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(54) **Title:** BIOLOGICALLY-ACTIVE RADIOLABELED CRY1FA AND RECEPTOR BINDING ASSAY METHODS

(57) **Abstract:** Cysteine-specific radiolabeled Cry1Fa protein retains insecticidal activity against insect pests and binds to insect brush border membrane vesicle receptors in a saturable manner. The biologically-active radiolabeled Cry1Fa protein is useful in competitive binding assays with other Cry toxins.



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Biologically-Active Radiolabeled Cry1Fa and Receptor Binding Assay Methods

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 61/423,844, filed December 16, 2010, the disclosure of which is hereby incorporated by reference in its entirety, including all figures, tables and amino acid or nucleic acid sequences.

BACKGROUND OF THE INVENTION

[0002] The general field of the invention is the area of life sciences, particularly agricultural sciences and testing methods. Specifically, the field of the invention is the biochemistry and mode of action of *Bacillus thuringiensis* crystalline (Cry) endotoxins and their interactions with insect Cry toxin receptors.

[0003] Cry insecticidal proteins are endotoxins produced by *Bacillus thuringiensis* (Bt), a Gram positive bacterium found globally distributed in different soil types. Various classes of Cry proteins are selectively toxic against certain insect pests. The endotoxin is typically found in the form of a crystalline protein located in large inclusion bodies of the bacterium. Cry toxins have considerable sequence diversity (Crickmore *et al.*, 1998; de Maagd *et al.*, 2003), but the majority of the toxins that have activity against lepidopteran pests are 130 kDa protoxins having a three domain active core toxin structure (de Maagd *et al.*, *supra.*).

[0004] The subject of the current invention relates to the Cry1Fa toxin, a three domain Cry protein. This toxin has demonstrated insecticidal activity against various lepidopteran insects, including *Spodoptera frugiperda* (J. E. Smith) (fall armyworm) and *Ostrinia nubilalis* (Hübner) (European corn borer), which are two of the most economically important insect pests of maize. Cry1Fa is the toxin component of two USDA deregulated transgenic plant incorporated pesticides know as event TC1507 in maize (HERCULEX[®]) and event 281-24-236 in cotton (WIDESTRIKE[®]).

[0005] Cry1Fa full-length holotoxin protein, like other three-domain Cry toxins, requires proteolytic cleavage at both the N-terminus and the C-terminus ends for activation of its insecticidal activity. The midguts of lepidopteran insects contain a variety of trypsin and chymotrypsin-like proteases that process the full length holotoxin to a core toxin structure having a size of approximately 68 kDa (Christeller *et al.*, 1992; Gatehouse *et al.*, 1997;

Bernardi *et al.*, 1996). The processing involves removal of approximately 28 amino acids from the N-terminus and approximately 530 amino acids from the C-terminus (protoxin segment), and the resulting core toxin segment is released and binds to specific receptors located within the insect gut.

[0006] Insects can develop resistance to the activity of Cry protein toxins through changes in midgut localized receptors that bind the Cry protein core toxin (Heckel *et al.*, 2007; Van Rie *et al.*, 1990b). Further, other mechanisms of resistance development have been documented, including: reduced activation of the protoxin, changes in the number of Cry receptors in the insect midgut, and the loss of the ability to respond to the toxin by formation of membrane pores that contribute to insect mortality (see Griffitts and Aroian, 2005; and Van Rie *et al.*, 1990b).

[0007] Prior to this invention, studies to characterize the binding of Cry1Fa core toxin protein to different insect receptors have not been reported. The reason being was that traditional radio-labeling methods involving oxidized iodine isotopes reacting with tyrosine residues in the Cry1Fa toxin produced radiolabeled core toxin protein that lost its ability to bind to receptors in brush border membrane vesicles (BBMVs) prepared from midguts of *Spodoptera exigua* and *Spodoptera frugiperda* (Luo *et al.*, 1999). Further, it was found that traditionally radiolabeled Cry1Fa core toxin protein lost its insecticidal activity and was inactive in diet bioassays against these *Spodoptera* species. Other methods of protein labeling such as fluorescent labeling, and other methods to measure ligand-receptor binding such as isothermal calorimetry, have been attempted, but have been found to be either too insensitive, or the optical methods too difficult to use due to the particulate properties of insect BBMVs.

[0008] Palmer *et al.* (1997) described an indirect method of radioactive labeling of proteins specifically at cysteine residues. In this method, an intermediate compound, fluorescein-5-maleimide, is first reacted with radio-iodine, then the radiolabeled fluorescein-5-maleimide is used to chemically modify the protein at available cysteine residues. This invention describes the use of the highly specific Palmer *et al.* method to radiolabel Cry1Fa protein by targeting a single cysteine residue (C205) located in Domain 1 of the Cry1Fa core toxin.

[0009] It was most surprising to find that the introduction of this steatorically cumbersome radiolabeled 5-maleimide into the Cry1Fa core toxin protein prepared by this method did not result in a loss of binding to its receptor or cause toxin inactivation. As a result, this non-

traditionally radiolabeled Cry1Fa core toxin maintained sufficient tertiary protein structure to retain both its insecticidal activity and its ability to bind specifically to receptors in BBMV preparations from a variety of insects.

[0010] The non-traditionally radiolabeled Cry1Fa protein was found to bind to receptors in a saturable manner, and was used in a competitive binding assay to determine if other Cry toxins compete with its binding. Using this assay it was demonstrated that field resistance to Cry1Fa toxin that developed in a population of *S. frugiperda* collected in Puerto Rico is due to the loss of the ability of receptors in BBMVs of these insects to bind Cry1Fa core toxin protein.

BRIEF DESCRIPTION OF THE FIGURES

[0011] Figure 1 is a bioassay of trypsin-truncated Cry1Fa and fluorescein-5-maleimide labeled, trypsin-truncated Cry1Fa against *S. frugiperda* larvae. The figure summarizes the results of insect diet bioassays with *Spodoptera frugiperda* neonate larvae using iodinated (nonradioactive) Cry1Fa core toxin protein prepared by the method of the current invention.

[0012] Figure 2 shows saturation binding curves of radio-iodinated Cry1Fa core toxin protein to European Corn Borer (ECB) BBMVs. The figure depicts the saturation binding of radioiodinated Cry1Fa core toxin protein prepared by the method of the current invention to BBMVs prepared from *Ostrinia nubilalis* larval midguts.

BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFICATIONS

[0013] SEQ ID NO:1 is the synthetic DNA sequence encoding Cry1Fa holotoxin.

[0014] SEQ ID NO:2 is the Cry1Fa holotoxin.

