VNIVERSITAT DE VALÈNCIA Facultat de Ciències Biològiques

Biochemical and molecular study of the *Bacillus thuringiensis* vegetative insecticidal proteins (Vip3A) mode of action in *Spodoptera* species

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Dr. JUAN FERRE, Catedrático de Universidad, del Departamento de Genética de la facultad de Ciencias Biológicas / ERI de Biotecnología y Biomedecina de la Universidad de València,

INFORMA: Que Dña. MAISSA CHAKROUN, licenciada en ciencia de la vida por la Facultad de Ciencias de Sfax (Tunez), ha realizado bajo mi dirección el trabajo de investigación recogido en esta memoria que lleva por título "Biochemical and molecular study of the *Bacillus thuringiensis* vegetative insecticidal proteins (Vip3A) mode of action in *Spodoptera* species" con el fin de optar al grado de Doctora por la Universitat de València dentro del programa de Doctorado en Biotecnología y,

AUTORIZA su presentación en el Departamento de Bioquimica y Biologia Molecular de la Facultad de Ciencias Biológicas para tal fin.

Para que así conste, y en cumplimiento de la legislación vigente, firmamos el presente certificado en Burjassot a 03 de septiembre de 2015.

Dr. Juan Ferré Manzanero

Para la realización de esta tesis, el autor ha sido beneficiario de una Beca de Santiago Grisolia para la Formación de Personal Investigador en centros de investigación de la comunidad valenciana, concedida por la Consellería d'Educació de la Generalitat Valenciana. El trabajo realizado ha sido subvencionado por los proyectos de investigación del Ministerio de Ciencia e Innovación con referencia AGL2009- 13340-C02-01, por Subvenciones ACOMP / 2009/313 y PROMETEO 2011/044 de la Generalitat Valenciana, una subvención de la Agencia Española de Cooperación Internacional (AECI, Ref. A / 017413/08), y por los fondos Europeos FEDER.

الحمد لله وكفى

أهدي هذه الرسالة إلى جميع أفراد عائلتي وخاصة أبي و أمي على كل ما قدموه من الحب والدعم طوال هذه الرحلة الملحمية

This thesis is dedicated to all the members of my family, especially my Mom and Dad, for all their love and support throughout this epic journey

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Summary:

Vip (Vegetative insecticidal protein) constitutes a new family of insecticidal proteins produced during the vegetative growth phase of different bacillus strains and mainly by *Bacillus thuringiensis* (*Bt*). This protein family accounts for 4 members: Vip1, Vip2, Vip3 and the recently described Vip4. The binary toxins Vip1 and Vip2 are found to be active to coleopteran and homopteran insects; Vip3 is active against lepidopterans, however, no target is yet known for Vip4. Determination of Vip1 and Vip2 mode of action was easy since they showed significant sequence homology with the well-known clostridial toxins C2 and C3 respectively. This was not the case of Vip3; no significant sequence homology was found with any of the described proteins in the data bases, and neither any characteristic domain with known toxic activity was found in its sequence. Thus their mode of action couldn't be deduced. In this work we study the main steps of the mode of action of Vip3A proteins in an attempt to extend the knowledge acquired so far.

In the present study we assess the stability of Vip3Aa through its purification process, after which we investigate two steps of its mode of action: the activation by the midgut juice of the susceptible insect strains *Spodoptera frugiperda* and *Spodoptera exigua* and the binding to its specific receptors localized in the midgut of *S. frugiperda*. The activity of Vip3Aa has been tested after different purification protocols using *S. frugiperda* as a control insect. It was found to be stable and retained full toxicity after the different biochemical steps used for its purification.

The bioassays results using the protoxin form of Vip3Aa showed pronounced differences in LC_{50} values (scored at 7 and 10 days) between *S. exigua* and *S. frugiperda*, the former being less susceptible. Strong growth inhibition was observed at 7 d and most live larvae were arrested in the first instar. LC_{50} values of ‘‘functional mortality’’ (dead larvae plus larvae remaining in the first instar), measured at 7 d, were similar or even lower than the LC_{50} values of mortality at 10 d. However, when the trypsin-activated Vip3Aa is used no growth inhibition was observed in either species, both were equally susceptible to this form. Processing of Vip3Aa protoxin to the activated form was faster with *S. frugiperda* midgut juice than with *S. exigua* midgut juice suggesting that the difference in the activation rate of Vip3Aa between the two species could be the basis of the difference in their susceptibility to the protoxin. In contrast, Vip3Ae was found to be equally toxic to 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. Results indicated 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. When processed with the midgut extract from both *Spodoptera* species, Vip3Aa and Vip3Ae protoxins were readily processed but no peptidase resistant core was observed under the experimental conditions used. 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 Vip3Aa toxicity.

The *in vivo* analysis of Vip3Aa binding to *S. frugiperda* midgut using immunofluorescence histological localization showed that Vip3Aa bound to the brush border membrane along the entire apical surface. The presence of fluorescence in the cytoplasm of epithelial cells seems to suggest internalization of Vip3Aa or a fragment of it. The *in vitro* analyses of Vip3Aa binding characteristics and parameters have been made possible by its successful radiolabeling. Heterologous competition using Vip3Ad, Vip3Ae, and Vip3Af as competitor proteins showed that they share the same binding site with Vip3Aa. In contrast, when using Cry1Ab and Cry1Ac as competitors, no competitive binding was observed. The present work will help to better understand the molecular basis of resistance to these toxins recently introduced in plants and thus to help design suitable insecticide resistance management strategies for continued use of Bt toxins in transgenic crops.

Resumen:

Las proteínas insecticidas vegetativas (Vip) constituyen una nueva familia de toxinas producidas durante la fase de crecimiento vegetativo de diferentes cepas de *Bacillus* y principalmente por *Bacillus thuringiensis* (Bt). Esta familia de proteínas está representada por 4 miembros: Vip1, Vip2, Vip3 y la recientemente descrita Vip4. Las toxinas binarias Vip1 y Vip2 son activas contra coleópteros y homópteros; las proteínas Vip3 son activas contra lepidópteros, sin embargo, los insectos diana para la proteína Vip4 no se conocen todavía. La determinación del modo de acción de Vip1 y Vip2 fue fácil ya que mostraron una homología de secuencia significativa con las toxinas clostridiales C2 y C3, respectivamente. Éste no fue el caso de Vip3. Las proteínas Vip3 no han mostrado ninguna homología de secuencia significativa con ninguna de las proteínas descritas en las bases de datos. Tampoco se encontró en su secuencia ningún dominio característico con actividad tóxica conocida, por lo que no se pudo inferir su modo de acción. En este trabajo se estudian las principales etapas del modo de acción de las proteínas Vip3A en un intento de ampliar los conocimientos adquiridos hasta el momento sobre estas proteinas.

En el presente estudio se evaluó la estabilidad de Vip3Aa respecto a diversos procesos de purificación, tras lo cual se investigó dos pasos de su modo de acción: la activación por el jugo intestinal de las especies susceptibles *Spodoptera frugiperda* y *Spodoptera exigua* y la unión a sus receptores específicos localizados en el intestino medio de *S. frugiperda*. El efecto de varios protocolos de purificación de Vip3Aa sobre su toxicidad fue analizado utilizando *S. frugiperda* como insecto control. La proteína Vip3Aa fue estable y retuvo total toxicidad después de los diferentes pasos bioquímicos usados para su purificación.

Los bioensayos utilizando la Vip3Aa en forma protoxina mostraron diferencias marcadas en los valores de LC_{50} (anotados a los 7 y 10 días) entre *S. exigua* (menos susceptible) y *S. frugiperda*. Se observó una fuerte inhibición del crecimiento a los 7 d y la mayoría de las larvas vivas detuvieron su crecimiento en el primer estadio larvario (L1). Los valores de LC_{50} de la " mortalidad funcional " (larvas muertas más larvas en L1), medidos a 7 d, fueron similares o incluso inferiores a los valores de la LC_{50} de la mortalidad a los 10 d. Sin embargo, cuando se utilizó la Vip3Aa activada por tripsina, no se observó inhibición del crecimiento, y ambas especies fueron igualmente susceptibles a esta forma. El procesado de la protoxina de Vip3Aa a la forma activada fue más rápido con el jugo intestinal de *S. frugiperda* que con el de *S. exigua*, lo que sugiere que la diferencia en la tasa de activación de Vip3Aa entre las dos especies podría ser la base de la diferencia de susceptibilidad a la protoxina. Por contra, Vip3Ae resultó ser igualmente tóxica para estas dos especies. Se

realizaron experimentos de proteolisis para estudiar la estabilidad de las proteínas Vip3A frente a las peptidasas intestinales y observar si las diferencias encontradas podrían explicar las diferencias en la toxicidad contra estas dos especies de *Spodoptera*. Los resultados indicaron que la activación de la protoxina y la degradación de la banda de 62 kDa tenía lugar a concentraciones más bajas de tripsina para Vip3Aa que para Vip3Ae. El efecto opuesto se observó con la quimotripsina. Cuando se usó jugo intestinal de ambas especies de *Spodoptera*, las protoxinas Vip3Aa y Vip3Ae fueron inmediatamente procesadas y no se encontró ningun péptido resistente a las peptidasas intestinales en las condiciones experimentales utilizadas. Los experimentos de digestión realizados con las serín peptidasas de *S. frugiperda* purificadas por cromatografía mostraron que la degradación del fragmento activo de las toxinas Vip3A se debe principalmente a la acción de la peptidasa de tipo quimotripsina catiónica. Aunque los patrones de digestión de las proteínas Vip3A no siempre se correlacionan con la toxicidad; la estabilidad del fragmento de 62 kDa a las peptidasas está de acuerdo con las diferencias intraspecíficas de la toxicidad de la proteína Vip3Aa.

La unión *in vivo* de Vip3Aa al intestino medio de *S. frugiperda* mediante la localización histológica por immuno-fluorescencia mostró que la Vip3Aa se une a lo largo de toda la superficie apical de la membrana del borde en cepillo de las células intestinales. La presencia ténue de fluorescencia verde en el citoplasma de las células epiteliales parece sugerir internalización de Vip3Aa o un fragmento de la misma. El éxito del radiomarcaje de Vip3Aa ha hecho posible el análisis *in vitro* de sus características y parámetros de unión. La competencia heteróloga de ^{125}I-Vip3Aa con Vip3Ad, Vip3Ae y Vip3Af mostró que estas proteínas comparten los mismos sitios de unión en *S. frugiperda*. Por contra, cuando se utilizaron Cry1Ab y Cry1Ac como competidores no se observó unión competitiva. El presente trabajo ayudará a comprender mejor la base molecular de la resistencia a las toxinas Vip3A introducidas recientemente en plantas y así ayudar a diseñar estrategias apropiadas de manejo de resistencia a los insecticidas para uso continuado de las toxinas *Bt* en cultivos transgénicos.

Introduction

Bacterial Vegetative Insecticidal Proteins (Vip) from entomopathogenic bacteria: A Review

The introduction of this thesis will be submitted to the journal of Microbiology and Molecular Biology Reviews.

1. Introduction:

1.1. Preface:

Entomopathogenic bacteria have an enormous potential for insect control and they can provide us with an arsenal of insecticidal compounds (de Maagd *et al.,* 2003). By far the most widely used insecticidal proteins are the Cry proteins from *Bacillus thuringiensis*. These proteins accumulate in the parasporal crystal at the time of sporulation and are released to the culture medium only after the cell wall disintegrates. Formulations based on *B. thuringiensis* crystals and spores have been successfully used to control a wide range of lepidopteran pests, some Coleoptera, blackflies and mosquito species (Sanchis 2011; Sanahuja *et al.,* 2011). The insecticidal potency of some Cry proteins is such that their respective *cry* genes have been transferred to plants, conferring total or very high protection against the most damaging pests (Estruch *et al.,* 1997; Shelton 2012; James 2014).

Despite the wide success of Cry proteins in insect control, some important pests were found to be very tolerant to Cry proteins, such as *Agrotis ipsilon* (Lepidoptera: Noctuidae) and *Diabrotica* spp. (Coleoptera: Chrysomelidae), which cause significant damage to the corn seedling. Screening programs aimed to evaluate insecticidal active principles in culture supernatants from *Bacillus* isolates succeeded in finding the culture supernatant of *Bacillus cereus* AB78 to produce 100% mortality to *Diabrotica virgifera virgifera* and *Diabrotica longicornis barberi*. The active principle in the supernatant was found to be proteinaceous. Anion exchange chromatography followed by SDS-PAGE showed that the insecticidal activity was due to two different proteins of 80 and 45 kDa, which were named Vip1Aa and Vip2Aa, respectively (from Vegetative Insecticidal Protein) (Warren 1997). Sequences with homology to their respective *vip1Aa* and *vip2Aa* genes were found in about 12% of the 463 *B. thuringiensis* strains tested. In the same study, the *B. thuringiensis* AB88 strain vegetative culture supernatant identified a 88.5 kDa protein highly toxic to *A. ipsilon* and other lepidopterans, which was named Vip3Aa (Estruch *et al.,* 1996). More recently, a new type, Vip4Aa, has been reported by direct sequence submission to the NCBI GenBank (accesion number AEB52299) and *in silico* analysis predicted a molecular mass of approx. 108 kDa (Palma *et al.,* 2014).

Alternative names for this kind of proteins were also given before the standardization by the Bt Toxin Nomenclature Committee (Crickmore *et al.,* 2015), such as Insecticidal Secreted Proteins (Isp), with the classes Isp1, Isp2 and Isp3 (NCBI GenBank acc. No. AJ871923, AJ871924, AJ872070), which are homologous to Vip1, Vip2 and Vip3,

respectively. It should be mentioned that another secreted insecticidal protein (named Sip) from *B. thuringiensis* has also been reported (Donovan *et al.,* 2006) which shares no homology to the Vip proteins and should not be mistaken with one of them.

To date, 15 Vip1, 20 Vip2, 101 Vip3 and one Vip4 proteins have been reported (Crickmore *et al.,* 2015). Figure 1 shows the dendrogram with the hierarchy of the Vip proteins based on their amino acid degree of identity. Vip1 and Vip2 act as a binary toxin for some coleopteran and Hemiptera and Vip3 is active against Lepidoptera (Estruch *et al.,* 1996; Ruiz de Escudero *et al.,* 2014). For the member of the new family Vip4, no target insects have been found as yet. In contrast to the Cry protein family, Vip1, Vip2 and Vip3 share almost no sequence homology among each other, being Vip1 and Vip4 the most similar (34% amino acid identity).

1.2. The binary Vip1/Vip2 toxin

In addition to *B. cereus* and *B. thuringiensis*, *vip1* and *vip2* genes have also been found in other bacterial species, such as *Lysinibacillus sphaericus* (formerly known as *Bacillus sphaericus*) and *Brevibacillus laterosporus* (Ruiu 2013; Schnepf *et al.,* 2005). Studies on the distribution of *vip1* and *vip2* genes have shown that they are found in around 10% of *B. thuringiensis* strains (Hernandez Rodriguez *et al.,* 2009; Yu *et al.,* 2011a; Shingote *et al.,* 2013a). These two genes are carried by the same operon and with two different open reading frames separated by an intergenic spacer of 4 to 16 bp placed in a 4 to 5 kb genomic sequence (Warren 1997; Shi *et al.,* 2007; Bi *et al.,* 2015) and in a megaplasmid (around 328 kb length) in *B. thuringiensis* strain IS5056 (Murawska *et al.,* 2013). At the time of writing this document the Bt Toxin Nomenclature data base listed the following *vip1* and *vip2* genes: 3 *vip1Aa*, 1 *vip1Ab*, 1 *vip1Ac*, 1 *vip1Ad*, 2 *vip1Ba*, 3 *vip1Bb*, 1 *vip1Bc*, 2 *vip1Ca*, and 1 *vip1Da*, and 3 *vip2Aa*, 1 *vip2Ab*, 2 *vip2Ac*, 1 *vip2Ad*, 3 *vip2Ae*, 2 *vip2Af*, 2 *vip2Ag*, 2 *vip2Ba*, and 4 *vip2Bb* (Crickmore *et al.*, 2015).

Vip1 and Vip2 proteins are expressed concomitantly and translation from the same transcript appears to be essential to ensure high levels of both proteins. They are produced during the vegetative growth phase of *B. thurigiensis* and their level remains high till after the sporulation stage. The gene transcripts are detected even at the starting of the logarithmic phase, following with maximum expression in the stationary phase and remaining at high levels in the sporulation stage (Bi *et al*., 2015; Estruch *et al.,* 1996; Shi *et al.,* 2004; Shi *et al.,* 2007).

1.2.1 Protein structure and function

Classical bacterial A-B toxins, such as cholera toxin, interact with cells as a complex composed by one or several polypeptides associated in solution. Alternatively, Gram positive bacilli from the genera *Clostridium* and *Bacillus* produce proteins with a synergistic binary mode of action in which the two proteins do not form an aggregate before binding to the cell surface (A+B toxins) (Barth *et al.*, 2004). The Vip1/Vip2 toxin is an example of A+B toxin related to mammalian toxins from *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium spiriforme* and *Bacillus anthracis*. Sequence homology with the mammalian toxins, the lack of toxicity of the individual proteins, and experiments of translational frameshift mutation of the *vip1* gene along with toxicity bioassays to susceptible insects confirmed the binary mode of action of these proteins (Warren 1997).

Sequence analysis of Vip1Aa and Vip2Aa proteins revealed the presence of an N-terminal signal peptide of about 30 and 50 amino acids respectively (Shi *et al.*, 2004; 2007; Warren *et al.*, 1998). The signal peptide was shown to be cleaved during secretion, rendering mature proteins of ca. 82 kDa (for Vip1Aa) and ca. 45 kDa (for Vip2Aa) (Warren, 1997; Bi *et al.*, 2015). Sequence alignment revealed that the N-terminal part of the Vip1 is highly conserved (identity of 75-91%) (Fig. 2). In contrast, the Vip1 C-terminal part is much less conserved (identity of 23-35%) (Warren, 1997; Shi *et al.*, 2004, 2007).

Vip1 has significant sequence identity with the binding component C2-II of the C2 *C. botulinum* toxin (29%), the Ib of the iota toxin from *C. perfringens* (31%), 33 to 38% of identity with *C. spiroforme* toxin and *B. anthracis* protective antigen, and with the toxin B of *C. difficile* at amino acids 142-569 (Shi *et al.*, 2004, 2007; Leubert *et al.*, 2006). Vip2 shares more than 30% sequence identity to the clostridial Rho-ADP-ribosylating exotoxin C3 (Han *et al.*, 1999). These homologies suggested that the Vip1 protein is the component called “B” and that the Vip2 protein is the “A” component of the binary toxin (Barth *et al.*, 2004). Vip1 would act as the binding and translocation component (channel forming protein) (Knapp *et al.*, 2002; Schmidt *et al.*, 1994, Blaustein *et al.*, 1989) and Vip2 would enter the cell and exert its toxic effect.

Vip2 is an NAD-dependent actin-ADP-ribosylating toxin (Jucovic *et al.*, 2008) and has two distinctive domains: the N-terminal domain from amino acids 60 to 265 and the C-terminal domain from amino acids 266 to 461 (Fig. 3) (Han *et al.*, 1999). Despite their limited sequence homology to each other, the crystallography structure analysis of Vip2 N- and C-terminal domains showed homology in their structure (Fig. 4). Each domain core is

formed mainly by the perpendicular packing of a five-stranded mixed β-sheet with a three-stranded antiparallel β-sheet. The three-stranded sheet is flanked by four consecutive α-helices and the five-stranded sheet by an additional α-helix (Han *et al.,* 1999). The overall fold of each domain resembles the catalytic domains of classical A-B toxins. In fact, crystal structure superposition of Vip2 and clostridial toxin C3, along with sequence alignment, suggests that the class of Vip2 binary toxins has arisen by single gene duplication of an ancestral ADP-ribosyltransyltransferase. This event would have been followed by further divergence by which the N-terminal domain would have lost the catalytic function and evolving into a binding component to finally give rise to a new protein family with ability to bind to other carrier proteins and thereby act as binary toxins (Han *et al.,* 1999; 2001; Barth *et al.,* 1998).

1.2.2 Insecticidal activity

The toxicity of Vip1 and Vip2 has been tested against a number of insect species belonging to the order Coleoptera (11 species), Lepidoptera (12 species), Diptera (2 species) Homoptera (1 species) and Nematoda (2 species) (Table 1). So far, toxicity has been found against 8 coleopteran species (Warren 1997; Boets *et al.,* 2011; Bi *et al.,* 2015; Shingote *et al.,* 2013b) and to the homopteran *Aphis gossypii* (Sattar and Maiti, 2011; Yu *et al.,* 2011b).

Vip1 and Vip2 acting as a binary toxin has been shown *in vivo* for Vip1Aa/Vip2Aa and Vip1Aa/Vip2Ab with *Diabrotica spp.* (Warren 1997; *Boets et al.,* 2011), for Vip1Ad/Vip2Ag with *Holotrichia oblita* (coleoptera: Melolonthidae), *Holotrichia parallela* (Coleoptera: Motschulsky), and *Anomala corpulenta* (Coleoptera: Rutelidae) (Bi *et al.,* 2015), for Vip1Da/Vip2Ad for *Anthonomus grandis* (Coleoptera: Curculionidae) and *Leptinotarsa decemlineata* (Coleoptera; Chrysomeloidea) (Boets *et al.,* 2011), for Vip1Ac/Vip2Ae and Vip1Ae/Vip2Ae with *A. gossypii* (Sattar and Maiti, 2011; Yu *et al.,* 2011b), and for an 88% homologous Vip1/Vip2 with *Sitophilus zeamais* (Coleoptera: Curculionidae) (Shingote *et al.,* 2013b). When both proteins were used in combination, they were effective, whereas when expressed alone, they exhibited minimal or no activity (Warren 1997; Boets *et al.,* 2011; Bi *et al.,* 2015; Yu *et al.,* 2011b; Sattar and Maiti 2011). Another interesting feature of these toxins comes from experiments combining different pairs of proteins. The pair Vip1Aa/Vip2Aa is active against *Diabrotica*, but this is not the case with the pair Vip1Ab/Vip2Ab. Interestingly, the pair Vip1Aa/Vip2Ab is active

whereas the pair Vip1Ab/Vip2Aa is not, suggesting that the lack of toxicity of the pair Vip1Ab/Vip2Ab to *D. virgifera* is due to the Vip1Ab component (Warren 1997).

1.2.3 Mode of action

The molecular mechanism of the insecticidal activity of Vip1/Vip2 toxin is not totally understood. The multistep process begins with the ingestion of the toxin by the larva, probably followed by the proteolytic activation in the midgut by trypsin-like proteases. The activated 66 kDa monomer of Vip1Ac has been shown to form oligomers containing seven Vip1 molecules (Leuber *et al.,* 2006). These oligomers would recognize specific receptors in the midgut brush border membrane and then insert into the membrane. Evidence that the Vip1 component is involved in receptor recognition was in part provided by the fact that Vip1Aa cannot be replaced by Vip1Ab without losing toxicity to *D. virgifera* (Warren 1997). Vip1Aa and Vip1Ab share 97 % homology at their N-terminal part and only 31% at the C-terminal, suggesting that the C-terminal domain of these proteins is involved in the insect specificity. The first receptor described for a Vip1 protein by ligand blot experiments was an approx. 50 kDa protein identified in *A. gossypii*, however no binding was observed to the BBMV of non susceptible insects (Sattar and Maiti, 2011).

In vitro experiments showed that Vip1 formed membrane pores in artificial lipid bilayer (Leuber *et al.,* 2006). The pores had two different conductance states, suggesting the simultaneous formation of two different channels. Vip1Ac channels are found to be asymmetric and moderately anion selective. The putative channel forming domain of Vip1 contains two negatively charged (E340 and E345) and two positively charged amino acids (K351 and H363) which are hypothesized to contribute to the selectivity of the channel (Leuber *et al.,* 2006). In contrast to the pores formed by Cry and Vip3 proteins, the pores formed by Vip1, in the absence of the Vip2 component, have no toxic effect to the susceptible insect (Leuber *et al.,* 2006).

The Vip1 pore would provide a channel for Vip2 to penetrate into the cells to exert its toxic action through the destabilization of the actin by preventing polymerization and thus inhibiting the microfilament network formation (Han *et al.,* 1999). The catalytic Vip2 domain would catalyze the transfer of the ADP-ribose group from NAD to the major cytoskeleton forming protein (actin), which would prevent actin polymerization (Han *et al.,* 1999; Jucovic *et al.,* 2008).

1.2.4 Expression in plants

Despite the economic importance of Vip1 and Vip2 as effective toxins against the major corn pest *D. virgifera*, the expression of the binary toxin *in planta* has not been possible knowing the cytolytic activity of the Vip2 protein. In fact, Vip2 expression in yeast resulted in serious developmental pathology and phenotypic alteration (Jucovic *et al.*, 2008). To overcome this problem, Jucovic *et al*. (2008) designed a new zymogene strategy which consisted in the expression of a zymogenic form of Vip2 called "ProVip2". The Vip2 proenzyme was obtained by extension of the C-terminal part of the protein in such a way that it masked the enzymatic activity. The C-terminal added peptide showed to be effectively eliminated by the proteolytic action of *D. virgifera* midgut enzymes, and insects on a diet containing ProVip2 transgenic corn and Vip1 were totally killed.

1.3. The Vip3 lepidopteran-active protein

Similarly to Vip1 and Vip2 proteins, Vip3 proteins are produced during the vegetative growth phase of *B. thurigiensis* and can be detected in culture supernatants from 15 h post-inoculation to beyond sporulation, which reflects their high stability (Estruch *et al.*, 1996; Mesrati *et al.*, 2005b). A study of the *vip3Aa16* gene reported that the transcription start point was located at 101 bp upstream the start codon and that the promoter -35 and -10 regions were very similar to the *B. subtilis* promoter σ^E and to the *B. thuringiensis* promoter σ^{35}, which strongly suggest that the *vip3Aa16* gene is transcribed by a holoenzyme E σ^{35} (Mesrati *et al.*, 2005b).

Genes coding for Vip3 proteins are commonly found among *B. thuringiensis* strains and hence some studies have found them even in 50 and up to 87% of the strains tested and in more than 90% of strains carrying cry1 and cry2 genes (Beard *et al.*, 2008; Espinasse *et al.*, 2003; Mesrati *et al.*, 2005a; Hernandez-Rodriguez *et al.*, 2009, Yu *et al.*, 2011a). *vip3* genes are about 2.4 kb long and they are normally carried in large plasmids (Wu *et al.*, 2004; Mesrati *et al.*, 2005a), though in some cases they have been proposed to be located in the bacterial chromosome (Franco Rivera *et al.*, 2004). Many screening strategies of *B. thuringiensis* isolates were performed with the aim of isolating new *vip3* genes (Loguercio *et al.*, 2002; Franco-Rivera *et al.*, 2003; Bhalla *et al.*, 2005; Rang *et al.*, 2005; Liu *et al.*, 2007;Sattar *et al.*, 2008; Abulreesh *et al.*, 2012; Asokan *et al.*, 2012;

Murawska *et al.*, 2013), at the time of writing this document there were 54 *vip3Aa*, 2 *vip3Ab*, 1 *vip3Ac*, 4 *vip3Ad*, 1 *vip3Ae*, 3 *vip3Af*, 15 *vip3Ag*, 1 *vip3Ah*, 1 *vip3Ai*, 2 *vip3Ba*, 3 *vip3Bb*, and 4 *vip3Ca* genes reported (Crickmore *et al.*, 2015). Therefore, most studies on the Vip3 proteins have been carried out with the most abundant Vip3Aa proteins and very little information is available on Vip3B and Vip3C proteins and on other less common Vip3A (Vip3Ab, Vip3Ac, etc.),. Unfortunately, early papers omitted the small letter in the Vip3 name, referring just to Vip3A. Although these studies were most likely carried out on Vip3Aa, in this review we have followed the nomenclature the authors' used whenever we found impossible to track down the identity of the protein by either the accession number or by any other means.

1.3.1 Protein structure and function

The number of amino acids in Vip3 proteins is around 787 with an average molecular mass of around 89 kDa. The N-terminal half of Vip3 proteins is highly conserved, while the C-terminal region is highly variable (Rang *et al.*, 2005; Wu *et al.*, 2007; Ruiz de Escudero *et al.*, 2014) (Fig. 5). Because of this reason it has been proposed that the C-terminus is related with target specificity (Wu *et al.*, 2007).

Vip3A proteins contain three cysteine residues. Point mutations have been introduced in each of the three residues in order to determine empirically the existence of disulfide bonds. The loss of activity was rather related with trypsin sensitivity than with the disruption of a disulfide bond (Dong *et al.*, 2012).

The N-terminus of Vip3 proteins contains a signal peptide which is responsible for the translocation of the protein to the periplasmic space across the cell membrane. It consists in a few positively charged amino acids, followed by a hydrophobic region, which are not removed after secretion from *B. thuringiensis* (Estruch *et al.*, 1996, Doss *et al.*, 2002, Chen *et al.*, 2003). Without a clear putative cleavage site, the signal peptide extent varies depending on the protein sequence and on the program used for prediction, and ranges from 11 to 28 amino acids (Estruch *et al.*, 1996; Doss *et al.*, 2002; Chen *et al.*, 2003). Since the secretion of proteins commonly implies the excision of the signal peptide, the secretion mechanism of Vip3 proteins is still unclear.

The highly conserved amino acid sequence of the N-terminal region of Vip3A proteins is an indication that this region must play an important role in either the protein folding or

by directly affecting binding to the membrane receptors. However, contradictory results have been obtained in experiments testing the insecticidal activity of mutant Vip3A proteins with deletions at the N-terminal end. Deletion of the first 198 amino acids (which corresponds to the 20 kDa proteolytic fragment described by Estruch *et al*., 2001) abolished the toxicity to *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Spodoptera exigua* (Lepidoptera: Noctuidae) (Li et al., 2007). Deletion of the 27 N-terminal amino acids from Vip3Aa rendered an inactive protein due to total loss of solubility (Chen *et al.,* 2003). The deletion of the 39 N-terminal amino acids from the Vip3Aa differentially affected the toxicity of this protein toward the two susceptible insect species: *Spodoptera littura* (Lepidoptera: Noctuidae) and *Chillo partellus* (Lepidoptera: Crambidae) (Selvapandiyan *et al.,* 2001). Contrarily to the above results, Gayen *et al.* (2012) found that deletion of the 200 N-terminal amino acids enhanced the insecticidal potency of the core active toxin about 2-3 folds against *H. armigera, A. ipsilon*, *Spodoptera littoralis* (Lepidoptera: Noctuidae) and *Scirpophaga incertulas* (Lepidoptera: Pyralidae). Similarly, in another study (Bhalla *et al.,* 2005), suppression of 33 amino acids from the Vip3Aa N-terminus caused no loss of toxicity against *S. littura, Plutella xylostella* (Lepidoptera: Plutellidae) and *Earias vitella* (Lepidoptera: Noctuidae).

The function of some C-terminus modifications has been also studied but without leading to a general conclusion. Usually the effect of the same change varies among different insect species, preventing a consensus about the contribution of certain regions or positions of Vip3A proteins to their toxicity (Selvapandiyan *et al.,* 2001; Chen *et al.,* 2003; Bhalla *et al.,* 2005; Li *et al.,* 2007; Gayen *et al.,* 2012). There is general agreement in that the last amino acids of the C-terminus are critical for the activity and stability of Vip3 proteins, since their deletion, substitution to non-conservative residues or addition of amino acids to the end of the protein completely abolish the protein activity (Selvapandiyan *et al.,* 2001; Gayen *et al.,* 2012) and the stability against proteases (Li *et al.,* 2007; Estruch *et al.,* 2001). A triple mutation at the C-terminus of Vip3Aa1 resulted in an unstable protein that was completely hydrolyzed by the midgut juice of *A. ipsilon* larvae but retained toxicity against Sf9 cells (Estruch *et al.,* 2001).

Analysis of the Vip3 protein sequences done by the authors for this review revealed the presence of a carbohydrate binding motif (CBM_4_9 superfamily, pfam02018) in all Vip3 proteins with the exception of Vip3Ba (Fig.6). The CBM spans from position 536 to a position near amino acid 652, with a consistent e-value between 10 e^{-4} and 10 e^{-17}, depending on the Vip3 protein considered (Marchler-Bauer *et al.,* 2011 & 2013 NCBI-CDD data base). The analysis of Vip3 sequences also revealed positive hits with different

multidomains in the N-terminal region, with lower e-values of around 10 e^{-4}, and with differences depending of the Vip3 protein considered (Fig. 6) (Marchler-Bauer *et al*., 2013 NCBI-CDD data base). No hydrophobic region susceptible to form a transmembrane domain was found besides the short succession of hydrophobic amino acids found in the signal peptide (Estruch *et al.*, 1996; Doss *et al.*, 2002).

Some differences exist between Vip3Aa1 and both the Vip3B and Vip3C proteins. These differences are distributed all along the protein sequence, although maximum divergence is found at the C-terminus, as occurs within Vip3A proteins. The N-terminus of the putative signal sequence of Vip3B and Vip3C is almost identical to that of all Vip3A toxins. The proteolytic processing sites are less conserved among the three Vip3 proteins, but major differences are found in the middle of the protein sequence; the insertion of 5 amino acids downstream the first processing site for Vip3C type and 17 amino acids downstream the second processing site for the Vip3B type may cause a change in the expected size of the toxin "active form" from 66 kDa to 69 kDa. The Vip3B inserted sequence consists in three repetitions of the pattern DCCEE, which is characterized by its high content of negatively charged amino acids (D and E) and cystein residues. From a total of 11 cystein residues found in Vip3B proteins, eight (78%) are located in this inserted sequence (Rang *et al.*, 2005; Palma *et al*. 2012). Whether the insertion of this repetitive pattern contributes to the limited insecticidal activity of these proteins is not known.

The conformational structure of Vip3 proteins has never been elucidated. Secondary structure prediction suggests that the N-terminal part is mainly constituted by α-helix structures whereas the essential component of the C-terminal part are β-helix structures and coils, which would be consistent with its proposed role in insect specificity (Rang *et al.*, 2005; Wu *et al.*, 2007). The fact that Vip3 proteins do not show homology to any protein outside their group prevents *in silico* modeling based on structure homology. Only a partial tertiary structure of the Vip3 protein corresponding to the last 200 amino acids has been modelled by homology to domain II of the Cry proteins (Wu *et al.*, 2007).

1.3.2 Insecticidal activity

Most information on the insecticidal activity of Vip3 proteins has been obtained with the most abundant proteins of the subclass Vip3Aa and very few data exist on the toxicity of Vip3B, Vip3C and other Vip3A different from the Vip3Aa subclass.

1.3.2.1 Insecticidal spectrum of Vip3 proteins

Vip3A proteins are toxic to a large number of lepidopteran insects. It is worth to mention that they are very active against insect species from the genus *Agrotis*, which are known to be tolerant to Cry proteins, and also to species from the genus *Spodoptera,* which are relatively little susceptible to Cry proteins (Van Frankenhuyzen & Nystrom, 2009). In this regard, it has been shown that deletion of the *vip3A* gene from the *B. thuringiensis* HD1 strain significantly decreased its toxicity toward *A. ipsilon* and *S. exigua* (Donovan *et al.,* 2001). On the other hand, other species susceptible to Cry proteins, such as *Ostrinia nubilalis* (Lepidoptera: Crambidae), *Culex quinquefasciatus* (Diptera: Culicidae) and *Chironomus tepperi* (Diptera: Chironomidae), are marginally or not susceptible to any Vip3A protein tested (Estruch *et al*., 1996; Yu *et al*., 1997; Doss *et al*., 2002; Yu *et.al*, 2012). With Vip3 proteins, depending on the Vip3 protein and the insect species considered, it is not uncommon to find that, while the mortality is reached at a high concentration of Vip3 protein, a strong growth inhibition (or even complete growth arrest) is observed at lower concentrations (Jamoussi *et al.*, 2009; Abdelkefi-Mesrati *et al.*, 2011a; Ben Hamadou-Charfi *et al.,* 2013; Palma *et al.,* 2012; Ruiz de Escudero *et al.*, 2014). Therefore, the "functional mortality" (dead insects plus those remaining at L1) represents better the effectivity of the Vip3 protein in those cases (Ali and Lutrell, 2011; Chakroun *et al.*, 2012; Ruiz de Escudero *et al.,* 2014).

