JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Highly Specific Enzyme-Linked Immunosorbent Assay for the Detection of Cry1Ac Insecticidal Crystal Protein in Transgenic WideStrike Cotton

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A highly selective enzyme-linked immunosorbent assay (ELISA) has been developed for the quantitative detection of the Cry1Ac protein expressed in transgenic cotton. Two Cry1Ac-specific monoclonal antibodies (MAb), Kbt and 158E6, were developed and selected to form a sandwich format ELISA. The MAb Kbt was used as a capture antibody, and 158E6 was conjugated with horseradish peroxidase and served as a detection antibody. The assay was optimized and validated with different cotton matrices. Tissues were extracted with phosphate-buffered saline containing 0.05% Tween 20 and 1% polyvinylpyrrolidone. The extract was then treated with trypsin to truncate full-length Cry1Ac into the core toxin for quantitation. The resulting assay has good accuracy and precision with a validated limit of quantitation ranging from 0.1 to 0.375 μg/g dry weight of cotton tissues. This assay is highly specific for Cry1Ac protein and has no cross-reactivity with the nontarget proteins tested such as Cry1Ab and Cry1F.

KEYWORDS: Cry1Ac; cotton; transgenic plant; ELISA; immunoassay

INTRODUCTION

Transgenic cotton, *Gossypium hirsutum* L., has been developed and commercialized by Dow AgroSciecnes LLC as WideStrike cotton, which expresses the Cry1Ac and Cry1F proteins derived from *Bacillus thuringiensis* (Bt) subspecies *kurstaki* and *aizawai*, respectively (*1*, *2*). Two cotton lines, Cry1Ac event 3006-210-23 and Cry1F event 281-24-236, were first developed and then crossed by conventional plant breeding to generate a combined trait product with the trade name WideStrike. The Cry1Ac event 3006-210-23 cotton plants were genetically modified by the introduction of a *cry*1Ac (synpro) gene, which was synthesized using the core Cry1Ac toxin with the addition of a nontoxic sequence derived from two other Cry1 proteins. This synthetic full-length gene was inserted into cotton plants, which subsequently expressed the Cry1Ac protein. The full-length Cry1Ac (synpro) protein is subject to proteolytic cleavage in the plant and insect gut, producing the insecticidally active Cry1Ac core toxin, which controls Lepidopteran insect pests such as tobacco budworm, *Heliothis virescens* (F.), cotton bollworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossipiella*). A rapid, selective, and sensitive method for monitoring protein levels in plant and related products is of significance for product quality control, environmental risk assessment, and other relevant studies (*3*, *4*). Because of the significant homology between Cry1Ac and Cry1Ab (*5*, *6*), which exists in other commercial transgenic crops, a simple and rapid assay able to distinguish the WideStrike Cry1Ac trait from other commercial traits is important for product stewardship. Moreover, the Cry1Ab δ-endotoxin is from soil bacterium *B. thuringiensis* and is common in nature and in commercial Bt spray formulations. A selective Cry1Ac assay is important for this recombinant protein's environmental monitoring and risk assessment in soil matrix. The antibody-based immunoassay has been widely used to detect transgenic proteins in a variety of applications including testing in the breeding process, testing for unapproved events, and determining GM content ensuring compliance with non-GM labeling requirements (*3*, *4*, *7*−*10*). There are several immunoassays commercially available for detecting Cry1Ab and Cry1Ac in both plate format and lateral flow strip format (*11*−*13*). Because both toxins have more than 80% amino acid sequence homology (*5*, *6*), all existing assays recognize both Cry1Ac and Cry1Ab proteins and lack the ability to differentiate one from the other. In this article, we described the development of a selective enzyme-linked immunosorbent assay (ELISA) for the detection of the Cry1Ac core toxin, which is one of two proteins expressed in WideStrike cotton. In addition, this ELISA method was further validated for the quantitation of the Cry1Ac protein in the tissues of transgenic cotton plants.

