SUMMARY

(In accordance with 40 CFR part 152, this summary is available for public release after registration)

STUDY TITLE

Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12 (AAD-12)

DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

B. W. Schafer

STUDY COMPLETED ON

22-Aug-2008

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

080140

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DATA REQUIREMENTS

Not Applicable

AUTHOR(S)

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STUDY COMPLETED ON

22-Aug-2008

PERFORMING LABORATORY

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LABORATORY STUDY ID

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-12

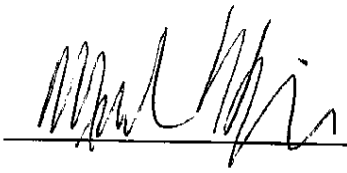
Title: Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12 (AAD-12)

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A)(B), or (C).*

Company: Dow AgroSciences LLC

Company Agent: M. S. Krieger

Title: Regulatory Manager

Signature: 

Date: 6 August 2008

*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12 (AAD-12)

Study Initiation Date: 03-Jun-2008

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the following exceptions: The GLP status of the commercial reference standards bovine serum albumin and protein molecular weight standards was unknown. The chain of custody of these standards was not monitored.

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22-AUG-2008
Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Compound: AAD-12 **Study ID:** 080140

Title: Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12 (AAD-12)

Study Initiation Date: 3 June 2008 **Study Completion Date:** 22 August 2008

GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
29 May 2008	3 June 2008	Protocol Review
11 June 2008	11 June 2008	Heat Inactivation of AAD-12, Activity Assay for AAD-12
18, 19, 20 August 2008	20 August 2008	Report & Raw Data, Test Substance Container and Sample Verification

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.

TFroggatt 22-Aug-2008

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Title: Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12 (AAD-12)

Study Director: B. W. Schafer

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TABLE OF CONTENTS

Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-12 (AAD-12)

ABSTRACT

Soybean has been modified by the insertion of the *aad-12* gene from *Delftia acidovorans* which encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The expressed AAD-12 protein is approximately 32 kDa in size.

The thermal stability of the AAD-12 protein was evaluated by heating protein solutions for 30 min at 50, 70 or 95 °C, or 20 min in an autoclave (120 °C @ ~117 kPa (~17 PSI)) in a phosphate based buffer. All heating conditions eliminated the enzymatic activity of the AAD-12 protein. The study also demonstrated that the AAD-12 protein is immunochemically less reactive when heated. When the AAD-12 was exposed to the heat conditions (50 – 95 °C) at ambient pressure, the protein lost more than 99% of its immunoreactivity, as measured by a polyclonal antibody sandwich ELISA. Gel electrophoresis analysis indicated that the molecular mass of the AAD-12 protein (approximately 32 kDa) was unchanged. However, autoclaving fragmented the AAD-12 protein and resulted in a >50% reduction in immunoreactivity. Together these data indicate that industrial processing of the soybean grain would degrade the primary structure of the AAD-12 protein reducing its immunoreactivity and eliminating its enzymatic activity.

ABBREVIATIONS

AAD-12	Aryloxyalkanoate Dioxygenase-12
AAP	4-aminoantipyrine
AAPPC	4-aminoantipyrine phenol complex
DAS	Dow AgroSciences LLC
ELISA	enzyme-linked immunosorbent assay
GLP	Good Laboratory Practice
HRP	horseradish peroxidase
kDa	kilodalton
kPa	kilopascal
min	minute
MW	molecular weight
OD	optical density
PAb	polyclonal antibodies
PBST	phosphate buffered saline with Tween 20, pH 7.4
PSI	pounds per square inch
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TSN	test substance number

INTRODUCTION

Soybean has been modified by the insertion of the *aad-12* gene from *Delftia acidovorans* which encodes the aryloxyalkanoate dioxygenase-12 (AAD-12) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The expressed AAD-12 protein is approximately 32 kDa in size.