Table 2 summarizes the results reported on the insecticidal activity of Vip3Aa proteins. Only the values of the protoxin form are given since there are no reports indicating differences in the insecticidal activity between the protoxin and the activated form (Ruiz de Escudero *et al* 2014), with the exception of Vip3Aa16 against *S. exigua* and Vip3Af1 against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Chakroun *et al.*, 2012; Hernández-Martínez *et al.*, 2013). Despite the very small differences among Vip3Aa sequences, some proteins may exhibit significant differences in toxicity to the same insect species (Selvapandiyan *et al.*, 2001; Ruiz de Escudero *et al.* 2014; Palma *et al,* 2013). For example, among all Vip3Aa proteins tested, only Vip3Aa1 and Vip3Aa14 have been described as low or non active against *H. armigera* (Table 2). Nonetheless, considering that most of the data in Table 2 were obtained in different laboratories, insecticidal differences are likely to come from factors other than slight differences in protein sequence, such as the protocol used for protein preparation, purity of the sample, method of quantification, bioassay conditions, or variability among insect populations. Hernández-Martínez *et al.* (2013) evidenced the decrease in the toxicity of Vip3A proteins after purification with metal-chelate chromatography. The effect of the method of purification depends on the

tandem protein - target species. This effect was also previously described for Cry proteins (Hernández-Rodríguez *et al.*, 2012). Ali and Lutrell (2011) found that insecticidal response of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) to Vip3Aa greatly varies among different batches of the same protein as well as with the buffer used. Besides, considerably variability was reported among several field and laboratory populations and within five consecutive seasons.

Table 3 summarizes the bioassay data on Vip3A proteins other than Vip3Aa, and Table 4 that of Vip3 proteins other than Vip3A.

1.3.2.2 Interactions with other insecticidal proteins

Synergism has been observed between Vip3Aa and Cyt2Aa proteins against *Chilo suppressalis* (Lepidoptera: Crambidae) and *S. exigua* after their co-expression in *E. coli*; contrarily, this protein combination was slightly antagonistic on *C. quinquefasciatus* (Yu *et al.,* 2012). Bergamasco *et al.,* (2013) reported synergism between Vip3A and Cry1Ia in three *Spodoptera* species (*S. frugiperda*, *S. albula* and *S. cosmioides* (Lepidoptera: Noctuidae)) but slight antagonism in *Spodoptera eridania* (Lepidoptera: Noctuidae). Antagonism between Vip3A, Cry1A and Cry1Ca proteins was described in *H. virescens* (Lemes *et al.,* 2014): antagonism was found in combinations of Cry1Ca with either Vip3Aa, Vip3Ae or Vip3Af, and of Vip3Af with either Cry1Aa or Cry1Ac. In the same study, Vip3Aa and Cry1Ca showed antagonism in *S. frugiperda* whereas the same combination was synergistic in *Diatraea saccharalis* (Lepidoptera: Crambidae).

The mechanism underlying synergism and antagonism is still unknown. For the antagonism between Vip3A and Cry1C proteins, Lemes *et al.,* (2014) hypothesized a physical interaction of the two proteins impairing the access of the binding epitopes to the membrane receptor. Conversely, synergism could be related to hetero-oligomer formation with better ability of membrane insertion or pore formation, as it has been previously proposed for Cry1Ac and Cry1Aa (Lee *et al.,* 1996).

1.3.2.3 Genetic engineered vip3A genes

Genetic engineering allows the creation of chimeric genes that code for parts of different proteins to obtain new ones with novel or improved properties. Knowledge of the domains of a protein is of great help in the design of chimeric proteins. Despite the lack of information on the tertiary structure of Vip3A proteins, two chimeras have been created by sequence swapping between *vip3Aa* and *vip3Ac* genes with the aim to increase host

specificity (Fang *et al.,* 2007). Chimeras between Vip3Aa and Vip3Ac were created by combining around 600 amino acids of the N-terminal region of one protein and around 180 amino acids of the C-terminal region of the other (Table 5). The two chimeric proteins had new toxicity properties: Vip3AcAa (with the N-terminal region of Vip3Ac) was more toxic than the two original proteins towards all the tested insects, causing even growth inhibition towards the Vip3A tolerant *O. nubilalis*, whereas Vip3AaAc was less toxic than its counterpart and the original proteins, and even completely lost the activity against *Bombyx mori* (Lepidoptera: Bombycidae) (Fang *et al.,* 2007) (Table 5). Li *et al.,* (2007) achived an 18-fold increase in toxicity against *S. exigua* by changing the last two amino acids of the chimeric Vip3AcAa protein (from IK to LR).

Similar attempts have been done combining *vip* and *cry* genes. Fusion of the *vip3Aa* gene with *cry1A* rendered a fusion protein that retained the toxicity of Cry1Ac, but partially lost that of Vip3Aa, possibly due to incorrect Vip3A folding (Saraswathy *et al.,* 2008). In another study, the *vip3Aa* gene was fused with the N-terminus of c*ry9Ca* and the resultant chimeric protein resulted more toxic than the individual proteins and the mix of them, probably because Vip3Aa increased the solubility of the Cry9Ca protein (Dong *et al.,* 2012). In an attempt to improve the Vip3Aa yield, a mutant *vip3Aa* gene (with the signal peptide deleted) was fused with the promoter and C-terminal half of Cry1C, with the result of a 9-fold increase in the expression of the recombinant protein which was concentrated in inclusion bodies. Unfortunately, this protein showed lower insecticidal activity than the original Vip3Aa protein against the insects tested, probably due to low solubilization or improper folding of the protein (Song *et al.,* 2008) (Table 5).

Another type of approach has been the introduction and expression of *vip3A* genes in *B thuringiensis* strains expressing different *cry* genes, to create new *B. thuringiensis* strains to be used in insecticidal formulations with broader spectrum of action. Commercial formulations of *B. thuringiensis* strains contain little amounts of Vip proteins, since the latter are secreted to the growth medium which is discarded during the processing of the formulation (Taborsky, 1992). This problem can be circumvented by directing the expression of the *vip3A* gene to the sporulation stage using sporulation-dependent promoters and specific transcription sequences from different *cry* genes (Arora *et al.,* 2003; Zhu *et al.,* 2005; Thamthiankul Chankhamhaengdecha *et al.,* 2008; Sellami *et al.,* 2011). The engineered strains in all these cases showed improved production of Vip3A proteins and higher toxicity toward the insects tested. Cloning and expression of the *vip3Aa* gene in *Pseudomonas fluorescens* has also been accomplished with the aim of producing sprayable

insecticides based on the Vip3A protein, either combined or not with Cry proteins (Hernández-Rodríguez *et al.,* 2013).

1.3.3 Mode of action

The study of the mode of action of the Vip3 proteins started soon after their discovery in 1996 by Estruch and coworkers, that suggested that Vip3 proteins most probably would exert its toxicity via a process different to the one proposed for the Cry proteins, based on the lack of structural homology of these two types of proteins (Estruch *et al.,* 1996). Despite being so different, both types of toxins exert their toxic action through apparently the same sequence of events: activation by midgut proteases, crossing the peritrophic membrane, binding to specific proteins in the apical membrane of the epithelial midgut cells, and pore formation (Lee *et al.,* 2003).

1.3.3.1. Behavioral and histopathological effects

The behavioral symptoms observed in susceptible insects after ingestion of Vip3A resemble the ones observed after Cry intoxication: feeding cessation, loss of gut peristaltism, and overall paralysis of the insect (Yu *et al.,* 1997). After ingestion of the Vip3Aa protein, the analysis of gut cross sections of susceptible insects show extensive damage in the midgut, with disrupted, swollen and/or lysed epithelial cells and leakage of cellular material to the lumen (Yu *et al.,* 1997; Doss, 2009; Abdelkefi-Mesrati *et al.,* 2011a; Abdelkefi-Mesrati *et al.,* 2011b; Ben Hamadou-Charfi *et al.,* 2013; Sellami *et al.,* 2015; Boukedi *et al.,* 2015). No damage was observed either in the foregut or in the hindgut, nor in the midgut of non susceptible insects (Yu *et al.,* 1997).

1.3.3.2. Proteolytic Processing

In vitro proteolysis of the full length Vip3A using insect midgut juice showed that these proteins are processed to several major proteolytic products, generally of about 62-66, 45, 33 and 22 kDa (Yu *et al.,* 1997, Abdelkefi-Mesrati *et al.,* 2011a; Abdelkefi-Mesrati *et al.,* 2011b; Ben Hamadou-Charfi *et al.,* 2013; Sellami *et al.,* 2015). The 22 kDa fragment corresponds to the N-terminal part of the protein (from amino acids 1 to 198), the 66 kDa fragment to the rest of the protein (from amino acid 199 to the end), and the 45 and 33 kDa fragments are thought to be derived from the 66 kDa portion (Estruch and Yu, 2001). The minimal toxic fragment of the protein has also been studied. Although an early study

claimed that the minimal fragment that retained insecticidal activity after proteolysis was the 33 kDa fragment (Estruch and Yu, 2001), the subsequent studies are in favor of the 62-66 kDa fragment as being the Vip3A toxic core (Lee *et al.*, 2003; Lee *et al*., 2006; Li *et al.*, 2007; Liu *et al.*, 2011; Abdelkefi-Mesrati *et al.*, 2011a; 2011b; Chakroun *et al.*, 2012; Gayen *et al.*, 2012 Hernández-Martínez *et al.*, 2013; Ben Hamadou-Charfi *et al.*, 2013; Caccia *et al.*, 2014; Sellami *et al.*, 2015).

There are two major proteolytic processing sites described in the primary structure of the Vip3A proteins. Processing sites are regions of few amino acids enriched in lysine residues. The first site is located at lysine K198 in Vip3Aa1 and is thought to release two fragments: a 22 kDa fragment which corresponds to the N-terminus of the protoxin, and a 66 kDa fragment ranging from amino acid 200 to the end, which is assumed to be the toxin active form. The second proteolytic site is located at position 455 and releases a fragment of about 33 kDa from amino acid 200 to 455 (Rang *et al.*, 2005; Estruch and Yu., 2001). Estruch and Yu (2001) proposed the 33 kDa fragment to be the minimal toxic core of the Vip3A protein.

In contrast to Cry proteins, Vip3A proteins do not have a protease-resistant core. Incubation of either Vip3Aa or Vip3Ae at different times and concentrations of commercial serine-proteases or insect midgut juice shows the unstable nature of the 62 kDa fragment, which starts to break down even before all the protoxin is being processed (Caccia *et al.*, 2014; Sellami *et al.*, 2015; Yu *et al.*, 1997; Abdelkefi-Mesrati *et al.*, 2011b; Ben Hamadou-Charfi *et al.*, 2013). Partial purification of peptidase activities from the *S. frugiperda* midgut juice showed that cationic trypsin-like and anionic chymotrypsin-like peptidases were involved in the formation of the Vip3A 62 kDa fragment, whereas cationic chymochypsin-like peptidases participated in its further processing (Caccia *et al.*, 2014). Interestingly, the 20 kDa fragment produced upon proteolytic processing of the Vip3Aa protoxin generally co-purifies with the 62 kDa fragment, suggesting that, after activation of the full length protein, the two fragments remain together (Chakroun and Ferré, 2014).

Results from bioassays using the full length protein and the trypsin-activated form of Vip3A proteins have shown that, in general, the in vitro proteolytic activation does not make a big difference in the insect toxicity and specificity (Chakroun *et al.*, 2012; Ruiz de Escudero *et al.*, 2014). There was also found that the midgut juice of a non-susceptible insect (*O. nubilalis*) could process Vip3A *in vitro* to a 65 kDa fragment that was fully toxic when fed to susceptible insects (Yu *et al.*, 1997). However, in some cases the rate of processing of the full length protein has been proposed to account for differences of

toxicity of a given Vip3A protein to different insect species (Abdelkefi-Mesrati *et al.*, 2011b; Chakroun *et al.*, 2012; Caccia *et al.*, 2014). Indeed, some studies have shown that differences in mortality disappeared when the trypsin activated protein was used instead of the full length protein (Chackroun *et al.*, 2012, Hernández-Martínez *et al.*, 2013).

1.3.3.3. Binding to the larval midgut epithelium

In vivo immunolocalization studies have shown that Vip3A binds to the apical microvilli from midgut epithelial cells (Yu *et al.*, 1997, Chakroun and Ferré 2014). Specific binding to the brush border membrane vesicles (BBMV)

Interestingly, Vip3Aa also binds specifically to the BBMV of the non susceptible *O. nubilalis* (Lee *et al.*, 2003), which indicates that binding is necessary but not sufficient to produce toxicity. Quantitative binding parameters were obtained using the ^{125}I-labeled protein (Chakroun and Ferré 2014). Specific binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV was found to be saturable, mostly irreversible and differentially affected by the presence of divalent cations. Vip3A proteins were also found to have lower affinity but higher number of binding sites compared with the Cry1A and Cry2A proteins. Interestingly, homologous competition showed that both the 62 kDa and the 20 kDa fragments of the trypsin-activated ^{125}I-Vip3Aa bound to BBMV and both were competed by the addition of non-labeled Vip3Aa. By contrast, Lui *et al.* (2011), using biotin-labeled Vip3Aa found that only the 62 kDa was able to bind to the BBMV of *H. armigera* and that the 20 kDa was found exclusively in the supernatant of the binding reaction.

Competition binding assays showed absence of shared binding sites between Vip3Aa and Cry1Ac, Cry1Ab, Cry1Fa, Cry2Ae and Cry2Ab which had been confirmed in several insect species, and between Vip3Af and both, Cry1Ab and Cry1F in *S. frugiperda* (Lee *et al.*, 2006; Sena *et al.*, 2009; Liu *et al.*, 2011, Gouffon *et al.*, 2011; Ben Hamadou-Charfi *et al.*, 2013, Chakroun and Ferré 2014). The only exception found was described by Bergamasco and coworkers (Bergamasco *et al.*, 2013), that reported partial competition of Cry1Ia protein for the Vip3Aa binding sites in *S. eridania* BBMV, but not in *S. frugiperda*, *S. albula* and *S. cosmioides* BBMV. Yet, Vip3Aa and its covariant Vip3Ae, Vip3Af and even the non active Vip3Ad share the same binding sites in *S. frugiperda* with no significant differences between their binding parameters (Chakroun and Ferré 2014).

Interaction of Vip3Aa with the BBMV of the susceptible insects involves specific binding molecules different from the ones recognized by Cry1A proteins. Ligand blot analyses revealed that Vip3A recognized 80 and 110 kDa proteins in *Manduca Sexta*

(Lepidoptera, Sphingidae), while Cry1Ab bound to proteins of 120 and 210 kDa (Lee *et al.*, 2003). The same study showed that Vip3A was unable to bind to the purified APN and the cadherin ectodomain RTB from *M. sexta*, both membrane proteins known to bind Cry proteins (Lee *et al.*, 2003). In *Prays oleae* (Lepidoptera: Yponomeutidae) and *Agrotis segetum* (Lepidoptera: Noctuidae) Vip3Aa bound to a 65 kDa protein, while Cry1Ac bound to a 210 kDa band in *P. oleae* and to a 120 kDa band in *A. segetum* (Abdelkefi-Mesrati *et al.*, 2009; Ben Hamadou-Charfi *et al.*, 2013). In *S. littoralis*, Vip3Aa bound proteins of 55 and 100 kDa (Abdelkefi-Mesrati *et al.*, 2011a), and in *Ephestia kuehniella* (Lepidoptera: Pyralidae), *S. frugiperda*, *S. albula*, *S.cosmioides*, and *S. eridania*, to a protein of 65 kDa (Abdelkefi-Mesrati *et al.*, 2011b, Bergamasco *et al.*, 2013), to which Cry1Ia also bound in the four *Spodoptera* species (Bergamasco *et al.*, 2013).

Very few studies have addressed the identity of the Vip3A binding molecules in the insect midgut. Two Vip3Aa binding molecules have been identified so far using the yeast two hybrid system. The first one was a 48 kDa protein from *A. ipsilon* with homology to a family of extracellular glycoproteins called tenascins, which could be associated with apoptotic processes (Estruch and Yu, 2001). The second one is the S2 ribosomal protein from *S. litura* (Singh *et al.*, 2010). It was identified as a Vip3A toxicity mediating interacting partner in Sf21 cells. Silencing of the *S2* gene reduced the toxicity of the Vip3A to both in Sf21 cells and in fifth-instar *S. litura* larvae. Both S2 and Vip3Aa co-precipitated in pull down assays and co-localized in the surface and cytoplasm of Sf21 cells (Singh *et al.*, 2010). How this S2-Vip3A protein interaction could trigger the lysis of the cells was not explained and remains unknown. In *H. armigera*, the molecules that bind to Vip3Aa were found to be slightly associated with lipid rafts (Liu *et al.*, 2011).

In an attempt to understand how midgut cells respond to the intoxication by Vip3 proteins, gene expression profiles of *S. exigua* larvae treated with a sublethal dose of Vip3Aa were obtained using a genome-wide microarray that included more than 29000 unigenes (Bel *et al.*, 2013). No alteration in the expression levels of the two Vip3A binding proteins described above (S2 and the tenascin X-tox-like protein) was found. Genes related with the mode of action of the Cry proteins were also analyzed and only minor differences in expression were found. Authors concluded that, most probably, the lack of significant changes in transcription was either because the genes analyzed are either not involved in the Vip3 mode of action or the mechanisms of defense against Vip3A toxins do not rely on the regulation of the members involved in the mode of action.

1.3.3.4. Pore formation

Despite the absence of any pore forming domain sequence in the Vip3 proteins, the pore formation activity of the trypsinized or midgut juice activated protein has been demonstrated by voltage clamping assays with dissected midguts of *M. sexta* (Lepidoptera: Sphingidae) and also in planar lipid bilayers, in opposition to the full length Vip3A protein that was unable to form them (Lee *et al.,* 2003). These ion channels are capable to destroy the transmembrane potential, are voltage independent and cation selective (Lee *et al.*, 2003). The pore forming ability of the activated Vip3A has been also demonstrated by fluorescence quenching using *H. armigera* BBMV (Liu *et al.,* 2011). Besides, Vip3A ion channels are restricted to susceptible insects and have been found to have biophysical properties that differ from those of Cry1Ab in *M. sexta* (Lee *et al.*, 2003).

1.3.4 Resistance and cross-resistance

Very few cases have been reported on resistance to Vip3 proteins. Laboratory selection of a *H. virescens* colony lead to 2040-fold resistance to Vip3A compared to the Vip-Unsel population (Pickett, 2009). Resistance was found to be polygenic with possible paternal influence and ranged from almost completely recessive to incompletely dominant; fitness costs were temperature dependent, with reduced mating success, fecundity and fertility (Gulzar *et al.,* 2012). The presence of Vip3Aa resistance alleles in field populations was studied in *H. armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae) in Australia using the F2 screening (Mahon *et al*., 2012). Results showed that resistance alleles existed in both insect species as natural polymorphisms at a relatively high frequency (0.027 and 0.008 respectively), above mutation rates normally encountered (Mahon *et al.,* 2012). Interestingly, within each species, the resistance of two different F2 families was due to alleles at the same locus, and resistance was found to be essentially recessive, most probably conferred by a single gene, and did not confer cross-resistance to Cry1Ac or Cry2Ab (Mahon *et al.,* 2012). Further studies on the *H. punctigera* resistant strain confirmed that there was no linkage between the Vip3A and the Cry2Ab resistance loci (Walsh *et al.*, 2014).

The increased use of Vip3 toxins in pyramided Bt crops, to improve both pest control and resistance management, sparked interest in the evaluation of cross-resistance between Cry and Vip3A proteins (Kurtz, 2010). So far, no significant cross-resistance between these two classes of proteins has been described. Vip3Aa was found to be equally toxic to a susceptible and to three Cry resistant *H. virescens* strains YHD2, resistant to Cry1Ac, Cry1F and slightly cross resistant to Cry2A, and CXC and KCBhyb, both resistant to

Cry1Ac, Cry1Aa, Cry1Ab, Cry1F and Cry2Aa2) (Jackson *et al.*, 2007). A Cry1Ac resistant strain of *H zea* (AR) showed cross-resistance to Cry1Ab but not to Vip3A, Cry2Aa2 or Cry2Ab2 (Anilkumar *et al.*, 2008). A study on two *H. armigera* populations from Cry1Ac-cotton planting regions in China showed lack of significant correlation between responses to Vip3Aa and Cry1Ac, suggesting little or no cross-resistance between these two toxins (An *et al.*, 2010). Cross-resistance to Vip3A has also been studied in two *S. frugiperda* Cry1F resistant populations, one collected from Bt-maize fields in Puerto Rico and the other in southeast USA. Both populations were very susceptible to Vip3Aa, indicating absence of cross resistance between Vip3Aa and Cry1F proteins (Vélez *et al.*, 2013; Huang *et al.*, 2014). A study using a different Vip3A protein, Vip3Ac, showed that it was equally toxic to a susceptible and a Cry1Ac-resistant *Trichoplusia ni* (Lepidoptera: Noctuidae) strain (Fang *et al.*, 2007). However, in this case the resistant strain was slightly less susceptible to Vip3Aa (resistance ratio of 2.1) and to two Vip3A chimeric proteins (resistance ratios of 1.8 and 3.2) (Fang *et al.*, 2007).

1.3.5 Expression in plants

The *vip3Aa* gene has been successfully introduced in cotton and in corn, and later combined with other *cry* genes to confer higher protection and delay insect resistance (http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm). VipCot™ and Agrisure Viptera™ were registered in the USA in 2008 and 2009, respectively (Syngenta Seeds, Inc.). The former is the result of event COT102 in cotton, which produces the Vip3Aa19 protein (http://www.isaaa.org/gmapprovaldatabase/gene/default.asp?GeneID=24&Gene=vip3A(a); http://en.biosafetyscanner.org/schedaevento.php?evento=208), whereas the latter contains the event MIR162 in corn, which produces the Vip3Aa20 protein (http://iaspub.epa.gov/apex/pesticides/f?p=CHEMICALSEARCH:30#p). Both events have been pyramided with *cry1Ab* (VipCot™ Vip3Aa + mCry1Ab, and Agrisure Viptera™ Vip3Aa + Cry1Ab) and later with *cry1Fa* (VipCot™ Vip3Aa + Cry1Ac + Cry1Fa, and Agrisure Viptera™ Vip3Aa + Cry1Ab + Cry1Fa) to confer a wider and more robust protection against Lepidoptera (Kurtz *et al.*, 2007; Adamczyk *et al.*, 2008; Burkness *et al.*, 2010). Furthermore, the corn event MIR162 has been stacked with other *cry* genes expressing proteins active against Coleoptera (Cry3A and eCry3.1Ab) to confer protection against these two insect orders (Carrière *et al.*, 2015) A three-year study on the field performance of VipCot™ expressing just the Vip3Aa protein indicated that the plants were

highly efficacious against *H. armigera* early in the season and that the efficacy declined as the season progressed, though not so drastically as Cry1Ac in Bollgard™ or Ingard™ cotton (Llewellyn *et al.*, 2007). In 2015, the first modified Vip3A, with improved toxicity, has been introduced in tobacco conferring almost total protection toward *H. armigera*, *A. ipsilon* and *S. littoralis* (Gayen *et al.*, 2015).

Cotton has also been transformed with a synthetic *vip3A* gene fused to a chloroplast transit peptide coding sequence (Wu *et al.*, 2011). The Vip3A protein accumulated in the chloroplasts and its concentration in the plant was higher than in plants transformed with just the synthetic gene. Transformed plants provoked 100% mortality to larvae of *S. frugiperda*, *S. exigua* and *H. zea.*

1.4 References:

Abdelkefi-Mesrati L, Boukedi H, Dammak-Karray M, Sellami-Boudawara T, Jaoua S, Tounsi S. 2011a. Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of *Spodoptera littoralis*. J Invertebr Pathol **106**: 250-254.

Abdelkefi-Mesrati L, Boukedi H, Chakroun M, Kamoun F, Azzouz H, Tounsi S, Rouis S, Jaoua S. 2011b. Investigation of the steps involved in the difference of susceptibility of *Ephestia kuehniella* and *Spodoptera littoralis* to the *Bacillus thuringiensis* Vip3Aa16 toxin. J Invertebr Pathol **107**:198-201.

Abdelkefi-Mesrati L, Rouis S, Sellami S, Jaoua S. 2009. *Prays oleae* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3LB differs from that of Cry1Ac toxin. Mol Biotechnol **43**:15–19.

Abulreesh HH, Osman GE, Assaeedi AS. 2012. Characterization of insecticidal genes of *Bacillus thuringiensis* strains isolated from arid environments. Indian J Microbiol **52**:500-3.

Adamczyk Jr JJ, Mahaffey JS. 2008. Efficacy of Vip3A and Cry1Ab transgenic traits in cotton against various lepidopteran pests. Fla Entomol **91**:570-575.

Ali MI, Luttrell RG. 2011. Susceptibility of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) to Vip3A insecticidal protein expressed in VipCot™ cotton. J Invertebr Pathol **108**:76-84.

An J, Gao Y, Wu K, Gould F, Gao J, Shen Z, Lei C. 2010. Vip3Aa tolerance response of *Helicoverpa armigera* populations from a Cry1Ac cotton planting region. J Econ Entomol **103**:2169-73.

Anilkumar KJ, Rodrigo-Simón A, Ferré J, Pusztai-Carey M, Sivasupramaniam S, Moar WJ. 2008. Production and characterization of *Bacillus thuringiensis* Cry1Ac-resistant cotton bollworm *Helicoverpa zea* (Boddie). Appl Environ Microbiol **74**:462-9.

Arora N, Selvapandiyan A, Agrawal N, Bhatnagar RK. 2003. Relocating expression of vegetative insecticidal protein into mother cell of *Bacillus thuringiensis*. Biochem Biophys Res Commun **310**:158-62.

Asokan R, Mahadeva Swamy HM, Arora K. 2012. Screening, diversity and partial sequence comparison of vegetative insecticidal protein (*vip3A*) genes in the local isolates of *Bacillus thuringiensis* Berliner. Curr Microbiol **64**:365-70.

Baranek J, Kaznowski A, Konecka E, Naimov S. 2015. Activity of vegetative insecticidal proteins Vip3Aa58 and Vip3Aa59 of *Bacillus thuringiensis* against lepidopteran pests. J Invertebr Pathol **130**:72-81.

Barth H, Aktories K, Popoff MR, Stiles BG. 2004. Binary bacterial toxins: Biochemistry, biology, and applications of common *Clostridium* and *Bacillus* Proteins. Microbiol Mol Biol Rev **68**:373-402.

Barth H, Hofmann F, Olenik C, Just I, Aktories K. 1998. The N-terminal part of the enzyme component (C2I) of the binary *Clostridium botulinum* C2 toxin interacts with the binding component C2II and functions as a carrier system for a Rho ADP-ribosylating C3-like fusion toxin. Infect Immun **66**:1364–1369.

Beard CE, Court L, Boets A, Mourant R, Van Rie J, Akhurst RJ. 2008. Unusually high frequency of genes encoding vegetative insecticidal proteins in an Australian *Bacillus thuringiensis* collection. Curr Microbiol **57**:195-9.

Bel Y, Jakubowska AK, Costa J, Herrero S, Escriche B. 2013. Comprehensive analysis of gene expression profiles of the beet armyworm *Spodoptera exigua* larvae challenged with *Bacillus thuringiensis* Vip3Aa toxin. PLoS One **8**:e81927.

Ben Hamadou-Charfi D, Boukedi H, Abdelkefi-Mesrati L, Tounsi S, Jaoua S. 2013. *Agrotis segetum* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa16 differs from that of Cry1Ac toxin. J Invertebr Pathol **114**:139-43.

Bergamasco VB, Mendes DR, Fernandes OA, Desidério JA, Lemos MV. 2013. *Bacillus thuringiensis* Cry1Ia10 and Vip3Aa protein interactions and their toxicity in *Spodoptera* spp. (Lepidoptera). J Invertebr Pathol **112**:152-8.

Bhalla R, Dalal M, Panguluri SK, Jagadish B, Mandaokar AD, Singh AK, Kumar PA. 2005. Isolation, characterization and expression of a novel vegetative insecticidal protein gene of *Bacillus thuringiensis*. FEMS Microbiol Lett **243**:467-72.

Bi Y, Zhang Y, Shu C, Crickmore N, Wang Q, Du L, Song F, Zhang J. 2015. Genomic sequencing identifies novel *Bacillus thuringiensis* Vip1/Vip2 binary and Cry8 toxins

that have high toxicity to *Scarabaeoidea* larvae. Appl Microbiol Biotechnol **99**:753-60.

Blaustein RO, Koehler TM, Collier RJ, Finkelstein A. 1989. Anthrax toxin: channel-forming activity of protective antigen in planar phospholipid bilayers. Proc Natl Acad Sci U S A **86**:2209–2213.

Boets A, Arnaut G, Van Rie J, Damme N. (2011). Toxins. U.S. Patent 7,919,609 B2, April 2011.

Boukedi H, Ben Khedher S, Triki N, Kamoun F, Saadaoui I, Chakroun M, Tounsi S, Abdelkefi-Mesrati L. 2015. Overproduction of the *Bacillus thuringiensis* Vip3Aa16 toxin and study of its insecticidal activity against the carob moth *Ectomyelois ceratoniae*. J Invertebr Pathol **127**:127-129.

Burkness EC, Dively G, Patton T, Morey AC, Hutchison WD. 2010. Novel Vip3A *Bacillus thuringiensis* (Bt) maize approaches high-dose efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae) under field conditions: Implications for resistance management. GM Crops **1**:337-343.

Caccia S, Chakroun M, Vinokurov K, Ferré J. 2014. Proteolytic processing of *Bacillus thuringiensis* Vip3A proteins by two *Spodoptera* species. J Insect Physiol **67**:76-84.

Carrière Y, Crickmore N, Tabashnik BE. 2015. Optimizing pyramided transgenic *Bt* crops for sustainable pest management. Nat Biotech **33**:161-8.

Chakroun M, Bel Y, Caccia S, Abdelkefi-Mesrati L, Escriche B, Ferré J. 2012. Susceptibility of *Spodoptera frugiperda* and *S. exigua* to *Bacillus thuringiensis* Vip3Aa insecticidal protein. J Invertebr Pathol **110**:334-9.

Chakroun M, Ferré J. 2014. *In vivo* and *in vitro* binding of Vip3Aa to *Spodoptera frugiperda* midgut and characterization of binding sites by ^{125}I radiolabeling. Appl Environ Microbiol **80**:6258-65.

Chen J, Yu J, Tang L, Tang M, Shi Y, Pang Y. 2003. Comparison of the expression of *Bacillus thuringiensis* full-length and N-terminally truncated *vip3A* gene in *Escherichia coli*. J Appl Microbiol **95**:310-6.

Crickmore N, Baum J, Bravo A, Lereclus D, Narva K, Sampson K, Schnepf E, Sun M, Zeigler DR. 2014. "*Bacillus thuringiensis* toxin nomenclature" http://www.btnomenclature.info/ (accessed on 05 July 2015).

De Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE. 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu Rev Genet **37**:409–33.

Donovan WP, Engleman JT, Donovan JC, Baum JA, Bunkers GJ, Chi DJ, Clinton WP, English L, Heck GR, Ilagan OM. 2006. Discovery and characterization of Sip1A: A novel secreted protein from *Bacillus thuringiensis* with activity against coleopteran larvae. Appl Microbiol Biotechnol **72**: 713–719.

Donovan WP, Donovan JC, Engleman JT. 2001. Gene knockout demonstrates that *vip3A* contributes to the pathogenesis of *Bacillus thuringiensis* toward *Agrotis ipsilon* and *Spodoptera exigua*. J Invertebr Pathol **78**:45-51.

Dong F, Shi R, Zhang S, Zhan T, Wu G, Shen J, Liu Z. 2012. Fusing the vegetative insecticidal protein Vip3Aa7 and the N terminus of Cry9Ca improves toxicity against *Plutella xylostella* larvae. Appl Microbiol Biotechnol **96**:921-9.

Doss VA, Kumar KA, Jayakumar R, Sekar V. 2002. Cloning and expression of the vegetative insecticidal protein (*vip3V*) gene of *Bacillus thuringiensis* in *Escherichia coli*. Protein Expr Purif **26**:82-8.

Environmental Protection Agency (EPA). 2003. *Bacillus thuringiensis* VIP3A insect control protein as expressed in event COT102; notice of filing a pesticide petition to establish an exemption from the requirement of a tolerance for a certain pesticide chemical in or on food. Federal register **68**:66422-66425.

Espinasse S, Chaufaux J, Buisson C, Perchat S, Gohar M, Bourguet D, Sanchis V. 2003. Occurrence and linkage between secreted insecticidal toxins in natural isolates of *Bacillus thuringiensis*. Curr Microbiol **47**:501-7.

Estruch JJ, Yu CG. 2001. Plant pest control. U S Patent No. 6,291,156 B1.

Estruch JJ, Carozzi NB, Desai N, Duck NB, Warren GW, Koziel MG. 1997. Transgenic plants: An emerging approach to pest control. Nat Biotechnol **15**:137-41.

Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc Natl Acad Sci U S A **93**:5389-5394.

Fang J, Xu X, Wang P, Zhao JZ, Shelton AM, Cheng J, Feng MG, Shen Z. 2007. Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl Environ Microbiol **73**:956-96.

Feitelson JS, Schnepf HE, Narva KE, Stockhoff BA, Schmeits J, Loewer D, Dullum CJ, Muller-cohn J, Stamp L, Morrill G, Finstad-lee S. 2003. Pesticidal toxins and nucleotide sequences which encode these toxins. U S Patent No. 6,656,908,B2.

Figueiredo CS, Marucci SC, Tezza RID, Lemos MVF, Desidério JA. 2013. Caracterização do gene *vip3A* e toxicidade da proteína Vip3Aa50 à lagarta-do-cartucho e à lagarta-da-soja. Pesq Agropec Bras **48**:1220-1227.

Franco-Rivera A, Benintende G, Cozzi J, Baizabal-Aguirre VM, Valdez-Alarcón JJ, López-Meza JE. 2004. Molecular characterization of *Bacillus thuringiensis* strains from Argentina. Antonie Van Leeuwenhoek **86**:87-92.

Gayen S, Hossain MA, Sen SK. 2012. Identification of the bioactive core component of the insecticidal Vip3A toxin peptide of *Bacillus thuringiensis*. J Plant Biochem Biotechnol **21**:S128-S135.

Gayen S, Samanta MK, Hossain MA, Mandal CC, Sen SK. 2015. A deletion mutant *ndv200* of the *Bacillus thuringiensis vip3BR* insecticidal toxin gene is a prospective candidate for the next generation of genetically modified crop plants resistant to lepidopteran insect damage. Planta **242**:269-81.

Gouffon C, Van Rie J, Jansens S, Jurat-Fuentes JL. 2011. Binding sites for *Bacillus thuringiensis* Cry2Ae toxin on heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin. Appl Environ Microbiol **77**:3182-8.

Gulzar A, Pickett B, Sayyed AH, Wright DJ. 2012. Effect of temperature on the fitness of a Vip3A resistant population of *Heliothis virescens* (Lepidoptera:Noctuidae). J Econ Entomol **105**:964-70.

Han S, Arvai AS, Clancy SB, Tainer JA. 2001. Crystal structure and novel recognition motif of rho ADP-ribosylating C3 exoenzyme from *Clostridium botulinum*: Structural insights for recognition specificity and catalysis. J Mol Biol **305**:95-107.

Han S, Craig JA, Putnam CD, Carozzi NB, Tainer JA. 1999. Evolution and mechanism from structures of an ADP-ribosylating toxin and NAD complex. Nat Struct Biol **6**:932-936.

Hernández-Martínez P, Hernández-Rodríguez CS, Van Rie J, Escriche B, Ferré J. 2013. Insecticidal activity of Vip3Aa, Vip3Ad, Vip3Ae, and Vip3Af from *Bacillus thuringiensis* against lepidopteran corn pests. J Invertebr Pathol **113**:78-81.

