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Characterization of Aryloxyalkanoate Dioxygenase-12, a Nonheme Fe(II)/α-Ketoglutarate-Dependent Dioxygenase, Expressed in Transgenic Soybean and *Pseudomonas fluorescens*

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Supporting Information

ABSTRACT: Aryloxyalkanoate dioxygenase-12 (AAD-12) was discovered from the soil bacterium *Delftia acidovorans* MC1 and is a nonheme Fe(II)/α-ketoglutarate-dependent dioxygenase, which can impart herbicide tolerance to transgenic plants by catalyzing the degradation of certain phenoxyacetate, pyridyloxyacetate, and aryloxyphenoxypropionate herbicides.[1] The development of commercial herbicide-tolerant crops, in particular AAD-12-containing soybean, has prompted the need for large quantities of the enzyme for safety testing. To accomplish this, the enzyme was produced in *Pseudomonas fluorescens* (*Pf*) and purified to near homogeneity. A small amount of AAD-12 was partially purified from transgenic soybean and through various analytical, biochemical, and *in vitro* activity analyses demonstrated to be equivalent to the *Pf*-generated enzyme. Furthermore, results from *in vitro* kinetic analyses using a variety of plant endogenous compounds revealed activity with *trans*-cinnamate and indole-3-acetic acid (IAA). The catalytic efficiencies (k_{cat}/K_m) of AAD-12 using *trans*-cinnamate (51.5 M^{-1} s^{-1}) and IAA (8.2 M^{-1} s^{-1}) as substrates were very poor when compared to the efficiencies of plant endogenous enzymes. The results suggest that the presence of AAD-12 in transgenic soybean would not likely have an impact on major plant metabolic pathways.

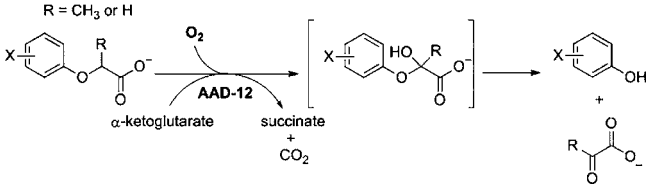
KEYWORDS: *herbicide tolerance, AAD-12, dioxygenase, nonheme Fe(II), Enlist weed control system*

INTRODUCTION

Soybean has recently been modified by insertion of the *aad-12* gene from *Delftia acidovorans* MC1.[1,2] The aryloxyalkanoate dioxygenase-12 (AAD-12) protein has been shown to confer tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) in plants. Characterization of the *aad-12* gene and the resulting translated protein is of significant importance in the safety assessment of transgenic crops.[3] For safety testing of the recombinant AAD-12 protein, large quantities are required for full characterization. Because the concentration of the AAD-12 protein in soybean is extremely low, it is technically infeasible to obtain large quantities of the active protein from transgenic plants.[4] As an alternative strategy, the protein was heterologously expressed in *Pseudomonas fluorescens* (*Pf*), which enabled the purification of a sufficient quantity of the enzyme for the current studies. However, to use the *Pf*-derived protein in safety assessment studies, biochemical and functional equivalence with the plant-derived protein needed to be established.[5]

AAD-12 is a nonheme Fe(II)/α-ketoglutarate-dependent dioxygenase, which catalyzes the degradation of various aryloxyalkanoate herbicides (Scheme 1).[1] Prior *in vitro* kinetic analyses demonstrated that AAD-12 (also known as SdpA) exhibits high catalytic efficiencies toward phenoxyalkanoate substrates.[1,6] Interestingly, AAD-12 can also utilize much larger structures such as aryloxyphenoxypropionates (AOPPs) and nitrogen-containing pyridyloxyalkanoates.[1] Studies have shown that the enzyme enantioselectively oxygenates the *S*-isomer of

Scheme 1. General Reaction Catalyzed by AAD-12



phenoxypropionate and aryloxyphenoxypropionates. The most efficiently utilized substrates reported are *S*-dichlorprop and *S*-2-(4-chloro-2-methylphenoxy)propionate (*S*-MCPP).[1,6] While monosubstituted compounds such as 2- and 4-chlorophenoxypropionates are substrates, the catalytic efficiencies are diminished significantly, suggesting the halogenation pattern on the phenoxy ring is important for substrate recognition. This notion was further substantiated by assessing the activity toward 3-phenoxypropionic acid and (*R*,*S*)-2-(2,4,5-trichlorophenoxy)propionic acid, of which neither had measurable activity under the conditions described.[6] Considerable activity was obtained using 2,4-D (a phenoxyacetate) as a substrate; however, the observed 30−50% decrease in efficiency suggests that the α-methyl group is one of the major binding

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determinants for an efficient substrate.[1,6] Finally, extending the alkanoate chain by two carbon atoms (2,4-dichlorophenoxybutyrate) completely abolished activity. The available *in vitro* substrate specificity studies for AAD-12 and *in silico* homology modeling of SdpA from *Sphingomonas herbicidovorans* MH (~64% amino acid identity to AAD-12) suggest that the following three structural elements are important for productive substrate binding and catalysis: (1) a two- or three-carbon chain that terminates in a free carboxylate (i.e., acetates or propionates), (2) a site amenable to oxidation directly between the phenoxy oxygen and the terminal carboxylate, and (3) a phenoxy or aryloxyphenoxy group.[1,6,7]

