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An α-amylase is a novel receptor for *Bacillus thuringiensis* ssp. *israelensis* Cry4Ba and Cry11Aa toxins in the malaria vector mosquito *Anopheles albimanus* (*Diptera*: *Culicidae*)

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Summary

Bacillus thuringiensis ssp. *israelensis* (*Bti*) produces four Cry toxins (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa), and two Cyt proteins (Cyt1Aa and Cyt2Ba), toxic to mosquito-larvae of the genus *Aedes, Anopheles* and *Culex*, important human disease vectors that transmit dengue virus, malaria and filarial parasites respectively. Previous work showed that *Bti* is highly toxic to *Anopheles albimanus*, the main vector for transmission of malaria in Mexico. In this work, we analysed the toxicity of isolated Cry proteins of *Bti* and identified an *An. albimanus* midgut protein as a putative Cry4Ba and Cry11Aa receptor molecule. Biossays showed that Cry4Ba and Cry11Aa of *Bti* are toxic to *An. albimanus* larvae. Ligand blot assays indicated that a 70 kDa glycosylphosphatidylinositolanchored protein present in midgut brush border membrane vesicles of *An. albimanus* interacts with Cry4Ba and Cry11Aa toxins. This protein was identified as an α-amylase by mass spectrometry and enzymatic activity assays. The cDNA that codes for the α-amylase was cloned by means of 5′- and 3′-RACE experiments. Recombinant α-amylase expressed in *Escherichia coli* specifically binds Cry4Ba and Cry11Aa toxins.

Introduction

Due to their ability to spread diseases like malaria, yellow fever, dengue and lymphatic filariasis among others, mosquitoes are deadly disease vectors, causing millions of deaths, and represent one of the biggest public-health threats. It is estimated that mosquitoes transmit disease to 700 000 000 people each year (WHO, 1996). For instance, malaria causes up to three million deaths and five billion clinical cases each year, with 90% of them occurring in Africa (Breman *et al*., 2004).

One way to stop the spread of mosquito-borne diseases is to control vector populations.Acommon control strategy has been the use of chemical compounds that not only harm the environment but lead to the appearance of resistant mosquitoes. One

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environmentally friendly alternative method to control these insects is the use of Cry toxins from *Bacillus thuringiensis* (*Bt*) (Bravo *et al.*, 2007).

Toxin crystals from the mosquitocidal strain *Bacillus thuringiensis* ssp. *israelensis* (*Bti*) are composed of four major insecticidal Cry proteins (Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa) and two Cyt proteins (Cyt1Aa and Cyt2Ba), whose genes are found on a large plasmid (Berry *et al.*, 2002). Although each toxin, by itself, displays low toxicity, their combined toxic effect is synergistic towards mosquito larvae, as shown by the interaction between Cry11Aa and Cyt1Aa proteins (Crickmore *et al.*, 1995; Perez *et al.*, 2005).

An emerging model has been drawn to explain the toxicity of Cry proteins to lepidopteran insects (Bravo *et al.*, 2004; Bravo *et al.*, 2007). After crystal solubilization the protoxins released are activated by proteases found in the larval midgut, followed by the binding of toxins to the primary receptor cadherin (Vadlamudi *et al.*, 1995). Binding to cadherin induces the cleavage of helix α-1 located at the N-terminal end of the toxin, facilitating its oligomerization (Gomez *et al.*, 2002). Then, the toxin oligomer binds to a secondary receptor such as the enzymes aminopeptidase N (APN) (Knight *et al.*, 1994; Bravo *et al.*, 2004; Gomez *et al.*, 2006), and alkaline phosphatase (ALP) (Jurat-Fuentes and Adang, 2004), which are membrane anchored by a glycosylphosphatidylinositol (GPI) group. Finally, the oligomeric toxin forms a pore in the membrane that leads to cell death (Rausell *et al.*, 2004; Pardo-Lopez *et al.*, 2006).

It has been reported that Cry toxins active against dipteran larvae bind similar proteins to the receptors identified in lepidopteran insects. The dipteran specific Cry11Ba toxin binds to an APN from *Anopheles quadrimaculatus* (Abdullah *et al.*, 2006), Cry11Aa and Cry4Ba interact with an ALP from *Aedes aegypti* (Fernandez *et al.*, 2006; Bayyareddy *et al.*, 2009; Fernandez *et al.*, 2009), and Cry4Ba and Cry11Aa are recognized by a cadherin-like protein from *Anopheles gambiae* and *Ae. aegypti* respectively (Hua *et al.*, 2008; Chen *et al.*, 2009).

In this work, we describe the identification and cloning of an α-amylase from the midgut of *An. albimanus*, the principal vector of malaria in Mexico. This α-amylase specifically binds Cry4Ba and Cry11Aa toxins. Ligand blot, 2D PAGE, MS identification, affinity chromatography and enzymatic activity assays were used to characterize this α-amylase as a putative *Bt* toxin receptor in mosquito larvae.

Results and discussion

Anopheles albimanus larvae sensitivity to *Bti* mosquitocidal toxins

As a first step to identify protein molecules that bind Cry toxins present in the midgut of *An. albimanus* fourth instar larvae, we performed bioassays to determine the toxicity of the parasporal crystals composed of individual Cry4Ba, Cry4Aa, Cry11Aa and Cyt1Aa toxins from *Bti*. As shown in Table 1, the parasporal crystals of *Bti* exhibited a high toxicity. In the case of the individual toxins, the most toxic protein was Cry4Ba, followed by Cry11Aa. Cry4Aa and Cyt1Aa showed no toxicity at the highest concentration of toxin tested.

Although it has been previously reported that *An. albimanus* is sensitive to the parasporal crystal of *Bti* (Ibarra *et al.*, 2003), this is the first time that the toxicity displayed by individual components of the *Bti* crystal has been assessed. We found that individual toxins of *Bti* are less toxic than their combination, probably due to the synergism observed between the different Cry and Cyt proteins, as observed originally for Cry11Aa and Cyt1Aa proteins in *Ae. aegypti* (Crickmore *et al.*, 1995; Perez *et al.*, 2005).

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The toxicity values of different Cry toxins reported here for *An. albimanus* are similar to those found for *An. stephensi*: Cry4Ba and Cry11Aa proteins were highly toxic to this insect (Delecluse *et al.*, 1993; Poncet *et al.*, 1995; Revina *et al.*, 2004), while Cry4Aa and Cyt1Aa showed low toxicity towards this mosquito larvae (Delecluse *et al.*, 1993; Poncet *et al.*, 1995; Thiery *et al.*, 1997).

Cry4Ba and Cry11Aa toxins bind to a 70 kDa protein in *An. albimanus* brush border membrane vesicle proteins

It has been reported that some receptors for Cry toxins are GPI-anchored membrane proteins in *An. quadrimaculatus* and *Ae. aegypti* larvae (Abdullah *et al.*, 2006; Fernandez *et al.*, 2006). We performed ligand blot binding assays of brush border membrane vesicle (BBMV) proteins released after treatment with phosphatidylinositolspecific phospholipase C (PI-PLC), which specifically cleaves proteins that are anchored to the membrane by a GPI anchor. As depicted in Fig. 1A, Cry4Ba and Cry11Aa toxins bound a GPI-anchored protein of approximately 70 kDa. Although two other minor bands are visible on the ligand blot for both toxins, they probably represent degradation products.