DETAILED DESCRIPTION OF THE INVENTION

[0015] Three structural domains of the Cry1Fa protein are located within the “tryptic core” that is resistant to further trypsin digestion. Domain I is composed of seven α -helices which are believed to insert into the membrane of the insect midgut and form a pore-like structure (Ballester *et al.*, 1999). Domain II consists of three antiparallel β -sheets, and Domain III forms a β -sandwich structure (Pigott and Ellar, 2007). Exposed regions of domains II and III are believed to interact with specific receptors located on the luminal surface of the insect midgut and bind tightly to these receptors (Bravo *et al.*, 2007; de Maagd *et al.*, 1996; de Maagd *et al.*, 1999; Gomez *et al.*, 2006; Herrero *et al.*, 2004; Lee *et al.*,

1999). Binding of the toxin to a receptor is a necessary requirement for insecticidal activity, and provides the specificity and selectivity of the toxins (Pigott and Ellar, 2007; Rausell *et al.*, 2004). The specificity of activity of different Cry proteins against different insect species has been shown to be related in part to differences in receptors found in different insects (Gomez *et al.*, 2003; Gomez *et al.*, 2007; Van Rie *et al.*, 1990a).

[0016] Quantitative biochemical assays provide a means to measure the interaction of Cry toxins with receptor proteins found in insect midguts. In saturation type binding assays, the labeled Cry toxin protein (ligand) is incubated in buffer solutions that promote the binding of the Cry toxin protein to an insect receptor protein. In one embodiment of a saturation binding assay, a fixed amount of insect receptor protein (commonly brush border membrane vesicles prepared from insect midguts) is mixed (in separate tubes) with increasing amounts of iodinated Cry toxin protein, and allowed to react for a set amount of time. Unbound Cry protein (that is, not bound to an insect receptor protein) is separated from bound protein by one of various methods, and the amount of radioactivity in the bound protein fraction provides an indication of the amount of Cry protein bound to the insect receptor. One skilled in the field of biochemistry will realize that an observation of little or no bound radioactivity will indicate the lack of a receptor for the particular Cry toxin being studied in the insect species from which the receptor preparation was made.

[0017] A second embodiment of a binding assay is a competitive assay. In a competitive binding assay, the radioactive Cry toxin protein is mixed with an excess of a second, nonradioactive Cry protein, and the Cry proteins are allowed to bind under standard conditions to insect receptor proteins. If the second, non radioactive Cry toxin protein is able to compete with the radioactive Cry toxin protein for binding to the insect receptor, then the radioactive protein will be displaced from the receptor binding site, and little radioactivity will be recovered in the bound state. If the nonradioactive and the radioactive Cry toxin proteins bind to different insect receptor proteins, then they will not compete with one another in binding to the same insect receptor protein. In this instance, the amount of radioactivity recovered in the bound protein fraction will be the same, or nearly the same, as when the nonradioactive second Cry toxin protein was not present in the binding reaction. Various methods are available to quantify the amount of radioactivity in the bound protein fraction.

[0018] Competitive binding assays using ^{125}I -Cry1Fa core toxin protein and either nonradioactive Cry1Fa core toxin protein (control reaction) or Cry1Ab core toxin protein

demonstrated that both of the nonradioactive core Cry toxin proteins were able to displace the labeled Cry1Fa core toxin from its receptor site in BBMV's from *S. frugiperda*. By contrast, the competitive binding assay demonstrated that that Cry1Ca core toxin protein did not compete with Cry1Fa core toxin binding. The results of these binding assays demonstrate that the insect resistance to Cry1Fa toxin observed in field studies in a population of *S. frugiperda* collected in Puerto Rico can be explained by the inability of receptors in brush border membrane vesicles from these insects to bind Cry1Fa core toxin.

[0019] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[0020] Nucleic acid sequences are presented in the standard 5' to 3' direction, and protein sequences are presented in the standard amino (N) terminal to carboxy (C) terminal direction.

[0021] Unless specifically indicated or implied, the terms “a”, “an”, and “the” signify “at least one” as used herein.

[0022] Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted. All temperatures are in degrees Celsius.

EXAMPLE 1

Construction of expression plasmids encoding Cry1Fa toxin protein and expression in bacterial hosts

[0023] Standard cloning methods (as provided by, for example, Sambrook *et al.*, (1989) and Ausubel *et al.*, (1997), and updates thereof) were used in the construction of pDAB1817, a *Pseudomonas fluorescens* (*Pf*) expression plasmid engineered to produce a chimeric toxin protein (herein referred to as Cry1Fa toxin; SEQ ID NO:2) comprised of a Cry1Fa core toxin segment (amino acids 1 to 603) and a Cry1Ab protoxin segment (amino acids 604 to 1148), encoded by a plant-optimized coding sequence (CDS; SEQ ID NO:1).

[0024] pDAB1817 is derived from pMYC1803 (US Patent No. 7338794). The basic cloning strategy entailed ligating a DNA fragment, flanked by *SpeI* and *KpnI* restriction enzyme recognition sites and containing the Cry1Fa toxin CDS, to the large fragment of pMYC1803 prepared by cleaving pMYC1803 DNA with *SpeI* and *KpnI* restriction enzymes.

Plasmid pMYC1803 is a medium copy plasmid derived from the RSF1010-based plasmid pTJS260 (see US Patent No. 5169760), and carries a regulated tetracycline resistance marker and the replication and mobilization loci from the RSF1010 plasmid. By this means, the Cry1Fa toxin CDS was placed under the expression control of the *Ptac* promoter and the *rrnBT1T2* terminator from plasmid pKK223-3 (PL Pharmacia, Milwaukee, WI). The expression plasmid pDAB1817 was transformed by electroporation into *P. fluorescens* strain MB217 (a derivative of strain MB101; *P. fluorescens* biovar I), with selection for resistance to tetracycline. Recombinant colonies were identified by restriction digestion of miniprep plasmid DNA.

[0025] Growth and Expression Analysis in Fermentors: Production of Cry1Fa toxin protein for biochemical manipulation and insect bioassay was accomplished by fermentor-grown *P. fluorescens* expression strain isolate DR1649. A seed culture was grown 20 hours (final Optical Density at 600 nm = 14) at 32° in a shake flask containing 600 mL of Ps20 medium supplemented with 15 µg/mL tetracycline, and was used to inoculate 6.6 L of DGMp2.2 medium with tetracycline in a 20 L fermentor tank (New Brunswick Scientific BioFlo 4500, Edison, NJ). Fermentation was carried out at 32° with agitation at 200 to 1000 rpm. Details of the microbiological manipulations for *P. fluorescens* are available in Squires *et al.* (2004), US Patent Application No. 20060008877, US Patent No. 7681799, US Patent Application No. 20080058262, and Huang *et al.* (2007), incorporated herein by reference. Glycerol was batch-fed periodically at 10 gm/L in response to dissolved oxygen concentration. Expression of the Cry1Fa toxin CDS via the *Ptac* promoter was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG). Cultures were monitored throughout the post induction fermentation time to determine cell density, the level of target gene expression, and other parameters. For SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and immunoblot analyses, aliquots of 0.1 mL of whole fermentation broth were frozen for subsequent analysis. At the final time point at 48 hours after IPTG induction (Optical Density at 600 nm = 222; final culture volume = 13.5 L), cells from 4 L of culture were harvested by centrifugation at 10000 × g for 90 min. The cell pellets were frozen at -20° or -80° for further processing.