Raw soybeans can not be used in animal and human food as they contain many anti-nutritional factors and allergens. Fortunately, many of the factors can be deactivated, modified or reduced through proper heat-treatment. In soybean industrial processing, high heat treatments are usually involved in the extrusion or toasting processes. For example, when soybeans are passed through a dry extruder the temperature is ~120 to 150 °C. This extrusion process destroys the anti-nutrient factors and improves the nutritional quality of the soybean meal. Soybean meal is the most valuable component obtained from processing the soybean, ranging from 50-75 percent of its value (depending on relative prices of soybean oil and meal). By far, soybean meal is the world's most important protein feed, accounting for nearly 65 percent of world supplies. Livestock feeds account for 98 percent of soybean meal consumption, with the remainder used in human foods such as bakery ingredients and meat substitutes.

The objective of this study was to determine the stability of the AAD-12 protein after 30 minutes exposure to heat at 50, 70 or 95 °C, or autoclaving (120 °C at ~117 kPa) for 20 minutes. Stability was measured based on the loss of protein immunoreactivity and enzymatic activity. Immunoreactivity was determined using a polyclonal antibody based sandwich ELISA specific for the AAD-12 protein. Enzymatic activity was measured using a colorimetric assay which measures the conversion of dichlorphenoxyacetate to 2, 4-dichlorophenol and glyoxylate. The stability of the primary protein structure was determined by SDS-PAGE and western blot analysis.

MATERIALS AND METHODS

Test Substances

The recombinant AAD-12 protein (Lot Number: 466-028A) was produced and purified from *P. fluorescens* by the DAS Supply R & D group in Indianapolis, IN. The protein preparation was sent to the Test Substance Coordinator at Dow AgroSciences also located in Indianapolis. The material was designated TSN030732-0002. The purity was determined to be 35.3% (Schafer, 2008a).

Reference Substances

1. The commercially available reference substances used are listed in the following table:

Reference Substance	Product Name	Lot Number	Assay	Reference
Bovine Serum Albumin	Pre-Diluted Protein Assay Standards: Bovine Serum Albumin (BSA) Set	FH71884A	Biochemical/ SDS-PAGE	Pierce Cat #: 23208
Molecular Weight Markers	Mark 12 Unstained Standard	399893	Biochemical/ SDS-PAGE	Invitrogen Cat #: LC5677, Molecular Weight Markers of 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5, 14.4, 6.0, 3.5 and 2.5 kDa
Prestained Molecular Weight Markers	Novex Sharp Protein Standard	419493	Biochemical/ SDS-PAGE & Western Blot	Invitrogen Cat #: LC5800, Approximate Molecular Weight Markers of 260, 160, 110, 80, 60, 50, 40, 30, 20, 15, 10 and 3.5 kDa

Heat Treatment

The AAD-12 protein was dissolved in PBST (Sigma Chemical, Cat #: P3563) at 1 mg of powder per mL of buffer by weighing 10.9 mg of lyophilized powder and adding 10.9 mL of buffer (353

µg AAD-12/mL). The solution was vortexed and aliquoted into 4 separate 1-mL aliquots. The original sample was held on ice and the others were heated at 50, 70 or 95 °C for 30 min. Another sample was autoclaved at 120 °C (~117 kPa) for 20 minutes in an Amsco 3041 Eagle autoclave (ID #: 3061663). After heat treatment, all samples were immediately placed on ice and assayed by ELISA, SDS-PAGE and western blot. In addition the protein activity was assayed by a colorimetric enzyme assay.

