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Comparison and Validation of Methods To Quantify Cry1Ab Toxin from *Bacillus thuringiensis* for Standardization of Insect Bioassays▽

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Standardization of toxin preparations derived from *Bacillus thuringiensis* (Berliner) used in laboratory bioassays is critical for accurately assessing possible changes in the susceptibility of field populations of target pests. Different methods were evaluated to quantify Cry1Ab, the toxin expressed by 80% of the commercially available transgenic maize that targets the European corn borer, *Ostrinia nubilalis* (Hübner). We compared three methods of quantification on three different toxin preparations from independent sources: enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis and densitometry (SDS-PAGE/densitometry), and the Bradford assay for total protein. The results were compared to those obtained by immunoblot analysis and with the results of toxin bioassays against susceptible laboratory colonies of *O. nubilalis*. The Bradford method resulted in statistically higher estimates than either ELISA or SDS-PAGE/densitometry but also provided the lowest coefficients of variation (CVs) for estimates of the Cry1Ab concentration (from 2.4 to 5.4%). The CV of estimates obtained by ELISA ranged from 12.8 to 26.5%, whereas the CV of estimates obtained by SDS-PAGE/densitometry ranged from 0.2 to 15.4%. We standardized toxin concentration by using SDS-PAGE/densitometry, which is the only method specific for the 65-kDa Cry1Ab protein and is not confounded by impurities detected by ELISA and Bradford assay for total protein. Bioassays with standardized Cry1Ab preparations based on SDS-PAGE/densitometry showed no significant differences in LC_{50} values, although there were significant differences in growth inhibition for two of the three Cry1Ab preparations. However, the variation in larval weight caused by toxin source was only 4% of the total variation, and we conclude that standardization of Cry1Ab production and quantification by SDS-PAGE/densitometry may improve data consistency in monitoring efforts to identify changes in insect susceptibility to Cry1Ab.

Transgenic plants expressing toxins from *Bacillus thuringiensis* (Berliner) (Bt plants) have high efficacy against target pests and represent an important alternative to conventional insecticides. However, the widespread use of this technology has generated concerns due to the potential for increased selection intensity and because insect resistance to *B. thuringiensis* formulations has already been reported both in the laboratory (13, 19) and among populations of target pests where *B. thuringiensis* formulations have been used in a pest management setting (8, 13, 20).

To prevent or at least reduce the rate of resistance development to Bt plants in target pest species, the U.S. Environmental Protection Agency has imposed rigorous regulatory requirements that mandate certain practices related to resistance management (1). One of the primary requirements for registration includes monitoring susceptibility of field populations of target pests to verify potential changes in susceptibility to Cry toxins (22). Maize, *Zea mays* L., expressing Cry toxins comprises 40% of the total area of maize production in United States (21), and Cry1Ab is the toxin expressed by 80% of the commercially available transgenic maize that targets the European corn borer, *Ostrinia nubilalis* (Hübner). Although susceptibility to Cry1Ab has been monitored since 1995, differences between batches of fermentation products or between formulated Bt insecticides and purified toxins have led to inconsistency in the measured bioactivity (B. D. Siegfried, unpublished). This inconsistency complicates the assessment of changes in insect susceptibility as estimated by 50% lethal concentration (LC_{50}) values generated by probit mortality curves or diagnostic concentrations (18). Differences in the bioactivity of Cry1Ab have been attributed to impurity of batches and to the lack of standardized protocols for Cry1Ab production and quantification.

Several methods have been used to quantify Cry1Ab, including enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis–densitometry (SDS-PAGE/densitometry), and total protein assays such as the Bradford assay. The latter two methods have been used to obtain relative estimates of Cry1Ab concentration (3, 4, 15). However, there has been limited information regarding conditions used during determinations of Cry1Ab concentration, and differences among protocols for Cry1Ab production may lead to inconsistent bioassay results. Moreover, estimates in-

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volving the Bradford assay may be affected by protein impurities and nonprotein components (2), and several factors may affect the estimates of protein concentration determined by SDS-PAGE/densitometry, including the reducing agent used to denature proteins, fixing solutions, and staining and destaining procedures (10, 14).