[0026] SDS-PAGE Analysis of fermentation samples: Frozen fermentor cell broths (0.1 mL) were diluted in chilled water by 5 fold and 200 µL were sonicated in ice for 10 minutes using a Branson 250 Sonifier (Branson Ultrasonics, Danbury CT) using a 1/8 inch diameter

micro tip with a constant output of 20 units. The lysates were centrifuged at 14000 rpm for 20 minutes (4°) and the supernatants removed (soluble fraction). The pellets were then resuspended in 200 µL of phosphate buffered saline (PBS; 11.9 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, pH7.4; Sigma-Aldrich, St. Louis, MO). Further dilutions of both the soluble and insoluble fractions of an equivalent of up to 20-fold of the original fermentation broth were performed in PBS. These fractions were then mixed 1:1 with Laemmli sample buffer (Bio-Rad Inc., Hercules, CA;) containing 5% β-mercaptoethanol and boiled for 5 minutes prior to loading 10 µL to 20 µL on a Criterion 10% Bis-Tris gel with MOPS buffer (Bio-Rad Inc.). Gels were stained with Simply Blue SafeStain™ (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

[0027] Immunoblotting: Standard biochemical methods (as provided by, for example, Sambrook *et al.*, (1989) and Ausubel *et al.*, (1997), and updates thereof) were used for protein isolations and immunoblot analyses. Samples were prepared and proteins were separated by electrophoresis through NuPAGE 4-12% Bis-Tris gels in MES running buffer according to the manufacturer's suggested protocol for denaturing electrophoresis (Invitrogen). Proteins were transferred onto nitrocellulose membrane for 80 min. at 30 V in NuPAGE transfer buffer. Blots were blocked for 1 hour at room temperature in 5% milk/PBST (PBS with 0.05% Tween-20) and then probed with primary antibody (specific for Cry1Fa core toxin segment) and then secondary antibodies for one hour each at room temperature in blocking solution, with rinsing in between each antibody for 15 minutes in PBST. Development of blots was done using Pierce's ECL Western blotting substrate according the manufacturer's protocol (Thermo Fisher Scientific, Rockford, IL).

EXAMPLE 2

Purification of Cry1Fa core toxin protein from *Pseudomonas*-produced inclusion bodies

[0028] Inclusion body preparations: Protein inclusion bodies (IB) were prepared from *P. fluorescens* DR1649 cells that contained insoluble Cry1Fa toxin protein, as demonstrated by SDS-PAGE and MALDI-MS (Matrix Assisted Laser Desorption/Ionization Mass Spectrometry). A MALDI-R (Reflectron) mass spectrometer from Micromass (Milford, MA) was used to measure peptide masses in accordance with the manufacturer's prescribed methods and recommendations. Frozen cell pellets were thawed in a room temperature water

bath. The cells were resuspended to 10% w/v in lysis buffer (50 mM Tris HCl, pH7.5; 200 mM NaCl; 5% Glycerol; 2 mM EDTA disodium salt (Ethylenediaminetetraacetic acid); 0.5% Triton X-100; and 1mM DTT (dithiothreitol—added just prior to use)). 25 mL of protease inhibitor cocktail (Sigma-Aldrich) was added for every 100 gm of cell paste treated. The slurry was passed twice through a Microfluidics Microfluidizer at 12000+ psi (Microfluidics Intern. Corp., Newton, MA). The lysate was centrifuged at 18000 x g at 4° for 30 min, and the supernatant was retained. The inclusion pellet was washed in lysis buffer without protease inhibitor cocktail until no bacterial odor remained (usually 2 or 3 washes), by gently homogenizing using a spatula or mechanical mixer (to about 10% w/v solids) and centrifuging as described. The resultant supernatants and final pellet were retained and stored at -20°. Small aliquots of each sample were taken and stored in a microcentrifuge tube for SDS-PAGE analysis. The inclusion body preparation pellets were then freeze-dried using a Virtis Advantage freeze dryer (Viopharma Process Systems LTD, Winchester, UK) at ambient shelf temperature and a maximum vacuum drawn for 2 days. The resulting powders were then stored at -20° until analyzed.

[0029] SDS-PAGE analysis and quantitation of IB preparations: Twenty-five mg of IB pellet were resuspended in 1 mL of HTS buffer (20 mM Tris HCL, pH7.5; 50 mM NaCl; 5% v/v glycerol, 10 mM EDTA disodium salt; 0.5% v/v Triton X-100) and sonicated as above for 1 minute on ice. The resuspended samples were then diluted 1:1 in Laemmli sample buffer containing 0.2 M DTT. The samples were then diluted 10X and 20X with Laemmli sample buffer without DTT and 10 µL were loaded onto a Criterion 18 well 10% Bis-Tris gel (Bio-Rad Inc.) run with 1X NuPAGE MES buffer (Invitrogen). Gels were run for 5 min at 100V and then 45 min at 200V. Gels were rinsed and washed in water for 20 min and stained with Simply Blue SafeStain™. Quantification of target bands was done by comparing densitometric values for the bands against a series of Bovine Serum Albumin (BSA) standard samples run on the same gel, and scanned to generate a densitometric standard curve.

[0030] Truncation and purification of Cry1Fa core toxin protein: Purified inclusion bodies were washed with sterile water, then solubilized in 20 mM CAPS, pH11 (3-(cyclohexamino)1-propanesulfonic acid) containing 10 mM DTT by rocking the protein solution for 1 hr at room temperature. After removing insoluble material by centrifugation (30000 x g for 30 min at 4°), trypsin treated with 0.5% (w/v) tosyl phenylalanyl chloromethyl ketone (TPCK) (Sigma-Aldrich) was added to the supernatant. This solution was incubated with mixing for 1 hr at room temperature, filtered and concentrated 5-fold using an Amicon

Ultra-15 regenerated cellulose centrifugal filter device (30000 Molecular Weight Cutoff; Millipore), then loaded onto a Pharmacia Mono Q 1010 column equilibrated with 20 mM CAPS pH10.5. After washing the loaded column with 2 column volumes of buffer, the truncated toxin protein was eluted using a linear gradient of 0 to 0.5 M NaCl in 20 mM CAPS pH10.5 in 15 column volumes at a flow rate of 1.0 mL/min. Purified trypsin-truncated Cry1Fa core toxin eluted at about 0.2 M to 0.3 M NaCl. The purity of the proteins was checked by SDS-PAGE with visualization using Coomassie brilliant blue dye (below). In some cases, the combined fractions of the purified toxin were concentrated and loaded onto a Superose 6 column (1.6 cm dia., 60 cm long; GE Healthcare Life Sciences), and further purified by size exclusion chromatography. Fractions comprising a single peak corresponding to the monomeric size of the truncated core toxin (*ca.* 68 kDa) were combined and concentrated, resulting in a preparation that was greater than 95% homogeneous for a protein having a molecular weight of about 68 kDa, as judged by SDS-PAGE.

