Immunodetection of Cry2Ab2 and Cry1A.105 Proteins in corn Grain from MON89034 following Heat Treatment

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Immunodetection of Cry2Ab2 and Cry1A.105 Proteins in Corn Grain from MON 89034 Following Heat Treatment

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Performing Laboratory

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Sponsor Representative: [signature] Date: 11/9/05

Study Director: [signature] Date: 11/9/05

Quality Assurance Statement

Study Title: Immunodetection of Cry2Ab2 and Cry1A.105 Proteins in Corn Grain from MON 89034 Following Heat Treatment

Study Number: 05-01-39-27

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates of Inspection / Audit	Phase	Date Reported To: Study Director	Date Reported To: Management
8/18/2005	Heat Stability	8/19/2005	8/19/2005
8/18/2005	Western Blot	8/22/2005	8/22/2005
9/27/2005	Raw Data Audit	9/30/2005	9/30/2005
11/07/2005	Draft Report Review	11/09/2005	11/09/2005

Joan M. Rejda-Heath
Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

November 9, 2005
Date

Study Information

Study Number: 05-01-39-27

Title: Immunodetection of Cry2Ab2 and Cry1A.105 Proteins in Corn Grain from MON 89034 Following Heat Treatment

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Records Retention: All study-specific raw data, protocol, final report, and facility records will be retained at Monsanto, St. Louis.

Sample Retention: Any study samples to be retained, will be stored at Monsanto, St. Louis.

Study Initiation Date: August 15, 2005
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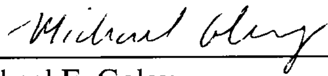
Study Certification

The results reported in this final report accurately reflect the data generated under study number 05-01-39-27.

Approved By:



11/9/05

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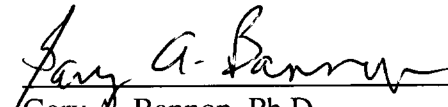
Date



11-09-2005

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Abbreviations[1] and Definitions

APS	Analytical protein standard
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CFR	Code of Federal Regulations
COA	Certificate of Analysis
DTT	DL-Dithiothreitol
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
LOD	Limit of detection
MALDI-TOF	Matrix-assisted laser desorption ionization time of flight
MS	Mass spectrometry
NFDM	Non-fat dry milk
NLS	N-Lauroyl sarcosine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline containing Tween-20
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
ppm	Parts per million
PVDF	Polyvinylidene difluoride
SOP	Standard operating procedure
US	United States
VOI	Verification of Identity

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time, etc., are used without definition according to the format described in “Instructions to Authors” in The Journal of Biological Chemistry.

1.0 Summary

Monsanto has developed corn, MON 89034, which produces the Cry1A.105 and Cry2Ab2 insecticidal proteins and is protected from feeding damage caused by European corn borer and other lepidopteran insect pests. Cry1A.105 is a modified *Bacillus thuringiensis* (*B.t.*) Cry1A protein with 93.6% overall amino acid sequence identity to the Cry1Ac protein. Cry2Ab2 is a *B.t.* (subsp. *kurstaki*) protein. The combination of the Cry2Ab2 and Cry1A.105 insecticidal proteins in a single plant provides better insect control and offers an additional insect-resistance management tool.

The purpose of this study was to assess the immunodetectability of the Cry2Ab2 and Cry1A.105 proteins in corn grain from MON 89034 following heat treatment. Test grain (MON 89034) and conventional control grain were ground and then heated in an oven to simulate the heating process used commercially to process corn grain. Heated and unheated samples of test and control grain were extracted with two buffers: 50 mM CAPS (referred to herein as CAPS buffer) and 50 mM CAPS containing 2% N-Lauroyl sarcosine (referred to herein as NLS buffer). The extracts were analyzed using the western blot method to detect the presence of the Cry2Ab2 and Cry1A.105 proteins. Image analyses of immunoreactive bands on the blot films were used to semi-quantitatively estimate the amount of immunoreactive Cry2Ab2 and Cry1A.105 proteins in the heated and unheated test and control grain extracts.

This study demonstrates that the amount of immunodetectable Cry2Ab2 protein present in either CAPS or NLS buffer extracts of MON 89034 after heating was below the lower LOD, or had decreased by greater than or equal to 77% and 70% relative to their original values, respectively. Likewise, this study demonstrates that the amount of immunodetectable Cry1A.105 protein present in either CAPS or NLS buffer extracts of MON 89034 after heating was below the lower LOD, or had decreased by greater than or equal to 94% and 78% relative to their original values, respectively. These results clearly demonstrate that the heating of ground corn grain, in a manner similar to the conditions employed for commercial processing, results in the loss of immunodetectable Cry2Ab2 and Cry1A.105 proteins. This loss is likely due to protein degradation or aggregation into an insoluble complex as a result of heat treatment.

2.0 Introduction

Monsanto has developed corn, MON 89034, which produces the Cry1A.105 and Cry2Ab2 insecticidal proteins and is protected from feeding damage caused by European corn borer and other lepidopteran insect pests. Cry1A.105 is a modified *Bacillus thuringiensis* (*B.t.*) Cry1A protein with 93.6% overall amino acid sequence identity to the Cry1Ac protein. Cry2Ab2 is a *B.t.* (subsp. *kurstaki*) protein. The combination of the

Cry2Ab2 and Cry1A.105 insecticidal proteins in a single plant provides better insect control and offers an additional insect-resistance management tool.

Food crops that have been developed through agricultural biotechnology for commercial use are thoroughly assessed for their safety. A key issue in the safety assessment of such crops is the potential allergenicity of the proteins introduced into food crops. One aspect of this assessment includes evaluation of the heat stability of the protein of interest. Manufacturing process steps, such as fermentation, heat treatment, or chemical treatment, may alter the allergenic potential of food by changing the physicochemical properties of proteins. For example, heat treatment completely eliminates the allergenic potential of the patatin protein in potato (Koppelman et al., 2002) and chitinases present in fruits (Sanchez-Monge et al., 2000). Heat treatment may also alter the digestibility of the proteins, thus, changing the potential allergenicity of food (Takagi et al., 2003). On the other hand, a number of food allergens, such as peanut allergen Ara h 1 (Koppelman et al., 1999), and the lipid-transfer proteins present in peach (Brenna et al., 2000) and maize (Pastorello et al., 2003), are extremely stable to heat treatment.

This study was performed to evaluate how heat treatment, which simulates the heating steps employed during the processing of corn grain (Rooney and Serna-Saldivar, 1994), affects the immunodetectability of the Cry2Ab2 and Cry1A.105 proteins present in MON 89034.

3.0 Purpose

The purpose of this study was to assess the immunodetectability of the Cry2Ab2 and Cry1A.105 proteins in corn grain from MON89034 following heat treatment.

4.0 Materials

4.1 Test Substance

The test substance for this study was MON 89034. In this study, test grain containing Cry2Ab2 and Cry1A.105 proteins was evaluated. The test grain (LIMS 04ZMGRO00393 produced from seed lot number GLP-0404-14916-S) was harvested from plants grown in the field under Production Plan 04-01-39-22. Records of the test grain production were archived with Production Plan 04-01-39-22. Test grain was stored at room temperature prior to grinding for the study.

4.2 Control Substance

The control substance was conventional corn with a genetic background similar to the test substance. The control grain (LIMS 04ZMGRO00399 produced from seed lot number GLP-0404-15002-S) was harvested from plants grown in the field under Production Plan 04-01-39-22. Records of the control grain production were archived with Production Plan 04-01-39-22. Control grain was stored at room temperature prior to grinding for the study.

4.3 Reference Substance

There were two reference standards in this study: Cry2Ab2 protein and Cry1A.105 protein. Cry2Ab2 protein (APS lot 20-100071) was produced in and purified from *E. coli*. The *E. coli*-produced Cry2Ab2 protein is referred to as Cry2Ab2.820 in the Certificate of Analysis. This reference standard was stored until use in a –80 °C freezer in a buffer solution [50mM CAPS, pH 11, and 2 mM DTT] at a total protein concentration of 0.5 mg/ml based upon amino acid analysis.

Cry1A.105 protein (APS lot 20-100073) was produced in and purified from *E. coli*. This reference standard was stored until use in a –80 °C freezer in a buffer solution [25 mM CAPS, pH ~10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT] at a total protein concentration of 1.2 mg/ml based upon amino acid analysis.

Analytical reference standards (e.g. molecular weight markers for SDS-PAGE) used in this study are documented in the study data and are described in this final report.

4.4 Characterization of the Test, Control, and Reference Substances

Prior to study initiation, the presence or absence of MON 89034 in the test and control substances was verified by PCR analyses. Characterization documentation for the test substance is archived under lot GLP-0404-14916-S and for the control substance under lot GLP-0404-15002-S. Copies of the certificate of analysis (COA) for the test and control substances starting seed and verification of identity (VOI) for the harvested test and control seed are archived with this study.

Prior to study initiation, the reference protein standards were characterized under Characterization Plans 20-100071 and 20-100073. The following properties were determined for both the Cry2Ab2 and the Cry1A.105 proteins: identity (N-terminal sequencing, matrix-assisted laser desorption ionization time of flight mass spectrometry [MALDI-TOF MS], immunodetection), concentration (amino

acid composition), purity (SDS-PAGE/densitometry), molecular weight (SDS-PAGE/densitometry, MALDI-TOF MS), activity (insect bioassay) and short-term stability (SDS-PAGE/densitometry). Copies of the COAs for the Cry2Ab2 and Cry1A.105 proteins are archived with this study.

Copies of the information sheets for the molecular weight markers used in SDS-PAGE are archived with this study.