MATERIALS AND METHODS

Material. The Cry1F, Cry1Ac (full-length and truncated forms), Cry1Ab, Cry34Ab1, Cry35Ab1, protein conferring tolerance to bialaphos (BAR), and phosphinothrin acetyltransferase (PAT) pure proteins used in this study were expressed in transgenic *Pseudomonas fluorescens* strains and purified at Dow AgroSciences LLC (Indianapolis, IN). Lyophilized transgenic and nontransgenic control cotton tissue

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10.1021/jf070664t CCC: $37.00
Published on Web 06/27/2007

samples were from the Regulatory Laboratories of Dow AgroSciences LLC. Common biochemical and chemical reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). ELISA experiments were performed in 96 well microplates (Nunc, Roskilde, Denmark), and the absorbances were measured with a Vmax microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Antibody Development. Polyclonal and monoclonal antibodies (PAbs and MAbs) were developed and screened for Cry1Ac specific ELISA.

PAbs. The PAbs were generated at Strategic Biosolution Inc. (Newark, DE) using its internal protocols. Six rabbits (130329-01, -02, and -03 and 130334-01, -02, and and -03) were inoculated with varying doses of truncated Cry1Ac protein. Rabbit test bleeds were screened for activity against Cry1Ac truncated protein in a direct bind screening assay and later confirmed in a heterologous sandwich assay using a Cry1Ac specific monoclonal antibody (MAb). Antisera from all six rabbits were pooled and purified by Protein A affinity chromatography to a purity of >95%.

MAbs. The anti-Cry1Ac MAbs were generated at Strategic Biosolution Inc. (Newark, DE) using its internal protocols and Dow AgroSciences LLC. Forty (40) mice were immunized with varying doses of truncated Cry1Ac protein. Six mouse fusions were conducted generating 12 direct bind positive MAbs. The MAbs were evaluated in 21 homologous and heterologous combinations with a pooled sample of rabbit PAb in a sandwich format ELISA against Cry1Ac. Six antibody pairs with higher titer, better sensitivity, and higher signal-to-noise ratio were chosen for further evaluation. The combination of antibodies with the best selectivity, especially against Cry1Ac protein, was selected for optimization. Monoclonal antibodies KBT and 158E6 were chosen for development. The purified MAb (158E6) was then conjugated with horse radish peroxidase (HRP) (*14*).

Assay Development. Protein A purified anti-Cry1Ac MAb (KBT) was coated on microtiter plates to evaluate the optimal coating concentration, coating buffer constituents, and pH. Similarly, anti-Cry1Ac (158-E6)−HRP conjugates were formulated at different concentrations and were evaluated in the assay to discern optimal ratio and concentration for the desired performance. The resulting assay procedure is described below (*12*): Briefly, 50 μL of standards or samples was incubated with 50 μL of enzyme-conjugated anti-Cry1Ac monoclonal antibodies in the wells of a 96 well plate coated with anti-Cry1Ac monoclonal antibodies in a sandwich ELISA format for 60 min. At the end of an incubation period, the unbound reagents were removed from the plate by washing with phosphate-buffered saline with 0.05% Tween 20 (PBST) five times. Enzyme substrate solution (100 μL of TMB solution) was then added and incubated for 15−20 min and stopped by addition of an acidic stop solution (50 μL of 2 M HCl). The absorbance was measured at dual wavelengths of 450−650 nm. All experiments were conducted in triplicate or duplicate. Standard curves were obtained by plotting absorbance against the analyte concentration. The calibration curve for the Cry1Ac ELISA was modeled using a quadratic curve regression of the known concentration of the standard solutions and their subsequent absorbance (optical density, OD). The following formula was used for calculation:

$$y = A + Bx + Cx^2$$

where y is the absorbance value (OD) and x is the antigen concentration.

Assay Specificity. Each nontarget protein (Cry1Ab, Cry1F, Cry34Ab1, Cry35Ab1, PAT, and BAR) and full-length Cry1Ac was tested at a concentration range from 0 to 10000 μg/L in assay buffer. The cross-reactivity (CR) was tested following the procedure described in Shan et al. (*15*). The concentration−response curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four-parameter logistic equation:

$$y = \{(A - D)/[1 + (x/C)^B]\} + D$$

where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the concentration of analyte giving 50% responses (RC_{50}), and D is the minimum absorbance for no analyte. CR values were calculated as follows:

$$CR\ \% = (RC_{50}\ \text{of truncated Cry1Ac}/RC_{50}\ \text{of target protein}) \times 100$$

Matrix Effects/Buffer Selection. To investigate matrix effects from cotton tissue samples and identify a suitable buffer system, different buffer systems including PBST only or PBST with a variable amount of PVP were tested in this study. In each buffer system, a Cry1Ac standard curve was prepared using solutions containing different concentrations of matrix extract such as 1×, 3×, and 9× dilutions and compared with the curve in assay buffer. These dilutions were analyzed on the same plate. A mean OD difference of greater than 15% between the observed (matrix fortified) and the theoretical (control) for each standard concentration level was considered indicative of a significant matrix effect.