Hernández-Rodríguez CS, Boets A, Van Rie J, Ferré J. 2009. Screening and identification of *vip* genes in *Bacillus thuringiensis* strains. J Appl Microbiol **107**: 219-225.

Hernández-Rodríguez CS, Ruiz de Escudero I, Asensio AC, Ferré J, Caballero P. 2013. Encapsulation of the *Bacillus thuringiensis* secretable toxins Vip3Aa and Cry1Ia in *Pseudomonas fluorescens.* Biol Control **66**:159-165.

Hernández-Rodríguez CS, Hernández-Martínez P, Van Rie J, Escriche B, Ferré J. 2012. Specific binding of radiolabeled Cry1Fa insecticidal protein from *Bacillus thuringiensis* to midgut sites in lepidopteran species. Appl Environ Microbiol **78**:4048-50.

Huang F, Qureshi JA, Meagher RL. Jr, Reisig DD, Head GP, Andow DA, Ni X, Kerns D, Buntin GD, Niu Y, Yang F, Dangal V. 2014. Cry1F resistance in fall armyworm *Spodoptera frugiperda*: Single gene versus pyramided Bt maize. PLoS One **9**:e112958.

Jackson RE, Marcus MA, Gould F, Bradley JR Jr, Van Duyn JW. 2007. Cross-resistance responses of Cry1Ac-selected *Heliothis virescens* (Lepidoptera: Noctuidae) to the *Bacillus thuringiensis* protein Vip3A. J Econ Entomol **100**:180-186.

Jamoussi K, Sellami S, Abdelkefi-Mesrati L, Givaudan A, Jaoua S. 2009. Heterologous expression of *Bacillus thuringiensis* vegetative insecticidal protein-encoding gene *vip3LB* in *Photorhabdus temperata* strain K122 and oral toxicity against the Lepidoptera *Ephestia kuehniella* and *Spodoptera littoralis*. Mol Biotechnol **43**:97-103.

Jucovic M, Walters FS, Warren GW, Palekar NV, Chen JS. 2008. From enzyme to zymogen: Engineering Vip2, an ADP-ribosyltransferase from *Bacillus cereus*, for conditional toxicity. Protein Eng Des Sel **21**:631–638.

James C. 2014. Global status of commercialized biotech/GM crops: 2014. *ISAAA Brief* No. 49. ISAAA: Ithaca, NY.

Knapp O, Benz R, Gibert M, Marvaud JC, Popoff MR. 2002. Interaction of *Clostridium perfringens* iota-toxin with lipid bilayer membranes. Demonstration of channel formation by the activated binding component Ib and channel block by the enzyme component Ia. J Biol Chem **277**:6143-52.

Kurtz RW. 2010. A review of Vip3A mode of action and effects on Bt Cry protein-resistant colonies of lepidopteran larvae. Southwest Entomol **35**:391-394.

Kurtz RW, McCaffery A, O'Reilly D. 2007. Insect resistance management for Syngenta's VipCot transgenic cotton. J Invertebr Pathol **95**:227-30.

Lee MK, Curtiss A, Alcantara E, Dean DH. 1996. Synergistic effect of the *Bacillus thuringiensis* toxins CryIAa and CryIAc on the gypsy moth, *Lymantria dispar*. Appl Environ Microbiol **62**:583-6.

Lee MK, Miles P, Chen JS. 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem Biophys Res Commun **339**:1043-1047.

Lee MK, Walters FS, Hart H, Palekar N, Chen JS. 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa differs from that of Cry1Ab delta-endotoxin. Appl Environ Microbiol **69**:4648-4657.

Lemes AR, Davolos CC, Legori PC, Fernandes OA, Ferré J, Lemos MV, Desiderio JA. 2014. Synergism and antagonism between *Bacillus thuringiensis* Vip3A and Cry1 proteins in *Heliothis virescens*, *Diatraea saccharalis* and *Spodoptera frugiperda*. PLoS One **9**:e107196.

Leuber M, Orlik F, Schiffler B, Sickmann A, Benz R. 2006. Vegetative insecticidal protein (Vip1Ac) of *Bacillus thuringiensis* HD201: Evidence for oligomer and channel formation. Biochemistry **45**:283-8.

Li C, Xua N, Huanga X, Wanga W, Chenga J, Wuc K, Shen Z. 2007. *Bacillus thuringiensis* Vip3 mutant proteins: Insecticidal activity and trypsin sensitivity. Biocontrol Sci Technol **17**:699-708.

Liu J, Song F, Zhang J, Liu R, He K, Tan J, Huang D. 2007. Identification of *vip3A*-type genes from *Bacillus thuringiensis* strains and characterization of a novel *vip3A*-type gene. Lett Appl Microbiol. **45**:432-438.

Liu JG, Yang AZ, Shen XH, Hua BG, Shi GL. 2011. Specific binding of activated Vip3Aa10 to *Helicoverpa armigera* brush border membrane vesicles results in pore formation. J Invertebr Pathol **108**:92-97.

Llewellyn DJ, Mares CL, Fitt, GP. 2007. Field performance and seasonal changes in the efficacy against *Helicoverpa armigera* (Hübner) of transgenic cotton expressing the insecticidal protein vip3A. Agric For Entomol **9**: 93–101.

Loguercio LL, Barreto ML, Rocha TL, Santos CG, Teixeira FF, Paiva E. 2002. Combined analysis of supernatant-based feeding bioassays and PCR as a first-tier screening strategy for Vip-derived activities in *Bacillus thuringiensis* strains effective against tropical fall armyworm. J Appl Microbiol **93**:269-77.

Mahon RJ, Downes SJ, James B. 2012. Vip3A resistance alleles exist at high levels in Australian targets before release of cotton expressing this toxin. PLoS One. **7**:e39192.

Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH. 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res. **39**:D225-9. (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (accessed on 26 January 2015).

Mesrati LA, Tounsi S, Jaoua S. 2005b. Characterization of a novel *vip3*-type gene from *Bacillus thuringiensis* and evidence of its presence on a large plasmid. FEMS Microbiol Lett. **244**:353-8.

Mesrati LA, Tounsi S, Kamoun F, Jaoua S. 2005a. Identification of a promoter for the vegetative insecticidal protein-encoding gene ***vip3LB*** from *Bacillus thuringiensis*. FEMS Microbiol Lett **247**:101-4.

Murawska E, Fiedoruk K, Bideshi DK, Swiecicka I. 2013. Complete genome sequence of *Bacillus thuringiensis* subsp. *thuringiensis* strain IS5056, an isolate highly toxic to *Trichoplusia ni*. Genome Announc **1**:e00108-13.

Palma L, Hernández-Rodríguez CS, Maeztu M, Hernández-Martínez P, Ruiz de Escudero I, Escriche B, Muñoz D, Van Rie J, Ferré J, Caballero P. 2012. Vip3C, a novel class of vegetative insecticidal proteins from *Bacillus thuringiensis*. Appl Environ Microbiol **78**:7163-5.

Palma L, Muñoz D, Berry C, Murillo J, Caballero P. 2014. *Bacillus thuringiensis* toxins: An overview of their biocidal activity. Toxins (Basel) **6**:3296-325.

Palma L, Ruiz de Escudero I, Maeztu M, Caballero P, Muñoz D. 2013. Screening of *vip* genes from a Spanish *Bacillus thuringiensis* collection and characterization of two Vip3 proteins highly toxic to five lepidopteran crop pests. Biol Control **66**:141-149.

Pickett B. 2009. Studies on resistance to vegetative (Vip3A) and crystal (Cry1A) insecticidal toxins of *Bacillus thuringiensis* in *Heliothis virescens* (Fabricius). Ph.D. thesis. Imperial College London, London, United Kingdom.

Rang C, Gil, Neisner N, Van Rie J, Frutos R. 2005. Novel Vip3-Related Protein from *Bacillus thuringiensis*. Appl Environ Microbiol **71**:6276-81.

Rice WC. 1999. Specific primers for the detection of *vip3A* insecticidal gene. Lett Appl Microbiol **28**: 378-382.

Ruiu L. 2013. *Brevibacillus laterosporus*, a pathogen of invertebrates and a broad-spectrum antimicrobial species. Insects **4**:476-492.

Ruiz de Escudero I, Banyuls N, Bel Y, Maeztu M, Escriche B, Muñoz D, Caballero P, Ferré J. 2014. A screening of five *Bacillus thuringiensis* Vip3A proteins for their activity against lepidopteran pests. J Invertebr Pathol **113**:78-81.

Sanchis V. 2011. From microbial sprays to insect-resistant transgenic plants: History of the biospesticide *Bacillus thuringiensis*. A review. Agron Sustain Dev **31**: 217-231.

Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P. 2011. *Bacillus thuringiensis*: A century of research, development and commercial applications. Plant Biotechnol J **9**:283-300.

Saraswathy N, Nain V, Sushmita K, Kumar PA. 2008. A fusion gene encoding two different insecticidal proteins of *Bacillus thuringiensis.* Indian J Biotechnol **7**:204-209.

Sattar S, Maiti MK. 2011. Molecular characterization of a novel vegetative insecticidal protein from *Bacillus thuringiensis* effective against sap-sucking insect pest. J Microbiol Biotechnol **21**: 937-946.

Schmid A, Benz R, Just I, Aktories, K. 1994. Interaction of *Clostridium botulinum* C2 toxin with lipid bilayer membranes; Formation of cation-selective channels and inhibition of channel function by chloroquine. J Biol Chem **269**:16706–16711.

Schnepf HE, Narva KE, Stockhoff BA, Lee SF, Walz M, Sturgis B. 2005. Pesticidal toxins and genes from *Bacillus laterosporus* strains. U S Patent No. 6,956,116.

Schnepf HE, Narva KE, Stockhoff BA, Lee SF, Waltz M, Sturgis B. 2003. Pesticidal toxins and genes from *Bacillus laterosporus* strains. U S Patent No. 6,605,701 B2.

Sellami S, Jamoussi K, Dabbeche E, Jaoua S. 2011. Increase of the *Bacillus thuringiensis* secreted toxicity against lepidopteron larvae by homologous expression of the *vip3LB* gene during sporulation stage. Curr Microbiol **63**:289-94.

Sellami S, Cherif M, Abdelkefi-Mesrati L, Tounsi S, Jamoussi K. 2015. Toxicity, activation process, and histopathological effect of *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa16 on *Tuta absoluta*. Appl Biochem Biotechnol **175**:1992-9.

Sena JA, Hernández-Rodríguez CS, Ferré J. 2009. Interaction of *Bacillus thuringiensis* Cry1 and Vip3Aa proteins with *Spodoptera frugiperda* midgut binding sites. Appl Environ Microbiol **75**: 2236-2237.

Selvapandiyan A, Arora N, Rajagopal R, Jalali SK, Venkatesan T, Singh SP, Bhatnagar RK. 2001. Toxicity analysis of N- and C-terminus-deleted vegetative insecticidal protein from *Bacillus thuringiensis*. Appl Environ Microbiol **67**:5855-8.

Shingote PR, Moharil MP, Dhumale DR, Jadhav PV, Satpute NS, Dudhare MS. 2013b. Screening of *vip1*/*vip2* binary toxin gene and its isolation and cloning from local *Bacillus thuringiensis* isolates. Sci Asia **39**: 620–624.

Shi Y, Ma W, Yuan M, Sun F, Pang Y. 2007. Cloning of *vip1*/*vip2* genes and expression of Vip1Ca/Vip2Ac proteins in *Bacillus thuringiensis*. World J Microbiol Biotechnol **23**:501–507.

Shi Y, Xu W, Yuan M, Tang M, Chen J, Pang Y. 2004. Expression of *vip1*/*vip2* genes in *Escherichia coli* and *Bacillus thuringiensis* and the analysis of their signal peptides. J Appl Microbiol **97**:757–765.

Shelton AM. 2012. Genetically engineered vegetables expressing proteins from *Bacillus thuringiensis* for insect resistance: Successes, disappointments, challenges and ways to move forward. GM Crops Food **3**:1-9.

Shingote PR, Moharil MP, Dhumale DR, Deshmukh AG, Jadhav PV, Dudhare MS, Satpute NS. 2013a. Distribution of *vip* genes, protein profiling and determination of entomopathogenic potential of local isolates of *Bacillus thuringiensis.* Bt Res **4**:14-20.

Singh G, Sachdev B, Sharma N, Seth R, Bhatnagar RK. 2010. Interaction of *Bacillus thuringiensis* vegetative insecticidal protein with ribosomal S2 protein triggers larvicidal activity in *Spodoptera frugiperda*. Appl Environ Microbiol **76**:7202–7209.

Song R, Peng D, Yu Z, Sun M. 2008. Carboxy-terminal half of Cry1C can help vegetative insecticidal protein to form inclusion bodies in the mother cell of *Bacillus thuringiensis*. Appl Microbiol Biotechnol **80**:647-54.

Taborsky V. 1992. Small-scale processing of microbial pesticides. FAO Agr Serv Bull **96**:1-90.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 1997. MEGA5: molecular 1433 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and 1434 maximum parsimony methods. Mol Biol Evol. **28**:2731-9.

Thamthiankul Chankhamhaengdecha S, Tantichodok A, Panbangred W. 2008. Spore stage expression of a vegetative insecticidal gene increase toxicity of *Bacillus thuringiensis* subsp. *aizawai* SP41 against *Spodoptera exigua*. J Biotechnol **136**:122-8.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 2011. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25**:4876-82.

Van Frankenhuyzen K, Nystrom C. 2009 "The *Bacillus thuringiensis* toxin specificity database" http://www.glfc.cfs.nrcan.gc.ca/bacillus (accessed on 05 July 2015).

Vélez AM, Spencer TA, Alves AP, Moellenbeck D, Meagher RL, Chirakkal H, Siegfried BD. 2013. Inheritance of Cry1F resistance, cross-resistance and frequency of resistant alleles in *Spodoptera frugiperda* (Lepidoptera: Noctuidae). Bull Entomol Res **103**:700-13.

Walsh TK, Downes SJ, Gascoyne J, James W, Parker T, Armstrong J, Mahon RJ. 2014. Dual Cry2Ab and Vip3A resistant strains of *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae); testing linkage between loci and monitoring of allele frequencies. J Econ Entomol **107**:1610-7.

Warren GW. 1997. Vegetative insecticidal proteins: novel proteins for control of corn pests, p 109-121. *In* Carozzi NB, Koziel M (ed), Advances in insect control, the role of transgenic plants. Taylors & Francis Ltd, London.

Warren GW, Koziel MG, Mullin MA, Nye GJ, Carr B, Desai NM, Kostichka K, Duck NB, Estruch J. 1998. Auxiliary proteins for enhancing the insecticidal activity of pesticidal proteins. U S Patent No. 5,770,696.

Wu J, Luo X, Zhang X, Shi Y, Tian Y. 2011. Development of insect-resistant transgenic cotton with chimeric TVip3A* accumulating in chloroplasts. Transgenic Res **20**:963-973.

Wu J, Zhao F, Bai J, Deng G, Qin S, Bao Q. 2007. Evidence for positive darwinian selection of *vip* gene in *Bacillus thuringiensis*. J Genet Genomics **34**:649-60.

Wu ZL, Guo WY, Qiu JZ, Huang TP, Li XB, Guan X. 2004. Cloning and localization of ***vip3A*** gene of *Bacillus thuringiensis*. Biotechnol Lett **26**:1425-8.

Yu X, Liu T, Liang X, Tang C, Zhu J, Wang S, Li S, Deng Q, Wang L, Zheng A, Li P. 2011b. Rapid detection of *vip1*-type genes from *Bacillus cereus* and characterization of a novel *vip* binary toxin gene. FEMS Microbiol Lett **325**:30-6.

Yu X, Zheng A, Zhu J, Wang S, Wang L, Deng Q, Li S, Liu H, Li P. 2011a. Characterization of vegetative insecticidal protein *vip* genes of *Bacillus thuringiensis* from Sichuan Basin in China. Curr Microbiol **62**: 752-757.

Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa lyses midgut epithelium cells of susceptible insects. Appl Environ Microbiol **63**: 532-536.

Yu X, Liu T, Sun Z, Guan P, Zhu J, Wang S, Li S, Deng Q, Wang L, Zheng A, Li P. 2012. Co-expression and synergism analysis of Vip3Aa29 and Cyt2Aa3 insecticidal proteins from *Bacillus thuringiensis*. Curr Microbiol **64**:326-31.

Zhu C, Ruan L, Peng D, Yu Z, Sun M. 2005. Vegetative insecticidal protein enhancing the toxicity of *Bacillus thuringiensis* subsp *kurstaki* against *Spodoptera exigua*. Lett Appl Microbiol **42**:109-14.

Table 1: Activity spectrum of individual and combination of Vip1 and Vip2 protoxins on neonate larvae reported in the literature.

Protein	**Insect order**	**Insect species**	**Activity/LC_{50}**	**Reference**
Vip1Aa	Coleoptera	*D. virgifera*	NA	Warren 1997
Vip1Ac	Coleoptera	*H. oblita*	NA	Yu *et al.*, 2011
		T. molitor	NA	Shi *et al.*, 2004
		C. suppressalis	NA	Yu *et al.*, 2011
	Lepidoptera	*H. armigera*	NA	Yu *et al.*, 2011
		S. exigua	NA	Shi *et al.*, 2004
		S. litura	NA	Yu *et al.*, 2011
	Diptera	*C.*	NA	Shi *et al.*, 2004
	Homoptera	*quinquefasciatus*	NA	Yu *et al.*, 2011
		A. gossypii	NA	Shi *et al.*, 2004
Vip1Ad	Coleoptera	*A. corpulenta*	NA	Bi *et al.*, 2015
		H. oblita	NA	Bi *et al.*, 2015
		H. parallela	NA	Bi *et al.*, 2015
Vip1Ae	Homoptera	*A. gossypii*	NA	Sattar and Maiti, 2011
Vip1Da	Coleoptera	*D. virgifera*	NA	Boets *et al*., 2011
Vip2Aa	Coleoptera	*D. virgifera*	NA	Warren 1997
Vip2Ac	Coleoptera	*T. molitor*	NA	Shi *et al.*, 2004
	Lepidoptera	*H. armigera*	NA	Shi *et al.*, 2004
		S. exigua	NA	Shi *et al.*, 2004
		S. litura	NA	Shi *et al.*, 2004
Vip2Ad	Coleoptera	*D. virgifera*	NA	Boets *et al*., 2011
Vip2Ae	Coleoptera	*H. oblita*	NA	Yu *et al.*, 2011
		T. molitor	NA	Yu *et al.*, 2011
	Lepidoptera	*C. suppressalis*	NA	Yu *et al.*, 2011
		H. armigera	NA	Yu *et al.*, 2011
		S. exigua	NA	Yu *et al.*, 2011
	Diptera	*C.*	NA	Yu *et al.*, 2011
	Homoptera	*quinquefasciatus*	NA	Sattar and Maiti, 2011
		A. gossypii		
			NA	Yu *et al.*, 2011
Vip2Ag	Coleoptera	*A. corpulenta*	NA	Bi *et al.*, 2015
		H. oblita	NA	Bi *et al.*, 2015
		H. parallela	NA	Bi *et al.*, 2015

Vip1Aa+Vip2Aa	Coleoptera	*D. longicornis*	+++ (NI)	Warren 1997
		D. undecimpunctata	+ (NI)	Warren 1997
		D. virgifera	+++ (40/20 ng/g diet)*	Warren 1997
		L. decemlineata	NA	Warren 1997
	Lepidoptera	*T. molitor*	NA	Warren 1997
		A. ipsilon	NA	Warren 1997
		H. virescens	NA	Warren 1997
		H. zea	NA	Warren 1997
		M. sexta	NA	Warren 1997
		O. nubilalis	NA	Warren 1997
		S. exigua	NA	Warren 1997
	Diptera	*S. frugiperda*	NA	Warren 1997
		C. pipiens	NA	
Vip1Aa+Vip2Ab	Coleoptera	*D. virgifera*	+++ (NI)	Warren 1997
Vip1Ab+ Vip2Aa	Coleoptera	*D. virgifera*	NA	Warren 1997
Vip1Ab+ Vip2Ab	Coleoptera	*D. virgifera*	NA	Warren 1997
Vip1Ac+Vip2Ac	Coleoptera	*T. molitor*	NA	Shi *et al.*, 2004
	Lepidoptera	*H. armigera*	NA	Shi *et al.*, 2004
		S. exigua	NA	Shi *et al.*, 2004
		S. litura	NA	Shi *et al.*, 2004
Vip1Ac+Vip2Ae	Coleoptera	*H. oblita*	NA	Yu *et al.*, 2011
		T. molitor	NA	Yu *et al.*, 2011
	Lepidoptera	*C. suppressalis*	NA	Yu *et al.*, 2011
		H. armigera	NA	Yu *et al.*, 2011
		S. exigua	NA	Yu *et al.*, 2011
	Diptera	*C. quinquefasciatus*	NA	Yu *et al.*, 2011
	Homoptera	*A. gossypii*	+++ (87.5 ng/ml)	Yu *et al.*, 2011
Vip1Ad+Vip2Ag	Coleoptera	*A. corpulenta*	+++ (220 ng/g soil)	Bi *et al.*, 2015
		H. oblita	+++ (120 ng/g soil)	Bi *et al.*, 2015
		H. parallela	+++ (80 ng/g soil)	Bi *et al.*, 2015
Vip1Ae+Vip2Ae	Homoptera	*A. gossypii*	++ (96/481 ng/ml)*	Sattar and Maiti, 2011
Vip1Ca+Vip2Aa	Coleoptera	*T. molitor*	NA	Shi *et al*., 2007
	Lepidoptera	*H. armigera*	NA	Shi *et al*., 2007
		S. exigua	NA	Shi *et al*., 2007
		S. litura	NA	Shi *et al*., 2007
	Diptera	*C. quinquefaciatus*	NA	Shi *et al*., 2007

Vip1Da+Vip2Ad	Coleoptera	*A. grandis*	+ (207 µg/ml)	
		D. barberi	+++ (213 ng/ml)	Boets *et al*., 2011
		D. undecimpunctata	++ (4.91 µg/ml)	Boets *et al*., 2011
				Boets *et al*., 2011
		D. virgifera	+++ (437 ng/ml)	Boets *et al*., 2011
		L. decemlineata	+++ (37 ng/ml)	Boets *et al*., 2011
	Lepidoptera	*H. virescens*	NA	Boets *et al*., 2011
		H. zea	NA	Boets *et al*., 2011
		M. sexta	NA	Boets *et al*., 2011
		O. nubilalis	NA	Boets *et al*., 2011
		S. frugiperda	NA	Boets *et al*., 2011
		S. littoralis	NA	Boets *et al*., 2011
		S. nonagrioides	NA	Boets *et al*., 2011
88% similarity to vip2Ac and vip1Ac genes	Coleoptera	*S. zeamais*	++ (NI)	Shingote *et al.*, 2013b
Vip1/Vip2	Nematoda	*P. pacificus*	NA	Iatsenko *et al.*, 2014
		C. elegans	NA	
Vip1Ba1-Vip2Ba1	Coleoptera	*D. virgifera*	+++ (NI)	Schnepf *et al.*, 2003
Vip1Aa2-Vip2Aa2	Coleoptera	*D. virgifera*	+++ (NI)	Feitelson *et al.*, 2003
	Lepidoptera	*H. virescens*	NA	
		H. zea	NA	
Vip1Bb1-Vip2Bb1	Coleoptera	*D. virgifera*	+++ (NI)	Feitelson *et al.*, 2003
	Lepidoptera	*H. virescens*	NA	
		H. zea	NA	

*Proportion of Vip1/Vip2 that give 50% of the mortality.
The number of "+" marks indicated the activity level.
NA: Not Active.
NI: No information.
Diabrotica undecimopunctata
Diabrotica barberi
Anomala corpulenta
Holotrichia parallela
Holotrichia oblita
Tenebrio Molitor
Leptinotarsa decemlineata
Culex pipiens
Anthonomus grandi

Sesamia nonagrioides
Sitophilus zeamais
Pristionchus pacificus
Caenorhabditis elegans

Table 2: Activity spectrum and relative toxicity of Vip3Aa protoxins reported in the literature. When it is not mentioned, LC_{50} is given in ng/cm².

Protein	**Insect species**	**Larval instar**	**Assay type**	**LC_{50}**	**Scoring time (d)**	**Reference**
Vip3A	*A .ipsilon*	2^{nd}-3^{rd}	Diet Inc.	<200.0	2	Yu *et al.,*
	O. nubilalis	2^{nd}-3^{rd}	Diet Inc.	NA	2	1997
	S. frugiperda	2^{nd}-3^{rd}	Diet Inc.	<200.0	2	
	H. armigera	Neonate	Diet Surf.	155	7	Liao *et al.,*
	H. punctigera	Neonate	Diet Surf.	22	7	2002
	H. virescens	Neonate	Diet Inc.	A	7	
	H. zea	Neonate	Diet Inc.	A	7	
	A. ipsilon	1st	Diet Surf.	17.1	5	Lee *et al.,*
	D. plexippus	1st	Diet Surf.	NA	5	2003
	H. zea	1st	Diet Surf.	112.5	5	
	M. sexta	1st	Diet Surf.	176.3	5	
	O.nubilalis	1st	Diet Surf.	NA	5	
	S. frugiperda	1st	Diet Surf.	55.9	5	
	H. armigera	Neonate	Diet Inc	89	5	Gayen *et al.,*
	A.ipsilon	Neonate	Diet Inc	63	5	2012
	S.littoralis	Neonate	Diet Inc	36	5	and 2015
	S. incertulas	Neonate	Diet Inc	60	5	
Vip3Aa1	*A. ípsilon*	Neonate	Diet Inc.	<28	6	Estruch *et al.,*
	H. virescens	Neonate	Diet Inc.	<420	6	1996
	H. zea	Neonate	Diet Inc.	≥420	6	
	O. nubilalis	Neonate	Diet Inc.	>420	6	
	S. exigua	Neonate	Diet Inc.	<28	6	
	S. frugiperda	Neonate	Diet Inc.	<70	6	
	B. mori	Neonate	Diet. Surf.	1986.1	7	Fang *et al.,* 2007
	H. zea	Neonate	Diet. Surf.	27.7	7	
	S. frugiperda	Neonate	Diet. Surf.	6.9	7	
	S. frugiperda	Neonate	Diet. Surf.	49.3	7	Sena *et al.,* 2009
	A. ipsilon	Neonate	Diet. Surf.	14	7	Hernández-Martínez *et*
	S. frugiperda	Neonate	Diet.	620	7	*al.,* 2013

			Surf.			
	H. armigera	Neonate	Diet Surf.	1660	7	Ruiz de
	L. botrana	Neonate	Diet Inc.	1.3 µg/ml	7	Escudero *et*
	M. Brassicae	Neonate	Diet Surf.	14.4	7	*al.*, 2014
	S. littoralis	Neonate	Diet Surf.	4.0	7	
Vip3Aa7	*H. armigera*	Neonate	Leaf Surf.	35.6 ng/ml	3	Song *et al.*,
	P. xylostella	3rd	Diet Inc.	28.9 ng/ml	3	2008
	S. exigua	Neonate	Diet Inc.	46.1 ng/ml	7	
	P xylostella	3rd	Leaf Surf.	4.9	3	Dong *et al.*, 2012a,b
Vip3Aa9	*A .ipsilon*	1st	Leaf Surf.	2165	1	Selvapandiyan
	C. partellus	1st	Leaf Surf.	8	1	*et al.*, 2001
	P. opercullela	1st	Leaf Surf.	370	1	
	P xylostella	1st	Leaf Surf.	36	1	
	S. litura	1st	Leaf Surf.	5	1	
Vip3Aa10	*A .ipsilon*	Neonate/1st	Diet Surf.	80.7	6	Doss *et al.*,
	B. mori	Neonate/1st	Diet Surf.	NA	6	2002
	C.quinquefaciatus	Neonate/1st	Water	NA	6	
	H. armigera	Neonate/1st	Diet Surf.	325.2	6	
	P. xylostella	Neonate/1st	Leaf Surf.	220.7	6	
	S. litura	Neonate/1st	Diet Surf.	45.4	6	
Vip3Aa11	*H. armigera*	1st	Diet Inc.	25.7	7	Liu *et al.*,
				ng/mg	7	2007
	O. furnicalis	1st	Diet Inc.	720 µg/ml	4	
	P. xylostella	1st	Leaf Surf.	4.2 mg/ml	7	
	S. exigua	1st	Diet Inc.	1.3 ng/mg		
Vip3Aa13	*H. armigera*	Neonate	Diet Inc.	160 ng/ml	2	Chen *et al.*,
	S. exigua	Neonate	Diet Inc.	740 ng/ml	2	2003
	S. litura	Neonate	Diet Inc.	270 ng/ml	2	
Vip3Aa14	*A. vitella*	Neonate	Leaf Surf.	794	3	Bhalla *et al.*,
	H. armigera	Neonate	Leaf Surf.	NA	3	2005
	P. brassicae	Neonate	Leaf Surf.	NA	3	
	P. xylostella	Neonate	Leaf Surf.	120	3	
	S. litura	Neonate	Leaf Surf.	12	3	
	H. armigera	Neonate	Diet Inc.	NA	3	Saraswathy *et*
	P. xylostella	Neonate	Leaf Surf.	NA	3	*al.*, 2008
	S. litura	Neonate	Leaf Surf.	0.1	3	
Vip3Aa16	*P. oleae*	3rd	Leaf Surf.	A	5	Abdelkefi *et*
	S. littoralis	1st	Diet Surf.	305	6	*al.*, 2009
						Abdelkefi *et*

						al., 2011a
	E. kuehniella	1st	Diet Inc	36	6	Abdelkefi *et al.*, 2011b
	S. exigua	Neonate	Diet Surf.	2600	7	Chakroun *et al.*, 2012
				290	10	
	S. frugiperda	Neonate	Diet Surf.	340	7	
				24	10	
	A .segetum	1st	Diet Surf.	86	6	Ben Hamadou *et al.*, 2013
	T. absoluta	3rd	Leaf Surf.	335	3	Sellami *et al.*, 2015
	E. ceratoniae	Neonate	Diet Inc	40*	5	Boukedi *et al.*, 2015
Vip3Aa19	*H. armigera*	1st	Diet Inc.	24.1ng/mg	7	Liu *et al.*,
	O. furnicalis	1st	Diet Inc.	>100µg/ml	7	2007
	P. xylostella	1st	Leaf Surf.	59.8µg/ml	4	
	S. exigua	1st	Diet Inc.	1.4 ng/mg	7	
Vip3Aa29	*C.quinquefaciatus*	-	In water	NA	2	Yu *et al.*,
	C. suppersalis	-	Diet Inc.	24.0µg/ml	5	2012
	C. tepperi	-	In water	NA		
	H .armigera	-	Diet Inc.	(IC_{50})	2	
				22.6µg/ml	5	
	S. exigua	-	Diet Inc.	36.6µg/ml	5	
Vip3Aa43	*S. albula*	Neonate	Diet Surf.	3.9	7	Bergamasco *et al.*, 2013
	S. cosmioides	Neonate	Diet Surf.	2.8	7	
	S. eridania	Neonate	Diet Surf.	3.4	7	
	S. frugiperda	Neonate	Diet Surf.	24.7	7	
Vip3Aa45	*C. chalcites*	Neonate	Diet Surf.	1044.6	7	Palma *et al.*,
	L. botrana	Neonate	Diet Inc..	1.96µg/ml	7	2013
	M. brassicae	Neonate	Diet Surf.	39.7	7	
	S. exigua	Neonate	Diet Surf.	119.7	7	
	S. littoralis	Neonate	Diet Surf.	18.7	7	
Vip3Aa50	*A. gemmatalis*	Neonate	Diet Surf.	20.3	7	Figueiredo *et al.*, 2013
	S. frugiperda	Neonate	Diet Surf.	79.6	7	

*Althought the LC_{50} value is given in ng/cm², the bioassay was performed using diet incorporation.
IC_{50}: Inhibition Concentration (50).
NA: Not Active.
A: Active.
Diet Inc.: Diet incorporation.

Diet Surf: Diet surface contamination.
Leaf Surf: Leaf surface contamination.
-: not specified.
Helicoverpa puntigera
Danaus plexippus
Ectomyelois ceratoniae
Chrysodeixis chalcites
Earias vitella
Ostrinia furnacalis
Pieris brassicae
Tuta absoluta
Mamestra brassicae
Lobesia botrana
Phthorimea opercullela
Anticarsia gemmatalis

Table 3: Activity spectrum and relative toxicity of Vip3A variants proteins reported in the literature up to date. Bioassays mortality was recorded at 7 days. When it is not mentioned, LC_{50} is given in ng/cm².

Protein	Insect species	Larval Instar	Assay type	LC_{50}	Reference
Vip3Ab1	*A .ipsilon*	Neonate	Diet Surf.	62	Ruiz de Escudero *et al.*, 2014
	S. exigua	Neonate	Diet Surf.	597	
	S. frugiperda	Neonate	Diet Surf.	2020	
	S. littoralis	Neonate	Diet Surf.	163	
Vip3Ac1	*A. gambiae*	-	-	NA	Fang *et al.*, 2007
	B. mori	Neonate	Diet Surf.	44.8	
	D. virgifera	-	-	NA	
	H. zea	Neonate	Diet Surf.	133.7	
	O. nubilalis	Neonate	Diet Surf.	NA	
	S. frugiperda	Neonate	Diet Surf.	11.6	
Vip3Ad2	*A. ipsilon*	Neonate	Diet Surf.	>4000	Hernández Martínez *et al.*,
	S. frugiperda	Neonate	Diet Surf.	>4000	2013
Vip3Ae1	*A. ipsilon*	Neonate	Diet Surf.	4	Hernández Martínez *et al.*,
	S. frugiperda	Neonate	Diet Surf.	28	2013
	S. exigua	Neonate	Diet Surf.	11.1	Caccia *et al.*, 2014
	S. frugiperda	Neonate	Diet Surf.	20	
	H. armígera	Neonate	Diet Surf.	4460	Ruiz de Escudero *et al.*, 2014
	L. botrana	Neonate	Diet Inc.	0.2 µg/ml	
	M. brassicae	Neonate	Diet Surf.	258	
	S. littoralis	Neonate	Diet Surf.	8	
Vip3Af1	*S. frugiperda*	Neonate	Diet. Surf.	49.3	Sena *et al.*, 2009
	A. ipsilon	Neonate	Diet Surf.	18	Hernández Martínez *et al.*,
	S. frugiperda	Neonate	Diet Surf.	60	2013
	H. armígera	Neonate	Diet Surf.	840	Ruiz de Escudero *et al.*, 2014
	L. botrana	Neonate	Diet Inc.	0.8 µg/ml	
	M. brassicae	Neonate	Diet Surf.	6	
	S. littoralis	Neonate	Diet Surf.	43.2	
Vip3Ag4	*C. chalcites*	Neonate	Diet Surf.	45.5	Palma *et al.*, 2013
	L. botrana	Neonate	Diet Inc.	1.1	
	M. brassicae	Neonate	Diet Surf.	>2500	
	S. exigua	Neonate	Diet Surf.	265.2	
	S. littoralis	Neonate	Diet Surf.	34.9	

-: not specified.

NA: Not Active.

Diet Inc.: Diet incorporation.
Diet Surf: Diet surface contamination.
Anopheles gambiae
Lobesia botrana
Chrysodeixis chalcites
Mamestra brassicae

Table 4: Activity spectrum and relative toxicity of Vip3B and Vip3C protein families reported in the literature. When it is not mentioned, LC_{50} is given in ng/cm².