SDS-PAGE and Western Blot

SDS-PAGE was performed with Bio-Rad Criterion gels (Bio-Rad Cat #:345-0124) fitted in a Criterion Cell gel module (Cat #: 165-6001). To visualize the total AAD-12 protein in solution the samples were vortexed and 2 µL of each treatment was added to 18 µL of Laemmli sample buffer (Bio-Rad Cat #: 161-0737 containing 5% freshly added 2-mercaptoethanol Bio-Rad Cat #: 161-0710) and heated for 5 minutes at ~100 °C. To determine the amount of soluble AAD-12 protein in solution, the samples were centrifuged at >20,000g for 5 min, to pellet any insoluble protein, and 3 µL was mixed with 27 µL of Laemmli buffer and processed as described earlier. After a brief centrifugation, 20 µL (~706 ng of AAD-12) of the supernatants were loaded directly on the gel to be stained for total protein. For the western blot, 5 µL (~177 ng of AAD-12) of the supernatant was loaded on the gel. To serve as a reference substance, ~700 ng of bovine serum albumin (Pierce, Cat #: 23208) was mixed with 14.4 µL of Laemmli buffer and processed as described earlier. The electrophoresis was conducted at a constant voltage of 180 V for ~45 minutes using XT MES buffer (Bio-Rad, Cat #: 161-0789). After separation, the gel was cut in half and one half was stained with Pierce GelCode Blue protein stain (Cat #: 24592). The remaining half was electro-blotted to a nitrocellulose membrane (Bio-Rad, Cat #:162-0213) with a mini trans-blot electrophoretic transfer cell (Bio-Rad Cat#: 170-3935) for 60 minutes under a constant charge of 100 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad (Cat #: 161-0734). For immunodetection, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (Strategic Biosolution Inc., Newark, DE, Protein A purified antibody Lot #: DAS F1197-167-2, 4.3 mg/mL). A conjugate of goat anti-rabbit IgG

(H+L) and horseradish peroxidase (Pierce, Cat #: 31460) was used as the secondary antibody. GE Healthcare chemiluminescent substrate (Cat #: RPN2132) was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to Classic Blue Film (MidSci, Cat #: BX810) for various time points and subsequently developed with an All-Pro 100 Plus film developer.

ELISA Assay of Heated and Non-heated AAD-12 Proteins

After treatment, all samples were kept on ice until analysis by a microtiter plate ELISA as described in the Appendix (Page 22). The samples (both vortexed total solution and centrifuged soluble fraction) were diluted to 160 ng/mL with PBST and further serially diluted (1:1) prior to being loaded into the wells of the ELISA plates. The assay used a sequential double antibody sandwich ELISA format. An aliquot of each sample (both vortexed and clarified supernatant) was incubated with an immobilized anti-AAD-12 polyclonal antibody in the wells of a coated plate, and then the unbound samples were removed from the plate by washing with PBST. An excess amount of enzyme-conjugated polyclonal antibody was added to the wells and incubated at room temperature. These antibodies bind with the target protein in the wells and form a "sandwich" with the immobilized antibodies. The presence of target protein was detected by incubating the wells with enzyme substrate, generating a colored product. Since the target protein is bound in the antibody sandwich, the level of color development is related to the concentration of target protein in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm minus absorbance at 650 nm was measured using a microtiter plate reader.

Activity Assay of Heated and Non-heated AAD-12 Proteins

The AAD-12 protein activity was measured by a modified enzyme assay based on the procedure described in Fukumori and Hausinger (1993). In the presence of Fe(II), the AAD-12 protein catalyzes the conversion of dichlorphenoxyacetate to 2,4-dichlorophenol and glyoxylate

concomitant with the decomposition of α-ketoglutarate to form succinate and carbon dioxide. The resulting phenol is measured with an AAPPC assay or the Emerson reaction (Emerson, 1943). Phenols react with 4-aminoantipyrine in the presence of alkaline oxidizing agents (potassium ferricyanide) at a pH of 10.0 to form a stable reddish-brown antipyrine dye (AAPPC). The amount of color produced is a function of the concentration of phenols and was measured with a microplate reader (Molecular Devices, Model #: SPECTRAmax 190 ROM v3.13) at 510 nm.

Statistical Treatment

Statistical treatment of the data in this study consisted of calculation of means and standard deviations of the replicated treatments.

RESULTS AND DISCUSSION

Heat Treatment

To test heat lability of the AAD-12 protein, four treatment conditions were evaluated in this study. Proteins are usually sensitive to high temperatures and the degree of denaturing is dependent upon the temperature and duration of the heat treatment. Three of the heat treatments (50, 70 and 95 °C) bracketed common temperatures that are known to inactivate AAD proteins (Schafer, 2008b). In addition, an autoclaving treatment (120 °C, 117 kPa) was added to mimic the roasting temperatures involved in the treatment of soybeans (~110 and 170 °C). These elevated temperatures are required because raw soybeans can not be used in animal and human food as they contain many anti-nutritional factors and allergens (Prachayawarakorn, *et al,* 2006). Fortunately, many of the factors are deactivated, modified or reduced through proper heat-treatment. In soybean industrial processing, high heat treatments are usually involved in the extrusion or toasting processes. For example, when soybeans are passed through a dry extruder

the temperature is ~120 to 150 °C. This extrusion process destroys the anti-nutrient factors and improves the nutritional quality of the soybean meal.