5.0 Experimental Design

Test and control grain were ground to a powder. The ground test and control grain that received the heat treatment are referred to as the "heated" samples. The ground test and control grain that did not receive the heat treatment are referred to as the "unheated" samples. Proteins were extracted from heated and unheated samples using 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS) and CAPS buffer containing N-Lauroyl sarcosine (NLS). Extracts were subjected to SDS-PAGE and analyzed by the western blot method for the presence of either the Cry2Ab2 or the Cry1A.105 protein. Concurrently, the western blot lower LOD for the protein being analyzed was determined by spiking the appropriate reference standard into unheated control extract. In addition, the reference standard spiked into unheated control extracts was run on each heat stability sample gel to positively identify the positions of the Cry2Ab2 or Cry1A.105 proteins on the western blot. Also, molecular weight markers were run on each gel to help estimate the molecular weight of each immunoreactive band and assess protein transfer to the blot membrane. The level of signal from the immunoreactive bands representing the full-length proteins of interest was determined for all heated and unheated test samples, except for samples where signal was not observed. The heat treatment was considered to have impact on the immunodetectability of the Cry2Ab2 and Cry1A.105 proteins in ground corn grain prepared from MON 89034 if a ≥20% difference in immunoreactivity, expressed as ppm (ng protein per mg ground corn grain), was observed between heated and unheated samples.

6.0 Methods

The effect of heat treatment on the immunologically detectable levels of the Cry2Ab2 and Cry1A.105 proteins in corn grain from MON 89034 was evaluated using the western blot method and image analysis of blot films. Test and control grain was heated in a forced air electric oven at approximately 204°C for 20 minutes to simulate heat processing used commercially to process corn grain. Heated and unheated samples were extracted with two separate buffers: a non-denaturing CAPS buffer (referred to as CAPS buffer) and a denaturing CAPS buffer containing N-Lauroyl sarcosine (referred to as NLS buffer). CAPS buffer represented a relatively mild, non-denaturing, and reducing aqueous extraction buffer. NLS buffer represented a denaturing and reducing aqueous

extraction buffer. Extracted proteins were separated by SDS-PAGE and analyzed for the presence of either the Cry2Ab2 or Cry1A.105 proteins by the western blot method using the anti-Cry2Ab2 and anti-Cry1A.105 specific antibodies, respectively. The standard curves, representing different quantities of either the Cry2Ab2 or Cry1A.105 reference standards that were spiked into the CAPS and NLS buffer extracts of unheated control grain, were prepared and analyzed by western blot method (see Sections 6.4, 6.5, 6.6, and 6.7). The amounts of Cry2Ab2 and Cry1A.105 proteins present in the CAPS and NLS buffer extracts of unheated and heated test grain was estimated using these standard curves.

6.1 Grinding of Corn Grain

Approximately 10 g of both the test and control grain were ground to a powder using a Harbil high-speed paint shaker. The ground corn grain was then stored in a -80°C freezer until use.

6.2 Heat Treatment of Ground Corn Grain

Water was added to a portion of the ground corn grain in the ratio of 1 part of water to 3 parts of ground corn grain (25% v/w). These mixtures were heated at a temperature of approximately 204°C for 20 minutes in a programmable forced air electric oven. Following the heat treatment, the heated corn grain samples were stored in a -80°C freezer until protein extraction.

6.3 Protein Extraction

Extractions were performed on the heated and unheated grain using two separate buffers: CAPS buffer [50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine, pH 10.8] and NLS buffer [2% (w/v) N-Lauroyl sarcosine in 50 mM CAPS, 1 mM EDTA, 10 mM DTT, 1 mM PMSF, 2 mM benzamidine, pH 10.8]. Heated and unheated test and control grain were extracted in CAPS and NLS buffer at 1:3 weight-to-volume (w/v) ratios and clarified by centrifugation. CAPS and NLS extractions were performed for 60 min in a 4°C refrigerator with gentle rocking on a Nutator. A 300 µl sub-sample of each unheated clarified extract was then diluted 1.25-fold by the addition of 75 µl 5× Loading Buffer [0.3125 M Tris, 10% (w/v) SDS, 50% (v/v) glycerol, 25% (v/v) 2-mercaptoethanol, 0.025% (w/v) Bromophenol Blue]. A 200 µl sub-sample of each heated clarified extract was diluted 1.25-fold by the addition of 50 µl 5× Loading Buffer. The diluted extracts were heated for 5 min at 96.3 °C and then stored in a -80 ºC freezer.

The table below summarizes the coding system that was used to identify grain during the heat treatment procedure and extracts during the western blot analysis.

<table>
<tr><th>Sample</th><th>Treatment</th><th>Treatment Code</th><th>Extraction Buffer</th><th>Extract Code</th></tr>
<tr><td rowspan="4">ground control grain</td><td rowspan="2">Unheated</td><td rowspan="2">C-UH</td><td>CAPS</td><td>C-UH-CAPS</td></tr>
<tr><td>NLS</td><td>C-UH-NLS</td></tr>
<tr><td rowspan="2">Heated</td><td rowspan="2">C-H</td><td>CAPS</td><td>C-H-CAPS</td></tr>
<tr><td>NLS</td><td>C-H-NLS</td></tr>
<tr><td rowspan="4">ground test grain</td><td rowspan="2">Unheated</td><td rowspan="2">T-UH</td><td>CAPS</td><td>T-UH-CAPS</td></tr>
<tr><td>NLS</td><td>T-UH-NLS</td></tr>
<tr><td rowspan="2">Heated</td><td rowspan="2">T-H</td><td>CAPS</td><td>T-H-CAPS</td></tr>
<tr><td>NLS</td><td>T-H-NLS</td></tr>
</table>

6.4 Cry2Ab2 Western Blot Analysis

Aliquots of the test and control samples, and control samples containing a Cry2Ab2 reference standard spike were heated for 5 minutes at 96.4-96.8 °C and then separated by SDS-PAGE using pre-cast 12-well Tris-glycine 10% polyacrylamide mini-gels and Tris-glycine SDS running buffer (Invitrogen and Bio-Rad, respectively). Electrophoresis was performed at 175 V for 62 min. Following electrophoresis, proteins were electrotransferred to a PVDF membrane (Invitrogen) for 60 min at a constant voltage of 25 V. Control extracts were analyzed on each gel to account for any nonspecific binding of the antibody. Pre-stained molecular weight markers (Precision Plus Dual Color, Bio-Rad) were used to verify electrotransfer of proteins to the membrane and to estimate the molecular weight of immunoreactive bands. A lane containing the control extract that was spiked with Cry2Ab2 reference standard was included on each gel to positively identify the position of the Cry2Ab2 protein on the blots.

All membranes were blocked overnight at ~4°C with 5% (w/v) non-fat dry milk (NFDM) in PBST. The membranes were incubated with a previously characterized goat anti–Cry2Ab2 antibody (lot 7227632) at a dilution of 1:3000 in 1% (v/v) NFDM in PBST for 1 h. Incubation in the primary antibody and all subsequent steps were performed at room temperature. Excess antibody was removed by three 10 min washes with PBST. The membranes were incubated with horseradish peroxidase (HRP)–conjugated anti-goat IgG (Sigma Chemical Co) at a dilution of 1:10,000 in 1% (v/v) NFDM in PBST for 1 h and washed again with PBST (three 10 min washes). Immunoreactive bands on the

membrane were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia) by exposing the membrane to Hyperfilm ECL film (Amersham Pharmacia) for 30 sec, 1 min, and 2.5 min. Films were developed using a Konica SRX101A automated film processor.

To account for any matrix effect on immunodetectability of the Cry2Ab2 protein, the Cry2Ab2 LOD was determined by spiking 5 µl aliquots of various dilutions of the Cry2Ab2 reference standard into 25 µl aliquots of the unheated control extracts. The LOD was defined as the lowest detectable standard visible on the blots. Gels to determine the LOD were run concurrently with the gels for the test and control extracts and subjected to the same western blot procedure as described above. The following amounts of Cry2Ab2 reference standard were loaded into lanes for the LOD western blot analysis: 40, 30, 20, 10, 5, 2.5, and 1 ng.

6.5 Image Analysis of Cry2Ab2 Immunoreactive Bands on Films

Image analysis of immunoreactive bands on the films was conducted using a Bio-Rad model GS-800 calibrated imaging densitometer supplied with Quantity One software (version 4.4.0). The 2.5 min exposure film was analyzed for the blots assessing the immunodetectability of Cry2Ab2 protein in CAPS buffer extracts, while the 1 min exposure film was analyzed for the blots assessing the immunodetectability of Cry2Ab2 protein in NLS buffer extracts. The levels of signals for immunoreactive bands on the blot films were determined as band volumes (average band OD × band area in mm^2). Due to the close proximity of the bands to one another in adjacent lanes, the software was unable to recognize the bands as separate entities automatically; therefore, all bands were drawn manually. Consistent with the intent of the LOD blots, six visible reference standard bands (representing different amounts of Cry2Ab2 reference standard spiked into CAPS buffer extracts of the unheated control extract) and five visible reference standard bands (representing different amounts of Cry2Ab2 reference standard spiked into NLS buffer extracts of the unheated control extract) were plotted versus adjusted band volume data using the Quantity One software. Using a linear fit, coefficients of determination (R^2) of 0.981 and 0.967 were obtained for the CAPS and NLS buffer extracts, respectively. These values exceeded the minimum acceptance criteria of $R^2 \geq 0.95$ established in SOP BR-ME-0932-02. Based on the acceptability of the standard curves, the volume quantity data for protein bands detected in the test grain extracts were converted to nanograms (ng) of protein using the FORECAST worksheet function of Microsoft Excel software (SP3 Version 10.6730.6718). The quantity of detected protein for each test grain extract was divided by the mg of ground corn grain represented by the sample loaded to calculate the concentration of Cry2Ab2 protein in parts per million (ppm).