[0031] SDS-PAGE analysis of purified Cry1Fa core toxin: SDS-PAGE analysis of trypsin truncated Cry1Fa toxin protein was conducted under reducing and denaturing conditions by the method of Laemmli (as per Sambrook *et al.*, *supra*). Reduction of the proteins was achieved using 5% β -mercaptoethanol, and heat denaturation was performed at 90° for 5 minutes in the presence of 2% SDS. Proteins were loaded into wells of a 4% to 20% Tris-glycine polyacrylamide gel (Invitrogen) and separated at 200 volts for 60 minutes. Protein bands were stained with Coomassie Brilliant Blue R-250 (Bio-Rad) for one hour, and then destained with a solution of 5% methanol in 7% acetic acid in the presence of cellulose sponges. The gels were imaged and analyzed using a Bio-Rad Fluro-S Multi Imager™ with Quantity One™ imaging software. Relative molecular weights of the protein bands were determined by including a sample of BenchMark™ Protein Ladder (Life Technologies, Rockville, MD) or See Blue™ prestained molecular weight marker (Invitrogen) in one well of the gel.

EXAMPLE 3

Preparation of specifically radio-iodinated Cry1Fa core toxin protein

[0032] Iodination of Cry1Fa core toxin protein: Previous work demonstrated that iodination of Cry1Fa toxin protein destroyed the capacity of the iodinated protein to bind to its receptor(s) in insect brush border membrane vesicles (BBMVs), as well as the insect

toxicity of the protein (Luo *et al.*, 1999). In that study, Cry1Fa toxin protein was radio-iodinated using a standard iodination bead method (Pierce Iodination Beads; Thermo Fisher Scientific), and binding studies revealed that the protein had lost all of its ability for specific binding to receptor(s) in BBMVs from *S. exigua* and *S. frugiperda*. Further, when non-radiolabeled NaI was used to iodinate Cry1Fa toxin protein employing the Iodination Bead method, the iodinated Cry1Fa was found to have lost its insecticidal activity against *Spodoptera* larvae in diet bioassays.

[0033] Iodination of proteins by Iodination Bead protocols takes place at positions ortho to the hydroxyl group on tyrosine; mono- or di-substitution can occur. Thus, the positions of iodination depend on the placement of tyrosine residues within the subject protein, and multiple iodinations may have the consequence of disruption of the protein's structure and/or function. It is noted that the Cry1Fa core toxin segment comprises 20 tyrosine residues, which may serve as iodination targets.

[0034] This example teaches an alternative method for labeling the Cry1Fa core toxin protein with radioactive iodine. Subsequent Examples teach that the radio-labeled core toxin protein binds to insect BBMVs, and that iodinated Cry1Fa core toxin protein is active in insect diet bioassays. Further, these Examples teach that trypsin truncated Cry1Fa core toxin protein can be specifically fluorescently labeled at the cysteine corresponding to the C205 residue of the Cry1Fa toxin protein using fluorescein-5-maleimide, and that the fluorescently labeled protein is biologically active, causing insect mortality at dosages equal to those of nonlabeled, trypsin truncated Cry1Fa core toxin.

[0035] The Cry1Fa core toxin segment contains three cysteine amino acid residues, at positions 9, 14, and 205. Truncation of the protein by trypsin treatment removes C9 and C14, providing a core toxin segment that retains the cysteine corresponding to the C205 residue. Palmer *et al.* (1997) demonstrated that the phenyl rings of fluorescein-5-maleimide can be radio-iodinated and then reacted with proteins that contain sulfhydryl groups (*e.g.* as provided by free cysteine residues), resulting in alkylation of the free cysteines in the protein, and thus providing a radioactively labeled protein. The trypsin-truncated Cry1Fa core toxin contains a single cysteine residue at the position corresponding to C205, and thus provides a substrate for alkylation and radiolabeling of the protein at a single (specific) site.

[0036] Fluorescein-5-maleimide (F5-M) was dissolved to 10 mM in DMSO (Dimethyl Sulfoxide), then diluted to 1 mM in phosphate buffered saline (PBS; 20 mM sodium phosphate, 0.15 M NaCl, pH7.5), as determined by the molar extinction coefficient of F 5-M

($68,000 \text{ M}^{-1}\text{cm}^{-1}$). To a $70 \mu\text{L}$ solution of PBS containing two Pierce Iodination Beads (Thermo Fisher Scientific), 0.5 mCi of Na^{125}I was added behind lead shielding. (An analogous procedure was performed using non-radioactive NaI to prepare (iodinated, non radioactive) fluorescently labeled Cry1Fa core toxin protein.) The solution was allowed to mix at room temperature for 5 min, then $10 \mu\text{L}$ of the 1 mM F 5-M solution were added. After reacting for 10 min, the solution was removed from the iodination by pipetting and $2 \mu\text{g}$ of highly purified trypsin-truncated Cry1Fa core toxin protein in PBS were added to the solution. The protein was incubated at 4° with the iodinated F 5-M solution for 48 hrs, when the reaction was terminated by adding β -mercaptoethanol to 14 mM final concentration. The reaction mixture was added to a Zebra™ spin column (Invitrogen) equilibrated in 20 mM CAPS, 150 mM KCl, pH9, and centrifuged at $1500 \times g$ for 2 min to separate non-reacted iodinated dye from the protein. The ^{125}I radiolabeled fluorescein-Cry1Fa core toxin protein was counted in a gamma counter to determine its specific radioactivity, assuming 80% recovery of the input toxin protein.

[0037] The specific activity of the radiolabeled Cry1Fa core toxin protein was approximately $1.1 \mu\text{Ci}/\mu\text{g}$ protein. This specific activity was low compared to typical expected levels of labeling of similarly-sized proteins using the Iodination Bead procedure. This is presumably due to a single site of labeling (corresponding to C205), and to the (possibly) relative inaccessibility of that site, which is presumed to be located deep within Domain I of the core toxin segment (based upon localization using a comparison crystal structure of the Cry1Aa protein).

[0038] The radiolabeled protein was also characterized by SDS-PAGE and visualized by phosphor-imaging to validate that the radioactivity measured was covalently associated with the Cry1Fa core toxin protein. Radio-purity of the radio-iodinated Cry1Fa core toxin protein (and subsequent detection of Cry1Fa bound to its BBMV receptor) was determined by SDS-PAGE, phosphor-imaging and gamma counting. Coomassie stained SDS-PAGE gels were imaged by wrapping them in Mylar™ film ($12 \mu\text{m}$ thick), and exposing them under a Molecular Dynamics (Sunnyvale, CA) storage phosphor screen ($35 \text{ cm} \times 43 \text{ cm}$) for 1 hour. The plates were developed using a Molecular Dynamics Storm 820 phosphor-imager and the image analyzed using ImageQuant™ software. The radioactive band along with areas immediately above and below the band were cut from the gel using a razor blade and counted in a gamma counter. Radioactivity was detected in the Cry1Fa core toxin protein band and in areas below the band. No radioactivity was detected in the gel region above the Cry1Fa core

toxin protein band. Some radioactivity was detectable in the gel region below the Cry1Fa core toxin protein band (*i.e.* fragments smaller than the Cry1Fa core toxin protein). These radioactive contaminants likely represent degradation products of the Cry1Fa core toxin protein.

[0039] Fluorescently labeled (non-radioactive, iodinated) Cry1Fa core toxin protein thus prepared was used in insect diet feeding bioassays to demonstrate that insect toxicity had not been affected, and radio-iodinated (fluorescently labeled) Cry1Fa core toxin protein was used in binding studies with BBMV preparations to demonstrate that receptor binding activities had not been affected.