To assure accurate assessment of susceptibility to Cry1Ab using insect bioassays, it is critical to establish technical specifications for protocols used to quantify different Cry1Ab preparations. The objective of the present research was to evaluate and compare different methods of Cry1Ab quantification based on the precision and relative ability to estimate Cry1Ab in batches obtained from independent sources. To determine the accuracy of quantification methods, we also assessed the susceptibility of *O. nubilalis* neonates to different Cry1Ab batches by using standard bioassay techniques.

MATERIALS AND METHODS

Cry1Ab sources. (i) Cry1Ab from the University of Nebraska, Lincoln (UNL). The Cry1Ab gene was expressed in *Escherichia coli* host strain JM103 by using the expression vector pKK223-3. The *E. coli* strain was provided by the *Bacillus* Genetic Stock Center (http://www.bgsc.org/). Cry1Ab protoxin was obtained from *E. coli* fermentation products by a modification of the method described by Lee et al. (11). The solubilized protein was digested with bovine pancreatic trypsin, and insoluble material was removed by centrifugation. The Cry1Ab preparation was dialyzed against 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.0) by using a 10,000 MW Slide-A-Lyzer dialysis cassette (Pierce Chemical, Rockford, IL).

(ii) Cry1Ab from Auburn University (AU). Cry1Ab protoxin was expressed in the XL1-Blue strain of *E. coli* as a single gene product using plasmid pBD-140 (provided by R. A. deMaagd, Plant Research International, Wageningen, The Netherlands). Inclusion bodies containing Cry1Ab protoxin were dissolved and treated with trypsin, and the activated Cry1Ab toxin was isolated by using high-performance liquid chromatography (16). Purified Cry1Ab toxin was desalted, lyophilized, and stored at −80°C. Prior to bioassays, the protein was solubilized in 50 mM sodium carbonate buffer (pH 10.0) at 37°C and then vortex mixed until small particles were no longer visible.

(iii) Cry1Ab from Monsanto Company (St. Louis, MO). The Cry1Ab was purified from a spore-crystal paste produced by fermentation of *B. thuringiensis* subsp. *kurstaki*. The full-length Cry1Ab protein was solubilized at a high pH in the presence of reducing agent and protease inhibitors at 4°C. The soluble protein was treated with bovine pancreatic trypsin at 4°C until ca. 90% was converted to the trypsin-resistant core protein, which was purified in a 1.2-liter Q Sepharose FastFlow column. The trypsin-resistant core protein was eluted as a single symmetrical peak by using a gradient of sodium chloride. Peak fractions were pooled and dialyzed against 50 mM carbonate-bicarbonate buffer (pH 10.25) with 50 mM sodium chloride.

Quantification methods. (i) Bradford assay. The Bradford assay has been reported to exhibit slight nonlinearity in the absorbance response, which is attributed to an overlap in the spectrum of the two different color forms of the dye (2). Therefore, the Cry1Ab concentration estimates were determined by using the range of standard concentrations that provides a linear response between 0 and 600 μg of protein/ml. Bovine serum albumin (BSA), standard grade at 2,000 μg/ml (Pierce Chemical), was diluted in 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.0) according to the manufacturer's instructions. The concentrations of standards were 25, 125, 250, and 500 μg/ml. Absorbance readings at 595 nm were determined with a microplate reader (Becton Dickinson Labware, Franklin Lakes, NJ) after duplicates of standards and unknowns had been incubated for 10 min with Coomassie G-250 dye (Coomassie Plus; Pierce Chemical). Determinations were replicated at least four times for each preparation. The Coomassie Plus reagent was at room temperature before incubation with standards and unknowns.

(ii) ELISA. The Bt-Cry1Ab/Cry1Ac ELISA kit was obtained from Abraxis (Warminster, PA) and stored at 4°C before use. Unknowns were diluted in sample extraction-dilution buffer provided with the kit. Centrifuge tubes (1.5 ml) were used to prepare 1-ml dilutions. Cry1Ab batches were diluted to achieve a total dilution factor of 200,000, which was necessary to fit unknown Cry1Ab concentrations into the range of standards provided with the kit (0.0 to 4.0 ng/ml). The kit is provided with removable strips containing eight wells each. Determinations were performed in two strips where a blank, four Cry1Ab standards, and three diluted Cry1Ab samples were assayed in duplicate. The dilution buffer and other kit components were at room temperature 30 min before assays. Blanks, Cry1Ab standards, diluted Cry1Ab preparations, and reagents were taken from vials and placed into PCR cap strips before being transferred into ELISA strips. Volumes were transferred to ELISA strips with a multichannel pipette to ensure the same incubation time in each well. Determinations were replicated at least three times. Incubation and washing steps were done according to the manufacturer's instructions.