6.6 Cry1A.105 Western Blot Analysis

Aliquots of the test and control samples, and control samples containing a Cry1A.105 reference standard spike were heated for 5 minutes at 98.1-99.1 °C and then separated by SDS-PAGE using pre-cast 12-well Tris-glycine 4-20% polyacrylamide gradient mini-gels and Tris-glycine SDS running buffer (Invitrogen and Bio-Rad, respectively). Electrophoresis was performed at 125 V for 90 min followed by 150 V for 15 min. Following electrophoresis, proteins were electrotransferred to a PVDF membrane (Invitrogen) for 60 min at a constant voltage of 25 V. Control extracts were analyzed on each gel to account for any nonspecific binding of the antibody. Pre-stained molecular weight markers (Precision Plus Dual Color, Bio-Rad) were used to verify electrotransfer of proteins to the membrane and estimate the molecular weight of immunoreactive bands. A lane containing the control extract that was spiked with Cry1A.105 reference standard was included on each gel to positively identify the position of the Cry1A.105 protein on the blots.

All membranes were blocked overnight at ~4°C with 5% (w/v) non-fat dry milk (NFDM) in PBST. The membranes were incubated with a previously characterized rabbit anti–Cry1A.105 antibody (lot 070705JL) at a dilution of 1:2000 in 1% (v/v) NFDM in PBST for 1 h. Incubation in the primary antibody and all subsequent steps were performed at room temperature. Excess antibody was removed by three 10 min washes with PBST. The membranes were incubated with horseradish peroxidase (HRP)–conjugated anti-rabbit IgG (Sigma Chemical Co) at a dilution of 1:10,000 in 1% (v/v) NFDM in PBST for 1 h and washed again with PBST (three 10 min washes). Immunoreactive bands on the membrane were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia) by exposing the membrane to Hyperfilm ECL film (Amersham Pharmacia) for 2.5 min, 4 min, and 5 min. Films were developed using a Konica SRX101A automated film processor.

To account for any matrix effect on immunodetectability of the Cry1A.105 protein, the Cry1A.105 LOD was determined by spiking 5 µl aliquots of various dilutions of the Cry1A.105 reference standard into 25 µl aliquots of the unheated control extracts. The LOD was defined as the lowest detectable standard visible on the blots. Gels to determine the LOD were run concurrently with the gels for the test and control extracts and subjected to the same western blot procedure as described above. The following amounts of Cry1A.105 reference standard were loaded into lanes for the LOD western blot analysis: 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 ng.

6.7 Image Analysis of Cry1A.105 Immunoreactive Bands on Films

Image analysis of immunoreactive bands on the films was conducted using a Bio-Rad model GS-800 calibrated imaging densitometer supplied with Quantity One software (version 4.4.0). The 5 min exposure film was analyzed for the blots assessing the immunodetectability of Cry1A.105 protein in CAPS buffer extracts, while the 4 min exposure film was analyzed for the blots assessing the immunodetectability of Cry1A.105 protein in NLS buffer extracts. The levels of signals for immunoreactive bands on the blot films were determined as band volumes (average band OD × band area in mm^2). Due to the close proximity of the bands to one another in adjacent lanes, the software was unable to recognize the bands as separate entities automatically; therefore, all bands were drawn manually. Consistent with the intent of the LOD blots, six visible reference standard bands (representing different amounts of Cry1A.105 reference standard spiked into either CAPS or NLS buffer extracts of the unheated control extract) were plotted versus adjusted band volume data using the Quantity One software. Using a linear fit, coefficients of determination (R^2) of 0.998 and 0.990 were obtained for the CAPS and NLS buffer extracts, respectively. These values exceeded the minimum acceptance criteria of $R^2 \geq 0.95$ established in SOP BR-ME-0932-02. Based on the acceptability of the standard curves, the volume quantity data for protein bands detected in the test grain extracts were converted to nanograms (ng) of protein using the FORECAST worksheet function of Microsoft Excel software (SP3 Version 10.6730.6718). The quantity of detected protein for each test grain extract was divided by the mg of ground corn grain represented by the amount of sample loaded to calculate the concentration of Cry1A.105 protein in parts per million (ppm).

7.0 Data Rejected or Not Reported

A set of CAPS and NLS buffer extracts were rejected because the 5× Loading Buffer that was used to perform the 1.25-fold dilution was not prepared correctly. The protein extraction procedure was repeated using a different lot of 5× Loading Buffer. Additionally, the various films that were visually determined to produce sub-optimal band intensity and contrast were not scanned. No further analysis was performed on these films.

8.0 Control of Bias

Appropriate controls and standards were included with each analysis. Unheated control extracts were spiked with either of the two reference standards to account for any possible influences of the matrix on the detection of the Cry2Ab2 and Cry1A.105

proteins. For each protein analyzed, heat stability gels and LOD gels were analyzed concurrently to eliminate run-to-run variation.

9.0 Results and Discussion

9.1 Analysis of the Cry2Ab2 Protein Levels in CAPS Buffer Extracts of Heated and Unheated Corn Grain

An immunoreactive band on the western blot with an approximate molecular weight of 61 kDa was identified as the full-length Cry2Ab2 protein (Fig. 1, lanes 2, 4 and 5). A variety of additional bands of both higher and lower molecular weight were observed in all unheated sample lanes (Fig. 1, lanes 2, 4, 5, and 9). These additional bands represent proteins of unknown identity that were present in the crude sample extracts in sufficient quantities to react non-specifically with either the primary or secondary antibodies. Based on the standard curve, the amount of full-length Cry2Ab2 protein in the unheated test grain extracts was estimated to be 6.00 ng and 10.06 ng in 5 and 10 µl of 1.25-fold diluted extract loads, respectively.

Neither Cry2Ab2 protein nor the aforementioned non-specifically reacting proteins were detected in the heated test grain extract (Fig.1, lanes 7 and 8). The heated and unheated control grain extracts were assessed concurrently with the test grain extract to confirm antibody specificity. As expected, Cry2Ab2 protein was not detected in the unheated or heated control grain extracts (Fig. 1, lanes 9 and 10).

A western blot was run concurrently to assess the LOD of the full-length Cry2Ab2 reference standard spiked into the CAPS buffer extract of unheated control grain. The LOD was defined as the lowest detectable reference standard visible on blots. The LOD was determined as described in Section 6.4 and was visually estimated to be 2.5 ng (Fig. 2, lane 9).

Calculations of the level of immunodetectable full-length Cry2Ab2 protein in the test grain extracts in ppm (equivalent to ng protein per mg ground corn grain or µg per g ground corn grain) and the effect of heat treatment on the level of immunodetectable protein are summarized in Table 1. A total of 0.30 g of the ground corn grain was extracted in a total volume of 0.9 ml CAPS buffer, and then 200 µl or 300 µl of clarified extract was diluted 1.25-fold by the addition of 50 µl or 75 µl 5× Loading Buffer, respectively. This dilution yielded a final concentration of approximately 0.2664 g tissue per ml of loading buffer containing extract or 0.2664 mg tissue per µl of loading buffer containing extract. Based on image analysis, 6.00 ng and 10.06 ng of Cry2Ab2 protein were detected

in 5 μl and 10 μl of 1.25-fold diluted extract, representing 1.332 mg and 2.664 mg of tissue, respectively. Therefore, the estimated protein concentration of the CAPS buffer extractable Cry2Ab2 protein in the unheated ground test grain was 4.50 ppm and 3.78 ppm calculated for the 5 μl and 10 μl loads, respectively:

$$\frac{6.00\ ng\ Cry2Ab2\ protein}{1.332\ \ mg\ tissue} = 4.50\ \frac{ng}{mg}\ or\ 4.50\ ppm\ of\ \ Cry2Ab2\ \ protein$$

$$\frac{10.06\, ng\ Cry2Ab2\ protein}{2.664\, mg\ tissue} = 3.78\ \frac{ng}{mg}\ or\ 3.78\ ppm\ of\ \ Cry2Ab2\ \ protein$$

The average concentration of the CAPS buffer extractable Cry2Ab2 protein in the unheated ground test grain was 4.14 ppm.

The LOD was estimated to be 2.5 ng of the Cry2Ab2 protein detected in diluted control grain extract representing 2.664 mg of tissue. Therefore, the LOD was calculated to be 0.938 ppm:

$$\frac{2.5\ ng\ Cry2Ab2\ protein}{2.664\ mg\ tissue} = 0.938\frac{ng}{mg}\ or\ 0.938\ \ ppm\ of\ \ Cry2Ab2\ \ protein$$

The LOD value represents 22.7% of the total protein loaded:

$$\frac{0.938\ ppm}{4.14\ ppm} = 0.227\ or\ 22.7\%$$

Because Cry2Ab2 protein was not observed in the CAPS extract of the heated test grain, it can be inferred that as a result of heat treatment the level of immunodetectable Cry2Ab2 protein was below the LOD. Thus, immunodetectable Cry2Ab2 protein had declined by an amount greater than or equal to 77% $(100\% - 22.7\% = 77.3\%)$ relative to its original calculated level. Given that no immunoreactive signal is observed in lanes containing heated grain extract, it is likely that the decrease in immunodetectable Cry2Ab2 protein following heating is greater than 77%. However, due to the low concentration of Cry2Ab2 protein in the ground corn grain (4.14 ppm) and the relatively high LOD of this protein spiked into the unheated control matrix (0.938 ppm), the measurable protein reduction by heat treatment was limited by the technique.