In this work we found an inverse relationship between binding of Cry4Ba and Cry11Aa toxins to their putative receptor and the toxicity displayed towards *An. albimanus* larvae. We observed that Cry4Ba is almost seven times more toxic than Cry11Aa, while binding to the 70 kDa protein is higher for Cry11Aa than for Cry4Ba. This is similar to what have been reported for Cry1Ab toxin that shows a higher toxicity towards *Lymantria dispar* (gypsy moth) than Cry1Ac, even though Cry1Ac shows a higher affinity towards its receptor (Wolfersberger, 1990). Pore formation in insect midgut cells involves the binding to the membrane and insertion into the membrane of Bt toxins, which have been recognized as reversible and irreversible steps of the mechanism of action of the toxins respectively (Liang *et al.*, 1995). In this context, toxicity was found to be directly correlated to the rate constant of irreversible binding (Liang *et al.*, 1995). As we have not measured the rate constant of irreversible binding for Cry4Ba and Cry11Aa toxins to the 70 kDa protein, the discrepancy between binding and toxicity shown may be only apparent.

Identification of the 70 kDa protein band by mass spectrometry

When the PI-PLC-treated fraction of BBMV was subjected to an SDS-PAGE, we observed a very prominent band of 70 kDa, the same size of the protein bound by Cry4Ba and Cry11Aa toxins (Fig. 1B). By *de novo* sequencing of the 70 kDa protein, the amino acid sequence (DIT FEETQDPQAANTNPDVYQQFTR) was obtained. This sequence was analysed by BLAST (Altschul *et al.*, 1997), showing high identity to α-amylase enzymes from other mosquitoes like *Ae. aegypti*, *Culex quinquefasciatus* and *An. gambiae* (GenBank accession numbers XP_001660907, XP_001863693 and XP_001689203).

As a way to confirm that the 70 kDa protein is the protein that is recognized by Cry4Ba toxin, we performed a 2D SDS-PAGE of the protein sample obtained after treatment with PI-PLC, followed by a ligand blot assay using biotin-labelled Cry4Ba toxin. The Cry4Ba toxin bound a 70 kDa protein that shows an acidic pI (Fig. 2B). The corresponding spot on a colloidal Coomasie blue stained 2D gel was picked up and sequenced by LC-MS/MS (Fig. 2A). As a result, sequences of three additional peptides of 12, 19 and 16 amino acids were obtained. The peptide sequences identified were VILD FVPNHTSDEHEWFVK, ANLEQTMLWYGN and YTQN LPECYDLIYDWR, which upon BLAST showed high identity to α-amylases, as the first identified peptide.

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Analysis of α-glucosidase activity of the α-amylase protein purified by Cry4Ba affinity chromatography

Our peptide sequences showed that the 70 kDa protein bound by Cry4Ba shows similarity to the α-amylase family of glycosyl hydrolases. As α-glucosidase enzymes belong to the α-amylase family, an in-gel α-glucosidase assay was performed with a protein sample obtained after treatment of BBMV proteins with PI-PLC-enzyme. We observed α-glucosidase activity in a 70 kDa band as shown in Fig. 3. In order to assess if the protein that shows α-glucosidase activity is able to bind Cry4Ba toxin, we performed an affinity chromatography purification using an agarose-Cry4Ba column. The 70 kDa protein that is retained in the column displayed α-glucosidase activity (Fig. 3), supporting the idea that an α-amylase protein is a binding molecule of the Cry4Ba toxin.

cDNA cloning of Aamy1, an α-amylase from *An. albimanus* larvae midgut that binds to Cry4Ba toxin

To clone the cDNA encoding the α-amylase we performed 5′ and 3′ rapid amplification of cDNA ends (RACE) experiments. Primer oligonucleotides were designed based on the sequence of one of the peptides identified by LC-MS/MS as described in *Experimental procedures*. Total mRNA was purified from midguts of fourth instar larvae of *An. albimanus*. Two fragments of 1305 bp and 750 bp were cloned as described in *Experimental procedures* that represent the 5′ RACE and 3′ RACE amplicons respectively. Both fragments were sequenced in both strands. The resulting DNA sequence, consisting of 1997 bp in length, codes for a protein of 605 amino acids (Fig. 4). Additionally, 53 bp and 111 bp of DNA sequence of the 5′ and 3′ untranslated regions were obtained respectively.

Characterization of Aamy1 protein

The Aamy1 protein composed of 605 amino acids has a predicted molecular weight of 69 kDa and a pI of 4.94. In addition, all of the four identified peptides by MS are present in this amino acid sequence (Fig. 4). The protein shows a cleavage site for a putative N-terminal leader peptide between G21 and K22, as well as an ω-site for GPI lipid anchoring (S584) (Fig. 4). It also shows sites for potential N-glycosylation (N134 and N288) (Fig. 4). Furthermore, Aamy1 protein displayed conserved domains characteristic of α-amylases (Marchler-Bauer *et al.*, 2009), including seven conserved blocks distinctive of the α-amylase family of enzymes (see Fig. 4) (Janeček, S., 1997). Several residues that are highly conserved in this enzyme family are found on Aamy1 amino acid sequence: residues D230, E296 and D364, which are present in the active site of the enzyme; histidines H135, H234 and H363, which are thought to be involved in substrate binding and, finally, residues N134, D200 and H234 that potentially bind calcium ions (Fig. 4) (Janeček, S. (1997).

All these characteristics are consistent with the proposed identification of the 70 kDa protein as an α-amylase that binds to Cry4Ba and Cry11Aa toxins.

Heterologous expression of the cDNA that codes for the α-amylase that binds to Cry4Ba and Cry11Aa toxins from *Bti*

To determine if the cloned α-amylase corresponds to the Cry4Ba binding protein identified in BBMV from *An. albimanus*, we assembled by PCR the α-amylase coding region devoid of the signal peptide and sequences downstream of the GPI-anchoring site, and cloned it into pGEX-4T-3 expression vector to obtain construct pAamy1. Recombinant protein was expressed in *Escherichia coli* as a fusion protein with glutathione transferase as described in *Experimental procedures*. We observed the expression of a protein of apparent molecular weight of 90 kDa, consistent with the expected size of the recombinant fusion protein. Ligand blot experiments showed that the 90 kDa protein bound biotin-labelled Cry4Ba and

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Cry11Aa toxins, but did not bind biotin-labelled Cry3Aa toxin, known to be non-toxic to *An. albimanus* (Fig. 5A). Besides the 90 kDa band, two other proteins of 34 and 20 kDa were observed (Fig. 5A). A control experiment in which a sample of the cellular extract from *E. coli* containing an empty vector showed the same 34 and 20 kDa bands, indicating that these bands are not recognized by biotin-labelled Cry4Ba nor Cry11Aa, but represent a detection artifact (Fig. 5A).

In order to assess the specificity of binding of Cry4Ba toxin to the recombinant 90 kDa protein, a homologous competition experiment was performed. Binding was shown to be specific since a 50-fold excess of unlabelled Cry4Ba protein abolished the binding of biotin-labelled Cry4Ba toxin to the 90 kDa protein (Fig. 5B).