EXAMPLE 4 Insect diet feeding bioassays

[0040] Trypsin-truncated Cry1Fa core toxin proteins, either non-radioactive iodine fluorescein-5-maleimide labeled, or unlabeled, were individually tested for their insecticidal activity in a top load diet bioassay against larvae of *Spodoptera frugiperda* (fall armyworm; FAW). Larvae of FAW were hatched from eggs obtained from a colony maintained by a commercial insectary (Benzon Research Inc., Carlisle, PA).

[0041] The bioassays were conducted in 128-well plastic trays specifically designed for insect bioassays (C-D International, Pitman, NJ). Each well contained 0.5 mL of Multi-species Lepidoptera diet (Southland Products, Lake Village, AR). A 40 μ L aliquot of the purified Cry1Fa core toxin protein, diluted to various concentrations in 10 mM CAPS, pH10.5, or 40 μ L of a control solution, were delivered by pipette onto the diet surface of each well (26.7 μ L/cm²). Sixteen wells were tested per sample. The negative control was a CAPS buffer solution blank containing no protein. Positive controls included preparations of full length Cry1Fa toxin. The treated trays were held in a fume hood until the liquid on the diet surface had evaporated or was absorbed into the diet. Diet concentrations of the Cry1F toxin proteins were calculated as the amount (ng) of Cry1F toxin protein per square centimeter of surface area in the well (1.5 cm²).

[0042] Within a few hours of eclosion, individual larvae were picked up with a moistened camel hair brush and deposited on the treated diet, one larva per well. The infested wells were then sealed with adhesive sheets of clear plastic that are vented to allow gas exchange (C-D International, Pitman, NJ). Bioassay trays were held under controlled environmental

conditions (28°, about 40% Relative Humidity, 16:8 (Light:Dark)) for 5 days, after which time the total number of insects exposed to each protein sample, the number of dead insects, and the weight of surviving insects were recorded. Percent mortality and percent growth inhibition were calculated for each treatment. Growth inhibition (GI) was calculated as follows:

$$GI = [1 - (TWIT/TNIT)/(TWIBC/TNIBC)]$$

where TWIT is the Total Weight of Insects in the Treatment,

TNIT is the Total Number of Insects in the Treatment

TWIBC is the Total Weight of Insects in the Background Check (Buffer control), and

TNIBC is the Total Number of Insects in the Background Check (Buffer control).

The GI₅₀ was determined to be the concentration of Cry1Fa toxin protein in the diet at which the GI value was 50%; *i.e.* the growth of the insects on Cry1Fa-containing diet was only half that of the insects on diet with no Cry1Fa protein, as calculated above. The LC₅₀ (50% Lethal Concentration; calculated as the concentration of Cry1Fa toxin protein in the diet at which 50% of test insects were killed) was not determined, as very little mortality was observed during the 5 day experimental period. Statistical analysis (One-way ANOVA) is done using JMP software (SAS, Cary, NC).

[0043] The labeled and non-labeled Cry1Fa core toxin proteins were approximately equally active at inhibiting growth of *S. frugiperda* larvae, causing fifty percent growth inhibition at concentrations between 33 ng/cm² and 100 ng/cm² (Figure 1). This result shows that labeling of trypsin truncated Cry1Fa core toxin protein with iodinated fluorescein-5-maleimide does not result in loss of insecticidal activity.

EXAMPLE 5

Preparation of brush border membrane vesicles (BBMVs)

[0044] Preparation of Solubilized BBMVs: Last instar larvae of *S. frugiperda* and *Ostrinia nubilalis* (European corn borer) were held without food overnight and then dissected after chilling on ice for 15 minutes. The midgut tissue was removed from the body cavity, leaving behind the hindgut attached to the integument. The midgut was placed in 9X volume of ice cold homogenization buffer (17 mM Tris base, pH7.5, 300 mM mannitol, and 5 mM

EGTA (Ethylene glycol tetraacetic acid)), supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich P-2714) diluted as recommended by the supplier. The final concentration of cocktail components (in μM) were AEBSF (500), EDTA (250), Bestatin (32), E-64 (0.35), Leupeptin (0.25), and Aprotinin (0.075). The tissue was homogenized with 15 strokes of a glass tissue homogenizer. BBMV s were prepared by the MgCl_2 precipitation method of Wolfersberger (1993). Briefly, an equal volume of a 24 mM MgCl_2 solution was mixed with the midgut homogenate, stirred for 5 minutes and allowed to stand on ice for 20 min. The solution was centrifuged at $2500 \times g$ for 15 min at 4° . The supernatant was saved and the pellet suspended into 0.5X volume of homogenization buffer and centrifuged again. The two supernatants were combined, centrifuged at $27000 \times g$ for 30 min at 4° to form the BBMV fraction. The pellet was suspended into BBMV Storage Buffer (10 mM HEPES pH7.4 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 130 mM KCl, 10% glycerol) to a concentration of about 3 mg/mL protein. Protein concentration was determined by using the Bradford method with BSA as the standard (Bradford, 1976). The leucine-p-nitroanilide assay for aminopeptidase N determination was made prior to freezing the samples using a Sigma-Aldrich assay kit following the manufacturer's instructions. The specific activity of this marker enzyme in the BBMV fraction typically increased 7-fold compared to that found in the midgut homogenate fraction. The BBMV preparations were dispensed into 250 μL samples, flash frozen in liquid nitrogen, and stored at -80° .

EXAMPLE 6

Iodinated Cry1Fa core toxin protein binding assays to BBMV s

[0045] Binding of ^{125}I -Cry1Fa core toxin protein to BBMV s: To determine the optimal amount of BBMV protein to use in competitive binding assays, a saturation curve was generated. ^{125}I radiolabeled Cry1Fa core toxin protein (0.5 nM) was incubated for 1 hr at 28° with *O. nubilalis* (ECB) BBMV proteins in concentrations ranging from 0 to 500 $\mu\text{g/mL}$ in binding buffer (8 mM NaH_2PO_4 , 2 mM KH_2PO_4 , 150 mM NaCl, 0.1% BSA, pH7.4). Total volume was 0.5 mL. Bound ^{125}I -Cry1Fa core toxin protein was separated from unbound material by sampling 150 μL of the reaction mixture in triplicate and centrifuging the samples at $14000 \times g$ for 8 min at room temperature. The supernatant was gently removed, and the pellet washed three times with ice cold binding buffer. The bottom of the centrifuge tube containing the pellet was cut off and placed into a 13 x 75-mm glass culture tube. Each

sample was counted for 10 min in a gamma counter. The value of counts-per-minute (CPM) minus background (reactions with no added protein) was plotted versus BBMV protein concentration. The optimal range of BBMV protein to use in each binding reaction was determined to be 100 µg/mL to 150 µg/mL.