SDS-PAGE and Western Blot Analysis

In the toxicology-lot preparation of *P. fluorescens*-produced AAD-12 (TSN030732-0002), the major protein band, as visualized on Coomassie stained SDS-PAGE gels, is approximately 32 kDa (Schafer, 2008). In the SDS-PAGE analysis, the AAD-12 protein held at 4 °C, or heated for 30 min at 50, 70 or 95 °C, showed that the protein molecular weights was as expected and the density of protein bands were essentially unchanged (Figure 1, Lanes 1-4). However, when the same heated samples were centrifuged prior to addition of Laemmli buffer, the protein was undetectable by SDS-PAGE and western blot, suggesting they form insoluble aggregates upon heating (Figure 1, Lanes 8-10 and 13-16). The 4 °C treatment remained unchanged (Figure 1, Lanes 1, 7 and 12). The autoclaved sample showed cleavage of the AAD-12 protein to undetectable fragments (Figure 1, Lanes 5 and 11).

ELISA Analysis of the Heat Treated AAD-12 Proteins

The immunoreactivity of each heated sample was measured using a standard curve generated with the sample held at 4 °C. The ELISA results are listed in Table 1. The AAD-12 protein lost >99% of its immunoreactivity when treated at 50, 70 or 95 °C for 30 minutes. These results indicate that almost all of the epitopes (i.e., binding sites) for the polyclonal antibody used in the AAD-12 sandwich ELISA format were destroyed once the protein was mildly heated. When the sample was autoclaved, immunoreactivity was reduced by over 50%. Since it was noted in the SDS-PAGE and western blot that the protein was insoluble after heating, both the total protein (vortexed) and the soluble fractions were tested in the polyclonal-antibody ELISA. The soluble fraction of the autoclaved sample (centrifuged for 20,000g for 5 min) contained 100% of the immunoreactivity of the original total protein solution. SDS-PAGE gel and western blot (using

the same polyclonal antibody as that used in the ELISA) of the soluble fraction indicated that the immunoreactive species was <2.5 kDa (Figure 1).

Activity Assay of Heat Treated AAD-12 Proteins

The AAD-12 protein activity was measured by a modified enzyme assay based on the procedure described in Fukumori and Hausinger (1993). The activities of the heated samples were compared to the sample held at 4 °C (Table 2). As expected, the enzyme activity was eliminated across all heat treatments. This result correlates well with the western blot as an insoluble protein aggregate is not expected to contain enzymatic activity. Therefore it can be concluded that the AAD-12 protein is functionally unstable when heated.

CONCLUSIONS

The thermal stability of AAD-12 protein was evaluated by heating protein solutions for 30 min at 50, 70 or 95 °C, or autoclaving for 20 min at 120 °C and ~117 kPa. The study demonstrated that the AAD-12 protein had significantly reduced immunoreactivity and is inactivated when heated. Under the milder temperature regimes, the AAD-12 protein lost more than 99% of its immunoreactivity based on a PAb sandwich ELISA, while the autoclaved sample lost over 50% of its original immunoreactivity. The protein lost 100% of its enzymatic activity at all temperatures tested. Gel electrophoresis analysis indicated that the molecular mass of the AAD-12 protein (approximately 32 kDa) was unchanged after heat treatment at 50, 70 or 95 °C. Autoclaving resulted in fragmentation of the AAD-12 protein to undetectable levels. These data indicate that the industrial grain processes would largely inactivate and/or significantly degrade the AAD-12 protein.

ARCHIVING

The protocol, raw data, and the original version of the final report are all filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, Indiana 46268-1054.