Protein Truncation. The Cry1Ac assay predominantly detects the truncated form, with only 53% CR to the full-length form. In plant tissue, the protein is expressed as the full-length protein and with partial truncation by plant proteases. Therefore, both forms of the protein often coexist in plant tissue samples. To measure total Cry1Ac in its truncated form, a proteolysis step prior to assay was developed. The truncation conditions were optimized using different trypsin concentrations, truncation times, and temperatures. Transgenic cotton tissue samples were used for the optimization.

Method Validation. The resulting ELISA was validated for lyophilized cotton plant tissues, including leaf, pollen, boll, and whole plant. Assay sensitivity, extraction efficiency, accuracy, and precision were evaluated.

Extraction Efficiency. To achieve the best extraction efficiency without compromising the assay simplicity, the optimal extraction buffer to sample mass ratio was investigated for each cotton matrix. Different sample sizes and buffer volumes were tested in the study. A series of five extractions were performed on transgenic cotton tissues known to express Cry1Ac. Briefly, 1.5 mL of assay buffer (PBST/PVP) was added to the tissue sample with two 1/8 in. stainless steel beads. The sample was then extracted in a Geno-Grinder (Certiprep, Metuchen, NJ) or a bead beater for 2 min (at 500 strokes per minute) and subsequently centrifuged at 10000 rpm for 5 min. The supernatant was transferred to an empty tube, and another 1.5 mL of buffer was added to the pellet, and the extraction process was repeated. This procedure was repeated three more times, to obtain five consecutive extractions. The concentration of Cry1Ac in each extraction was determined using a Cry1Ac ELISA after a trypsin truncation step. The apparent efficiency of the tissue extraction process was determined by comparison of Cry1Ac protein in the first extract relative to the total Cry1Ac protein in all five extracts. The final pellet was extracted with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and tested by Western blot to confirm any residual Cry1Ac protein.

Accuracy. To determine the method accuracy, different amounts of Cry1Ac protein were fortified in plant matrices, and it was then extracted and measured by Cry1Ac ELISA. In brief, an aliquot of negative control plant tissue was fortified with Cry1Ac protein solution [at 0.05 (or 0.075 for seed), 0.1, 0.25, and 0.8 (or 1.2 for seed) μg/g dry weight tissue] and then extracted once with assay buffer (as described above). After centrifugation, the supernatant was tested with Cry1Ac ELISA. A minimum of five sets of experiment (each includes four fortified concentrations and six matrices; see Table 4) were performed and analyzed. The fortified concentrations were served as true values, and the method accuracy was determined by comparing the measured value with the fortified value (true value), which was expressed as percent recovery (Table 4).

Precision. Method precision was evaluated using the results of experiments with fortified control whole plant (pollination stage) matrix that was analyzed by three analysts on two different days. The control sample extracts were fortified with four levels of Cry1Ac standard (theoretical concentrations at 0.10, 0.25, 0.40, and 0.80 μg/g dry weight). Each level of fortified extract was run in duplicate on each ELISA plate. The mean measured concentration, standard deviation (SD), and percent coefficient of variation (% CV) were calculated for each sample on each day. Positive tissue samples were also analyzed to assess the method precision by multiple analysts on different days in a span of 12 months.

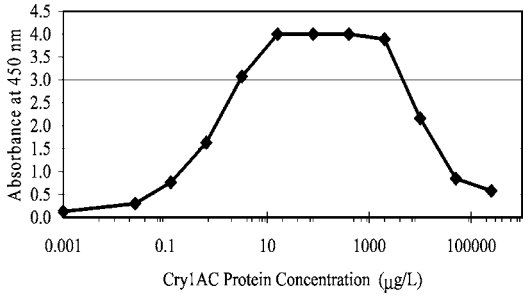

Figure 1. Evaluation of the Cry1Ac assay for the "hook" effect (a reduction in absorbance response with increasing dose of protein).

