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Identification of Cry48Aa/Cry49Aa toxin ligands in the midgut of *Culex quinquefasciatus* larvae

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Abstract

A binary mosquitocidal toxin composed of a three-domain Cry-like toxin (Cry48Aa) and a binary-like toxin (Cry49Aa) was identified in *Lysinibacillus sphaericus*. Cry48Aa/Cry49Aa has action on *Culex quinquefasciatus* larvae, in particular, to those that are resistant to the Bin Binary toxin, which is the major insecticidal factor from *L. sphaericus*-based biolarvicides, indicating that Cry48Aa/Cry49Aa interacts with distinct target sites in the midgut and can overcome Bin toxin resistance. This study aimed to identify Cry48Aa/Cry49Aa ligands in *C. quinquefasciatus* midgut through binding assays and mass spectrometry. Several proteins, mostly from 50 to 120 kDa, bound to the Cry48Aa/Cry49Aa toxin were revealed by toxin overlay and pull-down assays. These proteins were identified against the *C. quinquefasciatus* genome and after analysis a set of 49 proteins were selected which includes midgut bound proteins such as aminopeptidases, amylases, alkaline phosphatases in addition to molecules from other classes that can be potentially involved in this toxin's mode of action. Among these, some proteins are orthologs of Cry receptors previously identified in mosquito larvae, as candidate receptors for Cry48Aa/Cry49Aa toxin. Further investigation is needed to evaluate the specificity of their interactions and their possible role as receptors.

Keywords: *Lysinibacillus sphaericus*, Binary toxin, Cry, biolarvicides, receptors, mosquito.

1. Introduction

Lysinibacillus sphaericus is an entomopathogen that can produce crystals containing protoxins with high and selective activity against mosquito larvae, in particular those from the *Culex pipiens* complex. Some insecticidal proteins have been identified in *L. sphaericus* strains (Allievi et al., 2014; Berry, 2012) and the Binary crystal protoxin (Bin), which was the first mosquitocidal factor characterized, remains the active principle of the commercial larvicides based on this bacterium (Silva Filha et al., 2014). Bin is a heterodimer composed of BinA (42 kDa) and BinB (51 kDa) polypeptides which are produced at high levels in equimolar concentrations by some strains (Charles et al., 1996). Bin achieves the optimal activity only when both components are present, which characterizes its binary nature (Nicolas et al., 1993). Its mode of action has been mostly studied in species from the *Culex pipiens* complex and, after ingestion and proteolytic processing of protoxins, the active BinB subunit is responsible for specifically binding to the Cpm1/Cqm1 α-glucosidases that act as midgut receptors (Darboux et al., 2001; Romão et al., 2006; Silva-Filha et al., 1999), while the BinA component is associated with cell toxicity (Nicolas et al., 1993). Resistance of *C. pipiens* and *C. quinquefasciatus* larvae to Bin toxin has been recorded (Mulla et al., 2003; Nielsen-Leroux et al., 2002; Rao et al., 1995; Wirth et al., 2000; Yuan et al., 2000) due to mutations in genes encoding the receptors, which lead to the production of truncated or non-functional proteins and cause the failure of Bin toxin binding on the midgut epithelium. This has been the major resistance mechanism documented and *cqm1*/*cpm1* alleles causing such failures have been reported (Chalegre et al., 2012; Chalegre et al., 2015; Darboux et al., 2007; Darboux et al., 2002; Guo et al., 2013; Romão et al., 2006).

Resistance to *L. sphaericus* based on loss of Bin toxin binding highlights the need to characterize molecules with distinct modes of action. Investigation of *L. sphaericus* strain IAB59

71 began soon after the first reports of Bin-based resistance since this strain is toxic to Bin-resistant
72 larvae, suggesting the production of a novel insecticidal factor that can overcome resistance to
73 Bin toxin (Nielsen-LeRoux et al., 2001; Pei et al., 2002; Yuan et al., 2003). Jones et al. (2007)
74 identified the insecticidal factor as Cry48Aa (135 kDa) and Cry49Aa (53 kDa), which are also
75 produced as protoxins in small crystalline inclusions during sporulation. Cry48Aa/Cry49Aa is
76 considered a new binary toxin produced by *L. sphaericus* since neither the Cry48Aa nor
77 Cry49Aa component shows toxicity to larvae alone (Jones et al., 2007). They can act in synergy
78 forming the complex Cry48Aa/Cry49Aa through the N-terminal portion of the Cry49Aa subunit
79 (Guo et al., 2016). These toxins have comparable toxicity to Bin against *C. quinquefasciatus*, and
80 remain active to Bin-resistant larvae, when both Cry toxins are produced as recombinant proteins
81 and are administered in equimolar ratios (Jones et al., 2008). Unfortunately, native strains are
82 deficient in expression of Cry48Aa and do not attain the optimal 1:1 Cry48:Cry49 ratio required
83 for high toxicity, which accounts for why strains such as IAB59 can produce both binary toxins,
84 but are not more toxic than strains producing only Bin toxin.
85 The Cry48Aa component of the toxin belongs to the three-domain (3D) structural
86 family of Cry proteins with 33% amino acid identity with the Cry4Aa toxin from *Bacillus*
87 *thuringiensis* svar. *israelensis* (Bti) (Boonserm et al., 2006; Jones et al., 2007). Cry49Aa is part
88 of the group Bin-toxin-like proteins (Toxin–10 family) with about 30% identity to both subunits
89 of the Bin toxin from *L. sphaericus*, in addition to comparable identity to Cry36 (34%) and
90 Cry35 (20%) produced by *B. thuringiensis* strains (Berry, 2012; Jones et al., 2007). The initial
91 steps of the mode of action of Cry48Aa and Cry49Aa are similar to that of the Bin toxin
92 comprising ingestion of crystals, solubilization under alkaline pH and proteolytic activation of
93 protoxins into toxins (Jones et al., 2008), interaction with midgut (de Melo et al., 2009; Guo et

al., 2016) followed by cytopathological effects which appear similar to those produced by a synergistic mixture of Cry-like and Bin-like toxins (de Melo et al., 2009). However, the identity of ligands and receptors in the larval midgut that underlie toxic action and larval mortality is still unknown. The investigation of this specific step of the mode of action is strategic since Cry48Aa/Cry49Aa is toxic to Bin-resistant *C*. *quinquefasciatus* lacking the midgut receptors (Cqm1) for Bin toxin (de Melo et al., 2009; Pei et al., 2002) which indicates that the Cry48/Cry49 toxin complex interacts with distinct molecules mediating toxicity to larvae. Recently it was shown that both subunits display the ability to bind to the *C*. *quinquefasciatus* larval midgut (Guo et al., 2016). In this context, the major goal of the present study was to identify potential ligands for Cry48Aa/Cry49Aa in the midgut of *C*. *quinquefasciatus* larvae and contribute to the understanding of the mode of action of this mosquitocidal toxin.

2. Materials and methods

2.1 Preparation of Cry toxins

Cry48Aa and Cry49Aa were produced individually in the acrystalliferous *Bacillus thuringiensis* svar. *israelensis* strain 4Q7 transformed with plasmids pSTAB135 and pHTP49, which carry genes encoding the respective toxins (Jones et al., 2007). Cultures were grown in sporulation medium (de Barjac and Lecadet, 1976) supplemented with 1% glucose and erythromycin (25 µg/ml), under agitation (200 rpm) at 30°C, for 72 h until reaching sporulation (≥80%). Spore-crystal biomass was centrifuged, sequentially washed with 1M NaCl/10 mM EDTA pH 8.0 and 10 mM EDTA pH 8.0, and stored at -80°C. **Crystal/spores from both recombinant Bt strains were also processed together**. **For this purpose biomass containing spore-crystals of each protein were combined (1:1 wt/wt), solubilized (50 mM NaOH, 30°C, 1 h, at 150 rpm), the**

supernatant containing both solubilized proteins was separated by centrifugation (21.000*g*, 4°C, 30 min) and the pH was adjusted to ≈8.5 using 0.1 M HCl. Combined protoxins were activated with pancreatic bovine trypsin (1:100 wt/wt, 30°C, 1 h). Supernatant, containing the activated proteins, was centrifuged as described above and then dialyzed (0.02 M sodium phosphate, pH 8, 4°C, 16 h). Protein concentration was determined according to Bradford (1976) using the Biorad reagent (Biorad, Hercules, CA, USA) and a bovine serum albumin standard curve. Size and the integrity of proteins were analyzed in 10% SDS-PAGE. Activated proteins were stored at -80°C. Moreover, in this study Cry49Aa protein fused to a C-terminal poly-histidine tag (Cry49Aa-His) was individually produced in *Escherichia coli* T7 express cells (New England Biolabs, Beverly, Ma, USA). For this purpose, the *cry49Aa* gene was amplified from pHTP49 (described above) using specific primers containing *Bam*HI (bold) and *Not*I (underlined) restriction sites (Fwd 5'-CGA**GGATCC**ATGGAAAATCAAATAAAAGAAGAATTTAAC-3', Rev 5'-CGAGCGGCCGCATTATAATATGGCTTTGAATTTTCATG-3') and subsequently cloned into the expression vector pET21a® (Novagen, USA). Antibodies against the His-tag were used to track binding of the Cry48Aa/Cry49Aa mix through the Cry49-His. Attempts to produce Cry48Aa in *E. coli* in order to have a suitable expression to evaluate this toxin were not successful. For Cry49Aa cultures of transformed T7 express cells were grown using Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml) (under agitation, 200 rpm, 37°C until reaching an OD_{600} of 0.5), and induced with IPTG (0.1 mM, 30°C, 4 h). Cultures were centrifuged (21.500*g*, 4°C, 10 min), the cell pellets were re-suspended in phosphate-buffered saline pH 7.4 (PBS), sonicated, and Triton X-100 was added to a final 1% vol/vol. The samples were centrifuged (1700*g*, 30 min, 4°C) and proteins from the supernatants were purified using

140 the Ni-NTA resin (Qiagen, Hilden, Germany), according to the manufacturer's instructions.
141 Proteins were eluted with 1M imidazole in wash buffer (50 mM sodium phosphate-buffer, 300
142 mM NaCl, 10% glycerol, pH 6, 4°C, 1 h) and dialyzed (50 mM dibasic sodium phosphate, 5 mM
143 monobasic sodium phosphate, 50 mM NaCl, 0.1% Triton x-100, 5% glycerol, pH 7.8, 4°C,
144 overnight). Protein integrity and concentration were verified, as previously described. The
145 purified Cry49Aa-His from *E. coli* was combined with solubilized Cry48Aa from Bt in 1:2 ratio
146 (µg protein: µg protein) and the mixed sample was subjected to *in vitro* processing, as previously
147 described. The concentration and integrity of the activated mix of proteins was verified by 10%
148 SDS-PAGE and then it was stored at -80°C. **Cry49Aa fused to glutathione S-transferase**
149 **(Cry49Aa-GST) was also individually produced in *E. coli* BL21 Star™ (DE3) cells**
150 **(ThermoFischer Scientific, Waltham, MA, USA). *cry49Aa* gene was amplified from**
151 **pHTP49 using the following primers containing *Bam*HI and *Not*I sites as described** (Fwd
152 5'-CGA**GGATCC**ATGGAAAATCAAATAAAAGAAGAATTTAAC-3', Rev 5'-
153 CGAGCGGCCGCTTAATTATAATATGGCTTTGAATTTTCATG -3')**. The BinB subunit of**
154 **the binary toxin fused to glutathione S-transferase (BinB-GST) was also produced**
155 **according to Romão et al. (2006). Further steps to produce both purified GST proteins are**
156 **also described in that study.**
157
158 *2.2 Mosquito strain*
159 Fourth instar larvae of the CqSLab *Culex quinquefasciatus* strain were used in this study. This
160 colony has been maintained in the insectarium of the Instituto Aggeu Magalhães/FIOCRUZ for
161 more than five years under controlled conditions of 26 ±1°C, 70% relative humidity, and a
162 12h:12h (light/dark) photoperiod. CqSLab is a laboratory reference colony susceptible to

insecticidal compounds. Larvae were reared in dechlorinated water and fed on cat food. Adults were maintained on a 10% sugar solution, and females were also artificially fed with rabbit blood.