REFERENCES

Emerson, E. (1943) The condensation of aminoantipirene. A new colour test for phenolic compounds. *J. Organomet. Chem.* 8**:**417–428

Fukumori, F. and Hausinger, R. P. (1993) Purification and Characterization of 2, 4-Dichlorophenoxyacetate/α-Ketoglutarate Dioxygenase. *J. Biol. Chem*. 268, 15:24300-24317

Schafer, B. W. (2008a) Certificate of analysis for the Test/Reference/Control Substance: Aryloxyalkanoate dioxygenase (abbreviation: AAD-12) – TSN030732. Report Number: BIOT08-161536 (Unpublished).

Schafer, B. W. (2008b) Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1. Report Number: 080059 (Unpublished).

Prachayawarakorn, S., Prachayawasin, P. and Soponronnarit S. (2006) Heating process of soybean using hot-air and superheated-steam fluidized-bed dryers. *LWT - Food Science and Technology*. 39, 7:770-778

Table 1. Relative Immunoreactivity of Heat-Treated AAD-12 Protein

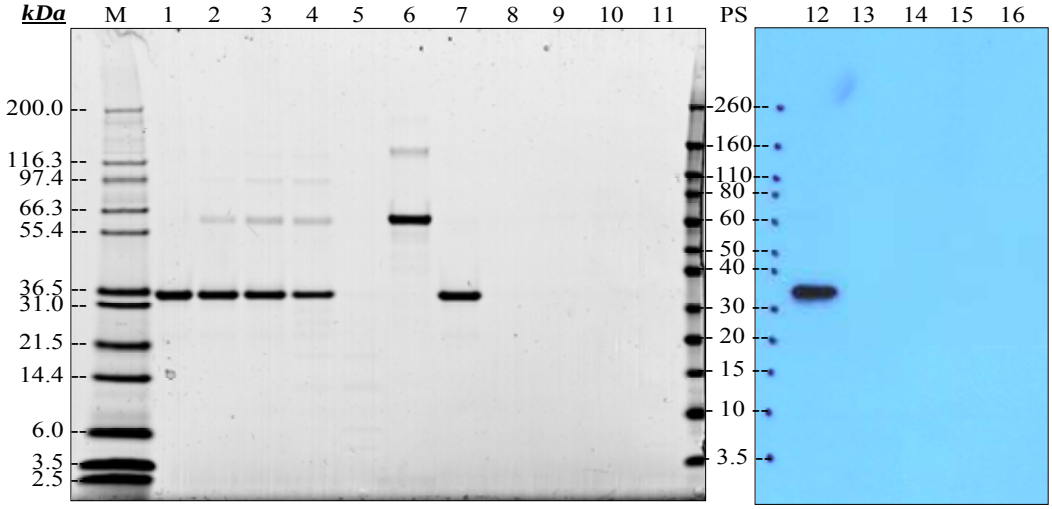
Treatment Temperature	Time (min)	% Immunoreactivity
4 °C	NA	100.0*
50 °C	30	0.0
70 °C	30	0.0
95 °C	30	0.7
120 °C (autoclave)	20	49.8

Note: The 4 °C sample was normalized to 100% immunoreactivity. The results are averaged from ELISA readings of serial dilutions of the AAD-12 protein. % immunoreactivity = ((avg. measured AAD-12 in solution) / (theoretical AAD-12 in solution) x 100)

Table 2. Summary of AAD-12 Enzyme Activity Results

Treatment Temperature	Time (min)	% Enzymatic Activity
4 °C	NA	100.0
50 °C	30	0.0
70 °C	30	0.0
95 °C	30	0.0
120 °C (autoclave)	20	0.0

Note: The relative activity of the 4 °C treatment is designated as 100%. The results are averaged from enzymatic assay absorbance readings of serial dilutions of the AAD-12. % enzymatic activity = (OD heat treatment - blank) / (OD 4 °C - blank) x 100.