False Positive and False Negative. All cotton matrices were tested for false-positive and false-negative occurrences. At least five unfortified control cotton samples and five samples fortified at the target limit of detection (LOD) were analyzed for each tissue to determine the false-positive and false-negative rates. A false-positive result occurs when residue at or above the established LOD is found in a sample known to be free of analyte. A false negative occurs when no residue is detected in a sample fortified at the LOD.

RESULTS AND DISCUSSION

Antibody Development and Assay Optimization. Titers of all six polyclonal antisera were tested against truncated Cry1Ac protein as a coating antigen, and all raw antisera from terminal bleeds showed high titers (data not shown). Antisera 130329-01, -02, and -03 showed slightly better sensitivity than antisera 130330-01, -02, and -03. However, because these six antisera had similar titers, they were pooled, purified, and further evaluated in a heterologous sandwich assay using selected MAbs (KBT and 158E6). Similarly, the MAbs were screened for titer and tested against truncated Cry1Ac protein using the PAb sandwich format. Three MAbs (158E6, 158E7, and KBT) showed the greatest sensitivity and were further evaluated in nine homologous and heterologous combinations in a sandwich format ELISA. Both selectivity (specific to Cry1Ac) and sensitivity were used as antibody pair screening criteria. Finally, the MAbs KBT (as capture Ab) and 158E6 (as detection Ab and conjugated with HRP) pair were selected and purified for further assay development. A Cry1Ac kit based on this combination was developed and assembled at SDI (Newark, DE), which is commercially available at SDI (catalog number 7140220) (*12*).

Table 1. Summary of CR for Cry1Ac Assay

protein	RC_{50}[a] (μg/L)	CR (%)[b]
Cry1Ac-truncated	10.35	100
Cry1Ac-full length	19.50	53
Cry1F	>10000	0
Cry1Ab	>10000	0
Cry34Ab1	>10000	0
Cry35Ab1	>10000	0
Bar	>10000	0
Pat	>10000	0

[a] RC_{50}, the concentration of analyte giving a 50% OD_{max} response. [b] CR, (RC_{50} of Cry1Ac-truncated/RC_{50} of target protein) $\times$ 100.

Sandwich format immunoassays, where the sample and second antibody are incubated in the coated plate simultaneously, are susceptible to a phenomenon known as the "hook" effect. This is seen as a reduction in absorbance response with increasing concentration of protein above a peak in the OD. The resulting Cry1Ac assay was evaluated for hook effects as illustrated in Figure 1. In this study, the initiation of the hook effect in the Cry1Ac assay was reported in the 20000−50000 μg/L concentration range. These data suggest that if the concentration of Cry1Ac in the plant tissue extract is expected to be greater than 20000−50000 μg/L, sample dilution will be required to quantitatively determine the Cry1Ac concentration. The final standard curve was defined from 0 to 10 μg/L with a lower standard concentration at 0.5 μg/L. A typical Cry1Ac ELISA standard curve is shown in Figure 2.

Specificity. The MAb KBT−185E6 antibody pair-based immunoassay was highly selective for the Cry1Ac truncated protein. The results of the CR and interference assessments are shown in Table 1. The proteins tested for potential CR were Cry1Ab, Cry1F, Cry34Ab1, Cry35Ab1, PAT, BAR, and full-length Cry1Ac. Full-length Cry1Ac protein is detected by this ELISA system with a CR of 53%.

Matrix Effects/Buffer Selection. Cotton plant tissues usually have a strong matrix effect due to the presence of a large variety and quantity of phenolics and quinones, which may adversely affect protein extraction, antibody−antigen binding, and enzyme activities (*16*, *17*). To minimize matrix effects, polymers such as polyvinylpyrrolidone (PVP) are usually added to the extracting media as a phenol adsorbent or quinone scavenger. PVP is effective in binding those phenolic compounds that form strong