Aamy1 constitutes a novel class of receptor for Cry toxins

The protein bound by Cry4Ba and Cry11Aa toxins has homology to the α-amylase protein family, also known as family 13 of glycoside hydrolases that groups together a large number of enzymes that act on a(1–4) glycosidic linkages present on starch, glycogen and related polysaccharides (Janeček, S., 1997; Stam *et al.*, 2006).

Other members of the α-amylase family protein have been reported as receptors for bacterial toxins. This is the case for the α-amylases of *C. pipiens* and *An. gambiae* that were identified as the binding molecules for the binary toxin of *Bacillus sphaericus* (Silva-Filha *et al.*, 1999; Romao *et al.*, 2006; Opota *et al.*, 2008). Although the Aamy1 protein is also an α-amylase, it shows only moderate sequence identity to the α-amylases found as receptors for the binary toxin.

It has been proposed that Cry toxins active against lepidopteran insects interact with a primary receptor (cadherin). Such interaction promotes the proteolytic cleavage of the N-terminal helix α-1, inducing toxin oligomerization. A second interaction between toxin oligomers and GPIanchored proteins as APN or ALP promotes the insertion of the oligomers into lipid rafts membrane, forming pores that cause cell lysis. Although less is known in the case of dipteran larvae, it has been suggested that mosquitocidal specific Cry toxins may follow a similar mode of action (Soberon *et al.*, 2007). In *An. gambiae*, cadherin is a functional receptor for Cry4Ba and GPI-anchored proteins, such as APN and ALP, have been shown to bind Cry4Ba and Cry11Aa in *An. quadrimaculatus* and *Ae. aegypti* respectively (Abdullah *et al.*, 2006; Fernandez *et al.*, 2006; 2009; Hua *et al.*, 2008; Bayyareddy *et al.*, 2009; Chen *et al.*, 2009). Furthermore, Cry11Aa toxin oligomerizes in the presence of *Ae. aegypti* BBMV proteins, leading to pore formation (Perez *et al.*, 2007). In the same way, it has been shown that Cry4Ba toxin is able to form oligomers and insert into lipidic vesicles (Likitvivatanavong *et al.*, 2006).

The α-amylase identified in this work constitutes a new class of receptor molecules for the Cry toxins. Further research on the interaction between monomeric or oligomeric structures of Cry4Ba or Cry11Aa toxins and the α-amylase is needed to determine the role that this receptor is playing in the mechanism of action of these toxins. In addition, more studies should be carried on to know if there are other protein receptors involved in the toxicity of Cry4Ba and Cry11Aa toxins towards *An. albimanus*.

Experimental procedures

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2. *Bt* strains were grown in nutrient broth sporulation medium at 200 r.p.m and 30°C (Lereclus *et al.*, 1995). The medium was supplemented with erythromycin, as follows: for strains containing

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Cry4Ba and Cry4Aa, 5 μg ml^{-1}; Cry11Aa, 25 μg ml^{-1}; Cry11Ba, 50 μg ml^{-1} and Cyt1Aa, 10 μg ml^{-1}. For *E. coli* strains LB medium was used for growth supplemented with ampicillin at the concentration of 100 μg ml^{-1}.

Insects

Mosquitoes were reared at 28°C, 75% humidity and 12/12 light/dark photoperiod. Guts from fourth instar larvae were dissected under a stereomicroscope and stored at −80°C in MET (300 mM mannitol, 5 mM EGTA and 200 mM Tris, pH 7.4), supplemented with 1 mM PMSF and 1 mM DTT, until used.

Purification and labelling of Cry and Cyt proteins

Spores and crystals produced in *Bt* strains harbouring plasmids that express only Cry4Ba, Cry4Aa, Cry11Aa or Cyt1Aa toxins, wild-type *Bti* or *B. thuringiensis* var. *tenebrionis* strain producing Cry3Aa toxin were harvested and washed three times with 0.3 M NaCl, 0.01 M EDTA, pH 8.0. The pellet was suspended in 0.1% Triton X-100, 300 mM NaCl, 20 mM Tris-HCl pH 7.2, and inclusions were purified by sucrose gradient centrifugation (Thomas and Ellar, 1983). Purified Cry4Ba inclusions were solubilized in carbonate buffer (sodium bicarbonate/carbonate 0.1 M, pH 9.5) supplemented with 0.2% β-mercaptoethanol (pH 10.5), and activated with 1:20 w/w trypsin (Sigma-Aldrich), for 16 h at 37°C. Purified Cry11Aa inclusions were solubilized in 100 mM NaOH and activated with 1:50 w/w trypsin for 2 h at 25°C. Toxins were dyalized overnight in borate buffer (boric acid 0.05 M, NaOH 0.05 M and NaCl 0.15 M, pH 8.6). Toxins were biotin-labelled using biotinyl-N-hydroxysuccinimide ester according to the manufacturer's instructions (Amersham).

Mosquito larvae bioassay

Mosquitocidal bioassays with spore/crystal suspensions from *B. thuringiensis* strains producing only Cry4Ba, Cry4Aa, Cry11Aa and Cyt1Aa toxins were performed against *An. albimanus* fourth instar larvae in 100 ml of dechlorinated water. Positive (*Bti* strain) and negative controls (dechlorinated water) were included in the bioassay, and larvae were examined 24 h after treatment. The mean lethal concentration (LC_{50}) was estimated by Probit analysis using statistical parameters after three independent assays (Finney, 1971).

Preparation of brush border membrane vesicles from *An. albimanus* larvae

Brush border membrane vesicles from dissected midguts of fourth instar *An. albimanus* larvae were prepared as reported and stored until used (Nielsen-LeRoux and Charles, 1992).

Phospholipase C-treatment of BBMV

Anopheles albimanus BBMV (300 μg) were suspended in 300 μl of PBS [45 mM NaCl, 2.8 mM NaH_2PO_4 (H_2O), 3.8 mM Na_2HPO_4 ($7H_2O$), pH 7.2] PI-PLC (phospholipase-C, 3 units) from *Bacillus cereus* (Boehringer-Mannheim) was added to the BBMV suspension and incubated for 90 min at 30°C, as previously reported (Lorence *et al.*, 1997).

Toxin blots

PI-PLC-treated BBMV proteins (10 μg) were separated in 1D or 2D 12% SDS-PAGE and electroblotted to PVDF Immobilon membranes (Amersham) at 350 mA by 45 min using transfer buffer (20% methanol, 25 mM Tris-base, 192 mM glycine). After blocking the membrane with 1% BSA, the membranes were incubated for 2 h with 10 nM of biotin-labelled Cry4Ba, Cry11Aa or Cry3Aa. Blots were washed three times using PBS containing 0.1% Tween-20. The bound protein was detected by the use of streptavidin-peroxidase conjugate and developed with SuperSignal chemiluminescence substrate (Pierce).

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Affinity chromatography purification of Cry4Ba binding proteins

Agarose–streptavidin (200 μl; Sigma–Aldrich) was incubated with 7 μg of biotinylated-Cry4Ba toxin overnight at 4°C. After incubation, the matrix was washed 10 times in PBS to remove any unbound protein. The solution containing GPIanchored proteins (42 μg) was incubated in batch with 200 μl of Cry4Ba–agarose column and was washed four times with PBS, pH 7.6. The bound proteins were then eluted by boiling for 5 min in 100 μl of sample buffer [0.1% SDS (w/v), 10% glycerol (v/v), 62.5 mM Tris-HCl pH 6.8 and 0.041% of bromophenol blue(w/v)]. After centrifugation, the supernatant was recovered and samples were stored at −20°C until used.