A: SDS-PAGE Gel Stained for Total Protein

B: Western blot probed with α–AAD-12 PAb

Notes: SDS-PAGE was performed with 4 – 12 % XT Bio-Rad Criterion gels fitted in a Criterion Cell gel module. To visualize the total AAD-12 protein in solution, the samples were vortexed well and each treatment was mixed with Laemmli sample buffer (containing 5% freshly added 2-mercaptoethanol) and heated for 5 minutes at ~100 °C. To determine the amount of AAD-12 protein in solution, the samples were centrifuged at >20,000xg for 5 min and mixed with Laemmli buffer and processed as described earlier. A reference substance, bovine serum albumin, was loaded on the gel to monitor running conditions. The electrophoresis was conducted at a constant voltage of 180 V for ~45 minutes using Bio-Rad XT MES buffer. After separation, the gel was cut in half and one half was stained with Pierce GelCode Blue protein stain. The remaining half was electro-blotted to a nitrocellulose membrane for 60 minutes under a constant charge of 100 volts. For immunodetection, the membrane was probed with an AAD-12 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-167-2, 4.3 mg/mL). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody. GE Healthcare chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to film for various time points and subsequently developed with a film developer.

Lane	***Sample***	***Amount Loaded***
M	Invitrogen Mark 12 MW markers	10 µL
1	AAD-12 (TSN030732) held @ 4 °C (total protein)	706ng
2	AAD-12 (TSN030732) heated @ 50 °C, 30 min (total protein)	706ng
3	AAD-12 (TSN030732) heated @ 70 °C, 30 min (total protein)	706ng
4	AAD-12 (TSN030732) heated @ 95 °C, 30 min (total protein)	706ng
5	AAD-12 (TSN030732) autoclaved, 20 min (total protein)	706ng
6	Bovine serum albumin	700ng
7	AAD-12 (TSN030732) held @ 4 °C (soluble protein)	706ng
8	AAD-12 (TSN030732) heated @ 50 °C, 30 min (soluble protein)	706ng
9	AAD-12 (TSN030732) heated @ 70 °C, 30 min (soluble protein)	706ng
10	AAD-12 (TSN030732) heated @ 95 °C, 30 min (soluble protein)	706ng
11	AAD-12 (TSN030732) autoclaved, 20 min (soluble protein)	706ng
PS	Invitrogen Novex Sharp Prestained MW markers	10 µL
12	AAD-12 (TSN030732) held @ 4 °C (soluble protein)	177ng
13	AAD-12 (TSN030732) heated @ 50 °C, 30 min (soluble protein)	177ng
14	AAD-12 (TSN030732) heated @ 70 °C, 30 min (soluble protein)	177ng
15	AAD-12 (TSN030732) heated @ 95 °C, 30 min (soluble protein)	177ng
16	AAD-12 (TSN030732) autoclaved, 20 min (soluble protein)	177ng

Figure 1. SDS-PAGE and Western Blot of Heat-Treated AAD-12 Protein

APPENDIX

Simplified Quantitative ELISA Protocol for AAD-12 in Maize Leaf Tissue

Dow AgroSciences LLC
Indianapolis, IN 46268

1. GENERAL REQUIREMENTS

A. Materials

- Bead, 1/8" chrome steel, catalog number BS-0125-C, Small Parts Inc., Miami Lakes, FL or equivalent.
- Cap, for 2.0-mL conical tube, catalog number 02-681-361, Fisher Scientific or equivalent
- Multi-channel pipettor, 12-channel, 10-300 µL
- Pipette tips, various sizes
- Plate covers or equivalent
- Reagent reservoirs, non-sterile
- Single channel pipettors of various sizes, 10 µL-1.0 mL
- Tubes, polypropylene, 5 mL
- Tube, 15-mL polypropylene centrifuge with cap
- Tube, 2.0-mL conical micro-centrifuge, catalog number 02-681-344, Fisher Scientific
- U-bottom plates, nonbinding 96-well, BD Falcon catalog no. 35-3918 or equivalent

B. Equipment

- Balance, analytical, Model AB54-S, Mettler Instrument Corporation or equivalent
- Centrifuge, capable of holding 2-mL Eppendorf tubes, Eppendorf–5417C or equivalent
- Freezer, capable of maintaining –20 °C, Model 75F, U-Line Corporation, Milwaukee, WI or equivalent
- Plate reader, capable of reading 450 nm, Molecular Devices catalog no. 0200-2018 or equivalent

