JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Cry34Ab1 and Cry35Ab1 Insecticidal Crystal Proteins Expressed in Transgenic Corn Plants and *Pseudomonas fluorescens*

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Cry34Ab1 and Cry35Ab1 proteins, identified from *Bacillus thuringiensis* strain PS149B1, act together to control corn rootworms. Transgenic corn lines coexpressing the two proteins were developed to protect corn against rootworm damage. Large quantities of the two proteins were needed to conduct studies required for assessing the safety of this transgenic corn crop. Because it was technically infeasible to obtain sufficient quantities of high purity Cry34Ab1 and Cry35Ab1 proteins from the transgenic corn plants, the proteins were produced using a recombinant *Pseudomonas fluorescens* (Pf) production system. The two proteins from both the transgenic corn and the Pf were purified and characterized. The proteins from each host had the expected molecular mass and were immunoreactive to specific antibodies in enzyme-linked immunosorbent assay and Western blot analysis. Data from N-terminal sequencing, tryptic peptide mass fingerprinting, internal peptide sequencing, and biological activity provided direct evidence that the Cry34Ab1 and Cry35Ab1 proteins produced in Pf and transgenic corn were, respectively, comparable or equivalent molecules. In addition, neither protein had detectable glycosylation regardless of the host.

KEYWORDS: Rootworm resistance corn; genetically modified plant; *Bacillus thuringiensis*; binary toxin; Cry34Ab1; Cry35Ab1; protein characterization; truncation

INTRODUCTION

Two proteins identified from *Bacillus thuringiensis* (Bt) strain PS149B1 were shown to act together as a binary insecticidal crystal protein against corn rootworm (*1*−*4*). The two proteins, approximately 14 and 44 kDa in size, were designated by the International Bt Nomenclature Committee as Cry34Ab1 and Cry35Ab1, respectively (*4*, *5*). Transgenic corn lines were developed by Dow AgroSciences LLC and Pioneer Hi-Bred International, Inc. through the insertion of the two genes encoding Cry34Ab1 and Cry35Ab1 proteins (*3*). Field tests of the transgenic corn demonstrated protection from root damage by western corn rootworm, *Diabrotica virgifera virgifera* LeConte. The level of protection exceeded that provided by chemical insecticides (*3*). It is anticipated that commercialization of these transgenic rootworm protection corn lines would bring significant benefit to corn growers and the environment by reducing the use of chemical pesticides while protecting the corn yields from a devastating pest.

Characterization of the transgenes and the gene products (i.e., the recombinant proteins) is of significant importance in the safety assessment of transgenic crops (*6*). For food, feed, and environmental safety assessments of the transgenic corn lines expressing Cry34Ab1 and Cry35Ab1 proteins, large quantities of the two proteins were required to conduct tests and, ultimately, to assess the risk of toxicity, allergenicity, and development of insect resistance. Because expression of the Cry34Ab1 and Cry35Ab1 proteins in corn was relatively low, it was technically infeasible to obtain large quantities of the proteins from transgenic corn plants. Alternatively, the proteins could be produced efficiently and purified in large quantities using a proprietary recombinant *Pseudomonas fluorescens* (Pf) production system (*4*). The proteins produced by Pf could then be used in safety tests as a surrogate for the plant-produced proteins. The objective of the current study was to purify and characterize the Cry34Ab1 and Cry35Ab1 proteins from the transgenic corn plants and Pf and examine the equivalency or comparability of the Cry34Ab1 and Cry35Ab1 proteins expressed by the two host organisms.

MATERIALS AND METHODS

Plant Materials. Seeds or frozen leaves of 11 transgenic corn events transformed with *cry*34Ab1 and *cry*35Ab1 genes were obtained from the Dow AgroSciences field station in Huxley, Iowa. Key differences in the transformation constructs and the methods of transformation for

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10.1021/jf048744+ CCC: $27.50 © 2004 American Chemical Society
Published on Web 12/08/2004

Table 1. Lead Events of the Cry34Ab1/Cry35Ab1 Transgenic Corn

event code	transformation vector	transformation method	Cry34Ab1 promoter	Cry35Ab1 promoter
5638	PHP12560	particle gun	UbiZm1[a]	UbiZm1
5639	PHP12560	particle gun	UbiZm1	UbiZm1
15344	PHP14352	agrobacterium	UbiZm1	UbiZm1
DAS-45214-4	PHP17662	agrobacterium	UbiZm1	TA peroxidase[b]
DAS-45216-6	PHP17662	agrobacterium	UbiZm1	TA peroxidase
42.1.18	PHP17662	agrobacterium	UbiZm1	TA peroxidase
DAS-59110-4	PHP17662	agrobacterium	UbiZm1	TA peroxidase
DAS-59132-8	PHP17662	agrobacterium	UbiZm1	TA peroxidase
DAS-59122-7	PHP17662	agrobacterium	UbiZm1	TA peroxidase
DAS-06317-5	PHP17662	agrobacterium	UbiZm1	TA peroxidase
DAS-71133-3	PHP17658	agrobacterium	UbiZm1	rice actin[c]

[a] *Zea mays* ubiquitin promoter (*7*). [b] *Triticum aestivum* peroxidase promoter (*8*). [c] *Oryza sativa* actin promoter (*9*).

these events are listed in **Table 1**. Upon receipt, frozen leaves were kept in a −80 °C freezer until analysis. The transgenic seeds, together with the seeds of nontransgenic control corn lines, were planted in pots at Dow AgroSciences greenhouse in Indianapolis, IN. Approximately 2 months after planting, the mature leaves of the greenhouse-grown corn plants were harvested for analysis.

Enzyme-Linked Immunosorbent Assay (ELISA). Leaf tissue was homogenized in phosphate-buffered saline with Tween (PBST) containing 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20 (pH 7.4). The homogenate was centrifuged, and the supernatant was saved for analysis. The concentrations of Cry34Ab1 and Cry35Ab1 proteins in the extracts were determined by Cry34Ab1 and Cry35Ab1 microtiter plate ELISA kits, respectively.

Protein Purification from Corn Plants. Mature leaves from the transgenic corn plants were harvested, ground in liquid nitrogen, and extracted with PBST containing a protease inhibitor cocktail for plant cell extracts (Sigma, catalog P-9599). The homogenate was clarified by filtration and centrifugation at 4 °C. Solid ammonium sulfate was slowly added to the supernatant to about 85% saturation to precipitate the proteins. The proteins were recovered by centrifugation at 20000*g* for 20 min at 4 °C, and the pellet was resuspended in PBST. Residual ammonium sulfate in the supernatant was removed using Econo-Pac 10DG desalting columns (Bio-Rad).