In-gel α-amylase assay

PI-PLC-treated BBMV proteins and Cry4Ba affinity chromatography purified proteins were run in a SDS-12% PAGE, at 4°C, 20 mA, for 75 min. The gel was washed three times with Triton X-100 for 20 min at room temperature and incubated with the substrate in the dark [100 mM citrate/phosphate, pH 6.5, 2 mM 4-methylumbelliferyl-α-D-glucopyranoside (Fluka)], at 37°C with gentle agitation until the signal was detected, and was visualized under UV light (Silva-Filha *et al.*, 1999).

2D PAGE

A sample (10 μg) of PI-PLC-treated BBMV proteins was precipitated with 2D Clean Up Kit (GE Amersham Biosciences). For the first dimension, precipitated proteins were suspended in hydration buffer [8 M urea, 2% w/v CHAPS, 15 mM DTT and 0.5% v/v IPG buffer pH 3–10 (GE Amersham Biosciences)]. IEF was performed by use of IPG strips (7 cm, pH 3–10; GE Amersham Biosciences) that were hydrated overnight and focused for 4 h at 6500 V at 20°C under mineral oil. The IPG strips were first incubated for 10 min in equilibration buffer I [6 M urea, 30% glycerol, 2% SDS and 1% DTT (w/v) in 0.05 M Tris-HCl buffer pH 8.8], and then with equilibration buffer II [6 M urea, 30% glycerol, 2% SDS and 4% iodoacetamide (w/v) in 0.05 M Tris-HCl buffer, pH 8.8]. After equilibration, strips were electrophoresed in a 12% SDS-PAGE for the second dimension separation, by running at 15 mA, 4°C for 2 h. Protein spots were visualized by staining with colloidal Coomasie blue (Candiano *et al.*, 2004).

In-gel trypsin digestion

The protein was digested in-gel following a protocol by Shevchenko and colleagues (1996). The protein spot of interest was excised from a colloidal Coomasie blue stained SDSPAGE and reduced with DTT, alkylated with iodoacetamide and in-gel digested with trypsin (Promega; Fitchburg, MA). After extraction with 50% v/v ACN and 5% v/v acetic acid, the protein mixture was desalted with Zip Tip (Millipore; Billerica, MA, USA) and applied to the LC-MS/MS system.

LC-MS/MS analysis

Samples were applied into an ESI-IT LCQ mass spectrometer (Thermo Fisher; San José, CA) equipped with a nanospray source. At first, the fluid flow speed was 10 ml min^{-1} and then it was reduced to 500 nl by means of a flow splitter (20 × 1), before the nanospray ionization system. The applied ionization voltage was 1.7 kV and the temperature of the heating capillary was maintained at 137°C. For the collisionally induced dissociation experiments, a nanospray capillary PicoFrit column (5 mm Biobasic C18, 300 Angstrom pore; New Objective, Woburn, MA) was loaded with the protein digestion mixture, using a fragmentation source of 25 V and a collision energy normalized between 35% and 45%. The experiments were performed with the ion detection in the positive mode and the wideband

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activated. The MS/MS spectra from enzymatically generated peptides was analysed by using the SEQUEST software (Eng *et al*., 1994).

Synthesis of cDNA, cloning and expression of a recombinant clone in *E. coli*

Total RNA from dissected midguts of fourth instar *An. albimanus* larvae were prepared with the RNeasy kit (Qiagen). By back-translating the sequence obtained by *de novo* sequencing of the 70 kDa protein spot (DITFEETQD PQAANTNPDVYQQFTR) and by use of the *An. albimanus* codon usage (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=7167), degenerate primers were designed for performing 5′ and 3′ rapid amplification of cDNA ends (RACE) experiments in order to clone the cDNA for the α-amylase using the SMART RACE cDNA Amplification kit (Clontech). The primers used were *Glu5* (AACTGCTGRTA SACKTCSGGGTTSGTGTTVGC) for 5′ RACE and *Glu3* (ATYACSTTCGARGARACSCAGGAYCCSCAGGC) for 3′ RACE (using the following code, *R*: A/G; *S*: C/G; *Y*: C/T; *K*: G/T; *V*: A/C/G). Both 5′ and 3′ RACE products were cloned into pCR2.1-TOPO (Invitrogen), to obtain plasmids p1.3–2 and pISO-1, carrying inserts of 1.3 kb (5′ RACE) and 0.75 kb (3′ RACE) respectively. In order to complete the nucleotide sequence of both strands of DNA, primers *GluA* (CGACAGC CAGTACTACCTTCAC) and *GluB* (CCTCGTACACATGGT TGATCG) were used. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number GQ344953.

For obtaining a clone to be expressed in *E. coli* that lacks the signal peptide and the GPI-anchoring site, a megaprimer method (Sarkar and SomMer, 1990) was followed to amplify a region from amino acids 24–582 of Aamy1 protein. First, the clone containing the 5′ RACE was used as a template for PCR amplification using primers Frag1a (GATCCGAATTCG GCACCGGGCCGGCAGTCGCAC, containing an EcoRI site that is shown underlined) and Frag1c (GAACTGCTGGTA CACGTCCGGGTTCG). Amplification conditions were 94°C for 2 min, then 30 cycles (94°C for 30 s, 50°C for 1 min, 68°C for 1 min) followed by an extension at 68°C for 10 min, using Advantage2 Polymerase (Clontech). The resultant amplicon was gel purified by minElute PCR purification kit (Qiagen) and used as a ‘megaprimer’ on a second amplicon that was obtained using as template the clone containing the 3′ RACE using primers Frag2D (ACATTACGTTCGAGGAGACGCAG GAC) and Frag2C (TCGATGCGGCCGCTGGAAATCT CAAACACAACCGCATCG, containing a NotI cleavage site that is shown underlined). Amplification conditions were 94°C for 2 min, then 30 cycles (94°C for 30 s, 60°C for 1 min, 68°C for 1 min) followed by an extension at 68°C for 10 min, using Vent Polymerase (New England Biolabs). Both amplicons were gel purified by minElute PCR purification kit (Qiagen). Finally, a third PCR reaction was performed using both amplicons that overlap by 68 bp (from nucleotides 1270 to 1337, which code for amino acids 406–428 of the Aamy1 protein sequence shown in Fig. 4). First, both amplicons were annealed at 55°C for 10 min, extended at 68°C for 10 min and amplified by using Frag1a-Frag2C primers and Advantage2 Polymerase. Amplification conditions were 94°C for 2 min, then 30 cycles (94°C for 30 s, 55°C for 1 min, 68°C for 3 min) followed by an extension at 68°C for 10 min. The resultant amplicon of 1.7 kb was digested with enzymes EcoRI and NotI and cloned into pGEX-4T-3 (GE Amersham Biosciences), digested with the same enzymes.