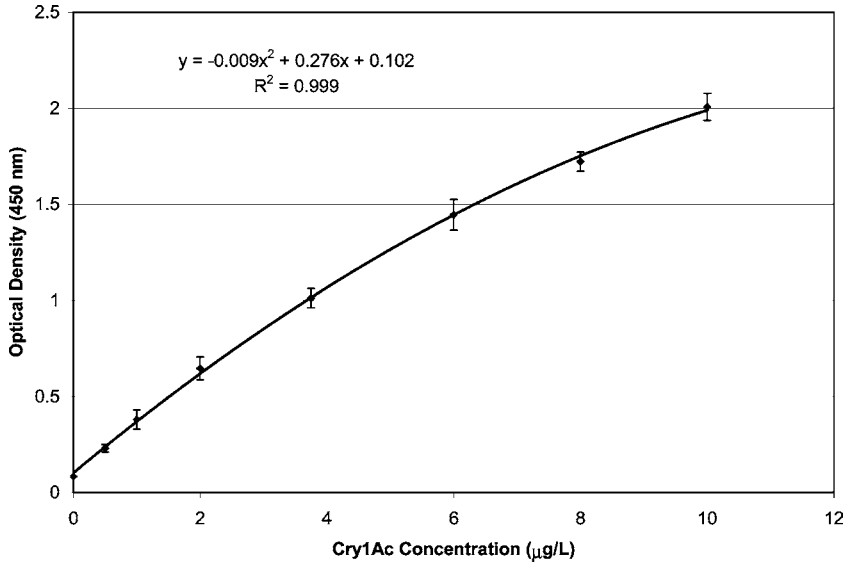

Figure 2. Cry1Ac ELISA calibration curve. This standard curve represents the average of 24 curves.

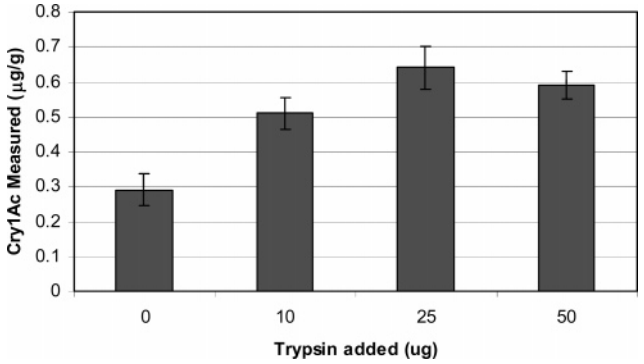

Figure 3. Effects of trypsin concentration on Cry1Ac truncation rate. An extract of whole plant at preharvest was used for this testing. An 800 μL extract (from 10 mg of tissue) was incubated for 60 min at 37 °C with varying amounts of trypsin and then assayed with ELISA. The Cry1Ac concentration measured is expressed as μg/g dry tissue weight.

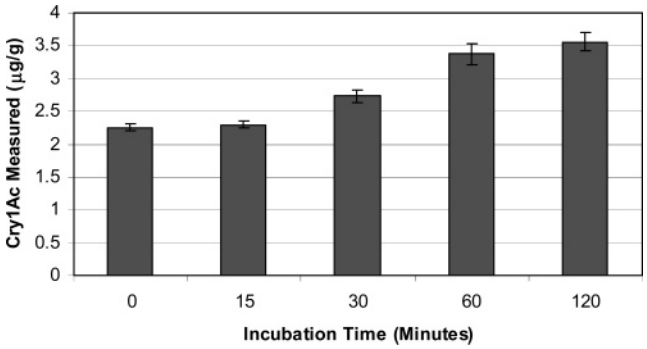

Figure 4. Effects of incubation time and temperature on Cry1Ac truncation rate. An extract of whole plant at pollination was used for this testing. Extracts (950 μL) with trypsin (30 μL or 30 μg) were incubated for 0–120 min at 37 °C and stopped with 20 μL of 10 mM PMSF and then assayed with ELISA.

hydrogen-bonded complexes. In this study, the effect of PVP on the assay and matrix and the optimal concentration of PVP in extraction buffer were investigated. Testing with whole plant extracts indicated that addition of PVP in the buffer system can significantly reduce matrix effects. A high concentration of PVP (2% or higher) suppressed assay OD values, while a low concentration of PVP such as <0.5% did not effectively eliminate the matrix effects. Taking into account these effects of PVP, the optimized PBST extraction and assay buffer contained a final concentration of 1% PVP.