Further purification of the Cry34Ab1 and Cry35Ab1 proteins was conducted using immunoaffinity chromatography. The polyclonal antibodies were generated by inoculating recombinant Pf-produced Cry34Ab1 or Cry35Ab1 into New Zealand female white rabbits. Rabbit test bleeds were screened for activity against the antigens in a direct bind screening assay. Antisera from the rabbits were pooled and purified by Protein A affinity chromatography to a purity of >95%. The immunoaffinity columns were generated by coupling Cry34Ab1 or Cry35Ab1 protein specific polyclonal antibodies to CNBr-activated Sepharose 4 FF gel (Amersham Biosciences). The antibody-coupled affinity gels were packed into two disposable syringelike columns. The bed volume for each column was approximately 5 mL, and the antibody ligand density was 4−6 mg per mL gel.

The protein solution following desalting was applied in batches to the Cry34Ab1 antibody-coupled column, which was preequilibrated with buffer A (20 mM sodium phosphate, 0.5 M NaCl, and 0.05% Tween 20, pH 7.2). The flow-through solution was collected and loaded onto the Cry35Ab1 antibody-coupled column, which was also preequilibrated with buffer A. After loading, both columns were washed with 15 mL of buffer A, and the bound proteins were eluted with 15 mL of buffer B (pH 2.9, prepared by mixing 840 mL of 0.1 M citric acid with 160 mL of 0.2 M dibasic sodium phosphate). The eluates were concentrated with centrifugal filter devices [molecular mass (MW) cutoff, 5 kDa; Millipore].

Protein Purification from Pf. Recombinant microbial Cry34Ab1 and Cry35Ab1 proteins were expressed as crystalline proteins in two separate strains of Pf (strain MR1253 and strain MR1256, respectively). After fermentation, the Pf cells were pelleted and resuspended in a lysis buffer (pH 7.5) containing 50 mM Tris, 0.2 M NaCl, 5% glycerol, 1 mM dithiothreitol, 20 mM ethylenediamine tetraacetate (EDTA), 0.5% Triton X-100, and 0.6 mg/mL lysozyme. The cells were incubated at 37 °C briefly and then held on ice for 1 h. Magnesium chloride and deoxyribonuclease I were added to final concentrations of 60 mM and 0.25 mg/mL, respectively. After the cells were incubated at 4 °C overnight, the cell lysate was homogenized and centrifuged at 10000*g* for 20 min at 4 °C. The pelleted crystalline proteins were washed with the lysis buffer, homogenized, and reprecipitated by centrifugation. The final pellet was resuspended in H_2O and lyophilized.

Isolation and purification of the full-length and a truncated Cry35Ab1 were conducted using cation exchange chromatography. The lyophilized Cry35Ab1 was resuspended in buffer C (20 mM sodium citrate, pH 3.3) and loaded onto a HiTrap SP column (Amersham Biosciences, column volume = 5 mL), which was preequilibrated with buffer C. Elution was achieved with a linear gradient to 100% of buffer D (20 mM sodium citrate, 1 M NaCl, pH 3.3) in 75 mL volume using an ATKA FPLC liquid chromatography system (Amersham BioSciences). Fractions containing the proteins of interest were pooled and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

SDS−PAGE and Western Blotting. In conducting gel and blot analyses, protein samples were mixed with Laemmli sample buffer (*10*) containing 5% freshly added β-mercaptoethanol and boiled for 5 min at 100 °C before loading onto precast gradient gels. For protein solutions in low pH, after mixing with Laemmli sample buffer, the pH of the mixture needed to be further adjusted to neutral by adding 1.0 M stock Tris solution. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB).

In Western blot analysis, rabbit polyclonal antibodies for Cry34Ab1 or Cry35Ab1 were used as the primary antibodies. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) (Pierce) was used as the secondary antibody. A chemiluminescent substrate (Amersham Biosciences, catalog RPN2132) was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to CL-XPosure (Pierce) detection film for various time points and subsequently developed with a Konica SR-X film developer.

Treatment of Cry35Ab1 with Serine Proteinases. The susceptibility of the isolated full-length Pf Cry35Ab1 to trypsin, chymotrypsin, and endoproteinase Glu-C was examined. The reaction was carried out in separate microcentrifuge tubes, each containing 280 μL of 133 mM Tris-HCl buffer (pH 8), 75 μg of Cry35Ab1, and 25 μg of the proteinase tested. All three proteinases were of sequencing grade purity (Roche and Promega). The reaction cocktail was incubated in a 26 °C waterbath for 30 min, and at the end of the incubation, the serine proteinases were deactivated by adding 0.5 M freshly prepared phenylmethylsulfonyl fluoride stock solution to a final concentration of 10 mM. SDS−PAGE was performed immediately following the treatments.

Insect Bioassay. The growth inhibition of the Pf-expressed Cry34Ab1 and Cry35Ab1 on southern corn rootworm (SCR) was tested by diet overlay bioassays (*11*). The assays were performed using Cry34Ab1 alone and Cry34Ab1 combined with either the full-length Cry35Ab1 or the truncated Cry35Ab1. Each well of the bioassay trays contained 0.5 mL of diet (*12*) and had a surface area of 1.5 cm^2. Protein samples were formulated in 10 mM potassium phosphate buffer (pH 7.5), and 50 μL of the protein solution was pipetted into each well. Buffer controls were included in each bioassay. A single neonate SCR was placed in each well. Sixteen SCR neonates were included per bioassay, and 10 bioassays were conducted per dosage. The insects were held at 26 °C in the dark after inoculation. Mortality and live insect mass data were recorded after 5 or 6 days.

Detection of Glycosylation. The purified Cry34Ab1 and Cry35Ab1 proteins from the transgenic corn and Pf were separated from minor impurities by SDS−PAGE. After electrophoresis, the gels were stained with a GelCode glycoprotein staining kit (Pierce). After the staining, only glycoproteins were visible, appearing as magenta bands on the gel with a light pink background. Simultaneously, an identical gel was stained with CBB to visualize all protein bands on the gel. HRP and soybean trypsin inhibitor were used as a positive and a negative control for glycoproteins, respectively.