2.3 Midgut brush border membrane fractions

Midgut apical membrane enriched preparations, called brush border membrane fractions (BBMFs), were prepared with batches of whole frozen (-80°C) 4th instar larvae (**5 g**) as described by (Silva-Filha et al., 1997). BBMFs were solubilized with 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate) according to Silva-Filha et al. (1999). BBMF and CHAPS-solubilized proteins (CHAPS-extract) were stored at -80°C. Protein concentration was determined as described in section 2.1 and the enrichment of proteins from apical cell membranes was evaluated through the detection of α-glucosidase activity (EC 3.2.1.20), according to Ferreira et al. (2014).

2.4 Homologous competition binding assays

Competition assays were performed to evaluate the capacity of radiolabeled (^{125}I) individual Cry48Aa, Cry49Aa and a mixture of these toxins to bind to BBMF, according to Silva-Filha et al. (1997). Labeled individual toxins or a mixture of Cry48Aa/Cry49Aa (1:1) (10 nM) were incubated with BBMF proteins (25 μg) in the absence, or in the presence of increasing concentrations (3, 10, 30, 100, 300, 1000, 3000 nM), of each homologous unlabeled toxin used as competitor, for 16 h at room temperature (RT). After incubation, ^{125}I-toxins bound to BBMF were separated through centrifugation (21,000*g*, 15 min, 4 °C), sediments were rinsed twice with PBS, added to a scintillation cocktail and analyzed using

a scintillation beta counter. Each experimental point was repeated at least four times and the inhibitory concentration of the competitor that reduces the binding response by half (IC_{50}) was determined using GraphPad Prism™ software (GraphPad, La Jolla, CA, USA).

2.5 Toxin overlay assays

As an initial approach to identify the *C*. *quinquefasciatus* midgut proteins that bind to Cry48Aa/Cry49Aa-His toxin, proteins (30-40 µg) from BBMF and CHAPS-extracts were separated on 10% SDS-PAGE and transferred to nitrocellulose Protran® membranes (GE Healthcare, Germany). Membranes were first incubated in TBS-T buffer (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20), containing 5% nonfat dry milk at RT for 1h and then with a mix (100 µg) of Cry48Aa/Cry49Aa-His activated toxins (16 h at 4°C). Unbound toxins were removed by washing with TBS-T buffer (4x 15 min at RT). Membranes were then incubated with a primary monoclonal serum raised against poly-histidine (Sigma-Aldrich, St Louis, MO, USA) (1:5.000, 1 h at RT) followed by washings and incubation with the secondary serum raised against mouse IgG conjugated to horseradish peroxidase (1:10.000, 1 h at RT). After washing, membranes were subjected to chemiluminescence detection using Luminata Forte® (Millipore, Billerica, MA, USA) **to detect binding of the Cry48Aa/Cry49Aa mix through the Cry49-His bound to midgut proteins**. Similar membranes with midgut proteins, but without incubation with the Cry48Aa/Cry49a-His mix, were subjected to immunodetection and were used as negative controls. Assays were conducted in triplicate.

2.6 Pull-down assays

Protein–protein binding assays were also performed using a mix of Cry48Aa/Cry49Aa activated toxins immobilized on CNBr activated sepharose 4B® (GE Healthcare, Uppsala, Sweden) beads adapted from the protocol described by Zhou et al. (2016). Briefly, activated Cry48Aa/Cry49Aa mix (1 mg) was immobilized on beads (500 µL), for 16h at 4°C. After washings (0.2 M $NaHCO_3$ pH 8.3) potential remaining active groups on the resin were blocked (glycine 0.1 M pH 8, 6h at RT) and the coupled Cry48Aa/Cry49Aa beads were re-suspended in a final volume of 500 µL of phosphate-buffered saline (pH 7.4) and stored at 4°C. Pull-down assays were then conducted using Cry48Aa/Cry49Aa beads (50 µL) and CHAPS-extracts (50 µg) incubated for 2 h at 4°C. After incubation, unbound proteins were removed by centrifugation (400*g*, 30 s, 4°C) and beads were washed five times with 500 µL of phosphate-buffered saline pH 7.4/ 1 M NaCl, followed by five washes with 500 µL of phosphate-buffered saline pH 7.4. Proteins that remained bound to the Cry48Aa/Cry49Aa beads were solubilized in Laemmli buffer boiled for 10 min and visualized in 10% SDS-PAGE. Samples of CNBr sepharose beads coupled with 0.1 M Tris-HCl buffer (pH 8.5) were submitted to pull-down assays with CHAPS-extracts and used as negative controls. Gels were stained with Coomassie blue or PlusOne Silver Staining kit® (GE Healthcare). At least three gels for each staining were analyzed.

A second set of pull-down assays was conducted using recombinant Cry49Aa and BinB toxins produced by *E. coli* fused to glutathione S-transferase (GST) and immobilized on glutathione-sepharose 4B™ beads (GE Healthcare, Uppsala, Sweden), according to Romão et al. (2006). For the assays the Cry49Aa-GST beads were pre-incubated with activated recombinant Cry48Aa toxin from Bt for 1 h at RT. After this, Cry49Aa-GST beads were recovered by centrifugation (1.500 *g*, 2 min, 4ºC) and washed three times with BB3 buffer (100 mM KCl /1 mM $MgCl_2$/50 mM HEPES/0.2% Nonidet P-40®/5% glycerol). Sf9 cell

culture medium samples enriched with Cqm1 recombinant protein were obtained as described in Ferreira et al. (2014) and Cqm1 content in samples was estimated based on a standard curve of purified Cqm1 protein immunodetected with an antibody raised against Cqm1 (Romão et al., 2006). Medium samples containing Cqm1 protein (0.015-1.5 μg) were incubated with equivalent amounts (~10 μg) of BinB beads, or Cry49Aa beads, or GST beads (negative control) for 2 h, at RT in BB3 buffer. After incubation beads samples, washed and recovered as described above, were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and subjected to immunoblotting with the antibody anti Cqm1.

2.7 Mass spectrometry analysis

Protein samples from a silver stained gel were sectioned into eight parts according to the molecular weight range and sent for LC-MS/MS analysis in the Proteomics and Mass Spectrometry Facility (PAMS) from the University of Georgia. In-gel digestions of these bands were performed. The tryptic peptides were analyzed by an Orbitrap Elite mass spectrometer coupling with a Proxeon nanoLC system (Thermo Scientific, Waltham, MA, USA). The data-dependent acquisition (DDA) Top 8 method was used to acquire MS data. Protein identification and characterization of modifications were performed using Thermo Proteome Discoverer (version 1.4) with Mascot (Matrix Science, London, UK). The NCBI proteome reference database for *C*. *quinquefasciatus* was downloaded on October 10, 2016. The searched protein database was complete, but redundant; the 39,875 entry database was composed of 18,883 entries from the NCBI reference protein database for *C*. *quinquefasciatus* and Refseq, UniProt and EMBL *C*. *quinquefasciatus* protein entries.

254

255 **3. Results**

256 *3.1 Production of Cry toxins*

257 Crystal/spore samples produced individually in the recombinant Bt strains were the sources of
258 Cry49Aa (≈53 kDa) and Cry48Aa (≈135 kDa), yielding protoxins with their expected molecular
259 weights (Fig. 1A, lanes 1 and 3). *In vitro* processing of Cry49Aa protoxin produced a major
260 polypeptide of ≈44 kDa while Cry48Aa processing resulted in fragments of ≈68 and 46 kDa
261 (Fig. 1A, lanes 2 and 4). Crystal/spores from both recombinant Bt strains were also processed
262 together, **as described in section 2.1**, and **the mix of activated proteins showed a similar**
263 **activation pattern to that of the individually processed toxins (Fig. 1A, lane 5). The mix of**
264 **activated Cry48Aa/Cry49Aa toxins was employed for pull-down assays, based on the**
265 **results of competition binding assays described below in section 3.2**. Cry49Aa protoxin
266 containing a poly-histidine tag (Cry49Aa-His) was also produced in *E. coli* as a recombinant
267 protein of ≈53 kDa (Fig. 1B, lane 1). A mix of protoxins produced in Bt and *E. coli*
268 (Cry48Aa/Cry49Aa-His) respectively, (Fig. 1B lane 2) processed *in vitro* exhibited the pattern
269 (Fig. 1B, lane 3) (Cry48Aa, ≈68 and 46 kDa; Cry49Aa ≈44 kDa) as observed before (Fig. 1A,
270 lane 5). Similarly, this Cry48Aa/Cry49Aa-His mixture of activated toxins was employed to
271 perform overlay assays. **Cry49Aa-GST and BinB-GST were successfully produced and**
272 **showed a expected molecular of around 80 kDa (data not shown).**

273

274 *3.2 Cry toxins binding to midgut proteins*

275 BBMFs used in assays showed a protein concentration of 5.1±0.8 µg/µl and the enrichment of α-
276 glucosidase activity in the BBMF, compared to the initial whole larvae extract used, was 3.3±0.9

277 fold. **First, homologous competition assays were performed to evaluate the binding capacity**
278 **of Cry48Aa and Cry49Aa toxins to *C. quinquefasciatus* midgut brush border proteins**
279 **(BBMF). Individual labelled toxins and the mixture of labeled Cry48Aa/Cry49Aa bound**
280 **specifically to BBMF and were displaced in presence of the respective unlabeled**
281 **homologous competitors (Fig. S1). Labeled Cry48Aa/Cry49Aa showed a lower IC_{50} (41**
282 **nM), compared to those observed for Cry48Aa (83 nM) and Cry49Aa (95 nM) individual**
283 **labeled toxins (Fig. S1), indicating that the binding affinity of the Cry48Aa/Cry49Aa mix to**
284 ***C. quinquefasciatus* BBMF is higher than the individual toxins.**