(iii) SDS-PAGE/densitometry. As a reference for protein quantification, BSA (>98%, ELISA grade; Sigma-Aldrich) was diluted to 600, 400, 267, 178, and 119 μg/ml in 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.0), with phenylmethylsulfonyl fluoride at 0.5 mM to minimize protein degradation. The concentrations were selected to prevent excessive protein loading, which reduces the sharpness of bands. BSA has a high content of cysteine residues and may undergo oxidation during electrophoresis (10), which also reduces the sharpness of the bands and ultimately compromises the quantification of band volume and intensity. To prevent oxidation during electrophoresis, BSA and Cry1Ab proteins were denatured by reduction-alkylation (10). Standards and samples (5 μl each) were mixed with 10 μl of Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 5 mM dithiothreitol and denatured for 3 min at 90°C. Iodacetamide was added to make the final concentration 12.5 mM on samples and standards and incubated at 90°C for 1 min. Standards and samples of the three Cry1Ab batches were then immediately subjected to SDS-PAGE in 12% polyacrylamide Tris-HCl Ready Gels (Bio-Rad) for 2 h at 80 V and ambient temperature. This polyacrylamide percentage provides little background staining with Brilliant Blue G-colloidal stain (14). Gels were replicated four times. After electrophoresis, gels were washed for 30 s in double-distilled water to eliminate electrode buffer. Before staining, gels were fixed in 12% trichloroacetic acid and 3.5% 5-sulfosalicyclic acid (Sigma-Aldrich). After 30 min, gels were rinsed with double-distilled water and stained in 50 ml of staining suspension containing 0.1% (wt/vol) Brilliant Blue G, 0.29 M phosphoric acid, and 16% saturated ammonium sulfate (Sigma-Aldrich) for 2 h and then rinsed in 10% acetic acid in 25% (vol/vol) methanol for 30 s with shaking. Gels were then rinsed with 25% methanol three times to remove acetic acid and destained in 25% methanol for up to 24 h. After destaining for at least 1 h, the gels were photographed by using Gel Doc 2000 documentation system (Bio-Rad). Each photograph was analyzed densitometrically with Quantity One 4.2.3. software (Bio-Rad).

Statistical analysis. The precision of each quantification method was compared based on the calculated coefficient of variation for concentration estimates obtained for each Cry1Ab batch. To test whether quantification methods generated different estimates of Cry1Ab concentration, the three methods were compared in a randomized block design with subsampling. Each independent Cry1Ab batch was considered a block, and the average amount of Cry1Ab quantified by each method was compared across all batches. Therefore, the main factor in the analysis was the quantification method used to quantify the three Cry1Ab batches. The data were first tested for normality by the methods of Shapiro-Wilk and the normal probability plots using the PROC UNIVARIATE procedure of the SAS software (version 9.1; SAS Institute, Inc., Cary, NC). The data were further analyzed with a one-way analysis of variance using the PROC MIXED procedure, and treatment means were separated by using LSMEANS tests at $\alpha = 0.05$.

Validation of quantification results. (i) Immunoblotting with anti-Cry1Ab. Immunoblot analysis was used to test for Cry1Ab-related impurities and to determine whether small quantities of Cry1Ab generate similar signal intensities. Cry1Ab concentrations were standardized based on SDS-PAGE/densitometry results. Duplicates of standardized concentrations of each Cry1Ab batch (200 ng) were separated by SDS-PAGE as described previously (9), electroblotted onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 90 min using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad), and blocked for 2 h at room temperature with phosphate-buffered saline (pH 8.0) containing 5% nonfat dry milk powder, 5% glycerol, and 0.5% Tween 20. The PVDF membrane was incubated with polyclonal rabbit anti-Cry1Ab (1:2,500; provided by the Monsanto Company), washed three times, and then incubated with goat anti-rabbit-AP (1:10,000) and washed three times with blocking buffer. The PVDF membrane was washed with assay buffer (Tropix, Inc., Bedford, MA), and detection was performed with the CDP-Star chemiluminescence kit (Tropix, Inc.). The membranes were then washed, exposed to Kodak BioMax film (Eastman Kodak, Rochester, NY), and developed according to the manufacturer's instructions. Films were photographed by using Gel Doc 2000 documentation system (Bio-Rad) and analyzed densitometrically by using Quantity One 4.2.3. software (Bio-Rad) as described previously.