Protein MW Determination by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF

MS). The isolated full-length and truncated Pf Cry35Ab1 protein samples were diafiltrated with H_2O (in centrifugal filter devices, 10 kDa MW cutoff, Millipore) and mixed with a matrix solution in a 1:1 ratio (v/v). The matrix solution was prepared by suspending 5 mg of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Aldrich) in 0.5 mL of 50% acetonitrile and 0.05% trifluoroacetic acid (TFA). The mixture of protein and matrix was spotted on a stainless steel MALDI sample plate and allowed to air-dry. Mass spectral analyses were performed on a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems). External calibration was performed using Proteomass protein MALDI-MS calibration kit (Sigma). All mass spectra for MW (mass) estimation were collected in the positive linear mode using delayed extraction. Each spectrum obtained was a result of an average of 300 laser shots.

Tryptic Peptide Mass Fingerprinting. Purified Cry34Ab1 and Cry35Ab1 proteins from transgenic corn and Pf were denatured by heating at 100 °C for 5 min, followed by SDS−PAGE. The respective protein bands were excised from the gel and placed into siliconized microcentrifuge tubes and destained with 25% acetonitrile in 12.5 mM ammonium bicarbonate. The gel pieces were dried in a Speed-Vac and digested with sequencing grade trypsin (Promega) at 37 °C for 5 h. The digestion was stopped by adding 10% formic acid, and the supernatant was transferred to a clean siliconized tube and stored at −20 °C. Before MALDI-TOF MS analysis, the solutions were thawed and the peptides were purified with C18 Zip Tip (Millipore).

Mass spectral analyses of the peptides in the trypsin digests were performed on a Voyager-DE STR MALDI-TOF mass spectrometer. Samples were prepared by mixing 1 μL of the peptides with 1 μL of the MALDI matrix solution of saturated α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.1% TFA, and the samples were then spotted onto the MALDI sample plate and allowed to air dry. External calibration was performed by using angiotensin I, adrenocorticotropic hormone (ACTH) (clip 1−17), ACTH (clip 18−39), and ACTH (clip 7−38). All mass spectra were collected in the positive reflector mode using delayed extraction. The mass data recorded were input into PAWS software (Genomic Solutions) to search for matched peptides.

N-Terminal Sequencing. The purified preparations of Cry34Ab1 and Cry35Ab1 from both transgenic corn and Pf were further separated from any minor impurities by SDS−PAGE and were electroblotted onto a polyvinyldifluoride (PVDF) membrane in 10 mM 3-(cyclohexylamino)-1-propanesulfonic (CAPS) and 20% methanol (MeOH) (pH 11.0). Proteins on the membrane were stained with GelCode Blue Stain (Pierce), and the Cry34Ab1 and Cry35Ab1 protein bands were excised and extensively rinsed with water. The N-terminal sequences were determined using a Procise Protein Sequencer (Applied Biosystems).

Internal Peptide Sequencing. Sequencing of selective peptides generated by trypsin digestion of the Pf or corn event 5638-derived Cry34Ab1 and Cry35Ab1 was performed using a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micromass). Following the MALDI-TOF MS analysis, the trypsin digest from each protein sample was diluted with 50% MeOH and 0.5% formic acid. Approximately 5 μL of the resulting solution was loaded into a nanovial (e.g., nanoflow type A 6028623 probe tip, Micromass) and installed on the nanospray source of the Q-TOF mass spectrometer. Positive electrospray ionization was used to ionize the peptides, and both MS and tandem mass spectrometry (MS/MS) spectra were acquired. Selection of MS/MS precursor ions was based on the peptides observed in the MS spectra of each sample. Precursor ion isolation for MS/MS analysis was approximately 5 amu, and MS/MS spectra were averaged at collision energies ranging from 10 to 50 eV to produce the best sequence coverage in the product ion spectrum. The resulting spectra were processed using the MaxEnt3 software (Micromass) to convert multiply charged ions and isotopic clusters into a more readily interpretive monoisotopic, singly charged ion series. These spectra were then used for amino acid sequence interpretation.

RESULTS

During the development of Cry34Ab1/Cry35Ab1 transgenic corn, 11 lead events were selected for extensive protein characterization (**Table 1**). The proteins were characterized by the protein expression level (ELISA), molecular size, immunorecognition, N-terminal sequencing, peptide mass fingerprinting, and examination of glycosylation. Internal peptide sequencing was also conducted for one of the events (event 5638). Except for the expression levels of the two proteins, the overall characterization results on the Cry34Ab1 and Cry35Ab1 proteins were similar across these transgenic events despite the differences in the genetic modification methods used in generating these events (**Table 1**). The protein characterization data from event DAS-45216-6 are presented in this paper unless otherwise specified.

Protein MW and Immunorecognition. On the basis of the sequences of the native *cry*34Ab1 gene transformed into Pf and the plant-optimized *cry*34Ab1 transgene transformed into corn, the amino acid sequence of Cry34Ab1 from the transgenic corn and Pf was predicted to be the same. The Cry34Ab1 (GenBank accession AAG41671) consists of 123 amino acids with a MW of approximately 13.6 kDa. On the basis of the sequences of the corresponding *cry*35Ab1 genes transformed into Pf and corn, a slight difference in amino acid sequence was expected between the transgenic corn Cry35Ab1 (383 amino acids, MW 43.8 kDa, GenBank accession AAG41672) and the Pf Cry35Ab1 (385 amino acids, MW 44.0 kDa). Specifically, the difference involves a total of four amino acid residues at the C terminus: K^{376} in corn vs I^{376} in Pf, Y^{381} in corn vs H^{381} in Pf, and two extra lysines (K) in Pf Cry35Ab1 (**Figure 1**). This C-terminal end in the recombinant Pf Cry35Ab1 is homologous to the C terminus of Cry35Aa1 protein of Bt strain 80JJ1 (*4*). During the early research stage, the primers used in the PCR (Polymerase Chain Reaction) to amplify the *cry*35Ab1 gene for Pf cloning were designed to also amplify the *cry*35Aa1 gene. Slight modifications were introduced in the fragment of *cry*35Ab1 gene during the PCR amplification, resulting in the four alternate amino acid residues.

Western blot analysis showed that the Cry34Ab1 and Cry35Ab1 proteins produced by both the transgenic corn and the Pf had the expected MWs and were reactive to the respective antibodies specific to the two proteins (**Figures 2** and **3**). As expected, the slight MW difference (0.2 kDa) between the Cry35Ab1 produced by the transgenic corn and Pf was indistinguishable on the blots.