9.2 Analysis of the Cry2Ab2 Protein Levels in NLS Buffer Extracts of Heated and Unheated Corn Grain

Results obtained from the NLS buffer extracts are similar to the results obtained from the CAPS buffer extracts. An immunoreactive band on the western blot with an approximate molecular weight of 61 kDa was identified as the full-length Cry2Ab2 protein (Fig. 3, lanes 2, 4 and 5). A variety of additional bands of both higher and lower molecular weight were observed in all unheated sample lanes (Fig. 3, lanes 2, 4, 5, and 9). These additional bands represent proteins of unknown identity that were present in the crude sample extracts in sufficient quantities to react non-specifically with either the primary or secondary antibodies. Based on the standard curve, the amount of full-length Cry2Ab2 protein in the unheated test grain extracts was estimated to be 7.61 ng and 18.09 ng in 5 and 10 µl of 1.25-fold diluted extract loads, respectively.

Neither Cry2Ab2 protein nor the aforementioned non-specifically reacting proteins were detected in the heated test grain extract (Fig. 3, lanes 7 and 8). The heated and unheated control grain extracts were assessed concurrently with the test grain extract to confirm antibody specificity. As expected, Cry2Ab2 protein was not detected in the unheated or heated control grain extracts (Fig. 3, lanes 9 and 10). However, faint bands of slightly higher and lower molecular weight, the products of proteins of unknown identity, were observed in the unheated control grain extract (Fig. 3, lane 9).

A western blot was run concurrently to assess the LOD of the full-length Cry2Ab2 reference standard spiked into the NLS buffer extract of unheated control grain. The LOD was defined as the lowest detectable reference standard visible on blots. The LOD was determined as described in Section 6.4 and was visually estimated to be 5 ng (Fig. 4, lane 8). The faint bands of slightly higher molecular weight that were observed in the remaining lanes interfered with the decreasing amount of spiked full-length Cry2Ab2 reference standard, and as such were not considered in the LOD determination (Fig. 4, lanes 9 and 10).

Calculations of the level of immunodetectable full-length Cry2Ab2 protein in the test grain extracts in ppm (equivalent to ng protein per mg ground corn grain or µg per g ground corn grain) and the effect of heat treatment on the level of immunodetectable protein are summarized in Table 1. A total of 0.30 g of the ground corn grain was extracted in a total volume of 0.9 ml NLS buffer, and then 200 µl or 300 µl of clarified extract was diluted 1.25-fold by the addition of 50 µl or 75 µl 5× Loading Buffer, respectively. This dilution yielded a final concentration of approximately 0.2664 g tissue per ml of loading buffer containing extract or 0.2664 mg tissue per µl of loading buffer containing extract. Based on image analysis, 7.61 ng and 18.09 ng of the Cry2Ab2 protein was

detected in 5 µl and 10 µl of 1.25-fold diluted extract, representing 1.332 mg and 2.664 mg of tissue, respectively. Therefore, the estimated protein concentration of the NLS buffer extractable Cry2Ab2 protein in the unheated ground test grain was 5.71 ppm and 6.79 ppm calculated for the 5 µl and 10 µl loads, respectively:

$$\frac{7.61\ ng\ Cry2Ab2\ protein}{1.332\ \ mg\ tissue} = 5.71\ \frac{ng}{mg}\ or\ 5.71\ ppm\ of\ \ Cry2Ab2\ \ protein$$

$$\frac{18.09\ ng\ Cry2Ab2\ protein}{2.664\ mg\ tissue} = 6.79\ \frac{ng}{mg}\ or\ 6.79\ ppm\ of\ \ Cry2Ab2\ \ protein$$

The average concentration of the NLS buffer extractable Cry2Ab2 protein in the unheated ground test grain was 6.25 ppm.

The LOD was estimated to be 5 ng of the Cry2Ab2 protein detected in diluted control grain extract representing 2.664 mg of tissue. Therefore, the LOD was calculated to be 1.877 ppm:

$$\frac{5\ \ ng\ Cry2Ab2\ protein}{2.664\ \ mg\ tissue} = 1.877\frac{ng}{mg}\ or\ 1.877\ \ ppm\ of\ \ Cry2Ab2\ \ protein$$

The LOD value represents 30.0% of the total protein loaded:

$$\frac{1.877\ ppm}{6.25\ ppm} = 0.300\ or\ 30.0\%$$

Because Cry2Ab2 protein was not observed in the NLS extract of the heated test grain, it can be inferred that as a result of heat treatment the level of immunodetectable Cry2Ab2 protein was below the LOD. Thus, immunodetectable Cry2Ab2 protein had declined by an amount greater than or equal to 70% $(100\% - 30.0\% = 70.0\%)$ relative to its original calculated level. Given that no immunoreactive signal is observed in lanes containing heated grain extract, it is likely that the decrease in immunodetectable Cry2Ab2 protein following heating is greater than 70%. However, due to the low concentration of Cry2Ab2 protein in the ground corn grain (6.25 ppm) and the relatively high LOD of this protein spiked into the unheated control matrix (1.877 ppm), the measurable protein reduction by heat treatment was limited by the technique.

9.3 Analysis of the Cry1A.105 Protein Levels in CAPS Buffer Extracts of Heated and Unheated Corn Grain

An immunoreactive band on the western blot with an approximate molecular weight of 131 kDa was identified as the full-length Cry1A.105 protein (Fig. 5, lanes 2, 4 and 5). A variety of additional bands of lower molecular weight were observed in all unheated sample lanes (Fig. 5, lanes 2, 4, 5, and 9). These additional bands represent proteins of unknown identity that were present in the crude sample extracts in sufficient quantities to react non-specifically with either the primary or secondary antibodies. Based on the standard curve, the amount of full-length Cry1A.105 protein in the unheated test grain extracts was estimated to be 2.06 ng and 3.81 ng in 5 and 10 µl of 1.25-fold diluted extract loads, respectively.

Neither Cry1A.105 protein nor the aforementioned non-specifically reacting proteins were detected in the heated test grain extract (Fig.5, lanes 7 and 8). The heated and unheated control grain extracts were assessed concurrently with the test grain extract to confirm antibody specificity. As expected, Cry1A.105 protein was not detected in the unheated or heated control grain extracts (Fig. 5, lanes 9 and 10).

A western blot was run concurrently to assess the LOD of the full-length Cry1A.105 reference standard spiked into the CAPS buffer extract of unheated control grain. The LOD was defined as the lowest detectable reference standard visible on blots. The LOD was determined as described in Section 6.6 and was visually estimated to be 0.25 ng (Fig. 6, lane 9).

Calculations of the level of immunodetectable full-length Cry1A.105 protein in the test grain extracts in ppm (equivalent to ng protein per mg ground corn grain or µg per g ground corn grain) and the effect of heat treatment on the level of immunodetectable protein are summarized in Table 2. A total of 0.30 g of the ground corn grain was extracted in a total volume of 0.9 ml CAPS buffer, and then 200 µl or 300 µl of clarified extract was diluted 1.25-fold by the addition of 50 µl or 75 µl 5× Loading Buffer, respectively. This dilution yielded a final concentration of approximately 0.2664 g tissue per ml loading buffer containing extract or 0.2664 mg tissue per µl of loading buffer containing extract. Based on image analysis, 2.06 ng and 3.81 ng of the Cry1A.105 protein was detected in 5 µl and 10 µl of 1.25-fold diluted extract, representing 1.332 mg and 2.664 mg of tissue, respectively. Therefore, the estimated protein concentration of the CAPS buffer extractable Cry1A.105 protein in the unheated ground test grain was 1.55 ppm and 1.43 ppm calculated for the 5 µl and 10 µl loads, respectively:

$$\frac{2.06\ ng\ Cry1A.105\ protein}{1.332\ \ mg\ tissue} = 1.55\ \frac{ng}{mg}\ or\ 1.55\ ppm\ of\ \ Cry1A.105\ \ protein$$

$$\frac{3.81\ ng\ Cry1A.105\ protein}{2.664\ mg\ tissue} = 1.43\ \frac{ng}{mg}\ or\ 1.43\ ppm\ of\ \ Cry1A.105\ \ protein$$

The average concentration of the CAPS buffer extractable Cry1A.105 protein in the unheated ground test grain was 1.49 ppm.

The LOD was estimated to be 0.25 ng of the Cry1A.105 protein detected in diluted control grain extract representing 2.664 mg of tissue. Therefore, the LOD was calculated to be 0.094 ppm:

$$\frac{0.25\ ng\ Cry1A.105\ protein}{2.664\ mg\ tissue} = 0.094\frac{ng}{mg}\ or\ 0.094\ \ ppm\ of\ \ Cry1A.105\ \ protein$$

The LOD value represents 6.3% of the total protein loaded:

$$\frac{0.094\ ppm}{1.49\ ppm} = 0.063\ or\ 6.3\%$$

Because Cry1A.105 protein was not observed in the CAPS extract of the heated test grain, it can be inferred that as a result of heat treatment the level of immunodetectable Cry1A.105 protein was below the LOD. Thus, immunodetectable Cry1A.105 protein had declined by an amount greater than or equal to 94% $(100\% - 6.3\% = 93.7\%)$ relative to its original calculated level.

9.4 Analysis of the Cry1A.105 Protein Levels in NLS Buffer Extracts of Heated and Unheated Corn Grain

Results obtained from the NLS buffer extracts are similar to the results obtained from the CAPS buffer extracts. An immunoreactive band on the western blot with an approximate molecular weight of 131 kDa was identified as the full-length Cry1A.105 protein (Fig. 7, lanes 2, 4 and 5). A variety of additional bands of lower molecular weight were observed in all unheated sample lanes (Fig. 7, lanes 2, 4, 5, and 9). These bands represent additional proteins of unknown identity that were present in the crude sample extracts in sufficient quantities to react non-specifically with either the primary or secondary antibodies. Based on the standard curve, the amount of full-length Cry1A.105 protein in the unheated test grain extracts was estimated to be 0.56 ng and 1.17 ng in 5 and 10 µl of 1.25-fold diluted extract loads, respectively.