285 The binding ability of the activated Cry48Aa/Cry49Aa-His mixture to the *C.*
286 *quinquefasciatus* BBMF proteins was investigated through overlay assays. Cry48Aa/Cry49Aa-
287 His mix recognized midgut proteins of about 52, 58, 65, 73, 80, 90, 100-125 kDa, **based on the**
288 **immunodetection of bound Cry49Aa-His toxin** (Fig. 2A, lanes 1, 3). **Most of the proteins**
289 **observed in these BBMF samples were also detected in solubilized** midgut proteins (CHAPS-
290 extract) (Fig. 2A, lanes 2, 4). Cry49Aa-His, included among the SDS-PAGE samples as positive
291 control, was recognized by the monoclonal antibody raised against the poly-histidine tail of this
292 protein (Fig. 2A, lane 5). In parallel the immunodetection of midgut proteins without incubation
293 with the Cry48Aa/Cry49Aa-His mix, was used as a negative control. In this assay two major
294 proteins (≈44 and 48 kDa) from BBMF and CHAPS-extract (Fig. 2B, lanes 1-4) were recognized
295 by the anti-His antibody and they were discarded from further analysis as potential binding
296 proteins as well as proteins whose molecular weights were lower than 40-50 kDa since they
297 seemed to be a result of non-specific binding, as observed on negative control blot overlays.
298
299 *3.3 Identification of Cry48Aa/Cry49Aa binding proteins*

300 A proteomic approach was performed to identify Cry48Aa/Cry49Aa binding proteins from *C*.
301 *quinquefasciatus* solubilized BBMF proteins. Pull-down assays were performed between midgut
302 CHAPS-solubilized proteins (Fig. 3, lane 1) and activated Cry48Aa/Cry49Aa toxins immobilized
303 on CNBr-beads (Fig. 3, lane 2). Pulled-down, i.e. extracted proteins were separated by 10%
304 SDS-PAGE and then visualized by silver staining. Separation of proteins from the
305 Cry48Aa/Cry49Aa beads alone (not incubated with gut extracts) showed major bands of ≈ 68, 46
306 and 44 kDa, a profile similar to the activated Cry48Aa/Cry49Aa toxins (Fig. 1A, lane 5). A band
307 of ≈ 90 kDa is possibly a Cry49Aa dimer that is stable under the conditions of this assay. Midgut
308 proteins that bound to the immobilized toxins (Fig. 3, lane 3) showed apparent molecular weights
309 consistent with those immunodetected as potential ligands in the overlay assays (Fig. 2A, lanes
310 1-4). When midgut solubilized proteins were incubated with CNBr beads that had been prepared
311 by incubation with Tris buffer only (without Cry48Aa/Cry49Aa), used as a negative control, no
312 proteins bound to the beads were visualized by silver staining (data not shown). Lane 3 from the
313 pull-down assay (Fig. 3) was cut into eight sections that were subjected to LC-MS/MS analysis.
314 A list of 266 *C*. *quinquefasciatus* proteins from gel sections (1-8) with a significance score
315 higher than 67 and the number of unique peptides greater than 2 as threshold, is presented in the
316 supplementary table 1 (Table S1). From this dataset a group of 49 proteins was selected (Table
317 1), in most cases, because they belong to a class previously reported as Cry receptors/ligands, or
318 they were already cited as molecules potentially involved in the mode of action of those toxins.
319 Proteins that were detected in more than one gel section were cited in that section corresponding
320 to its expected molecular weight (Table 1). When a protein occurred in sections where the
321 molecular weight range differs from the predicted weight, they were cited in the section where
322 they displayed the highest scores. Gel section 8 (30-45 kDa) displayed the highest number of

identified proteins and this is likely to be related to the presence of polypeptides resulting from the degradation of higher molecular weight proteins. Several selected proteins from Table 1 belong to protein classes that have been described as functional receptors to 3-domain Cry toxins, including aminopeptidases (APN), maltases and alkaline phosphatases (ALPs). Eight of these proteins are orthologs of functional receptors for Cry toxins from *B. thuringiensis* svar. *israelensis* or *B. thuringiensis* svar. *jegathesan* previously identified in *Aedes aegypti* or *Anopheles gambiae* larvae (Table 2). These proteins have molecular weights that were consistent with those of the bands detected in the binding assays. APN molecules were detected in all sections analysed regardless of the fact that their predicted molecular weights are greater than 100 kDa. Maltases including α-glucosidases also were found in almost all sections and the *C. quinquefasciatus* maltase 1 (Cqm1), the receptor of the Bin toxin, was detected in sections 5 and 6. ALPs, on the other hand, were exclusively detected in section 5. Proteins already described as ligands to Cry toxins as apolipophorin, actin, dipeptidyl-peptidase, glyceraldehyde-3-phosphate dehydrogenase, glucosil transferase, myosin-Id, prohibitin, ATP synthase (alpha or beta subunit), and V-ATP synthase (subunit E or H) were found with high score in one or more sections analyzed. In addition, proteins not previously characterized as Cry binding proteins were identified such as aldehyde dehydrogenase, calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type, carboxylesterase-6, carboxypeptidase A1, fasciclin, maltose phosphorylase, panthetheinase, sodium-potassium-transporting ATPase alpha chain, sodium-potassium-dependent ATPase beta-2 subunit, transferrin, truncated ER mannose-binding lectin, and vanin-like protein 1.

The identification of Cqm1 among the ligands led to the investigation of its role in the mode of action of Cry48Aa/Cry49Aa since this toxin is active against *L. sphaericus*

resistant larvae due to the loss of Cqm1 (de Melo et al., 2009). Pull-down assays between recombinant Cqm1 protein and Cry49Aa-GST immobilized on sepharose beads previously pre-incubated with Cry48Aa, was compared with the respective assay between Cqm1 and the BinB subunit. Cqm1 bound to BinB-GST, as expected, while Cqm1 bound to Cry49Aa-GST but binding was only detected using Cqm1 amounts about 30-fold higher, compared to that used to detect binding to BinB (Fig. S2).

4. Discussion

Three-domain Cry toxins might require midgut processing for their insecticidal activity and Bin protoxins are also converted in this way (Berry, 2012; Tabashnik et al., 2015). In this study a mixture of Cry48Aa and Cry49Aa protoxins processed *in vitro* yielded a pattern of activated toxins similar to that observed for individually processed toxins (Jones et al., 2008). **These activated individual subunits showed the capacity to bind to *C. quinquefasciatus* midgut, in agreement with Guo et al. (2016). The IC_{50} values determined in our work were higher but it is likely that differences in the methodology, biotinylated Cry toxins and fresh BBMF employed by Guo et al. (2016), might have improved the resolution of these binding assays. On the other hand our study showed that the Cry48Aa/Cry49Aa mixture bound with higher affinity than the individual subunits and this indicated that both Cry48Aa and Cry49Aa toxins are required to attain maximal binding affinity to *C. quinquefasciatus* midgut, in contrast to Bin binary toxin whose binding ability relies exclusively on the BinB subunit (Charles et al., 1997).** Cry48Aa/Cry49Aa interacts with larval midgut and our study shows a set of *C. quinquefasciatus* proteins that bound to Cry48Aa/Cry49Aa toxin using overlay and pull-down assays. Although our study showed midgut ligands when both Cry48Aa/Cry49Aa

toxins were employed in the assays, the role of each toxin for this interaction requires further investigation.

Cry48Aa/Cry49Aa ligands identified in this study include molecules belonging to protein classes previously characterized as receptors of other Cry toxins, plus other proteins that may be potentially involved in the mode of action. Several forms of APNs/metalloproteases were detected as ligands, which is consistent with previous studies that have demonstrated that APNs act as Cry toxin receptors. In mosquito larvae, for instance, APNs were identified as Cry11Ba, Cry11Aa and Cry4Ba receptors in *Anopheles quadrimaculatus* (Abdullah et al., 2006), *An. gambiae* (Zhang et al., 2008) and *Aedes aegypti* (Aroonkesorn et al., 2015; Chen et al., 2009; Chen et al., 2013). The apparent sizes of several identified APNs suggested proteolytic degradation and/or formation of dimers with high-molecular weight. Some of these *C. quinquefasciatus* APNs are orthologs, with more than 60% identity, to known Cry receptors cited above, which reinforces their possible role as Cry48Aa/Cry49Aa receptors. Maltases, including α-glucosidases and α-amylases, although less numerous than APNs, were also identified and proteins from this class have been reported as receptors for Cry11Aa and Cry4Ba toxins in *An. albimanus* (Fernandez-Luna et al., 2010) and Cry11Ba toxin in *An. gambiae* (Zhang et al., 2013). Ortholog α-glucosidases Cpm1 and Cqm1 are proven, and Agm3 is a putative receptor of Bin toxin in *C. pipiens*, *C. quinquefasciatus* and *An. gambiae*, respectively (Darboux et al., 2001; Opota et al., 2008; Romão et al., 2006). Agm3 was also reported as a receptor to Cry11Ba toxin in *An. gambiae* (Zhang et al., 2013) and Cqm1, the Bin receptor in *C. quinquefasciatus,* was identified among the Cry48Aa/Cry49Aa ligands in this study. Cry48Aa/Cry49Aa is active against Bin-resistant larvae deprived of the Cqm1 α-glucosidase and this finding might indicate that Cry48Aa/Cry49Aa binding to Cqm1 is not specific or that the toxin can bind alternative

receptors without dependence on Cqm1. **Comparative binding assays performed in this study confirmed that Cqm1 binding to Cry48Aa/Cry49Aa is much more limited than that observed to the BinB subunit from the Bin toxin**. In contrast to APNs and maltases, only three alkaline phosphatases (ALPs) were detected exclusively in gel section 5 (50-62 kDa) and one of them is an ortholog of an *Ae*. *aegypti* ALP which is a binding protein for Cry4Ba (Bayyareddy et al., 2009). ALPs have been identified as receptors for Cry11Aa and Cry4Ba in *Ae*. *aegypti* (Dechklar et al., 2011; Fernández et al., 2006) and Cry11Ba in *An*. *gambiae* (Hua et al., 2009). Cadherins play a major role for the binding and oligomerization of some 3-domain Cry toxins (Bravo et al., 2004) but they were not identified in this study. This could be due the low abundance of cadherins in mosquito midgut and their relative instability in brush border preparations. Whether or not cadherins have a role in Cry48/49 action merits more attention.

Some proteins identified in our study were shown to be associated with lipid rafts that are enriched in glycosphingolipids, cholesterol and GPI-anchored proteins; functionally they are proposed to be involved in signal transduction, sorting and trafficking of proteins and pathogens (Bayyareddy et al., 2012). Among them apolipophorin, identified in this study, was previously found as a ligand for the Cry8Ea toxin (Shu et al., 2015), glucosyl transferase was identified as binding protein for Cry1Aa (Zhou et al., 2016), prohibitin was detected as ligand for Cry4Ba and Cry3Aa (Kuadkitkan et al., 2012; Ochoa-Campuzano et al., 2013). Other proteins associated with lipid rafts of *Ae*. *aegypti* were identified in our study including calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type, carboxylesterase-6, fasciclin, maltose phosphorylase and transferrin, however, their functional relevance to the Cry toxin mode of action is unknown (Bayyareddy et al., 2012). Vanin-like protein 1 and pantetheinase are members of the Vanin family that can be expressed as membrane-associated proteins (Pitari et

al., 2000). Vanin-1 proteins, as well as aldehyde dehydrogenases, are associated with lipid rafts in *Ae*. *aegypti* and although their function has been scarcely studied, it could be related to the regulation of responses to oxidative stress, detoxification processes and recycling (Bayyareddy et al., 2012; Pitari et al., 2000; Popova-Butler and Dean, 2009).