In Cry35Ab1 preparations from both corn and Pf, two immunoreactive protein bands with apparently different MWs were observed (**Figure 3**). These two Cry35Ab1 protein bands were isolated with cation exchange chromatography from the Pf expressed Cry35Ab1 preparation (**Figure 4**). Because of the limitation of SDS−PAGE in determining accurate MW of proteins, MALDI-TOF MS was employed to measure the MW of the two purified protein bands. On the basis of the expected amino acid sequence of the Pf Cry35Ab1, the MW of an intact, full-length Pf Cry35Ab1 is 44026 Da. The measured value of the upper Cry35Ab1 band by MALDI-TOF MS was 44041 Da, essentially matching the expected MW of the full-length Pf Cry35Ab1 within the expected accuracy range of the equipment. The measured MW of the lower band was 40306 Da, which is 3735 Da smaller than the full-length Cry35Ab1.

The faster migrating Cry35Ab1 immunoreactive protein was predicted to be a proteolytic truncated form of the slower migrating protein. To test this hypothesis, the isolated full-length Cry35Ab1 was subjected to treatment by three serine proteinases. The result demonstrated that the full-length Cry35Ab1 was sensitive to chymotrypsin and endoproteinase Glu-C, resulting in the formation of truncated form(s) which, on SDS−PAGE,

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corn    (1) MLDTNKVYEISNHANGLYAATYLSLDDSGVSLMNKNDDDIDDYNLKWFLF
Pf      (1) MLDTNKVYEISNHANGLYAATYLSLDDSGVSLMNKNDDDIDDYNLKWFLF

corn   (51) PIDDDQYIITSYAANNCKVWNVNNDKINVSTYSSTNSIQKWQIKANGSSY
Pf     (51) PIDDDQYIITSYAANNCKVWNVNNDKINVSTYSSTNSIQKWQIKANGSSY

corn  (101) VIQSDNGKVLTAGTGQALGLIRLTDESSNNPNQQWNLTSVQTIQLPQKPI
Pf    (101) VIQSDNGKVLTAGTGQALGLIRLTDESSNNPNQQWNLTSVQTIQLPQKPI

corn  (151) IDTKLKDYPKYSPTGNIDNGTSPQLMGWTLVPCIMVNDPNIDKNTQIKTT
Pf    (151) IDTKLKDYPKYSPTGNIDNGTSPQLMGWTLVPCIMVNDPNIDKNTQIKTT

corn  (201) PYYILKKYQYWQRAVGSNVALRPHEKKSYTYEWGTEIDQKTTIINTLGFQ
Pf    (201) PYYILKKYQYWQRAVGSNVALRPHEKKSYTYEWGTEIDQKTTIINTLGFQ

corn  (251) INIDSGMKFDIPEVGGGTDEIKTQLNEELKIEYSHETKIMEKYQEQSEID
Pf    (251) INIDSGMKFDIPEVGGGTDEIKTQLNEELKIEYSHETKIMEKYQEQSEID

corn  (301) NPTDQSMNSIGFLTITSLELYRYNGSEIRIMQIQTSDNDTYNVTSYPNHQ
Pf    (301) NPTDQSMNSIGFLTITSLELYRYNGSEIRIMQIQTSDNDTYNVTSYPNHQ

corn  (351) QALLLLTNHSYEEVEEITNIPKSTLKKLKKYYF--
Pf    (351) QALLLLTNHSYEEVEEITNIPKSTLIKLKKHYFKK
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Figure 1. Alignment of the amino acid sequence of Cry35Ab1 protein comparing the transgenic corn-expressed Cry35Ab1 and the recombinant Pf MR1256-expressed Cry35Ab1.

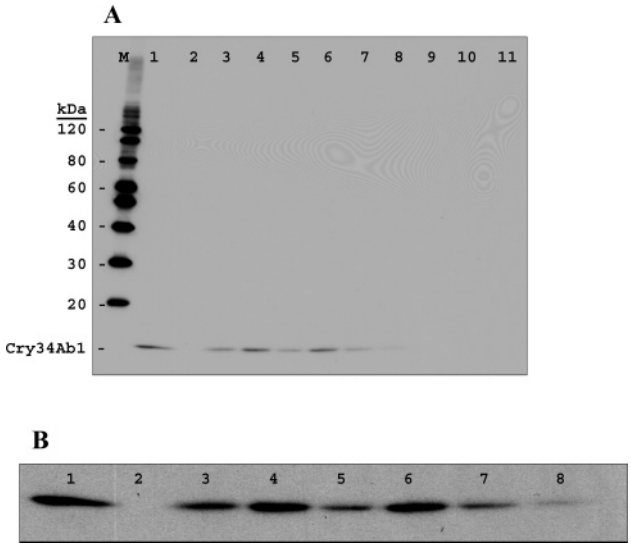

Figure 2. (**A**) Western blot of Cry34Ab1 protein expressed by transgenic corn and recombinant Pf. (**B**) Zoomed (4×) area showing the immunoreactive protein bands. Lane M, MagicMark MW markers (Invitrogen, visible on the blot because the markers have an IgG binding site from protein G, which recognizes the secondary antibody); lane 1, Pf Cry34Ab1 (2.7 ng); lane 2, blank; lanes 3 and 4, event 42.1.18; lanes 5 and 6, event DAS-59122-7; lanes 7 and 8, event DAS-59132-8; lane 9, TriChromRanger prestained MW markers (Pierce, invisible on Western blot film, visible on stained SDS–PAGE only); and lanes 10 and 11, two different nontransgenic corn lines.

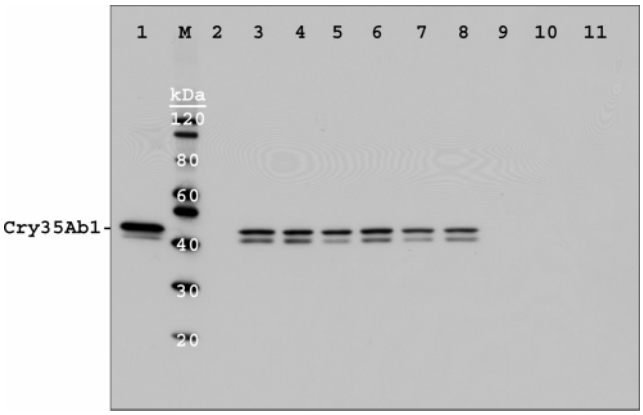

Figure 3. Western blot of Cry35Ab1 protein expressed by transgenic corn and recombinant Pf. Lane 1, Pf Cry35Ab1 (1.9 ng); lane M, MagicMark MW markers; lane 2, blank; lanes 3 and 4, event 42.1.18; lanes 5 and 6, event DAS-59122-7; lanes 7 and 8, event DAS-59132-8; lane 9, TriChromRanger prestained MW markers (invisible on Western blot film); and lanes 10 and 11, two different nontransgenic corn lines.