Expression of the recombinant construct pAamy1 in *E. coli*

For expression in *E. coli*, plasmid pAamy1 that codes for Aamy1 protein devoid of signal peptide and the sequence downstream of the GPI-anchoring site was transformed into strain JM101 (New England Biolabs). From a single colony, a 5 ml overnight culture was grown on LB Amp. The next day, 20 ml of LB Amp was inoculated with 2 ml of the overnight culture and grown for 3 h. Then, IPTG was added to a final concentration of 0.1 mM and

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incubated for 2 h at 37°C and 180 r.p.m. Cells were centrifuged at 20 800 *g* for 15 min at 4°C. The cells were suspended by vortex in 5 ml of Nonidet-40 (Sigma). The solution was incubated at room temperature for 10 min. After incubation, the solution was centrifuged for 15 min at 20 800 *g* and 4°C. The pellet was suspended in 4 ml of PBS. Protein samples (5 μg) were run in a PAGE-SDS 12% acrylamide gel at 30 mA. Finally, the gel was electroblotted to PVDF membranes and analysed by ligand blot with biotin-labelled Cry4Ba, Cry11Aa or Cry3Aa toxins.

Binding specificity of the recombinant protein

A sample of 5 μg of the insoluble fraction of an *E. coli* extract containing the recombinant protein was run in a PAGE-SDS 12% acrylamide gel at 30 mA and electroblotted to PVDF membranes, which were incubated with 10 nM of biotin-labelled Cry4Ba toxin in the presence or absence of 500 nM of unlabelled Cry4Ba toxin.

Bioinformatic analysis

Blast-p searches were done in the Blast server (http://blast.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997). For the prediction of the N-terminal signal sequence, we used the program SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen *et al.*, 2004). Prediction of potential C-terminal GPI modification sites was done with big-PI predictor (http://mendel.imp.ac.at/sat/gpi/gpi_server.html) (Eisenhaber *et al.*, 1999). Asparagines that could potentially be N-glycosylated were predicted using the program NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetGlyc/) (Blom *et al.*, 2004).

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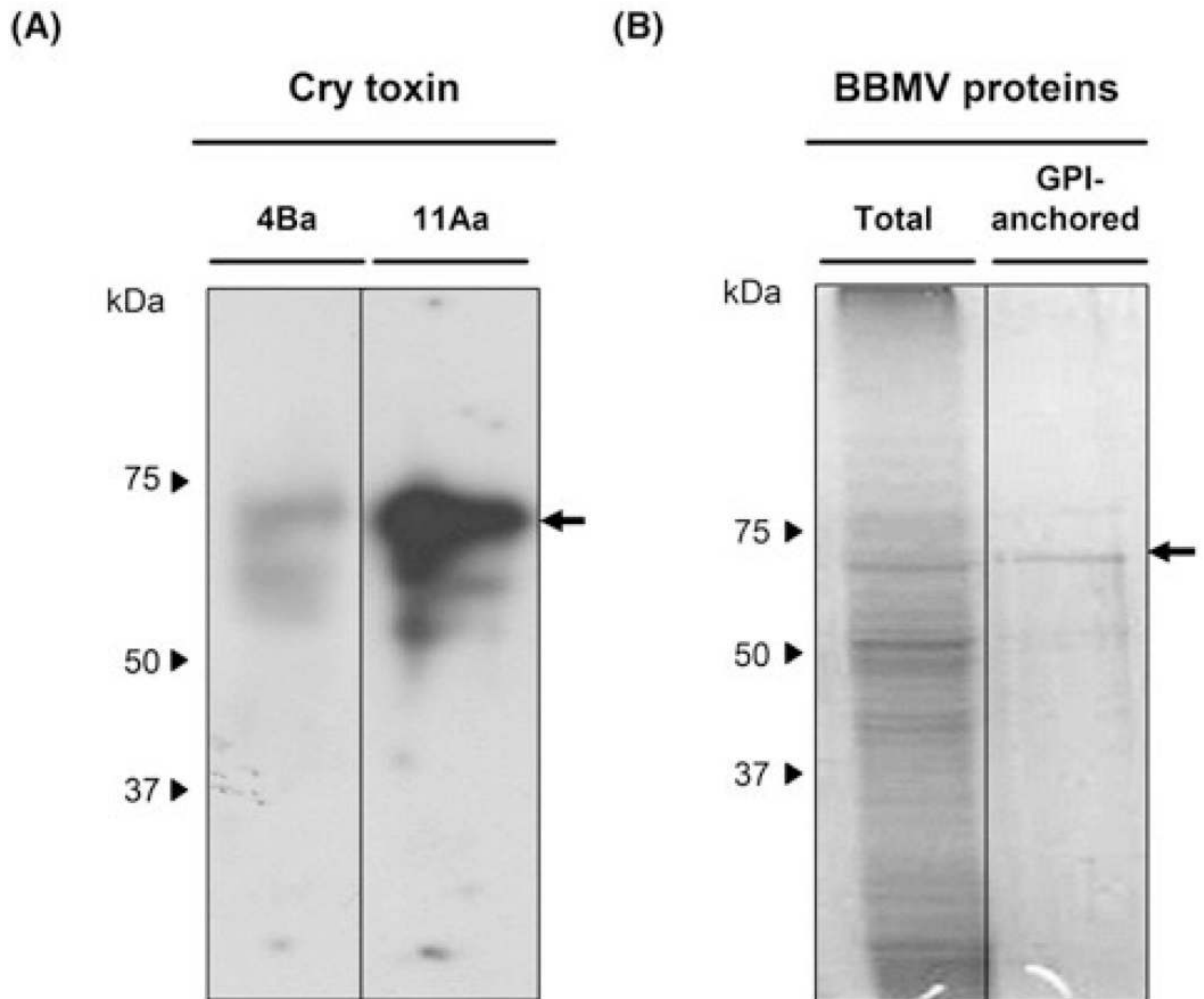

Fig. 1.

A 70 kDa GPI-anchored protein that interacts with Cry4Ba and Cry11Aa toxins is an α-amylase.

A. Cry4Ba and Cry11Aa toxins bind aGPI-anchored protein. BBMVs isolated from*An. albimanus* midgut larvae were treated withphospholipase C to release the GPI-anchoredprotein fraction, which was subjected toSDS-PAGE and electroblotted. Proteins thatbound biotin-labelled Cry4Ba and Cry11Aatoxins were detected by a ligand blot assay.

B. The main component of the BBMVGPI-anchored protein fraction is a 70 kDaα-amylase. BBMV total proteins and BBMVproteins anchored by GPI were run in aSDS-PAGE and stained with Coomasie blue.Arrow shows the protein band that wassequenced by LC-MS/MS.