Several proteins identified as integral membrane components according to their Gene Ontology (GO) classifications were extracted by Cry48Aa/49Aa beads including sodium-potassium-transporting ATPase alpha chain, sodium-potassium-dependent ATPase beta-2 subunit, and truncated ER mannose-binding lectin. Dipeptidyl-peptidase, a membrane component found in our study, was already identified as a Cry1Aa ligand (Zhou et al., 2016). Consistent with other studies that detected intracellular proteins forming part of the cell cytoskeleton as Cry toxin ligands, some, such as actin, myosin and glyceraldehyde-3-phosphate dehydrogenase (Bayyareddy et al., 2009; Chen et al., 2010; Krishnamoorthy et al., 2007; Shu et al., 2015; Zhou et al., 2016), were also found in this investigation. However, further studies remain to be performed to understand the function of these proteins in Cry48Aa/Cry49Aa mode of action. Among the mitochondrial proteins detected in our study the most relevant were V-ATPases, as they have been identified as Cry toxin binding proteins in different insects, although their localization on the cytoplasmic side of the plasma membrane seems inconsistent with a direct receptor function (Bayyareddy et al., 2009; Chen et al., 2010; Krishnamoorthy et al., 2007). Detection of the α- and β-ATP synthases is also consistent with the literature as they are reported as Cry4Ba and Cry1Ac toxin ligands (Bayyareddy et al., 2009; Zhou et al., 2016). However, the ATP synthase complex is related with ATP generation in mitochondrial membrane and their presence has been considered as evidence of contamination in midgut preparations.

This study reveals a set of ligands of the Cry48Aa/Cry49Aa toxin in *C. quinquefasciatus* that are described as molecules involved in the mode of action of Cry toxins in different target insects. Some of them were identified as toxin ligands in binding assays and it is possible that they could be involved in the intracellular mode of action of Cry toxins rather than being membrane receptors. Molecules such as APNs, ALPS and maltases have been characterized as toxin receptors based on their localization as membrane-bound proteins and their capacity to bind specifically to the toxins with high affinity, as monomers or oligomers, in order to display toxicity in insect midguts. This study provides evidence of binding ligands for Cry48Aa/Cry49Aa toxin in *C. quinquefasciatus* midgut and further work is necessary to elucidate their role on its mode of action since the unique composition of Cry48Aa/Cry49Aa, consisting of a 3D-Cry like and Bin-like subunits, could display distinct features compared to models already described for toxins from these groups.

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460 **Supplementary data**

461 **Table S1.** Complete data set of *Culex quinquefasciatus* midgut proteins found as ligands of
462 Cry48Aa/Cry49Aa toxin through pull-down assays and identification by mass spectrometry.

463

464 **Figure S1. Homologous competition binding assays between labeled (^{125}I-) Cry48Aa,**
465 **Cry49Aa, or a mixture of both toxins (10 nM) with midgut brush border membrane**
466 **fractions (25 μg) from *Culex quinquefasciatus* larvae in the absence, or in the presence, of**
467 **respective unlabeled toxins (3-3000 nM). Maximum binding corresponds to the binding**
468 **observed in the absence of competitor. The competitor concentration that displaces 50% of**
469 **the ^{125}I-bound toxin (IC_{50}) is indicated. Each point is the mean of, at least, four**
470 **experimental replicates.**

471

472 **Figure S2. Pull-down assay to evaluate the binding of the recombinant Cqm1 protein to the**
473 **recombinant Cry48Aa/Cry49Aa-GST toxin (A) , BinB-GST toxin (B) or GST (C, negative**
474 **control) immobilized on sepharose beads. After incubation, beads were washed and bound**
475 **proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and**
476 **subjected to immunodetection with an antibody raised against Cqm1 protein. P. Cqm1**
477 **protein (0.15 μg). MW molecular weight in kDa.**

478

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659 **FIGURE LEGENDS**

660

661 **Fig. 1**. Cry48Aa and Cry49Aa recombinant proteins separated in 10% SDS-PAGE and
662 visualized with Coomassie blue. **A.** Proteins expressed in *Bacillus thuringiensis* svar. *israelensis*
663 4Q7 strain, Cry49Aa solubilized (1) and activated (2), Cry48Aa solubilized (3) and activated (4),
664 mixture of Cry48Aa/Cry49Aa activated together (5). **B**. Cry49Aa-His produced in *Escherichia*
665 *coli* (1), mix of protoxins Cry48Aa/Cry49Aa-His (2), mix Cry48Aa/Cry49Aa-His activated
666 together (3). MW molecular weight in kDa. * protoxins and their activated forms.

667

668 **Fig. 2.** Overlay assays performed between *Culex quinquefasciatus* midgut proteins and
669 Cry48Aa/Cry49Aa-His. Midgut proteins from BBMV (30-40 μg, lanes 1, 3) and after
670 solubilization with CHAPS (30-40 μg, lanes 2, 4) were separated on 10% SDS-PAGE,
671 transferred to a nitrocellulose membrane, incubated with activated Cry48Aa/Cry49Aa-His (2 μg)
672 (A) or without toxin (B) and subjected to immunodetection with a monoclonal anti-poly-histidine
673 antibody to detect bound Cry49-His. Sample of Cry49-His protein (lane 5). MW molecular
674 weight in kDa. Major proteins detected (*) and those (º) observed in negative control (B).

675

676 **Fig. 3.** Proteins identified by pull-down assay. Pull-down assays were performed between
677 solubilized *Culex quinquefasciatus* migdut proteins (A) and a mix of activated
678 Cry48Aa/Cry49Aa toxins immobilized on CNBr-beads (B) and resulting bound proteins to toxin
679 beads (C). Samples were separated in 10% SDS-PAGE and silver stained. MW molecular weight
680 in kDa. The eight sections from lane 3 were subjected to mass spectrometry. Cry49Aa putative
681 dimer (*).

Table 1. Selected *Culex quinquefasciatus* midgut proteins detected as ligands of Cry48Aa/Cry49Aa toxin through pull-down assays and identification by LC-MS/MS.

Section	Accession no.	Description	Score	Coverage (%)	No. unique peptides	Predicted MW (kDa)	Detection sections
1 (110-180 kDa)	EDS27419.1[a]	protease m1 zinc metalloprotease	365.96	18.00	9	113.2	2,3,4,5,7,8
	EDS27892.1	calcium-transporting ATPase sarcoplasmic/ER type	146.88	7.74	4	88.8	2,4
2 (90-110 kDa)	EDS27418.1[a]	protease m1 zinc metalloprotease	93.25	11.25	6	101.2	
	EDS40798.1	CHP[c]	78.46	2.96	2	100.1	
	EDS36841.1[a]	aminopeptidase N	74.67	8.38	3	105.0	
3 (70-90 kDa)	EDS38951.1[a]	alpha-glucosidase	619.19	40.83	25	69.4	4,5,6,8
	EDS30018.1	apolipophorins	374.88	6.41	18	366.8	4,5
	EDS38952.1	alpha-glucosidase	374.85	25.37	12	70.6	4,5
	EDS32575.1	maltose phosphorylase	277.49	18.29	10	85.4	
	EDS45210.1	dipeptidyl peptidase 4	250.82	19.19	12	84.1	
	EDS32578.1	maltose phosphorylase	206.18	15.58	11	85.1	
	EDS32127.1	alpha-glucosidase	199.35	20.66	10	70.7	
	EDS32576.1	maltose phosphorylase	172.15	7.89	5	84.8	
	EDS26147.1	sodium/potassium-transporting ATPase alpha chain	97.64	5.44	3	80.3	5,6,7,8
	EDS35643.1	glutamyl aminopeptidase	93.53	6.02	5	116.5	

	EDS37148.1	dipeptidyl-peptidase	88.24	6.05	5	88.2	2
4 (62-70 kDa)	EDS38950.1[a]	maltase 1	183.05	11.26	6	69.4	5
	EDS45922.1	pantetheinase	113.26	16.47	9	57.1	
5 (50-62 kDa)	EDS35272.1	CHP[c]: ATP synthase subunit alpha	1914.86	59.17	40	59.3	6,8
	EDS27254.1	ATP synthase beta subunit	1100.18	56.16	21	54.6	2,6,8
	EDS29323.1	alkaline phosphatase	563.81	27.30	14	62.9	
	ABC59609.1[b]	maltase 1 (Cqm1)	528.27	29.31	18	66.2	6
	EDS39442.1	V-type ATP synthase beta chain	515.83	38.82	15	54.7	8
	EDS29322.1[a]	alkaline phosphatase	485.67	17.04	13	100.6	
	EDS29972.1	ATP synthase alpha subunit vacuolar	474.63	31.76	15	68.1	3,4,5,6,8
	EDS27420.1[a]	protease m1 zinc metalloprotease	215.56	8.23	6	102.7	4
	EDS35286.1	CHP: Aldehyde dehydrogenase	200.25	12.86	2	58.1	
	EDS29320.1	alkaline phosphatase	186.96	15.91	5	28.9	
	EDS45921.1	Vanin-like protein 1	166.86	7.21	4	61.6	
	EDS28386.1	transferrin	158.64	15.15	5	51.4	4
	EDS42579.1	glucosyl transferase	98.98	6.01	3	58.2	
	EDS44961.1	myosin-Id	113.40	3.18	2	77.9	6
	EDS31007.1	aminopeptidase N	68.48	4.43	4	105.4	
6 (48-50 kDa)	EDS31006.1[a]	aminopeptidase N	984.52	13.66	23	210.0	2,3,5,8
	EDS27170.1	truncated ER mannose-binding lectin	279.17	18.26	8	53.4	5,7,8

	EDS35706.1	vacuolar ATP synthase subunit H	243.87	15.19	6	54.5	8
7 (45-48 kDa)	EDS44431.1	fasciclin	71.84	7.08	2	48.4	
8 (30-45 kDa)	EDS26297.1	Ca-transporting ATPase sarcoplasmic/ER type	2335.41	32.46	4	109.1	3,5,6
	EDS44094.1	actin 1	1699.07	48.94	3	41.7	
	EDS25844.1	actin-2	1689.34	47.61	6	41.6	3,5
	EDS38275.1	CHP [c]	755.20	34.77	10	33.0	3,5
	EDS28370.1	Na/K-dependent ATPase beta-2 subunit	602.81	34.05	10	37.9	
	EDS28367.1	Na/K-dependent ATPase beta-2 subunit	521.83	35.65	10	36.2	
	EDS36304.1	Na/K-dependent ATPase beta-2 subunit	447.15	24.66	7	33.4	
	EDS29666.1	vacuolar ATP synthase subunit e	389.43	27.88	7	25.8	
	EDS45475.1	CHP: Glyceraldehyde-3-phosphate dehydrogenase	225.43	20.78	6	35.4	
	EDS28502.1	carboxylesterase-6	177.38	5.06	3	71.1	6
	EDS26618.1	prohibitin-2	164.97	15.38	5	33.1	
	EDS34662.1	carboxypeptidase A1	96.08	9.18	3	48.4	

[a] Orthologs identified in *Aedes aegypti* or *Anopheles* species known as functional receptors to Cry toxins, details are shown in Table 2.