Matrix effects were evaluated by comparing standard curves generated from dilutions of Cry1Ac that were not fortified with matrix with those that were fortified. Three different matrix dilutions, 1×, 3×, and 9×, were tested for each matrix. Tissue-dependent matrix effects were found in seed at 3× dilutions and in leaf, bolls, and whole plant (pollination and preharvest) at 1× dilutions. No matrix effect was observed for pollen. A 2× or 3× dilution is needed for tissues of leaf, bolls, and whole plant (pollination and preharvest) to minimize the matrix effects, while a 4× dilution is suggested for a cotton seed matrix.

Protein Truncation. According to the specificity study, the CR of the assay to full-length Cry1Ac is 53%. Although a synthetic full-length gene was genetically engineered in cotton plants and the primary protein expressed in the cotton is full-length Cry1Ac, a portion of the protein pool is subjected to truncation by plant proteases. Previous studies have shown that both forms of Cry1Ac protein coexist in the transgenic cotton plants (personal communication, Dr. Y. Gao of Dow AgroSciences LLC). Therefore, the conversion of full-length Cry1Ac to the truncated form is required for quantifying the total active Cry1Ac in a sample. Trypsin was used as the truncation protease and evaluated for the optimal concentration in the system. To terminate the truncation at the end of incubation, both α-toluenesulfonyl fluoride (PMSF) and soybean trypsin II inhibitor

Table 2. Summary of Extraction Efficiencies with Different Buffer–Tissue Ratios[a]

buffer (μL)/ tissue (mg) ratio	relative amount of Cry1Ac extracted[b]					
	bolls	leaf	pollen	whole plant, pollination	whole plant, preharvest	seed
60		0.58	0.26	0.80		0.73
75	0.89	0.66	0.60	1.03	0.91	0.93
100		0.70	0.71	1.06		0.98
125		0.77	0.78	0.91		0.85
150	0.95	0.87	0.94	1.00	1.02	0.85
250	1.00	0.90	0.93	0.98	1.00	1.00
300		1.00	1.00	1.00		

[a] In a 2 mL tube, different amounts of tissue samples were weighed, and two metal beads and 1.5 mL of PBST/PVP were added to each tube. The tubes were extracted in a Geno/Grinder for 3 min, and then, it was centrifuged at 10000 rpm for 5 min. The supernatant was then trypsin treated and assayed by ELISA. [b] The Cry1Ac concentration of the highest buffer/tissue ratio extract of each tissue was assigned as 1.00.

Table 3. Summary of Extraction Efficiency Results

tissue	mean Cry1Ac measured (μg/g)	mean extraction efficiency[a] (%)	extraction efficiency range (%)	SD	% CV
boll	0.76	84	83–85	0.98	1.16
leaf	1.86	81	78–87	4.48	5.56
pollen	1.29	89	88–92	1.81	2.03
seed	0.62	92	91–93	1.45	1.58
whole plant, at pollination	0.91	82	79–85	2.33	2.84
whole plant, at preharvest	0.70	91	88–92	1.58	1.74

[a] Extraction efficiency represents the percent target protein extracted in the first extraction (the sum of Cry1Ac protein measured in all five extractions is used as the total Cry1Ac protein in the sample). A total of five sets of experiment were performed for each matrix.

Table 4. Summary of Accuracy Results

matrix	fortification level μg/g dry weight	recovery rate (%)			
		mean	range	CV %	n
boll	0.8	85	78–92	6.6	5
	0.25	82	70–90	9.7	5
	0.1	80	70–91	12.2	5
leaf	0.8	81	74–92	8.1	7
	0.25	77	65–89	13.0	7
	0.1	84	77–93	4.9	10
pollen	0.8	86	75–94	8.7	5
	0.25	88	80–97	8.2	5
	0.1	82	76–92	8.0	5
seed	1.2	76	72–81	5.1	5
	0.375	66	53–79	15.4	7
whole plant, at pollination	0.8	79	66–91	11.0	7
	0.25	74	67–83	7.7	7
	0.1	67	55–78	12.3	7
whole plant, at preharvest	0.8	74	65–88	11.4	7
	0.25	68	60–86	14.8	7
	0.1	64	50–79	16.7	7