Neither Cry1A.105 protein nor the aforementioned non-specifically reacting proteins were detected in the heated test grain extract (Fig. 7, lanes 7 and 8). The heated and unheated control grain extracts were assessed concurrently with the test grain extract to confirm antibody specificity. As expected, Cry1A.105 protein was not detected in the unheated or heated control grain extracts (Fig. 7, lanes 9 and 10).

A western blot was run concurrently to assess the LOD of the full-length Cry1A.105 reference standard spiked into the NLS buffer extract of unheated control grain. The LOD was defined as the lowest detectable reference standard visible on blots. The LOD was determined as described in Section 6.6 and was visually estimated to be 0.25 ng (Fig. 8, lane 9).

Calculations of the level of immunodetectable full-length Cry1A.105 protein in the test grain extracts in ppm (equivalent to ng protein per mg ground corn grain or µg per g ground corn grain) and the effect of heat treatment on the level of immunodetectable protein are summarized in Table 2. A total of 0.30 g of the ground corn grain was extracted in a total volume of 0.9 ml NLS buffer, and then 200 µl or 300 µl of clarified extract was diluted 1.25-fold by the addition of 50 µl or 75 µl 5× Loading Buffer, respectively. This dilution yielded a final concentration of approximately 0.2664 g tissue per ml of loading buffer containing extract or 0.2664 mg tissue per µl of loading buffer containing extract. Based on image analysis, 0.56 ng and 1.17 ng of the Cry1A.105 protein was detected in 5 µl and 10 µl of 1.25-fold diluted extract, representing 1.332 mg and 2.664 mg of tissue, respectively. Therefore, the estimated protein concentration of the NLS buffer extractable Cry1A.105 protein in the unheated ground test grain was 0.42 ppm and 0.44 ppm calculated for the 5 µl and 10 µl loads, respectively:

$$\frac{0.56\ ng\ Cry1A.105\ protein}{1.332\ mg\ tissue} = 0.42\ \frac{ng}{mg}\ or\ 0.42\ ppm\ of\ Cry1A.105\ protein$$

$$\frac{1.17\ ng\ Cry1A.105\ protein}{2.664\ mg\ tissue} = 0.44\ \frac{ng}{mg}\ or\ 0.44\ ppm\ of\ Cry1A.105\ protein$$

The average concentration of the NLS buffer extractable Cry1A.105 protein in the unheated ground test grain was 0.43 ppm.

The LOD was estimated to be 0.25 ng of the Cry1A.105 protein detected in diluted control grain extract representing 2.664 mg of tissue. Therefore, the LOD was calculated to be 0.094 ppm:

$$\frac{0.25\ ng\ Cry1A.105\ protein}{2.664\ mg\ tissue} = 0.094\frac{ng}{mg}\ or\ 0.094\ ppm\ of\ Cry1A.105\ protein$$

The LOD value represents 21.9% of the total protein loaded:

$$\frac{0.094\ ppm}{0.43\ ppm} = 0.219\ or\ 21.9\%$$

Because Cry1A.105 protein was not observed in the NLS extract of the heated test grain, it can be inferred that as a result of heat treatment the level of immunodetectable Cry1A.105 protein was below the LOD. Thus, immunodetectable Cry1A.105 protein had declined by an amount greater than or equal to 78% $(100\% - 21.9\% = 78.1\%)$ relative to its original calculated level. Given that no immunoreactive signal is observed in lanes containing heated grain extract, it is likely that the decrease in immunodetectable Cry1A.105 protein following heating is greater than 78%. However, due to the very low concentration of Cry1A.105 protein in the ground corn grain (0.43 ppm) the measurable protein reduction by heat treatment was limited by the technique.

10.0 Conclusions

This study demonstrates that the amount of immunodetectable Cry2Ab2 protein present in either CAPS or NLS buffer extracts of MON 89034 after heating was below the lower LOD, or had decreased by greater than or equal to 77% and 70% relative to their original values, respectively. Likewise, this study demonstrates that the amount of immunodetectable Cry1A.105 protein present in either CAPS or NLS buffer extracts of MON 89034 after heating was below the lower LOD, or had decreased by greater than or equal to 94% and 78% relative to their original values, respectively. These results clearly demonstrate that the heating of ground corn grain, in a manner similar to the conditions employed for commercial processing, results in the loss of immunodetectable Cry2Ab2 and Cry1A.105 proteins. This loss is likely due to protein degradation or aggregation into an insoluble complex as a result of heat treatment.

11.0 References

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Table 1. Summary of the Cry2Ab2 Protein Concentrations in CAPS and NLS Extracts of Heated and Unheated Test Grain from MON 89034

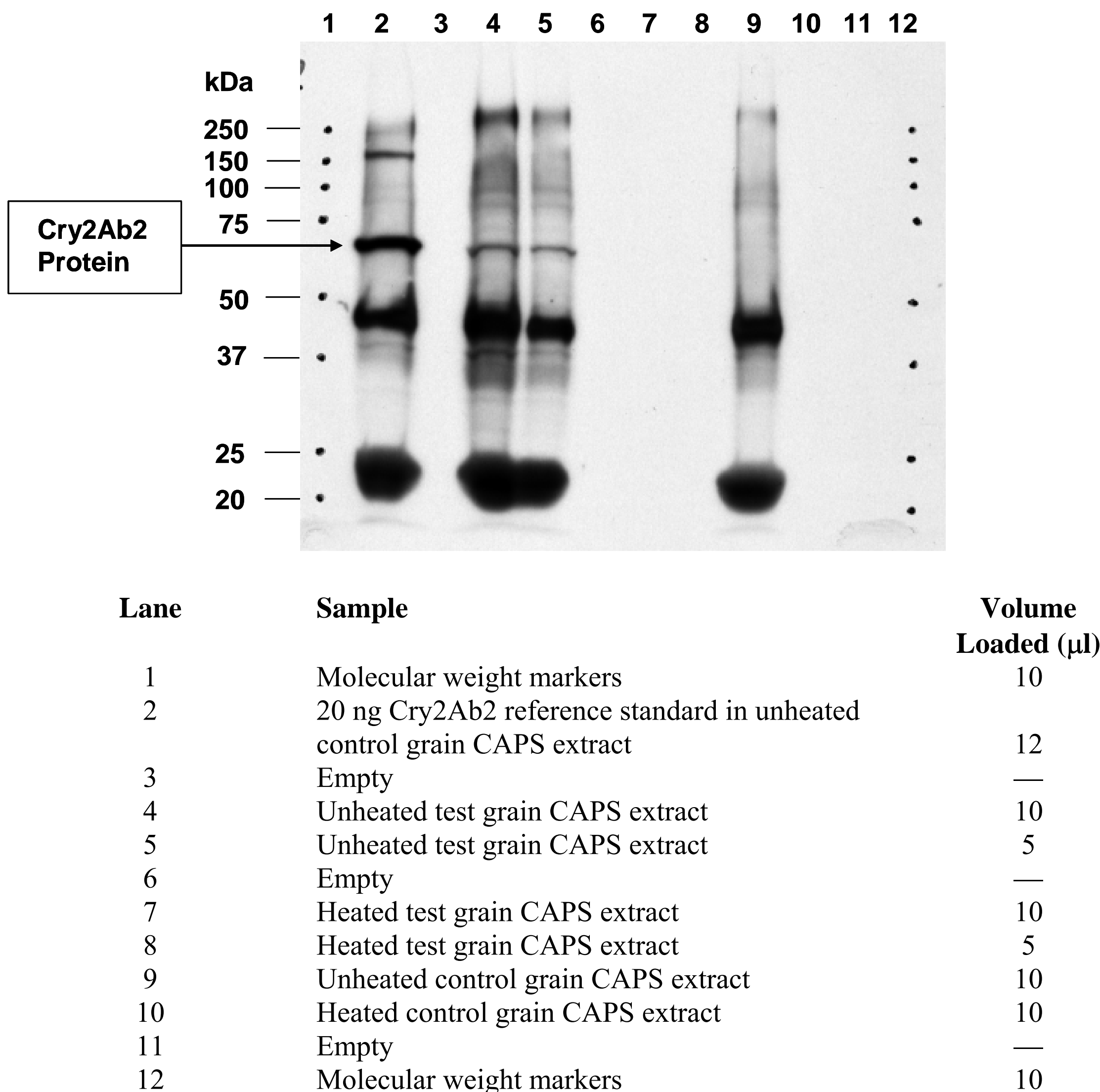
Extraction buffer	Lower LOD		Heat treatment of ground grain	Test grain extract quantitation				
	ng	ppm		Extract volume loaded (μl)	Cry2Ab2 detected (ng)	Tissue amount loaded (mg)	Cry2Ab2 concentration (ppm)	Average Cry2Ab2 concentration (ppm)
CAPS	2.5	0.938	Unheated	5	6.00	1.332	4.50	4.14
				10	10.06	2.664	3.78	
			Heated	5	not detected[1]	1.332	<LOD	<LOD
				10	not detected[1]	2.664	<LOD	
NLS	5	1.877	Unheated	5	7.61	1.332	5.71	6.25
				10	18.09	2.664	6.79	
			Heated	5	not detected[1]	1.332	<LOD	<LOD
				10	not detected[1]	2.664	<LOD	

[1] Signal from Cry2Ab2 protein was not observed on the western blot films.

