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Report Title

Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1

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	Report / File Number(s)
	080059

Materials

DED Code	Formulation No.	Formulation Type	Product Name	Active Ingredient(s)
				AAD-1

Keywords

Aryloxyalkanoate Dioxygenase-1, Heat Lability

Compound Number(s)	Batch/Lot Number(s)	Bayer Codes

Lab Notebooks	Language(s)	Performing Laboratory Name
		Biotechnology Regulatory Sciences

Data Requirement(s) (Guidelines)

None

Geo Area and Country (s) of Study		Protocol Number(s)	Study Number(s)	Published?	Date Study Completed
NA	USA	080059		NO	23Jul2008
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	N/A		N/A

Method Technique(s)

Reviewer(s)

S. K. Embrey, R. A. Herman, G. Shan, K. A. Clayton

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-1

DATA REQUIREMENTS

None

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STUDY COMPLETED ON

23-Jul-2008

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LABORATORY STUDY ID

080059

Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1

SUMMARY

Corn has been modified by the insertion of the *aad*-1 gene from *Shingomonas herbicidivorans* which encodes the aryloxyalkanoate dioxygenase-1 (AAD-1) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The expressed AAD-1 protein is approximately 33 kDa in size.

The thermal stability of the AAD-1 protein was evaluated by heating protein solutions for 30 min at 50, 70 and 95 °C and 20 min in an autoclave (120 °C @ ~117 kPa (~17 PSI)) in a phosphate based buffer. All heating conditions virtually eliminated the enzymatic activity of the AAD-1 protein. The study also demonstrated that the AAD-1 protein is immunochemically less reactive when heated. When the AAD-1 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-1 protein (approximately 33 kDa) was unchanged. However, autoclaving resulted in significant degradation of the primary structure of the AAD-1 protein and a >50% reduction in immunoreactivity. Together these data indicate that industrial processing of the grain would significantly degrade the primary structure of the protein reducing its immunoreactivity and eliminating its enzymatic activity.

STUDY TITLE

Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1

DATA REQUIREMENTS

None

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: AAD-1

Title: Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1

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: P. L. Hunst

Title: Regulatory Manager

Signature: Penny L. Hunst

Date: 10-July-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-1

Study Initiation Date: 08-Apr-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, such as, bovine serum albumin and protein molecular weight standards were unknown. The chain of custody of these standards was not monitored.

Penny K. Hunst — 10-July-2008
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Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Compound: AAD-1 **Study ID:** 080059

Title: Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1

Study Initiation Date: 8 April 2008 **Study Completion Date:** 23 July 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
7 April 2008	8 April 2008	Protocol Review
15 April 2008	18 April 2008	Sample Preparation and Heat Treatment; SDS-PAGE
9 May 2008	12 May 2008	Sample Preparation and Heat Treatment; SDS-PAGE & Western Blot
2, 7, 8 July 2008	9 July 2008	Final Report and Raw Data; Test Substance Container & 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.

T Froggatt 23 July 2008

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TABLE OF CONTENTS

Effect of Heat Treatment on a Recombinant Aryloxyalkanoate Dioxygenase-1

ABSTRACT

Corn has been modified by the insertion of the *aad*-1 gene from *Shingomonas herbicidivorans* which encodes the aryloxyalkanoate dioxygenase-1 (AAD-1) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The expressed AAD-1 protein is approximately 33 kDa in size.

The thermal stability of the AAD-1 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 virtually eliminated the enzymatic activity of the AAD-1 protein. The study also demonstrated that the AAD-1 protein is immunochemically less reactive when heated. When the AAD-1 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-1 protein (approximately 33 kDa) was unchanged. However, autoclaving resulted in significant degradation of the primary structure of the AAD-1 protein and resulted in a >50% reduction in immunoreactivity. Together these data indicate that industrial processing of the grain would degrade the primary structure of the protein reducing its immunoreactivity and eliminating its enzymatic activity.

ABBREVIATIONS

AAD-1	Aryloxyalkanoate Dioxygenase-1
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

Corn has been modified by the insertion of the *aad*-1 gene from *Shingomonas herbicidivorans* which encodes the aryloxyalkanoate dioxygenase-1 (AAD-1) protein. The trait confers tolerance to 2,4-dichlorophenoxyacetic acid and aryloxyphenoxypropionate (commonly referred to as "fop" herbicides such as quizalofop) herbicides and may be used as a selectable marker during plant transformation and in breeding nurseries. The expressed AAD-1 protein is approximately 33 kDa in size.

In the food industry, corn grain is usually processed into a wide variety of by-products via wet milling or dry milling processes, with the wet milling industry being the second largest user of corn grain after animal feed. In corn industrial processing, multiple steps of heat treatment are usually involved in both milling processes. For example, temperatures of 50 to 91 °C are used in the production of steep water liquor, germ, hull, gluten, and starch fractions from wet milled grain. During the dry milling process, the grain is exposed to temperatures up to 79 °C during preparation of the grits, meal, hull, flour, and solvent extracted germ. Processing of oil and soapstock fractions from dry and wet milling involves exposure to temperatures of 90 - 104 °C with chemicals such as hexane and NaOH. The most extreme temperature exposure is in pressure cookers sold in the U.S. In 1917, the USDA set the standard internal pressure of pressure cooker to approximately 100 kPa (~15 PSI) over atmospheric pressure. At this pressure boost, water boils at ~125 °C (257 °F).

The objective of this study was to determine the stability of the AAD-1 protein after 30 minutes exposure to heat at 50, 70, 95 °C and to 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-1 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 coomassie staining of the protein by SDS-PAGE and western blot analysis.

MATERIALS AND METHODS

Test Substances

The recombinant AAD-1 protein (Lot Number: 480-15 (Batch 2)) 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 TSN105930. The purity was determined to be 36.1% (Schafer, 2008).

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/ 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-1 protein was dissolved in PBST (Sigma Chemical, Cat #: P3563) at 1 mg of powder per mL of buffer by weighing 9.1 mg of lyophilized powder and adding 9.1 mL of buffer (361 µg AAD-1/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-1 protein in solution the samples were vortexed well and 2 µL of each treatment was added to 18 µL of 2x 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-1 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 (~720 ng of AAD-1) of the supernatants were loaded directly on the gel to be stained for total protein. For the western blot, 5 µL (~180 ng of AAD-1) of the supernatant was loaded on the gel. To serve as a reference substance, 750 ng of bovine serum albumin (Pierce, Cat #: 23208) was mixed with 14 µL of Laemmli buffer and processed as described earlier. The electrophoresis was conducted at a constant voltage of 200 V for ~30 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 #: 161-0771). For immunodetection, the membrane was probed with an AAD-1 specific polyclonal rabbit antibody (Strategic Biosolution Inc., Newark, DE, Protein A purified rabbit polyclonal antibody Lot #: DAS F1197-151, 1.6 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-1 Proteins

After treatment, all samples were kept on ice until analysis by a microtiter plate ELISA as described in the Appendix (Page 42). 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-1 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-1 Proteins

The AAD-1 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-1 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-1 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 heat treatments bracketed common temperatures that occur in the corn milling process (Tagliani, *et al*, 2002 and Robb, 2002). In corn industrial processing, multiple steps of heat treatment are usually involved in dry- and wet-milling processes. For example, temperatures of 50 to 91 °C are used in the production of steep water liquor, germ, hull, gluten, and starch fractions from wet milled grain (May, 1999). During the dry milling process, the grain is exposed to temperatures up to 79 °C during

preparation of the grits, meal, hull, flour, and solvent extracted germ. Processing of oil and soapstock fractions from dry and wet milling involves exposure to temperatures of 90 - 104 °C with chemicals such as hexane and NaOH. The most extreme temperature exposure is in pressure cookers sold in the U.S. In 1917, the USDA set the standard internal pressure of pressure cookers to approximately 100 kPa (15 PSI) over atmospheric pressure. At this pressure boost, water boils at 125 °C (257 °F). Thus, the treatment at 120 °C and ~117 kPa for 20 minutes (autoclaving) was conducted as an extreme condition to examine the protein lability following pressure cooking.

SDS-PAGE and Western Blot Analysis

In the toxicology-lot preparation of *P. fluorescens*-produced AAD-1 (TSN105930), the major protein band, as visualized on Coomassie stained SDS-PAGE gels, is approximately 33 kDa (Schafer, 2008). In the SDS-PAGE analysis, the AAD-1 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 both SDS-PAGE and western blot, suggesting they form insoluble aggregates upon heating (Figure 1, Lanes 8-10 and 13-15). The 4 °C treatment remained unchanged (Figure 1, Lanes 1, 7 and 12). The autoclaved sample showed extensive degradation of the AAD-1 protein and the remaining intact protein was also insoluble as it was removed by centrifugation (Figure 1, Lanes 5, 11 and 16).

ELISA Analysis of the Heat Treated AAD-1 Proteins

The immunoreactivity of each heated sample was compared to the sample held at 4 °C. The ELISA results are listed in Table 1. Under the milder temperature regimes, the AAD-1 protein lost almost all of its immunoreactivity based on a PAb sandwich ELISA, while the autoclaved sample lost ~60% of its original immunoreactivity. These results indicate that almost all of the

epitopes (i.e., binding sites) for the polyclonal antibody used in the AAD-1 sandwich ELISA format were destroyed once the protein was mildly heated (one dilution contained a slight signal just above the ELISA background). When the sample was autoclaved, immunoreactivity was reduced by ~ 60%. 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 greater than 92% of the immunoreactivity of the vortexed 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-1 Protein

The AAD-1 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 virtually eliminated across the heat treatments. The 95 °C treatment showed 97% reduction in the enzymatic activity while the other heat treatments resulted in complete inactivation of the AAD-1 protein. 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-1 protein is functionally unstable when heated.

CONCLUSIONS

The thermal stability of AAD-1 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-1 protein has significantly reduced immunoreactivity and is unstable when heated. Under the milder temperature regimes, the AAD-1 protein virtually lost all of its immunoreactivity based on a PAb sandwich ELISA, while the autoclaved sample lost ~ 60% of its original immunoreactivity. The protein lost greater than 9% of its enzymatic activity at all

temperatures tested. Gel electrophoresis analysis indicated that the molecular mass of the AAD-1 protein (approximately 33 kDa) was unchanged after heat treatment at 50, 70 or 95 °C. Autoclaving resulted in significant degradation of the primary structure of the AAD-1 protein. These data indicate that the industrial grain milling processes would largely inactivate and/or significantly degrade the AAD-1 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.