TABLE 1. Cry1Ab content in different sources obtained by three quantification methods

Toxin source	Method	Cry1Ab concn (μg/ml) ± SE	CV (%)
University of Nebraska	Bradford	409.8 ± 6.1	4.5
	ELISA	311.4 ± 29.2	26.5
	SDS-PAGE/densitometry	279.3 ± 8.0	5.7
Auburn University	Bradford	315.3 ± 2.6	2.4
	ELISA	293.7 ± 12.8	10.6
	SDS-PAGE/densitometry	369.9 ± 28.5	15.4
Monsanto Company	Bradford	365.9 ± 5.3	5.4
	ELISA	352.3 ± 16.0	12.8
	SDS-PAGE/densitometry	338.1 ± 0.4	0.2

(ii) Insect strains and bioassay. The three Cry1Ab batches were tested against two susceptible strains of European corn borer. One strain originated in 2005 from a field collection of ~900 individuals from Iowa and Nebraska and was reared for 10 generations before bioassays (F10). The second strain originated from a field collection of ~500 individuals from the Lombardia region of northern Italy and has been reared in the absence of selection for more than 100 generations (F100). Bioassay methods described by Marçon et al. (12) were used to test the effectiveness of the three different Cry1Ab batches previously described with each quantified based on SDS-PAGE/densitometry. Neonate larvae (<24 h after eclosing) were exposed to seven concentrations of each Cry1Ab toxin (0.1, 0.3, 0.9, 2.7, 8.0, 24.0, and 72.0 ng/cm^2). Each toxin was tested in four replicates in two separate dilutions which were prepared using 0.1% Triton X-100 nonionic detergent to obtain uniform spreading over the diet surface. Dilutions were tested on two different dates, and 512 insects in total were tested for each toxin-strain combination. Mortality and larval weights were recorded after 7 days. Concentration-mortality curves for each toxin-strain combination was obtained by probit analysis (5) using POLO-PC (LeOra Software, Berkeley, CA). Larval weights were transformed to percent growth inhibition relative to the controls, and these data were analyzed by nonlinear regression fitted to a probit model (12).

To verify the relative importance of variation associated with the use of different toxin sources, we conducted a hierarchical analysis of variance (PROC NESTED) using SAS software (version 9.1; SAS Institute) to quantify variance components associated with the inhibitory response of larvae exposed to two Cry1Ab concentrations (0.3 and 0.9 ng/cm^2). Larval weight after 7 days was used to estimate variance components associated with toxin source, dilution, strain, and day. We used a balanced design where 16 larvae were tested per toxin-dilution-strain-day combination. The weight of larva exposed to diet treated with 0.1% Triton X-100 nonionic detergent was used as a control.

RESULTS

Quantification methods. Significant differences in Cry1Ab concentration-estimates were detected among the three methods of quantification ($F = 5.64$, df = 61, $P < 0.0057$). A comparison across all toxin sources showed that the Bradford assay produced statistically higher estimates (365.57 ± 9.00) than the ELISA (321.44 ± 10.69; $t = 3.16$, df = 61, $P = 0.0025$) and SDS-PAGE/densitometry (328.26 ± 15.12; $t = 2.12$, df = 61, $P = 0.0381$). There were no significant differences detected between estimates obtained by ELISA and SDS-PAGE/densitometry ($t = -0.37$, df = 61, $P = 0.7137$). The average concentration and coefficient of variation (CV) estimated for the three methods tested are presented in Table 1. For two Cry1Ab batches (UNL and Monsanto Company), the concentration estimates determined by ELISA were higher than the concentration estimates determined by SDS-PAGE/densitometry. The Bradford assay showed the lowest CVs for estimates of Cry1Ab concentration, which ranged from 2.4 to 5.4%. The CV of estimates obtained by ELISA ranged from 12.8 to 26.5%, whereas the CV of estimates obtained by SDS-PAGE/densitometry ranged from 0.2 to 15.4%. These results indicate that ELISA exhibited the lowest precision among the three methods tested.