[0046] Binding kinetics: To determine the binding kinetics, a saturation curve was generated. Briefly, 150 µg/mL of ECB BBMV proteins were incubated for 1 hr at 28° with increasing concentrations (ranging from 0.5 nM to 20 nM) of ¹²⁵I-Cry1Fa core toxin protein. Total binding was determined by sampling 150 µL of each concentration in triplicate, followed by centrifuging and counting as described above. The triplicate values were averaged. Non-specific binding was determined in the same manner, with the addition of 1,000 nM of non-radioactive (competitor) Cry1Fa core toxin protein. This concentration was at least 50-fold higher than the highest concentration of the radiolabeled Cry1Fa core toxin protein used; thus it would be expected to bind to all available receptor sites, while displacing the radiolabeled Cry1Fa core toxin protein. Specific binding was calculated as the difference in CPM (minus background) between total binding and non-specific binding. It was determined (Figure 2) that ¹²⁵I-Cry1Fa core toxin protein specifically bound to the ECB BBMV proteins in a saturable manner, with a K_d of 11 nM and a B_{max} of 37 fmoles/mg of ECB BBMV proteins. The level of non-specific binding was nearly 70% of total binding; however the level of specific binding clearly saturated, showing that the interaction was a receptor-mediated binding response.

EXAMPLE 7

Competition binding assays with BBMVs

[0047] A "pull-down" type receptor assay was used to determine the amount of competition for binding to receptor sites between radiolabeled Cry1Fa core toxin protein and other (non labeled) Cry toxin proteins. In this assay the relative molecular size of the bound ligand with receptor was characterized by separation by SDS-PAGE, and the amount of radiolabeled ligand bound to the BBMV receptor was measured by detection of radioactivity in the gel by phosphor-imaging. Homologous and heterologous competition binding assays were conducted using 100 µg/mL or 150 µg/mL of BBMV protein (from various insect sources) and 0.5 nM or 2.5 nM of ¹²⁵I-Cry1Fa core toxin protein. The competitive ligand (*i.e.* 1000 nM of non-radiolabeled Cry toxin protein) was added to the reaction mixture at the

same time as the radioactive ligand to assure true competitive binding interactions. Incubations were carried out for 1 hr at 28°, and the ^{125}I -Cry1Fa core toxin protein bound to its BBMV receptor(s) was separated from nonbound protein by centrifuging the reaction mixture as described above. The pellet was washed three times with ice cold binding buffer, then solubilized by adding 25 μL of 2X Laemmli buffer with 5% β -mercaptoethanol, and rapidly mixing the sample for 10 min at 95°. The sample was centrifuged and duplicate samples were loaded onto a 4% to 20% Tris glycine polyacrylamide gel and separated by SDS-PAGE. The amount of ^{125}I -Cry1Fa core toxin protein bound to receptor was measured by phosphor-imaging of the gel and densitometry of the bands corresponding to radioactive Cry1Fa core toxin protein after a 3 day exposure of the plate.

[0048] In a control reaction, 0.5 nM of ^{125}I -Cry1Fa core toxin protein was incubated with *S. frugiperda* (FAW) BBMV proteins for 1 hr, and the amount of ^{125}I -Cry1Fa protein that bound to the BBMVs was measured by SDS-PAGE and phosphor-imaging as above. A concurrent experiment, to which was added 1000 nM of unlabeled Cry1Fa core toxin protein, demonstrated that this addition of a 2000-fold excess concentration of unlabeled Cry1Fa core toxin protein completely eliminated the binding of the radiolabeled Cry1Fa protein. These results demonstrated that this assay effectively measures the ability of a Cry toxin protein to displace the binding of Cry1Fa core toxin protein to its FAW BBMV receptor(s). In a similar manner, it was determined that 1000 nM of Cry1Ab core toxin protein completely eliminated the binding of ^{125}I -Cry1Fa core toxin protein to FAW BBMV proteins. This result is consistent with other reports indicating that the Cry1Ab toxin protein and Cry1Fa toxin protein share similar receptor binding sites (Banks *et al.*, 2001; Hernandez and Ferre, 2005).

[0049] Further, it was found that there was no competition for binding to FAW BBMV receptors between 1000 nM of Cry1Ca core toxin protein and 0.5 nM of ^{125}I -Cry1Fa core toxin protein, indicating that the Cry1Ca protein binds to a receptor different from the Cry1Fa receptor(s). This result is consistent with other studies conducted with *Diatraea saccharalis* (sugarcane borer) BBMVs, in which it was found that excess Cry1Fa protein did not displace biotinylated Cry1Ca protein from its receptor(s).

EXAMPLE 8

Cry1Fa core toxin binding to BBMVs prepared from
FAW insects resistant to Cry1Fa toxin

[0050] Pull-down competitive binding assays were performed using BBMV prepared from insect larvae that are resistant to intoxication by ingested Cry1Fa toxin. Cry1Fa-resistant larvae of *S. frugiperda* (rFAW) were hatched from eggs harvested from a proprietary colony (Dow AgroSciences LLC, Indianapolis, IN). The rFAW larvae are more than 30-fold resistant to the toxicity of Cry1Fa toxin. BBMV were prepared from rFAW larvae in the same manner as from their Cry1Fa-sensitive counterparts.

[0051] As shown above, BBMV from wild type (*i.e.* Cry1Fa-sensitive) FAW larvae bound ^{125}I -Cry1Fa, and the binding could be displaced by 2000-fold addition of non-labeled Cry1Fa protein. In contrast, BBMV prepared from rFAW larvae and used in pull-down binding assays at 100 $\mu\text{g/mL}$ with 2.5 nM ^{125}I -Cry1Fa did not bind any of the ^{125}I -Cry1Fa core toxin protein. These results indicate that the resistance to Cry1Fa toxin in rFAW may be due to the inability of the Cry1Fa receptor(s) in the BBMV to bind Cry1Fa core toxin protein. Alternatively, the resistance may be a result of absence of the receptors from the insects. In any case, it appears that the mechanism of resistance to Cry1Fa toxin in these rFAW larvae is receptor based.

EXAMPLE 9

Cry1Fa core toxin binding to BBMV prepared from ECB insects resistant to Cry1Fa toxin

[0052] Pull-down competitive binding assays were performed using BBMV prepared from insect larvae that are resistant to intoxication by ingested Cry1Fa toxin. Cry1Fa-resistant larvae of *O. nubilalis* (rECB) were hatched from eggs harvested from a proprietary colony (Dow AgroSciences LLC, Indianapolis, IN). The rECB larvae are more than 30-fold resistant to the toxicity of Cry1Fa toxin. BBMV were prepared from rECB larvae in the same manner as from their Cry1Fa-sensitive counterparts.

[0053] Pull-down experiments were conducted using 100 $\mu\text{g/mL}$ of BBMV prepared from wild type ECB larvae (*i.e.* Cry1Fa-sensitive) and Cry1Fa-resistant (rECB) larvae, and using 2.5 nM of ^{125}I -Cry1Fa core toxin protein. The radioiodinated protein was bound to BBMV from ECB larvae, but to a lesser extent than was seen for binding to BBMV from FAW larvae. Non-radiolabeled (competitor) Cry1Fa protein competed with the binding of ^{125}I -Cry1Fa to ECB BBMV but the competition was less than that which was measured in competition experiments using FAW BBMV.