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

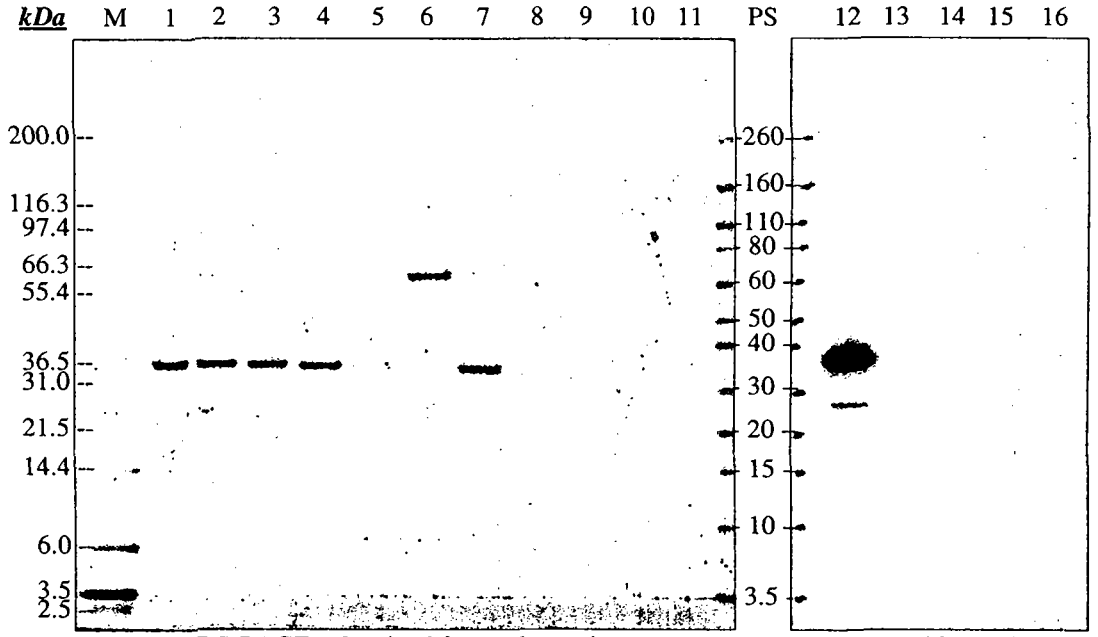
Treatment Temperature	Time (min)	% Immunoreactivity
4 °C	NA	100.0*
50 °C	30	0.0
70 °C	30	0.0
95 °C	30	0.2
120 °C (autoclave)	20	40.8

Notes: The 4 °C sample was normalized to 100% immunoreactivity. The results are averaged from two separate ELISA readings of serial dilutions of the AAD-1 protein. % immunoreactivity = ((avg. measured AAD-1 in solution) / (theoretical AAD-1 in solution) x 100)

Table 2. Summary of AAD-1 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	3.0
120 °C (autoclave)	20	0.0

Notes: 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-1. % enzymatic activity = (OD heat treatment - blank) / (OD 4 °C - blank) x 100



Panel A: SDS-PAGE gel stained for total protein

B: Western blot probed with α-AAD-1 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-1 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-1 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 200 V for ~30 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-1 specific polyclonal rabbit antibody (Protein A purified: Lot #: DAS F1197-151, 1.6 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	5 µL
1	AAD-1 (TSN105930) held @ 4 °C (total protein)	720ng
2	AAD-1 (TSN105930) heated @ 50 °C, 30 min (total protein)	720ng
3	AAD-1 (TSN105930) heated @ 70 °C, 30 min (total protein)	720ng
4	AAD-1 (TSN105930) heated @ 95 °C, 30 min (total protein)	720ng
5	AAD-1 (TSN105930) autoclaved, 20 min (total protein)	720ng
6	Bovine serum albumin	750ng
7	AAD-1 (TSN105930) held @ 4 °C (soluble protein)	720ng
8	AAD-1 (TSN105930) heated @ 50 °C, 30 min (soluble protein)	720ng
9	AAD-1 (TSN105930) heated @ 70 °C, 30 min (soluble protein)	720ng
10	AAD-1 (TSN105930) heated @ 95 °C, 30 min (soluble protein)	720ng
11	AAD-1 (TSN105930) autoclaved, 20 min (soluble protein)	720ng
PS	Invitrogen Novex Sharp Prestained MW markers	10 µL
12	AAD-1 (TSN105930) held @ 4 °C (soluble protein)	180ng
13	AAD-1 (TSN105930) heated @ 50 °C, 30 min (soluble protein)	180ng
14	AAD-1 (TSN105930) heated @ 70 °C, 30 min (soluble protein)	180ng
15	AAD-1 (TSN105930) heated @ 95 °C, 30 min (soluble protein)	180ng
16	AAD-1 (TSN105930) autoclaved, 20 min (soluble protein)	180ng

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

APPENDIX

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF TRINITY COLLEGE]

THE CONDENSATION OF AMINOANTIPYRINE. II. A NEW COLOR TEST FOR PHENOLIC COMPOUNDS

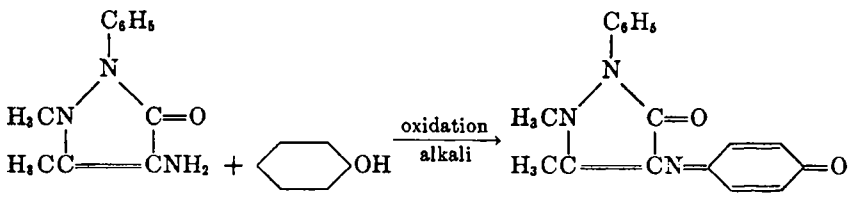
EDGAR EMERSON

Received June 15, 1943

I. REACTIONS OF PHENOLS

Aminoantipyrine was found to condense with aromatic amines (1) in the presence of acid oxidizing reagents. The reaction is general for primary, secondary, and tertiary aromatic amines with the exceptions noted. Phenols do not react under the conditions described. However, when alkaline oxidizing agents are used, primary and secondary aromatic amines react with aminoantipyrine to form intensely colored compounds, and under these same conditions phenols also react.

Phenol and aminoantipyrine in an ammoniacal solution containing a trace of cupric ion form a dark red precipitate when air is blown through the solution (2).



This technique for the production of colored derivatives of phenols is not well suited for general laboratory procedure. Alkaline potassium ferricyanide is a better reagent because of its greater sensitivity. The reaction is analogous to that of *p*-aminophenol, *p*-phenylenediamine, *p*-methylaminoaniline, and *p*-dimethylaminoaniline with phenols.

Aminoantipyrine is a sensitive reagent for detecting phenols. Phenol can be detected in one part in over eight million, *o*-cresol in one part in two million, and *m*-cresol in one part in over six and one-half million. In addition to being sensitive, aminoantipyrine has the advantage of giving negative blanks. Alkaline solutions of this amine alone do not yield highly colored products when treated with oxidizing agents. The reaction with phenols is clean cut; any color, red to purple, is due to the reaction products of aminoantipyrine with the phenols, provided the alkalinity is sufficient to prevent the formation of antipyrine red. It must be understood that the hydroxy compound by itself does not produce colored oxidation products. Catechol, hydroquinone, and pyrogallol darken when treated with the oxidizing agent but where each test is completely controlled no confusion arises.

The phenols with only one OH group and no other acid groups form colored products soluble in chloroform. Introduction of these acid groups in the molecule prevent the dissolution of the colored products in chloroform. Thus the

sensitivity of the test for phenols producing chloroform-soluble colored products can be increased by concentration of the color in chloroform.

A study was made of numerous phenols[1] to determine the scope and limitations of the reaction. The results are shown in Table I.

On the basis of the results shown in Table I the following conclusions can be drawn:

1. There must be at least one free phenolic hydroxyl group in the molecule for a positive test.

2. Substituents in the position para to the hydroxyl group prevent the reaction except as follows: halogen, carboxyl, sulfonic acid, hydroxyl, and methoxyl. These groups are probably expelled.

3. A nitro group in the ortho position prevents reaction and a nitro group in the meta position inhibits the test but not completely.

4. Coupling of aminoantipyrine with the phenol takes place in the para position rather than in the ortho position.

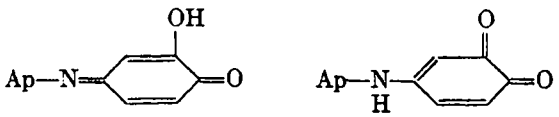
The fact that anisole and veratrole fail to give a positive test while phenol and guiacol do give one indicates that a free phenolic group is one of the prime requisites for a positive reaction.

That certain substituents are expelled from the para position may be deduced from information conveyed in Table I. *o*-Phenolsulfonic acid gives a positive test but the colored product formed is insoluble in chloroform. It is to be expected that a salt of an acid would be insoluble in chloroform. *p*-Phenolsulfonic acid also gives a positive test, but in this case the colored product formed is soluble in chloroform, a fact which indicates that the salt-forming group is no longer present in the molecule. Similar evidence is to be found in the series salicylic acid, *m*-hydroxybenzoic acid, and *p*-hydroxybenzoic acid. All three compounds give a positive test but only the colored product formed from the para compound is soluble in chloroform. Likewise, in the series catechol, resorcinol, and hydroquinone, the results are the same; only in the case of the para isomer is the colored product soluble in chloroform, a fact which indicates that the salt-forming group is expelled from the para position.

The results in the series of dihydroxybenzenes are not so conclusive as in the other two series. With hydroquinone no apparent coupling takes place if sodium hydroxide is used as an alkalizing agent. The stronger base seems to favor the oxidation of hydroquinone to quinhydrone and to quinone. When ammonium hydroxide is used there is a distinct positive test with the simultaneous formation of other colored oxidation products of hydroquinone. Alkalization with sodium bicarbonate results in a positive test obscured by very little color from other oxidation products of hydroquinone. It would seem from these facts that sodium bicarbonate is the best alkalizing agent to use. However, it is so weak

[1] The author expresses his thanks to the following firms which generously supplied samples: Calco Chemical Division of American Cyanamid, The Dow Chemical Company, Monsanto Chemical Company, the New York Quinine and Chemical Works, and Sharples Chemicals, Inc.

a base that it does not form salts with the free hydroxyl groups and consequently the colored products formed from catechol and resorcinol are also soluble in chloroform when this base is used. It is also to be noted that 4-tert.-butylcatechol gives a weak test and that the colored product is soluble in chloroform. It is soluble even if an excess of sodium hydroxide is used. In the catechol series the dyes might exist in one or both of the tautomeric forms.[2]



(I) (II)

Formula II would account for the solubility in chloroform of the dye even from an alkaline solution.

In the reaction of aromatic amines with aminoantipyrine no instance of expulsion has been observed when either alkaline or acid oxidizing agents are employed.

That the colored products are produced by aminoantipyrine coupling with the phenols in the para position may be deduced from the following facts. When the para position is blocked by an alkyl, aryl, ester, nitro, benzoyl, nitroso, or aldehyde groups, no color reaction takes place even though the ortho positions are unsubstituted. When the para position is substituted by carboxyl or sulfonic acid groups the colored products produced in the test are soluble in chloroform provided there is no other salt-forming group in the molecule. It is not likely that a group would be expelled from the para position followed by a coupling in an ortho position. If this were the case, then with 3,5-dibromo-4-hydroxybenzoic acid not only would the carboxyl group have to be expelled but also a bromine atom.