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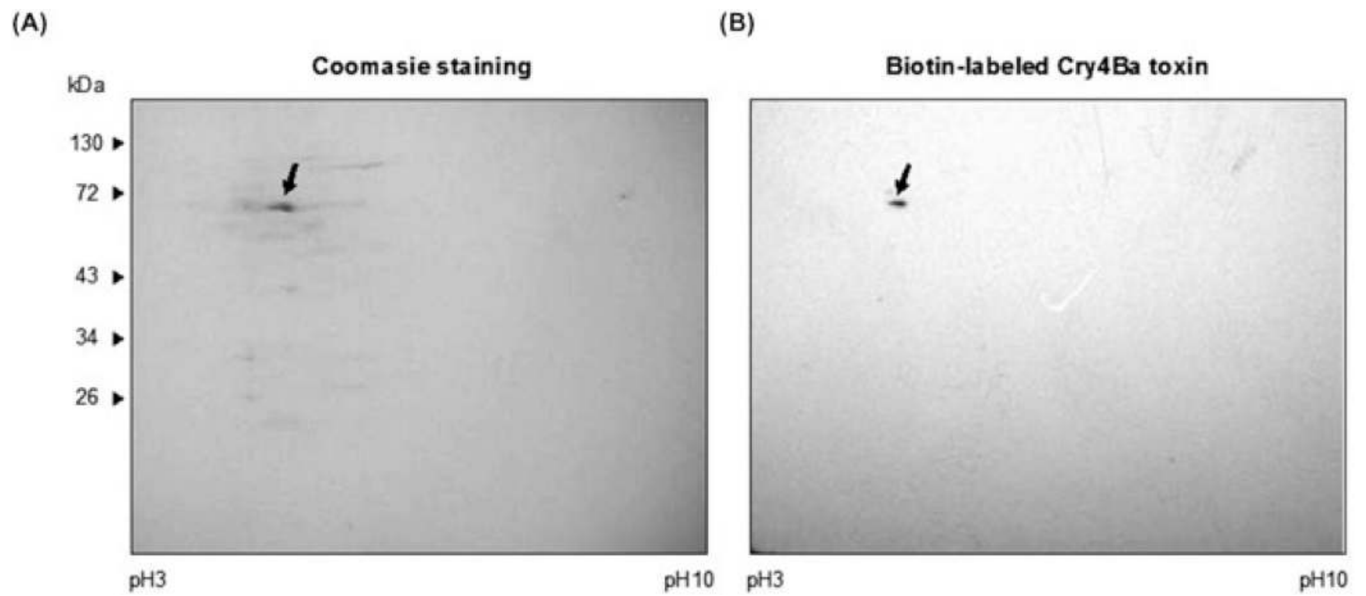

Fig. 2.
Cry4Ba toxin binds an acidic, 70 kDa GPI-anchored protein. Colloidal Coomasie blue stained 2D gel (A) and ligand blot using biotin-labelled Cry4Ba toxin (B). Arrow shows the spot that was subjected to LC-MS/MS sequencing.

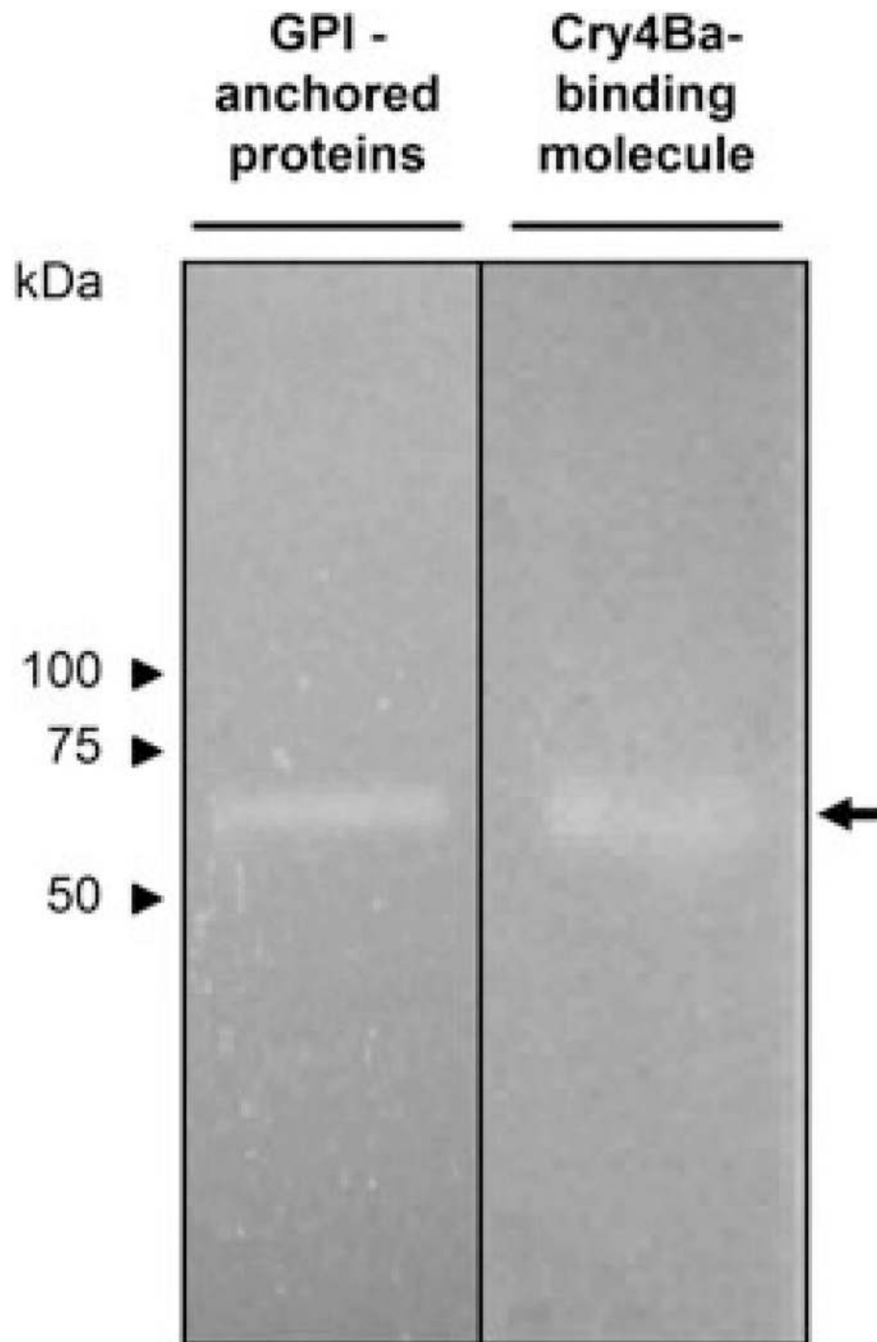

Fig. 3.

A Cry4Ba binding protein shows α-glucosidase activity. In-gel α-glucosidase assay of the BBMV GPI-anchored protein fraction and the protein bound by an Agarose-Cry4Ba column.

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   1 GGTATCGGAGATCGAGAGAGAGTGTGTGTACGAACGGTCTTCTCTAGCGCACGATGCGGT
                                                          m  r  l

  61 TGACGGTGAGCTTGCTGGTGGCGATCGGCCTTGGCATCATTGCGGTGGCCAGTGGAAAGA
   4  t  v  s  l  l  v  a  i  g  l  g  i  i  a  v  a  s  g  K  T

 121 CGGCACCGGGCCGGCAGTCGCACGATCACGGATCCCTCGACTGGTGGGAGGCGGGAGTGT
  24  A  P  G  R  Q  S  H  D  H  G  S  L  D  W  W  E  A  G  V  F

 181 TCTATCAAATCTATCCGCGCTCGTTCAAGGACAGTGACAACAATGGTGTCGGCGACCTGA
  44  Y  Q  I  Y  P  R  S  F  K  D  S  D  N  N  G  V  G  D  L  K

 241 AGGGTATTACGGAGAAGCTGAACCATTTGGTCGATCTCGGTATCGATGATGTGTGGCTCA
  64  G  I  T  E  K  L  N  H  L  V  D  L  G  I  D  D  V  W  L  S
                                                     I