Western blot with Cry1Ab batches. A Western blot of the Cry1Ab proteins from three independent preparations is shown in Fig. 1. Cry1Ab quantities were normalized based on SDS-PAGE/densitometry results, and the relative intensity of truncated Cry1Ab (65 kDa) was similar across all batches tested. The Cry1Ab antiserum also recognized peptide residues that were both larger and smaller in molecular mass than truncated Cry1Ab. The batch from UNL showed a faint band above 65 kDa. The number and intensity of bands <65 kDa indicates that toxins were different in Cry1Ab-related impurities.

Effectiveness of different Cry1Ab batches. Results of Cry1Ab bioassays with *O. nubilalis* strains exposed to three different preparations of Cry1Ab are presented in Table 2. For the F10 strain, the Cry1Ab LC_{50} values ranged from 6.1 ng/cm^2 (Monsanto) to 9.4 ng/cm^2 (AU). For the F100 strain, the Cry1Ab LC_{50} values ranged from 4.8 ng/cm^2 (UNL) to 6.0 ng/cm^2 (AU). There were no significant differences in LC_{50} values among Cry1Ab batches for each insect colony. However, comparisons of growth inhibition of both European corn borer strains indicate that 50% effective concentration (EC_{50}) values obtained with Cry1Ab from AU were significantly higher (F10, 0.73 ng/cm^2; F100, 0.69 ng/cm^2) than the EC_{50} values obtained with Cry1Ab from UNL (F10, 0.43 ng/cm^2; F100, 0.45 ng/cm^2) (Fig. 2).

Variance components. The hierarchical analysis of variance allowed quantification of the contribution of each factor for the entire variation in larval weight. Estimates of variance components for weights of larvae grown with an artificial diet treated with 0.3 and 0.9 ng of Cry1Ab/cm^2 are presented in Table 3. The control treatment indicated that 75% of the variation in larval weight is associated with the bioassay method. Similar variation also was observed at both Cry1Ab concentrations tested. The toxin source was responsible for ca.

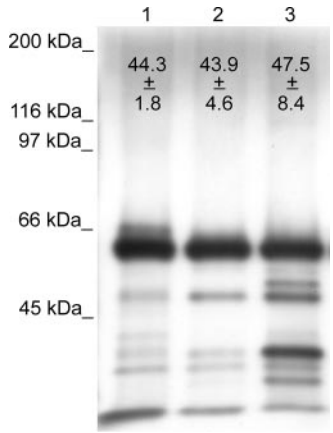

FIG. 1. Immunoblot analysis of three Cry1Ab batches. Lane 1, batch from UNL; lane 2, batch from Monsanto Company; lane 3, batch from AU. Proteins (200 ng) were loaded in each lane based on SDS-PAGE/densitometry quantification. Values indicate the volumes of 65-kDa Cry1Ab bands expressed as optical density multiplied by area in mm^2 (± standard error) detected by antibody.

TABLE 2. Susceptibility of two European corn borer populations to Cry1Ab toxins from three independent sources

Source and strain	No. of insects	Slope ± SE	Susceptibility (95% FL)[a]		χ^2 (df)
			LC_{50}	LC_{90}	
University of Nebraska					
F10	446	3.78 ± 0.49	7.4 (6.2–8.6)	16.3 (13.2–22.0)	0.8 (4)
F100	384	2.60 ± 0.25	4.8 (4.0–5.8)	15.0 (11.8–20.9)	0.2 (3)
Auburn University					
F10	319	2.57 ± 0.30	9.4 (4.7–15.8)	29.7 (17.3–113.5)	2.4 (2)
F100	447	2.11 ± 0.18	6.0 (4.2–8.7)	24.3 (15.5–49.8)	6.1 (4)
Monsanto Company					
F10	383	2.07 ± 0.20	6.1 (3.5–9.9)	25.5 (15.1–61.5)	4.5 (3)
F100	384	2.62 ± 0.25	5.6 (4.6–6.7)	17.3 (13.5–24.1)	2.2 (3)

[a] Expressed as nanograms of Cry1Ab/cm^2 of artificial diet. FL, fiducial limits.