The test described can also be used to detect aminoantipyrine in small amounts. Amidopyrine does not interfere with the test and thus small amounts of aminoantipyrine present as an impurity in amidopyrine can be detected.

II. REACTIONS OF NAPHTHOLS

Just as there are two distinct series of naphthols, the alpha and the beta series, so also there are two distinct series of colors, red for the alpha series and green for the beta series. Unless there is an acid group in the molecule, the colored products formed when the naphthol is oxidized in the presence of aminoantipyrine are insoluble in water and soluble in chloroform.

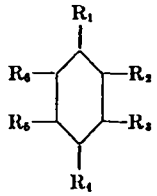
The red compounds are fairly stable in aqueous media and extremely so in chloroform, while the green ones are fugitive. In chloroform the green color disappears and on long standing changes to a yellow-red.

The results are indicated in Table II.

[2] Ap is used to denote the antipyryl radical.

TABLE I

THE RESULTS OF THE COLOR TEST OF AMINOANTIPYRINE WITH PHENOLS



COMPOUND	R_1	R_2	R_3	R_4	R_5	R_6	REACTION[a]
Anisole	OCH_3						0
Phenol	OH						3-S
o-Cresol	OH	CH_3					3-S
m-Cresol	OH		CH_3				3-S
p-Cresol	OH			CH_3			0
4-Hydroxy-1,3-dimethylbenzene	OH	CH_3		CH_3			0
4-Hydroxy-1,2-dimethylbenzene	OH		CH_3	CH_3			0
5-Hydroxy-1,3-dimethylbenzene	OH		CH_3		CH_3		3-S[b]
3-Hydroxy-1,4-dimethylbenzene	OH	CH_3			CH_3		3-S
Thymol	OH		CH_3			C_3H_7	3-S
Carvacrol	OH	CH_3			C_3H_7		3-S
p-tert. Amylphenol	OH			C_5H_{11}			0
p-tert. Butylphenol	OH			C_4H_9			0
p-tert. Butyl-*o*-cresol	OH	CH_3		C_4H_9			0
p-tert. Amyl-*o*-cresol	OH	CH_3		C_5H_{11}			0
o-Hydroxydiphenyl	OH	C_6H_5					3-S
p-Hydroxydiphenyl	OH			C_6H_5			0
o-Cyclohexylphenol	OH	C_6H_{11}					3-S

[a] The numbers refer to the strength of the test and the letters to the solubility of the colors in chloroform. Number 3 represents a strong test; 2, a moderate test; 1, a slight test; S, soluble; Ins, insoluble; SSl, slightly soluble.

[b] All positive tests produce varying shades of red except this one which is purple.

p-Cyclohexylphenol	OH			C_6H_{11}			0
Tyrosine	OH			CH_2NH_2COOH			0
o-Hydroxyacetophenone	OH	$COCH_3$					3-S
p-Hydroxyacetophenone	OH			$COCH_3$			0
2,4'-Dihydroxybenzophenone	OH	COC_6H_4OH					3-Ins.
4,4'-Dihydroxybenzophenone	OH			COC_6H_4OH			0
2-Chloro-5-hydroxytoluene	OH		CH_3	Cl			3-S
o-Chlorophenol	OH	Cl					3-S
p-Chlorophenol	OH			Cl			3-S
2,4-Dichlorophenol	OH	Cl		Cl			3-S
p-Bromophenol	OH			Br			3-S
2,6-Dibromophenol	OH	Br			Br		3-S
2,4,6-Tribromophenol	OH	Br		Br	Br		3-S
Veratrole	OCH_3	OCH_3					0
Guiacol	OH	OCH_3	OCH_3				3-S
Resorcinol monomethyl ether	OH		OCH_3				3-S
Hydroquinone monomethyl ether	OH			OCH_3			3-S
Eugenol	OH	OCH_3		$CH_2CH:CH_2$			0
iso-Eugenol	OH	OCH_3		$CH:CHCH_3$			0
Guiacol potassium sulphonate	OH	OCH_3			SO_3K		3-Ins.
o-Phenolsulphonic acid	OH	SO_3H					3-Ins.
p-Phenolsulphonic acid	OH			SO_3H			3-S
Salicylic acid	OH	COOH	COOH				3-Ins.
m-Hydroxybenzoic acid	OH						3-Ins.
p-Hydroxybenzoic acid	OH			COOH			3-S
Methyl *p*-hydroxybenzoate	OH			$COOCH_3$			0
Methyl salicylate	OH	$COOCH_3$					3-S
Salol	OH	$COOC_6H_5$					3-S
Salicylsalicylic acid	OH	$COOC_6H_4$ — $CO_2H(o)$					3-SSl
Salicylamide	OH	$CONH_2$					3-S
Salicylaldehyde	OH	CHO					3-S
p-Hydroxybenzaldehyde	OH			CHO			0

TABLE I—*Concluded*

COMPOUND	R_1	R_2	R_3	R_4	R_5	R_6	REACTION
Vanillin	OH	OCH_3		CHO			0
Vanillic acid	OH	OCH_3		COOH			3-S
3,5-Dibromo-4-hydroxybenzoic acid	OH	Br		COOH		Br	3-S
Catechol	OH	OH	OH				3-Ins.
Resorcinol	OH		OH				3-Ins.
Hydroquinone	OH			OH			1-S
Orcinol	OH		OH		CH_3		3-Ins.
Hexylresorcinol	OH	C_6H_{13}			OH		
4-tert.-Butylcatechol	OH	OH			C_4H_7		1-S
Epinephrine	OH	OH			$HNCH_3$ | $CHOHCH_3$		3
Phloroglucinol	OH		OH		OH		3-Ins.
Pyrogallol	OH	OH	OH				3-Ins.
o-Nitrophenol	OH	NO_2					0
m-Nitrophenol	OH		NO_2				2-S
p-Nitrophenol	OH			NO_2			0
p-Nitrosophenol	OH			NO			0
o-Aminophenol	OH	NH_2					0
m-Aminophenol	OH		NH_2				3-S
p-Aminophenol	OH			NH_2			0

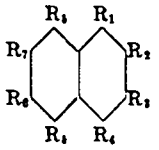
III. REACTIONS WITH HYDROXYPYRIDINES AND HYDROXYQUINOLINES[3]

(WITH HARRY H. BEACHAM[4])

Since many natural products and drugs are derivatives of pyridine and quinoline it seemed desirable to investigate the color reaction of aminoantipyrine

TABLE II

THE COLOR REACTIONS OF NAPHTHOLS WITH AMINOANTIPYRINE IN THE PRESENCE OF AMMONIACAL POTASSIUM FERRICYANIDE



SUBSTANCE	REACTION[a]	R_1	R_3	R_4	R_5	R_6	R_7	R_8
1-Naphthol	R—3—S	OH						
L Acid	R—3—Ins	OH			SO_3H			
Nevile and Winther's acid	R—3—S	OH		SO_3H				
Chromotropic acid	[b]	OH		SO_3H		SO_3H		OH
H Acid	[c]	OH		SO_3H		SO_3H		NH_2
Gamma Acid	R—1—Ins	OH		SO_3H			NH_2	
2-Naphthol	G—3—S		OH					
Schaeffer's acid	G—3—Ins		OH			SO_3H		
R Acid	G—3—Ins		OH	SO_3H		SO_3H		
G Acid, dipotassium salt	G—2—Ins		OH			SO_3K		SO_3K
3-Hydroxy-2-naphthoic acid	G—3—Ins		OH	COOH				
N-substituted 3-hydroxy-2-naphthamide	G—3—SSl		OH	CONHR[d]				

[a] The letters and numbers refer to the color of the solution, the strength of the test and to the solubility of the colored products in chloroform. R = red, G = green. The number 3 indicates a strong test, and 2 a moderate test. S = soluble, SSl = slightly soluble, and Ins = insoluble. Thus the designation G—3—SSl indicates that a strong green test was obtained and that the colored product is slightly soluble in chloroform.

[b] Brown changing to red-brown. Color insoluble in chloroform.

[c] Yellow-green turning to purple. Color insoluble in chloroform.

[d] R is C_6H_5, o-$CH_3C_6H_4$, or o-$CH_3OC_6H_4$. The anilide of 3-hydroxy-2-naphthoic acid gives only a weak test.

with hydroxy derivatives of these substances. In the series alpha, beta, and gamma hydroxypyridine, only the beta compound is unable to form a keto

[3] From part of the thesis of Harry H. Beacham submitted to the graduate faculty of Trinity College, in partial fulfillment of the requirements for the degree of Master of Science.

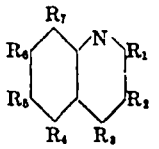
[4] Present address Eastman Kodak Company, Rochester, New York.

tautomer by the migration of the phenolic hydrogen to the nitrogen (3) and the beta compound is the only one which gives a positive test when treated with aminoantipyrine and alkaline potassium ferricyanide. The red solutions produced by this test are relatively stable compared to reddish-purple solutions produced when solutions of vitamin B_6 are tested.

When vitamin B_6 is tested, the color is extremely evanescent, appearing and disappearing in a matter of seconds. Only slight success toward stabilizing the color was achieved when the reaction was carried out in buffered solutions of

TABLE III

THE REACTION OF AMINOANTIPYRINE WITH HYDROXYQUINOLINES AND WITH HYDROXYQUINALDINES



COMPOUND	REACTION[a]	R_1	R_2	R_3	R_4	R_5	R_6	R_7
2-Hydroxyquinoline	0	OH						
3-Hydroxyquinaldine	G—1—Y	CH_3	OH					
4-Hydroxyquinaldine	0	CH_3		OH				
5-Hydroxyquinoline	R—2—R				OH			
6-Hydroxyquinoline	B—3—Y					OH		
7-Hydroxyquinoline	G—1—Y						OH	
8-Hydroxyquinoline	R—5—R							OH
7-Methyl-8-hydroxyquinoline	R—5—R						CH_3	OH
8-Hydroxyquinoline-5-sulfonic acid	R—4—R				SO_3H			OH

[a] The numbers in this column represent the strength of the test, 0 is negative and 5 represents a precipitate. The letters represent the colors of the solutions R, red; G, green; B, blue; Y, yellow. The colors of the aqueous solutions are represented by letters to the left of the numbers and the colors of the chloroform solutions are shown by letters to the right of the numbers. R—5—R indicates a very strong test in which the aqueous portion containing a precipitate is red. Extraction with chloroform produces a red chloroform layer.

varying *p*H. Just as the reaction of vitamin B_6 with quinonechloroimides is inhibited by borate ion, as reported by Scudi (4), so also do borates prevent the reaction of vitamin B_6 with aminoantipyrine.[5] Borates do not interfere with the aminoantipyrine test for phenols, naphthols, and 3-hydroxypyridine.