[b] *Culex quinquefasciatus* maltase 1 (Cqm1) receptor of the Binary toxin.

[c] CHP:Conserved hypothetical protein.

Table 2. *Culex quinquefasciatus* midgut proteins identified as potential ligands of Cry48Aa/Cry49Aa toxins and their orthologs in *Aedes aegypti* or *Anopheles* species that were described as receptors of other Diptera-active Cry toxins.

Accession no.	Description	Orthologs id	Identity (%)	Specie	Toxin	Reference
EDS31006.1	aminopeptidase N	AAEL008155	31.4	*Ae. aegypti*	Cry11Aa	(Chen et al., 2013)
EDS36841.1	aminopeptidase N	AAEL005808	63.6	*Ae. aegypti*	Cry4Ba	(Saengwiman et al., 2011)
EDS27418.1	protease m1 zinc metalloprotease	AAEL012783	70.9	*Ae. aegypti*	Cry4Ba	
EDS27419.1	protease m1 zinc metalloprotease	AAEL012778	65.1	*Ae. aegypti*	Cry11Aa	(Chen et al., 2009b)
					Cry4Ba	(Saengwiman et al., 2011)
EDS27420.1	protease m1 zinc metalloprotease	AAEL012776	63.4	*Ae. aegypti*	Cry4Ba	(Bayyareddy et al., 2009)
EDS38951.1	alpha-glucosidase	AALB015771	64.3	*An. albimanus*	Cry11Aa	(Fernandez-Luna et al., 2010)
		AGAP008963	64.9	*An. gambiae*	Cry11Ba	(Zhang et al., 2013)
EDS38950.1	maltase 1	AALB015771	61.3	*An. albimanus*	Cry11Aa	(Fernandez-Luna et al., 2010)
		AGAP008963	62.8	*An. gambiae*	Cry11Ba	(Zhang et al., 2013)
EDS29322.1	alkaline phosphatase	AAEL003313	67.1	*Ae. aegypti*	Cry4Ba	(Bayyareddy et al., 2009)

Table supplementary 1

Supplementary Fig. 1. Complete data set of *Culex quinquefasciatus* midgut proteins found as ligands of Cry48Aa/Cry49Aa toxin through pull-down assays and identification by mass spectrometry.

Section	Accession N°	Description	Score	Coverage (%)	N° Unique Peptides	N° AAs	Predicted MW [kDa]	pI
1	EDS27419.1	protease m1 zinc metalloprotease	365.96	18.00	9	1011	113.2	5.12
	EDS27892.1	calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	146.88	7.74	4	814	88.8	6.00
2	EDS31006.1	aminopeptidase N	316.58	14.69	13	1852	210.0	5.08
	EDS27419.1	protease m1 zinc metalloprotease	271.97	14.05	8	1011	113.2	5.12
	EDS27892.1	calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	144.70	13.27	5	814	88.8	6.00
	EDS27418.1	protease m1 zinc metalloprotease	93.25	11.25	6	898	101.2	5.14
	EDS37148.1	dipeptidyl-peptidase	90.93	10.09	4	793	88.2	6.47
	EDS40798.1	conserved hypothetical protein	78.46	2.96	2	911	100.1	7.30
	EDS41405.1	angiotensin-converting enzyme	78.22	3.09	2	1229	142.8	5.22
	EDS27254.1	ATP synthase beta subunit	77.51	8.22	2	511	54.6	5.12
	EDS36841.1	aminopeptidase N	74.67	8.38	3	919	105.0	5.01
3	EDS29972.1	ATP synthase alpha subunit vacuolar	744.99	51.63	23	614	68.1	5.39
	EDS27419.1	protease m1 zinc metalloprotease	632.33	26.51	20	1011	113.2	5.12
	EDS38951.1	alpha-glucosidase	619.19	40.83	25	605	69.4	5.20
	EDS39731.1	78 kDa glucose-regulated protein	415.13	28.31	16	657	72.3	5.20
	EDS30018.1	apolipophorins	374.88	6.41	18	3324	366.8	7.36
	EDS38952.1	alpha-glucosidase	374.85	25.37	12	611	70.6	5.31
	EDS35981.1	endoplasmin	354.28	25.69	20	794	91.0	4.98

EDS26297.1	calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type	346.17	14.97	13	995	109.1	5.59
EDS34040.1	fatty acid oxidation complex subunit alpha	329.63	27.69	19	744	79.7	9.06
EDS35048.1	conserved hypothetical protein	308.85	27.29	14	645	71.4	6.86
EDS26512.1	succinate dehydrogenase flavoprotein subunit. mitochondrial	278.70	19.21	9	661	72.1	6.46
EDS32575.1	maltose phosphorylase	277.49	18.29	10	760	85.4	5.78
EDS31006.1	aminopeptidase N	258.73	6.26	10	1852	210.0	5.08
EDS45210.1	dipeptidyl peptidase 4	250.82	19.19	12	745	84.1	5.01
EDS32578.1	maltose phosphorylase	206.18	15.58	11	764	85.1	5.67
EDS32127.1	alpha-glucosidase	199.35	20.66	10	610	70.7	4.92
EDS32576.1	maltose phosphorylase	172.15	7.89	5	760	84.8	6.37
EDS32138.1	heat shock 70 kDa protein cognate 4	140.16	9.16	3	655	71.4	5.52
EDS42649.1	long-chain-fatty-acid coa ligase	139.79	10.06	6	696	78.0	8.18
EDS39919.1	disulfide isomerase	135.33	11.16	5	493	55.4	4.91
EDS33460.1	carnitine O-palmitoyltransferase 2. mitochondrial	132.79	14.92	9	657	74.5	7.97
EDS42654.1	5' nucleotidase	120.83	11.54	5	546	59.9	4.84
EDS39080.1	beta-galactosidase	112.60	9.69	6	650	73.7	4.89
EDS36767.1	CD98hc amino acid transporter protein	112.54	6.73	4	639	70.2	5.08
EDS25844.1	actin-2	106.42	18.09	3	376	41.6	5.48
EDS40938.1	integrin alpha-ps	103.28	4.86	4	1112	124.5	5.53
EDS38275.1	conserved hypothetical protein	100.53	17.22	4	302	33.0	9.72
EDS26147.1	sodium/potassium-transporting ATPase alpha chain	97.64	5.44	3	735	80.3	5.67
EDS26794.1	conserved hypothetical protein	94.97	3.06	7	2324	249.4	4.96
EDS35643.1	glutamyl aminopeptidase	93.53	6.02	5	1030	116.5	6.25
EDS37148.1	dipeptidyl-peptidase	88.24	6.05	5	793	88.2	6.47

	EDS31874.1	neurotactin	84.61	5.83	4	857	94.2	5.01
	EDS35272.1	conserved hypothetical protein	76.96	8.53	4	551	59.3	8.94
	EDS36798.1	long-chain-fatty-acid coa ligase	76.18	6.47	4	649	72.3	7.93
	EDS28166.1	elongation factor 2	72.03	4.07	4	1031	114.4	6.71
	EDS31200.1	nodal modulator 3	70.21	2.88	2	868	93.8	6.54
4	EDS38950.1	maltase 1	382.03	37.42	20	604	69.4	4.94
	EDS27892.1	calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	206.55	13.76	10	814	88.8	6.00
	EDS28386.1	transferrin	176.12	21.21	8	462	51.4	6.67
	EDS38952.1	alpha-glucosidase	156.43	27.00	14	611	70.6	5.31
	EDS25721.1	electron transfer flavoprotein-ubiquinone oxidoreductase	139.13	16.31	9	607	66.1	6.54
	EDS36767.1	CD98hc amino acid transporter protein	132.75	11.42	7	639	70.2	5.08
	EDS39919.1	disulfide isomerase	129.60	8.72	4	493	55.4	4.91
	EDS38951.1	alpha-glucosidase	121.82	11.90	7	605	69.4	5.20
	EDS45922.1	pantetheinase	113.26	16.47	9	516	57.1	5.39
	EDS27419.1	protease m1 zinc metalloprotease	101.76	4.45	4	1011	113.2	5.12
	EDS27420.1	protease m1 zinc metalloprotease	99.42	9.34	7	899	102.7	5.59
	EDS29972.1	ATP synthase alpha subunit vacuolar	91.37	11.40	5	614	68.1	5.39
	EDS42654.1	5' nucleotidase	82.25	8.97	5	546	59.9	4.84
	EDS30018.1	apolipophorins	78.86	1.11	3	3324	366.8	7.36
5	EDS35272.1	conserved hypothetical protein - ATP synthase subunit alpha	1914.86	59.17	40	551	59.3	8.94
	EDS27254.1	ATP synthase beta subunit	1100.18	56.16	21	511	54.6	5.12
	BAI77924.1	cytochrome P450	923.53	48.39	24	496	56.3	7.69
	EDS31872.1	lactase-phlorizin hydrolase	779.33	44.92	22	532	61.4	5.20
	EDS26297.1	calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type	660.27	22.61	2	995	109.1	5.59

EDS27419.1	protease m1 zinc metalloprotease	596.77	20.57	17	1011	113.2	5.12
EDS29323.1	alkaline phosphatase	563.81	27.30	14	564	62.9	5.01
ABC59609.1	maltase 1	528.27	29.31	18	580	66.2	5.95
EDS39442.1	V-type ATP synthase beta chain	515.83	38.82	15	492	54.7	5.49
EDS29322.1	alkaline phosphatase	485.67	17.04	13	904	100.6	5.63
EDS36767.1	CD98hc amino acid transporter protein	485.29	25.20	17	639	70.2	5.08
EDS29972.1	ATP synthase alpha subunit vacuolar	474.63	31.76	15	614	68.1	5.39
EDS31870.1	lactase-phlorizin hydrolase	453.52	19.35	11	920	105.6	5.27
EDS35287.1	conserved hypothetical protein	416.13	26.74	8	460	51.2	7.08
EDS37279.1	croquemort	375.07	24.31	10	469	52.6	4.67
EDS42910.1	disulfide isomerase	347.91	35.54	14	484	53.8	6.25
EDS38951.1	alpha-glucosidase	339.73	20.83	12	605	69.4	5.20
EDS25844.1	actin-2	313.78	37.77	4	376	41.6	5.48
EDS45363.1	cytochrome P450	310.42	30.46	16	522	58.6	7.15
EDS36420.1	cytochrome p450 family protein 44A1	286.50	27.05	13	499	57.6	8.62
EDS34125.1	conserved hypothetical protein	271.63	31.91	2	376	41.8	5.48
EDS38514.1	sarcalumenin	248.99	8.66	7	958	107.6	3.93
EDS31006.1	aminopeptidase N	241.43	5.62	9	1852	210.0	5.08
EDS35981.1	endoplasmin	235.94	14.99	10	794	91.0	4.98
EDS27504.1	5' nucleotidase	219.42	18.77	9	554	60.8	5.53
EDS27420.1	protease m1 zinc metalloprotease	215.56	8.23	6	899	102.7	5.59
EDS27170.1	truncated ER mannose-binding lectin	213.76	13.59	6	471	53.4	6.05
EDS36419.1	cytochrome P450 26B1	211.90	12.83	6	499	57.1	8.37
EDS26147.1	sodium/potassium-transporting ATPase alpha chain	209.18	9.52	6	735	80.3	5.67