Table 5. Summary of Assay Precision Results-1

fortified protein in WP matrix (μg/g dry weight)	day 1 analyst 1	day 1 analyst 2	day 1 analyst 3	day 2 analyst 1	day 2 analyst 2	day 2 analyst 3	mean (μg/g)	SD	CV %
0.80	0.63	0.75	0.75	0.90	0.59	0.90	0.74	0.094	12.6
0.40	0.36	0.43	0.41	0.43	0.33	0.43	0.38	0.045	12.0
0.25	0.20	0.19	0.21	0.24	0.25	0.24	0.21	0.018	8.4
0.10	0.08	0.096	0.079	0.09	0.11	0.09	0.091	0.009	10.4

Table 6. Summary of Assay Precision Results-2

tissues[a]	mean (μg/g dry weight)	SD (μg/g dry weight)	% CV	range (μg/g dry weight)
leaf	1.779	0.180	10.1	1.52–2.03
boll	0.780	0.077	9.9	0.66–0.88
cotton seed	0.501	0.073	14.5	0.41–0.59
whole plant	0.869	0.099	11.4	0.70–1.02

[a] All samples were analyzed over 12 months, and a total of 8–10 data points were collected for each tissue.

are effective and do not interfere with the ELISA. Because of the reagent availability and suitability for high-throughput format, PMSF was chosen for further optimization. With transgenic whole plant (pollination stage) extract, approximately 25 μg of trypsin is required for a complete truncation of an 800 μL extract (Figure 3). The incubation condition was further investigated with different incubation times at ambient temperature and 37 °C. No significant truncation occurred at ambient temperature even for incubation of 4 h. However, Cry1Ac truncation was significantly accelerated when incubated at 37 °C, and the truncation rate increased with time (Figure 4). The rate of truncation reaches a plateau after a 60 min incubation. Finally, considering the assay simplicity and assaying time, a 60 min incubation at 37 °C was chosen for Cry1Ac extract truncation, and with each milliliter of extract containing 30 μg of trypsin, 20 μL of 10 mM PMSF was recommended as the stop solution.

Extraction Efficiency. The extraction of protein from plant tissues with high efficiency and consistency is critical for accurately determining total Cry1Ac protein levels in a sample. The extraction efficiency is dependent on sample, buffer, and buffer−tissue ratios. Different buffer−tissue ratios and sample sizes were studied for a variety of tissue samples. In general, a minimum buffer−tissue ratio is needed for effective tissue sample extraction, and the minimum ratio varies depending on tissue type (Table 2). On the basis of an extensive study with different cotton tissues, a minimum buffer−tissue ratio was established. A buffer−tissue ratio of 150 is needed for leaf and pollen, and a lower ratio of 75 is sufficient for other tissues: whole plant at pollination and preharvest stages, bolls, and seed. With an optimized buffer−tissue ratio, the majority of Cry1Ac protein in each tissue is extracted in the first extraction. The extraction efficiency was between 81 and 92% (Table 3). No Cry1Ac protein was found in the pellets after multiple extractions.

Accuracy. The method accuracy was assessed with Cry1Ac-fortified negative-control samples at different concentrations near the midpoint of the standard curve. Good recovery was achieved for each fortified concentration at or above the LOQ. The mean recoveries of Cry1Ac from leaf, pollen, bolls (late), seed, and whole plant (pollination and preharvest) tissues are shown in Table 4. Fortified at the LOQ level or above, the mean recoveries for all tissues ranged from 64 to 88% with a CV% of 16.7% or less.

Precision. Good interassay precision was observed when tested with fortified samples or positive-control samples by multiple analysts on different days. The precision data from whole plant (pollination) extract fortified at four levels (0.1, 0.25, 0.40, and 0.80 μg/g) are shown in Table 5. The interassay precision across all days and analysts was 10.4, 8.4, 12.0, and 12.6% CV for the whole plant (pollination) extracts fortified at 0.10, 0.25, 0.40, and 0.80 μg/g, respectively. The interday and interanalyst precisions for positive plant samples of leaf, boll, seed, and whole plant were 10.1, 9.9, 14.5, and 11.4% CV, respectively (Table 6).