[0054] Some binding of ^{125}I -Cry1Fa to rECB BBMV was detected, and the amount of binding was less than that measured using ECB BBMV. Non-radiolabeled (competitor) Cry1Fa protein did not completely compete with ^{125}I -Cry1Fa core toxin protein for binding to rECB BBMV. These data support earlier Cry1Fa binding studies done by Surface Plasmon Resonance using BBMV from ECB and rECB. Those studies showed that non-iodinated Cry1Fa toxin protein could bind to BBMV fractions prepared from either ECB or rECB larvae, but dissociation of the toxin from the ECB BBMV was slower than its dissociation from rECB BBMV. The ^{125}I -Cry1Fa core toxin protein binding detected in the pull-down type assays described here are not sufficiently quantitative to measure binding affinity, but do indicate that Cry1Fa toxin protein binds to rECB BBMV to a significant degree. Thus, resistance to Cry1Fa toxin in rECB may be due to less efficient binding of Cry1Fa core toxin protein to rECB BBMV receptor(s), as compared to binding to receptors of susceptible larvae. This could manifest as less efficacious for membrane pore formation, since the time that the toxin remains on the receptor is shorter than in susceptible insects.

[0055] Table 1 summarizes the relative binding results from assays using radio-iodinated Cry1Fa core toxin protein with BBMV preparations from various insect sources. Percentages indicate the relative amounts of radioactivity bound to BBMV, with, and without, nonradioactive competitor ligand. 100% values do not represent equal numbers; ECB BBMV bind less total Cry1Fa protein than do FAW BBMV.

Table 1				
	Relative Amounts of radio-iodinated Cry1Fa Protein Bound			
Non labeled Competitor* →	None	Cry1Fa	Cry1Ab	Cry1Ca
FAW BBMV	100%	0%	-4%	50%
rFAW BBMV	10%	NA**	Not done	Not done
ECB BBMV	100%	31%	Not done	Not done
rECB BBMV	64%	51%	Not done	Not done

*1000 nM of nonradioactive competitor Cry core toxin proteins added where appropriate

**N/A = Not Applicable, since very little ^{125}I -Cry1Fa core toxin protein was bound

[0056] This invention demonstrates that, surprisingly, Cry1Fa core toxin protein can be radio-labeled using ^{125}I labeled fluorescein-5-maleimide to specifically alkylate cysteine residues while maintaining insecticidal activity and receptor binding capability. The trypsin

truncated form of Cry1Fa core toxin contains only a single cysteine residue (corresponding to C205 within Domain I of Cry1Fa), which must comprise the site of radiolabeling. This region constitutes part of the pore structure of the protein and is not thought to be involved in receptor binding. One skilled in the field of protein biochemistry and structure will realize that molecules similar to fluorescein-5-maleimide, which contain a phenol ring attached to a maleimide function, could be iodinated by chloramine chemistry and could be used to iodinate specific cysteine residues in proteins such as Cry1Fa. In particular, analogs smaller than fluorescein-5-maleimide might be more accessible to the interior of Cry1Fa core toxin thus may result in greater specific radioactivity of the labeled protein.

[0057] The cysteine alkylated Cry1Fa protein produced herein is insecticidally active against FAW larvae. Unlabeled Cry1Fa protein and unlabeled Cry1Ab protein, but not unlabeled Cry1Ca protein, can displace the binding of radiolabeled Cry1Fa protein from FAW BBMV. Bioassay data demonstrates that Cry1Ca is active against both FAW and rFAW larvae, while Cry1Ab is less active against rFAW larvae. Thus, the biological activities of Cry1Fa, Cry1Ab, and Cry1Ca proteins against FAW and rFAW larvae are conveniently explained and predicted by the BBMV binding assay results. It is therefore one aspect of this invention that combinations of Cry proteins useful in Insect Management strategies may be predicted using competitive binding assays employing Cry1Fa protein radiolabeled by means of the methods of this invention.

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CLAIMS

We claim:

1. A protein selected from the group consisting of Cry1Fa holotoxin and Cry1Fa core toxin wherein said protein is labeled with a radioactive isotope of iodine and wherein said labeled protein retains the ability to selectively bind to receptors in brush border membrane vesicles prepared from midguts of *Spodoptera exigua* and *Spodoptera frugiperda*.
2. The protein of Claim 1 that is Cry1Fa holotoxin.
3. The protein of Claim 1 that is Cry1Fa core toxin.
4. A saturation-type binding assay method for determining binding activity of Cry1Fa core toxin to receptors on the midgut of a given insect species which comprises:
 - a) preparing a suspension containing a known concentration of brush border membrane vesicles isolated from midguts of the insect species;
 - b) adding, in separate containers, increasing amounts of the labeled Cry1Fa of Claim 3 to fixed amounts of the brush border membrane vesicle suspension under time and buffer conditions that permit the labeled Cry1Fa to bind to receptors on the brush border membrane vesicles;
 - c) separating the unbound labeled Cry1Fa from labeled Cry1Fa bound to the receptors on the brush border membrane vesicles; and
 - d) counting any radioactive signal emitted from the labeled Cry1Fa bound to the brush border membrane vesicles.
5. A competitive binding assay method for determining cross-reactivity which comprises:
 - a) preparing a suspension containing brush border membrane vesicles isolated from midguts of an insect species;
 - b) mixing an the labeled Cry1Fa of Claim 3 with an excess of a different unlabeled protein and the brush boarder membrane suspension under time and

buffer conditions that permit the labeled Cry1Fa and unlabeled protein to bind to receptors on the brush border membrane vesicles;

c) isolating the bound brush border membrane vesicles from unbound labeled Cry1Fa and unbound unlabeled protein mixture; and

d) comparing the radioactivity emitted from the bound brush border membrane vesicle fraction to the radioactivity emitted from the unbound mixture fraction.

6. The method of Claim 5 wherein the unlabeled protein is a Cry protein.

1/1

Figure 1

Bioassay of trypsin-truncated Cry1Fa and fluorescein-5-maleimide labeled, trypsin-truncated Cry1Fa against *S. frugiperda* larvae