EDS35286.1	conserved hypothetical protein	200.25	12.86	2	521	58.1	7.97
EDS38952.1	alpha-glucosidase	195.21	11.95	6	611	70.6	5.31
EDS41433.1	conserved hypothetical protein	188.63	30.43	3	115	13.3	9.25
EDS28872.1	disulfide-isomerase A6	187.22	19.95	7	436	47.5	5.49
EDS29320.1	alkaline phosphatase	186.96	15.91	5	264	28.9	9.32
EDS38275.1	conserved hypothetical protein	185.09	20.86	6	302	33.0	9.72
EDS38950.1	maltase 1	183.05	11.26	6	604	69.4	4.94
EDS38022.1	chitotriosidase-1	178.22	17.27	7	440	48.6	6.81
EDS34537.1	cytochrome P450 12b1. mitochondrial	177.44	8.19	4	525	60.0	7.74
EDS45921.1	Vanin-like protein 1	166.86	7.21	4	555	61.6	5.14
BAK26813.1	cytochrome P450	164.41	13.41	7	537	61.4	7.55
EDS28166.1	elongation factor 2	163.42	5.43	5	1031	114.4	6.71
EDS28386.1	transferrin	158.64	15.15	5	462	51.4	6.67
EDS32421.1	calnexin	158.29	13.41	7	589	66.6	4.65
EDS34510.1	cytochrome P450 9b2	157.50	11.42	5	534	61.3	7.90
EDS40900.1	saccharopine dehydrogenase domain-containing protein	156.83	11.63	5	430	47.7	8.47
EDS32097.1	prolylcarboxypeptidase	150.43	9.38	3	501	57.5	4.83
EDS38838.1	glutactin	150.03	10.70	5	570	63.2	5.94
EDS44930.1	l(2) long form	148.82	4.95	5	1294	148.8	4.58
EDS32098.1	prolylcarboxypeptidase	144.01	15.29	6	726	84.5	4.61
EDS25721.1	electron transfer flavoprotein-ubiquinone oxidoreductase	141.98	6.75	4	607	66.1	6.54
EDS27857.1	UDP-glucuronosyltransferase 2B4	141.38	10.56	4	521	59.0	7.62
AEN19673.1	cytochrome P405 CYP9J40	136.89	15.46	5	524	59.8	8.50
EDS28616.1	catalase	134.43	12.04	5	490	55.0	7.43

	EDS37300.1	olygosaccharyltransferase alpha subunit	128.00	10.46	5	459	51.8	8.22
	B0WYY2.1	RecName: Full=Moesin/ezrin/radixin homolog 1	123.67	9.62	6	572	67.7	5.68
	EDS26512.1	succinate dehydrogenase flavoprotein subunit. mitochondrial	122.42	6.05	3	661	72.1	6.46
	EDS45144.1	dolichyl-diphosphooligosaccharide protein glycotransferase	118.05	5.08	2	453	49.5	5.71
	BAI77921.1	cytochrome P450	116.88	13.41	6	507	57.4	8.46
	EDS44961.1	myosin-Id	113.40	3.18	2	692	77.9	9.14
	BAI77925.1	cytochrome P450	112.08	7.30	4	493	57.3	8.12
	EDS26138.1	cytochrome P450	109.67	6.79	2	501	57.3	7.53
	EDS43476.1	glycogen phosphorylase	106.39	2.73	2	842	96.6	6.37
	EDS30018.1	apolipophorins	105.93	0.72	2	3324	366.8	7.36
	EDS27182.1	prolylcarboxypeptidase	104.81	8.79	4	512	58.3	4.56
	EDS41432.1	conserved hypothetical protein	100.23	7.54	2	305	34.6	7.14
	EDS42579.1	glucosyl transferase	98.98	6.01	3	516	58.2	7.69
	EDS34040.1	fatty acid oxidation complex subunit alpha	97.04	6.45	4	744	79.7	9.06
	EDS35048.1	conserved hypothetical protein	90.01	8.99	5	645	71.4	6.86
	EDS31797.1	synaptotagmin	85.68	3.64	3	825	89.9	6.35
	EDS30010.1	conserved hypothetical protein	77.74	2.04	2	931	103.5	5.02
	EDS41303.1	24-dehydrocholesterol reductase	72.17	3.57	2	504	58.4	8.38
	EDS34513.1	cytochrome P450 9b2	71.72	3.33	2	540	61.8	6.49
	EDS43339.1	brain chitinase and chia	70.89	9.48	5	485	53.5	5.34
	EDS31007.1	aminopeptidase N	68.48	4.43	4	926	105.4	6.19
6	EDS31006.1	aminopeptidase N	984.52	13.66	23	1852	210.0	5.08
	EDS35272.1	conserved hypothetical protein - ATP synthase subunit alpha	613.20	32.30	16	551	59.3	8.94
	EDS27254.1	ATP synthase beta subunit	546.45	33.07	13	511	54.6	5.12

	EDS26297.1	calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type	380.57	13.67	2	995	109.1	5.59
	EDS38798.1	mitochondrial processing peptidase beta subunit	352.77	20.25	10	474	52.2	6.13
	EDS27170.1	truncated ER mannose-binding lectin	279.17	18.26	8	471	53.4	6.05
	EDS35706.1	vacuolar ATP synthase subunit H	243.87	15.19	6	474	54.5	6.14
	EDS34125.1	conserved hypothetical protein	211.62	31.65	3	376	41.8	5.48
	EDS37675.1	conserved hypothetical protein	210.59	11.50	7	887	98.8	5.76
	EDS36615.1	adipocyte plasma membrane-associated protein	182.45	8.56	6	841	92.2	8.06
	EDS28872.1	disulfide-isomerase A6	178.50	15.14	6	436	47.5	5.49
	EDS26631.1	juvenile hormone epoxide hydrolase 1	126.90	9.52	5	462	53.0	6.92
	EDS38951.1	alpha-glucosidase	121.02	7.77	4	605	69.4	5.20
	EDS45881.1	gamma glutamyl transpeptidase	118.93	4.49	2	579	62.2	5.20
	EDS26147.1	sodium/potassium-transporting ATPase alpha chain	117.12	5.44	3	735	80.3	5.67
	EDS32421.1	calnexin	92.60	7.98	4	589	66.6	4.65
	EDS26585.1	enolase	92.12	5.08	2	433	46.6	6.76
	EDS29972.1	ATP synthase alpha subunit vacuolar	91.18	4.40	2	614	68.1	5.39
	EDS42147.1	gram-negative bacteria binding protein	88.09	8.74	3	412	46.7	5.08
	EDS44961.1	myosin-Id	86.80	3.18	2	692	77.9	9.14
	EDS30648.1	arginine kinase	77.89	7.80	3	410	45.1	5.80
	ABC59609.1	maltase 1	76.87	4.14	2	580	66.2	5.95
	EDS28502.1	carboxylesterase-6	75.82	3.96	2	632	71.1	6.19
7	EDS26629.1	juvenile hormone epoxide hydrolase 1	206.65	25.16	7	457	51.7	8.41
	EDS42147.1	gram-negative bacteria binding protein	163.44	12.86	5	412	46.7	5.08
	EDS27093.1	trifunctional enzyme beta subunit	159.37	16.49	7	467	50.3	9.01
	EDS30648.1	arginine kinase	142.07	15.61	6	410	45.1	5.80

	EDS30010.1	conserved hypothetical protein	116.28	4.19	3	931	103.5	5.02
	EDS27170.1	truncated ER mannose-binding lectin	110.70	6.58	3	471	53.4	6.05
	EDS26190.1	adenosine diphosphatase	110.41	12.45	4	466	51.6	7.77
	EDS26631.1	juvenile hormone epoxide hydrolase 1	107.01	7.79	3	462	53.0	6.92
	EDS31071.1	polyadenylate-binding protein 1	85.94	4.62	2	628	69.7	9.47
	EDS26147.1	sodium/potassium-transporting ATPase alpha chain	82.21	4.76	3	735	80.3	5.67
	EDS44431.1	fasciclin	71.84	7.08	2	452	48.4	5.17
	EDS27419.1	protease m1 zinc metalloprotease	67.48	2.57	2	1011	113.2	5.12
8	EDS35272.1	conserved hypothetical protein - ATP synthase alpha subunit	2921.97	55.90	35	551	59.3	8.94
	EDS26297.1	calcium-transporting atpase sarcoplasmic/endoplasmic reticulum type	2335.41	32.46	4	995	109.1	5.59
	EDS34125.1	conserved hypothetical protein	2191.72	56.65	6	376	41.8	5.48
	EDS27254.1	ATP synthase beta subunit	2118.71	46.97	19	511	54.6	5.12
	EDS44094.1	actin 1	1699.07	48.94	3	376	41.7	5.39
	EDS25844.1	actin-2	1689.34	47.61	6	376	41.6	5.48
	EDS30322.1	ATP synthase gamma chain. mitochondrial	1211.21	54.21	17	297	32.8	8.88
	EDS29972.1	ATP synthase alpha subunit vacuolar	765.00	31.76	17	614	68.1	5.39
	EDS34040.1	fatty acid oxidation complex subunit alpha	760.17	26.34	18	744	79.7	9.06
	EDS38275.1	conserved hypothetical protein	755.20	34.77	10	302	33.0	9.72
	EDS26604.1	voltage-dependent anion-selective channel	652.29	45.39	11	282	30.7	8.56
	EDS28370.1	sodium/potassium-dependent ATPase beta-2 subunit	602.81	34.05	10	326	37.9	6.79
	EDS38514.1	sarcalumenin	579.44	13.57	12	958	107.6	3.93
	EDS28367.1	sodium/potassium-dependent ATPase beta-2 subunit	521.83	35.65	10	317	36.2	7.64
	EDS39731.1	78 kDa glucose-regulated protein	520.06	21.46	11	657	72.3	5.20
	EDS30029.1	3-demethylubiquinone-9 3-methyltransferase	494.92	30.07	9	306	34.5	6.93

