an approximately 62 kDa active toxin form that is able to cross the peritrophic membrane and bind to specific receptors on the brush border membrane of midgut epithelial cells (Lee et al., 2003, 2006; Sena et al., 2009; Abdelkefi-Mesrati et al., 2009). Similarly as with Cry toxins, all these steps finally lead to the disruption of midgut epithelial cells and death of the insect (Yu et al., 1997; Liu et al., 2011; Abdelkefi-Mesrati et al., 2011a,b). Although the model resembles that of δ-endotoxins involving a series of sequential steps finally leading to the formation of ion channels in midgut epithelial cells,

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Vip3Aa binds to membrane receptors different from those of Cry proteins (Lee et al., 2003, 2006; Abdelkefi-Mesrati et al., 2009; Sena et al., 2009; Liu et al., 2011).

Digestion of food proteins in lepidopteran larvae relies on extracellular serine peptidases contributing to about 95% of the total digestive proteolytic activity. Lepidopteran serine peptidases have high pH optimum (Terra and Ferreira, 1994; Srinivasan et al., 2006), which perfectly fits with the alkaline conditions of the lepidopteran midgut, and they are present in a multitude of different isoforms. For example, *Helicoverpa armigera* gut contains about 20 different types of active serine peptidase isoforms at any given moment (Christeller et al., 1992; Terra and Ferreira, 1994; Johnston et al., 1995; Bown et al., 1997; Gatehouse et al., 1997; Patankar et al., 2001; Lopes et al., 2006, 2009; Srinivasan et al., 2006). Among this array of isoforms, lepidopteran midgut lumen serine peptidases are mainly characterized by trypsin- and chymotrypsin-like activities and, to a lesser extent, by elastase activity. In *Spodoptera* species, trypsin, chymotrypsin, and elastase account respectively for 7%, 85% and 1% of the digestive peptidases activity, while exopeptidases associated to the brush border of the midgut epithelial cells account for the residual 6% (Srinivasan et al., 2006).

The role of lepidopteran serine peptidases in the pathology of Cry toxins has been extensively studied: Cry proteins are synthesized as inactive precursors, or protoxins, which are further processed into active toxins by both trypsins and chymotrypsins in the insect midgut (Rukmini et al., 2000). Such proteolytic activation plays an important role not only in the formation of the active toxin that is able to bind to epithelial midgut receptors, but has also been reported to be involved in the host range specificity of the different toxins (Haider et al., 1986, 1989; Haider and Ellar, 1989; Milne et al., 1990; Rukmini et al., 2000) and the development of Cry resistance in some insect species (Oppert et al., 1997; Ferré and Van Rie, 2002; Li et al., 2007; Ferré et al., 2008). Despite the many studies focusing on the interaction between luminal serine peptidases and Cry toxins in regard to the mode of action and resistance development, the role of insect midgut peptidases in Vip pathogenicity has not received much attention yet. In a previous study with Vip3Aa, it was suggested that differences in susceptibility between species might be explained by differences in the processing of the protoxin by midgut peptidases (Abdelkefi-Mesrati et al., 2011b).

In a previous publication, we described a marked difference in susceptibility to the Vip3Aa protoxin between *Spodoptera frugiperda* and *S. exigua* (Chakroun et al., 2012). In contrast, in the present work we have found that Vip3Ae protoxin is essentially equally toxic against these two species. Therefore, we have performed a detailed study of Vip3A protein proteolysis to establish whether the midgut digestive proteolytic complex is involved in defining the susceptibility differences in these two *Spodoptera* species to Vip3A proteins.

2. Materials and methods

2.1. Insects

The laboratory strain of *S. exigua* was kindly supplied by M. López-Ferber, INRA (St. Christol les Alés, France) and the laboratory strain of *S. frugiperda* was provided by P. Caballero, Universidad Pública de Navarra (Pamplona, Spain). Both strains were reared on artificial diet (Moar et al., 1995) at 25 ± 2 °C, with a relative humidity of 65 ± 5% and a photoperiod of 16:8 (light/dark).