4% of the variation in larval weight. The variation caused by toxin at 0.9 ng/cm^2 was significant at a P value of <0.05. Dilution and strain did not contribute significantly to variability in bioassay results. In contrast, the date of the assay was an important factor affecting variability in larval weight, causing ca. 20% of the total variation.

DISCUSSION

The results of this investigation provide a basis for establishing standardized methods for quantifying *B. thuringiensis* toxins among different preparations. In general, all three methods provided relatively consistent estimates of Cry1Ab concentration, but each technique had characteristics that limited accurate quantification of Cry1Ab preparations. The generally higher estimates obtained with the Bradford assay were not unexpected because the toxins tested were not 100% pure (Fig. 3). Although this method generally showed the highest precision, the presence of other proteins and peptide degradation products could compromise quantification estimates (2).

Our results indicate relatively high variation in estimates of Cry1Ab concentrations obtained by ELISA. Because ELISA kits are designed for the detection and quantification of trace amounts of toxin, a 200,000-fold dilution of stock solutions was necessary to obtain concentrations within the range of the standards used to generate standard curves. As a consequence, slight dilution errors may have caused higher variation in the concentration estimates (7). Although our study did not indicate significant differences between concentration estimates obtained by ELISA and SDS-PAGE/densitometry, the reaction of Cry1Ab antiserum with Cry1Ab-related impurities may also reduce the accuracy of estimates by ELISA. Western blots of the different Cry1Ab batches revealed immunologically reactive peptides other than the truncated Cry1Ab protein (Fig. 1), which could result in overestimates of Cry1Ab concentration. Concentration estimates obtained by SDS-PAGE/densitometry showed consistently lower CV's than ELISA (Table 1). This method uses aliquots taken directly from Cry1Ab stocks reducing errors caused by sample dilution. Moreover, SDS-PAGE/densitometry also allows quantification of specific protein bands of interest, eliminating interference of immunologically reactive Cry1Ab residues.

Although LC_{50} values were not significantly different among Cry1Ab batches, the EC_{50} values obtained with Cry1Ab from AU were ~1.6-fold higher than the EC_{50} values obtained with

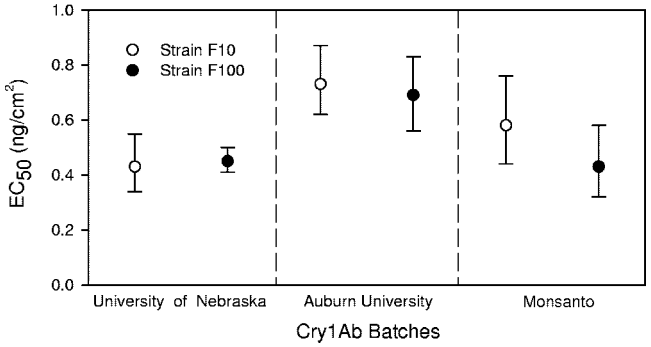

FIG. 2. Growth inhibition (EC_{50} values with 95% confidence intervals) of two susceptible European corn strains for three Cry1Ab batches: UNL, AU, and Monsanto Company.

TABLE 3. Source of variation in weight of European corn borer larvae growing in artificial diet untreated and treated with Cry1Ab toxins from different sources

Treatment group and source of variation	df	s^2	F ratio	P	% Variation[a]
Control (untreated)					
Strain	11	0.00	0.20	0.9938	0.00
Day	12	22.87	6.42	<0.0001	25.31†
Error	360	67.48			74.69
Treated (Cry1Ab, 0.3 ng/cm^2)					
Toxin	2	2.97	2.24	0.2537	3.67
Dilution	3	1.34	1.39	0.3345	1.65
Strain	6	0.00	0.82	0.5735	0.00
Day	12	12.78	4.20	<0.0001	15.77†
Error	360	63.96			78.92
Treated (Cry1Ab, 0.9 ng/cm^2)					
Toxin	2	1.91	9.76	0.0486	3.88*
Dilution	3	0.03	1.08	0.4253	0.07
Strain	6	0.00	0.14	0.9882	0.00
Day	12	9.27	4.91	<0.0001	18.86†
Error	360	37.94			77.19

[a] *, significant at $P < 0.05$; †, significant at $P < 0.01$.