EDS36550.1	ubiquinol-cytochrome c reductase complex core protein	493.72	21.41	10	439	45.3	8.84
EDS40274.1	40S ribosomal protein S3	484.58	34.80	9	250	27.5	9.61
EDS30648.1	arginine kinase	458.40	26.83	13	410	45.1	5.80
EDS26147.1	sodium/potassium-transporting ATPase alpha chain	449.65	17.41	11	735	80.3	5.67
EDS36304.1	sodium/potassium-dependent ATPase beta-2 subunit	447.15	24.66	7	292	33.4	6.24
EDS26512.1	succinate dehydrogenase flavoprotein subunit. mitochondrial	436.97	13.77	7	661	72.1	6.46
EDS37338.1	short-chain dehydrogenase	416.40	21.52	7	316	34.6	8.13
EDS29666.1	vacuolar ATP synthase subunit e	389.43	27.88	7	226	25.8	6.81
EDS27419.1	protease m1 zinc metalloprotease	377.47	11.28	10	1011	113.2	5.12
EDS34105.1	vacuolar ATP synthase subunit ac39	367.69	33.05	8	348	39.6	4.94
EDS28166.1	elongation factor 2	349.85	6.40	6	1031	114.4	6.71
EDS44958.1	myosin heavy chain	341.20	1.61	2	1927	219.3	5.97
EDS34461.1	60S ribosomal protein L5	314.24	16.50	6	297	34.0	9.73
EDS32198.1	vacuolar ATP synthase subunit C	303.68	17.99	10	528	60.0	6.13
EDS41621.1	40S ribosomal protein SA	303.08	14.54	3	282	30.8	4.91
EDS38023.1	bacteria responsive protein 1; AgBR1	300.66	14.03	5	449	49.2	8.43
EDS33081.1	60S acidic ribosomal protein P0	296.73	25.08	6	315	33.9	5.81
EDS35981.1	endoplasmin	291.51	15.11	10	794	91.0	4.98
EDS41216.1	anionic trypsin-2	281.48	12.12	3	297	31.8	7.88
EDS42199.1	proliferation-associated 2g4	280.01	13.82	5	434	46.8	8.22
AEN19673.1	cytochrome P405 CYP9J40	275.46	15.84	6	524	59.8	8.50
EDS44784.1	lysosomal aspartic protease	275.27	19.90	6	387	41.7	5.31
EDS36212.1	tubulin alpha-2 chain	268.68	9.35	3	449	49.8	5.14
BAI77924.1	cytochrome P450	257.61	19.76	9	496	56.3	7.69

EDS30010.1	conserved hypothetical protein	257.32	6.12	5	931	103.5	5.02
EDS27170.1	truncated ER mannose-binding lectin	253.46	12.31	6	471	53.4	6.05
EDS42768.1	steroid dehydrogenase	243.73	16.30	4	319	34.7	9.50
EDS34510.1	cytochrome P450 9b2	242.12	8.24	4	534	61.3	7.90
EDS42147.1	gram-negative bacteria binding protein	235.12	13.59	5	412	46.7	5.08
EDS45475.1	conserved hypothetical protein	225.43	20.78	6	332	35.4	8.25
EDS32263.1	serine-type enodpeptidase	224.32	19.59	3	296	32.0	5.02
EDS32138.1	heat shock 70 kDa protein cognate 4	218.49	10.53	4	655	71.4	5.52
EDS30841.1	conserved hypothetical protein	216.54	9.31	6	462	52.9	5.24
EDS33630.1	zinc carboxypeptidase A 1	193.62	14.65	4	430	48.9	5.19
EDS38373.1	activated protein kinase C receptor	189.62	28.30	9	311	34.9	7.88
EDS38397.1	soluble NSF attachment protein	177.44	17.75	5	293	33.0	5.85
EDS28502.1	carboxylesterase-6	177.38	5.06	3	632	71.1	6.19
EDS44930.1	l(2) long form	165.06	3.79	4	1294	148.8	4.58
EDS26618.1	prohibitin-2	164.97	15.38	5	299	33.1	9.67
EDS39442.1	V-type ATP synthase beta chain	162.82	9.15	4	492	54.7	5.49
EDS35346.1	membrane associated progesterone receptor	153.54	23.25	4	228	24.3	4.83
EDS31006.1	aminopeptidase N	152.31	2.21	4	1852	210.0	5.08
EDS37258.1	cytochrome P450 9b1	152.07	6.55	2	534	61.0	7.78
EDS33477.1	phosphatidylinositol transfer protein/retinal degeneration b protein	151.83	24.07	5	270	31.1	6.44
EDS34661.1	zinc carboxypeptidase	151.47	6.78	2	428	48.8	5.10
B0WN96.2	RecName: Full=40S ribosomal protein S3a	150.45	15.56	3	270	30.0	9.42
EDS35048.1	conserved hypothetical protein	147.19	12.25	6	645	71.4	6.86
EDS40443.1	mitochondrial cytochrome c1	146.62	18.30	4	306	33.2	8.94

EDS39919.1	disulfide isomerase	145.56	6.29	3	493	55.4	4.91
EDS31640.1	conserved hypothetical protein	145.27	20.90	5	244	26.7	6.65
EDS37912.1	UDP-glucuronosyltransferase 2C1	131.65	9.63	4	509	56.9	8.79
EDS35706.1	vacuolar ATP synthase subunit H	126.53	8.44	3	474	54.5	6.14
EDS27414.1	palmitoyl-protein thioesterase 1	126.32	8.70	2	299	34.0	6.39
EDS38951.1	alpha-glucosidase	121.69	8.93	5	605	69.4	5.20
EDS33030.1	cytochrome P450 4g15	120.09	4.42	4	566	64.6	8.53
EDS33238.1	fructose-bisphosphate aldolase	118.87	7.99	3	363	39.2	7.62
EDS44707.1	cytochrome P450 93A3	118.01	10.02	5	499	57.6	8.50
EDS34505.1	cytochrome P450 52A5	117.92	8.52	3	540	62.0	7.44
EDS34537.1	cytochrome P450 12b1. mitochondrial	112.63	7.43	4	525	60.0	7.74
EDS37463.1	polyserase-2	111.44	8.14	3	381	40.2	8.10
EDS43476.1	glycogen phosphorylase	103.80	5.46	5	842	96.6	6.37
EDS25938.1	succinate dehydrogenase	101.44	10.56	3	303	34.1	7.50
EDS28607.1	lipase	98.30	10.72	2	345	37.4	6.76
EDS34662.1	carboxypeptidase A1	96.08	9.18	3	425	48.4	5.19
EDS36767.1	CD98hc amino acid transporter protein	95.47	3.29	2	639	70.2	5.08
EDS44578.1	motor-protein	94.33	3.51	3	769	85.2	8.85
B0WYY2.1	RecName: Full=Moesin/ezrin/radixin homolog 1	93.26	3.32	2	572	67.7	5.68
EDS35443.1	60S ribosomal protein L14	90.05	13.41	2	179	20.7	11.15
EDS44962.1	myosin heavy chain	89.24	1.21	2	2068	237.3	5.85
EDS37025.1	disulfide-isomerase tigA	88.59	7.32	3	396	44.0	5.07
EDS38798.1	mitochondrial processing peptidase beta subunit	87.83	5.27	3	474	52.2	6.13
EDS28847.1	long-chain fatty acid transport protein 4	86.09	4.78	3	627	69.7	8.18

BAK26813.1	cytochrome P450	85.07	3.17	2	537	61.4	7.55
EDS38696.1	40S ribosomal protein S4	84.10	10.27	3	263	29.7	10.33
EDS33797.1	retinol dehydrogenase 14	82.53	7.01	2	328	36.1	8.81
EDS36124.1	NADH-cytochrome b5 reductase	81.51	7.84	3	319	35.1	8.43
EDS36469.1	multidrug resistance-associated protein 14	78.19	5.23	2	440	48.9	6.67
EDS29539.1	guanine nucleotide-binding protein subunit beta 1	76.74	10.59	2	340	37.2	6.58
EDS45227.1	DNA-J/hsp40	75.82	8.66	3	358	40.8	5.58
EDS39751.1	conserved hypothetical protein	74.80	7.04	3	412	47.9	8.07
EDS45747.1	plasma membrane calcium-transporting ATPase 2	71.69	3.18	3	1195	131.7	6.80
EDS35011.1	conserved hypothetical protein	70.43	5.05	2	436	50.1	5.39

Figure 1
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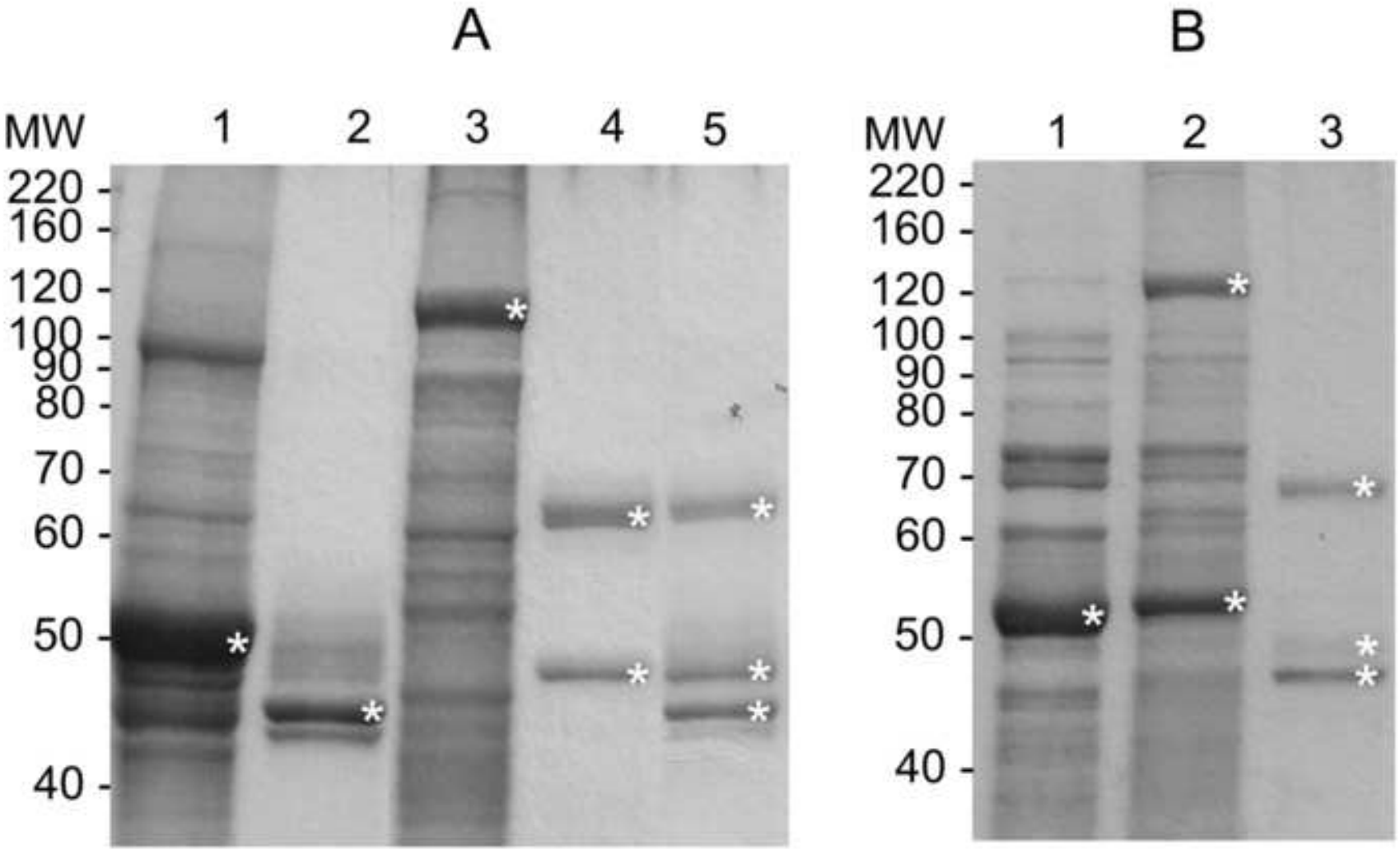