are not distinguishable from the 40306 Da Cry35Ab1 protein initially isolated (**Figure 5**). For the trypsin treatment, no apparent MW shift was seen based on SDS−PAGE (**Figure 5**, lane 5). In this experiment, two separate sources of sequencing grade trypsin (Promega and Roche) were used. We observed that when full-length Cry35Ab1 was incubated with a trypsin product of low purity (such as Sigma catalog T-7168), truncation would occur. This is likely due to the effect of a small amount of chymotrypsin impurities in the low-purity grade trypsin product rather than trypsin itself. In addition, it should be noted that in conducting the truncation experiments, after completion of the digestion treatment, it is pivotal to deactivate the proteinases before proceeding to SDS−PAGE analysis. If the proteinases are not deactivated, they can rapidly digest Cry35Ab1 once it becomes denatured upon mixing with the Laemmli sample buffer and being heated.

Potentiation Effect of Cry35Ab1 on Cry34Ab1. Herman et al. previously reported that Cry35Ab1 protein did not exhibit toxicity to SCR when applied alone (*11*). Although the Cry34Ab1 alone exerted an insecticidal effect, the activity was greatly enhanced by the presence of the Cry35Ab1 protein (*11*). This phenomenon can be described as a potentiation effect (*13*) of Cry35Ab1 on Cry34Ab1. Using either the full-length or the truncated Cry35Ab1, the current study demonstrated that the truncated Cry35Ab1 had a significantly stronger potentiation effect than the full-length Cry35Ab1 on the bioactivity of Cry34Ab1 (**Table 2**).

Purification of the Proteins from Plants. The expression levels of Cry34Ab1 and Cry35Ab1 proteins in leaves of the transgenic corn events were determined by ELISA. There were

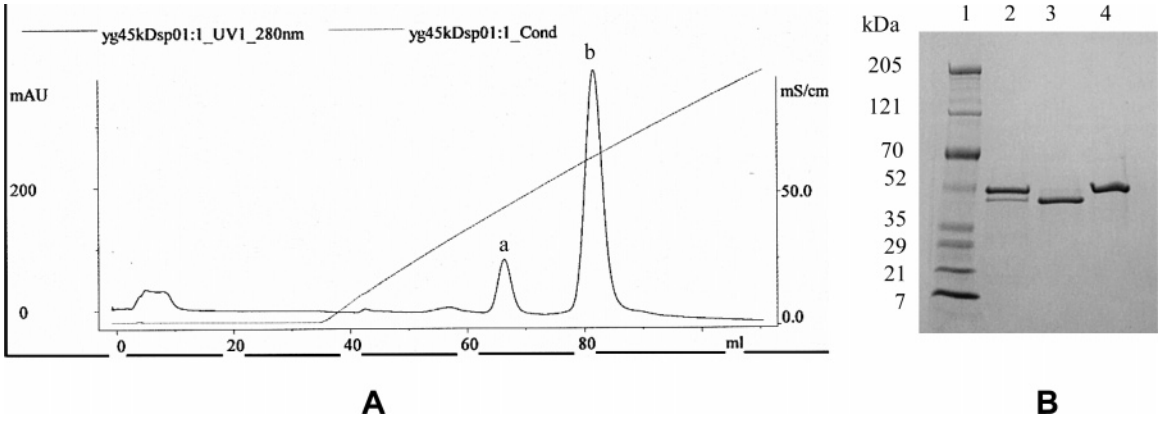

Figure 4. Isolation of the full-length and truncated form of Cry35Ab1 produced by recombinant Pf. (**A**) Chromatogram of HiTrap SP cation exchange chromatography. (**B**) SDS–PAGE (4–15% gradient) stained by CBB. Lane 1, prestained MW markers (Bio-Rad); lane 2, material before isolation; lane 3, pooled fractions of peak a; and lane 4, pooled fractions of peak b. Approximately 2 μg of protein was loaded in each lane.

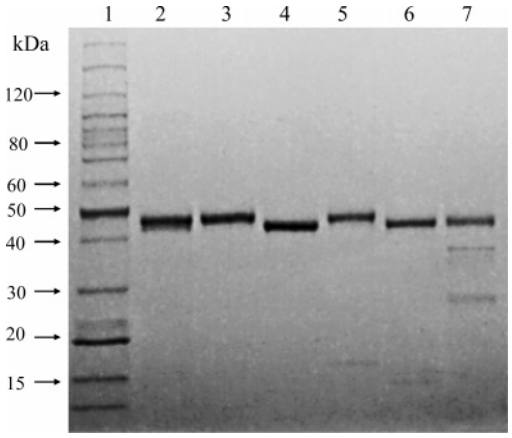

Figure 5. SDS–PAGE (4–20% gradient) of Pf-expressed Cry35Ab1 following proteinase treatment. Lane 1, BenchMark protein ladder (Invitrogen); lane 2, Cry35Ab1 preparation (mainly full-length with some truncated); lane 3, isolated full-length Cry35Ab1 (1.3 μg); lane 4, isolated truncated Cry35Ab1; lane 5, trypsin-treated full-length Cry35Ab1; lane 6, chymotrypsin-treated full-length Cry35Ab1; and lane 7, endoproteinase Glu-C-treated full-length Cry35Ab1.

Table 2. Growth Inhibition (GI) of SCR by Cry34Ab1 and Cry35Ab1

treatment	mean GI[a] (%)	Duncan grouping[b]
0.5 μg/cm^2 Cry34Ab1 only	24	A
0.5 μg/cm^2 Cry34Ab1 + 0.5 μg/cm^2 full-length Cry35Ab1	44	B
0.5 μg/cm^2 Cry34Ab1 + 0.5 μg/cm^2 truncated Cry35Ab1	56	C

[a] GI, growth inhibition calculated based on the reduction in total live insect mass in each treatment as compared with buffer control for each test (*11*). [b] Means with the same letter are not significantly different by Duncan's multiple range test (α = 0.05).

remarkable variations in protein expression levels between the corn events and individual plants. For the greenhouse-grown plants, the leaf expression levels for the various transgenic events and individual plants ranged from 20 to 200 ppm and from 10 to 70 ppm (on fresh weight basis) for Cry34Ab1 and Cry35Ab1, respectively. Because of the low expression levels of the Cry34Ab1 and Cry35Ab1, the protein profiles of the crude leaf extracts were indistinguishable between the transgenic and the nontransgenic corn lines on SDS−PAGE. Further characterization of the corn-produced Cry34Ab1 and Cry35Ab1 required purification of the two proteins. Proteins were extracted from the transgenic corn leaves and enriched by ammonium sulfate precipitation, and the Cry34Ab1 and Cry35Ab1 (including full-length and truncated forms) were purified to apparent homogeneity after immunoaffinity chromatography (**Figure 6A**, lanes 6 and 7).