 301 GCCCGGTCTTTACGTCGCCGATGGCCGATTTCGGGTACGACATCGCCGACTTCCGCTCGA
  84  P  V  F  T  S  P  M  A  D  F  G  Y  D  I  A  D  F  R  S  I

 361 TCGATCCACTGTTCGGTACGATGGCGGACCTGGACGCTATGATCGCGAAGGCAAAAGATC
 104  D  P  L  F  G  T  M  A  D  L  D  A  M  I  A  K  A  K  D  L

 421 TGGGCATCCGGGTGATACTGGACTTTGTGCCGAATCATACGAGCGATGAGCACGAGTGGT
 124  G  I  R  V  I  L  D  F  V  P  N  H  T  S  D  E  H  E  W  F
                              II

 481 TCGTGAAGGCAAAGAACAACGATCCCGCCTTCCGTGACTTTTACGTGTGGCGCGATCCGA
 144  V  K  A  K  N  N  D  P  A  F  R  D  F  Y  V  W  R  D  P  R

 541 GGGGAAACGCCGAGCCGAACAACTGGCAATCGGTCTTCCGCACGCCCGCCTGGACGAGGC
 164  G  N  A  E  P  N  N  W  Q  S  V  F  R  T  P  A  W  T  R  L

 601 TGCCAGGCGACAGCCAGTACTACCTTCACCAGTTCGACAAGAAGCAACCGGATCTGAACT
 184  P  G  D  S  Q  Y  Y  L  H  Q  F  D  K  K  Q  P  D  L  N  Y
                                               III

 661 ACCGGAATCAGCGCGTGAAGGACGAGATGGAGTCGATGATTCGGTTCTGGCTGGACAAGG
 204  R  N  Q  R  V  K  D  E  M  E  S  M  I  R  F  W  L  D  K  G

 721 GCGTCGATGGGTTCCGCATCGATGCGATCAACCATGTGTACGAGGATCCGCAGTTCCGCG
 224  V  D  G  F  R  I  D  A  I  N  H  V  Y  E  D  P  Q  F  R  D
                       IV

 781 ATGAGGAGCTGATCGATCCGAAAGGGGAGCTGATCTGGGAGAACCTGGACCACAAGTACA
 244  E  E  L  I  D  P  K  G  E  L  I  W  E  N  L  D  H  K  Y  T

 841 CGCAGAATCTGCCCGAGTGCTACGATCTGATCTACGATTGGCGCGACGTGTTTGATCAGT
 264  Q  N  L  P  E  C  Y  D  L  I  Y  D  W  R  D  V  F  D  Q  Y

 901 ACAAGGCGCGGGACAATGTGACGCGCCTGATGATGACGGAGGCGTACGCTAACCTGGAGC
 284  K  A  R  D  N  V  T  R  L  M  M  T  E  A  Y  A  N  L  E  Q
                                                  V

 961 AGACGATGCTGTGGTACGGTAACCCGCAGCGCAACCGCAAGGGTGCCCACATACCGTTCA
 304  T  M  L  W  Y  G  N  P  Q  R  N  R  K  G  A  H  I  P  F  N

1021 ACTTCGCCATGATCAACCGCCTGTCGAACGATTCGCGGGCCGGTGACTTCAAGGCGATCG
 324  F  A  M  I  N  R  L  S  N  D  S  R  A  G  D  F  K  A  I  V

1081 TCGACGAGTGGCTGGATGCGATGCCGGCCGGTCAGCAGGCGAACTGGGTGCTCGGTAATC
 344  D  E  W  L  D  A  M  P  A  G  Q  Q  A  N  W  V  L  G  N  H
                                                        VI
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1141 ACGATCGTCCCCGTATCGCTTCCCGCTTCGGTCGCGATCGGGCATCCAGCTTTGCCGTGC
 364  D   R   P   R   I   A   S   R   F   G   R   D   R   A   S   S   F   A   V   L

1201 TCGAGATGACGCTGCCCGGTATTGCGGTGGTGTACTACGGCGAGGAGATCGGCATGGAGG
 384  E   M   T   L   P   G   I   A   V   V   Y   Y   G   E   E   I   G   M   E   D
                                          VII
1261 ACAACCGGGACATTACGTTCGAGGAGACGCAGGACCCGCAGGCGGCTAACACGAACCCGG
 404  N   R   D   I   T   F   E   E   T   Q   D   P   Q   A   A   N   T   N   P   D

1321 ACGTGTACCAGCAGTTCACGCGCGATCCCGTGCGCACCCCGTTCCAGTGGGATGACACGG
 424  V   Y   Q   Q   F   T   R   D   P   V   R   T   P   F   Q   W   D   D   T   A

1381 CGTACGCCGGCTTTACGGGCGCAGCGGCACGGGAGACCTGGCTCCCGGTGCATCCCAACT
 444  Y   A   G   F   T   G   A   A   A   R   E   T   W   L   P   V   H   P   N   Y

1441 ATCGGCAGATTAATCTCGCGGCCCAGAAGGCCGCCCCGCAGAGCATGTTCAAGCTGTACC
 464  R   Q   I   N   L   A   A   Q   K   A   A   P   Q   S   M   F   K   L   Y   Q

1501 AGCGTTTGATTCAGCTGCGCAAGGGCGACACCTTCCGGTATGGTGACTACGAGTCGAAGG
 484  R   L   I   Q   L   R   K   G   D   T   F   R   Y   G   D   Y   E   S   K   V

1561 TGATGCTGAACAATGTGTTCGGCTATACGCGCACCCTCGAAGGTCACGAATCGTACGCCG
 504  M   L   N   N   V   F   G   Y   T   R   T   L   E   G   H   E   S   Y   A   V

1621 TGGTGGTCAATCTGAACGACAACGATGTGAACGTGAACCTGCAGGAGCTGCACCCCGATG
 524  V   V   N   L   N   D   N   D   V   N   V   N   L   Q   E   L   H   P   D   V

1681 TGGGGGCAGCGAAGGTTGTGCTCACGTCGCTCGATGCCAAGCTGAAGGAGGGTGACGAGA
 544  G   A   A   K   V   V   L   T   S   L   D   A   K   L   K   E   G   D   E   I

1741 TAACGGAAGTGTTCCACGTCATCGTCGGTGCGTACGATGCGGTTGTGTTTGAGATTTCCT
 564  T   E   V   F   H   V   I   V   G   A   Y   D   A   V   V   F   E   I   S   S

1801 CGTCCGCCAGTACGCTCGGCGTGTCGATGGTACTGATGCTGTTCGCTTCCATCCTGCGCT
 584  S   A   S   T   L   G   V   S   M   V   L   M   L   F   A   S   I   L   R   S