As might be expected, derivatives of 2- and 4-hydroxyquinolines fail to give

[5] If *p*-dimethylaminoaniline is used in place of aminoantipyrine in the test for vitamin B_6 the blue compound formed is soluble in chloroform in which solvent the color is stable for several hours. This test will detect vitamin B_6 in dilutions of between 1 part per million and 1 part per 10 million by visual methods.

the test because in both cases the position para to the hydroxyl group is blocked, in the 2-hydroxy compound by the fused benzene ring, and in the 4-hydroxy compound by the ring nitrogen.

The remaining hydroxy compounds give a positive test. It was expected that the 5- and 8-hydroxy compounds would give a red test in the same way that the 1-naphthols do and that the 3-, 6-, and 7-compounds would give a green test in the same way as the 2-naphthols. These expectations were realized. However, the 6-hydroxyquinoline gave a blue-green solution rather than the expected green. In general the test with compounds which produce green solutions is less sensitive than the test with compounds producing red solutions. Whereas the color of the red solutions is stable, the color of the green solutions fades rapidly to yellow. This change is hastened when the solution is shaken with chloroform. Attempts to capture the green color in chloroform always resulted in the appearance of a yellow chloroform layer.

It should be noted that the colored compound formed from 8-hydroxyquinoline-5-sulfonic acid is soluble in chloroform, a fact which indicates that the sulfonic acid group is expelled.

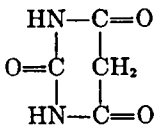
The results are summarized in Table III.

IV. REACTIONS WITH BARBITURATES, URACILS, HYDANTOINS, AND IMIDAZOLONS[6]

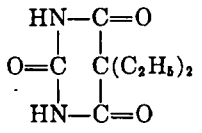
(WITH LINDLEY CLAIR BEEGLE[7])

An investigation was made of some barbiturates, uracils, hydantoins, and imidazolons in order to study further the scope and limitations of the aminoantipyrine color test.

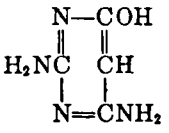
Barbituric acid (III) gives a positive test in which the red color formed is insoluble in chloroform. When the 5 position is substituted as in barbital (IV) no color is formed. Several commercial sedatives of the barbital type give a slight positive test, a fact which suggests that traces of barbituric acid may be present. When these compounds are purified they no longer give a positive test. The differences in the melting points of these commercial products and of the samples purified from them were not detectable by the usual laboratory procedure. The aminoantipyrine test would seem to offer a means of detecting traces of certain barbituric acids in sedatives which are derivatives of these acids.



(III)



(IV)



(V)

[6] From the thesis of Lindley Clair Beegle submitted to the Committee on Graduate Students at Trinity College in partial fulfillment of the requirements for the degree of Master of Science.

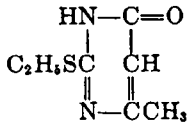
[7] Present address American Cyanamid Co., Stamford, Conn.

Apparently 5,5-disubstitution blocks the reaction of barbituric acid. In a similar manner substitution of a benzyl group in the 5 position inhibits the reaction. However, benzalbarbituric acid gives a positive test in which the red color formed is insoluble in chloroform. The apparent elimination of the benzal group finds no counterpart in any of the reactions previously studied although a similar reaction was noted in the pyrazolon series.

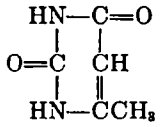
1,3-Diphenylbarbituric acid was prepared in order to determine the effect of large blocking groups on the nitrogen atoms. The compound gave a purplish-red test and the color formed was soluble in chloroform, a result which was to be expected since there was no longer a salt-forming group in the molecule.

If some of the carbonyl oxygen atoms of barbituric acid are replaced by imine groups the reaction still takes place. 2,4-Diamino-6-hydroxypyrimidine (V) couples with aminoantipyrine to give a red dye insoluble in chloroform. Instead of hydrolyzing readily in dilute acids, the dye takes on a blue-red color. The reaction also takes place in neutral or slightly acid solutions when ferric chloride is used as the oxidant. It seems likely that the reaction of this pyrimidine with aminoantipyrine is analogous to the reactions of the *m*-aminophenols (5).

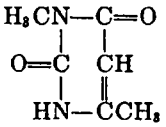
2-Ethylmercapto-4-methyluracil (VI), 4-methyluracil (VII), 1,4-dimethyluracil (VIII), and 1,3,4-trimethyluracil (IX) failed to react with aminoantipyrine.



(VI)

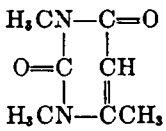


(VII)

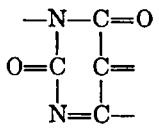


(VIII)

Compounds (VI) and (IX) are not capable of forming derivatives having a structure represented by formula (X) and were not expected to give the test. However, (VII) and VIII) theoretically are able to form derivatives of type (X). They failed to produce the least trace of color with aminoantipyrine and an oxidant. It may be that here we are dealing with a situation analogous to that encountered with 2-hydroxypyridine in which case no methylene derivative is possible, due either to the preference of the pyridone or zwitterion structure (3).



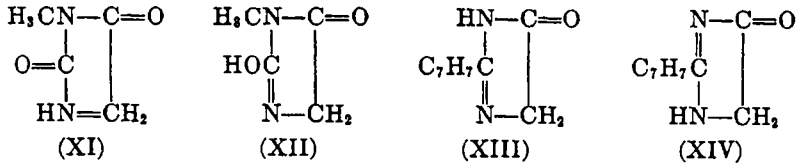
(IX)



(X)

It was believed that a compound should condense with aminoantipyrine to form highly colored products if there were alpha,beta double bonds on both sides of the coupling position as represented by the structure $=X-\underset{\|}{C}-Y=$ which must be in a ring system. Hydantoin and 3-methylhydantoin (XI)

might form this structure by tautomeric shift of the 1-hydrogen atom to the 2-carbonyl oxygen (XII).



(XI) (XII) (XIII) (XIV)

However, both compounds failed to give any trace of color when they were tested. Again it was thought that we might be dealing with an alpha-pyridone type of structure.

In order to avoid tautomerization of the group attached to the 2-carbon atom, the 2-hydroxy group was replaced by a benzyl radical. 2-Benzylimidazolon-5 (XIII) and 2-benzylimidazolon-4 (XIV) were prepared and tested. Neither one gave the least indication of a positive reaction.

While it appears that the ability of a compound to form the structure $=X-\underset{\|}{C}-Y=$ which must be included in a ring[8] is a necessary condition for a positive aminoantipyrine test, it is also apparent that this is not a sufficient condition. As yet no predictions can be made with any certainty as to which compounds will give the test.

EXPERIMENTAL

Compounds not commercially available were prepared according to directions found in the literature.

Procedure used for phenols and naphthols. All tests were carried out in the same general manner. A small quantity of the compound (about 10-20 mg.) was dissolved in 10 cc. of water to which 2-3 drops of 6 *N* ammonium hydroxide was added. If the substance did not dissolve in water or ammonium hydroxide it was dissolved first in a few drops of alcohol and then diluted with water to 10 cc. The solution was then divided into two equal portions and to one portion was added 2 drops of 2% aminoantipyrine solution, the other portion being kept as a control. Then to each of the tubes was added several drops of 8% potassium ferricyanide solution. A large excess of oxidizing agent is to be avoided because it may decolorize the dye formed. After the development of full color, 2 cc. of chloroform was added and the mixture shaken. The results are detailed in Tables I and II.

Determination of sensitivity of test. The general procedure used was as follows: a quantity of the phenol was weighed accurately on an analytical balance and dissolved in an equivalent amount of *M* NaOH solution and water added to make a solution of known volume. Then by a series of ten-fold dilutions a number of solutions were prepared and tested. Each test was carried out by the addition of 1 drop of 0.2% aminoantipyrine solution followed by 1 drop of 2% $K_4Fe(CN)_6$ in dil. NaOH [$K_4Fe(CN)_6$, 0.1395 g. + 0.30 ml. of *M* NaOH + 9.70 cc. water], to a 1-ml. portion. After the addition of the ferricyanide solution, 0.25 cc. of chloroform was added and the mixture shaken. By this method it was found that phenol could be detected in 1.2 parts per ten million. In the light of later work it seems probable that $NaHCO_3$ or Na_2CO_3 would be better to use than NaOH.

Procedure used in sections III and IV. A better procedure for testing the compounds was used in sections III and IV. Aqueous solutions of the compounds tested were made up

[8] Dibenzoylmethane, ethyl benzoylacetate, ethyl acetoacetate, diethyl malonate, and malonic acid give no test.

to 1 part in 10,000. To 2 ml. of each solution was added 1 drop of aminoantipyrine solution (13.6 g. per liter) followed by 1 drop of alkaline potassium ferricyanide (86.7 g. of $K_3Fe(CN)_6$, 18 ml. of conc'd NH_4OH and water to make 1 liter). After 5 minutes the color of the solution was noted, 0.5 ml. of chloroform added, the mixture shaken, and the color of the chloroform layer recorded.

Similar results were obtained when the oxidant was prepared with equivalent quantities of NaOH, Na_2CO_3, or $NaHCO_3$ in place of NH_4OH. Generally the sodium hydroxide was the least efficient alkali for color production.

The results obtained using other coupling amines instead of aminoantipyrine are recorded in the Chemistry Library of Trinity College (6).

Other compounds and mixtures tested. The following gave a positive test: 1-phenyl-3-methylpyrazolon-5, digitalis glucosides (Roche) (weak brownish test), urine, diketohydrindine,[9] and milk (very slight test).

The following gave a negative test: antipyrine, creatine, creatinine, 3,4-dimethyl-2-hydroxyfluorene, 1,4-dimethyl-2-hydroxyfluorenone, quinine, morphine, codeine, theobromine, uric acid, testosterone, theelol, thyroid (Schering), novatropine, benzedrine, thyroid gland emplets (Parke Davis), digilanid (Sandoz Chemical Works), ascorbic acid, dried blood plasma, saliva, aspirin, anthranol, anthrone, and thiophenol.

SUMMARY

1. A sensitive new color test for phenolic compounds is described. The color produced from vitamin B_6 is evanescent when aminoantipyrine is used but stable when *p*-dimethylaminoaniline is used.

2. Monohydric alpha-naphthols and the comparable hydroxyquinolines produce red colors while the monohydric beta-naphthols and the comparable quinolines produce green colors.

3. The probable structure of the colored products is discussed as well as the limitations of the reaction.

4. Aminoantipyrine as a reagent for phenolic compounds gives a well-defined test because alone it does not react with alkaline oxidizing agents to produce highly colored products as do the *p*-aminophenols and *p*-diamines.

HARTFORD, CONN.

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[9] This compound was prepared in this laboratory by Mr. Kenneth Kelly.