Protein	Insect species	Larval Instar	Assay type	LC50	Scoring time (d)	Reference
Vip3Ba1	*O. nubilalis*	Neonate	Diet Surf.	NA	7	Rang *et al.*,
	P. xylostella	2nd	Leaf Surf.	NA	7	2005
Vip3Bb2	*A. gossypii*	Nymphs	Diet Inc.	NA	7	Beard *et al.*,
	C. tepperi	4th	Liquid solution	NA	4	2008
	H. armígera	Neonate	Diet Surf.	A	7	
	H. punctigera	Neonate	Diet Surf.	A	7	
	T. castaneum	-	Diet Inc.	NA	10	
Vip3Ca3	*A. ipsilon*	Neonate	Diet Surf.	>4000	10	Palma *et*
	C. chalcites	Neonate	Diet Surf.	<400	10	*al.*, 2012
	H. armigera	Neonate	Diet Surf.	<4000	10	
	L. botrana	Neonate	Diet Inc.	>100 µg/ml	10	
	M. brassicae	Neonate	Diet Surf.	<4000	10	
	O. nubilalis	Neonate	Diet Surf.	>4000	10	
	S. exigua	Neonate	Diet Surf.	>4000	10	
	S. frugiperda	Neonate	Diet Surf.	>4000	10	
	S. littoralis	Neonate	Diet Surf.	<4000	10	
	T. ni	Neonate	Diet Surf.	<4000	10	

NA: Not Active.
A: Active. Diet Inc.: Diet incorporation.
Diet Surf.: Diet
surface contamination.
Leaf Surf.: Leaf surface contamination.
-: not specified.
Mamestra brassicae
Chrysodeixis chalcites
Lobesia botrana
Helicoverpa puntigera
Tribolium castaneum

Table 5: Genetic engineered Vip3A proteins and effects on insect toxicity.

Protein	Modification type	Position	Effect of the modification	Reference
Vip3AcAa	Domain swapping	Chimera of Vip3Ac N-t (600 aa) and Vip3Aa C-t (189 aa)	Gain of toxicity to *O. nubilalis* IA to *S. frugiperda, H. zea* and *B.mori*	Fang et al., 2007
Vip3AaAc	Domain swapping	Chimera of Vip3Aa1 N-t (610 aa) and Vip3Ac C-t (179 aa)	DA to *S. frugiperda, H. zea* LA to *B.mori*	Fang et al., 2007
Vip3Aa14	Protein fusion	Chimera of Vip3Aa14 and Cry1Ac	NE of Cry1Ac toxicity vs. *H. armigera, S. litura* and *P. xylostella* but DA of Vip3Aa14 toxicity vs *S. litura*	Saraswathy et al., 2008
Vip3Aa7	Gene promoter change and protein fusion	Chimera of Cry1C promoter with truncated Vip3Aa7 (39 aa deleted at N-t) and Cry1C C-t region	Higher yield of Vip3Aa7, Vip relocation in Bt inclusion bodies but DA to *P. xylostella, H. armigera* and *S. exigua*	Song et al., 2008
Vip3Aa7	Protein fusion	Chimera of Vip3Aa7 and Cry9Ca N-t	IA to *P. xylostella*	Dong et al., 2012

N-t: N-terminal region.
C-t: C terminal region.
DA: decrease of activity.
IA: increase of activity.
NE: no effect.
LA: loss of activity.

Figure 2: Dendogram of the Vip proteins based on their amino acid degree of identity. Amino acid sequences were aligned using the Clustal X interface (Thomson et al., 1997). The evolutionary distance was calculated by the maximum likelihood analysis and the tree was performed using the MEGA5 program (Tamura et al., 2011).

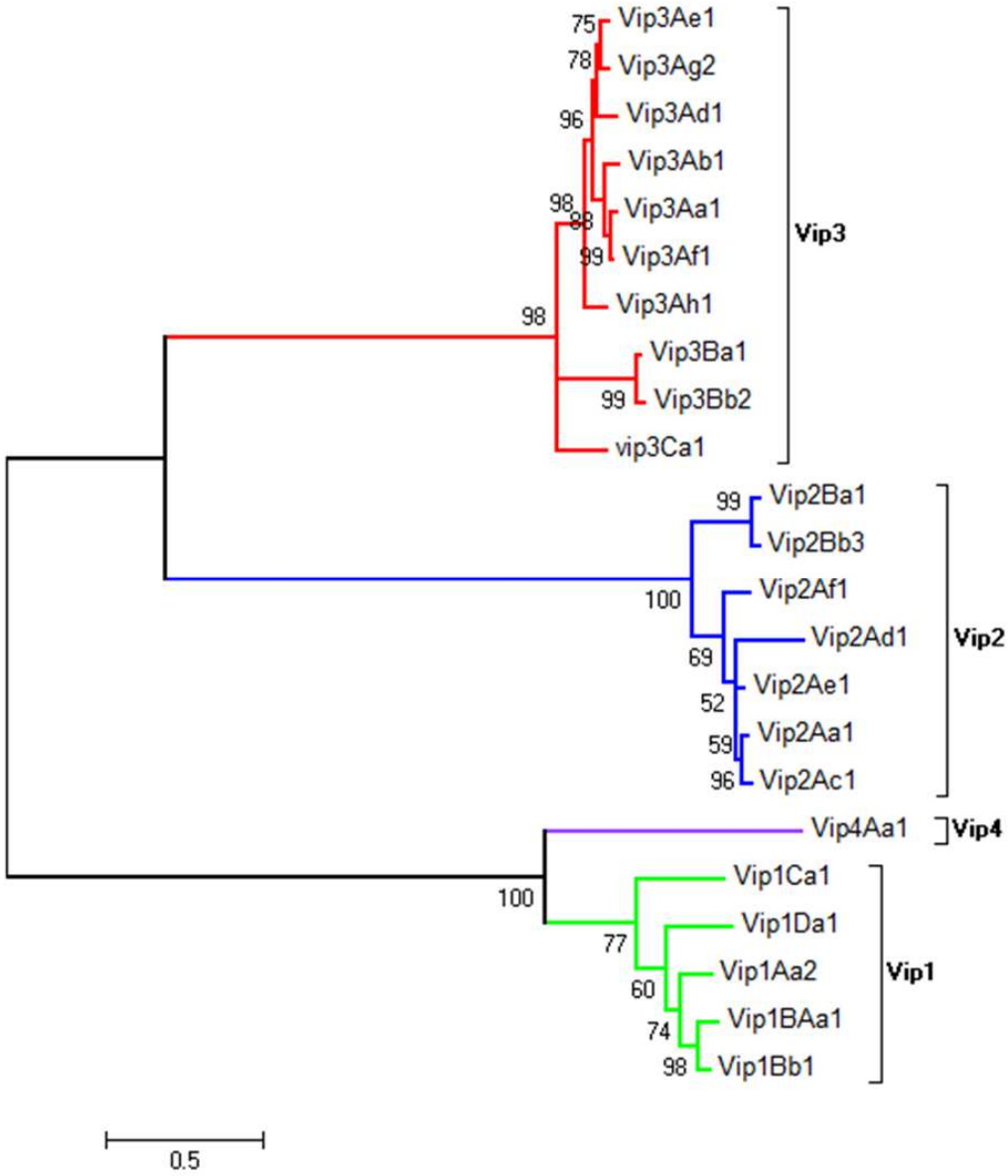

Figure 2: Multiple sequence alignment of the Vip1 proteins. Sequence identity is indicated by shading: black for 100%, dark gray for 80–100%, light gray for 60–80%, and white for less than 60% identity. Intervals of 10 amino acids are marked with "*"."SP": Signal Peptide. Accessions numbers of the protein sequences used in this analysis: Vip1Aa1 (available in patent US5770696, Seq. ID 5), Vip1Ab1 (available in patent US5770696, Seq ID 21), Vip1Ac1 (HM439098), Vip1Ad1 (JQ855505), Vip1Ba1 (AAR40886), Vip1Bb1 (AAR40282), Vip1Ca1 (AAO86514) and Vip1Da1 (CAI40767).


SP
Vip1Aa1
Vip1Ab1
Vip1Ac1
Vip1Ad1
Vip1Ba1
Vip1Bb1
Vip1Ca1
Vip1Da1

Figure 3: Multiple sequence alignment of the Vip2 proteins. Sequence identity is indicated by shading: black for 100%, dark gray for 80–100%, light gray for 60–80%, and white for less than 60% identity. Intervals of 10 amino acids are marked with "*"."SP": Signal Peptide. Domain N-terminal (N-Domain) and domain C-terminal (C-domain) are framed In boxes. Accessions numbers of the protein sequences used in this analysis: Vip2Aa1(1QS1A), Vip2Ab1 (Available in patent US5770696, Seq. ID 20), Vip2Ac1 (AAO86513), Vip2Ad1 (CAI40768), Vip2Ae1 (EF442245), Vip2Af1 (ACH42759), Vip2Ag1 (JQ855506), Vip2Ba1 (AAR40887) and Vip2Bb3 (AIA96500).

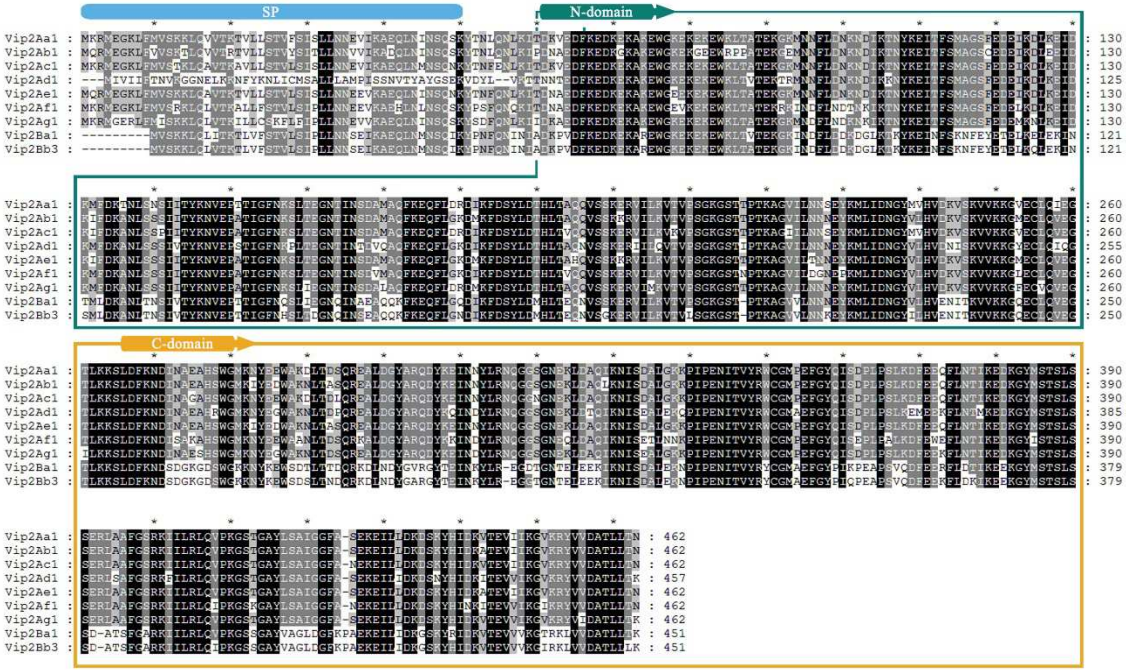

Figure 4: **a.** Vip2 Stereo view showning the NAD-binding site. In blue is the N-terminal domain, in orange the C-terminal domain and the NAD molecule (black bonds with nitrogen in blue, oxygen in red and phosphorus in green) is bound to the active site of the C-terminal domain. The labels N and C indicate the locations of the termini. **b.** Schematic drawing of Vip2 secondary structure illustrating the folding patterns of the two domains and nomenclature. (Han *et al.*, 1999)

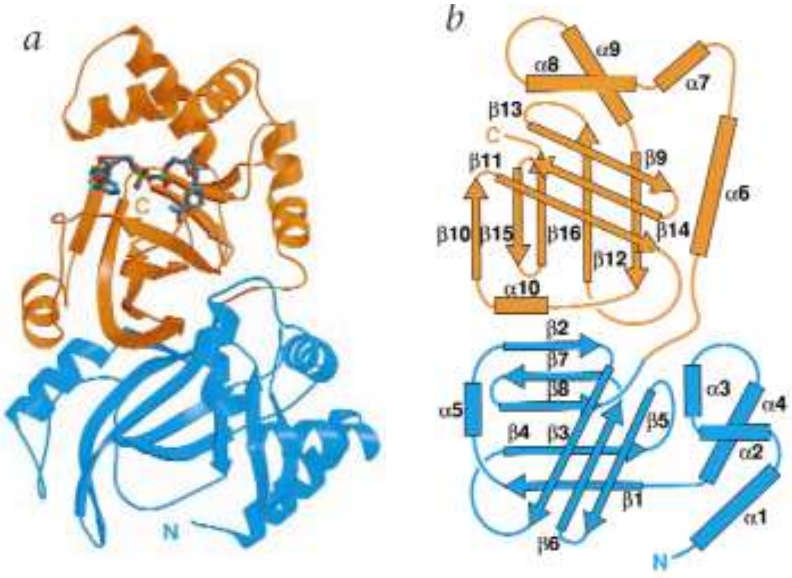

Figure 5: Multiple sequence alignment of the Vip3A proteins. Sequence identity is indicated by shading: black for 100%, dark gray for 80–100%, light gray for 60–80%, and white for less than 60% identity. “SP”: Signal Peptide (Rang *et al.*, 2005); “T”: 65 kDa fragment after proteolysis; “PPS1” and “PPS2”: first and second processing site, respectively (Rang *et al.*, 2005). Intervals of 10 amino acids are marked with “*”. Accession numbers of the protein sequences used in this analysis: Vip3Aa1 (AAC37036), Vip3Ab1 (AAR40284), Vip3Ac1 (available in patent US20040128716, PS49C Seq. ID 7), Vip3Ad2 (CAI43276), Vip3Ae1 (CAI43277), Vip3Af1 (CAI43275), Vip3Ag2 (ACL97352), Vip3Ah1 (ABH10614), Vip3Ai1 (KC156693), Vip3Aj1 (KF826717), Vip3Ba1 (AAV70653), Vip3Bb2 (ABO30520), Vip3Ca1 (ADZ46178).

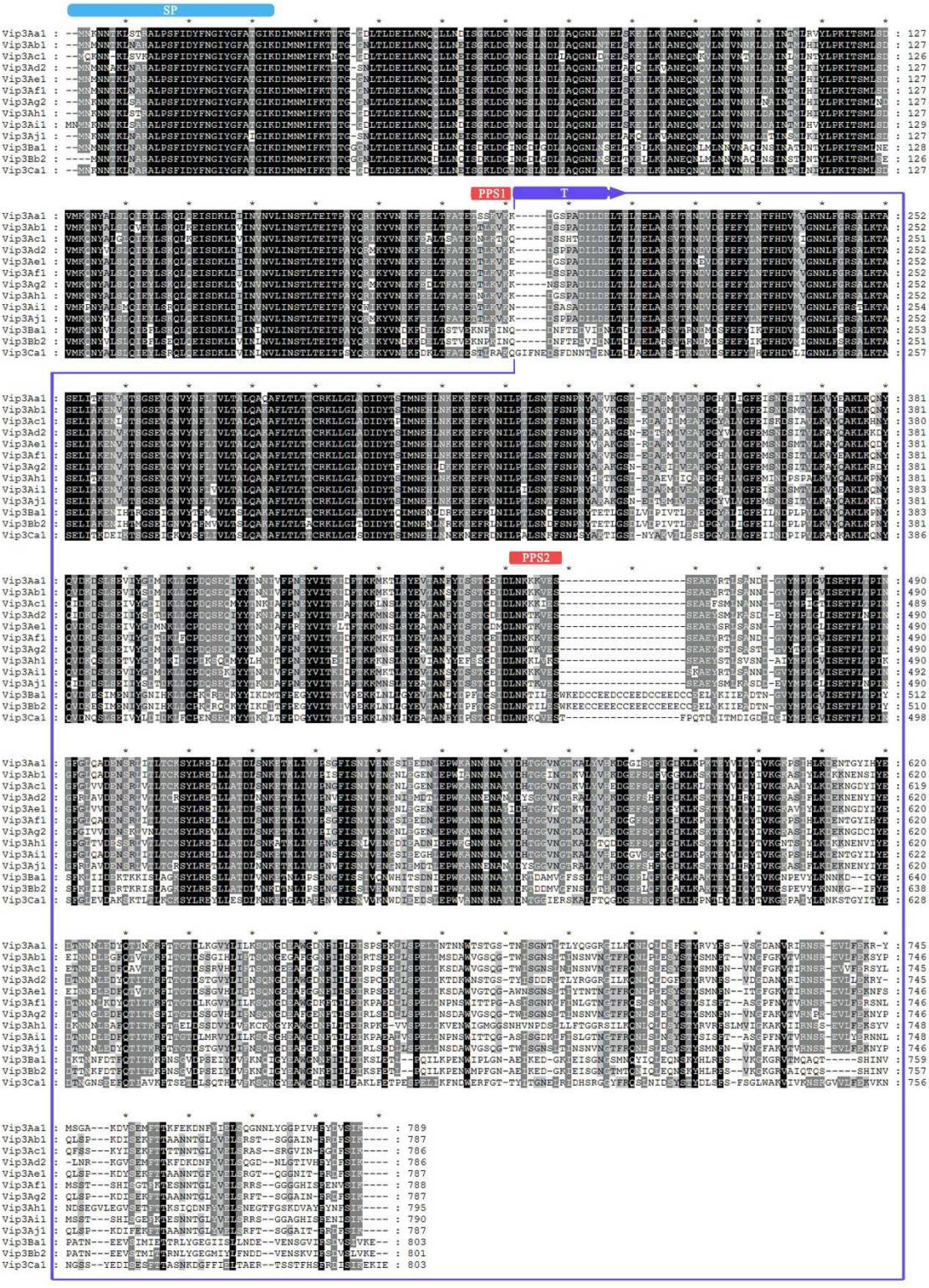
SP
PPS1
T
PPS2

Figure 6: Conserved Domain (CDD) Analysis of representative Vip3 proteins (Marchler-Bauer *et al*, 2011). Same sequences as in Figure 4 were used.

(COG1893: ApbA): Ketopantoate reductase motif.
(COG0840: Tar): Methyl-accepting chemotaxis protein motif.
(COG3264): Small conductance mechanosensitive channel motif.
(COG1511): Motif of a predicted protein membrane of unknown function.

Objectives
Objetivos

2. Objectives:

The overall aim of this project is to understand the mode of action of the Vip3A proteins. Knowledge of its mode of action will help to understand the molecular basis of resistance to these toxins recently introduced in plants. This information is also an essential tool during the design of effective pyramided *Bt*-crop and implementing strategies aiming at delaying resistance and cross-resistance to insecticide based on *Bt*-crops.

2.1 Collect all the knowleg acquired on Vip proteins up to date in a paper that would serve as review on these proteins (introduction of the thesis).

2.2 Monitoring of Vip3Aa stability under different preparation protocols and storage conditions using bioassays against *S. frugiperda* and analysis of the correlation between the difference of susceptibility of *S. exigua* and *S. frugiperda* to Vip3Aa and its activation by the midgut juice of both species (first paper).

2.3 Study of the processing of two Vip3A proteins by two spodoptera species and analysis of the role of the *S. frugiperda* trypsin-like and chymotrypsin–like midgut juice fraction in the activation and degradation of these proteins (second paper).

2.4 *In vivo* and *in vitro* binding of Vip3Aa to *S. frugiperda* midgut and characterization of its binding sites by ^{125}I-radiolabeling (third paper).

2. Objetivos:

El objetivo general de este proyecto es entender modo de acción de las proteínas Vip3A. El conocimiento de su modo de acción ayudará a comprender la base molecular de la resistencia a estas toxinas recientemente introducidas en plantas. Esta información es también una herramienta esencial a la hora de diseñar los cultivos *Bt* piramidados y la aplicación de estrategias encaminadas a retrasar la resistencia y resistencia cruzada a insecticidas en base a los cultivos *Bt*.

2.1. Reunir todos los conocimientos adquiridos hasta el día sobre las proteínas Vip en un documento que serviría como revisión de estas proteínas (introducción de la tesis).

2.2. Seguimiento de la estabilidad de Vip3Aa durante el proceso de su preparación utilizando diferentes protocolos de purificación y condiciones de almacenamiento por medio de bioensayos contra *S. frugiperda* y análisis de la correlación entre la diferencia de susceptibilidad de *S. exigua* y *S. frugiperda* a Vip3Aa y su activación por el jugo del intestino medio de ambas especies (primer artículo).

2.3. Estudio del proces*ado de dos pro*teínas Vip3A por el jugo intestinal de dos especies de *Spodoptera* y an*álisis de* la *función de l*as actividades de tipo tripsina y quimotripsina, purificadas a partir del intestino medio de *S. frugiperda*, en la activación y degradación de estas proteínas (segundo artículo).

2.4. Análisis de la unión *in vivo* y *in vitro* de la proteína Vip3Aa al intestino medio de *S. frugiperda* y caracterización de sus sitios de unión mediante marcaje con I^{125} (tercer artículo).

General discussion

3. General discussion:

B. thuringiensis is one of the modern agricultural defenses against plant eating insects and it has been used for decades in agronomical pest control. This popularity came from the high specificity of the mode of action of its delta-endotoxins. One way to maximize the effect of these insecticidal proteins is combining them whether in a *Bt* formulation or by plant expression. The use of commercial crops expressing *Bt* has increased during the last years and despite its high efficacy as a biological control agent there are still some concerns over the narrow spectrum of activity of the individual toxins and also the threat of emergence of resistance by insects species. One way to address these concerns is the search for of novel toxins with new insecticidal spectra. *B. thuringiensis* vegetative insecticidal protein (Vip3) is one of these toxins; it has shown to be significantly more active against several agronomically important insects than other Bt toxins, being *A. ipsilon* is the most illustrative example. An even more important feature of Vip3 proteins is that they share no sequence homology with the Cry toxins and have been found to exert their insecticidal activity using a different mode of action which makes them good candidates for the resistance management strategy. Knowing the importance of these proteins for the future of the biological control, this thesis has been dedicated to the analysis of the different aspects of the mode of action of these proteins for its better understanding.

3.1. Monitoring of Vip3Aa stability under different preparation protocols and storage conditions using bioassays against *S. frugiperda*.

Our first observation when we started working with these proteins was their unusual instability when compared with the Cry1 toxins. Vip3A proteins were found to precipitate largely at pH below 8, especially after affinity purification or after freezing. They showed better stability at high pH and when the sodium chloride concentration was above 100 mM. The main problem caused by the protein precipitation is that we couldn't reproduce efficiently our bioassays results since the precipitation was irreversible and the precipitated form was inactive. After having suffered for long time trying to solve this precipitation problem, we decided to assess the stability of these proteins under different purification protocols and storage conditions to be sure that its toxicity is not affected by these treatments. *S. frugiperda* was used as control pest for the analysis of the activity of the different Vip3Aa preparations because of its high susceptibility: crude lysate supernatant, ammonium sulfate precipitation, Ni-chelating affinity purification or ion-exchange chromatography purification; these preparations achieved different purity degrees of the Vip3Aa, the less pure was the ammonium sulfate precipitation and the purest preparation

was obtained with the chromatography purification. Vip3Aa was also tested under its activated form; this form was obtained after trypsinization of the protoxin and it resulted in a major product of 62 kDa considered to be the active core of Vip3A proteins.

Despite the difference in Vip3Aa purity among the different preparations, the bioassay results (scored at 7 days) indicated that the Vip3Aa maintained its toxicity independently of the purification protocol. However, the use of trypsin-activated Vip3Aa significantly decreased the LC_{50} values at 7 days compared with the protoxin sample, and the difference of LC_{50} between 7 and 10 days disappeared which suggest that the *in vivo* activation of the protoxin in *S. frugiperda* midgut is a limiting step for the protein toxicity.

3.2. Analysis of the correlation between difference of susceptibility of *S. exigua* and *S. frugiperda* to Vip3Aa and its activation by the midgut juice of both species.

Side by side bioassays of *S. frugiperda* and *S. exigua* with the Vip3Aa protoxin showed around 10-fold difference in their LC_{50} scored at 7 and 10 days, being *S. exigua* less susceptible to Vip3Aa. The growth of both insect species was strongly inhibited by the protoxin and when the functional mortality (dead larvae + first-instar arrested larvae) was considered, these differences disappeared. This means that *S. exigua* suffers a stronger growth inhibition than larvae from *S. frugiperda*, which compensates for the lower mortality of the former when the functional mortality is measured. Ali and Luttrell (2011), testing the toxicity of Vip3Aa, also found important differences between mortality and growth inhibition (10-fold) in *H. zea* but little differences in *H. virescens*, which indicates that the growth inhibition is not a general rule in the Vip3Aa mode of action but a peculiarity limited to some insect species.

The difference between mortality of *S. exigua* and *S. frugiperda* observed at 7 and 10 d completely disappeared when trypsin-activated Vip3Aa was used, which strongly suggest that the differences might be due to differences at the activation step. Indeed, a higher activation rate of Vip3Aa by the midgut juice of *S. frugiperda* was confirmed in a time course experiment; *S. exigua* midgut juice was found to be less efficient in activating the protoxin. Further analysis of the midgut juice of both Spodoptera species showed more variety of proteases and higher protease activity in *S. frugiperda* than in *S. exigua*, which could account for the faster processing of the protoxin in the former.

Difference in processing of the Vip3Aa protoxin has also been proposed to be a crucial factor in determining the difference of susceptibility between *Ephestia kuehniella* and *Spodoptera littoralis* (Abdelkefi-mesrati et al., 2011b). Defects in the proteolysis process of Cry proteins have been recognized to play a role in some cases of insect resistance to Cry1A proteins (Oppert *et al.*, 1994; Forcada *et al.*, 1996; Li *et al.*, 2004) and in one case, proteases were shown to be critical in determining the specificity of the activated toxin to different insect targets (Haider *et al.*, 1986).

3.3. Study of the processing of two Vip3A proteins by two Spodoptera species and analysis of the role of the dissected trypsin- and chymotrypsin–like fraction from the *S. frugiperda* midgut juice in the activation and degradation of these proteins

Vip3Ae, a new variant of the Vip3A proteins, has shown to be equally toxic to *S. exigua* and *S. frugiperda*. Therefore, we have performed a more detailed study of the Vip3A proteolysis to establish whether this step is involved in defining the susceptibility difference in these two Spodoptera species.

The proteolytic processing of Vip3Aa and Vip3Ae proteins was first analyzed using the commercial serine proteases: trypsin and chymotrypsin. Both toxins were susceptible to high concentrations of both proteases and in particular to chymotrypsin. In general, the 62 kDa toxin form of Vip3A was more efficiently produced by the action of trypsin than chymotrypsin, and the yield obtained of this form was greater for Vip3Aa than for Vip3Ae using either trypsin or chymotrypsin. The higher instability of Vip3Aa and Vip3Ae to chymotrypsin is in agreement with the predominance of the higher predicted cleavage sites of this peptidase compared to the trypsin.

Processing of Vip3Aa and Vip3Ae was also analyzed using midgut juice of *S. exigua* and *S. frugiperda*. The results showed that both proteins do not produce a protease resistant core, at least under the tested experimental conditions. Vip3Aa processing with *S. frugiperda* produced a higher yield of 62 kDa activated form in comparison with *S. exigua,* which is likely contributing to the higher susceptibility (12-fold) of this insect to Vip3Aa compared to *S. exigua*. However, this correlation is not observed in the case of Vip3Ae.

To better understand the role of the different midgut juice components and identify those in charge of activation and/or degradation of the Vip3A proteins, the midgut juice of

S. frugiperda was fractionated into its trypsin- and chymotrypsin-like luminal digestive peptidases. Using anion exchange- and size exclusion-chromatography three serine peptidase fractions were isolated: two chymotrypsin-like fractions, one cationic and one anionic and a cationic trypsin-like fraction. Vip3Aa digested with the cationic trypsin like fraction resulted in a major band of 62 kDa active toxin, which remained moderately stable to a wide range of peptidase concentrations. Vip3Ae gave the same pattern but with lower yield of 62 kDa band. No significant amount of 62 kDa band was obtained with either Vip3Aa or Vip3Ae when they were digested with cationic chymotrypsin-like fraction. These results suggest that the 62 kDa fragment accumulation is mainly due to the action of the cationic trypsin-like and the anionic chymotrypsin-like peptidases of *S. frugiperda*. However, cationic chymotrypsin-like activities mainly participate in their degradation.

According to the above results, Vip3Aa and Vip3Ae proteolysis, with either the commercial or the insect purified midgut peptidases, does not produce a peptidase resistant core, which is different to what happens with the *Bt* Cry1 protein. *In vitro* activation studies of the Cry1 proteins with insect peptidases showed that most of them are processed into stable fragments (Ogiwara *et al.,* 1992; Shao *et al.,* 1998; Lightwood *et al.,* 2000; Rukmini *et al.,* 2000; Miranda *et al.,* 2001; Siqueira *et al.,* 2004; Díaz Mendoza *et al.,* 2007; Dammak *et al.,* 2010; González-Cabrera *et al.,* 2013) even for incubation periods and midgut juice amounts higher than the ones we used in the present study. The few cases reported on Cry1 degradation by the midgut juice peptidases were associated with low susceptibility of the insect to the tested toxin (Ogiwara *et al.,* 1992; Shao *et al.,* 1998; Lightwood *et al*., 2000; Miranda *et al.,* 2001) except for Cry1Ia, an unconventional secreted δ-endotoxin toxic to *P. oleae* was rapidly degraded by the midgut juice of this insect, and no stable intermediate were observed (Dammak *et al.,* 2010).

In summary, no peptidase resistant core appears in the course of proteolytic processing of Vip3Aa and Vip3Ae protoxins by the action of different types of serine peptidases. The concentration of the active form of Vip proteins in the midgut seems to be the dynamic result of two antagonistic effects. The idea of the transient accumulation of the 62 kDa fragment as a major determinant of the toxicity of Vip3A proteins is in agreement with the kinetic study that showed that the accumulation of the 62 kDa Vip3Aa band was faster with *S. frugiperda* midgut juice than with *S. exigua* midgut juice (Chakroun *et al.* 2012). Therefore, according to the new results, insect interspecific differences at the level of midgut peptidases seem to be one key step in defining in vivo differences in susceptibility to Vip3 proteins.

3.4. *In vivo* and *in vitro* binding of Vip3Aa to *S. frugiperda* midgut and characterization of binding sites by ^{125}I-radiolabeling:

In this chapter we started by investigating the *in vivo* binding of Vip3Aa to its target tissue in *S. frugiperda*. Immunohistochemical analysis of Vip3Aa intoxicated larvae showed that binding mainly took place in the brush border membrane of the midgut epithelial cells, as had been described previously for *A. ipsilon* and *O. nubilalis* (Yu *et al.,* 1997). In our experimental conditions and incubation time no binding to the basal membrane nor to the peritrophic membrane was observed with Vip3Aa as had been described for the Cry proteins (Bravo *et al.,* 1992; Rodrigo-Simón *et al.,* 2006; Rouis *et al.,* 2008). In addition, green florescence was observed inside the midgut epithelial cells which could suggest possible internalization of the Vip3Aa or a fragment of it. Results from a previous study with Sf21 insect cells also suggested that Vip3Aa internalized after binding to the cell membrane (Singh *et al.,* 2010). Whether internalization of Vip3Aa is actually a step in the mode of action deserves further study.

Vip3Aa shares no sequence homology with Cry proteins and there has been demonstrated to be a good candidate for resistance management since it does not share binding sites with either Cry1A, Cry1F or Cry2 proteins in different insect species (Lee *et al.,* 2006; Sena *et al.,* 2009; Liu et al, 2011, Gouffon *et al.,* 2011; Ben Hamadou-Charfi *et al.,* 2013). So far, all studies on the binding of Vip3A proteins to the insect midgut have been done with biotinylated proteins, and thus, quantitative binding parameters were lacking. In this part of the thesis, successful radiolabeling of a Vip3 protein and its use to characterize the binding to BBMV is described for the first time. Specific binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV was shown by incubating a fixed amount of ^{125}I-Vip3Aa with increasing concentrations of BBMV and this was confirmed by autoradiography of the bound protein after separation from BBMV by SDS-PAGE. In both cases, around half of the total binding of the iodinated toxin was inhibited by the presence of an excess of unlabeled Vip3Aa. The 20-kDa fragment present in the sample of Vip3Aa used in radiolabeling was also competed by an excess of unlabeled Vip3Aa. This is due to the fact that this fragment remains tightly linked to the 62-kDa fragment. Saturation of Vip3Aa binding sites was shown by incubating a fixed amount of BBMV and increasing concentrations of the radiolabeled protein. Despite the fact that the affinity-purified Vip3Aa protoxin, after trypsin activation, showed strong toxicity against *S. frugiperda* (Chakroun *et al.,* 2012), this toxin preparation was found to be inappropriate for binding assays: no

specific binding could be obtained with the radiolabeled toxin (data not shown), and the unlabeled protein was unable to compete with radiolabeled Vip3Aa (anion-exchange purified), For Cry1 toxins, a direct correlation between irreversible binding, pore formation, and toxicity has been described in various cases (Ihara *et al.,* 1993; Liang *et al.,* 1995). Vip3A proteins have been shown to form pores in different susceptible insects, such as *M. sexta* and *H. armigera* (Liu *et al.,* 2011; Lee *et al.,* 2003), which indirectly indicates that binding of Vip3A to the BBMV from these insects is, at least in part, irreversible. Our study provides the first direct evidence of the irreversible binding of Vip3Aa to *S. frugiperda* BBMV.

Since this was the first time that radiolabeled Vip3Aa was used for binding assays, it was necessary to select first the conditions under which the binding to the *S. frugiperda* BBMV was optimum. As in one of the first studies with radiolabeled Cry proteins (Van Rie et al., 1989), the influence of pH, NaCl concentration, and incubation time was tested. Furthermore, the effect of the presence of EDTA or the type and concentration of divalent cations was investigated. Since the pH of the midgut of lepidopterans is known to be alkaline, the effect of pH was tested in the range from 7.4 to 9. The binding was shown to be dependent on the pH: the highest values of specific binding were obtained at the lowest pH. The NaCl concentration also had an influence on the specific binding of ^{125}I-Vip3Aa, most likely by stabilizing the Vip protein.

Hernández-Martínez et al. (Hernández-Martínez *et al.,* 2013) showed that the purification of two different Vip3A proteins using the metal chelation columns had a negative effect on their toxicity, and thus, EDTA was used to stabilize the toxin. However, the addition of the chelating agent EDTA in the binding reaction mixture of the ^{125}I-Vip3Aa decreased both the total and the specific binding to the BBMV, which indicated that the *in vitro* binding is sensitive to the presence of divalent cations. The concentration and type of the cations affected both the total and the specific binding of the ^{125}I-Vip3Aa. Mn^{2+} at 10 mM yielded the highest total binding, although all this binding was nonspecific. However, at 1 mM Mn^{2+}, despite that the total binding decreased comparatively, a substantial amount of specific binding was obtained. Addition of Cu^{2+} (0.1 or 1 mM), Mg^{2+} (10 mM), or Ca^{2+} (1 mM) had relatively small effects on the total binding; however, in all cases specific binding was decreased compared with when these ions were absent. These results are in contrast with the binding of Cry1Ab to *M. sexta* BBMV, which was not affected by the presence of either 5 mM EGTA or 10 mM Mn^{2+} or Ca^{2+} (Van Rie *et al.,* 1989). It is possible that some metal ions are required by some brush border membrane proteins involved in the Vip3Aa binding.

The displacement of the ^{125}I-Vip3Aa protein observed in the homologous competition experiment confirms the occurrence of a limited number of receptors for Vip3Aa that could be saturated, adding an excess of unlabeled toxin. The heterologous competition by Vip3Ad, Vip3Ae, and Vip3Af indicates that these three proteins also bind to the same sites as Vip3Aa. However, whether Vip3Aa competes for all of the binding sites recognized by Vip3Ad, Vip3Ae, or Vip3Af (i.e., the reciprocal competition experiments) has not been tested here. Competition of Vip3Aa with biotinylated Vip3Af had been shown previously in *S. frugiperda* (Sena *et al.,* 2009). The proteins Vip3Aa, Vip3Ae, and Vip3Af are known to be toxic to *S. frugiperda* (Hernández-Martínez *et al.,* 2013, Sena *et al.,* 2009, Chakroun *et al.,* 2012); however, Vip3Ad is nontoxic (Hernández-Martínez *et al.,* 2013). This result indicates, as occurs with Cry proteins, that binding of Vip proteins is necessary, though not sufficient, for toxicity.