- Refrigerator, capable of maintaining temperature at 2–8°C
- Vortex, Genie-2 Model, catalog number 12-812, Fisher Scientific or equivalent
- Shaker/Grinder, Model Geno/Grinder, catalog number 2000-115, Certiprep, Metuchen, New Jersey or eqivalent
- Washer, 96-well microplate, Model Elx 405, Bio-Tek Instruments, Inc. or equivalent

2. REAGENTS AND REAGENT PREPARATION

A. AAD-12 Microtiter Plate from Beacon Analytical Systems, Inc., Portland, ME 04103.

As a part of kit catalog number CPP 089, Store at 2-8 °C.

B. PBST, pH 7.4, catalog number P-3563, Sigma. Store at 2-8 °C.

C. Phosphate-Buffered Saline + 0.05% Tween-20 (PBST), pH 7.4. Store at 2-8 °C for up to 6 months. PBST powder may be used to prepare the buffer, catalog number P-3563, Sigma.

D. 30% Bovine Serum Albumin (BSA) solution, Immunohematology grade (Serologicals Corporation, Inc. 1-800-431-4505 Catalog No. 81-070 or equivalent. Store at 2-8 °C.

E. AAD-12 standard protein: Obtain AAD-12 microbial protein from Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268.

F. AAD-12 antibody-HRP conjugate provided by Beacon Analytical Systems Inc. Portland, ME 04103. Use with 1:400 dilution.

G. Assay Buffer: PBST plus 0.5% BSA (w/v) (PBST/BSA). PBST powder may be used to prepare the buffer, catalog number P-3563, Sigma.

- Add 1 mL of 30% BSA to the 30 mL PBST in the container;
- Mix well and it can be used for the day. Discard the remaining after the experiment.

H. Washing Buffer: PBST plus 0.05% Tween-20 (w/v). PBST powder may be used to prepare the buffer, catalog number P-3563, Sigma.

- Add 0.5 mL of Tween-20 to the 1 L PBST in the container;

- Mix well and store at room temperature. Discard the solution if any visible contamination is observed.

I. AAD-12 Stock Solution, 1000 ng/mL.

- Prepare AAD-12 liquid stock solution according to instruction from Dow AgroSciences LLC. It may need to be aliquoted and stored in freezer (such as -20ºC).
- Prepare a 1000-ng/mL stock solution based on the liquid standard concentration in PBST/BSA. Keep it in ice to be used within 2 hours. Discard if any visible contamination is observed. Discard the remainder AAD-12 stock; do not re-freeze it for reuse.

3. PROCEDURE

A. Bring ELISA kit reagents to 20-25 °C by removing them from the refrigerator at least 30 minutes prior to performing the assay.

B. Prepare the AAD-12 standards in Assay Buffer in 5-mL polystyrene tubes as follows. Vortex a few seconds before transferring to next dilution. Store the tubes on ice; prepare new standards for each assay immediately before adding samples to the plate. (Note: when transferring stock reference antigen, please rinse the pipet tip once in the destination solution after dispense)

Conc. of Stock Soln.	Aliquot of Stock Soln.	Starting Buffer Volume	Final Soln. Volume	Final Standard Conc.	Remaining Volume after Aliquot [a]
ng/mL	µL	µL	µL	ng/mL	µL
1000	160	840	1000	160.00	500
160.00	500	500	1000	80.00	500
80.00	500	500	1000	40.00	500
40.00	500	500	1000	20.00	500
20.00	500	500	1000	10.00	500
10.00	500	500	1000	5.00	500
5.00	500	500	1000	2.50	500
0	0	500	500	0	500

[a] The final solution volume is the remaining volume in the container after it has served as the stock solution for the next standard concentration and the relevant amount of solution is transferred.

C. Prepare test samples:

- For fresh leaf samples, make 4 leaf punches and place into 2-mL polypropylene tubes. Add two or three metal beads to each tube. Then add 0.80 mL of the Assay Buffer. Cap all the tubes.
- Extract the samples using the Geno/Grinder automatic shaker/grinder at a dial setting of 350 and the toggle switch at the 1X setting (approximately 1500 strokes per minute) for 3 minutes as one cycle. An alternative equivalent grinding or extraction method may be used.
- Centrifuge the samples at 14,000 (or greater) rpm for 5 minutes or until separated (no visible particles in the supernatant). The supernatant can be transferred to a separate tube or aliquoted for analysis. Keep the solution on ice and assay it within 2 hours.