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Purification and Characterization of 2,4-Dichlorophenoxyacetate/α-Ketoglutarate Dioxygenase*

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The *Alcaligenes eutrophus* 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase, encoded by the *tfdA* gene of plasmid pJP4, is an Fe(II)-dependent enzyme that catalyzes the conversion of 2,4-dichlorophenoxyacetate to 2,4-dichlorophenol and glyoxylate concomitant with the decomposition of α-ketoglutarate to form succinate and carbon dioxide (Fukumori, F., and Hausinger, R. P. (1993) *J. Bacteriol.* 175, 2083–2086). Using recombinant *Escherichia coli* cells that overexpress the *tfdA* gene, the thermolabile enzyme (stable only up to 30 °C) was purified to apparent homogeneity (specific activity of 16.9 μmol of substrate converted min^{-1} mg of $protein^{-1}$) by a simple two-step procedure. The native protein has an apparent M_r of 50,000 ± 2,500, consistent with a homodimeric structure. Ferrous ion is absolutely required for activity and cannot be replaced by several other divalent cations tested. Ascorbic acid stimulates dioxygenase activity and reduces the rate of enzyme inactivation by a metal ion-mediated process. The enzyme exhibits maximum activity at pH 6.5–7, however, it is stable over a pH range of 6.5–11. Although capable of hydroxylating a wide range of phenoxyacetates and related compounds, the enzyme exhibits the greatest affinity (K_m 17.5 ± 1.0 μM) and highest catalytic efficiency for 2,4-dichlorophenoxyacetate. Similarly, α-ketoglutarate is the preferred co-substrate (K_m 3.20 ± 0.54 μM) for the enzyme, but it can utilize a range of other α-ketoacids with lower efficiency. Results from chemical modification studies are consistent with the presence of multiple essential histidine residues in the enzyme.

The biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D),[1] a broadleaf herbicide, has been studied as a model for microbial decomposition of chloroaromatic compounds (1–3). *Alcaligenes eutrophus* JMP134 is the most extensively studied 2,4-D degrader and the pathway found in this microbe is shown in Fig. 1. The genes responsible for the first five steps in this pathway (*tfdA*, *-B*, *-C*, *-D*, and *-E*) have been localized to the transmissible plasmid, pJP4 (4). The nucleotide sequences of *tfdCDE* (5) exhibit extensive similarity to the well studied *clcABD* and *tcbCDE* chlorocatechol degradation genes (reviewed in Ref. 3) and similarly provide for the catabolism of dichlorocatechol. The sequence of the *tfdB* gene (5), encoding 2,4-DCP hydroxylase, also exhibits similarity to other genes involved in phenol hydroxylation (*e.g.* see Ref. 6). In contrast to the downstream genes, however, the sequence of the *tfdA* gene (7) does not exhibit obvious evolutionary relationships to any other known genes. The *tfdA* gene product (TfdA) generally has been referred to as 2,4-D monooxygenase; however, we recently demonstrated that the enzyme carries out a very different type of reaction (8) that is mechanistically related to reactions catalyzed by a group of α-KG-dependent dioxygenases (9). The reaction requires α-KG and Fe(II), in addition to 2,4-D and oxygen, and produces carbon dioxide, succinate, 2,4-DCP, and glyoxylate (Fig. 2).

Here, we detail the purification and describe the catalytic properties of 2,4-D/α-KG dioxygenase. In addition, we examine the substrate ranges of the enzyme for other phenoxyacetates and α-ketoacids. Furthermore, we describe evidence consistent with the presence of multiple essential histidine residues in the enzyme.

EXPERIMENTAL PROCEDURES

Chemicals—Organic chemicals were obtained from Sigma or Aldrich and were of the highest purity available. α-[1-^{14}C]KG (1.8 MBq/mmol) was obtained from DuPont NEN.

Media, Organisms, and Plasmid—Modified LB medium (10 g of Tryptone, 5 g of yeast extract, and 5 g of NaCl per liter (pH 7.2)) supplemented with 100 μg/ml of ampicillin was used to grow the host strain *Escherichia coli* JM109 (10). *A. eutrophus* JMP134 (4) was grown at 30 °C in 1/10 PTYG medium (0.25 g of peptone, 0.25 g of Tryptone, 0.5 g of yeast extract, 0.5 g of glucose, 0.03 g of $MgSO_4$, and 0.0035 g of $CaCl_2$ per liter (pH 7.2)) supplemented with 2.5 mM 2,4-D. Plasmid pUS311 (8) that encodes the *tfdA* gene had been generated from pKJS31 (7).

Purification of TfdA—Unless stated otherwise, all purification steps were done at 4 °C. Cultures (2 liters) of *E. coli* JM109(pUS311) were grown overnight at 30 °C with moderate aeration. Cells were harvested by centrifugation (6,000 × *g*) for 20 min and suspended in 25 ml of 20 mM Tris, 1 mM EDTA, 0.4 mg/liter leupeptin (pH 7.2). The cell suspensions were stored at −20 °C until use. Suspended cells were adjusted to 1 mM phenylmethylsulfonyl fluoride and passed through a French pressure cell at 120 MPa. Cell extracts were obtained after clarification of disrupted cells by centrifugation at 100,000 × *g* for 30 min. To the extract was added 20 mM Tris, 1 mM EDTA, 0.1 mg/liter leupeptin (pH 7.2) (Buffer A) containing 3.4 M $(NH_4)_2SO_4$ and the fraction precipitating between 1.0 and 1.7 M was collected. The precipitate was dissolved in 20 ml of Buffer A and dialyzed against 4 liters of the same buffer for 16 h. The solution was then chromatographed on a Mono Q 10/10 column (Pharmacia LKB Biotechnology Inc.) in Buffer A and eluted with a 100 ml of linear salt gradient to 0.2 M NaCl at room temperature (about 20 °C). TfdA was found to elute at approximately 0.05 M NaCl.

Enzyme Assays—2,4-D/α-KG dioxygenase was assayed at 30 °C. The typical assay mixture (total volume of 1 ml) contained 1 mM α-KG, 50 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, and 1 mM 2,4-D in 10 mM imidazole buffer (pH 6.75). Aliquots of the reaction mixture were quenched by the addition of EDTA. The concentration of released phenol derivative was

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[1] The abbreviations used are: 2,4-D, 2,4-dichlorophenoxyacetate; α-KG, α-ketoglutarate; 2,4-DCP, 2,4-dichlorophenol; DEP, diethylpyrocarbonate; TNBS, trinitrobenzenesulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; 2,4,5-T, 2,4,5-trichlorophenoxyacetate; Bis-tris, bis(2-hydoxyethyl)iminotris(hydroxymethyl)methane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

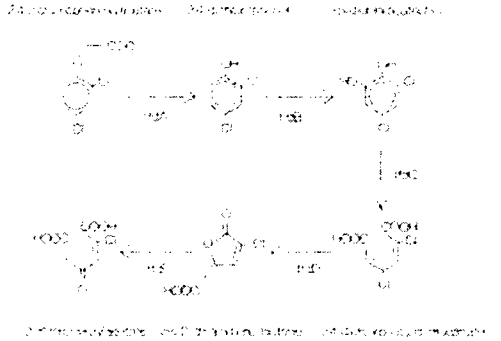

FIG. 1. **Pathway for degradation of 2,4-D in *A. eutrophus* JMP134.**

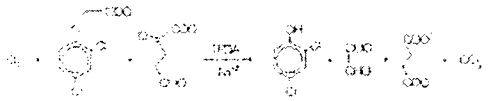

FIG. 2. **Overall reaction for 2,4-D/α-ketoglutarate dioxygenase.**

measured by a modification of the 4-aminoantipyrine method (11); to 1 ml of reaction mixture was added 100 μl of a pH 10 buffer solution (3.09 g of boric acid, 3.73 g of potassium chloride, and 44 ml of 1 N sodium hydroxide brought to 1 liter with distilled water), 10 μl of 2% 4-aminoantipyrine, and 10 μl of 8% potassium ferricyanide. The absorbance at 510 nm was determined after 20 min with a Gilford Response spectrophotometer. One unit of activity is defined as the amount of enzyme that forms 1 μmol of phenol derivative/min at 30 °C.

Examination of the kinetic parameters for 2,4-D and its analogues and for α-KG and its analogues involved holding the concentration of one substrate constant at 1 mM while varying the concentration of the substrate under consideration. For each of the phenoxyacetate derivatives the appropriate modified phenol compound was used as a standard in the spectrophotometric assay to account for their slightly different extinction coefficients. In contrast, 2,4-DCP served as the standard for kinetic evaluation of each of the α-ketoacids. Because of the extremely low K_m value for α-KG, special conditions were used with this substrate. Enzyme (0.1 μg) was incubated with α-[1-^{14}C]KG (45 kBq/mmol) under standard conditions for up to 1 min, the reaction was stopped by the addition of 1 N HCl, reaction mixtures were incubated for 1 h at room temperature with shaking to drive off the released carbon dioxide (α-KG is stable under the low pH conditions used in this experiment), and the remaining radioactivity in the solution was measured. Rates for α-KG utilization were calculated based on the released radioactivity. The capacity of the enzyme to react with several other non-phenoxyacetate substrates was assessed by monitoring spectroscopic changes or by high performance liquid chromatography (Hewlett-Packard HP-1050) methods using a C_{18} (octadecyl silane) reverse-phase column (4.0 × 250 mm) at a flow rate of 1.5 ml/min in various compositions of methanol, 0.1% H_3PO_4 in H_2O. For all compounds that served as substrate for the enzyme, apparent kinetic constants were obtained (12) from rates that were calculated by using linear regression analysis. Values of k_{cat} were based on a subunit M_r of 32,171 (7).

Analytical Methods—Protein concentration was measured (13) using bovine serum albumin as a standard. The native M_r of TfdA was estimated by gel filtration through a Superose 12 HR 10/30 column (Pharmacia; flow rate 15 ml/h) in 20 mM Tris, 1 mM EDTA, 100 mM NaCl (pH 7.2). The calibration proteins were thyroglobulin, M_r 670,000; bovine γ-globulin, M_r 158,000; chicken ovalbumin, M_r 44,000; equine myoglobin, M_r 17,000; and vitamin B-12, M_r 1,350 (Bio-Rad).

Gel Electrophoresis and Immunoblotting—Denaturing SDS-polyacrylamide gel electrophoresis was carried out on 12% polyacrylamide gels (14). Gels were stained with Coomassie Brilliant Blue. Standard proteins included phosphorylase *b*, M_r 92,500; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400 (Bio-Rad).

Antibodies were generated against purified TfdA in a white New Zealand rabbit, and the IgG fraction was obtained from the serum (15). Immunoblots were prepared by blotting the proteins onto nitrocellulose, probing with anti-TfdA rabbit IgG, and developing with goat anti-rabbit IgG-alkaline phosphatase conjugates (16).