Table 2. Summary of the Cry1A.105 Protein Concentrations in CAPS and NLS Extracts of Heated and Unheated Test Grain from MON 89034

Extraction buffer	Lower LOD		Heat treatment of ground grain	Test grain extract quantitation				
	ng	ppm		Extract volume loaded (µl)	Cry1A.105 detected (ng)	Tissue amount loaded (mg)	Cry1A.105 concentration (ppm)	Average Cry1A.105 concentration (ppm)
CAPS	0.25	0.094	Unheated	5	2.06	1.332	1.55	1.49
				10	3.81	2.664	1.43	
			Heated	5	not detected[1]	1.332	<LOD	<LOD
				10	not detected[1]	2.664	<LOD	
NLS	0.25	0.094	Unheated	5	0.56	1.332	0.42	0.43
				10	1.17	2.664	0.44	
			Heated	5	not detected[1]	1.332	<LOD	<LOD
				10	not detected[1]	2.664	<LOD	

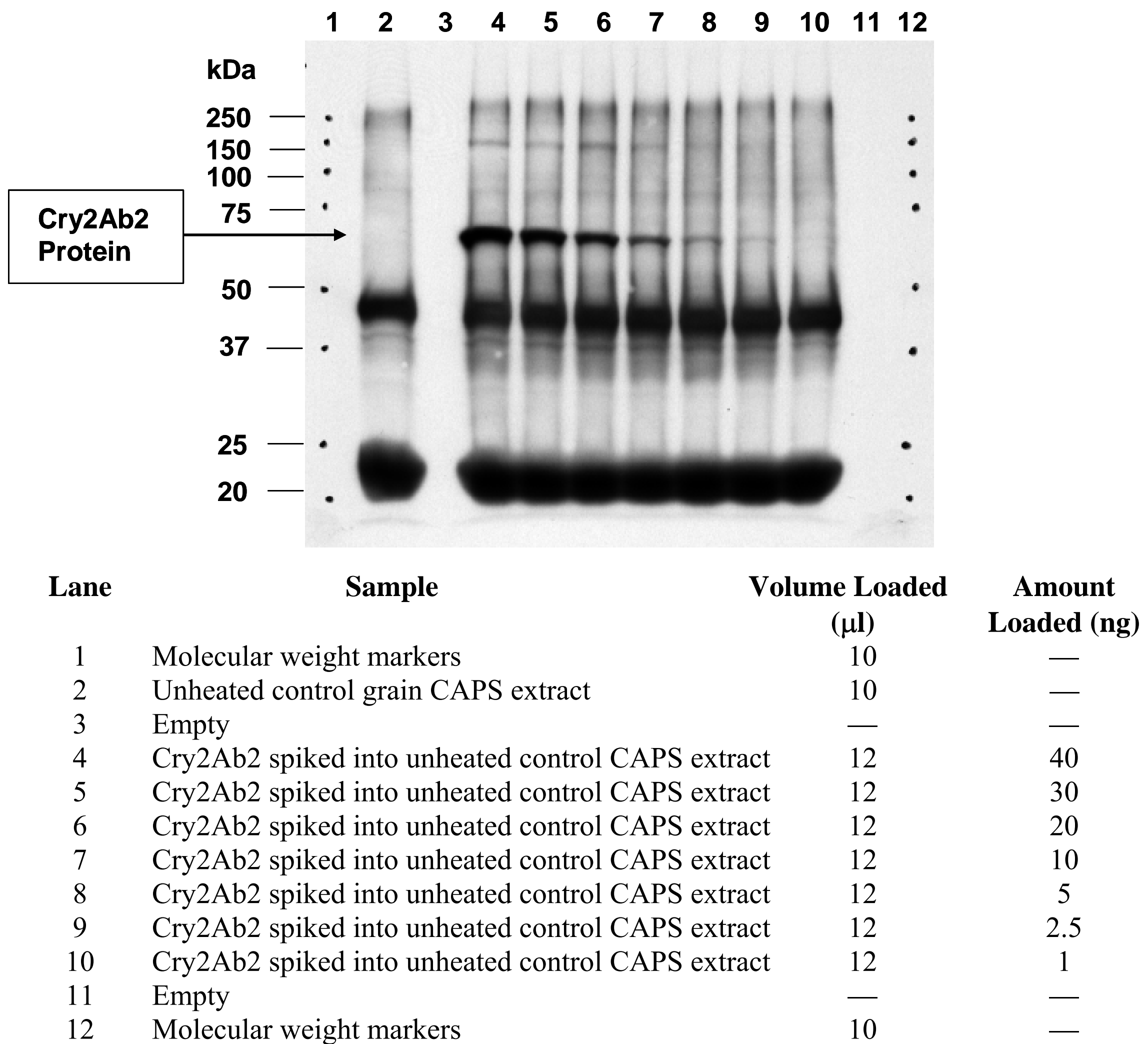
[1] Signal from Cry1A.105 protein was not observed on the western blot films.



Lane	Sample	Volume Loaded (µl)
1	Molecular weight markers	10
2	20 ng Cry2Ab2 reference standard in unheated control grain CAPS extract	12
3	Empty	—
4	Unheated test grain CAPS extract	10
5	Unheated test grain CAPS extract	5
6	Empty	—
7	Heated test grain CAPS extract	10
8	Heated test grain CAPS extract	5
9	Unheated control grain CAPS extract	10
10	Heated control grain CAPS extract	10
11	Empty	—
12	Molecular weight markers	10

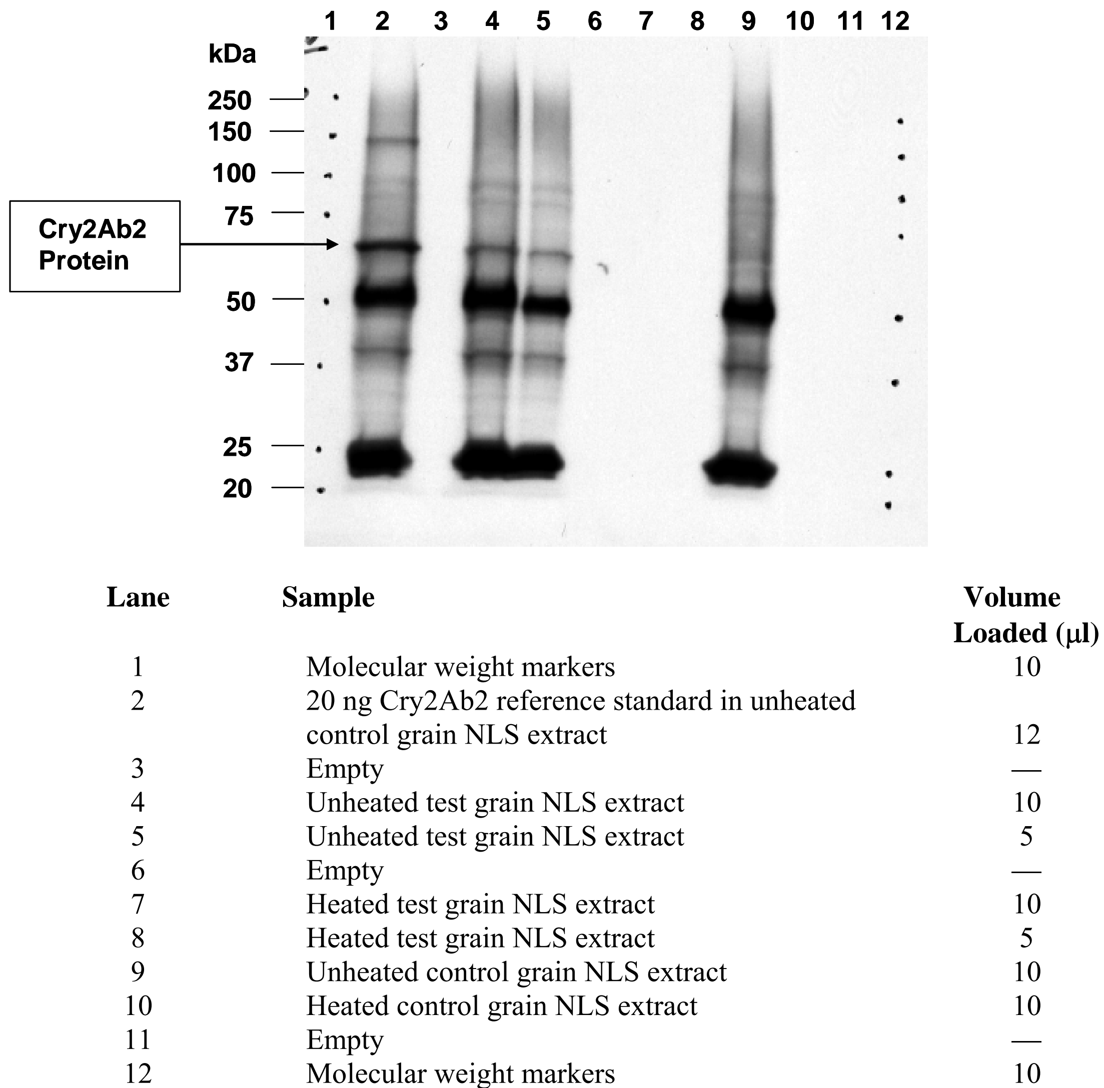
Figure 1. Western Blot Demonstrating the Effect of Heat Treatment on the Immunodetectability of the Cry2Ab2 Protein in CAPS Buffer Extracts of Heated and Unheated Test Grain from MON 89034

CAPS buffer extracts of the unheated and heated test and control grain were separated by a Tris-glycine 10% polyacrylamide gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 2.5 min.