Table 3. N-Terminal Sequencing

protein samples	N-terminal sequence[a]
Cry34Ab1, expected sequence	MSAREVHIDV
corn Cry34Ab1	SAREVHIDV
Pf Cry34Ab1	XAREVHIDV
Cry35Ab1, expected sequence	MLDTNKVYEI
corn Cry35Ab1 intact	MLDTNKVYEI
corn Cry35Ab1 truncated	MLDTNKVYEI
Pf Cry35Ab1 intact	MLDTNKVYEI
Pf Cry35Ab1 truncated	XLDTNKVYEI

[a] X indicates an uncertain amino acid assignment during the sequencing test.

Lack of Posttranslational Glycosylation. The native Cry34Ab1 and Cry35Ab1 proteins in Bt are not glycoproteins. However, there are several potential N-glycosylation sites in the sequence of both proteins (N at positions 12, 90, and 104 of Cry34Ab1 and N at positions 169, 324, 338, 342, and 358 of Cry35Ab1). The potential for glycosylation of the proteins in the transgenic hosts was examined. Using the GelCode glycoprotein staining kit, it was shown that Cry34Ab1 and Cry35Ab1 proteins from both the transgenic corn and the recombinant Pf had no detectable carbohydrates (**Figure 6**). This staining method is able to detect 0.625 ng of the glycoprotein avidin and 0.16 μg of HRP (Pierce technical information of the test kit). The amount of purified, corn-expressed Cry34Ab1 and Cry35Ab1 loaded on the gel was approximately 2 and 3 μg, respectively. Thus, the amount of corn-expressed Cry34Ab1 loaded on the gel was 3200 times higher than the detection limit for avidin and 12 times higher than that of HRP. The amount of corn-expressed Cry35Ab1 loaded was 4800 times higher than the detection limit for avidin and 19 times higher than that of HRP.

N-Terminal Sequencing. The amino acid residues at the N termini of Cry34Ab1 and Cry35Ab1 from both the transgenic corn and the Pf were sequenced. The results demonstrated that the N termini of the Cry34Ab1 and Cry35Ab1 proteins from the transgenic corn and Pf matched the expected sequences except for the terminal methionine or a single amino acid, which was not assigned during the sequencing tests (**Table 3**). Different transgenic corn events tested yielded the same conclusion. Both

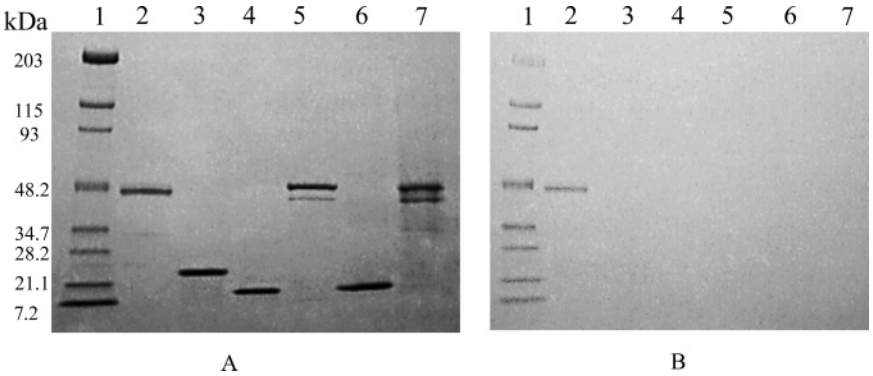

Figure 6. SDS–PAGE gels stained by CBB (**A**) or Pierce GelCode glycoprotein staining kit (**B**). Lane 1, prestained MW markers; lane 2, HRP (positive control of glycoprotein); lane 3, soybean trypsin inhibitor (negative control); lane 4, Pf Cry34Ab1 (1.7 μg); lane 5, Pf Cry35Ab1 (1.2 μg); lane 6, immunoaffinity-purified corn Cry34Ab1 (2 μg); and lane 7, immunoaffinity-purified corn Cry35Ab1 (3 μg).

Table 4. Tryptic Peptide Mass Data (*m*/*z* of Peptide Ion $[M + H]^+$) of Cry34Ab1 Protein

amino acid residues nos.	theoretical mass (*m*/*z*)	corn Cry34Ab1	Pf Cry34Ab1
5–13	1067.6	1067.5	1067.5
5–25	2419.3	2419.3	2419.2
14–25	1370.7	1370.7	1370.7
14–30	1869.0	1869.0	1868.9
33–44	1287.7	1287.6	1287.6
92–118	2943.4	2943.4	2943.3

the intact and the truncated Cry35Ab1 had an intact N-terminal sequence, suggesting that the truncation of Cry35Ab1 by the host (corn or Pf) proteases occurred at the C terminus. The result also showed that the N-terminal methionine (M) of Cry34Ab1 was removed in both hosts. This is not uncommon, as it is known that N-terminal methionine can be cleaved by methionine aminopeptidases in both prokaryotic and eukaryotic systems (*14*).

Tryptic Peptide Mass Fingerprinting. The masses of the detected peptides in the trypsin digests of heat-denatured Cry34Ab1 and Cry35Ab1 proteins were compared to those deduced in silico based on trypsin cleavage sites in the protein sequence. In the trypsin digests of Cry34Ab1 from both the transgenic corn and the Pf, six peptides were identified matching the theoretical deduced peptide masses of Cry34Ab1 (**Table 4**). These peptides together cover 52% of the Cry34Ab1 protein sequence. In the trypsin digests of the intact and truncated Cry35Ab1, 11−13 peptides were identified matching the theoretical deduced peptide masses of Cry35Ab1 (**Table 5**). These peptides together cover approximately 44−53% of the Cry35Ab1 protein sequence.