Assay Sensitivity. Following established guidelines (*18*), the limits of quantitation (LOQ) and detection (LOD) for the determination of truncated Cry1Ac were calculated using the SD from the results of the recovery samples fortified at the lowest standard concentration level of 0.05 μg/g. Because of matrix interference, cotton seed was tested at 0.112 μg/g. Because of the nature of immunoassay, blank samples were not used for the purpose of calculating assay LOD/LOQ. The LOQ was calculated as 10 times the SD (10*s*), and the LOD was calculated as three times the SD (3*s*) of the results (using a minimum of five samples). The results are summarized below and listed in Table 7. The calculated LODs and LOQs supported the method target LOD and LOQ for all matrices. The LOQs and LODs are varied depending on the tissue type. The validated LOQs and LODs for bolls (late), leaf, pollen, and whole plant (pollination and preharvest) are 0.1 and 0.025 μg/g, respectively. For cotton seed, the LOQ is 0.375 μg/g and the LOD is 0.075 μg/g. Unfortified control samples (matrix blanks) and samples fortified at the LOD (Table 7) were analyzed to determine the false-positive and false-negative rates. There were

Table 7. Summary of LOD and LOQ Calculation for Cry1Ac ELISA

tissue	fortified level (μg/g)	average recovery (μg/g)	SD (*s*)	*n*[a]	3× SD	target LOD[b] (μg/g)	10× SD	target LOQ[b] (μg/g)
boll	0.050	0.028	0.0049	6	0.015	0.025	0.049	0.10
leaf	0.050	0.030	0.0032	5	0.010	0.025	0.032	0.10
pollen	0.050	0.037	0.0030	5	0.009	0.025	0.030	0.10
seed	0.112	0.063	0.0138	11	0.041	0.075	0.138	0.375
whole plant (pollination)	0.050	0.032	0.0047	6	0.015	0.025	0.047	0.10
whole plant (preharvest)	0.050	0.023	0.0042	6	0.013	0.025	0.042	0.10

[a] *N* = number for replicates performed. [b] These LOD and LOQ are targeted LOD and LOQs.

no false positives from the unfortified control samples and no false negatives from the LOD fortified samples analyzed in this study.

Conclusions. A simple and selective Cry1Ac ELISA has been developed and optimized for determination of Cry1Ac protein in cotton tissues. PBST with 1% PVP was selected as the extraction and assay buffer to minimize matrix effects. The optimum extraction buffer-to-tissue mass ratios were determined at 75 or 150 depending on tissue type. To measure total Cry1Ac protein in a sample, the extract should be treated with trypsin for Cry1Ac truncation prior to ELISA. Each milliliter of extract should be incubated with at least 25 μg of trypsin for 60 min at 37 °C and terminated with 25 μL of 10 mM PMSF. The method was validated over the concentration range of 0.1−0.8 μg/g dry weight tissue with a validated LOQ of 0.1 μg/g for all cotton matrices except cotton seed, which has a validated range of 0.375−1.2 μg/g with an LOQ of 0.375 μg/g. The assay is specific for Cry1Ac protein, and there is no CR with the nontarget proteins tested. Slight matrix effects were detected in some tissues, with 2× to 4× dilutions recommended to minimize potential matrix effects. In addition, Cry1Ac protein was efficiently extracted from all cotton tissues. The assay was shown to have acceptable accuracy and precision, and no false-positive or false-negative results at the target LOD. This Cry1Ac ELISA method has been demonstrated to be suitable for quantitative measurement of the WideStrike-specific Cry1Ac protein in cotton tissues.

ABBREVIATIONS USED

BAR, protein conferring tolerance to bialaphos; CV, coefficient of variation; ELISA, enzyme linked immunosorbent assay; HRP, horse radish peroxidase; LOD, limit of detection; LOQ, lower limit of quantitation; MAb, monoclonal antibody; OD, optical density; PAb, polyclonal antibody; PAT, phosphinothrin acetyltransferase; PBST, phosphate buffered saline with 0.05% Tween 20; PMSF, α-toluenesulfonyl fluoride; PVP, polyvinylpyrrolidone.

ACKNOWLEDGMENT

We thank George Teaney and Dale Onisk of Strategic Diagnostic Inc. for their technical support in antibody production and screening and assay development. We acknowledge Rod Herman, Yong Gao, and Laura Tagliani at Regulatory Laboratories of Dow AgroSciences for providing technical support during the assay development and validation. We also thank Irene Gatti, Larry Sernyk, Kathryn Clayton, and John Cuffe of Dow AgroSciences for reviewing the manuscript.

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Received for review March 7, 2007. Revised manuscript received May 18, 2007. Accepted May 21, 2007. WideStrike is a trademark of Dow AgroSciences LLC.

JF070664T