The analysis of the binding parameters from the homologous and the heterologous competitions rendered K_d and R_t values similar for all four Vip3A proteins, with K_d values in the range of 6.1 to 22 nM and R_t values in the range of 48 to 76 pmol/mg of BBMV protein. These values are higher (around 10-fold) than the ones normally obtained for the Cry1A and Cry2A proteins (Gouffon *et al.,* 2011; Hernández-Rodríguez *et al.,* 2008; Garczynski *et al.,* 1991; Rang *et al.,* 2004; Caccia *et al.,* 2010), which indicates that Vip3A proteins have lower affinity but a higher number of binding sites in the BBMV than the Cry1A and Cry2A proteins. Lee et al. (Lee *et al.,* 2003) showed that the kinetics of pore formation of activated Vip3A was more than 8-fold slower than that of Cry1Ab (at equimolar concentrations) and that the kinetics did not change after a 10-fold increase in the Vip3A concentration. Lee et al. claimed that this could be due to the fact that saturation of functional binding sites of the Vip3A proteins was hard to reach.

When Cry1Ab and Cry1Ac were used as heterologous competitors, no displacement of ^{125}I-Vip3Aa occurred. This result, along with the competition of the Vip3A proteins for the same binding site found here and the results obtained in a previous study (Sena *et al.,* 2009), strongly suggests that Vip3A proteins do not share binding sites with Cry1A proteins in *S. frugiperda*. Lack of competition between Cry1A and Cry2A proteins and Vip3Aa had already been reported in three heliothine species (Lee *et al.,* 2006; Lui *et al.,* 2011; Gouffon *et al.,* 2011). The overall results suggest that these two classes of toxins (Vip3A and Cry1A/2A) use different receptors to bind to the brush border membrane of target insects.

In conclusion, the successful radiolabeling of Vip3Aa in this work opens up interesting perspectives for the future of binding studies with Vip3A proteins. Using radiolabeled Vip3Aa allowed us to estimate for the first time binding parameters for this protein. Furthermore, heterologous competition has revealed that Vip3Ad, Vip3Ae, and Vip3Af competed for the Vip3Aa binding sites. The absence of competition of Cry1Ac and Cry1Ab makes them appropriate candidates to be used in combination with Vip3A proteins in transgenic crops as a strategy to delay the evolution of resistance in insects.

References

Abdelkefi-Mesrati L, Boukedi H, Chakroun M, Kamoun F, Azzouz H, Tounsi S, Rouis S, Jaoua S. 2011b. Investigation of the steps involved in the difference of susceptibility of *Ephestia kuehniella* and *Spodoptera littoralis* to the *Bacillus thuringiensis* Vip3Aa16 toxin. J. Invertebr. Pathol 107:198-201.

Ali MI, Luttrell RG. 2011. Susceptibility of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) to Vip3A insecticidal protein expressed in VipCot™ cotton. J Invertebr Pathol 108:76-84.

Ben Hamadou-Charfi D, Boukedi H, Abdelkefi-Mesrati L, Tounsi S, Jaoua S. 2013. *Agrotis segetum* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa16 differs from that of Cry1Ac toxin. J Invertebr Pathol 114:139-43.

Bravo A, Jansens S, Peferoen M. 1992. Immunocytochemical localization of *Bacillus thuringiensis* isinsecticidal crystal proteins in intoxicated insects. J. Invertebr. Pathol. 60:237–246.

Caccia S, Hernández-Rodríguez CS, Mahon RJ, Downes S, James W, Bautsoens N, Van Rie J, Ferré J. 2010. Binding site alteration is responsible for field-isolated resistance to *Bacillus thuringiensis* Cry2A insecticidal proteins in two Helicoverpa species. PLoS One 5:e9975.

Chakroun M, Bel Y, Caccia S, Abdelkefi-Mesrati L, Escriche B, Ferré J. 2012. Susceptibility of *Spodoptera frugiperda* and *S. exigua* to *Bacillus thuringiensis* Vip3Aa insecticidal protein. J. Invertebr. Pathol. 110, 334–339.

Dammak M, Tounsi S, Rouis S, Jaoua, S. 2010. Comparative study of *Bacillus thuringiensis* Cry1Ia and Cry1Aa delta-endotoxins: Activation process and toxicity against *Prays oleae*. J. Invertebr. Pathol. 104, 39–43.

Díaz-Mendoza M, Farinós GP, Castañera P, Hernández-Crespo P, Ortego F. 2007. Proteolytic processing of native Cry1Ab toxin by midgut extracts and purified trypsins from the Mediterranean corn borer *Sesamia nonagrioides*. J.Insect Physiol. 53, 428–435.

Forcada C, Alcácer E, Garcerá MD, Martínez R. 1996. Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. Arch. Insect Biochem. Physiol. 31, 257–272.

González-Cabrera J, García M, Hernández-Crespo P, Farinós GP, Ortego F, Pedro Castañera P. 2013. Resistance to *Bt* maize in *Mythimna unipuncta* (Lepidoptera: Noctuidae) is mediated by alteration in Cry1Ab protein activation. Insect Biochem. Mol. Biol. 43, 635–643.

Gouffon C, Van Vliet A, Van Rie J, Jansens S, Jurat-Fuentes JL. 2011. Binding sites for *Bacillus thuringiensis* Cry2Ae toxin on heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin. Appl. Environ. Microbiol. 77:3182–3188.

Garczynski SF, Crim JW, Adang MJ. 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* delta-endotoxin by protein blot analysis. Appl. Environ. Microbiol. 57:2816–2820.

Haider MZ, Knowles BH, Ellar DJ. 1986. Specificity of *Bacillus thuringiensis* var. colmeri insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. 156:531–540.

Hernández-Martínez P, Hernández-Rodríguez CS, Van Rie J, Escriche B, Ferré J. 2013. Insecticidal activity of Vip3Aa, Vip3Ad, Vip3Ae, and Vip3Af from *Bacillus thuringiensis* against lepidopteran corn pests. J. Invertebr. Pathol. 113:78–81.

Hernández-Rodríguez CS, Van Vliet A, Bautsoens N, Van Rie J, Ferré J. 2008. Specific binding of *Bacillus thuringiensis* Cry2A insecticidal proteins to a common site in the midgut of *Helicoverpa* species. Appl. Environ. Microbiol. 74:7654–7659.

Ihara H, Kuroda E, Wadano A, Himeno M. 1993. Specific toxicity of δ-endotoxin from *Bacillus thuringiensis* to *Bombyx mori*. Biosci. Biotechnol. Biochem. 57:200–204.

Lee MK, Miles P, Chen JS. 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem. Biophys. Res. Commun. 339:1043–1047.

Lee MK, Walters FS, Hart H, Palekar N, Chen JS. 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa differs from that of Cry1Ab delta-endotoxin. Appl. Environ. Microbiol. 69:4648–4657.

Li H, Oppert B, Higgins RA, Huang F, Zhu KY, Buschman LL. 2004. Comparative analysis of proteinase activities of *Bacillus thuringiensis* resistant and susceptible *Ostrinia nubilalis* (Lepidoptera: Crambidae). Insect Biochem. Mol. Biol. 34:753–762.

Liang Y, Patel SS, Dean DH. 1995. Irreversible binding kinetics of *Bacillus thuringiensis* CryIA δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J. Biol. Chem. 270:24719–24724.

Lightwood DJ, Ellar DJ, Jarrett P. 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac delta-endotoxin. Appl. Environ. Microbiol. 66:5174–5181.

Liu J, Yang A, Shen X, Hua B, Shi G. 2011. Specific binding of activated Vip3Aa10 to *Helicoverpa armigera* brush border membrane vesicles results in pore formation. J. Invertebr. Pathol. 108:92–97.

Miranda R, Zamudio FZ, Bravo A. 2001. Processing of Cry1Ab d-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Mol. Biol. 31, 1155–1163.

Ogiwara K, Indrasith LS, Asano S, Hori H. 1992. Processing of δ-endotoxin from *Bacillus thuringiensis* subsp. kurstaki HD-1 and HD-73 by gut juices of various insect larvae. J. Invertebr. Pathol. 60, 121–126.

Oppert B, Kramer KJ, Johnson DE, MacIntosh SC, McGaughey WH. 1994. Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. Biochem. Biophys. Res. Commun. 198:940–947.

Rang C, Bergvingson D, Bohorova N, Hoisington D, Frutos R. 2004. Competition of *Bacillus thuringiensis* Cry1 toxins for midgut binding sites: a basis for the development and management of transgenic tropical maize resistant to several stem borers. Curr. Microbiol. 49:22–27.

Rodrigo-Simón A, de Maagd RA, Avilla C, Bakker PL, Molthoff J, González-Zamora JE, Ferré J. 2006. Lack of detrimental effects of *Bacillus thuringiensis* Cry toxins on the insect predator Chrysoperla carnea: a toxicological, histopathological, and biochemical analysis. Appl. Environ. Microbiol. 72:1595–1603.

Rukmini V, Reddy CY, Venkateswerlu G. 2000. *Bacillus thuringiensis* crystal δ-endotoxin: role of proteases in the conversion of protoxin to toxin. Biochimie. 82: 109–116.

Rouis S, Chakroun M, Jaoua S. 2008. Comparative study of *Bacillus thuringiensis* Cry1Aa and Cry1Ac delta-endotoxin activation, inactivation and in situ histopathological effect in *Ephestia kuehniella* (Lepidoptera: Pyralidae). Mol. Biotechnol. 38:233–239.

Shao Z, Cui Y, Liu X, Yi H, Ji J, Yu Z. 1998. Processing of delta-endotoxin of *Bacillus thuringiensis* subsp. kurstaki HD-1 in Heliothis armigera midgut juice and the effects of protease inhibitors. J. Invertebr. Pathol. 72, 73–81.

Siqueira HAA, Nickerson KW, Moellenbeck D, Siegfried BD. 2004. Activity of gut proteinases from Cry1Ab-selected colonies of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae). Pest Manag. Sci. 60, 1189–1196.

Sena JA, Hernández-Rodríguez CS, Ferré J. 2009. Interaction of *Bacillus thuringiensis* Cry1 and Vip3Aa proteins with *Spodoptera frugiperda* midgut binding sites. Appl. Environ. Microbiol. 75:2236–2237.

Singh G, Sachdev B, Sharma N, Seth R, Bhatnagar RK. 2010. Interaction of *Bacillus thuringiensis* vegetative insecticidal protein with ribosomal S2 protein triggers larvicidal activity in *Spodoptera frugiperda*. Appl. Environ. Microbiol. 76:7202–7209.

Van Rie J, Jansens S, Höfte H, Degheele D, Van Mellaert H. 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the midgut of target insects. Eur. J. Biochem. 186:239–247.

Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63:532–536.

Conclusions
Conclusiones

4. Conclusions:

4.1 Vip3Aa purification is a very critical step. Although the protoxin retains full toxicity after being subjected to different biochemical treatments, the nickel affinity purification of the Vip3Aa is not an appropriate purification protocol for the molecular study of its mode of action. During the purification process we also discovered that the Vip3Aa proteolytic products of 62 kDa 20 kDa are tightly linked in solution.

4.2 A limiting rate of protoxin activation seems to be the cause of the marked difference of susceptibility between *S. frugiperda* and *S. exigua* to Vip3Aa. The disappearance of these differences when the trypsin activated Vip3Aa is used strengthen this hypothesis.

4.3 Under our experimental conditions, the proteolytic processing of Vip3Aa and Vip3Ae by the serine proteases (trypsin and chymotrypsin) does not produce a peptidase resistant core, and the accumulation of the 62 kDa active form of Vip3A proteins is not always correlated with the difference of susceptibility between insect species.

4.4 Most likely, inside the midgut of the lepidopteran insects, the cationic trypsin-like and the anionic chymotrypsin-like peptidases are the ones contributing to the accumulation of the 62 kDa active toxin form, while the cationic chymotrypsin-like activities mainly participate in its degradation.

4.5 Vip3Aa binds specifically to the brush border membrane of *S. frugiperda* and is possibly internalized into the midgut epithelial cells.

4.6 The successful radiolabeling of Vip3Aa allowed us to estimate for the first time binding parameters for this protein. Vip3Aa binding is almost totally irreversible and is sensitive to many factors such as sodium chloride concentration, pH, the presence of the divalent cations.

4.7 Vip3Aa share binding sites in *S. frugiperda*, not only with the toxic Vip3Ae and Vip3Af, but also with the non toxic Vip3Ad, which indicates that the binding of Vip3 proteins is necessary but not sufficient for their toxicity.

4. Conclusiones:

4.1. La purificación de la proteína Vip3Aa es un paso crítico para el mantenimiento de su función. A pesar de que la protoxina retiene total toxicidad después de diferentes tratamientos bioquímicos, la purificación por afinidad a níquel de la Vip3Aa no es un protocolo de purificación apropiado para el estudio molecular del modo de acción. Durante el proceso de purificación también descubrimos que los productos de proteólisis de 62 kDa y 20 kDa de Vip3Aa están fuertemente unidos en solución.

4.2. Una velocidad limitante de activación de la protoxina parece ser la causa de la marcada diferencia de susceptibilidad entre *S. frugiperda* y *S. exigua* a la Vip3Aa. La desaparición de estas diferencias cuando se utiliza la Vip3Aa activada por tripsina refuerza esta hipótesis.

4.3. Bajo nuestras condiciones experimentales, el procesado proteolítico de Vip3Aa y Vip3Ae por las serín proteasas (tripsina y quimotripsina) no produce un núcleo resistente a las peptidasas, y la acumulación de la forma activa de 62 kDa de las proteínas Vip3A no siempre se correlaciona con la diferencia de susceptibilidad entre especies de insectos.

4.4. Muy probablemente, en el interior del intestino medio de los lepidópteros, las peptidasas de tipo tripsina catiónica y las peptidasas de tipo quimotripsina aniónica son las que contribuyen a la acumulación de la forma activa de 62 kDa, mientras que las actividades de tipo quimotripsina catiónica participan principalmente en su degradación

4.5. Vip3Aa se une específicamente a la membrana de borde en cepillo de *S. frugiperda* con posible internalizacion en las células epiteliales del intestino medio.

4.6. El éxito en el marcaje radioactivo de Vip3Aa permitió estimar por primera vez los parámetros de unión de esta proteína. La unión de Vip3Aa es casi totalmente irreversible y es sensible a muchos factores, tales como la concentración de cloruro de sodio, el pH y la presencia de los cationes divalentes.

4.7. Vip3Aa comparte sus sitios de unión en *S. frugiperda*, no sólo con las toxinas Vip3Ae y Vip3Af, sino también con la Vip3Ad que no es tóxica para este insecto, lo que indica que la unión de las proteínas Vip3 es necesaria pero no suficiente para su toxicidad.

Publications

First paper

Susceptibility of *Spodoptera frugiperda* and *S. exigua* to *Bacillus thuringiensis* Vip3Aa insecticidal protein.

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Susceptibility of *Spodoptera frugiperda* and *S. exigua* to *Bacillus thuringiensis* Vip3Aa insecticidal protein

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ABSTRACT

The Vip3Aa protein is an insecticidal protein secreted by *Bacillus thuringiensis* during the vegetative stage of growth. The activity of this protein has been tested after different steps/protocols of purification using *Spodoptera frugiperda* as a control insect. The results showed that the Vip3Aa protoxin was stable and retained full toxicity after being subjected to common biochemical steps used in protein purification. Bioassays with the protoxin in *S. frugiperda* and *S. exigua* showed pronounced differences in LC_{50} values when mortality was measured at 7 vs. 10 d. At 7 d most live larvae were arrested in their development. LC_{50} values of "functional mortality" (dead larvae plus larvae remaining in the first instar), measured at 7 d, were similar or even lower than the LC_{50} values of mortality at 10 d. This strong growth inhibition was not observed when testing the trypsin-activated protein (62 kDa) in either species. *S. exigua* was less susceptible than *S. frugiperda* to the protoxin form, with LC_{50} values around 10-fold higher. However, both species were equally susceptible to the trypsin-activated form. Processing of Vip3Aa protoxin to the activated form was faster with *S. frugiperda* midgut juice than with *S. exigua* midgut juice. The results strongly suggest that the differences in the rate of activation of the Vip3Aa protoxin between both species are the basis for the differences in susceptibility towards the protoxin form.

1. Introduction

Bacillus thuringiensis is a gram positive bacterium that produces two major categories of lepidopteran active proteins: The Cry proteins, which are produced at the end of the log phase and accumulate in the parasporal crystal, and the Vip proteins, which are produced during the vegetative stage of growth and are secreted into the culture medium. The Cry proteins are toxic to a variety of insect orders such as Lepidoptera, Diptera and Coleoptera. The mode of action of the Cry proteins involves solubilization of the crystal, processing of the protoxins by intestinal proteases, and recognition of a binding site on the midgut brush border membrane surface, followed by pore formation and cell lysis, leading ultimately to insect death (Schnepf et al., 1998). Two steps in the mode of action of the Cry proteins are considered key steps in their toxicity against susceptible larvae, the alteration in the proteolytic activation of the protoxin and the loss of binding of these proteins to receptors located in the brush border membrane. In fact, both have been described as mechanisms of resistance to these toxins (Ferré and Van Rie, 2002; Ferré et al., 2008).

The Vip proteins have no sequence homology with the Cry proteins. Vip proteins have been classified into four groups according to their sequence homology: Vip1, Vip2, Vip3, and Vip4 (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html). Vip1 and Vip2 act as binary toxins and are toxic to coleopterans (Shi et al., 2004). Vip3 proteins are toxic to lepidopterans and genes coding for this type of proteins have been found to be very common among *B. thuringiensis* isolates (Bhalla et al., 2005; Abdelkefi-Mesrati et al., 2005; Liu et al., 2007; Fang et al., 2007; Beard et al., 2008; Hernández-Rodríguez et al., 2009; Yu et al., 2010).

Vip3Aa proteins were the first ones discovered and the most common ones within the Vip3 family. They have been shown to have a broad insecticidal spectrum against a range of Lepidopteran pests, including *Agrotis ipsilon*, *Heliothis virescens*, *Helicoverpa zea*, *Helicoverpa armigera*, *Plutella xylostella*, *Spodoptera frugiperda*, *S. exigua*, and *S. litura*, among others (for a summary of toxicities, see Milne et al., 2008; van Frankenhuyzen and Nystrom, 2002).

Vip3Aa has been shown to kill larvae of susceptible insects by a series of steps that resemble those used by Cry proteins in their mode of action. Vip3Aa is secreted from the *B. thuringiensis* cell as a protoxin, which is partially processed by proteases in the larva midgut rendering the active toxin (Yu et al., 1997; Lee et al., 2003). This toxin then binds to specific receptors in the midgut membrane, which are different from those of Cry proteins (Lee et al.,

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2003, 2006; Sena et al., 2009; Abdelkefi-Mesrati et al., 2009; Liu et al., 2011). The bound toxin provokes the disruption of the midgut epithelial cells (Yu et al., 1997; Abdelkefi-Mesrati et al., 2011a,b) by the formation of pores in the apical membrane (Lee et al., 2003; Liu et al., 2011).

We have observed that Vip3A proteins are less stable than Cry proteins. Since toxicities of Vip3Aa among different laboratories may differ not only due to the different source of protein, but to differences in the protocols used for the preparation and storage of the protein sample, we first investigated the possible effect of different steps/protocols of purification on the activity of Vip3Aa16 using *S. frugiperda* as a control insect. In a second step, the susceptibility of *S. frugiperda* and *S. exigua* was compared using both the protoxin and the activated form of Vip3Aa. Strong differences in susceptibility between both species were observed when using the protoxin, but not when using the activated toxin. *In vitro* activation of Vip3Aa by midgut juice from both species was examined for the possible role of midgut proteases on the toxicity differences of Vip3Aa.

2. Materials and methods

2.1. Source of the Vip3Aa protein

BUPM95 is a *B. thuringiensis* subsp. *kurstaki* strain producing the Vip3Aa16 protoxin (Abdelkefi-Mesrati et al., 2005). The *vip3Aa16* gene was cloned and the corresponding protein fused to a six histidine-tail was overexpressed in recombinant *Escherichia coli* (Abdelkefi-Mesrati et al., 2009).

2.2. Expression, purification and activation of Vip3Aa

One single colony of *E. coli* BL21 harboring the pET plasmid with the *vip3Aa16* gene was inoculated in a preculture containing 3 ml of LB medium (100 μg/ml) ampicillin and (25 μg/ml) chloramphenicol, and grown at 37 °C with shaking (250 rpm) until OD_{600} was 0.4. The preculture was transferred to 400 ml LB medium containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml). When the OD_{600} reached 1.2, 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added for induction. The culture was grown overnight at 37 °C in a shaking incubator at 190 rpm. Cells were centrifuged at 4000g for 15 min. The pellet was resuspended in 20 mM Tris-HCl buffer, pH 8.6, containing 0.3 M NaCl, 0.3 mg/ml lysozyme, and 10 μg/ml DNAse, and incubated with shaking for 30 min at 37 °C. The pellet was then sonicated twice for 60 s, with a 10 s pause in between. The supernatant was collected following centrifugation at 17000g and then filtered through a 0.22 μm filter. This lysate supernatant was used in bioassays and for subsequent purification. The concentration of Vip3Aa protein was determined by densitometry after separation from other contaminant proteins by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS-PAGE).

Affinity chromatography purification of Vip3Aa was carried out using the HiTrap™ Chelating HP column (GE Healthcare). Imidazol was added to the lysate supernatant containing the Vip3Aa protein until reaching 10 mM. The solution was loaded on the column and eluted with elution buffer (50 mM phosphate buffer, pH 8.0, containing 0.3 M NaCl and 100 mM imidazol) and fractions (0.9 ml) were collected in tubes containing 100 μl of 50 mM EDTA. Fractions most concentrated in Vip3Aa were combined and dialyzed overnight against 20 mM Tris-HCl buffer, pH 9, 0.3 M NaCl. The concentration of Vip3Aa protein was determined by Bradford (1976).

Alternatively, Vip3Aa was purified by isoelectric point (pI) precipitation followed by anion-exchange chromatography. The pH of the lysate supernatant was lowered to 5.5 with 0.1 M acetic acid (predicted pI of 5.05 according to the *Protein Identification and Analysis Tools on the ExPASy Server*). The pellet was recovered by centrifugation and dialyzed overnight against 20 mM Tris-HCl, pH 9. After filtration, the dialysate was loaded on a HiTrap Q HP (5 ml bed volume) column equilibrated in 20 mM Tris-HCl, pH 9, using an ÄKTA explorer 100 chromatography system (GE Healthcare, UK). The proteins were eluted by a linear gradient of 1 M NaCl (0–80% in 100 ml). The concentration of Vip3Aa protein was determined by Bradford. The eluted Vip3Aa protein was stored at −20 °C.

Ammonium sulfate precipitation was also used in some batches of Vip3Aa for stability testing. Vip3Aa protoxin was precipitated by adding a saturated solution of ammonium sulfate until 70% salt by continuous stirring for 1 h at 4 °C. After centrifugation, the pellet was solubilized in 20 mM Tris-HCl, pH 8.6, 0.15 M NaCl, 5 mM EDTA and used in bioassays. The concentration of Vip3Aa protein was determined by densitometry after SDS-PAGE.

To activate the Vip3Aa protoxin to use in bioassays, either the supernatant crude lysate or the pI precipitated and dialyzed protoxin were incubated with 10% commercial trypsin (by weight referred to total protein in the mixture) at 37 °C for 2 h (after this time no further activation/degradation of the protein occurred). The reaction mixture was centrifuged at 16000g and the supernatant containing the activated Vip3Aa was stored at −20 °C until use.

2.3. Insect bioassays

First instar larvae of *S. frugiperda* and *S. exigua* were reared on artificial diet as described by Bell and Joachim (1976). The activity of Vip3Aa was determined by surface contamination assays. Seven different concentrations of Vip3Aa (serial dilutions) were tested for each treatment. A volume of 50 μl of the sample dilutions was applied on the artificial diet (in 2 cm^2 multiwell plates) and let dry. One first instar larvae was placed in each well, and 16 neonate larvae were used for each concentration. Mortality was scored at 7 and 10 d. In addition to mortality, larvae remaining in L1 after 7 d were also counted. The number of dead larvae plus larvae arrested at L1 was considered "functional mortality". Regression estimates of mortality and functional mortality were obtained using the POLO-PC probit analysis program (LeOra Software,. Berkeley, CA). LC_{50} values were considered significantly different if fiducial limits did not overlapped.

2.4. Midgut juice preparation and kinetics of Vip3Aa processing

S. frugiperda and *S. exigua* last instar larvae were immobilised on ice for 5 min and then longitudinally dissected to collect the peritrofic membrane together with the food bolus. For each sample, five peritrophic membranes with their food contents were homogenized in a microtube and centrifuged for 10 min at 16000*g* at 4 °C. The supernatant (midgut juice) was collected and stored at −80 °C in small aliquots. The total protein concentration was measured with the Bradford assay.

Vip3Aa protoxin (10 μg) was mixed with 5 μl of diluted midgut juice from either *S. exigua* or *S. frugiperda*, in a final volume of 25 μl. The reaction mixture was incubated at 30 °C for different times, and stopped by adding the electrophoresis loading buffer and heating at 99 °C for 10 min. Reaction products were separated by 12% SDS-PAGE and the gels stained with Coomassie blue.

2.5. Zymogram analysis

Proteins (20 μg) from midgut juice samples of *S. exigua* and *S. frugiperda* larvae were separated by non-denaturing SDS-PAGE

in a 12% Tris–glycine gel and processed for zymogram analysis as described by Li et al. (2004). The gel was incubated in 50 mM carbonate buffer pH 9.6, 10 mM DTT, for 15 min and then incubated for 4 h in the same buffer supplemented with 8 mg/ml of either milk powder (containing casein) or Vip3Aa lysate supernatant. Clear bands on a dark background, corresponding to protease activity in the gel, were revealed by Coomassie blue staining.

3. Results

3.1. Purification and trypsin-activation of Vip3Aa protoxin

Purification of Vip3Aa by affinity chromatography with Ni columns gave rise to inconsistent results in preliminary bioassays. The instability of Vip3Aa was also observed in crude extracts stored at −20 °C. For this reason, we tried different methods of sample preparation and purification to design protocols that did not affect adversely the activity of the Vip3Aa protein in bioassays.

The Vip3Aa protein in the crude lysate supernatant was readily purified to a high degree by affinity chromatography (Fig 1, lane 5).

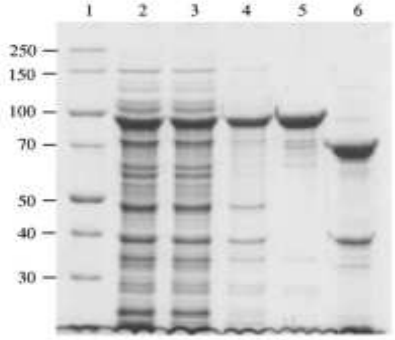

Fig. 1. Purity analysis of Vip3Aa after different preparation methods. Proteins were separated by SDS-PAGE and then stained with Coomassie blue. Lane 1: molecular weight markers; lane 2: supernatant of the crude lysate; lane 3: solubilized pellet after ammonium sulfate precipitation; lane 4: solubilized pellet after isoelectric point precipitation; lane 5: affinity-chromatography eluate; lane 6: trypsin-treated sample after isoelectric point precipitation.

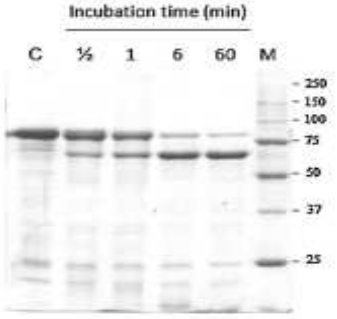

Fig. 2. Proteolytic activation of Vip3Aa protoxin by commercial trypsin. Affinity-purified Vip3Aa protoxin (10 μg) was mixed with bovine trypsin (1% by weight) and incubated at 30 °C for different times. Vip3Aa protoxin incubated for 20 min in the absence of trypsin was used as a control (C). M: Molecular weight markers. Reaction products were separated by SDS-PAGE and stained with Coomassie blue.

Also, a high degree of purity was achieved by lowering the pH to approach the isoelectric point of Vip3Aa (Fig. 1, lane 4). As expected, a low degree of purification was obtained by precipitation with 70% ammonium sulfate (Fig. 1, lane 3). Treatment with commercial trypsin served two purposes: to activate the Vip3Aa protoxin and to eliminate some of the contaminating proteins (Fig. 1, lane 6; Fig. 2).

Anion-exchange chromatography (after pI precipitation) separated most of the contaminating proteins and revealed two isoforms of Vip3Aa, both with similar molecular weight (indistinguishable by SDS-PAGE) (Fig. 3) but with obviously different net charge. The degree of purification of Vip3Aa (as determined by visual inspection of the contaminant bands by SDS-PAGE) was lower than that obtained by affinity chromatography but much higher than by either ammonium sulfate or pI precipitation (Fig. 1).

3.2. Factors affecting insecticidal activity against S. frugiperda

Mortality scored at 7 d showed no significant differences in LC_{50} values among different procedures to prepare the protoxin sample, with a weighted average of 203 ng/cm^2 (Table 1). Slopes of regression lines ranged from 0.66 to 1.31, with higher values for the more purified samples (with the exception of the chromatographic peak 2). The LC_{50} value of the trypsin-activated sample (41 ng/cm^2) was lower than those of the protoxin samples, with the fiducial limits overlapping only with the samples purified by anion-exchange chromatography.

When considering functional mortality at 7 d (both dead and L1 larvae), the LC_{50} values were much lower than just considering mortality. The weighted average was 13 ng/cm^2. Again, there were

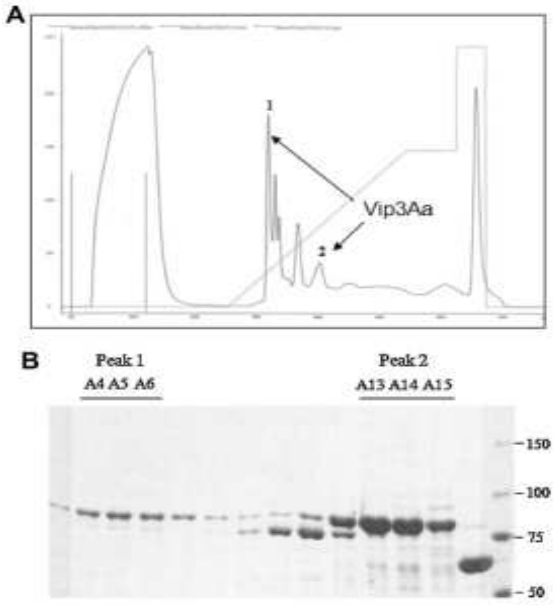

Fig. 3. Purification by anion-exchange chromatography. An *E. coli* extract containing Vip3Aa protoxin was purified by isoelectric point precipitation and then subjected to anion-exchange chromatography in a HiTrap Q column. (A) Chromatogram showing the absorbance at 280 nm and the NaCl gradient. (B) SDS-PAGE of the chromatography fractions stained with Coomassie blue.

Table 1
Effect of the preparation and activation of Vip3Aa on its insecticidal activity on *S. frugiperda*. LC_{50} values are given in ng of Vip protein per cm^2 of diet surface. Fiducial limits (95%) are given in parenthesis.

Sample preparation[a]	Mortality (7 d) LC_{50}	Functional mortality (7 d) LC_{50}	Mortality (10 d) LC_{50}
Supernatant of crude lysate	340 (160–1100) Slope = 0.87 ± 0.11	12 (6.0–25) Slope = 2.35 ± 0.44	24 (17–34) Slope = 1.80 ± 0.11
Ammonium sulfate purified	170 (110–250) Slope = 0.91 ± 0.07	14 (12–17) Slope = 2.50 ± 0.23	33 (22–49) Slope = 1.77 ± 0.18
Chromatographic peak 1	77 (36–170) Slope = 1.20 ± 0.16	15 (10–23) Slope = 1.90 ± 0.28	14 (7.1–26) Slope = 2.23 ± 0.27
Chromatographic peak 2	116 (57–270) Slope = 0.66 ± 0.12	11 (6.9–16) Slope = 1.51 ± 0.22	57 (36–92) Slope = 0.98 ± 0.09
Nickel purified	290 (160–540) Slope = 1.31 ± 0.26	–	78 (43–120) Slope = 2.01 ± 0.40
Trypsin-activated	41 (20–79) Slope = 1.23 ± 0.10	4.4 (0.6–13) Slope = 1.66 ± 0.15	12 (6.1–21) Slope = 2.16 ± 0.20

[a] Supernatant of crude lysate and ammonium sulfate purified samples were replicated from 3 to 7 times. The rest were replicated twice.

Table 2
Insecticidal activity of Vip3Aa on *S. exigua*. LC_{50} and fiducial limits (95%) values are given in ng of Vip protein per cm^2 of diet surface. Values are the mean of three to six replicates.

	Mortality (7 d)	Functional mortality (7 d)	Mortality (10 d)
Supernatant of crude lysate	2600 (1200–9100) Slope = 0.72 ± 0.09	33 (24–44) Slope = 4.59 ± 0.99	290 (160–610) Slope = 0.79 ± 0.08
Trypsin-activated	35 (5.9–200) Slope = 0.69 ± 0.10	1.7 (0.9–2.8) Slope = 1.84 ± 0.34	24 (4.7–80) Slope = 1.15 ± 0.15

no significant differences among protoxin samples and, in contrast to the values obtained just considering mortality, the LC_{50} values for the functional mortality were more homogeneous (range of 11–15) and with narrower fiducial limits. Slopes of regression lines were steeper, ranging from 1.51 to 2.50, with higher values for the less purified samples. The trypsin-activated sample was the sample with the lowest LC_{50} value (4.4 ng/cm^2), although it was not significantly different from those of the protoxin samples according to the fiducial limits.

Scoring mortality after 10 d had a marked effect on the LC_{50} values, which were considerably lower than those at 7 d and with narrower fiducial limits. Slopes of the regression lines were steeper than at 7 d. The trypsin-activated sample had the lowest LC_{50} value (12 ng/cm^2), being significantly different from those of some protoxin samples.

3.3. Insecticidal activity against S. exigua

The Vip3Aa protoxin (in crude lysate supernatant) was less active against *S. exigua*, with LC_{50} values of 2600 and 290 ng/cm^2 at 7 and 10 d, respectively (Table 2). These values are significantly different from those obtained for *S. frugiperda* (around 10-fold higher). In contrast, when considering either the functional mortality at 7 d of the protoxin or the mortality caused by the activated toxin (no matter the scoring model used), the LC_{50} values from both species were not significantly different from each other. This result suggests an inefficient processing of the protoxin *in vivo* in the *S. exigua* midgut, which is overcome when administering the activated toxin.

3.4. Activation of Vip3Aa protoxin by midgut juice proteases

Vip3Aa protoxin was incubated with diluted midgut juice from last instar larvae of *S. frugiperda* and *S. exigua* to determine whether differences in susceptibility to the protoxin were due to differences in the rate of activation. An appropriate dilution (1/250) of the midgut juice was chosen because an excess (a 1/50 dilution) completely degraded the 62 kDa band (data not shown). The results, at equal dilutions from both insect species, show that the midgut juice of *S. frugiperda* activated the Vip3Aa protoxin at a faster rate than the *S. exigua* midgut juice (Fig. 4).

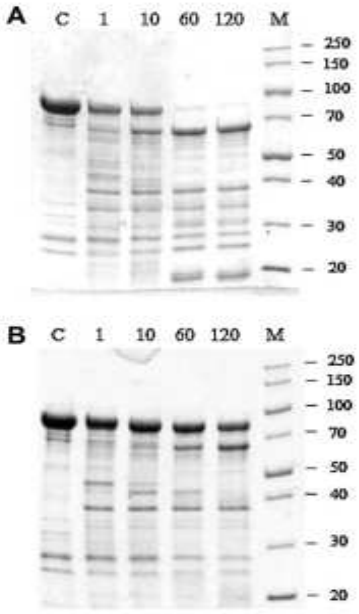

Fig. 4. Time course of proteolytic processing of Vip3Aa protoxin by midgut juice. Affinity-chromatography purified Vip3Aa protoxin (10 μg) (lane C) was incubated with 5 μl of a 1/250 dilution of midgut juice from *S. frugiperda* (A) and from *S. exigua* (B). Reaction products were separated by SDS-PAGE and the gels were stained with Coomassie blue. Incubations were carried out at 30 °C for different times (min). M: Molecular weight markers.