D. Add the AAD-12 samples to ELISA plate(s) as follows:

- Transfer the ELISA standard dilutions to Columns 1-3 on a non-binding 96-well U-bottom microtiter plate (approximately 130 µl/well). For each plate tested, run standard solutions in triplicate.
- Prepare sample dilutions as needed and transfer diluted samples to the non-binding 96-well microtiter plate (130 µL/well) containing the standard calibration solutions and record the location on the 96-well assay template sheet.

E. Transfer 100 µL of the ELISA standard solutions and diluted samples from the U-bottom microtiter plate to a pre-coated plate, keeping the same orientation as the samples are transferred to the pre-coated plate. Change pipette tips with each row.

F. Cover the pre-coated plate, and gently swirl on the benchtop or a plate shaker for approximately five seconds to mix. Allow to incubate at ambient temperature for 1 hour (± 5 min).

G. **Wash** the pre-coated plate 3-5 times by filling each well with PBST. Tap out excess liquid on a paper towel. It may be washed by plate washer.

H. Transfer 30 µL of AAD-12 antibody conjugate and add into 11.970 mL of assay buffer and mix well to make 12 mL of conjugate solution. Pipet 100 µL of the conjugate solution to each well of the antibody coated 96-well microtiter plate. Change pipette tips with each row.

I. Cover the plate and place it on a shaker and incubate at ambient temperature for 60 min (± 5 min).

J. **Wash** the plate 3-5 times by filling each well with washing buffer. Tap out excess liquid on a paper towel. It may be washed by plate washer.

K. Add 100 µL of the **TMB substrate** to each well of the reaction plate. Cover and gently mix. Allow to incubate at ambient temperature in the dark for 20±2 minutes.

L. Add 100 µL of Stop Solution to each well to stop the reaction. Mix the plate gently and read the absorbance at 450 nm minus 650 nm using the MAXline Vmax plate reader.

M. Save the raw data file and do the data analysis as described in Section 4.

4. Data Analysis and Calculation

A. Calibration Curve:

Absorbance values from the reference standards should be used to develop a calibration curve. SOFTmax PRO software or Microsoft Excel is used to provide necessary analysis and calculation. The calibration curve for the AAD-12 ELISA is constructed using a quadratic regression of the expected concentrations of the standards and their subsequent absorbance (optical density).

B. The equation fits the best parabola to the standard curve based on the equation:

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

Where:

y = mean absorbance value (OD)
x = reference standard concentration

C. Calculation of AAD-12 in Test Samples:

SOFTmax PRO or Microsoft Excel is used to calculate the AAD-12 concentration of each test sample. The predicted concentration is determined using the coefficients of the curve and optical density (OD) readings in the quadratic equation. The regression equation is applied as follows:

$$\text{Predicted concentration} = \frac{-B + \sqrt{B^2 - 4C*(A - OD)}}{2C}$$

The determined AAD-12 concentration of a test sample (i.e., the individual replicates of a single dilution) is obtained by multiplying the predicted concentration by the dilution factor used.

5. Criteria for Acceptance of an Analytical Batch

Each analytical batch shall meet the accept criteria in the procedure to be valid as listed below. If the data fail to meet these performance criteria, the analyst should evaluate the results, determine the potential source of the variation, and repeat the analysis if necessary.

Assay Buffer Blank	Absorbance 450-650 nm < 0.200
0-ng/mL standard	Absorbance 450-650 nm < 0.200
80-ng/mL standard	Absorbance 450-650 nm ≥ 0.80
Calibration curve	r^2 (Correlation of determination) > 0.990
All positive reference standards, OD	CV (OD) of triplicates ≤ 15%
Unknown or QC samples, solution	CV (OD) of replicates ≤ 20%
Quality control samples, solution (if applicable)	Measured value ≤ ±20% expected value