2.2. Preparation and purification of Vip3Aa and Vip3Ae proteins

The gene coding for the Vip3Ae protein (NCBI accession No. CAI43277) was kindly provided by Bayer BioScience N.V. (Ghent, Belgium), cloned in *Escherichia coli* WK6. Before expression, the gene had been modified to contain a His-tag sequence at the N-terminus of the protein to facilitate purification. Recombinant *E. coli* was grown at 37 °C in LB medium with 100 μg/ml ampicillin and Vip3Ae expression was induced with 1 mM isopropyl-β-D-thiogalacto pyranoside (IPTG). The culture was then centrifuged at 5000*g* for 15 min at 4 °C and the pellet was resuspended in lysis buffer (20 mM phosphate buffer pH 7.4, 500 mM NaCl, 3 mg/ml lysozyme, 10 μg/ml DNAse and 100 μM phenylmethylsulfonyl fluoride). After 30 min of incubation at 37 °C, the lysate was sonicated, stirred for 30 min at 4 °C and centrifuged (12,000*g* for 30 min at 4 °C). The Vip3Ae present in the supernatant was precipitated by 70% ammonium sulfate and then resuspended in PBS buffer and used for the bioassays. For proteolysis assays, the toxin was purified from the culture supernatant by means of immobilized metal ion absorption chromatography (IMAC) on Hi-Trap chelating HP column (GE Healthcare) charged with Ni^{2+}. Briefly, the supernatant was loaded onto columns equilibrated with 50 mM phosphate buffer, pH 8.0, containing10 mM imidazole. After washing with 50 mM phosphate buffer, pH 8.0, with 40 mM imidazole, the bound proteins were eluted with the same buffer containing100 mM imidazole. Fractions were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) to a final concentration of 5 mM. Finally, the Vip3Ae protoxin-containing fractions were dialyzed overnight at 4 °C against 20 mM Tris–HCl, pH 8.6, 150 mM NaCl and 5 mM EDTA.

The *vip3Aa16* gene, originally from *B. thuringiensis* subsp. *kurstaki* strain BUPM95, was kindly provided by the Laboratory of Biopesticides (Centre de Biotechnologie de Sfax, Tunisia), and had been cloned in pET vector in fusion with a His-tag, and then subcloned into BL21 *E. coli* strain for protein expression (Abdelkefi-Mesrati et al., 2009). *E. coli* BL21 was grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. Induction was done with 0.4 mM IPTG. Cells collection, lysate preparation and protein purification was performed as described above for Vip3Ae.

Vip3A protein concentration was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) as standard.

2.3. Bioassays with Vip3Ae

Overlay bioassays with artificial diet were carried out following the same protocol and with the same source of insects as those used in bioassays with Vip3Aa (Chakroun et al., 2012). Bioassays of Vip3Ae protoxin were performed in duplicate or triplicate using seven concentrations (from 1.8 to 1350 ng/cm^2) of Vip3Ae protoxin (supernatants of crude lysates) and a control with the buffer in which the insecticidal protein was dissolved. For each concentration and controls, 16 neonate larvae of *S. frugiperda* or *S. exigua* were used. Mortality was monitored after 10 days of larvae exposure at 25 ± 2 °C, with a relative humidity of 65 ± 5% and a photoperiod of 16:8 (light/dark). Median lethal concentrations (LC_{50}) were estimated from mortality data by probit analysis (POLO-PC; LeOra Software, 1987) and values were considered significantly different if their 95% fiducial limits (FL_{95}) did not overlap (Finney, 1971).

2.4. Spodoptera midgut content crude extracts isolation

Actively feeding last instar larvae from the two *Spodoptera* species (fifth and sixth instar larvae for *S. exigua* and *S. frugiperda*, respectively) were anesthetized on ice and then cut lengthwise to expose the midgut. To obtain the crude midgut content extracts for the Vip proteins activation experiments and digestive peptidases chromatographic separation, dissected midguts were carefully rinsed in cold 128 mM NaCl (to remove contaminating