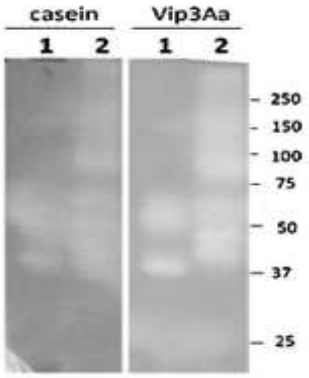

Fig. 5. Zymogram analysis of midgut juice proteases. Midgut juice proteases from *S. exigua* (lane 1) and *S. frugiperda* (lane 2) were separated in non-denaturing SDS-PAGE in a 12% Tris–glycine gel and their activity revealed using casein or Vip3Aa protoxin as substrates.

3.5. Zymogram analysis of midgut proteases

Zymograms from the midgut juice proteases from the two insect species were obtained using two types of proteins as substrates: casein (a general substrate) and Vip3Aa. Results were essentially the same using either substrate. However, the two insect samples showed a totally different pattern in several aspects: in the number of bands, their position and the overall intensity (Fig. 5).

4. Discussion

The Vip3Aa protoxin was subjected to different purification protocols (affinity chromatography and ion-exchange chromatography) and storage conditions (ammonium sulfate precipitation, and freezing) commonly used in a biochemistry laboratory. The aim was not to develop a purification protocol, but to check if any of the steps affected negatively the insecticidal activity of this protein. For this reason, we tested the following samples: *E. coli* crude lysate supernatant, ammonium sulfate precipitated protein, Ni-chelating affinity purified protein, and anion-exchange purified protein. It is noteworthy that the Vip3Aa protoxin eluted in two different fractions after anion-exchange chromatography. The two Vip3Aa isoforms very likely derived one from another by the action of *E. coli* enzymes during Vip3Aa production or cell lysis.

To test the insecticidal activity of the different preparations of protoxin, *S. frugiperda* was chosen because of its known susceptibility to this insecticidal protein (Estruch et al., 1996; Yu et al., 1997; Sena et al., 2009). The results indicate that the Vip3Aa protoxin maintains its insecticidal activity independently of the purification protocol used (Table 1). Moreover, trypsin-activated samples had lower LC_{50} values than protoxin samples, suggesting that the activation step is rate limiting.

Mortality of *S. frugiperda* scored at 7 d revealed two effects caused by the toxin: only part of the insects was killed, although a considerable proportion of larvae that remained alive were arrested in their development. Most of these larvae died before 10 d. This is the reason of the pronounced differences in LC_{50} values at 7 d vs. 10 d, and also why the values of the functional mortality at 7 d resemble those of mortality at 10 d. Such pronounced differences between mortality at 7 d vs. 10 d are not observed when testing the trypsin-activated protein, suggesting again that the activation step may be rate limiting.

Side by side bioassays with the two *Spodoptera* species show that *S. exigua* is more tolerant than *S. frugiperda* to the Vip3Aa protoxin. The former species also shares with the latter the dichotomy of mortality and severe growth inhibition when exposed to the protoxin. Both species have a significant difference in susceptibility to the protoxin when mortality is measured, however, in terms of functional mortality, they do not differ significantly. This indicates that larvae from *S. exigua* suffer a stronger growth inhibition than larvae from *S. frugiperda*, which compensates for the lower mortality of the former when the functional mortality is measured. The strong growth inhibition seems not be a general pattern of the effect of Vip3Aa, but a peculiarity limited only to some insect species. For example, important differences (around 10-fold) in LC_{50} values between mortality and functional mortality were found in *H. zea*, while minimum differences were found in *H. virescens* (Ali and Luttrell, 2011).

Differences in susceptibility between both species disappeared when testing the activated toxin, a strong indication that the differences might be due to differences at the activation step. The higher activation rate was confirmed with the results obtained with the time course experiment with Vip3Aa protoxin and midgut juice from both species, in which *S. exigua* midgut juice was shown to be less efficient in activating the protoxin than *S. frugiperda* midgut juice (Fig. 4). The zymogram study revealed more variety of proteases and higher protease activity in *S. frugiperda* than in *S. exigua* (Fig. 5), which could account for the faster processing of the protoxin in the former.

Differences in the processing of the Vip3Aa protoxin by *Ephestia kuehniella* and *S. littoralis* midgut juice have also been proposed to be crucial in determining the differences in susceptibility between both species (Abdelkefi-Mesrati et al., 2011b). The role of midgut proteases in activating Cry proteins have been recognized to play a role in some cases of insect resistance to Cry1A proteins (Oppert et al., 1994; Forcada et al., 1996; Li et al., 2004) and, at least in one case, proteases were shown to be critical in determining the specificity of the activated toxin to different insect targets (Haider et al., 1986).

Based on our results, the Vip3Aa protoxin retains full toxicity after being subjected to different biochemical treatments. The marked differences observed in the two insect species in the protoxin LC_{50} values when mortality was measured at 7 vs. 10 d seems to be due to the limiting rate of protoxin activation. These differences between 7 and 10 d disappear when using the trypsin-activated toxin. Furthermore, the differences in susceptibility between both species against the protoxin also disappeared when the activated toxin was tested instead. All these observations, along with the time course activation experiment, clearly point out that the protoxin activation is a critical step contributing to the toxicity of Vip3Aa to these two *Spodoptera* species.

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References

Abdelkefi-Mesrati, L., Tounsi, S., Jaoua, S., 2005. Characterization of a novel vip3-type gene from *Bacillus thuringiensis* and evidence of its presence on a large plasmid. FEMS Microbiol. Lett. 244, 353–358.

Abdelkefi-Mesrati, L., Rouis, S., Sellami, S., Jaoua, S., 2009. *Prays oleae* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3LB differs from that of Cry1Ac toxin. Mol. Biotechnol. 43, 15–19.

Abdelkefi-Mesrati, L., Boukedi, H., Dammak-Karray, M., Sellami-Boudawara, T., Jaoua, S., Tounsi, S., 2011a. Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of *Spodoptera littoralis*. J. Invertebr. Pathol. 106, 250–254.

Abdelkefi-Mesrati, L., Boukedi, H., Chakroun, M., Kamoun, F., Azzouz, H., Tounsi, S., Rouis, S., Jaoua, S., 2011b. Investigation of the steps involved in the difference of susceptibility of *Ephestia kuehniella* and *Spodoptera littoralis* to the *Bacillus thuringiensis* Vip3Aa16 toxin. J. Invertebr. Pathol. 107, 198–201.

Ali, M.I., Luttrell, R.G., 2011. Susceptibility of *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera: Noctuidae) to Vip3A insecticidal protein expressed in VipCot™ cotton. J. Invertebr. Pathol. 108, 76–84.

Beard, C.E., Court, L., Boets, A., Mourant, R., Van Rie, J., Akhurst, R.J., 2008. Unusually high frequency of genes encoding vegetative insecticidal proteins in an Australian *Bacillus thuringiensis* collection. Curr. Microbiol. 57, 195–199.

Bell, R.A., Joachim, F.G., 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Ann. Entomol. Soc. Am. 69, 365–373.

Bhalla, R., Dalal, M., Panguluri, S.K., Jagadish, B., Mandaokar, A.D., Singh, A.K., Kumar, P.A., 2005. Isolation, characterization and expression of a novel vegetative insecticidal protein gene of *Bacillus thuringiensis*. FEMS Microbiol. Lett. 243, 467–472.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. J. 72, 248–254.

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., Koziel, M.G., 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. USA 93, 5389–5394.

Fang, J., Xu, X., Wang, P., Zhao, J.Z., Shelton, A.M., Cheng, J., Feng, M.G., Shen, Z., 2007. Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl. Environ. Microbiol. 73, 956–961.

Ferré, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47, 501–533.

Ferré, J., Van Rie, J., MacIntosh, S.C., 2008. Insecticidal genetically modified crops and insect resistance management (IRM). In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.), Integration of Insect Resistant Genetically Modified Crops within IPM Programs. Springer, New York, pp. 41–85.

Forcada, C., Alcácer, E., Garcerá, M.D., Martínez, R., 1996. Differences in the midgut proteolytic activity of two *Heliothis virescens* strains, one susceptible and one resistant to *Bacillus thuringiensis* toxins. Arch. Insect Biochem. Physiol. 31, 257–272.

Haider, M.Z., Knowles, B.H., Ellar, D.J., 1986. Specificity of Bacillus thuringiensis var. colmeri insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. 156, 531–540.

Hernández-Rodríguez, C.S., Boets, A., Van Rie, J., Ferré, J., 2009. Screening and identification of vip genes in *Bacillus thuringiensis* strains. Appl. Microbiol. 107, 219–225.

Lee, M.K., Walters, F.S., Hart, H., Palekar, N., Chen, J.S., 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa differs from that of Cry1Ab delta-endotoxin. Appl. Environ. Microbiol. 69, 4648–4657.

Lee, M.K., Miles, P., Chen, J.S., 2006. Brush border membrane binding properties of Bacillus thuringiensis Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem. Biophys. Res. Commun. 339, 1043–1047.

Li, H., Oppert, B., Higgins, R.A., Huang, F., Zhu, K.Y., Buschman, L.L., 2004. Comparative analysis of proteinase activities of *Bacillus thuringiensis* resistant and susceptible *Ostrinia nubilalis* (Lepidoptera: Crambidae). Insect Biochem. Mol. Biol. 34, 753–762.

Liu, J., Song, F., Zhang, J., Liu, R., He, K., Tan, J., Huang, D., 2007. Identification of vip3Atype genes from *Bacillus thuringiensis* strains and characterization of a novel vip3A-type gene. Lett. Appl. Microbiol. 45, 432–438.

Liu, J., Yang, A., Shen, X., Hua, B., Shi, G., 2011. Specific binding of activated Vip3Aa10 to *Helicoverpa armigera* brush border membrane vesicles results in pore formation. J. Invertebr. Pathol. 108, 92–97.

Milne, R., Liu, Y., Gauthier, D., van Frankenhuyzen, K., 2008. Purification of Vip3Aa from *Bacillus thuringiensis* HD-1 and its contribution to toxicity of HD-1 to spruce budworm (*Choristoneura fumiferana*) and gypsy moth (*Lymantria dispar*) (Lepidoptera). J. Invertebr. Pathol. 99, 166–172.

Oppert, B., Kramer, K.J., Johnson, D.E., MacIntosh, S.C., McGaughey, W.H., 1994. Altered protoxin activation by midgut enzymes from a *Bacillus thuringiensis* resistant strain of *Plodia interpunctella*. Biochem. Biophys. Res. Commun. 198, 940–947.

Schnepf, H.E., Cricmore, N., Vanrie, J., Lereclus, D., Baum, J., Feitelson, J., Zfider, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775–806.

Sena, J.A., Hernández-Rodríguez, C.S., Ferré, J., 2009. Interaction of *Bacillus thuringiensis* Cry1 and Vip3Aa proteins with *Spodoptera frugiperda* midgut binding sites. Appl. Environ. Microbiol. 75, 2236–2237.

Shi, Y., Xu, W., Yuan, M., Tang, M., Chen, J., Pang, Y., 2004. Expression of vip1/vip2 genes in *Escherichia coli* and *Bacillus thuringiensis* and the analysis of their signal peptides. Appl. Microbiol. 97, 757–765.

van Frankenhuyzen, K., Nystrom, C., 2002. The *Bacillus thuringiensis* toxin specificity database. <http://www.glfc.forestry.ca/bacillus/>.

Yu, C.G., Mullins, M.A., Warren, G.W., Koziel, M.G., Estruch, J.J., 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63, 532–536.

Yu, X., Zheng, A., Zhu, J., Wang, S., Wang, L., Deng, Q., Li, S., Liu, H., Li, P., 2010. Characterization of vegetative insecticidal protein vip genes of *Bacillus thuringiensis* from Sichuan Basin in China. Curr. Microbiol. 62, 752–757.

Second paper

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

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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

hemolymph) and then placed on a piece of Parafilm. Midguts were carefully opened longitudinally and the content, within the intact peritrophic envelope, was removed and transferred into ice-cold centrifuge tubes. After centrifugation at 15,000g for 10 min at 4 °C, supernatants were immediately aliquoted, frozen in liquid nitrogen and stored at −80 °C until use.

2.5. Peptidase activity assay

Azocasein was used as a general peptidase substrate to determine the activities of commercial trypsin, commercial chymotrypsin, and larval midgut extracts (Vinokurov et al., 2009). The commercial peptidase or a midgut content extract aliquot diluted up to 100 µl with 20 mM Tris–HCl, pH 8.6, was incubated for 1 h at 30 °C with 100 µl of 0.5% azocasein made in the same buffer. The reaction was terminated by addition of trichloroacetic acid (TCA) up to 6% final concentration and the mixture was incubated for 30 min on ice to favor undigested substrate precipitation. Finally, the reaction mixture was clarified by centrifugation at 10.000g for 10 min. An equal volume of 1 M NaOH was added to a 100 µl aliquot of supernatant transferred to a cuvette and the absorbance was measured at 440 nm with Spectronic Genesys 5 spectrophotometer (Milton Roy) or with Multiskan Ascent plate reader (Thermo Labsystems). One unit of total proteolytic activity with azocasein was defined as the increase in absorbance by 0.1 unit per min per mg protein or per µl.

Specific proteolytic activity was assessed with synthetic substrates, including BzRpNA and SAAPFpNA (Bachem AG, Bubendorf, Switzerland), for trypsin- and chymotrypsin-like peptidase activity evaluation, respectively. Briefly, 5 µl of 10 mM substrate in dimethylformamide (DMF) was added to each well of a microtiter plate containing the enzyme aliquot diluted to 195 µl with reaction buffer (20 mM Tris–HCl, pH 8.6). Samples were incubated at 30 °C, and the absorbance of released *p*-nitroaniline was measured spectrophotometrically with Multiskan Ascent plate reader (Thermo Labsystems). Enzyme activity was calculated in µmol of *p*-nitroaniline produced per min.

2.6. Purification of trypsin and chymotrypsin peptidase fractions from midgut contents of S. frugiperda

Partial separation of *S. frugiperda* trypsin- and chymotrypsin-like peptidases was achieved by combination of anion-exchange and size-exclusion chromatography performed on ÄKTA 100 explorer system (Amersham Biosciences) with HiTrap Q HP and Superdex 75 columns (GE Healthcare). For the primary separation, midgut content extract (up to 0.5 mg total protein) was loaded onto a 1 ml HiTrap Q HP column equilibrated with 20 mM phosphate buffer, pH 6.9. The elution was accomplished by applying a linear gradient of 0–600 mM NaCl in the same buffer for 20 min at a flow rate of 1 ml/min. Chromatography fractions were assayed for trypsin- and chymotrypsin-like activities as it has been described previously. Two peaks with serine peptidase activity were detected (Fig. 1A). Fractions of peak 1, containing trypsin- and chymotrypsin-like activities, were not retained on the column (cationic peptidases), whereas peak 2 peptidases were bound onto the column and eluted by the NaCl gradient (anionic chymotrypsin-like peptidases). Since fractions of peak 2 showed mainly chymotrypsin-like activity, they were pooled, concentrated on 10 K Centricon ultrafiltration units (Millipore), and used without further purification for Vip3A protoxins hydrolysis. Fractions 3–7 (peak 1) were applied for subsequent separation of trypsin- and chymotrypsin-like activities on a size-exclusion chromatography column Superdex 75 equilibrated with 10 mM phosphate buffer, 150 mM NaCl, pH 6.8 (Fig 1B). Fractions showing either trypsin- or chymotrypsin-like activities (cationic trypsin- and chymotrypsin-like peptidases) were combined, concentrated by ultrafiltration and used for Vip3A protoxins hydrolysis.

2.7. Digestion of Vip3A protoxins by commercial and Spodoptera midgut content serine peptidases

Purified protoxins (9.5 µg) were mixed with different concentrations of either commercial bovine trypsin and chymotrypsin (Sigma Chemical Co., St. Louis, USA), midgut crude extracts, or serine peptidase chromatographic fractions, and incubated for 1 h at 30 °C. Incubation was stopped by addition of SDS-sample buffer (2.5% (w/v) SDS, 1% β-mercaptoethanol, 0.075% (w/v) bromophenol blue, 3.8 mM EDTA, 150 mM Tris–HCl pH 6.8, 750 mM sucrose) followed by heat denaturation at 99 °C for 10 min. Proteolysis products were then separated by 12% SDS–PAGE. Gels were stained with Coomassie blue R-250 dye to visualize the proteolysis products. The amount of protoxin and activated toxin was estimated densitometrically from the intensity of the corresponding bands in the gel with the 1D Manager ver. 2.0 programme (TDI Tecnología de Diagnóstico e Investigación).

3. Results

3.1. Toxicity of Vip3Ae against S. frugiperda and S. exigua larvae

Vip3Ae was found highly toxic to neonate larvae of *S. frugiperda* and *S. exigua*, with LC_{50} values not significantly different (20 and 11 ng/cm^2, respectively) (Table 1). This differs from protoxin Vip3Aa, being significantly more toxic (12-fold) to *S. frugiperda* than to *S. exigua*. Comparing the toxicity of Vip3Aa and Vip3Ae, they both are equally toxic against *S. frugiperda* (LC_{50} of 24 and 20 ng/cm^2, respectively), whereas Vip3Ae is more toxic to *S. exigua* than Vip3Aa (LC_{50} of 11 and 290 ng/cm^2, respectively).

3.2. Proteolysis of Vip3Aa and Vip3Ae protoxins by commercial trypsin and chymotrypsin

Affinity purified Vip3A full-length proteins were exposed to different concentrations of commercial bovine trypsin and chymotrypsin (Fig. 2A and B; Table 2). The proteolysis of the 90 kDa Vip3Aa protoxin with the two commercial enzymes led to a major digestion product which corresponded to a band of 62 kDa (Fig. 2A, Table 2). The fragment was susceptible to further digestion at increasing concentrations of trypsin higher than 0.2 mU and at concentrations of chymotrypsin higher than 2 mU. The proteolysis of the 88 kDa Vip3Ae protoxin with the two commercial enzymes also resulted in a 62 kDa major digestion product which was also susceptible to further digestion (Fig. 2B; Table 2). Accumulation of the 62 kDa active toxin form was maximal at 1–2 mU of trypsin and started to decline at higher enzyme amounts. Against chymotrypsin, Vip3Ae was relatively unstable, showing at 0.1–0.4 mU a maximal yield of active 62 kDa toxin form that was completely degraded at 10 mU of chymotrypsin. With both Vip3A proteins, the amount of 62 kDa band produced by chymotrypsin never reached the levels obtained with trypsin, which suggests that its degradation started even before activation was completed.

In silico prediction of trypsin and chymotrypsin cleavage sites with ExPASy Peptide Cutter tool showed that Vip3Aa has 78 and 173 potential hydrolysis sites for trypsin and chymotrypsin, respectively, while Vip3Ae has 75 sites for trypsin and 175 for chymotrypsin.

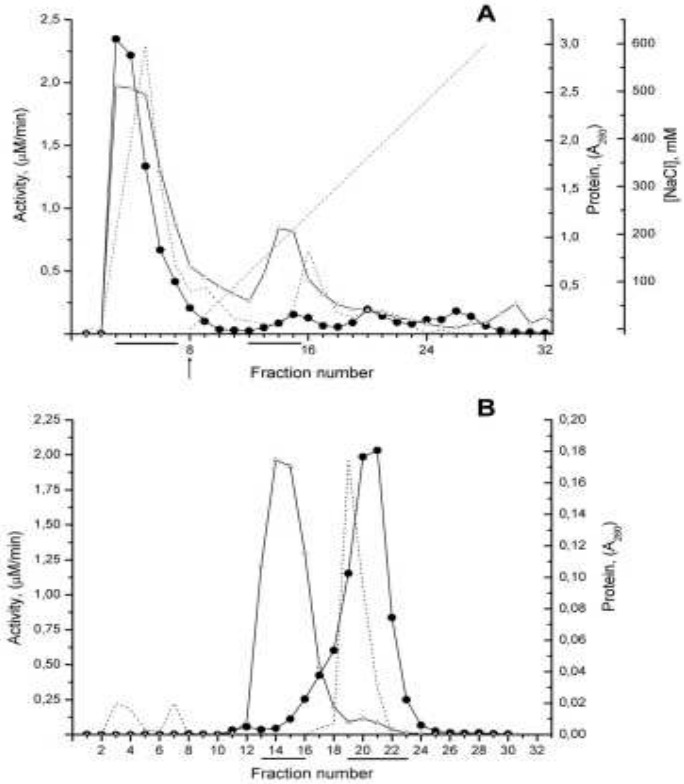

Fig. 1. Chromatographic separation of trypsin- and chymotrypsin-like peptidases from *S. frugiperda* crude midgut content extracts. (A) A sample of crude midgut content (total protein: 0.465 mg) was injected onto a 1 ml HiTrap Q HP column equilibrated with 20 mM phosphate buffer, pH 6.9. The elution was started by applying a linear gradient (marked by a vertical arrow) of 0–600 mM of NaCl (—) in the same buffer at a flow rate of 1 ml/min for 20 min. Underlined sets of active fractions were pooled and concentrated, and either used in Vip3A protoxin hydrolysis experiments (frs. 12–16) or directly applied for subsequent purification on a size-exclusion column (frs. 3–7). (B) The combined flowthrough (total protein: 0.05 mg) was applied onto a Superdex 75 column equilibrated with 10 mM phosphate buffer, 150 mM NaCl, pH 6.8. The amount of protein in the fractions was followed by absorbance at 280 nm (···) and the chymotrypsin- and trypsin-like activities of the elution fractions (underlined) were assayed with SAAPFpNA (–Δ–) and BzRpNA (–●–) peptidases substrates, respectively.

Table 1
Effect of *B. thuringiensis* Vip3A protoxins (supernatant of crude lysates) on larval mortality of two *Spodoptera* species after 10 days.

Protein	*S. frugiperda*			*S. exigua*		
	LC_{50}	FL_{95}	Slope	LC_{50}	FL_{95}	Slope
Vip3Aa*	24	(17–34)	1.80 ± 0.11	290	(160–610)	0.79 ± 0.08
Vip3Ae	20	(8–43)	1.25 ± 0.50	11.1	(6.6–16)	1.02 ± 0.08

Assays were performed with neonate larvae and values are given in ng per cm^2. Parameters were obtained with the POLO-PC program.
* Data published by Chakroun et al. (2012) and included here to help discussion.

3.3. Proteolysis of Vip3Aa and Vip3Ae protoxins by Spodoptera midgut content extracts

Digestion of Vip3A protoxins by crude midgut content extracts from *S. frugiperda* produced two major bands: a fragment of 62 kDa, the same as in the case of commercial serine peptidases, and a smaller product of approximately 47 kDa (Fig. 3A and B). In the case of Vip3Aa, the 62 kDa band accumulated until 0.7 mU of midgut extract activity equivalents (defined as azocaseinolytic activity units, see Section 2) and started to decrease at higher activity levels (Fig. 3A). In the case of the Vip3Ae protoxin (Fig. 3B), activation proceeded with an intermediate product with slightly lower molecular mass than the protoxin, which was not formed in the course of Vip3Aa activation. All proteolysis products formed were unstable at the highest concentrations of midgut content extract used for this study, being completely degraded at 70 mU.

The protoxins digestion by *S. exigua* midgut content extract (Fig. 4A and B) generated a similar pattern of hydrolysis products as described for *S. frugiperda*. However, the Vip3A protoxin hydrolysis by *S. exigua* peptidases was more efficient since both protoxin and 62 kDa active toxins were completely degraded even at 1.8 mU of midgut content extract.

3.4. Proteolysis of Vip3Aa and Vip3Ae protoxins by purified S. frugiperda midgut peptidases

As described above (see Section 2.6 of Materials and Methods), the fractionation of serine peptidases from *S. frugiperda* midgut

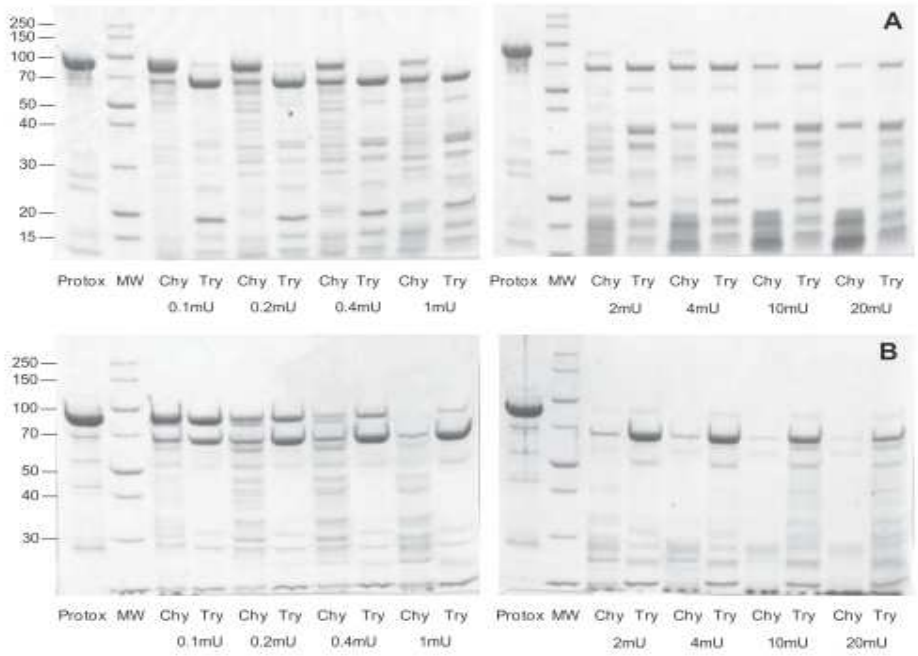

Fig. 2. Proteolysis with commercial trypsin (Try) and chymotrypsin (Chym) of Vip3Aa (A) and Vip3Ae (B) protoxins for 1 h at 30 °C. All reactions contained 9.5 μg of protoxin and different amounts of enzymes (see Section 2 for general peptidase activity unit determination). Molecular mass markers (MW) in kDa are indicated on the left of the figure.

Table 2
Densitometric estimation of the protoxin and 62 kDa bands from Vip3Aa (Fig. 2A) and Vip3Ae (Fig. 2B) produced by the action of different amounts (mU) of trypsin or chymotrypsin. (–): not measurable.

	Peptidase activity (mU)	0.1	0.2	0.4	1	2	4	10	20
Chymotrypsin	Vip3Aa protoxin (μg)	4.8	4.3	2.0	0.4	0.5	–	–	–
	Vip3Aa toxin (μg)	0.6	0.9	1.5	2.0	2.1	1.8	1.2	0.4
Trypsin	Vip3Aa protoxin (μg)	–	–	–	–	–	–	–	–
	Vip3Aa toxin (μg)	4.1	4.0	3.4	2.7	2.8	2.6	1.7	0.9
Chymotrypsin	Vip3Ae protoxin (μg)	4.8	1.4	0.2	–	–	–	–	–
	Vip3Ae toxin (μg)	1.2	1.3	1.4	0.6	0.7	0.4	–	–
Trypsin	Vip3Ae protoxin (μg)	4.3	2.5	1.4	0.3	–	–	–	–
	Vip3Ae toxin (μg)	2.3	3.4	4.4	4.8	4.9	4.4	3.1	1.9

content extracts allowed us to separate the 3 main digestive peptidase fractions; one cationic trypsin-like and two chymotrypsin-like peptidases (one cationic and one anionic) (Fig. 1). Treatment of Vip3Aa and Vip3Ae protoxins by the cationic trypsin-like fraction produced similar processing patterns (Fig. 5A and B). The 62 kDa fragment accumulated between 0.1 and 1.1 mU for Vip3Aa and 0.1–0.44 mU for Vip3Ae. In the case of Vip3Aa, proteolytic activation of protoxin with formation of 62 kDa active toxin band was more apparent allowing us to propose a stepped proteolytic processing in comparison with Vip3Ae that shows a similar intensity of hydrolysis products bands of different MW, probably due to unspecific cleavage in different parts of the more proteolytically labile molecule. Similar results were obtained with the anionic chymotrypsin-like fraction (Fig. 6A and B). When using the cationic chymotrypsin-like fraction with Vip3Aa and Vip3Ae, the 62 kDa band did not appear as the major intermediate (Fig. 7A and B). In the case of Vip3Aa, the 62 kDa band appeared only as a minor band whereas with Vip3Ae this band was completely absent in the gel and, instead, there appeared a major intermediate with a smaller molecular mass (ca. 56 kDa).

4. Discussion

Spodoptera larvae are mostly polyphagous pests attacking a variety of commercially important crops including cotton, rice, maize, legumes, grasses and ornamental plants all around the world. For this reason the control of these species is crucial for crop

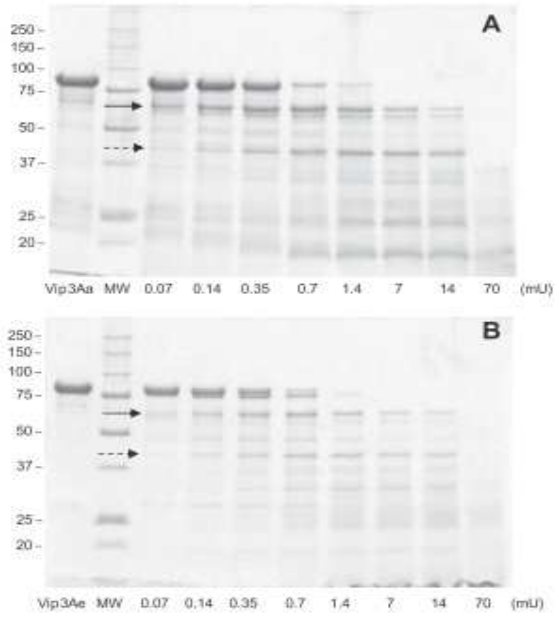

Fig. 3. Proteolysis of Vip3Aa (A) and Vip3Ae (B) protoxins by the crude midgut content extract from *S. frugiperda* for 1 h at 30 °C. All reactions contained 9.5 μg of protoxin and different amounts of midgut content extract (see Section 2 for general peptidase activity unit determination). Molecular mass markers (MW) in kDa are indicated on the left of the figure. Arrows indicate the expected position of the 62 kDa active toxin band (solid line) and that of the 47 kDa band (dotted line).

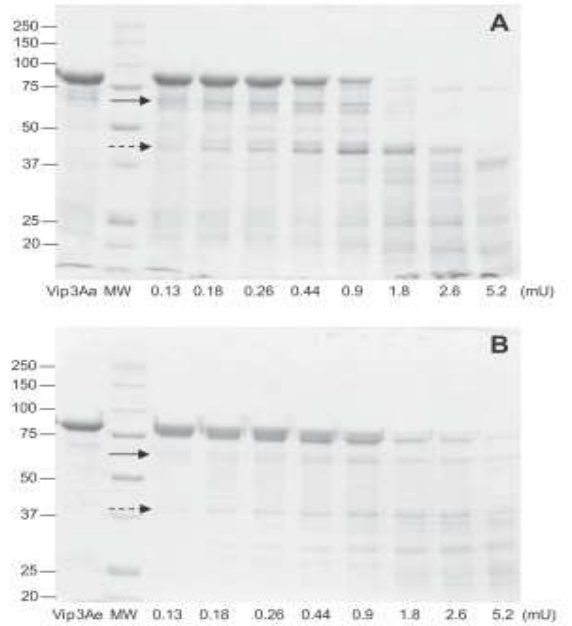

Fig. 4. Proteolysis of Vip3Aa (A) and Vip3Ae (B) protoxins by the crude midgut content extract from *S. exigua* for 1 h at 30 °C. All reactions contained 9.5 μg of protoxin and different amounts of midgut content extract (see Section 2 for general peptidase activity unit determination). Molecular mass markers (MW) in kDa are indicated on the left of the figure. Arrows indicate the expected position of the 62 kDa active toxin band (solid line) and the 47 kDa band (dotted line).

protection and Bt products have been largely used to minimize the damage caused by these pests. To preserve the efficacy of this biopesticide, effective strategies delaying resistance development must be adopted.

Vip insecticidal proteins were discovered in the 1990's (Estruch et al., 1996) and they still represent a relative novelty as part of the Bt arsenal. In fact, studies clarifying their mode of action are not abundant (Yu et al., 1997; Lee et al., 2003, 2006; Sena et al., 2009; Abdelkefi-Mesrati et al., 2011a,b; Liu et al., 2011). We have chosen two *Spodoptera* species (*S. frugiperda* and *S. exigua*) (present paper and Chakroun et al., 2012) with two Vip3A proteins for this study: Vip3Aa, a toxin that has been already tested with many insect species, and Vip3Ae, a protein that was discovered more recently. Both toxins are good candidates for the control of insect pests. Since differences in the Vip3Aa activation have been proposed to be involved in the susceptibility differences between lepidopteran species (Abdelkefi-Mesrati et al., 2011b; Chakroun et al., 2012) we decided to study in detail the proteolytic processing of Vip3Aa and Vip3Ae by serine peptidases, both commercial (bovine trypsin and chymotrypsin) and from the larval midgut of *S. frugiperda* and *S. exigua*.

Estruch and Yu (2001) indicated that Vip3Aa processing by midgut peptidases from the different lepidopteran species led to main products of approximately 66, 45, 33 and 22 kDa. However, further studies have found the 62 kDa fragment as the major product of the protoxin processing and thus it has been considered as the active form of this protein (Yu et al., 1997; Lee et al., 2003, 2006; Chakroun et al., 2012). It was shown that only 62 kDa Vip3A toxin form specifically binds to BBMV of *H. armigera* (Liu et al., 2011). Since binding to membrane receptors is a key step in the mode of action of Vip3A proteins, this is an additional confirmation that the 62 kDa form is the active toxin *in vivo*.

Activation with commercial trypsin and chymotrypsin clearly shows that the Vip3Aa and Vip3Ae 62 kDa toxin forms are susceptible to further digestion by both enzymes and, in particular, by chymotrypsin (Fig. 2A and B; Table 2). Activation of Vip3Aa with commercial peptidases showed that trypsin is more efficient in processing the protoxin which almost completely disappeared with 0.1 mU. However, with chymotypsin the protoxin band was more stable and appeared visible up to 4 mU of peptidase (Fig. 2A).Whereas trypsin activation yielded up to 4.1 μg of the 62 kDa toxin, chymotrypsin only yielded 2.1 μg at the most (Table 2). This difference in the yields of the active 62 kDa toxin form probably indicates its higher instability to chymotrypsin than to trypsin. This result is in agreement with the predominance of chymotrypsin cleavage sites predicted for Vip3Aa.

Activation of Vip3Ae with commercial enzymes (Fig. 2B; Table 2) showed that the protoxin band almost disappeared at 1 mU of either peptidase. Similarly to Vip3Aa, the yield of the 62 kDa band was higher with trypsin than with chymotrypsin, though with Vip3Ae such difference was even more pronounced. Also, the chymotrypsin cleavage sites predicted *in silico* for this toxin were more abundant than trypsin cleavage sites.