1861 CCCTGTTCTAGGTGAGGCGTGCGCTTTAACTTATTTTTCTCCCTCAAACGTCGTCTTTAC
 604  L   F   *

1921 TGAACGCACTTTCCATTAAACTAGACTGTAAATTGTATGCCGGGCCTTCTGTGCATGGTG

1981 ATAAAAAAAAAAAAAAA
```

Fig. 4.

Nucleotide sequence of *aamy1* gene and its deduced amino acid sequence. The initiation and stop codons are indicated by a star (✧) and an asterisk (*) respectively. Peptide sequences obtained from LC-MS/MS identification are underlined. The N-terminal hydrophobic domain that corresponds to the putative signal peptide is shown in lower case. The ω-site for GPI modification and the amino acid putatively implicated in the cleavage site is shown as a pentagon. Asparagines predicted to be N-glycosylated are encircled. Seven conserved regions (I to VII) characteristic of the α-amylase family proteins are boxed. The catalytically important residues, the histidines responsible for substrate binding and the

amino acids involved in binding of calcium ions are referred by diamonds, squares or a double underline respectively.The GenBank accession number of *aamy1* gene is GQ344953.

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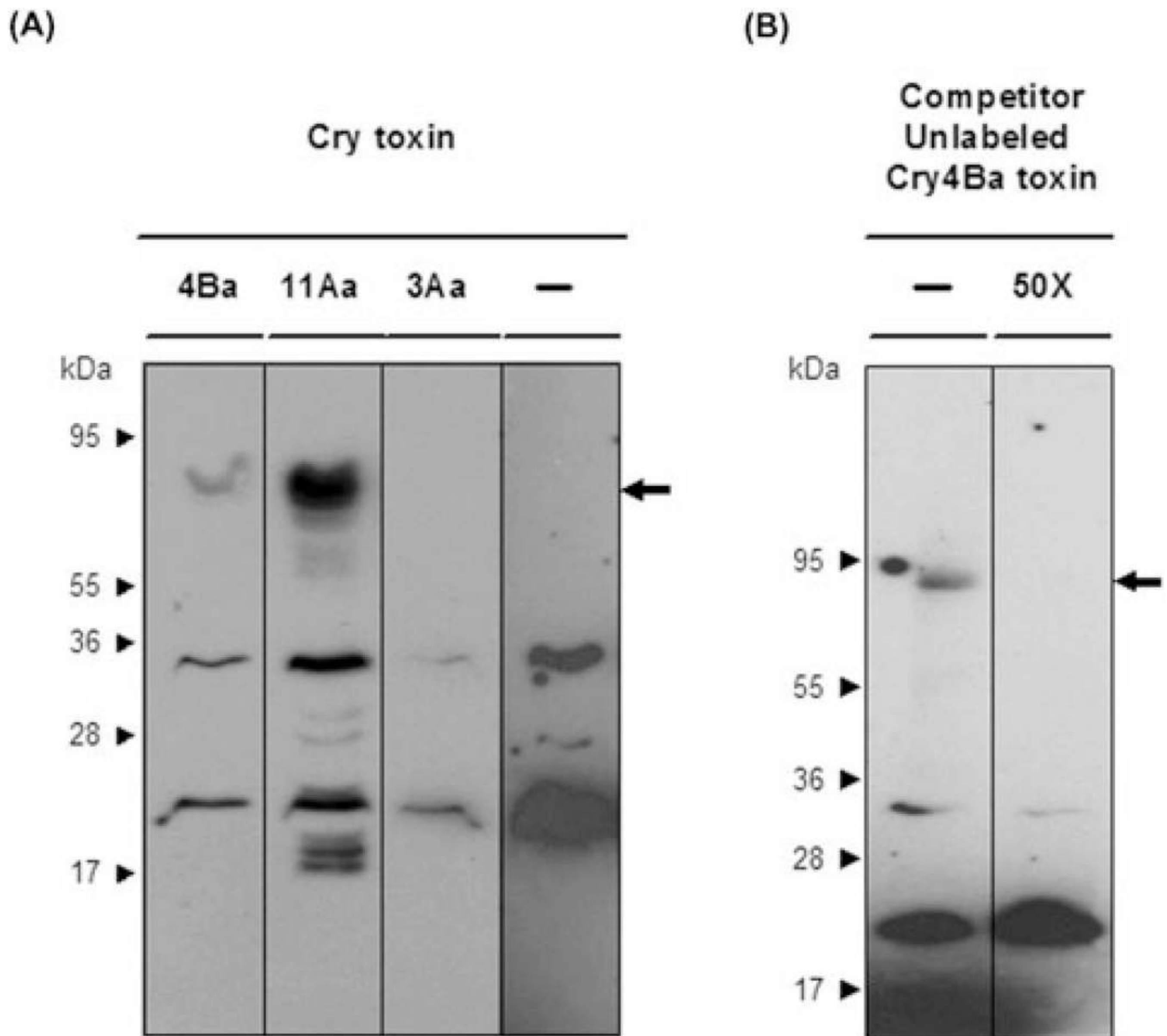

Fig. 5.
Specific binding of Cry4Ba toxin to heterologous expressed Aamy1 protein.
A. Ligand blot analysis of cell extracts of*E. coli* expressing Aamy1 protein usingbiotin-labelled Cry4Ba, Cry11Aa or Cry3Aatoxins. As a negative control, cell extractsprepared from *E. coli* cells transformed withan empty plasmid, in the absence of anybiotin-labelled Cry toxin but incubated withStreptavidin-peroxidase (lane marked with adash).
B. Homologous competition of Cry4Ba bindingto heterologous expressed Aamy1 protein.Ligand blot analysis of cell extracts of *E. coli*expressing Aamy1 protein usingbiotin-labelled Cry4Ba toxin in the absence orthe presence of an excess (50×) of unlabelledCry4Ba protein.

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Table 1

Anopheles albimanus fourth instar larvae sensitivity to different *Bti* Cry mosquitocidal toxins.

Toxin	LC_{50} (ng ml^{-1})
Cry4Ba, Cry 11Aa,Cry 4Aa, Cyt1Aa, Cyt2Ba	2 (1–3)[a]
Cry4Ba	130 (80–200)
Cry11Aa	900 (720–1120)
Cry4Aa	> 2 000
Cyt1Aa	> 10 000

[a]95% fiducial limits.

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Table 2

The bacterial strains and plasmids used in this study.

	Characteristics	Reference
Bt strains		
Bti HD916	Cry4Aa,Cry4Ba, Cry11Aa, Cry10Aa, Cyt1Aa, Cyt2Ba	Bacillus Genetic Stock Center (Columbus OH)
Bt CG6	*B. thuringensis* crystal negative strain	Chang *et al.* (1993)
4Q7	*Bti* crystal negative strain	D. Dean, The Ohio State University, USA
BTS1	*B. thuringiensis* var. *tenebrionis* strain containing Cry3Aa gene	M. Peferoen, Plant Genetic Systems, Belgium
Plasmids		
pWF26	Cry11Aa, *E*ry^R	Wu and Federeci (1995)
pHT606	Cry 4Aa, *Ery*^{R}	Delecluse *et al.* (1993)
pHT611	Cry4Ba, *Ery*^{R}	Delecluse *et al.* (1993)
pWF45	Cyt1Aa, *Ery*^{R}	Wu *et al.* (1994)
p1.3–2	1.3 kb fragment of *An. albimanus* α-amylase cloned into plasmid pCR2.1-TOPO, *Amp*^{R}*Km*^{R}	This work
pISO-1	0.75 kb fragment of *An. albimanus* α-amylase cloned into plasmid pCR2.1-TOPO, *Amp*^{R}*Km*^{R}	This work
pAamy1	*An. albimanus* α-amylase gene coding for Aamy1 protein cloned into expression vector pGEX-4T-3, *Amp*^{R}	This work

Ery^R, erythromycin resistant; *Amp*^{R}, ampicillin resistant; *Km*^{R}, kanamycin resistant.