Internal Peptide Sequencing. The peptide mixtures of trypsin-digested Cry34Ab1 and Cry35Ab1 from Pf and transgenic corn event 5638 were analyzed using a Q-TOF mass spectrometer. Full scan mass spectra of each sample were acquired and used to determine the selection of MS/MS precursor ions. MS/MS spectra of the observed peptide fragments were collected and used for internal sequencing. The doubly charged precursor ion $[M + 2H]^{2+}$ at *m*/*z* 644.3 (corresponding to the singly charged peptide ion $[M + H]^+$ at *m*/*z* 1287.6) was selected from the Cry34Ab1 digests of both transgenic corn and Pf, and the doubly charged precursor ion $[M + 2H]^{2+}$ at *m*/*z* of 685.4 (corresponding to the singly charged peptide ion $[M + H]^+$ at *m*/*z* 1369.6) was selected from the Cry35Ab1 digests of the transgenic corn and Pf.

In peptide MS/MS nomenclature, fragmentation of doubly charged precursor ions can lead to fragments of type a_n, b_n, and c_n if the charge is located at the N terminus and to fragments of type x_n, y_n, and z_n if the charge is retained on the C terminus.

Table 5. Tryptic Peptide Mass Data (*m*/*z* of Peptide Ion $[M + H]^+$) of Cry35Ab1 Protein

amino acid residues nos.	theoretical mass (*m*/*z*)	corn Cry35Ab1 full-length	corn Cry35Ab1 truncated	Pf Cry35Ab1 full-length	Pf Cry35Ab1 truncated
7–35	3146.5	ND[a]	ND	3146.2	3148.3
7–46	4466.1	4465.3	ND	4467.5	ND
47–68	2637.9	ND	ND	ND	2638.3
69–76	988.1	ND	ND	988.0	988.0
69–90	2511.2	2511.5	2511.3	ND	ND
109–122	1369.8	1369.9	1369.9	1369.6	1369.6
123–154	3650.9	3651.3	3652.1	3651.0	3651.0
207–213	1071.5	1071.6	1071.6	1071.0	1071.0
208–213	943.4	943.5	943.5	943.0	943.0
214–226	1377.8	1377.8	1377.8	1377.6	1377.6
227–240	1747.8	1747.9	1747.9	1747.6	1747.6
228–240	1619.7	1619.8	1619.8	1619.5	1619.5
241–258	1966.0	ND	1966.1	1965.8	1965.8
259–272	1476.7	ND	1476.8	1476.5	1476.5
259–280	2432.2	ND	2432.3	ND	ND
259–288	3419.7	3419.9	ND	ND	ND
273–288	1962.0	ND	1962.0	ND	ND
293–322	3492.6	3493.0	ND	3493.0	ND
323–329	837.9	ND	ND	838.0	838.0

[a] ND, not detected.

Table 6. Interpretation of Amino Acid Sequence of the Doubly Charged Precursor Peptide Ion $[M + 2H]^{2+}$ at *m/z* 644.3 from the Trypsin Digest of Cry34Ab1 Protein[a]

fragment type	*m/z*											
A	74.1	**161.1**	258.1	359.2	473.2	572.3	643.3	757.4	872.4	1000.5	1113.6	
B	102.1	**189.1**	286.1	387.2	**501.2**	**600.3**	**671.3**	785.4	900.4	1028.5	**1141.5**	
C″	119.1	206.1	303.2	404.2	518.3	**617.3**	**688.4**	802.4	917.4	1045.5	1158.6	
	Thr	Ser	Pro	Thr	Asn	Val	Ala	Asn	Asp	Gln	Ile	Lys
x		1212.6	1125.6	1028.5	927.5	813.4	714.3	643.3	529.3	414.2	286.2	173.1
y″		**1186.6**	**1099.6**	**1002.5**	**901.5**	**787.4**	**688.4**	**617.3**	**503.3**	**388.3**	**260.2**	**147.2**
z		1169.6	**1082.5**	985.5	**884.4**	770.4	**671.3**	**600.3**	486.3	**371.2**	243.2	**130.1**

[a] Shown in the table is a complete list of possible theoretical fragments, and those detected by the MS/MS are bolded.

Table 7. Interpretation of Amino Acid Sequence of the Doubly Charged Precursor Peptide Ion $[M + 2H]^{2+}$ at *m/z* 685.4 from the Trypsin Digest of Cry35Ab1 Protein[a]

fragment type	*m/z*													
a	72.1	**185.2**	286.2	357.3	414.3	515.3	572.3	700.4	771.4	884.5	941.5	1054.6	1167.7	
b	100.1	**213.2**	**314.2**	385.2	442.3	543.3	600.3	728.4	799.4	912.5	969.5	1082.6	1195.7	
c″	117.1	230.2	331.2	402.3	459.3	560.3	617.4	745.4	816.5	929.5	986.6	1099.6	1212.7	
	Val	Leu	Thr	Ala	Gly	Thr	Gly	Gln	Ala	Leu	Gly	Leu	Ile	Arg
x		1296.7	1183.6	1082.6	1011.6	954.4	853.5	796.5	668.4	597.4	484.3	427.3	314.2	201.1
y″		1270.7	**1157.7**	**1056.6**	**985.6**	**928.6**	**827.5**	**770.5**	**642.4**	**571.4**	**458.3**	401.3	**288.2**	**175.1**
z		1253.7	1140.6	1039.6	968.6	911.5	810.5	753.5	625.4	554.4	441.3	384.3	271.2	158.1

[a] Shown in the table is a complete list of possible theoretical fragments, and those detected by the MS/MS are bolded.

If an extensive fragment ion series is obtained, the analyzed peptide can be sequenced because the adjacent signals differ by the masses of the individual amino acid residues. In the current study, the precursor ion for one major peptide fragment was selected from each protein digest. **Table 6** illustrates the detected and predicted MS/MS fragment ion series from the precursor ion $[M + 2H]^{2+}$ at *m/z* 644.3 of the Cry34Ab1 trypsin digest. The obtained peptide sequence, TSPTNVANDQIK, matched the theoretical sequence of Cry34Ab1 from residue T_{33} to K_{44}. **Table 7** illustrates the detected and predicted MS/MS fragment ion series from precursor ion $[M + 2H]^{2+}$ at *m/z* 685.4 of the Cry35Ab1 trypsin digest. The obtained peptide sequence, VLTAGTGQALGLIR, matched the theoretical sequence of Cry35Ab1 from residue V_{109} to R_{122}.

DISCUSSION

Characterization of the newly introduced protein(s) in genetically modified plants is a critical component for the food, feed, and environmental safety assessment. Large quantities (tens to hundreds of grams) of the transgenic proteins are required to perform toxicological and other safety oriented studies (*6*). Ideally, the protein to be used in safety tests would be directly purified from the transgenic plants. However, in many cases, it is technically impossible to obtain sufficient quantities of the subject protein in high purity from the transgenic plants due to the low expression levels. Consequently, government regulatory agencies accept the use of an equivalent protein from an alternate source, such as one produced in a high expression system like bacteria (*15*, *16*).