Regarding the relative stability against the commercial peptidases of the two Vip3A proteins (both the protoxin and the activated forms), Vip3Ae was found more tolerant to the action of trypsin than Vip3Aa. The Vip3Aa protoxin showed comparatively

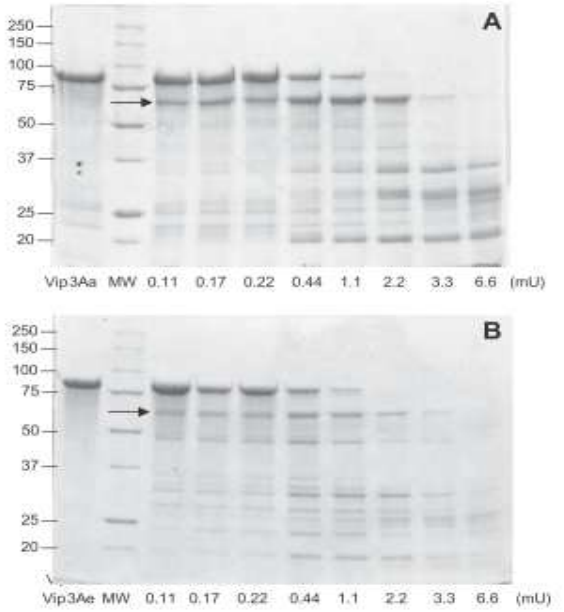

Fig. 5. Proteolysis of Vip3Aa (A) and Vip3Ae (B) protoxins by chromatography purified cationic trypsin-like peptidase from *S. frugiperda* midgut content extract (see Section 2.6). Proteolysis was performed for 1 h at 30 °C. The reaction mixture contained 9.5 μg of protoxin and different dilutions of trypsin-like peptidase. Molecular mass markers (MW) in kDa are indicated on the left of the figure. Arrows indicate the expected position of the 62 kDa band of active toxin.

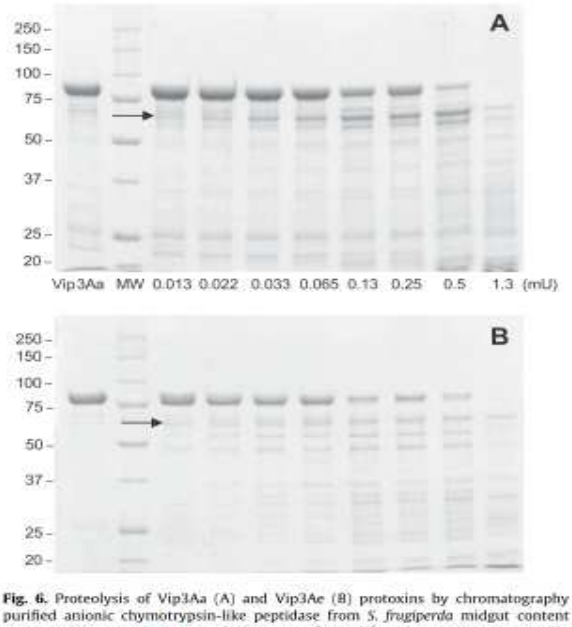

Fig. 6. Proteolysis of Vip3Aa (A) and Vip3Ae (B) protoxins by chromatography purified anionic chymotrypsin-like peptidase from *S. frugiperda* midgut content extract (see Section 2.6). Proteolysis was performed for 1 h at 30 °C. The reaction mixture contained 9.5 μg of protoxin and different dilutions of anionic chymotrypsin-like peptidase. Molecular mass markers (MW) in kDa is indicated on the left of the figure. Arrows indicate the expected position of the 62 kDa band of active toxin.

higher resistance against chymotrypsin, however its 62 kDa activated form was more easily degraded by chymotrypsin than by trypsin (Fig. 2; Table 2).

Vip3Aa and Vip3Ae protoxins were readily processed by midgut luminal serine peptidases from the two *Spodoptera* species (Figs. 3 and 4). The results indicate that, at least under the current *in vitro* conditions, the proteolytic activation of both proteins does not produce a protease resistant core. The comparatively higher yield of the 62 kDa activated form of Vip3Aa toxin produced by *S. frugiperda* midgut peptidases is likely to contribute to the higher susceptibility (12-fold) of this insect against this protein compared to *S. exigua* (Table 1). However, this correlation is not observed in the case of Vip3Ae.

The attempt of separation of the luminal digestive peptidases of *S. frugiperda* was performed to assign their role in the activation of the protoxin and degradation of the 62 kDa active toxin form. Three serine peptidase fractions (one cationic trypsin-like and two chymotrypsin-like peptidases, one anionic and one cationic) were isolated from the midgut content of *S. frugiperda* larvae (Fig. 1). Digestion of Vip3Aa by the cationic trypsin-like fraction and the anionic chymotrypsin-like fraction resulted in a major band of 62 kDa which remained moderately stable at a wide range of peptidase amounts (Figs. 5A and 6A). Vip3Ae gave similar patterns, but with a lower yield of the 62 kDa active toxin (Figs. 5B and 6B). Fractions with cationic chymotrypsin-like activity digested Vip3A protoxins without generating significant amounts of the 62 kDa band of active toxin which, in the case of Vip3Ae, was not even detectable (Fig. 7). These results suggest that the cationic trypsin-like and the anionic chymotrypsin-like peptidases of *S. frugiperda* are the ones contributing to the accumulation of the 62 kDa active toxin form while the cationic chymotrypsin-like activities mainly participate in its degradation.

In summary, according to our results, no peptidase resistant core appears during in *vitro* proteolytic processing of Vip3Aa and Vip3Ae protoxins by the action of different types of serine peptidases. This result is highly relevant as we can conclude that the activation of Vip3A proteins differs from that of the major class of Bt proteins active against lepidopterans (Cry1 toxins), whose active cores are comparatively more stable to insect's serine peptidases in *vitro* (Rukmini et al., 2000). *In vitro* activation studies showed that Cry1 protoxins are processed by midgut juice into relatively stable fragments (Ogiwara et al., 1992; Shao et al., 1998; Lightwood et al., 2000; Miranda et al., 2001; Siqueira et al., 2004; Díaz Mendoza et al., 2007; Dammak et al., 2010; González-Cabrera et al., 2013) with a few exceptions (Shao et al., 1998; Dammak et al., 2010). In *in vitro* activation studies, further proteolysis or degradation of the Cry protein by midgut juice is often associated with low susceptibility of the insect for the tested protein (Ogiwara et al., 1992; Shao et al., 1998; Lightwood et al., 2000; Miranda et al., 2001) but, in all cases, a consistent accumulation of the active fragment was observed, even for incubation periods and midgut juice amounts greater than the ones used in the present study. A pattern of Cry1 degradation similar to the one described herein for Vip3A proteins was observed only in one case (Dammak et al., 2010). Dammack and collegues performed the *in vitro* activation of Cry1Aa and Cry1Ia proteins by *Prays oleae* midgut juice. Cry1Ia is an "unconventional" Cry protein since it is a secreted δ-endotoxin, whereas Cry1Aa is a typical one that accumulates in the parasporal crystal. Interestingly, while Cry1Aa was

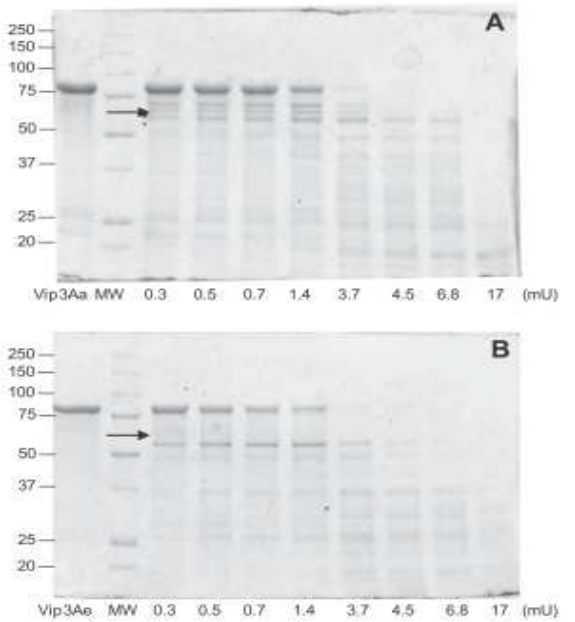

Fig. 7. Proteolysis of Vip3Aa (A) and Vip3Ae (B) protoxins by chromatography purified cationic chymotrypsin-like peptidase from *S. frugiperda* midgut content extract (see Section 2.6). Proteolysis was performed for 1 h at 30 °C. The reaction mixture contained 9.5 μg of protoxin and different dilutions of cationic chymotrypsin-like peptidase. Molecular mass markers (MW) in kDa are indicated on the left of the figure. Arrows indicate the expected position of the 62 kDa band of active toxin.

processed into a stable active core, Cry1Ia was strongly degraded and no stable intermediates were observed.

The transient accumulation of the 62 kDa fragment as a major determinant of the toxicity of Vip3Aa protein is in agreement with our previous kinetic studies showing that the accumulation of the 62 kDa Vip3Aa band was faster with *S. frugiperda* midgut juice than with *S. exigua* midgut juice (Chakroun et al., 2012). Therefore, according to our results, insect interspecific differences at the quantitative and qualitative levels of midgut digestive peptidases seem to be involved in defining *in vivo* differences in susceptibility to Vip3A proteins.

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References

Abdelkefi-Mesrati, L., Rouis, S., Sellami, S., Jaoua, S., 2009. *Prays oleae* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3LB differs from that of Cry1Ac toxin. Mol. Biotechnol. 43, 15–19.

Abdelkefi-Mesrati, L., Boukedi, H., Dammak-Karray, M., Sellami-Boudawara, T., Jaoua, S., Tounsi, S., 2011a. Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of *Spodoptera littoralis*. J. Invertebr. Pathol. 106, 250–254.

Abdelkefi-Mesrati, L., Boukedi, H., Chakroun, M., Kamoun, F., Azzouz, H., Tounsi, S., Rouis, S., Jaoua, S., 2011b. Investigation of the steps involved in the difference of susceptibility of *Ephestia kuehniella* and *Spodoptera littoralis* to the *Bacillus thuringiensis* Vip3Aa16 toxin. J. Invertebr. Pathol. 107, 198–201.

Ben Hamadou-Carfi, D., Boukedi, H., Abdelkefi-Mesrati, L., Tounsi, S., Jaoua, S., 2013. *Agrotis segetum* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa16 differs from that of Cry1Ac toxin. J. Invertebr. Pathol. 114, 139–143.

Bhalla, R., Dalal, M., Panguluri, S.K., Jagadish, B., Mandaokar, A.D., Singh, A.K., Kumar, P.A., 2005. Isolation, characterization and expression of a novel vegetative insecticidal protein gene of *Bacillus thuringiensis*. FEMS Microbiol. Lett. 243, 467–472.

Bown, D.P., Wilkinson, H.S., Gatehouse, J.A., 1997. Differentially regulated inhibitor sensitive and insensitive protease genes from the phytophagous pest, *Helicoverpa armigera*, are members of complex multigene families. Insect Biochem. Mol. Biol. 27, 625–638.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chakroun, M., Bel, Y., Caccia, S., Abdelkefi-Mesrati, L., Escriche, B., Ferré, J., 2012. Susceptibility of *Spodoptera frugiperda* and *S. exigua* to *Bacillus thuringiensis* Vip3Aa insecticidal protein. J. Invertebr. Pathol. 110, 334–339.

Christeller, J.T., Laing, W.A., Markwick, N.P., Burgess, E.P.J., 1992. Midgut proteases activities in 12 phytophagous lepidopteran larvae: dietary and protease inhibitor interactions. Insect Biochem. Mol. Biol. 22, 735–746.

Dammak, M., Tounsi, S., Rouis, S., Jaoua, S., 2010. Comparative study of *Bacillus thuringiensis* Cry1Ia and Cry1Aa delta-endotoxins: Activation process and toxicity against *Prays oleae*. J. Invertebr. Pathol. 104, 39–43.

Díaz-Mendoza, M., Farinós, G.P., Castañera, P., Hernández-Crespo, P., Ortego, F., 2007. Proteolytic processing of native Cry1Ab toxin by midgut extracts and purified trypsins from the Mediterranean corn borer *Sesamia nonagrioides*. J. Insect Physiol. 53, 428–435.

Estruch, J.J., Yu, C.G., 2001. Plant pest control. US Patent No. 6,291,156 B1.

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., Koziel, M.G., 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. USA 93, 5389–5394.

Fang, J., Xu, X., Wang, P., Zhao, J.Z., Shelton, A.M., Cheng, J., Feng, M.G., Shen, Z., 2007. Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl. Environ. Microbiol. 73, 956–996.

Ferré, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Ann. Rev. Entomol. 47, 501–533.

Ferré, J., Van Rie, J., Macintosh, S.C., 2008. Insecticidal genetically modified crops and insect resistance management (IRM). In: Romeis, J., Shelton, A.M., Kennedy, G.G. (Eds.), Integration of Insect Resistant Genetically Modified Crops Within IPM programs. Springer, New York, pp. 41–85.

Finney, D.J., 1971. Probit Analysis, third ed. Cambridge University, Cambridge (UK).

van Frankenhuyzen, K., Nystrom, C., 2002. The *Bacillus thuringiensis* toxin specificity database. <http://www.glfc.cfs.nrcan.gc.ca/Bacillus/btsearch.cfm>.

Gatehouse, L.N., Shannon, A.L., Burgess, E.P.J., Christeller, J.T., 1997. Characterization of major midgut proteinase cDNAs from *Helicoverpa armigera* larvae and changes in gene expression in response to four proteinase inhibitors in the diet. Insect Biochem. Mol. Biol. 27, 929–944.

González-Cabrera, J., García, M., Hernández-Crespo, P., Farinós, G.P., Ortego, F., Pedro Castañera, P., 2013. Resistance to Bt maize in *Mythimna unipuncta* (Lepidoptera: Noctuidae) is mediated by alteration in Cry1Ab protein activation. Insect Biochem. Mol. Biol. 43, 635–643.

Haider, M.Z., Ellar, D.J., 1989. Functional mapping of an entomocidal δ-endotoxin. Single aminoacid changes produced by site directed mutagenesis influence toxicity and specificity of the protein. J. Mol. Biol. 208, 183–194.

Haider, M.Z., Knowles, B.H., Ellar, D.J., 1986. Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by larval gut proteases. Eur. J. Biochem. 156, 531–540.

Haider, M.Z., Smith, G.P., Ellar, D.J., 1989. Detection of the toxic coding fragments and an insect specificity region of a dual toxicity *Bacillus thuringiensis* crystal protein gene. FEMS Microbiol. Lett. 58, 157–164.

Hernández-Rodríguez, C.S., Boets, A., Van Rie, J., Ferré, J., 2009. Screening and identification of *vip* genes in *Bacillus thuringiensis* strains. Appl. Microbiol. 107, 219–225.

Johnston, K.A., Lee, M.J., Brough, C., Hilder, V.A., Gatehouse, A.M.R., Gatehouse, J., 1995. Protease activities in the larval midgut of *Heliothis virescens*: evidence for trypsin and chymotrypsin-like enzymes. Insect Biochem. Mol. Biol. 25, 375–383.

Lee, M.K., Walters, F.S., Hart, H., Palekar, N., Chen, J.S., 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa differs from that of Cry1Ab delta-endotoxin. Appl. Environ. Microbiol. 69, 4648–4657.

Lee, M.K., Miles, P., Chen, J.S., 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem. Biophys. Res. Commun. 339, 1043–1047.

Li, H., Buschman, L.L., Huang, F., Zhu, K.Y., Bonning, B., Oppert, B., 2007. DiPel-selected *Ostrinia nubilalis*, larvae are not resistant to transgenic corn expressing *Bacillus thuringiensis* Cry1Ab. J. Econ. Entomol. 100, 1862–1870.

Lightwood, D.J., Ellar, D.J., Jarrett, P., 2000. Role of proteolysis in determining potency of *Bacillus thuringiensis* Cry1Ac delta-endotoxin. Appl. Environ. Microbiol. 66, 5174–5181.

Liu, J., Song, F., Zhang, J., Liu, R., He, K., Tan, J., Huang, D., 2007. Identification of *vip3A* type genes from *Bacillus thuringiensis* strains and characterization of a novel *vip3A*-type gene. Lett. Appl. Microbiol. 45, 432–438.

Liu, J., Yang, A., Shen, X., Hua, B., Shi, G., 2011. Specific binding of activated Vip3Aa10 to *Helicoverpa armigera* brush border membrane vesicles results in pore formation. J. Invertebr. Pathol. 108, 92–97.

Lopes, A.R., Juliano, M.A., Marana, S.R., Juliano, L., Terra, W.R., 2006. Substrate specificity of insect trypsins and the role of their subsites in catalysis. Insect Biochem. Mol. Biol. 36, 130–140.

Lopes, A.R., Sato, P.M., Terra, W.R., 2009. Insect chymotrypsins: chloromethyl ketone inactivation and substrate specificity relative to possible coevolutional adaptation of insects and plants. Arch. Insect Biochem. Physiol. 70, 188–203.

Milne, R., Ge, A.Z., Rivers, D., Dean, D.H., 1990. Specificity of insecticidal crystal proteins. In: Hickle, L.A., Fitch, W.L. (Eds.), Analytical Chemistry of *Bacillus thuringiensis*. American Chemical Society, Washington D.C, pp. 22–35.

Milne, R., Liu, Y., Gauthier, D., van Frankenhuyzen, K., 2008. Purification of Vip3Aa from *Bacillus thuringiensis* HD-1 and its contribution to toxicity of HD-1 to spruce budworm (*Choristoneura fumiferana*) and gypsy moth (*Lymantria dispar*) (Lepidoptera). J. Invertebr. Pathol. 99, 166–172.

Miranda, R., Zamudio, F.Z., Bravo, A., 2001. Processing of Cry1Ab δ-endotoxin from *Bacillus thuringiensis* by *Manduca sexta* and *Spodoptera frugiperda* midgut proteases: role in protoxin activation and toxin inactivation. Insect Biochem. Mol. Biol. 31, 1155–1163.

Moar, W.J., Pusztai-Carey, M., Van Faassen, H., Bosh, D., Frutis, R., Rang, C., Luo, K., Adang, M.J., 1995. Development of *Bacillus thuringiensis* Cry1C resistance by *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. 95, 2086–2092.

Ogiwara, K., Indrasith, L.S., Asano, S., Hori, H., 1992. Processing of δ-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 and HD-73 by gut juices of various insect larvae. J. Invertebr. Pathol. 60, 121–126.

Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H., 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J. Biol. Chem. 272, 23473–23476.

Patankar, A.G., Giri, A.P., Harsulkar, A.M., Sainani, M.N., Deshpande, V.V., Ranjekar, P.K., Gupta, V.S., 2001. Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. Insect Biochem. Mol. Biol. 31, 453–464.

Raybould, A., Quemada, H., 2010. Bt crops and food security in developing countries: realised benefits, sustainable use and lowering barriers to adoption. Food Sec. 2, 247–259.

Rukmini, V., Reddy, C.Y., Venkateswerlu, G., 2000. *Bacillus thuringiensis* crystal δ-endotoxin: role of proteases in the conversion of protoxin to toxin. Biochimie 82, 109–116.

Sattar, S., Maiti, M.K., 2011. Molecular characterization of a novel vegetative insecticidal protein from *Bacillus thuringiensis* effective against sap-sucking insect pest. J. Microbiol. Biotechnol. 21, 937–946.

Sellami, S., Jamoussi, K., Dabbeche, E., Jaoua, S., 2011. Increase of the *Bacillus thuringiensis* secreted toxicity against lepidopteron larvae by homologous expression of the *vip3LB* gene during sporulation stage. Curr. Microbiol. 63, 289–294.

Sena, J.A., Hernández-Rodríguez, C.S., Ferré, J., 2009. Interaction of *Bacillus thuringiensis* Cry1 and Vip3Aa proteins with *Spodoptera frugiperda* midgut binding sites. Appl. Environ. Microbiol. 75, 2236–2237.

Shao, Z., Cui, Y., Liu, X., Yi, H., Ji, J., Yu, Z., 1998. Processing of delta-endotoxin of *Bacillus thuringiensis* subsp. kurstaki HD-1 in *Heliothis armigera* midgut juice and the effects of protease inhibitors. J. Invertebr. Pathol. 72, 73–81.

Shi, Y., Xu, W., Yuan, M., Tang, M., Chen, J., Pang, Y., 2004. Expression of *vip1/vip2* genes in *Escherichia coli* and *Bacillus thuringiensis* and the analysis of their signal peptides. Appl. Microbiol. 97, 757–765.

Siqueira, H.A.A., Nickerson, K.W., Moellenbeck, D., Siegfried, B.D., 2004. Activity of gut proteinases from Cry1Ab-selected colonies of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae). Pest Manag. Sci. 60, 1189–1196.

Software, Le.Ora., 1987. POLO-PC. A user's manual for Probit or Logit analysis. LeOra Software, Berkeley, CA.

Srinivasan, A., Giri, A.P., Gupta, V.S., 2006. Structural and functional diversities in lepidopteran serine proteases. Cell. Mol. Biol. Lett. 11, 132–154.

Terra, W.R., Ferreira, C., 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109B, 1–62.

Vinokurov, K.S., Elpidina, E.N., Zhuzhikov, D.P., Oppert, B., Kodrik, D., Senhal, F., 2009. Digestive proteolysis organization in two closely related tenebrionid beetles: red flour beetle (*Tribolium castaneum*) and confused flour beetle (*Tribolium confusum*). Arch. Insect Biochem. Physiol. 70, 254–279.

Yu, C.G., Mullins, M.A., Warren, G.W., Koziel, M.G., Estruch, J.J., 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. 63, 532–536.

Yu, X., Zheng, A., Zhu, J., Wang, S., Wang, L., Deng, Q., Li, S., Liu, H., Li, P., 2010. Characterization of vegetative insecticidal protein *vip* genes of *Bacillus thuringiensis* from Sichuan Basin in China. Curr. Microbiol. 62, 752–757.

Third paper

In Vivo and *In Vitro* Binding of Vip3Aa to *Spodoptera frugiperda* Midgut and Characterization of Binding Sites by ^{125}I Radiolabeling.

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In Vivo and *In Vitro* Binding of Vip3Aa to *Spodoptera frugiperda* Midgut and Characterization of Binding Sites by ^{125}I Radiolabeling

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Bacillus thuringiensis **vegetative insecticidal proteins (Vip3A) have been recently introduced in important crops as a strategy to delay the emerging resistance to the existing Cry toxins. The mode of action of Vip3A proteins has been studied in *Spodoptera frugiperda* with the aim of characterizing their binding to the insect midgut. Immunofluorescence histological localization of Vip3Aa in the midgut of intoxicated larvae showed that Vip3Aa bound to the brush border membrane along the entire apical surface. The presence of fluorescence in the cytoplasm of epithelial cells seems to suggest internalization of Vip3Aa or a fragment of it. Successful radiolabeling and optimization of the binding protocol for the ^{125}I-Vip3Aa to *S. frugiperda* brush border membrane vesicles (BBMV) allowed the determination of binding parameters of Vip3A proteins for the first time. Heterologous competition using Vip3Ad, Vip3Ae, and Vip3Af as competitor proteins showed that they share the same binding site with Vip3Aa. In contrast, when using Cry1Ab and Cry1Ac as competitors, no competitive binding was observed, which makes them appropriate candidates to be used in combination with Vip3A proteins in transgenic crops.**

Cry proteins produced by *Bacillus thuringiensis* are the active components of the most widely used biopesticides in biological control. They have been used in spray formulations for more than 60 years in forestry and agriculture. The importance of Cry proteins has increased dramatically following the introduction of *cry* genes into a number of major crops (known as Bt crops), mainly maize and cotton, to make them resistant to insect attack. Development of insect resistance to *B. thuringiensis* insecticidal proteins is an important concern for the long-term use of both spray products and Bt crops. In the last decade, high levels of insect resistance raised against Bt crops have been identified in several lepidopteran pests (1–5).

A different class of insecticidal proteins from *B. thuringiensis* are the Vip (vegetative insecticidal proteins) proteins, which have been referred to as second generation insecticidal proteins. These proteins were initially found to be secreted into the medium during the vegetative growth phase of this bacterium, and they were discovered much later than Cry proteins (6). Vip proteins share no sequence or structural homology with the Cry proteins, and those belonging to the Vip3A class are active against a wide range of lepidopteran insects (7, 8). One interesting feature presented by the Vip3A proteins is that they extend their activity to some agronomically important pests that have little or no susceptibility to *B. thuringiensis* Cry proteins, such as the black cutworm, *Agrotis ipsilon* (6, 8). Moreover, studies so far on the mode of action of Vip3A proteins have revealed differences from that of Cry proteins: in particular, they seem to bind to different binding sites from those targeted by the Cry proteins (9–12).

The above-mentioned characteristics of Vip3A proteins make them interesting candidates to complement Cry proteins in Bt crops to broaden the insecticidal spectrum and for resistance management purposes. For this reason, several agro-biotech companies, such as Dow Agrosciences, Bayer CropScience, and Syngenta, have shown an interest in introducing the *vip3A* genes in plants, to combine them with the already transferred *cry* genes (13, 14).

Vip3A proteins are molecules of around 88 to 90 kDa that, once ingested by lepidopteran larvae, are processed by the intestinal serine peptidases to a number of proteolytic fragments. Only the 62-kDa fragment of Vip3Aa has been shown to bind to brush border membrane vesicles (BBMV) from *Helicoverpa armigera* (11) and is considered to be the active form of the toxin (8, 9, 15, 16). After crossing the peritrophic membrane, the activated toxin specifically binds to the brush border membrane and forms pores (11, 15). All of these steps give rise to the paralysis and complete degeneration of the gut epithelium cells and eventually to the insect's death (17, 18, 19).

In the present work, we show the *in vivo* binding of Vip3Aa to the brush border membrane of the midgut epithelium cells of *Spodoptera frugiperda* using immunofluorescence. We also set up the conditions for the *in vitro* binding of the radiolabeled Vip3Aa to BBMV of this insect, testing different conditions of pH, sodium chloride, chelating agents, and concentrations of divalent cations. Radiolabeling of Vip3Aa has allowed us to estimate quantitative binding parameters of a Vip3A protein for the first time and to perform heterologous competition experiments among Vip3Aa and a number of Cry and Vip3A proteins to determine whether they share binding sites.

MATERIALS AND METHODS

Source of *B. thuringiensis* Vip3A and Cry1 proteins. Vip3Aa was prepared from recombinant *Escherichia coli* BL21 expressing the *vip3Aa16* gene from the *B. thuringiensis* subsp. *kurstaki* BUPM95 strain (20). The genes encoding the Vip3Ad (NCBI accession no. CAI43276), Vip3Ae (NCBI accession no. CAI43277), and Vip3Af (NCBI accession no. CAI43275) proteins were kindly supplied by Bayer CropScience N.V. (Ghent, Belgium); these proteins were expressed in pMac5-8 in *E. coli* (21). Cry1Ab was obtained from *B. thuringiensis* recombinant strain EG7077

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(Ecogen, Inc.) and Cry1Ac from recombinant *E. coli* strain XL-1 (kindly supplied by Ruud de Maagd).

Toxin preparation. Conditions for bacterial culture and expression of Vip3A proteins from recombinant *E. coli* strains were described before for Vip3Aa (16). For Vip3Ad, Vip3Ae, and Vip3Af, the protocol by Ruiz de Escudero (22) was followed. The Vip3Aa protein used to intoxicate the larvae was purified by using a HiTrap chelating high-performance (HP) column (GE Healthcare) equilibrated with Ni^{2+}. The lysate supernatant of the induced *E. coli* cells carrying the *vip3Aa* gene was loaded in the preequilibrated column and eluted with (50 mM phosphate buffer [pH 8.0], containing 0.3 M NaCl and 200 mM imidazole) elution buffer. Fractions (1 ml) were collected in tubes containing 5 mM EDTA, and the more concentrated ones were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 9)–0.3 M NaCl.

Since we found out that the Vip3Aa protein purified with the protocol described above was not suitable for binding assays, a different purification protocol was used to prepare Vip3A proteins for radiolabeling and competition assays. Expressed Vip3A proteins were partially purified and trypsin activated as follows. After cell lysis and centrifugation, the pH of the lysate supernatant was lowered to 5.5 with 0.1 M acetic acid. The pellet was recovered by centrifugation and dissolved in 20 mM Tris-HCl–150 mM NaCl (pH 7.4). Vip3A protoxins were incubated with 1% trypsin (wt/wt) for 2 h at 37°C. Activated toxins obtained at this point were used as competitors in the binding assays. Vip3Aa to be used for labeling was further purified by anion-exchange chromatography. After overnight dialysis against 20 mM Tris-HCl, (pH 9), Vip3Aa was purified on a HiTrap Q HP (5-ml bed volume) column equilibrated in the same dialysis buffer, using an ÄKTA explorer 100 chromatography system (GE Healthcare, United Kingdom). Proteins were eluted with a 100-ml linear gradient (0 to 80%) of 1 M NaCl.

Cry1Ac expression, solubilization from *E. coli* inclusion bodies, and trypsin treatment were performed as described before (23). Cry1Ab was solubilized from *B. thuringiensis* parasporal crystals, trypsin activated, and chromatography purified as described elsewhere (24).

Immunohistochemical localization of Vip3Aa toxin. Third or second instar larvae of *S. frugiperda* were starved overnight before being exposed to 1.2 mg/ml Vip3Aa protoxin (purified in a HiTrap chelating HP column) in a solution of Fluorella blue in 50% sucrose. After 1 h of exposure, larvae with blue-stained midgut were transferred to 4% paraformaldehyde in phosphate-buffered saline (PBS) (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl [pH 7.4]) and incubated for 2 days at 4°C with gentle shaking, and then for 2 additional days in 30% sucrose in PBS. Whole larvae were fixed to a support and coated with Tissue Tech gel (Sakura, Japan). The gel was allowed to solidify at −30°C. Sections of 10 μm were prepared using the cryostat microtome Leica CM 1510S. Slides with the tissue sections were stored at −20°C until used.

Tissue sections were washed three times with buffer A (PBS, 0.5% bovine serum albumin [BSA], 0.3% Triton) for 10 min and blocked with the same buffer supplemented with 5% fetal bovine serum (FBS) for 30 min in a humid chamber. Sections were then coated with the anti-Vip3Aa protoxin polyclonal antibody for 2 days at 4°C in a humid chamber. Unbound antibodies were washed off with three rinses using buffer A (10 min each), and slides were subsequently incubated with a mix of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (1:1,000 dilution) and phalloidin (1:1,000 dilution) in blocking buffer for an additional 2 h. After the slides were washed with buffer A, coverslips were mounted using Vectashield mounting medium with DAPI (4′,6-diamidino-2-phenylindole) from Vector Laboratories. The specimens were then examined using a Leica DMI2500 microscope equipped with a digital color camera (Leica DFC300 FX). Fluorescent images acquired in the red, blue, and green channels were merged using ImageJ software (25).

Radiolabeling of Vip3Aa. Iodination was performed twice using two different batches of Vip3Aa by the chloramine T method (24, 26). Trypsin-activated and anion-exchange-purified Vip3Aa protein (25 μg) was mixed with 0.5 mCi of ^{125}I and 1/3 (vol/vol) 18 mM chloramine T. The excess of free ^{125}I was separated from the labeled protein using a PD10 desalting column (GE HealthCare). The purity of the ^{125}I-labeled Vip3Aa was checked by analyzing the elution fractions by SDS-PAGE with further exposure of the dry gel to an X-ray film. The specific activity of the labeled protein was 1.1 mCi/mg in both cases. The first batch was used for the optimization of the binding parameters and the saturation experiment, and the second batch was used for the rest of experiments.

BBMV preparation. Last instar larvae of *S. frugiperda* were dissected, and the midguts were frozen in liquid nitrogen and preserved at −80°C until required. Brush border membrane vesicles (BBMV) were prepared by the differential magnesium precipitation method (27), frozen in liquid nitrogen, and stored at −80°C. The protein concentration in the BBMV preparations was determined by Bradford (28) using bovine serum albumin (BSA) as a standard.

Binding assays with ^{125}I-labeled Vip3Aa. For all binding assays, ^{125}I-Vip3Aa was incubated with BBMV at room temperature in a 0.1-ml final volume of PBS or Tris buffer containing 0.1% BSA. The reaction was stopped by centrifuging the tubes at 16,000 × *g* for 10 min at 4°C, and the pellet was washed once with 500 μl of cold buffer. The radioactivity retained in the pellet was measured in a model 2480 WIZARD^2 gamma counter. An excess of unlabeled Vip3Aa toxin (300-fold) was used to estimate the nonspecific binding.

To determine the appropriate amount of BBMV to use, a fixed amount of ^{125}I-Vip3Aa (1.2 nM) was mixed with increasing concentrations of BBMV in PBS with 0.1% BSA. For all subsequent experiments, 20 μg/ml of BBMV was chosen. For the time course experiment, BBMV were mixed with 1.2 nM ^{125}I-Vip3Aa and incubated for 15, 30, 60, 90, and 120 min in PBS–0.1% BSA. For saturation experiments (three replicates), BBMV were incubated for 90 min with increasing amounts of ^{125}I-Vip3Aa in PBS–0.1% BSA. To test the effect of pH, NaCl concentration, EDTA and the presence of divalent cations, two independent replicates were performed. To avoid precipitation of some divalent cations, Tris buffer was used instead of phosphate buffer.

The conditions chosen to perform the competition binding assays were 1.2 nM ^{125}I-Vip3Aa (second labeled batch), 20 μg/ml BBMV, a 90-min incubation time, and increasing concentrations of unlabeled competitor in a 0.1-ml final volume of Tris binding buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM $MnCl_2$, 0.1% BSA). Equilibrium dissociation constants (K_d) and the concentration of binding sites (R_t) were estimated using the LIGAND software (29).

To estimate the ratio of reversible and irreversible binding within the specific binding of Vip3Aa, the procedure described by Park et al. (30) was followed. The experiment consisted of three samples, each containing 1.2 nM ^{125}I-Vip3Aa and 2 μg of BBMV in Tris binding buffer, incubated at room temperature for 3 h. One sample was used to determine the total binding. Another sample, to which an excess of unlabeled Vip3Aa (300-fold) was added at the start of the assay, was used to determine the nonspecific binding. The third sample was used to estimate the ratio of reversible/irreversible binding by means of adding the excess of Vip3Aa 1.5 h after the start of the assay.

RESULTS

***In vivo* binding of Vip3Aa to *S. frugiperda* midgut.** Midgut sections from *S. frugiperda* larvae, either exposed or nonexposed to Vip3Aa protoxin, were observed under a fluorescence microscope to investigate the fate of Vip3Aa after ingestion (Fig. 1). In the midgut of Vip3Aa-fed larvae, an intense green color, which corresponded to the toxin, was observed along the entire midgut apical surface, which was still intact (Fig. 1B). Furthermore, some green fluorescence could be observed nonhomogeneously distributed in the cytoplasm of the epithelial cells. The insect cuticle also showed intense green fluorescence, most probably due to residual Vip3Aa that adhered to the larva surface during its exposure to the protoxin. Nuclei were stained in blue and the basement mem-

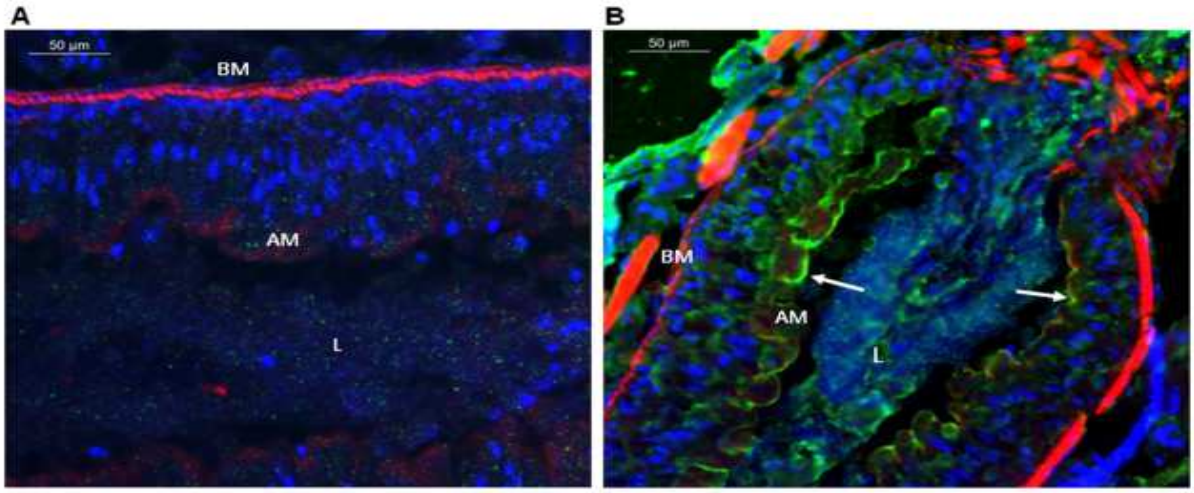

FIG 1 Immunolocalization of Vip3Aa in midgut tissue sections (10 μm) after *in vivo* ingestion by *S. frugiperda* larvae. Binding of Vip3Aa was revealed by Alexa Fluor-conjugated secondary antibody (green) using fluorescence microscopy. Nuclei were stained with DAPI (blue), and the apical and the basal membranes were stained with phalloidin (red). Magnification × 400. (A) Larvae not exposed to Vip3Aa; (B) larvae that ingested Vip3Aa. BM, basal membrane; AM, apical membrane; L, gut lumen. White arrows show the Vip3Aa protein bound to the midgut apical membrane.

brane and the apical membrane microvilli in red. In the control sections of the nonexposed larvae, the overall midgut epithelium structure was found to be intact and well organized. No green fluorescence was detected either inside the cells or in the apical membrane of the nonexposed larvae (Fig. 1A).