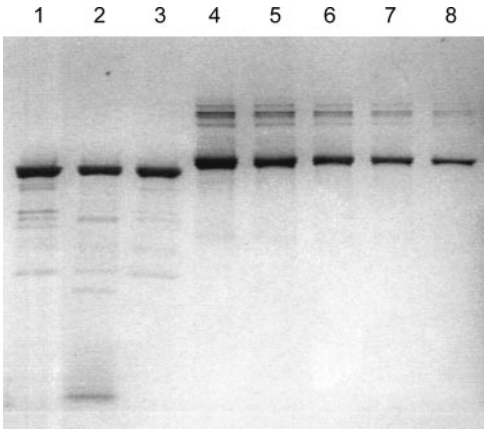

FIG. 3. SDS-PAGE of Cry1Ab batches and BSA standards. Lane 1, batch from AU; lane 2, batch from UNL; lane 3, batch from Monsanto Company, lanes 4 to 8. BSA standards: 3.0, 2.0, 1.33, 0.89, and 0.59 μg.

Cry1Ab from UNL for both strains tested (Fig. 2). A study with three European corn borer populations from Germany indicated that two toxin batches caused a ninefold difference in LC_{50} values, although the Cry1Ab concentrations of the different batches were not compared before bioassays (18). These results suggest that Cry1Ab sources may cause pseudovariation in susceptibility whenever a new Cry1Ab batch is introduced. Such variation may decrease the sensitivity and reliability of monitoring efforts and lead to an incorrect assessment of natural variation.

The potency of insecticides is often measured by LC_{50} and EC_{50} estimates because the tolerance distribution is symmetrical for all populations. However, potency comparisons using EC_{50} estimates that are based on the weights of exposed subjects are more useful to identify the relative potency of toxic preparations. Because larval weight is a continuous variable, it more accurately reflects the relationship between the dose transferred and the magnitude of the response (6). Therefore, LC_{50} estimates are less sensitive than EC_{50} estimates for detecting small differences in potency of Cry1Ab toxins, as shown in the present study.

Natural variation in European corn borer susceptibility, errors in Cry1Ab quantification, difficulties in the solubilization of lyophilized Cry1Ab preparations, or inherent differences in the toxicity of Cry1Ab batches may contribute to variation in EC_{50} values obtained in bioassays. Moreover, bioassay methods are associated with a degree of variation caused by other factors, including cohort, dilution, or environmental conditions (6, 17). The strains tested had similar susceptibilities to Cry1Ab, and the hierarchical analysis of variance did not reveal a significant variance for the strain component (Table 3). This result was not unexpected because both strains showed a similar degree of genetic variability in response to Cry1Ab toxins, which is indicated by the similarity among slopes and LC_{50} values of probit mortality curves (Table 2). The ~20% variation associated with bioassay data is likely explained by slight differences in environmental conditions or, more importantly, differences in cohorts used in bioassays. Interestingly, the toxin batch was responsible for only 4% variation in larval weight, which indicates the importance of the standardization methods.

Comparisons of EC_{50} values indicate that the Cry1Ab preparation from Auburn University exhibited slightly lower potencies than the other preparations tested. This reduced bioactivity may be related to incomplete solubilization because this was the only preparation to be lyophilized after purification. Comparisons among bioassays with *Heliothis virescens* (Fabricius) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) at different laboratories using a single Cry1Ac batch indicate that solubilization of lyophilized Cry1Ac preparations can introduce variation in bioassays (W. J. Moar, unpublished results).

The methods described here provide a starting point for developing methods to standardize different *B. thuringiensis* toxin preparations. Quantification involving SDS-PAGE/densitometry provides a means to determine the purity and standardize the concentrations among different Cry1Ab preparations. Bioassays using well-established susceptible insect strains can be used to further validate determinations of concentrations among different preparations (18). Moreover, the methods provide means to determine whether degradation during long-term storage at −80°C has caused a reduction in Cry1Ab bioactivity over time.

The present study is the first report of bioassays with Cry1Ab preparations produced and purified at independent laboratories showing similar bioactivity toward susceptible insects. Because the batches were obtained using different fermentation protocols and because we were able to identify small variations caused by toxin source, we conclude that standardization of Cry1Ab production and quantification may improve data consistency in monitoring efforts to identify changes in susceptibility of target pests to Cry1Ab toxin from *B. thuringiensis*.

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