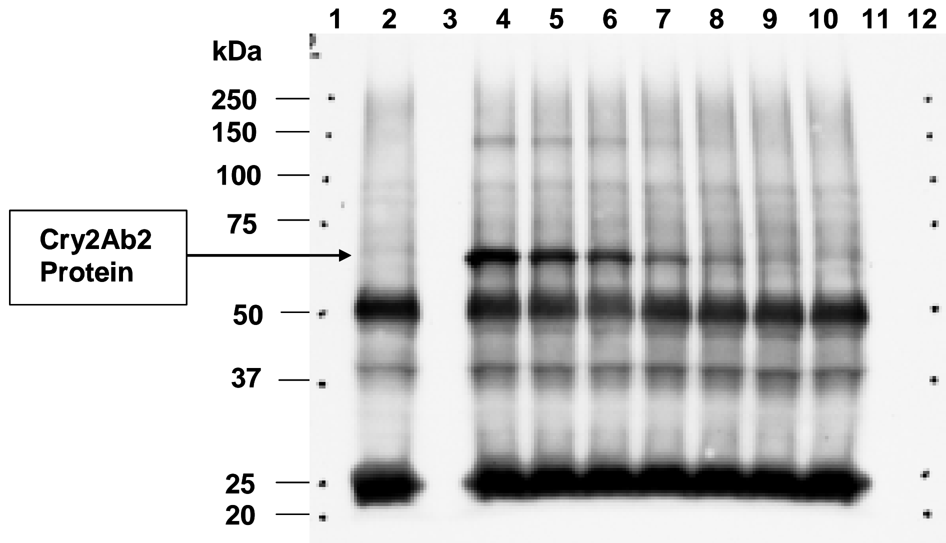
Lane	Sample	Volume Loaded (µl)	Amount Loaded (ng)
1	Molecular weight markers	10	—
2	Unheated control grain CAPS extract	10	—
3	Empty	—	—
4	Cry2Ab2 spiked into unheated control CAPS extract	12	40
5	Cry2Ab2 spiked into unheated control CAPS extract	12	30
6	Cry2Ab2 spiked into unheated control CAPS extract	12	20
7	Cry2Ab2 spiked into unheated control CAPS extract	12	10
8	Cry2Ab2 spiked into unheated control CAPS extract	12	5
9	Cry2Ab2 spiked into unheated control CAPS extract	12	2.5
10	Cry2Ab2 spiked into unheated control CAPS extract	12	1
11	Empty	—	—
12	Molecular weight markers	10	—

Figure 2. Western Blot Demonstrating Lower Limit of Detection for the Cry2Ab2 Reference Standard Spiked into the CAPS Buffer Extract of Unheated Control Grain
CAPS buffer extracts of the unheated control grain that were spiked with different amounts of Cry2Ab2 reference standard were separated by a Tris-glycine 10% polyacrylamide gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Amount Loaded (ng) refers to the quantity of the Cry2Ab2 reference standard loaded on the gel. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 2.5 min.



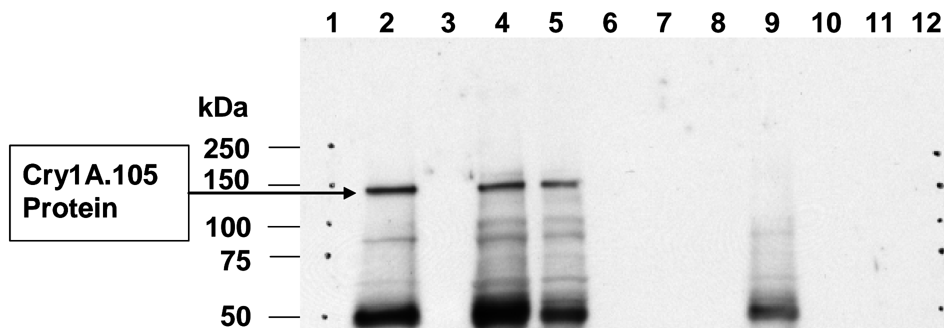
Lane	Sample	Volume Loaded (μl)
1	Molecular weight markers	10
2	20 ng Cry2Ab2 reference standard in unheated control grain NLS extract	12
3	Empty	—
4	Unheated test grain NLS extract	10
5	Unheated test grain NLS extract	5
6	Empty	—
7	Heated test grain NLS extract	10
8	Heated test grain NLS extract	5
9	Unheated control grain NLS extract	10
10	Heated control grain NLS extract	10
11	Empty	—
12	Molecular weight markers	10

Figure 3. Western Blot Demonstrating the Effect of Heat Treatment on the Immunodetection of the Cry2Ab2 Protein in NLS Buffer Extracts of Heated and Unheated Test Grain from MON 89034
NLS buffer extracts of the unheated and heated test and control grain were separated by a Tris-glycine 10% polyacrylamide gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 1 min.



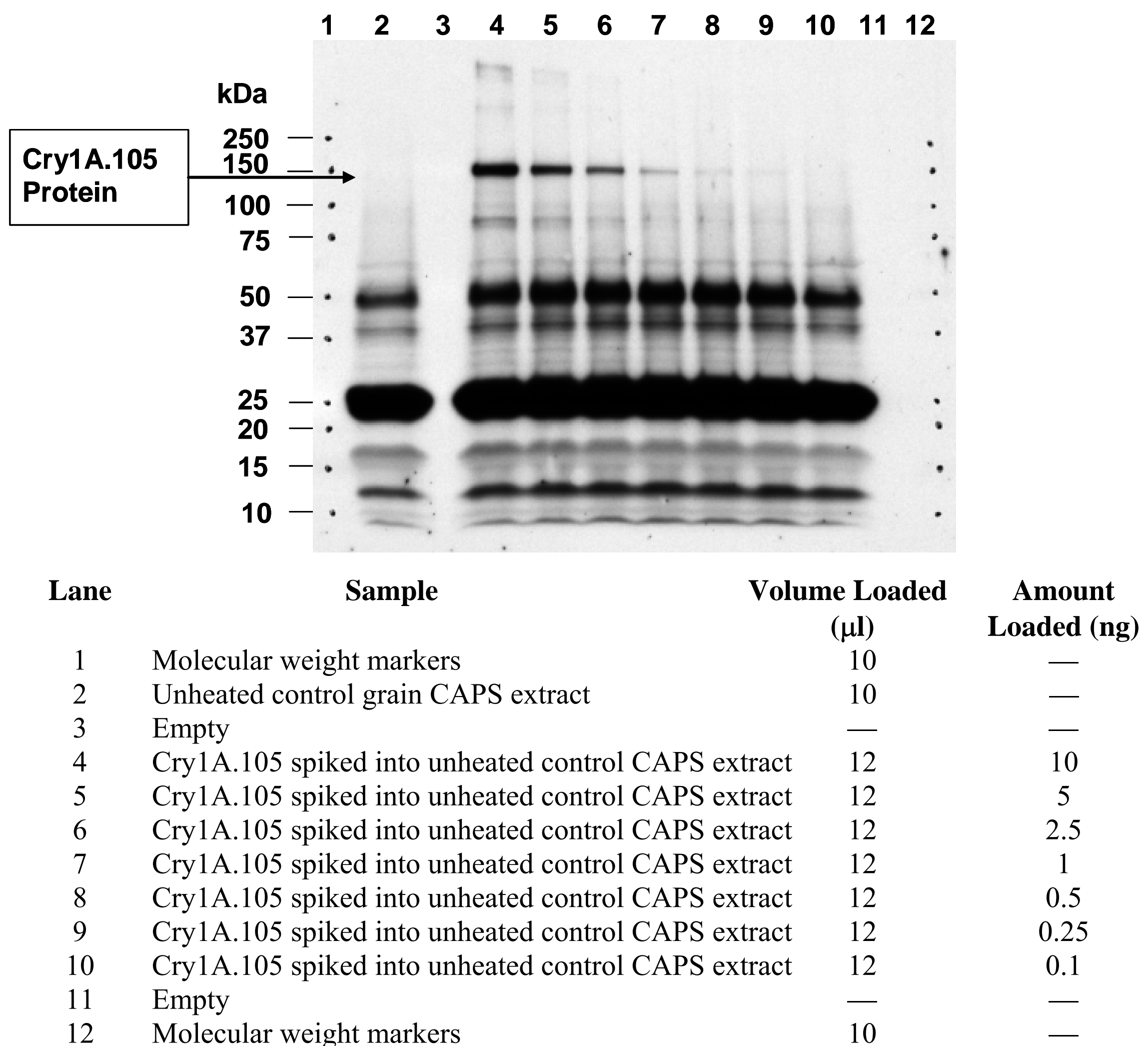
Lane	Sample	Volume Loaded (µl)	Amount Loaded (ng)
1	Molecular weight markers	10	—
2	Unheated control grain NLS extract	10	—
3	Empty	—	—
4	Cry2Ab2 spiked into unheated control NLS extract	12	40
5	Cry2Ab2 spiked into unheated control NLS extract	12	30
6	Cry2Ab2 spiked into unheated control NLS extract	12	20
7	Cry2Ab2 spiked into unheated control NLS extract	12	10
8	Cry2Ab2 spiked into unheated control NLS extract	12	5
9	Cry2Ab2 spiked into unheated control NLS extract	12	2.5
10	Cry2Ab2 spiked into unheated control NLS extract	12	1
11	Empty	—	—
12	Molecular weight markers	10	—

Figure 4. Western Blot Demonstrating Lower Limit of Detection for the Cry2Ab2 Reference Standard Spiked into the NLS Buffer Extract of Unheated Control Grain
NLS buffer extracts of the unheated control grain that were spiked with different amounts of Cry2Ab2 reference standard were separated by a Tris-glycine 10% polyacrylamide gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.4. Amount Loaded (ng) refers to the quantity of the Cry2Ab2 reference standard loaded on the gel. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 1 min.