Purification of trypsin-activated Vip3Aa for radiolabeling and binding assays. Vip3Aa was purified by isoelectric point precipitation, followed by trypsin activation and then anion-exchange chromatography (Fig. 2A). To further purify the 62-kDa fragment, the chromatographic fraction containing the highest concentration of Vip3Aa was loaded into a gel filtration column (Superdex 75 10/300 GL; GE Healthcare). The analysis of the different chromatographic fractions showed that the 62-kDa peptide and the approximately 20-kDa peptide eluted together (Fig. 2B), indicating that they remain attached after trypsinization. Since we were unable to separate these two peptides even using 1 mM dithiothreitol (DTT) (31), the preparation after anion-exchange chromatography was used for radiolabeling and competition assays.

***In vitro* binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV.** Specific binding was firstly shown by incubating a fixed amount of ^{125}I-Vip3Aa (1.2 nM) with increasing concentrations of BBMV, in the presence or absence of excess of unlabeled Vip3Aa (Fig. 3). Around 10% of ^{125}I-Vip3Aa used in the assay bound to the BBMV, of which approximately 50% was specific.

A second experiment was done, which consisted of the separation by SDS-PAGE of the proteins in the pellet after incubation of ^{125}I-Vip3Aa with the BBMV and subsequent autoradiography (Fig. 4). Two radioactive peptides bound to the BBMV, one of 62 kDa (which corresponded to the molecular mass of the activated Vip3Aa toxin) and the second of approximately 20 kDa. Western blot analysis with a Vip3Aa-specific antibody showed that this peptide corresponded to a Vip3Aa proteolytic fragment (data not shown). As shown in Fig. 4, an excess of unlabeled Vip3Aa displaced the binding of both ^{125}I-Vip3Aa peptides, indicating that both the activated Vip3Aa and the 20-kDa fragment bind to the BBMV specifically.

To show that binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV was saturable, a fixed amount of BBMV from *S. frugiperda* was incubated with increasing concentrations of ^{125}I-Vip3Aa. As shown in Fig. 5, the plot of the specific binding versus input ^{125}I-Vip3Aa exhibited an asymptotic curve, in which saturation was observed at concentrations above 50 nM.

The analysis of the irreversible and reversible components of the Vip3Aa-specific binding showed that most part of this binding was irreversible (over 87% of the specific binding) (Fig. 6).

Optimization of binding conditions. A time course experiment was performed to determine the time required for the binding reaction to reach equilibrium. Binding increased rapidly over the first 30 min, reached equilibrium at about 90 min, and remained stable for at least 2 h.

To select the best conditions to obtain the highest total binding while maintaining the nonspecific binding to a minimum, the influence of the incubation time, pH, NaCl concentration, EDTA, and the presence of divalent cations was studied (Table 1). The effect of pH was analyzed in the range of 7.4 to 9.0. The results showed that binding was pH dependent, with both the total and the specific binding decreasing as the pH increased. The concentration of NaCl also influenced the amount of total binding and the proportion of specific binding, with the optimum between 100 and 150 mM. For subsequent optimization assays, a pH of 7.4 and a concentration of NaCl of 150 mM were chosen.

Results in our laboratory have shown that the presence of some divalent cations, such as Ni^{2+}, significantly reduced the toxicity of

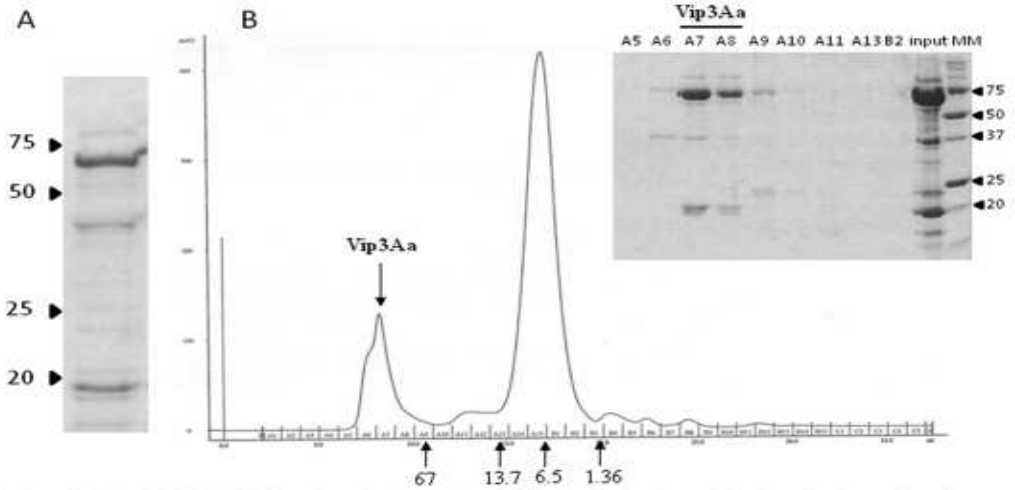

FIG 2 Purification of Vip3Aa. (A) SDS-PAGE of trypsin-activated Vip3Aa purified by isoelectric point precipitation and anion-exchange chromatography. (B) Gel filtration chromatography (Superdex 75 10/300 GL) of the anion-exchange-purified Vip3Aa. The inset shows the elution fractions as revealed by SDS-PAGE. The arrows below the chromatogram indicate the elution volume of molecular mass (MM) standards in kDa. The arrowheads by the electrophoresis gels indicate the molecular mass markers in kDa.

Vip3Ae and Vip3Af toward *S. frugiperda* (8). For this reason, the effect of the addition of the chelating agent EDTA to the binding reaction and the type and concentration of several divalent cations was tested (Table 1). The results showed that EDTA reduced both the total binding and the proportion of specific binding. The presence of some divalent cations, such as Mn^{2+} and Zn^{2+}, drastically increased the total binding. However, the proportion of specific binding was strongly influenced by the concentration of the divalent cation, as could be observed with Mn^{2+} and Cu^{2+}. Based on these results, 1 mM $MnCl_2$ was included in the binding reaction in further assays with *S. frugiperda* BBMV.

Competition binding assays. Homologous competition was performed with a fixed amount of ^{125}I-Vip3Aa and increasing concentrations of unlabeled Vip3Aa (Fig. 7). The analysis of the data indicated that the curve fitted one binding-site model of high affinity (equilibrium dissociation constant [K_d] of 15 ± 2 nM) and a relatively high concentration of binding sites (R_t = 54 ± 7 pmol/mg BBMV protein). It is worth mentioning that the activated VipAa that had been purified by a Ni^{2+}-loaded chelating column did not compete with ^{125}I-Vip3Aa (Fig. 7).

To find out whether Vip3Aa shares binding sites with other *B. thuringiensis* toxins, binding assays with ^{125}I-Vip3Aa and increasing concentrations of unlabeled competitors were performed (heterologous competition). No competition was observed when Cry1Ac or Cry1Ab was used as a competitor (Fig. 7). However, Vip3Ad, Vip3Ae, and Vip3Af completely displaced the specific

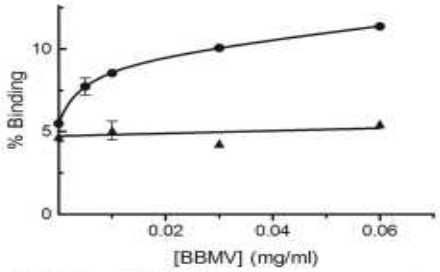

FIG 3 Specific binding of ^{125}I-Vip3Aa at increasing concentrations of *S. frugiperda* BBMV. Data points are the mean of two replicates. The nonspecific binding was calculated using an excess of unlabeled Vip3Aa toxin. ●, total binding; ▲, nonspecific binding.

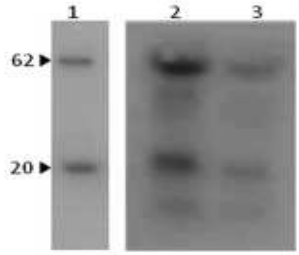

FIG 4 Autoradiography of ^{125}I-Vip3Aa bound to BBMV from *S. frugiperda*. ^{125}I-Vip3Aa was incubated with BBMV in the absence (lane 2) or presence (lane 3) of a 300-fold excess of unlabeled Vip3Aa. The pellet obtained after centrifugation of the reaction mixture was subjected to SDS-PAGE and exposed to X-ray film for a week. Lane 1, labeled toxin used in the assay (input). Arrowheads indicate the approximate molecular masses of the labeled peptides in kDa.

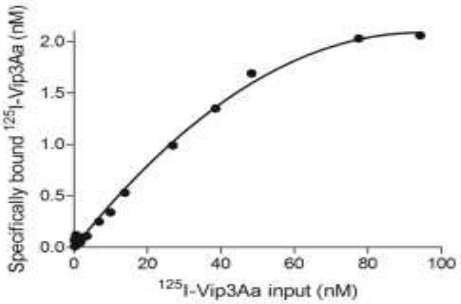

FIG 5 Saturation of ^{125}I-Vip3Aa binding to *S. frugiperda* BBMV. A fixed amount of BBMV (2 μg) was incubated with increasing amounts of ^{125}I-Vip3Aa for 90 min. The reaction was stopped by centrifugation, and the radioactivity retained in the pellet was measured. Specific binding was determined by subtracting nonspecific binding from the total binding.

binding of ^{125}I-Vip3Aa (Fig. 8). These results indicate that the four Vip3A proteins share binding sites in *S. frugiperda* and that Cry1Ab and Cry1Ac do not bind to these sites. The estimation of K_d and R_t for the competing proteins from the heterologous data indicated that binding parameters obtained for all Vip3A proteins were comparable (Table 2).

DISCUSSION

Vip3 proteins are considered a new generation of insecticidal proteins from *B. thuringiensis* as they do not share any type of sequence homology with the Cry toxins (6, 15). Vip3Aa has been recently introduced in important crops not only in an effort to widen their protection against lepidopteran pests but also as part of a strategy of resistance management in a response to the emerging resistance to the existing Cry toxins (3, 5). In addition to having different activity spectra from Cry1A and Cry2A proteins, Vip3A proteins have been shown to target distinct receptors in the insect midgut (9, 15, 32). Their discovery in 1996 was the starting point for the search for new *vip3* genes, with more than 80 *vip3A* genes identified to date (33). Different Vip3A proteins have been shown to differ in their insecticidal spectra (8, 22). Because of the increasing interest in this new class of insecticidal proteins, it becomes important to study their mode of action in more detail.

TABLE 1 Influence of pH, NaCl concentration, chelating agents, and the presence or absence of divalent cations on the binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV[a]

Buffer[b]	Binding condition				^{125}I-Vip3Aa binding (% of input)		Ratio of specific to total binding
	pH	NaCl concn (mM)	Divalent cation	EDTA concn (mM)	Specific	Total	
A	9	150			0	6.3	0
	8.2				2.5	7.1	0.35
B	7.4	150			4.1	8.5	0.48
		0			1.9	5.8	0.33
		50			3.5	7.5	0.47
		100			3.8	8.5	0.45
		300			2.7	8.1	0.33
		500			1.8	7.5	0.24
		150		5	2.8	7.2	0.39
A	7.4	150	$MnCl_2$ (10 mM)		0	21.8	0
			$MnCl_2$ (1 mM)		5.5	13.1	0.42
			$CuSO_4$ (1 mM)		0	8.2	0
			$CuSO_4$ (0.1 mM)		2.5	8.2	0.30
			$ZnCl_2$ (1 mM)		4.2	10.8	0.39
			$MgCl_2$ (10 mM)		1.2	6.9	0.17
			$CaCl_2$ (1 mM)		2.4	7.0	0.34

[a] Values are the means of at least two replicates.
[b] Buffer A is 20 mM Tris–0.1% BSA, and buffer B is 10 mM Na_2HPO_4/NaH_2PO_4-0.1% BSA.

As a first approach to the study of the binding of the Vip3Aa to the midgut of *S. frugiperda*, immunohistochemical analysis of Vip3Aa showed that binding took place in the brush border membrane of the midgut epithelial cells (Fig. 1), as had been described previously in *Agrotis ipsilon* and *Ostrinia nubilalis* larvae (17). Unlike the Cry toxins, there was no visible binding of the Vip3Aa toxin to the basal membrane of the midgut epithelial cells or to the peritrophic membrane (34, 35, 36). In addition, some green fluorescence could be observed inside the epithelial cells, which could suggest internalization of Vip3Aa or a fragment of it. Results from a previous study with Sf21 insect cells also suggested that Vip3Aa internalized after binding to the cell membrane (37). Whether

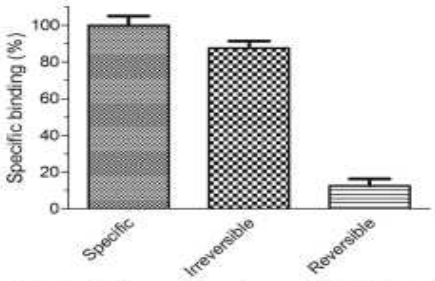

FIG 6 Contribution of the reversible and irreversible binding to the specific binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV.

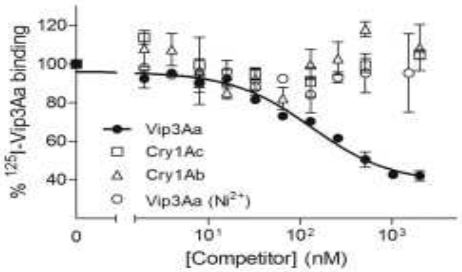

FIG 7 Binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV at increasing concentrations of unlabeled competitor. Each data point represents the mean of two independent replicates.

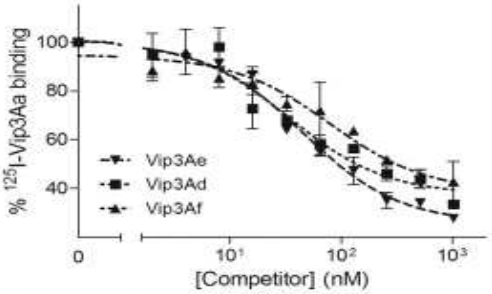

FIG 8 Heterologous competition of Vip3A proteins with ^{125}I-Vip3Aa and *S. frugiperda* BBMV. Each data point represents the mean of two independent replicates.

internalization of Vip3Aa is actually a step in the mode of action deserves further study.

So far, all studies on the binding of Vip3A proteins to the insect midgut have been done with biotinylated Vip3A proteins, and thus, quantitative parameters of Vip3A binding were lacking. In the present work, successful radiolabeling of a Vip3 protein and its use to show saturable and specific binding to BBMV are described for the first time. Specific binding of ^{125}I-Vip3Aa to *S. frugiperda* BBMV was shown by incubating a fixed amount of ^{125}I-Vip3Aa with increasing concentrations of BBMV (Fig. 3) and confirmed by autoradiography of the bound protein after separation from BBMV by SDS-PAGE (Fig. 4). In both cases, around half of the total binding of the iodinated toxin was inhibited by the presence of an excess of unlabeled Vip3Aa. The 20-kDa fragment present in the sample of Vip3Aa used in radiolabeling also showed competition by an excess of unlabeled Vip3Aa. This is due to the fact that this fragment remains tightly linked to the 62-kDa fragment (Fig. 2B). Saturation of Vip3Aa binding sites was shown by incubating a fixed amount of BBMV and increasing concentrations of the radiolabeled protein (Fig. 5). Despite the fact that the affinity-purified Vip3Aa protoxin, after trypsin activation, showed strong toxicity against *S. frugiperda* (16), this toxin preparation was found to be inappropriate for binding assays: no specific binding could be obtained with the radiolabeled toxin (data not shown), and the unlabeled protein was unable to compete with radiolabeled Vip3Aa (anion-exchange purified) (Fig. 7).

For Cry1 toxins, a direct correlation between irreversible binding, pore formation, and toxicity has been described in various cases (38, 39). Vip3A proteins have been shown to form pores in different susceptible insects, such as *M. sexta* and *H. armigera* (11, 15), which indirectly indicates that binding of Vip3A to the BBMV from these insects is, at least in part, irreversible. Our study provides the first direct evidence of the irreversible binding of Vip3Aa to *S. frugiperda* BBMV (Fig. 6).

Since this was the first time that radiolabeled Vip3Aa was used for binding assays, it was necessary to first select the conditions under which the binding to the *S. frugiperda* BBMV was optimum. As in one of the first studies with radiolabeled Cry proteins (26), the influence of pH, NaCl concentration, and incubation time was tested. Furthermore, the effect of the presence of EDTA or the type and concentration of divalent cations was investigated. Since the pH of the midgut of lepidopterans is known to be alkaline, the effect of pH was tested in the range from 7.4 to 9. The binding was shown to be dependent on the pH: the higher values of specific binding were obtained at the lowest pH. The NaCl concentration also had an influence on the specific binding of ^{125}I-Vip3Aa, most likely by stabilizing the Vip protein.

Hernández-Martínez et al. (8) showed that the purification of two different Vip3A proteins using the metal chelation columns exerted negative effect on their toxicity, and thus, EDTA was used to stabilize the toxin. However, the addition of the chelating agent EDTA in the binding reaction mixture of the ^{125}I-Vip3Aa decreased both the total and the specific binding to the BBMV (Table 1), which indicated that the *in vitro* binding is sensitive to the presence of divalent cations. The concentration and type of the cations affected both the total and the specific binding of the ^{125}I-Vip3Aa. Mn^{2+} at 10 mM yielded the highest total binding, although all this binding was nonspecific. However, at 1 mM Mn^{2+}, despite that total binding decreased comparatively, a substantial amount of specific binding was obtained. Addition of Cu^{2+} (0.1 or 1 mM), Mg^{2+} (10 mM), or Ca^{2+} (1 mM) had relatively small effects on the total binding; however, in all cases specific binding was decreased compared with when these ions were absent. These results are in contrast with the binding of Cry1Ab to *Manduca sexta* BBMV, which was not affected by the presence of either 5 mM EGTA or 10 mM Mg^{2+} or Ca^{2+} (26). It is possible that some metal ions are required by some brush border membrane proteins involved in the Vip3Aa binding.

The displacement of the ^{125}I-Vip3Aa protein observed in the homologous competition experiment confirms the occurrence of a limited number of receptors for Vip3Aa that could be saturated, adding an excess of unlabeled toxin. The heterologous competition by Vip3Ad, Vip3Ae, and Vip3Af indicates that these three proteins also bind to the same sites as Vip3Aa. However, whether Vip3Aa competes for all of the binding sites recognized by Vip3Ad, Vip3Ae, or Vip3Af (i.e., the reciprocal competition experiments) has not been tested here. Competition of Vip3Aa with biotinylated Vip3Af had been shown previously in *S. frugiperda* (10). The proteins Vip3Aa, Vip3Ae, and Vip3Af are known to be toxic to *S. frugiperda* (8, 10, 16); however, Vip3Ad is nontoxic (8). This result indicates, as occurs with Cry proteins, that binding of Vip proteins is necessary, though not sufficient, for toxicity.

The analysis of the binding parameters from the homologous and the heterologous competitions rendered K_d and R_t values similar for all four Vip3A proteins, with K_d values in the range of 6.1 to 22 nM and R_t values in the range of 48 to 76 pmol/mg of BBMV protein. These values are higher (around 10-fold) than the ones normally obtained for the Cry1A and Cry2A proteins (12, 24, 40–42), which indicates that Vip3A proteins have lower affinity

TABLE 2 K_d and R_t of Vip3A proteins with BBMV from *S. frugiperda*[a]

Protein	Mean ± SEM	
	K_d (nM)	R_t (pmol/mg)
Vip3Aa	15 ± 2	54 ± 7
Vip3Ad	17 ± 3	76 ± 17
Vip3Ae	6.1 ± 1.1	48 ± 8
Vip3Af	22 ± 5	72 ± 14

[a] Equilibrium constants for Vip3Aa were estimated from homologous competition (three independent replicates), and those for Vip3Ad, Vip3Ae, and Vip3Af were estimated from heterologous competition (two independent replicates).

but a higher number of binding sites in the BBMV than the Cry1A and Cry2A proteins. Lee et al. (15) showed that the kinetics of pore formation of activated Vip3A was more than 8-fold slower than that of Cry1Ab (at equimolar concentrations) and that the kinetics did not change after a 10-fold increase in the Vip3A concentration. Lee et al. claimed that this could be due to the fact that saturation of functional binding sites of the Vip3A proteins was hard to reach.

When Cry1Ab and Cry1Ac were used as heterologous competitors, no displacement of ^{125}I-Vip3Aa occurred. This result, along with the competition of the Vip3A proteins for the same binding site found here and the results obtained in a previous study (10), strongly suggests that Vip3A proteins do not share binding sites with Cry1A proteins in *S. frugiperda*. Lack of competition between Cry1A and Cry2A proteins and Vip3Aa had already been reported in three heliothine species (9, 11, 12). The overall results suggest that these two classes of toxins (Vip3A and Cry1A/2A) use different receptors to bind to the brush border membrane of target insects.

In conclusion, the successful radiolabeling of Vip3Aa in this work opens up interesting perspectives for the future of binding studies with Vip3A proteins. Using radiolabeled Vip3Aa allowed us to estimate for the first time binding parameters for this protein. Furthermore, heterologous competition has revealed that Vip3Ad, Vip3Ae, and Vip3Af competed for the Vip3Aa binding sites. The absence of competition of Cry1Ac and Cry1Ab makes them appropriate candidates to be used in combination with Vip3A proteins in transgenic crops as a strategy to delay the evolution of resistance in insects.

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REFERENCES

1. **Tabashnik BE, Sisterson MS, Ellsworth PC, Dennehy TJ, Antilla L, Liesner L, Whitlow M, Staten RT, Fabrick JA, Unnithan GC, Yelich AJ, Ellers-Kirk C, Harpold VS, Li X, Carrière Y.** 2010. Suppressing resistance to *Bt* cotton with sterile insect releases. Nat. Biotechnol. **28:**1304–1307. http://dx.doi.org/10.1038/nbt.1704.
2. **Dhurua S, Gujar GT.** 2011. Field-evolved resistance to *Bt* toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), from India. Pest Manag. Sci. **67:**898–903. http://dx.doi.org/10.1002/ps.2127.
3. **Downes S, Mahon R.** 2012. Evolution, ecology and management of resistance in *Helicoverpa* spp. to *Bt* cotton in Australia. J. Invertebr. Pathol. **110:**281–286. http://dx.doi.org/10.1016/j.jip.2012.04.005.
4. **Storer NP, Kubiszak ME, King JE, Thompson GD, Santos AC.** 2012. Status of resistance to Bt maize in *Spodoptera frugiperda*: lessons from Puerto Rico. J. Invertebr. Pathol. **110:**294–300. http://dx.doi.org/10.1016/j.jip.2012.04.007.
5. **Tabashnik BE, Wu K, Wu Y.** 2012. Early detection of field-evolved resistance to *Bt* cotton in china: cotton bollworm and pink bollworm. J. Invertebr. Pathol. **110:**301–306. http://dx.doi.org/10.1016/j.jip.2012.04.008.
6. **Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG.** 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. U. S. A. **93:**5389–5394. http://dx.doi.org/10.1073/pnas.93.11.5389.
7. **Milne R, Liu Y, Gauthier D, van Frankenhuyzen K.** 2008. Purification of Vip3Aa from *Bacillus thuringiensis* HD-1 and its contribution to toxicity of HD-1 to spruce budworm (*Choristoneura fumiferana*) and gypsy moth (*Lymantria dispar*) (Lepidoptera). J. Invertebr. Pathol. **99:**166–172. http://dx.doi.org/10.1016/j.jip.2008.05.002.
8. **Hernández-Martínez P, Hernández-Rodriguez CS, Van Rie J, Escriche B, Ferré J.** 2013. Insecticidal activity of Vip3Aa, Vip3Ad, Vip3Ae, and Vip3Af from *Bacillus thuringiensis* against lepidopteran corn pests. J. Invertebr. Pathol. **113:**78–81. http://dx.doi.org/10.1016/j.jip.2013.02.001.
9. **Lee MK, Miles P, Chen JS.** 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem. Biophys. Res. Commun. **339:**1043–1047. http://dx.doi.org/10.1016/j.bbrc.2005.11.112.
10. **Sena JA, Hernández-Rodríguez CS, Ferré J.** 2009. Interaction of *Bacillus thuringiensis* Cry1 and Vip3Aa proteins with *Spodoptera frugiperda* midgut binding sites. Appl. Environ. Microbiol. **75:**2236–2237. http://dx.doi.org/10.1128/AEM.02342-08.
11. **Liu J, Yang A, Shen X, Hua B, Shi G.** 2011. Specific binding of activated Vip3Aa10 to *Helicoverpa armigera* brush border membrane vesicles results in pore formation. J. Invertebr. Pathol. **108:**92–97. http://dx.doi.org/10.1016/j.jip.2011.07.007.
12. **Gouffon C, Van Vliet A, Van Rie J, Jansens S, Jurat-Fuentes JL.** 2011. Binding sites for *Bacillus thuringiensis* Cry2Ae toxin on heliothine brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin. Appl. Environ. Microbiol. **77:**3182–3188. http://dx.doi.org/10.1128/AEM.02791-10.
13. **Anonymous.** 2010. Syngenta, Dow AgroSciences sign cotton technology licensing agreements. http://www.syngenta.com/global/corporate/en/news-center/news-releases/Pages/en-100105.aspx.
14. **Anonymous.** 2010. Syngenta and Bayer CropScience sign global cotton technology licensing agreement. http://www.syngenta.com/global/corporate/en/news-center/news-releases/Pages/en-100714.aspx.
15. **Lee MK, Walters FS, Hart H, Palekar N, Chen JS.** 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa differs from that of Cry1Ab delta-endotoxin. Appl. Environ. Microbiol. **69:**4648–4657. http://dx.doi.org/10.1128/AEM.69.8.4648-4657.2003.
16. **Chakroun M, Bel Y, Caccia S, Abdelkefi-Mesrati L, Escriche B, Ferré J.** 2012. Susceptibility of *Spodoptera frugiperda* and *S. exigua* to *Bacillus thuringiensis* Vip3Aa insecticidal protein. J. Invertebr. Pathol. **110:**334–339. http://dx.doi.org/10.1016/j.jip.2012.03.021.
17. **Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ.** 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3Aa lyses midgut epithelium cells of susceptible insects. Appl. Environ. Microbiol. **63:**532–536.
18. **Abdelkefi-Mesrati L, Boukedi H, Dammak-Karray M, Sellami-Boudawara T, Jaoua S, Tounsi S.** 2011. Study of the *Bacillus thuringiensis* Vip3Aa16 histopathological effects and determination of its putative binding proteins in the midgut of *Spodoptera littoralis*. J. Invertebr. Pathol. **106:**250–254. http://dx.doi.org/10.1016/j.jip.2010.10.002.
19. **Abdelkefi-Mesrati L, Boukedi H, Chakroun M, Kamoun F, Azzouz H, Tounsi S, Rouis S, Jaoua S.** 2011. Investigation of the steps involved in the difference of susceptibility of *Ephestia kuehniella* and *Spodoptera littoralis* to the *Bacillus thuringiensis* Vip3Aa16 toxin. J. Invertebr. Pathol. **107:**198–201. http://dx.doi.org/10.1016/j.jip.2011.05.014.
20. **Mesrati AL, Tounsi S, Jaoua S.** 2005. Characterization of a novel *vip3*-type gene from *Bacillus thuringiensis* and evidence of its presence on a large plasmid. FEMS Microbiol. Lett. **244:**353–358. http://dx.doi.org/10.1016/j.femsle.2005.02.007.
21. **Stanssens P, Opsomer C, McKeown YM, Kramer W, Zabeau M, Fritz HJ.** 1989. Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers. Nucleic Acids Res. **17:**4441–4454. http://dx.doi.org/10.1093/nar/17.12.4441.
22. **Ruiz de Escudero I, Banyuls N, Bel Y, Maeztu M, Escriche B, Muñoz D, Caballero P, Ferré J.** 2014. A screening of five *Bacillus thuringiensis* Vip3A proteins for their activity against lepidopteran pests. J. Invertebr. Pathol. **117:**51–55. http://dx.doi.org/10.1016/j.jip.2014.01.006.
23. **Bosch D, Schipper B, van der Kleij H, de Maagd RA, Stiekema WJ.** 1994. Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. Nat. Biotechnol. **12:**915–918. http://dx.doi.org/10.1038/nbt0994-915.
24. **Hernández-Rodríguez CS, Van Vliet A, Bautsoens N, Van Rie J, Ferré J.** 2008. Specific binding of *Bacillus thuringiensis* Cry2A insecticidal pro-

teins to a common site in the midgut of *Helicoverpa* species. Appl. Environ. Microbiol. 74:7654–7659. http://dx.doi.org/10.1128/AEM.01373-08.
25. **Schneider CA, Rasband WS, Eliceiri KW.** 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9:671–675. http://dx.doi.org/10.1038/nmeth.2089.
26. **Van Rie J, Jansens S, Höfte H, Degheele D, Van Mellaert H.** 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur. J. Biochem. 186:239–247.
27. **Wolfersberger MG, Luthy P, Maurer P, Parenti P, Sacchi VF, Giordana B, Hanozet GM.** 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly (*Pieris brassicae*). Comp. Biochem. Physiol. 86A:301–308.
28. **Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.
29. **Munson P, Rodbard D.** 1980. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107:220–239. http://dx.doi.org/10.1016/0003-2697(80)90515-1.
30. **Park Y, González-Martínez RM, Navarro-Cerrillo G, Chakroun M, Kim Y, Ziarsolo P, Blanca J, Cañizares J, Ferré J, Herrero S.** 2014. ABCC transporters as receptors for *Bacillus thuringiensis* toxins revealed by bulk segregant analysis of a Bt-resistant *Spodoptera exigua* colony. BMC Biol. 12:46. http://dx.doi.org/10.1186/1741-7007-12-46.
31. **Zhao JZ, Collins HL, Tang JD, Cao J, Earle ED, Roush RT, Herrero S, Escriche B, Ferré J, Shelton AM.** 2000. Development and characterization of diamondback moth resistance to transgenic broccoli expressing high levels of Cry1C. Appl. Environ. Microbiol. 66:3784–3789. http://dx.doi.org/10.1128/AEM.66.9.3784-3789.2000.
32. **Abdelkefi-Mesrati L, Rouis S, Sellami S, Jaoua S.** 2009. *Prays oleae* midgut putative receptor of *Bacillus thuringiensis* vegetative insecticidal protein Vip3LB differs from that of Cry1Ac toxin. Mol. Biotechnol. 43:15–19. http://dx.doi.org/10.1007/s12033-009-9178-4.
33. **Crickmore N, Baum J, Bravo A, Lereclus D, Narva K, Sampson K, Schnepf E, Sun M, Zeigler DR.** 2013. *Bacillus thuringiensis* toxin nomenclature. http://www.btnomenclature.info/.
34. **Bravo A, Jansens S, Peferoen M.** 1992. Immunocytochemical localization of *Bacillus thuringiensis* isinsecticidal crystal proteins in intoxicated insects. J. Invertebr. Pathol. 60:237–246. http://dx.doi.org/10.1016/0022-2011(92)90004-N.
35. **Rodrigo-Simón A, de Maagd RA, Avilla C, Bakker PL, Molthoff J, González-Zamora JE, Ferré J.** 2006. Lack of detrimental effects of *Bacillus thuringiensis* Cry toxins on the insect predator *Chrysoperla carnea*: a toxicological, histopathological, and biochemical analysis. Appl. Environ. Microbiol. 72:1595–1603. http://dx.doi.org/10.1128/AEM.72.2.1595-1603.2006.
36. **Rouis S, Chakroun M, Jaoua S.** 2008. Comparative study of *Bacillus thuringiensis* Cry1Aa and Cry1Ac delta-endotoxin activation, inactivation and in situ histopathological effect in *Ephestia kuehniella* (Lepidoptera: Pyralidae). Mol. Biotechnol. 38:233–239. http://dx.doi.org/10.1007/s12033-007-9021-8.
37. **Singh G, Sachdev B, Sharma N, Seth R, Bhatnagar RK.** 2010. Interaction of *Bacillus thuringiensis* vegetative insecticidal protein with ribosomal S2 protein triggers larvicidal activity in *Spodoptera frugiperda*. Appl. Environ. Microbiol. 76:7202–7209. http://dx.doi.org/10.1128/AEM.01552-10.
38. **Ihara H, Kuroda E, Wadano A, Himeno M.** 1993. Specific toxicity of δ-endotoxin from *Bacillus thuringiensis* to *Bombyx mori*. Biosci. Biotechnol. Biochem. 57:200–204. http://dx.doi.org/10.1271/bbb.57.200.
39. **Liang Y, Patel SS, Dean DH.** 1995. Irreversible binding kinetics of *Bacillus thuringiensis* CryIA δ-endotoxins to gypsy moth brush border membrane vesicles is directly correlated to toxicity. J. Biol. Chem. 270:24719–24724. http://dx.doi.org/10.1074/jbc.270.42.24719.
40. **Garczynski SF, Crim JW, Adang MJ.** 1991. Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* delta-endotoxin by protein blot analysis. Appl. Environ. Microbiol. 57:2816–2820.
41. **Rang C, Bergvingson D, Bohorova N, Hoisington D, Frutos R.** 2004. Competition of *Bacillus thuringiensis* Cry1 toxins for midgut binding sites: a basis for the development and management of transgenic tropical maize resistant to several stemborers. Curr. Microbiol. 49:22–27. http://dx.doi.org/10.1007/s00284-003-4258-3.
42. **Caccia S, Hernández-Rodríguez CS, Mahon RJ, Downes S, James W, Bautsoens N, Van Rie J, Ferré J.** 2010. Binding site alteration is responsible for field-isolated resistance to *Bacillus thuringiensis* Cry2A insecticidal proteins in two *Helicoverpa* species. PLoS One 5:e9975. http://dx.doi.org/10.1371/journal.pone.0009975.

Fourth paper

Bacterial Vegetative Insecticidal Proteins (Vip) from entomopathogenic bacteria: A Review.

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Bacterial Vegetative Insecticidal Proteins (Vip) from entomopathogenic bacteria: A Review.

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ABSTRACT

Entomopathogenic bacteria produce, in addition to insecticidal proteins that accumulate in inclusion bodies or parasporal crystals (proteins Cry and Cyt), insecticidal proteins that are secreted to the culture media. Among the latter, the most important ones are the Vip proteins, which are divided into four families according to their amino acid identity. The Vip1 and Vip2 proteins act as a binary toxin and are toxic to some Coleoptera and Homoptera. The Vip1 component is thought to bind to receptors in the membrane of the insect midgut and the Vip2 component enters the cell where it displays its ADP-ribosyltransferase activity against actin, preventing microfilament formation. Vip3 has no sequence similarity to Vip1 or Vip2 and is toxic to a wide variety of Lepidoptera. Its mode of action has been shown to be similar to that of Cry proteins in terms of proteolytic activation, binding to the midgut epithelial membrane and pore formation, though Vip3A proteins do not share binding sites with Cry proteins. This last property makes them good candidates to be combined with Cry proteins in transgenic plants (Bt-crops) to prevent or delay insect resistance, in addition to broaden the insecticidal spectrum. There are already commercially grown varieties of Bt-cotton and Bt-maize that express the Vip3Aa protein in combination with Cry proteins. For the most recently reported Vip4 family, no target insects have been found as yet.

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