Chemical Modification Studies—DEP was freshly diluted with ethanol and its concentration was determined by using 10 mM imidazole (pH 7.0) based on the increase in absorbance at 240 nm ($\epsilon = 3000\ M^{-1}\ cm^{-1}$) (17). Reactions were carried out by incubating the enzyme (1.2 μM) with DEP and other additions as indicated in 10 mM MOPS buffer (pH 6.5) at room temperature (21–22 °C). Aliquots (25 μl) were withdrawn at various time intervals, the reactions were quenched with 755 μl of 13.3 mM imidazole buffer (pH 6.75), and residual activities were determined as in the standard procedure. Reactivation of the DEP-modified TfdA (treated for 30 min with 1 mM DEP) was examined after adjusting the solution to 10 mM imidazole and 0.5 M hydroxylamine (using neutralized reagent). TfdA activity at various times was assessed after removal of excess reagent by using a Bio-Rad 10 DG desalting column. Inactivation studies of the enzyme (15 μM) by various other reagents, dissolved in H_2O, were performed in 10 mM imidazole or 10 mM MOPS buffers (pH 6.75). Aliquots (2 μl) were withdrawn at various time intervals and residual activities were determined as described above.

RESULTS

Purification of TfdA—2,4-D/α-KG dioxygenase was purified to over 95% homogeneity from *E. coli* JM109(pUS311) with 56% yield by using the 2-step procedure summarized in Table I. Comparison of the isolated protein to cell extracts from the recombinant strain by denaturing gel electrophoresis (Fig. 3) demonstrated that the cells produced the *tfdA* gene product as more than 10% of the total protein. Gel filtration chromatography was used to estimate a M_r of 50,000 ± 2,500 for the native enzyme, consistent with a homodimeric structure (subunit M_r of 32,171; Ref. 7).

The presence of protease inhibitors during early stages of TfdA purification enhances the stability of the enzyme by pre-

TABLE I
Purification of 2,4-D/α-ketoglutarate dioxygenase

Purification step	Total protein	Total activity	Specific activity	Purification
	mg	*units (%)*	*units/mg*	*fold*
Cell extracts	750	1690 (100)	2.2	1
Ammonium sulfate precipitation	225	1400 (83)	6.2	2.8
Mono Q	58	980 (58)	16.9	7.7

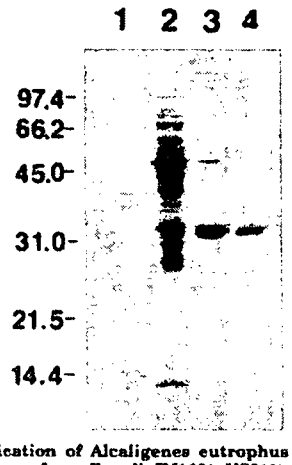

FIG. 3. **Purification of Alcaligenes eutrophus JMP134 2,4-D/α-KG dioxygenase from *E. coli* JM109(pUS311).** Protein samples were analyzed by denaturing gel electrophoresis on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue. *Lane 1*, molecular weight markers with the relative molecular mass values indicated in kilodaltons on the *left margin*; *lane 2*, supernatant of disrupted cells of *E. coli* JM109(pUS311); *lane 3*, fraction precipitated from 1–1.7 M ammonium sulfate; *lane 4*, fraction containing purified TfdA as eluted from MonoQ ion exchange column.

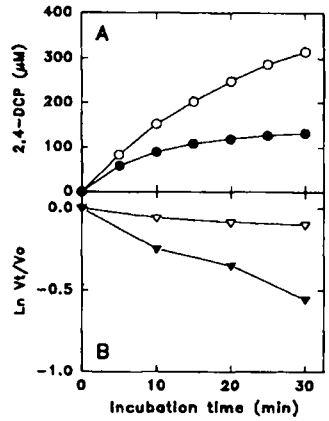

FIG. 4. **Time- and ferrous ion-dependent inactivation of 2,4-D/α-KG dioxygenase.** *A*, Progress curves of 2,4-DCP formation for TfdA (1 μg/ml) incubated in the standard assay with (○) and without (●) ascorbate. *B*, TfdA (2 μg/ml) was incubated at room temperature in 10 mM imidazole buffer (pH 6.75) with no additions (▽), and with 100 μM ferrous ion and 100 μM ascorbate (▼). Aliquots were withdrawn over time and assayed under standard conditions.

venting conversion of the subunit to an inactive TfdA fragment of apparent M_r 27,000. Whereas NH_2-terminal sequence analysis of the non-degraded subunit revealed the residues expected from DNA sequence analysis (S-V-V-A-N-; Ref. 7), the amino-terminal sequence of the proteolytic fragment of TfdA (F-L-Y-A-E-L-) was consistent with hydrolytic cleavage after arginine (residue 77) and loss of the NH_2-terminal fragment. By using anti-TfdA IgG in Western blot analysis of various samples, the conversion was found to occur after cell disruption rather than during the cell cultivation period. The same methods were used to demonstrate that the M_r of 32,000 form of the protein was present in cell extracts of *A. eutrophus* JMP134.

Maintenance of low temperatures during bacterial growth and enzyme isolation also is important for obtaining high levels of activity. As previously described (8), TfdA forms aggregates in *E. coli* JM109(pUS311) cells that are grown at 37 °C but remains soluble for cells grown at 30 °C. Indeed, we have observed that colonies grown at the higher temperature are turbid, whereas those grown at the more moderate temperature remain clear. Furthermore, the purified 2,4-D/α-KG dioxygenase was found to be very sensitive to thermal inactivation. At a protein concentration of 0.3 μM, TfdA remains stable only up to 30 °C and is completely inactivated within 5 min at 40 °C. In contrast, the purified enzyme was found to be stable at 4 °C for at least several weeks after purification.

Catalytic Properties of TfdA—As previously noted (8), activity of 2,4-D/α-KG dioxygenase requires ferrous ion and is enhanced by ascorbic acid. The ferrous ion requirement could not be met by other divalent metal ions including Co(II), Cu(II), Li(II), Mg(II), Mn(II), Ni(II), and Zn(II) when added as sulfate salts at a concentration of 100 μM. As shown by the time-dependent decrease in enzyme activity in Fig. 4*A*, however, ferrous ion alone is unable to sustain enzyme catalysis over long time periods. The rate of activity loss was greatly reduced (although not completely eliminated) by inclusion of ascorbic acid in the assay. The ascorbic acid serves to prevent inactivation rather than to reactivate the enzyme as shown by studies in which addition of ascorbate to partially active enzyme in the ferrous ion-alone treatment failed to restore full activity. Similarly, activity could not be recovered from inactive enzyme that was dialyzed against 1 mM EDTA and/or 1 mM dithiothreitol and then reassayed in the presence of ferrous ion plus ascorbate. Catalytic turnover of the enzyme was not required for inactivation as shown by the loss of activity during enzyme incubation with ferrous ion prior to addition of substrate (Fig. 4*B*). Although not completely characterized, the inactivation clearly results from a metal ion-mediated event as demonstrated by the retention of activity when the enzyme is stored in the absence of metal ions and the presence of EDTA. To minimize enzyme inactivation in the kinetic studies described below, reactions were initiated by addition of enzyme to assay mixtures and sufficiently short assay periods were used to calculate initial rates. The concentration dependences for ferrous ion and ascorbate on initial 2,4-D/α-KG dioxygenase rates are shown in Fig. 5.

The pH dependence of 2,4-D/α-KG dioxygenase was examined. The enzyme activity exhibits a narrow pH optimum of 6.5–7.0 (Fig. 6*A*). In contrast, TfdA is stable over a wide pH

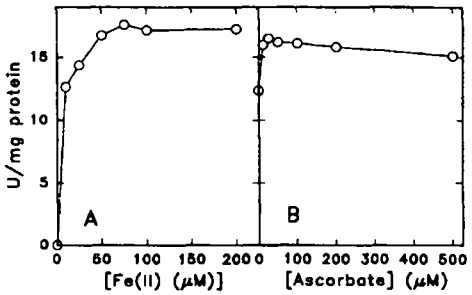

FIG. 5. **Effect of Fe(II) and ascorbic acid concentrations on the activity of 2,4-D/α-KG dioxygenase.** *A*, Fe(II) concentration dependence on 2,4-D/α-KG dioxygenase activity; *B*, ascorbic acid concentration dependence on enzyme activity.

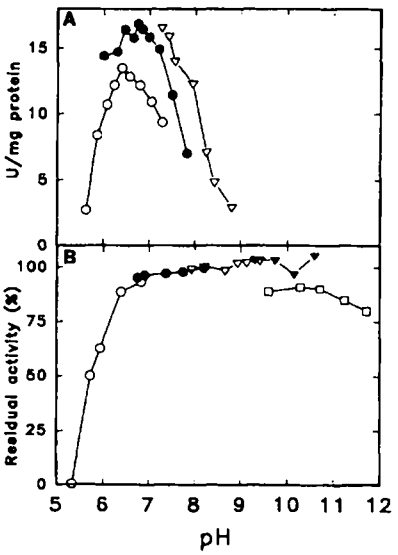

FIG. 6. **pH dependence of 2,4-D/α-KG dioxygenase.** *A*, enzyme activity. The enzyme assays were done in the following buffers at a concentration of 5 mM: Bis-Tris, pH 5.6–7.3 (○); imidazole, pH 6.0–7.6 (●); Tris, pH 7.3–8.8 (▽), all at 30 °C. *B*, enzyme stability. The residual enzyme activity after incubation at various pH values for 20 h at 4 °C was determined by using standard assay conditions. The enzyme was incubated in the following buffers at a concentration of 10 mM: MES, pH 5.3–6.8 (○); imidazole, pH 6.7–8.2 (●); Tris, pH 7.9–9.4 (▽); CHES, pH 9.3–10.6 (▼); CAPS, pH 9.6–11.7 (□).

TABLE II
Phenoxyacetate substrate specificity and kinetic parameters for 2,4-D/α-ketoglutarate dioxygenase

All experiments were performed at 30 °C in 10 mM imidazole buffer (pH 6.75) containing 1 mM α-KG, 50 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, and the indicated substrates over the indicated concentration ranges.

Substrate (concentration range studied)	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	$min^{-1} \cdot mM^{-1}$ (%)
Phenoxyacetate (200–1000 μM)	460 ± 23	443 ± 10	960 (3)
2-Chlorophenoxyacetate (40–200 μM)	110 ± 6.5	380 ± 11	3,450 (11)
4-Chlorophenoxyacetate (40–200 μM)	117 ± 6.2	595 ± 15	5,090 (17)
2,3-Dichlorophenoxyacetate (50–200 μM)	102 ± 8.5	288 ± 12	2,820 (9)
2,4-Dichlorophenoxyacetate (10–50 μM)	17.5 ± 1.0	529 ± 16	30,200 (100)
3,4-Dichlorophenoxyacetate (50–500 μM)	219 ± 7.6	307 ± 5	1,400 (5)
2,4,5-Trichlorophenoxyacetate (20–150 μM)	59.6 ± 3.4	96 ± 3	1,610 (5)
4-Chloro-2-methylphenoxyacetate (20–150 μM)	89.0 ± 8.7	233 ± 12	2,620 (9)
DL-2-Phenoxypropionate (0.5–5 mM)	1170 ± 120	5.1 ± 0.2	4.4 (–)
2-(2,4-Dichlorophenoxy)propionate (0.05–1 mM)	191 ± 6.7	61 ± 3	320 (1)
3-Phenoxypropionate (5–25 mM)	12900 ± 3200	3.2 ± 0.4	0.25 (–)

range (although it is totally inactivated by acidic conditions (pH < 6.5)) as shown by measuring the remaining activity after incubation in various pH buffers at 4 °C for 20 h (Fig. 6*B*).