According to the U.S. Environmental Protection Agency (EPA), the tests to be conducted in examination of the equivalence of bacterium and plant produced proteins (for insect resistant transgenic crops) include molecular sizing by SDS−PAGE, immunorecognition using ELISA and Western blot analysis, N-terminal amino acid sequencing, confirmation of the lack of glycosylation in the plant-produced protein, and bioactivity against a range of insects (*17*). The current study demonstrated that the Cry34Ab1 and Cry35Ab1 proteins produced by the transgenic corn lines were equivalent to those produced by the recombinant Pf in terms of MW, immunorecognition by Cry34Ab1 or Cry35Ab1 specific antibodies (**Figures 2** and **3**), N-terminal sequence (**Table 3**), and the lack of glycosylation (**Figure 6**). A separate study also concluded that the Cry34Ab1 and Cry35Ab1 toxins produced by both the Pf and the transgenic corn events demonstrated a similar bioactivity profile against a range of insect species (*18*).

We also performed tryptic peptide mass fingerprinting with MALDI-TOF MS (**Tables 4** and **5**) and sequencing of two internal peptides with MS/MS (**Tables 6** and **7**) to provide additional evidence supporting the equivalence of the proteins from the two sources. Mass spectra of the peptide mixture resulting from the digestion of a denatured protein by an enzyme (most commonly trypsin) provide a peptide mass fingerprint (PMF) of a protein with great specificity (*19*). In general, a protein identification made by peptide mass fingerprinting is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five peptide matches (*20*). In our experiments, the detected peptide coverage was 52% with six peptide matches and 44−53% with 11−13 peptides matches for Cry34Ab1 and Cry35Ab1 proteins, respectively (**Tables 4** and **5**), suggesting the recombinant Cry34Ab1 and Cry35Ab1 proteins produced by the corn and Pf were expressed correctly as intended.

Because of many factors, 100% sequence coverage with a single enzyme digestion is not feasible for most proteins. These factors include the frequency of the specific cleavage sites in the sequence, conditions of the digestion, peptide recovery of postdigestion purification, and the response of the mass spectrometer. In addition, it is not uncommon for a given peptide to be detected in one experiment but not in another. Detectability of a peptide is concentration-dependent and peptide specific. It could also be influenced by other factors such as signal suppression by other components in the sample mixture. During MALDI, the matrix molecules absorb the energy from the laser light and transfer it to peptides, which can become ionized. Only ionized peptides are detected by the mass spectrometer, and not

all of the peptides can be ionized with equal efficiency. Typically, hydrophilic peptides are ionized more efficiently than hydrophobic ones. Also, signals for less efficiently ionized peptides can be suppressed by other dominant and readily ionized peptides. Therefore, for a specific peptide, if the amount in the mixture is not dominant or if it is difficult to ionize, it might be detected in one experiment but be suppressed and not detected in another. This effect was observed for several of the peptides of Cry35Ab1 (**Table 5**).

Herman et al. previously reported that Cry34Ab1 alone was active against corn rootworms, while Cry35Ab1 alone was not. Adding Cry35Ab1 to Cry34Ab1 significantly increased the activity of Cry34Ab1 (*11*). While details around the mode of action of Cry34Ab1 and Cry35Ab1 toxins remain to be elucidated, the current study demonstrated that a C-terminal fragment of Cry35Ab1 was not required for its potentiation effect on Cry34Ab1. In fact, the 40 kDa protease resistant truncated form of Cry35Ab1 produced even a greater potentiation effect as compared to the 44 kDa full-length Cry35Ab1 (**Table 2**). On the basis of the MW difference (3735 kDa) between the full-length and the truncated Cry35Ab1 measured by MALDI-TOF MS, it could be calculated that the truncation stopped at residue L_{354}. This cleavage site was confirmed by a separate study on C-terminal sequencing of the truncated 40 kDa protein (Dow AgroSciences, unpublished). In vitro digestion tests demonstrated that serine proteinases, such as chymotrypsin and endoproteinase Glu-C, could readily cleave full-length Cry35Ab1 into stable truncated form(s) with a similar MW as the 40 kDa form directly isolated from the Pf-produced preparation (**Figure 5**). The truncated form is resistant to further protease cleavage when it is in a native state. Once the protein is denatured, it can be fully digested into small peptides by these serine proteinases as shown in the tryptic peptide mass fingerprinting analysis (**Table 5**).

Finally, although there is a difference of four amino acid residues between the transgenic corn- and Pf-expressed Cry35Ab1, the four residues are located at the C terminus (**Figure 1**). These residues are not required for the function of Cry35Ab1 (**Table 2**), and they can be easily cleaved by proteases in the plants, Pf, and very likely in the guts of susceptible insects. Therefore, despite this slight difference in sequence, the Cry35Ab1 produced by the corn and Pf is functionally equivalent.

In conclusion, the results of the current study demonstrate that the Cry34Ab1 and Cry35Ab1 proteins produced by the transgenic corn lines are equivalent to those produced by recombinant Pf.

ABBREVIATIONS USED

ACTH, adrenocorticotropic hormone; Bt, *Bacillus thuringiensis*; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CBB, Coomassie brilliant blue; CHCA, α-cyano-4-hydroxycinnamic acid; EDTA, ethylenediamine tetraacetate; ELISA, enzyme-linked immunosorbent assay; EPA, Environmental Protection Agency; HRP, horseradish peroxidase; *m*/*z*, mass-to-charge ratio; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MeOH, methanol; MS/MS, tandem mass spectrometry; MW, molecular mass; PBST, phosphate-buffered saline with Tween 20; Pf, *Pseudomonas fluorescens*; PMF, peptide mass fingerprint; PVDF, polyvinyldifluoride; Q-TOF, quadrupole time-of-flight; SCR, southern corn rootworm; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

ACKNOWLEDGMENT

We are indebted to Josh Russell for providing the recombinant *Pseudomonas*-derived proteins. We are grateful to Penny Hunst, Steve Evans, James Bing, and Katherine Armstrong of Dow AgroSciences for their support. We are also thankful to the many scientists at Pioneer Hi-Bred International for their collaboration.

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Received for review July 27, 2004. Revised manuscript received October 28, 2004. Accepted October 28, 2004.

JF048744+