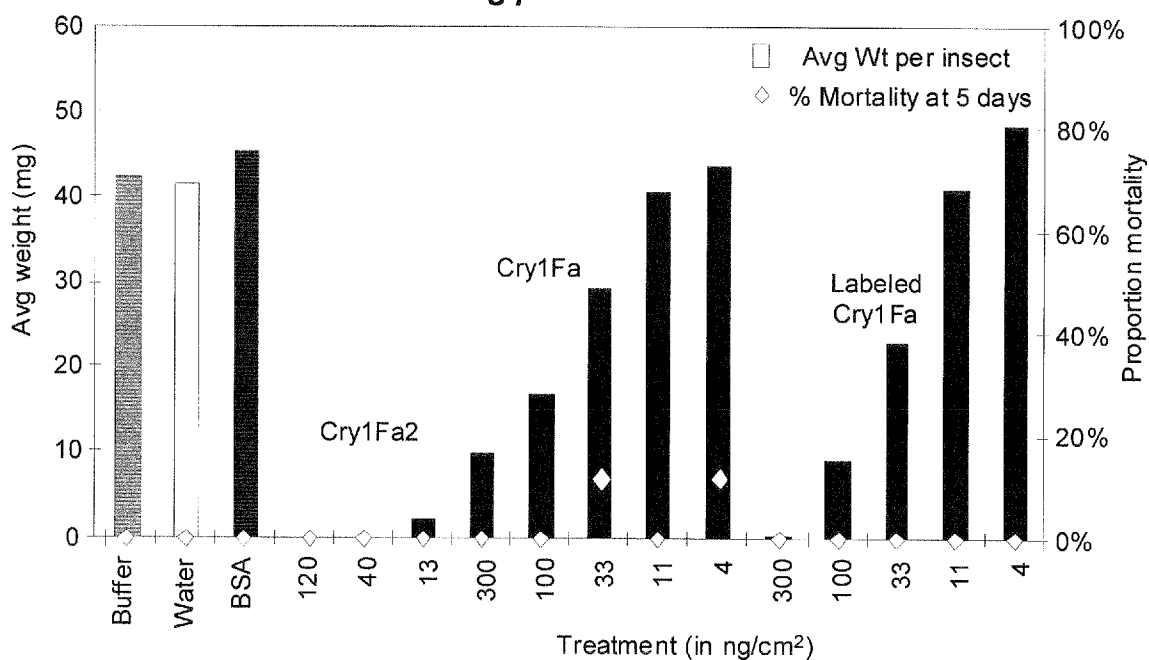
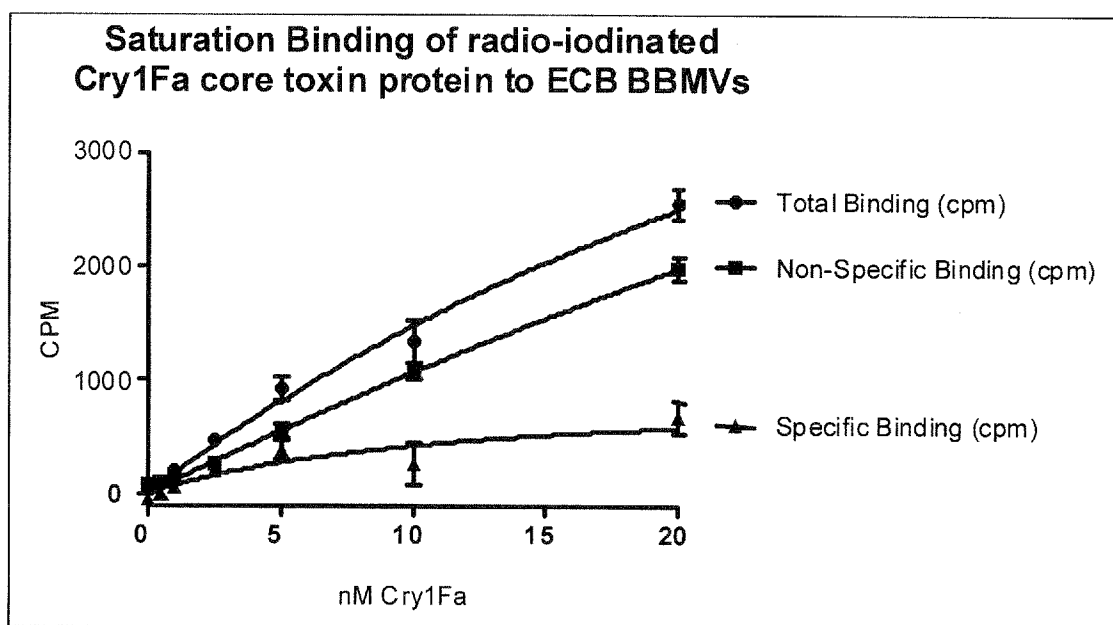


Figure 2



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/65322

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/00; C12Q 1/00 (2012.01)

USPC - 530/350; 435/4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C07K 14/00; C12Q 1/00 (2012.01)

USPC: 530/350; 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST (DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=NO; OP=ADJ), Google Scholar, Google Patents

Search Terms Used: Cry1Fa, holotoxin, core, radiolabel\$, radioactiv\$, iodinat\$, iodine, exigua, frugiperda, brush border membrane vesicles, BBMV, competitive, saturation, proteolyt\$, cleav\$, 28 amino acids, Choma, Bietlot, Lightwood, Bravo, Narva, etc.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	Luo et al. Toxicity, Binding, and Permeability Analyses of Four <i>Bacillus thuringiensis</i> Cry1 delta-Endotoxins Using Brush Border Membrane Vesicles of <i>Spodoptera exigua</i> and <i>Spodoptera frugiperda</i> . APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 1999, Vol. 65, No. 2, p. 457-464. esp: abstract; pg 458 first column, first full paragraph; pg 458 first column, section entitled "Iodination"; pg 459 second column, section entitled "Homologous and heterologous competition binding"; pg 458 second column, section entitled "Membrane vesicle binding assays"; pg 459 first column, section entitled "Maximal binding of labeled toxins to BBMV from <i>S. exigua</i> and <i>S. frugiperda</i> "; pg 463 first column, last paragraph; Fig. 2, Fig. 3	1, 2 ----- 3-6
Y	Risk Assessment and Risk Management Plan - Application for licence for dealings involving intentional release of a GMO into the environment -DIR 040/2003- Title: Agronomic assessment and seed increase of GM cotton expressing insecticidal genes from <i>Bacillus thuringiensis</i> . Applicant: Dow AgroSciences Australia Pty Ltd. November 2003. esp: pg 84 Section 3.3 item 52. [Available online from <www.ogtr.gov.au/internet/ogtr/publishing.nsf/content/dir040-4/\$file/dir040finalramp2.rtf>]	3-6
A	Jurat-Fuentes et al. Altered Glycosylation of 63- and 68-Kilodalton Microvillar Proteins in <i>Heliothis virescens</i> Correlates with Reduced Cry1 Toxin Binding, Decreased Pore Formation, and Increased Resistance to <i>Bacillus thuringiensis</i> Cry1 Toxins. Appl. Environ. Microbiol. 2002, 68(11):5711-5717. entire document.	3-6
A	Jurat-Fuentes et al. Importance of Cry1 delta-Endotoxin Domain II Loops for Binding Specificity in <i>Heliothis virescens</i> (L.). Appl. Environ. Microbiol. 2001 vol 67., no. 1, p. 323-329. entire document.	3-6

☒ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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Date of the actual completion of the international search

20 March 2012 (20.03.2012)

Date of mailing of the international search report

12 APR 2012

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/65322

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Bravo et al. N-terminal Activation Is an Essential Early Step in the Mechanism of Action of the Bacillus thuringiensis Cry1Ac Insecticidal Toxin. J Biol Chem 2002 Vol. 277, No. 27, pp. 23985-23987. entire document.	3-6
A	Choma et al. Unusual proteolysis of the protoxin and toxin from Bacillus thuringiensis - Structural implications. Eur. J. Biochem. (1990) 189, 523-527. entire document.	3-6
A	US 2005/0064386 A1 (Adang et al.) 24 March 2005 (24.03.2005) entire document.	1-6
A	US 2010/0017914 A1 (Hart et al.) 21 January 2010 (21.02.2010) entire document.	1-6