Substrate Range of TfdA—In addition to 2,4-D, the enzyme catalyzes the α-KG-dependent release of the expected phenol derivatives from several other phenoxyacetates (Table II). The enzyme exhibits greatest affinity and catalytic efficiency toward 2,4-D, however, many ring-substituted compounds are reasonable substrates with similar catalytic rates but lower affinity. Non-halogenated phenoxyacetate possesses a larger K_m value than do the halogenated substrates. Similarly, the K_m value for 2-phenoxypropionate is substantially larger than that of 2-(2,4-dichlorophenoxy)propionate. The additional methyl group in the side chain of these two compounds, however, greatly decreases their k_{cat} values for hydroxylation, perhaps due to the change from a secondary to a tertiary carbon atom. Although it is a very poor substrate 3-phenoxypropionate is hydroxylated by the enzyme, demonstrating that the substrate binding site can accommodate one extra methylene carbon in the side chain. In contrast, TfdA exhibits no activity toward analogues in which (i) the phenoxy group is two carbons atoms more distant from the carboxyl group (4-(2,4-dichlorophenoxy)-butyrate), (ii) the carboxyl and phenoxy groups are located at adjacent positions on an aromatic ring (2-phenoxybenzoate), (iii) the acid group is reduced (2-phenoxyethanol), or (iv) the phenoxy oxygen is replaced by a methylene group (hydrocinnamic acid). Similarly, the enzyme exhibits insignificant activity toward methyl esters of phenoxyacetate and 4-chlorophenoxyacetate. Although trace amounts of product phenols were observed (<0.5%), they may have arisen from uncatalyzed hydrolysis of the substrate, with subsequent enzymatic action on the phenoxyacetates, as the amount of product did not depend on the enzyme concentration. Finally, because other investigators have characterized a mechanistically related 4-hydroxyphenylpyruvate dioxygenase (17–19), we tested whether this compound could serve as a substrate for TfdA. We found that 4-hydroxyphenylpyruvate is not utilized by this enzyme.

The K_m and k_{cat} values for several α-ketoacids were determined to examine the specificity of the enzyme for α-KG (Table III). Although the enzyme exhibits a broad α-ketoacid substrate range, the highest affinity and greatest catalytic rate were observed for α-KG. The non-α-ketoacid carboxyl group is not required for recognition by the enzyme, but the K_m values for the two substrates possessing a second acidic group are significantly increased and the catalytic rates are greatly reduced compared to the other substrates. Addition of an extra methylene group between the α-ketoacid group and the free carboxyl group, as in α-ketoadipate, leads to small changes in the kinetic constants: a 6.4-fold increase in K_m and a 55% decrease in k_{cat} compared to α-KG. In contrast, removal of one of the methylene groups, as in oxalacetate, led to an ineffective substrate. Furthermore, using 25 μg of enzyme and 50-min incubation times, β-ketoglutarate, malonate, succinate, and glutarate (all up to 1 mM concentrations) were found to be incapable of supporting 2,4-D hydroxylation. Finally, in the absence of 2,4-D, no decomposition of α-KG was observed.

TABLE III
α-Ketoacid substrate specificity and kinetic parameters for 2,4-D/α-ketoglutarate dioxygenase

Enzyme reactions were performed at 30 °C in 10 mM imidazole buffer (pH 6.75) containing 1 mM 2,4-D, 50 μM ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$, and the indicated substrates over the indicated ranges of concentration.

Substrate (concentration range studied)	K_m	k_{cat}	k_{cat}/K_m
	μM	min^{-1}	$min^{-1} \cdot mM^{-1}$ (%)
α-KG (1.5–10 μM)	3.20 ± 0.54	643 ± 44	2.0×10^5 (100)
α-Ketoadipate (10–100 μM)	20.6 ± 1.1	290 ± 5	14100 (7.05)
Pyruvate (500–2500 μM)	1020 ± 86	58 ± 2	60 (0.03)
α-Ketobutyrate (150–800 μM)	464 ± 61	89 ± 6	190 (0.10)
α-Ketovalerate (300–1250 μM)	607 ± 47	404 ± 14	660 (0.33)
α-Ketocaproate (200–1000 μM)	583 ± 50	158 ± 7	270 (0.14)
α-Ketoisovalerate (200–1000 μM)	745 ± 36	16 ± 1	20 (0.01)

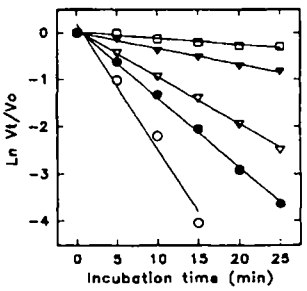

FIG. 7. **Kinetics of inactivation of 2,4-D/α-KG dioxygenase with DEP.** Pseudo-first-order plots for TfdA inactivation by DEP at concentrations of 0.13 mM (□), 0.27 mM (▼), 0.67 mM (▽), 1.0 mM (●) and 1.34 mM (○) in 10 mM MOPS buffer (pH 6.5, 21–22 °C). Ln (V_t/V_0) (where V_t is the velocity at time t and V_0 is the initial velocity) is shown as a function of time.

Chemical Modification of TfdA—DEP modification of TfdA in MOPS buffer (pH 6.75) resulted in the rapid loss of enzyme activity. A plot of the natural logarithm of remaining activity *versus* time at various concentrations of DEP exhibits pseudo first-order kinetics (Fig. 7). Replotting the apparent first-order

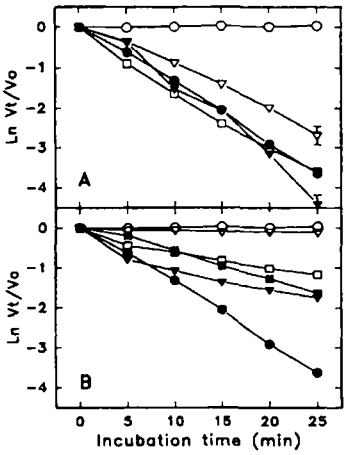

FIG. 8. **Substrate/ligand protection against DEP inactivation of 2,4-D/α-KG dioxygenase.** Residual activities are plotted against the incubation time. The enzyme was incubated with 1 mM DEP in 10 mM MOPS buffer (pH 6.5, 22 °C) in the presence of the indicated compounds. *A*, 1 mM 2,4-D (▽), 1 mM α-KG (▼), 0.5 mM Fe(II) and 0.5 mM ascorbate (□), and no addition (●). *B*, 1 mM 2,4-D, 0.5 mM Fe(II), and 0.5 mM ascorbate (▼), 1 mM α-KG, 0.5 mM of Fe(II), and 0.5 mM ascorbate (□), 1 mM 2,4-D and 1 mM α-KG (▽), 0.1 mM 2,4-D and 0.1 mM α-KG (■), and no addition (●). Activity was also measured in a control sample to which ethanol was added instead of DEP (○).

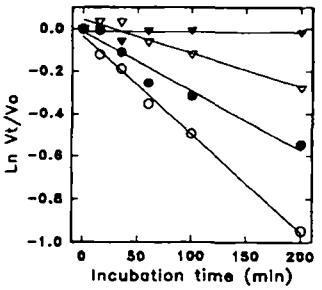

FIG. 9. **Kinetics of inactivation of 2,4-D/α-KG dioxygenase with TNBS.** Pseudo-first-order plots for inactivation of TfdA by TNBS at concentrations of 0 mM (▼), 0.5 mM (▽), 1 mM (●), and 2.5 mM (○) in 10 mM MOPS buffer (pH 6.75, 22 °C).

rate constants as a function of DEP concentration yields a straight line that provides a second-order rate constant of 6.14 M^{-1} s^{-1}. The ability of 2,4-D, α-KG, Fe(II) plus ascorbate, and combinations of these substances to protect the enzyme against DEP inactivation was examined. Whereas none of the individual compounds are able to significantly protect the enzyme from inactivation by DEP (Fig. 8*A*), the combinations of 2,4-D plus Fe(II) or α-KG with Fe(II) clearly decreases the inactivation rate (Fig. 8*B*). Furthermore, the combined presence of 2,4-D and α-KG is very effective in protecting the enzyme from inactivation by DEP.

Although commonly considered a histidine-selective reagent, DEP is known to react with tyrosine, lysine, and cysteine residues (17). We were unable to use the reported spectroscopic method (17) to determine whether a DEP-reactive tyrosine residue is present in TfdA because the DEP-modified protein forms a precipitate. This problem also prevented spectroscopic quantitation of the total DEP-reactive residues. Two approaches were used to assess whether the enzyme possesses a DEP-reactive lysine residue: hydroxylamine treatment of DEP-

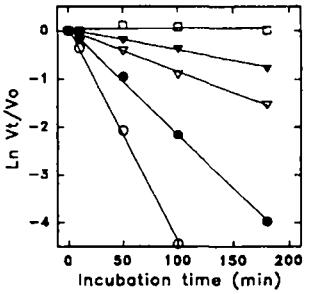

FIG. 10. **Kinetics of inactivation of 2,4-D/α-KG dioxygenase with EDC.** Pseudo-first-order plots for inactivation of TfdA by EDC at concentrations of 0 mM (□), 10 mM, (▼), 20 mM (▽), 50 mM (●), and 100 mM (○) in 10 mM MOPS buffer (pH 6.75, 22 °C).

modified protein and enzyme reactivity toward TNBS. Of the possible DEP-reactive residues usually only modified lysine or cysteine groups are stable to hydroxylamine treatment. Although we found that DEP-treated enzyme was incapable of being reactivated by 0.5 M hydroxylamine (not shown), it remains possible that one or more carbethoxyhistidine residues may be inaccessible to the reagent. The latter explanation is most consistent with our results using the lysine-selective reagent, TNBS. The enzyme was slowly inactivated by TNBS (Fig. 9), however, no protection from inactivation was observed when 1 mM α-KG, 1 mM 2,4-D, or 1 mM α-KG plus 1 mM 2,4-D was included in the reaction mixture. With regard to possible cysteine reactivity toward DEP, neither iodoacetamide (to 100 mM) nor *N*-ethylmaleimide (to 25 mM) inactivated the enzyme after 100 min of incubation. Similarly, 2,3-butanedione (to 100 mM) failed to inactivate the enzyme. In contrast, the carboxyl group-specific modifier, EDC, slowly inactivates the enzyme at high concentrations with pseudo-first-order kinetics (Fig. 10). A replot of the apparent rate constants *versus* EDC concentration is linear and allows calculation of the second-order rate constant of 0.018 M^{-1} s^{-1}. Analysis of substrate protection was impossible with this reagent since 2,4-D and α-KG both have carboxyl groups. These results are inconsistent with the presence of essential cysteine or arginine residues or of highly reactive lysine or carboxyl groups in the enzyme.