Lane	Sample	Volume Loaded (µl)
1	Molecular weight markers	10
2	2.5 ng Cry1A.105 reference standard in unheated control grain CAPS extract	12
3	Empty	—
4	Unheated test grain CAPS extract	10
5	Unheated test grain CAPS extract	5
6	Empty	—
7	Heated test grain CAPS extract	10
8	Heated test grain CAPS extract	5
9	Unheated control grain CAPS extract	10
10	Heated control grain CAPS extract	10
11	Empty	—
12	Molecular weight markers	10

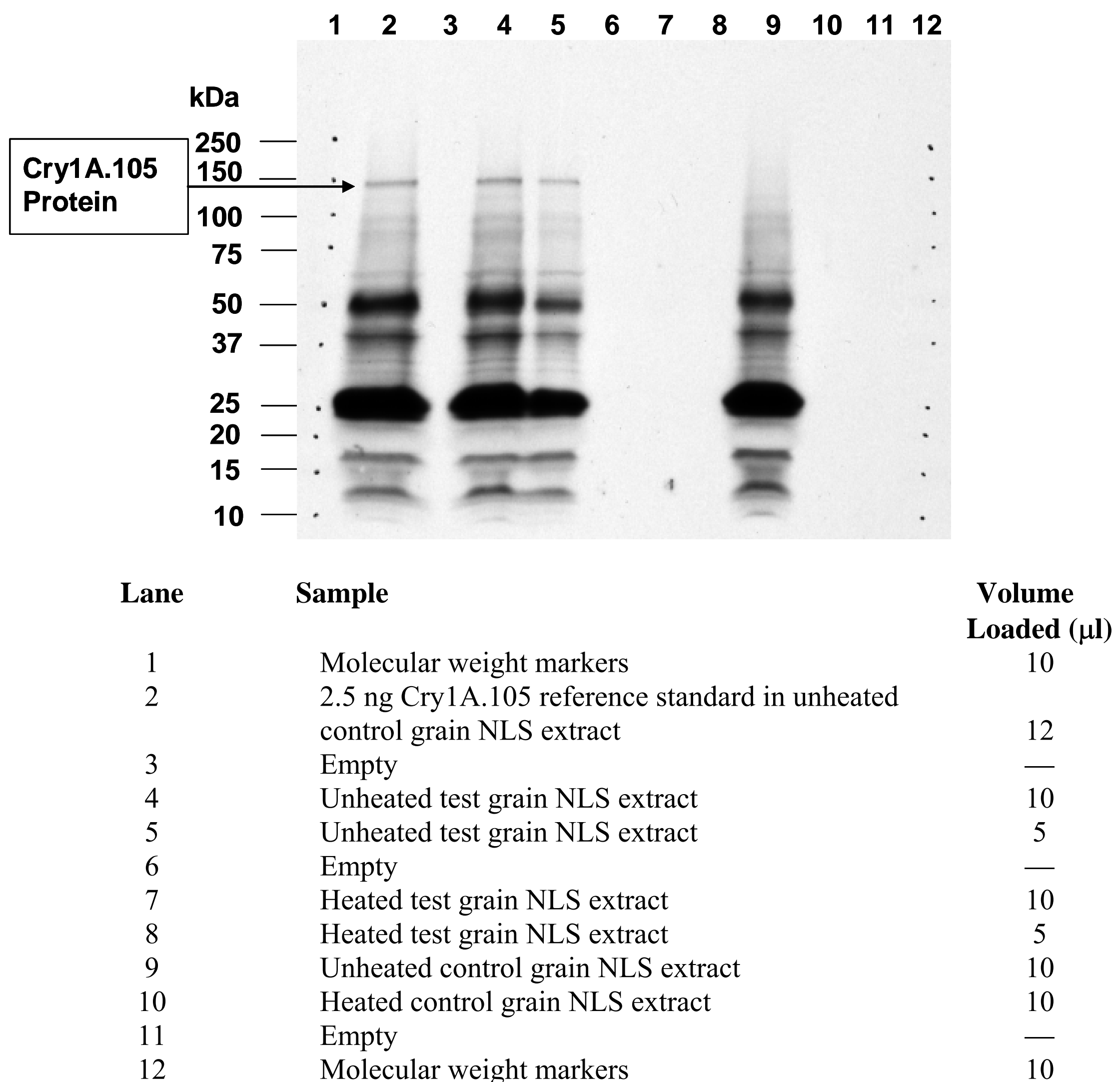
Figure 5. Western Blot Demonstrating the Effect of Heat Treatment on the Immunodetectability of the Cry1A.105 Protein in CAPS Buffer Extracts of Heated and Unheated Test Grain from MON 89034
CAPS buffer extracts of the unheated and heated test and control grain were separated by a Tris-glycine 4-20% polyacrylamide gradient gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.6. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 5 min.



Lane	Sample	Volume Loaded (µl)	Amount Loaded (ng)
1	Molecular weight markers	10	—
2	Unheated control grain CAPS extract	10	—
3	Empty	—	—
4	Cry1A.105 spiked into unheated control CAPS extract	12	10
5	Cry1A.105 spiked into unheated control CAPS extract	12	5
6	Cry1A.105 spiked into unheated control CAPS extract	12	2.5
7	Cry1A.105 spiked into unheated control CAPS extract	12	1
8	Cry1A.105 spiked into unheated control CAPS extract	12	0.5
9	Cry1A.105 spiked into unheated control CAPS extract	12	0.25
10	Cry1A.105 spiked into unheated control CAPS extract	12	0.1
11	Empty	—	—
12	Molecular weight markers	10	—

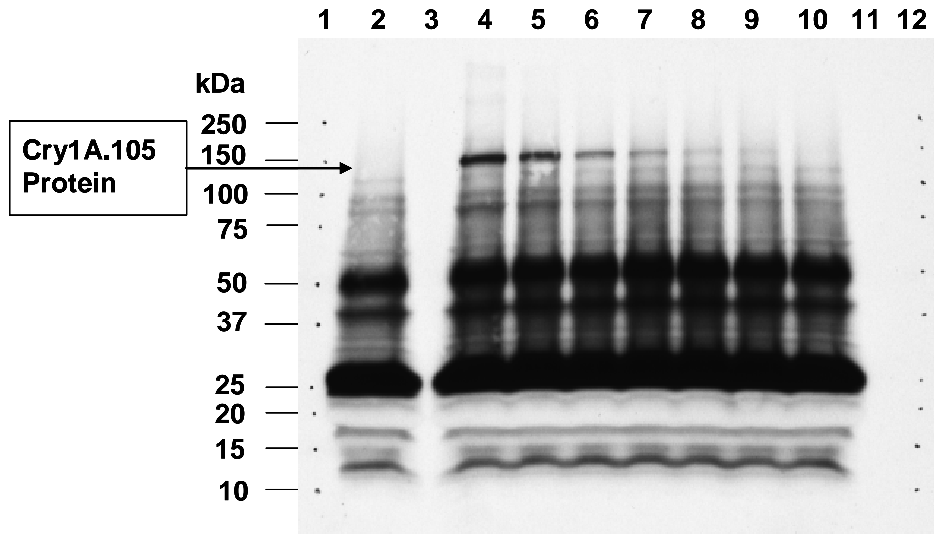
Figure 6. Western Blot Demonstrating Lower Limit of Detection for the Cry1A.105 Reference Standard Spiked into the CAPS Buffer Extract of Unheated Control Grain

CAPS buffer extracts of the unheated control grain that were spiked with different amounts of Cry1A.105 reference standard were separated by a Tris-glycine 4-20% polyacrylamide gradient gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.6. Amount Loaded (ng) refers to the quantity of the Cry1A.105 reference standard loaded on the gel. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 5 min.



Lane	Sample	Volume Loaded (µl)
1	Molecular weight markers	10
2	2.5 ng Cry1A.105 reference standard in unheated control grain NLS extract	12
3	Empty	—
4	Unheated test grain NLS extract	10
5	Unheated test grain NLS extract	5
6	Empty	—
7	Heated test grain NLS extract	10
8	Heated test grain NLS extract	5
9	Unheated control grain NLS extract	10
10	Heated control grain NLS extract	10
11	Empty	—
12	Molecular weight markers	10

Figure 7. Western Blot Demonstrating the Effect of Heat Treatment on the Immunodetection of the Cry1A.105 Protein in NLS Buffer Extracts of Heated and Unheated Test Grain from MON 89034
NLS buffer extracts of the unheated and heated test and control grain were separated by a Tris-glycine 4-20% polyacrylamide gradient gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.6. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 4 min.



Lane	Sample	Volume Loaded (µl)	Amount Loaded (ng)
1	Molecular weight markers	10	—
2	Unheated control grain NLS extract	10	—
3	Empty	—	—
4	Cry1A.105 spiked into unheated control NLS extract	12	10
5	Cry1A.105 spiked into unheated control NLS extract	12	5
6	Cry1A.105 spiked into unheated control NLS extract	12	2.5
7	Cry1A.105 spiked into unheated control NLS extract	12	1
8	Cry1A.105 spiked into unheated control NLS extract	12	0.5
9	Cry1A.105 spiked into unheated control NLS extract	12	0.25
10	Cry1A.105 spiked into unheated control NLS extract	12	0.1
11	Empty	—	—
12	Molecular weight markers	10	—

Figure 8. Western Blot Demonstrating Lower Limit of Detection for the Cry1A.105 Reference Standard Spiked into the NLS Buffer Extract of Unheated Control Grain
NLS buffer extracts of the unheated control grain that were spiked with different amounts of Cry1A.105 reference standard were separated by a Tris-glycine 4-20% polyacrylamide gradient gel under denaturing and reducing conditions and transferred to a PVDF membrane by electroblot. Western blot analysis was conducted as described in Section 6.6. Amount Loaded (ng) refers to the quantity of the Cry1A.105 reference standard loaded on the gel. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in lanes 1 and 12. Film exposure was 4 min.

Appendix

List of Applicable SOPs

BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0878-01	Plant Tissue Processing and Analyte Extraction in Various Matrices Using the Harbil 5G High-Speed Paint Shaker
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0932-02	Assessment of Immunoreactive Bands from Western Blots Exposed to X-Ray Films Using Bio-Rad GS-710 and GS-800 Densitometers