Figure 2

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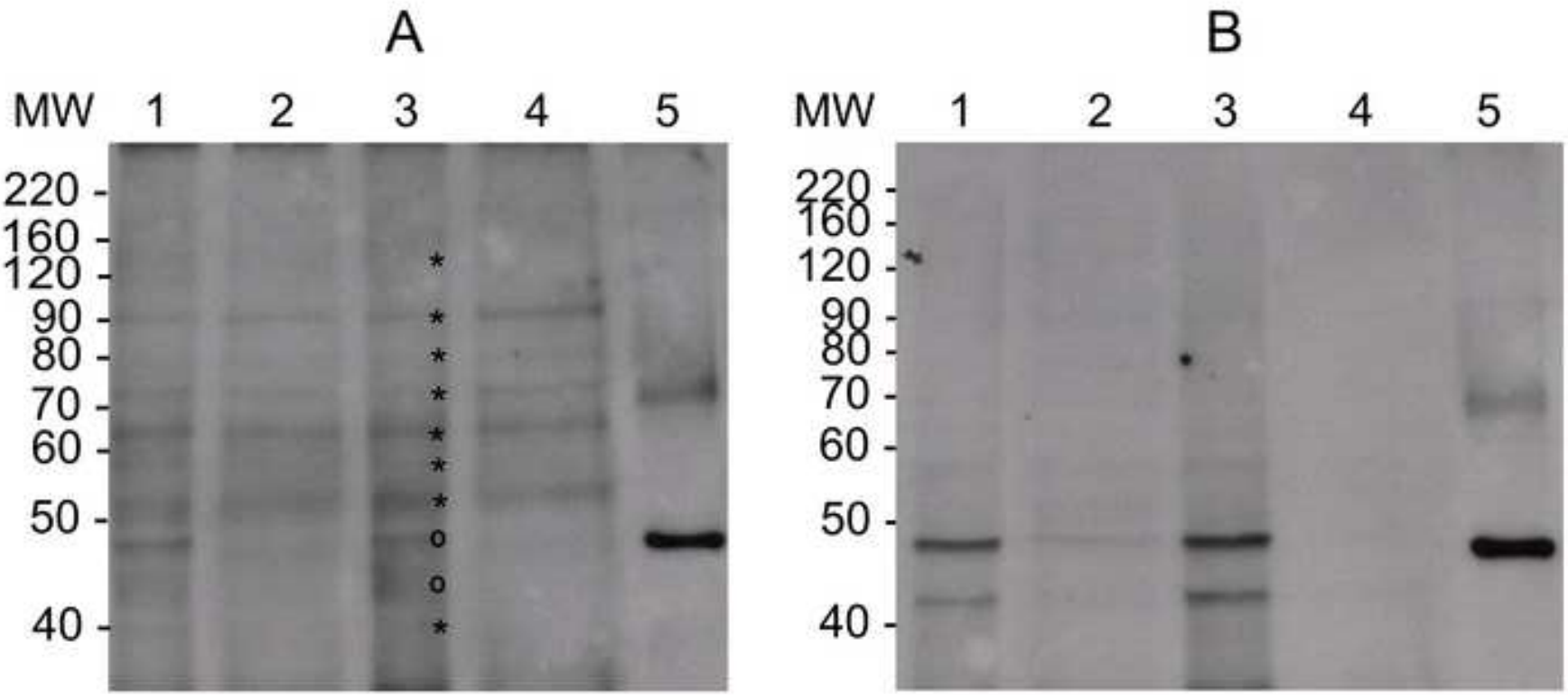

Figure 3
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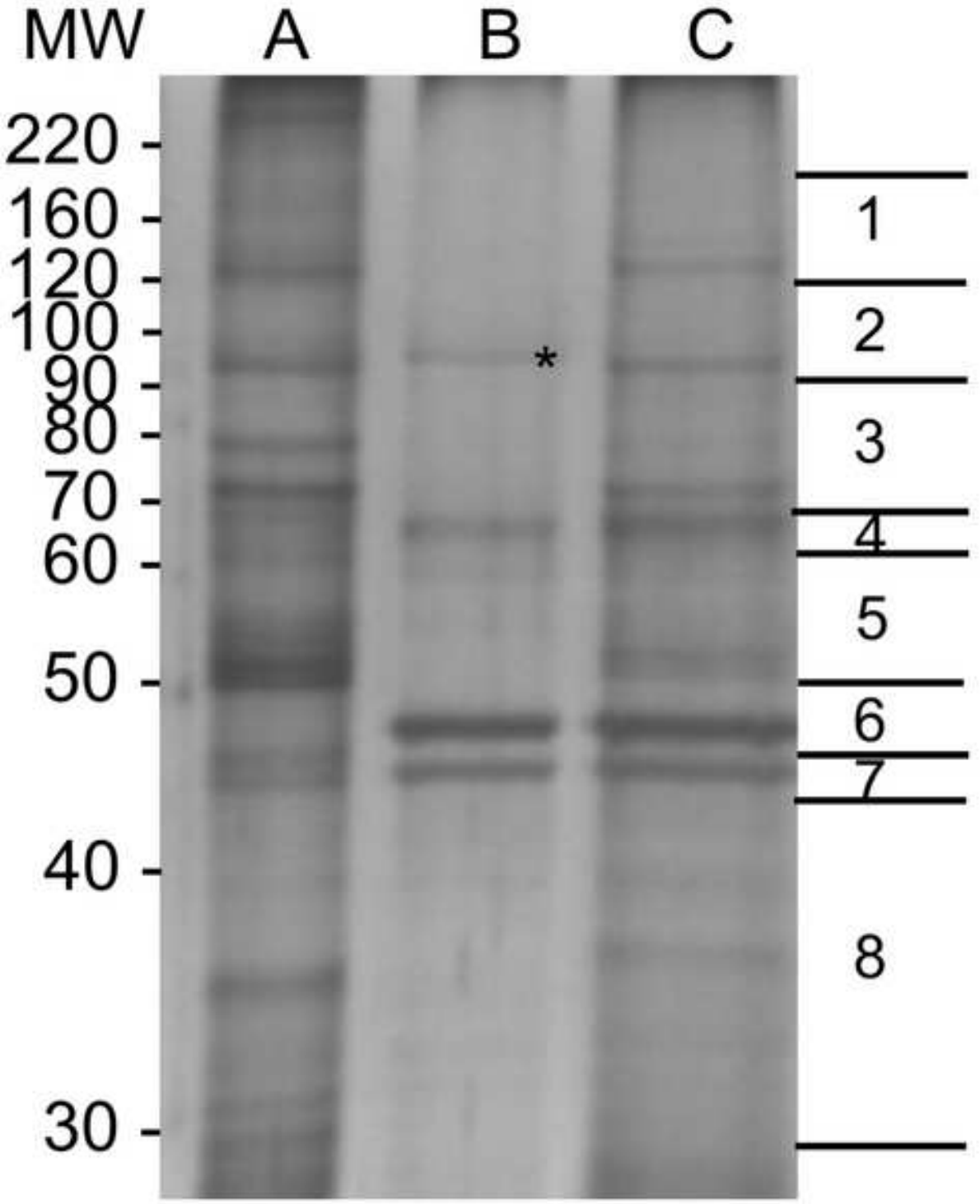
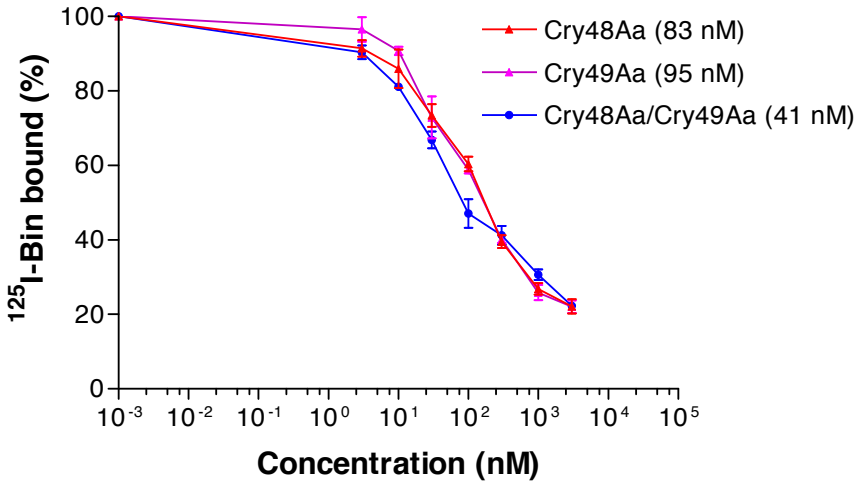
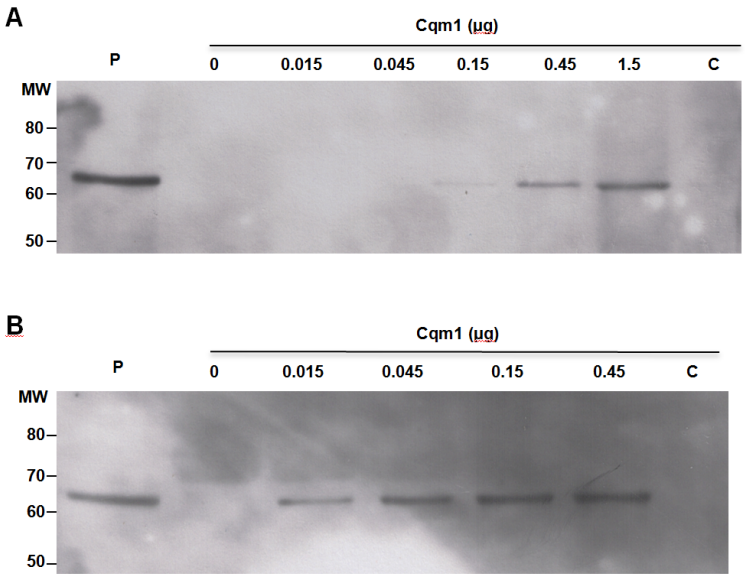


Figure supplementary 1



Supplementary Fig. 1. Homologous competition binding assays between labeled (^{125}I-) Cry48Aa, or Cry49Aa, or a mixture of both toxins (10 nM) with midgut brush border membrane fractions (25 μg) from *Culex quinquefasciatus* larvae in the absence, or in the presence, of the respective unlabeled toxins (3-3000 nM). Maximum binding corresponds to the binding observed in the absence of competitor. The competitor concentration that displaces 50% of the ^{125}I-bound toxin (IC_{50}) is indicated. Each point is the mean of, at least, four experimental replicates.

Figure supplementary 2



Supplementary Fig. 2. Pull-down assay to evaluate the binding of the recombinant Cqm1 protein to the recombinant Cry48Aa/Cry49Aa-GST toxin (A), BinB-GST toxin (B) or GST (C, negative control) immobilized on sepharose beads. After incubation, beads were washed and bound proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and subjected to immunodetection with an antibody raised against Cqm1 protein. P. Cqm1 protein (0.15 μg). MW molecular weight in kDa.