DISCUSSION

2,4-D/α-KG dioxygenase was purified to apparent homogeneity from recombinant *E. coli* cells by a simple two-step procedure and several properties of the enzyme were examined. This discussion will focus on comparison of its enzymic properties to that of other α-KG-dependent enzymes, the substrate specificity of the enzyme, and evidence consistent with the presence of multiple essential histidine residues in TfdA.

The specific requirements for α-KG and Fe(II), and the stimulation of activity by ascorbate, are typical characteristics of α-KG-dependent-dioxygenases (9). For example, prolyl 4-hydroxylase is relatively stable when assayed in the presence of ascorbate, whereas, it is rapidly inactivated in the absence of this reductant with 90% of the enzyme becoming inactive in approximately one minute corresponding to 15–30 turnovers (20, 21). In contrast, ascorbate-free 2,4-D/α-KG dioxygenase activity is much more stable with the enzyme carrying out approximately 4000 turnovers before becoming completely inactivated after 30–40 min. Inactivation of prolyl or lysyl hydroxylases is thought to involve a side reaction of these enzymes in which α-KG is decarboxylated in the absence of the peptide substrate leaving the metallocenter in an oxidized state that subsequently must be reduced by ascorbate (22, 23). TfdA,

however, does not appear to catalyze such an ascorbate-dependent decarboxylation of α-KG uncoupled from 2,4-D hydroxylation and enzyme inactivation occurs in the absence of either substrate. Rather than serving to reduce an oxidized enzyme-bound metal center, ascorbate appears to reduce the rate of some other metal ion-mediated enzyme inactivation process. The decrease in enzyme activity that is observed in assays containing both ferrous ion and ascorbate is not due to product inhibition as shown by the lack of any effect when 1 mM 2,4-DCP was included in assay mixtures containing various concentrations (10, 20, 50, and 100 μM) of 2,4-D.

Our results establish the broad substrate range for the hydroxylase reaction of the *tfdA*-encoded dioxygenase. Pieper *et al.* (24) reported that whole cells of *A. eutrophus* JMP134 that were deregulated for *tfdA* expression could degrade a series of 11 phenoxyacetates with chloro- and methyl-substitutions on the phenol ring (although only three of these compounds were growth substrates). Detailed kinetic studies of degradation rates, however, were impossible in their whole cell preparations because of substrate transport and other complications. Here, we were able to evaluate the kinetic constants for seven of the compounds examined by Pieper *et al.* (24) and four other substrates of TfdA. With regard to the observed substrate range of TfdA, it is important to emphasize that not all of these *in vitro* substrates are used by the *A. eutrophus* cells *in situ*. For example, we established that TfdA catalyzes conversion of 2,4,5-T to 2,4,5-trichlorophenol despite the reported inability of *A. eutrophus* JMP134 to catabolize it (25). These results suggest that either the strain can not transport 2,4,5-T or it lacks the ability to degrade 2,4,5-trichlorophenol. Whereas we were unable to detect utilization of 4-(2, 4-dichlorophenoxy)butyric acid by purified TfdA, a *Flavobacterium* species could degrade 4-(2,4-dichlorophenoxy)butyric acid as well as 2-(2,4-dichlorophenoxy)propionic acid, 2-(4-chloro-2-methylphenoxy)propionic acid, and 2,4-D (26). These results may indicate that the enzyme corresponding to TfdA in *Flavobacterium* has a different substrate range or that another enzyme is required; *e.g.* the four-carbon side chain of the substrate may be oxidized to remove two carbons and form 2,4-D. Similarly, we were unable to demonstrate utilization of the methyl ester of 2,4-D despite the known ability of numerous microorganisms to degrade this compound (27). It is likely that a separate enzyme synthesized by these microbes first hydrolyses the methyl ester to release 2,4-D that is subsequently metabolized by a TfdA-like enzyme. Examination of the kinetic values obtained for TfdA reveals that the rates of catalysis for the various substrates are generally quite similar with the exception of the sluggish rates observed with the propionates. Rather, the major differences in the catalytic efficiencies for these substrates involve their affinities for the enzyme. TfdA appears to prefer chlorinated substrates over the unsubstituted compounds. The position of the phenoxy groups at the C-2 carbon of an acid is very important for recognition, although one extra methylene group can be tolerated. The importance of the carboxyl group for substrate recognition is consistent with the presence of a positively charged residue in the 2,4-D binding site.

Although TfdA exhibits greatest efficiency with α-KG as the cosubstrate, many other α-ketoacids are able to be used and α-ketoadipate supports significant levels of hydroxylation. Similar utilization of α-ketoadipate has been reported in the reaction of some other α-KG-dependent dioxygenases such as prolyl 4-hydroxylase (28), but not in γ-butyrobetaine hydroxylase (29). The greatly enhanced affinity of α-ketoacids containing a second carboxyl group is consistent with the presence of a positively charged residue at the α-ketoacid binding site.

Chemical modification studies of TfdA were used to rule out the presence of essential and accessible cysteine or arginine residues and of highly reactive and essential lysine and carboxyl groups. In contrast, DEP rapidly inactivates the enzyme. Myllylä *et al.* (30) reported DEP-dependent inactivation of prolyl 4-hydroxylase and provided some evidence for substrate protection. In our more detailed analysis of substrate protection, neither 2,4-D, α-KG, nor Fe(II) alone was found to protect the enzyme from inactivation; yet, combinations of 2,4-D or α-KG with Fe(II) provide some protection, and a combination of 2,4-D and α-KG afforded nearly complete protection. These results are compatible with the presence of multiple essential histidine residues, as illustrated in Fig. 11, but do not rule out the possible DEP-reactivity of alternate residues. Consistent with the expected requirements for positive charges at the binding sites of 2,4-D and α-KG, we propose that essential histidine residues are present at the binding sites for both of these substrates. In addition, we propose that one or more additional histidine residues may be buried in the protein at the Fe(II) binding site, consistent with the known ability of histidine residues to function as metallocenter ligands. For example, spectroscopic analysis of isopenicillin N synthase, possibly a mechanistically related α-KG-dependent enzyme, suggested the presence of three histidine ligands to Fe(II) in that protein (31, 32). Binding of 2,4-D and α-KG would protect the histidine residues at the substrate binding sites and additionally may protect the Fe(II) ligands by steric constraints. Binding of either 2,4-D plus Fe(II) or α-KG and Fe(II) might lead to the observed reduced rate of inactivation. In contrast, addition of any one compound alone is unable to protect the three or more distinct sites of inactivation postulated in this model. Further studies clearly are required to assess the validity of this model.

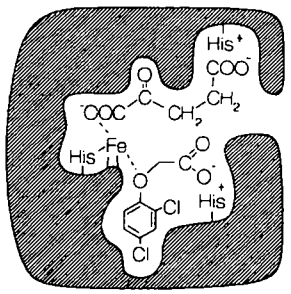

FIG. 11. **Model of the TfdA active site indicating potential roles of essential histidine residues.** The varied abilities of 2,4-D, α-KG, Fe(II), and mixtures of these compounds to protect against TfdA modification by DEP is interpretted in terms of a model that includes three distinct roles for histidine residues: assisting in the binding of 2,4-D, facilitating the binding of α-KG, and ligation of the metallocenter. The illustration is not meant to depict the Fe(II) coordination number or geometry, and the indicated Fe(II) coordination by the α-ketoacid group and the phenoxy oxygen is purely speculative at this time.

Acknowledgment—We thank Katherine Nummy for technical assistance.

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Simplified Quantitative PP ELISA Protocol for AAD1 in Maize Leaf Tissue

For use with AAD1 Microtiter Plate ELISA Test Kit, catalog number CPP 088, Beacon Analytical Systems, Inc.,

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. AAD1 Microtiter Plate ELISA Test Kit, catalog number CPP 088, Beacon Analytical Systems, Inc., Portland, ME 04103 (Phone 1-207-761-2199). Store at 2-8 °C. Contents:

- Antibody coated 96-well microtiter plates
- AAD1 Antibody Conjugate (l20 mL)
- Color Reagent (120 mL)

- Stop Solution (120 mL)
- AAD1 Microtiter Plate ELISA Assay User's Guide

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. Coating buffer, pH 9.6

a. Weigh 0.795 g Na_2CO_3, 1.465 g $NaHCO_3$, and 0.1g NaN_3 in a 500-mL container

b. Dissolve and dilute to almost 500 mL with distilled water,

c. Adjust pH to 9.6 and bring to final volume of 500 mL. Store at 2-8 °C.

F. Blocking solution and HRP conjugate diluent, 1% BSA in PBST

To prepare every 30 mL solution, add 1 mL of 30% BSA into 29 mL of PBST and mix well. Keep on ice and is good for one day.

G. AAD1 standard protein: Obtain AAD1 microbial protein from Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268.

H. 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.

I. 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.

J. AAD1 Stock Solution, 1000 ng/mL.

- Prepare AAD1 liquid stock solution according to instruction from Dow AgroSciences LLC. It may need to be aliquoted and stored in freezer (such as -20°C).
- 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 AAD1 stock; do not re-freeze it for reuse.

3. PROCEDURE

A. Coat the microtiter plate with AAD1 polyclonal antibody. Make a solution of AAD1 antibody that is 1:320 dilution in pH 9.6 carbonate coating buffer (final concentration at 5.0 µg/mL). Add 100 µL to each well of an ELISA microtiter plate. Cover the microtiter plate with a plate sealer and incubate overnight at 4 °C *or at 37 °C for 2 hours.*

B. Wash the coated microtiter plate 5 times with PBST and tap dry. The wash procedure involves flooding each well with washing buffer repeatedly to remove unbound reagents.

C. Block the plate by adding 220 µL Blocking solution in each well, and incubate for at least 2 hours at room temperature.

D. Wash the plate 5 times with PBST using an automatic plate washer. Tap out excess liquid on a paper towel. This blocked plate is ready for loading samples.

E. Prepare the AAD1 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	80	920	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
2.50	500	500	1000	1.25	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.

F. 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.

G. Add the AAD1 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.

H. 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.

I. 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).

J. **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.

K. Prepare the conjugate solution by diluting conjugate stock with 1% BSA solution for 250 times. (e.g., every 50 µL of stock added 12.450 mL of dilutent). Dispense 12 mL of AAD1 antibody conjugate solution per plate into a reagent basin.

L. Pipet 100 µL of the AAD1 antibody conjugate to each well of the antibody coated 96-well microtiter plate. **Discard any unused AAD1 antibody conjugate solution.**

M. Cover the plate with an adhesive plate sealer. Allow the microtiter plate to incubate at room temperature (20-30 °C) with shaking for 30 minutes.

N. **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.

O. 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 20-30 minutes.

P. 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.

Q. 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 AAD1 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 AAD1 in Test Samples:

SOFTmax PRO or Microsoft Excel is used to calculate the AAD1 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 AAD1 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