The objective of the current study was to purify and characterize AAD-12 produced in two unique transgenic soybean lines (DAS-68416-4 and DAS-444Ø6-6) and *Pf*. A series of immunological, bioanalytical, and biochemical techniques were used to investigate biochemical and functional equivalency. Furthermore, we examined the *in vitro* substrate specificity of *Pf*-derived AAD-12 (*Pf* AAD-12) toward both xenobiotic and endogenous plant compounds.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Chlorinated *trans*-cinnamate analogues and 3-phenylglycolate (3-PGA) were prepared at Dow AgroSciences (Indianapolis, IN, USA). Indole-3-glycolic acid (IGA) was prepared according to literature procedures.[8]

Transgenic Plant Materials. The seeds used to produce the AAD-12-containing tissue (events DAS-444Ø6-6 and DAS-68416-4) were from T4 or T5 generation seed that was homozygous for the presence of the AAD-12 transgene. Leaf punches from each individual plant were harvested fresh from the greenhouse and were analyzed by a lateral flow strip assay, as described below, to confirm the presence of the AAD-12 protein. After confirmation of the presence of the AAD-12 protein, leaf and root tissues were then harvested separately, lyophilized, ground to a fine powder, and stored at −80 °C until use.

Control Plant Materials. The control plants used in this study were nontransgenic soybean plants (*Glycine max* cv. Maverick) that were the same genotype as those transformed with the *aad-12* gene. Seeds of the Maverick soybean line were planted, grown, harvested, tested, and processed under the same conditions as the transgenic plants.

Purification of AAD-12 from Soybean. The AAD-12 protein was extracted with a Tris-based buffer (50 mM Tris-HCl pH 8.0, 20 mM sodium ascorbate, 10 mM sodium metabisulfite, 250 mM NaCl, 0.5% protease inhibitor cocktail (Sigma P8849)) by macerating lyophilized leaf or root tissues in a blender (~1 g of tissue per 10 mL of buffer). The blended material was strained through four layers of cheesecloth, and the tissue was re-extracted at the same buffer-to-tissue ratio. The cheesecloth filtrates were combined and centrifuged at 10000*g* for 20 min. The resulting supernatant was filtered through diatomaceous earth. Ammonium sulfate was slowly added to the extract to a concentration of 1 M. The extract was then stirred for 1 h at 4 °C followed by centrifugation at 10000*g* for 20 min. The supernatant was collected and filtered through a 0.2 μm nylon filter, and the extract was loaded onto a 5 mL Phenyl HP Hi-Trap column (GE Healthcare) equilibrated with 1 M ammonium sulfate and 50 mM Tris-HCl pH 8.0. The column was washed with 2 column volumes (CV) of the equilibration buffer, and the bound proteins were eluted with a 20 CV elution gradient to 100% 5 mM Tris-HCl pH 8.0. Five-milliliter fractions were collected and assayed for AAD-12 content by ELISA (Acadia BioScience, LLC) and by Western blot analysis. Fractions containing the AAD-12 protein were pooled, and polyvinylpolypyrrolidone (PVPP) was added to a final concentration of 3% at 4 °C. The sample was centrifuged at 10000*g* for 10 min. The resulting supernatant was applied onto an immunoaffinity column that consisted of covalently cross-linked anti-AAD-12 monoclonal antibody (mAb 539B304, lot #609.04-2-4) to a Protein A/G resin (Thermo Scientific). The affinity resin was equilibrated with 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 5% glycerol, pH 7.4 (equilibration buffer), and, the PVPP-treated sample was incubated with the resin on an orbital shaker for 1 h at room temperature. The resin was pelleted by centrifugation at 1000*g* for 1 min, transferred to a gravity flow column, and washed with 15 mL of equilibration buffer followed by 2.5 mL of conditioning buffer (Thermo Scientific). The bound AAD-12 protein was eluted with 6.5 mL of elution buffer (Thermo Scientific) into tubes containing 1 M Tris-HCl pH 8.0. Fractions were collected and analyzed by SDS-PAGE, Western blot, and ELISA. The flow-through from the column contained AAD-12 protein and was reapplied to the column and purified as previously described. Fractions containing the AAD-12 protein were pooled and buffer exchanged into 5 mM ammonium bicarbonate using a PD-10 column (GE Healthcare). Aliquots were collected, lyophilized, and used for subsequent analyses.

Overexpression and Purification of AAD-12 from *P. fluorescens*. Recombinant AAD-12 protein was expressed in *Pf* strain DC57. After fermentation, samples were harvested by centrifugation at 10000*g* for 90 min, and the cell paste was frozen at −80 °C for further downstream processing. The frozen cell paste containing the AAD-12 protein was thawed and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, and 1 mM AEBSF) at a ratio of 1 g of wet cell paste per 4 mL of lysis buffer (i.e., 20% w/v solids). Cells were lysed by two passages through a microfluidizer (Microfluidics Corporation) at 15 000 psi. Cell debris and unbroken cells were removed by centrifugation for 30 min at 15 000 rpm at 4 °C. The extract was filtered through a 0.2 μm filter and collected in a 50 L sterile bag (Stedim Biosystems, Inc.). The clarified lysate was applied to a Q Sepharose FF column (GE Healthcare), which was equilibrated with buffer A (20 mM Tris-HCl pH 8.0, 1 mM EDTA). The column was washed with 5 CV of buffer A and then further developed by stepping to 32.5% buffer B (20 mM Tris-HC1 pH 8.0 containing 0.27 M NaCl) followed by a linear gradient from 32.5% to 60% buffer B in 7 CV, followed by a hold step at 60% buffer B. Fractions were collected and analyzed for the presence of AAD-12 by SDS-PAGE analysis. Fractions containing AAD-12 with >80% purity were pooled. Pooled fractions were diluted to 3 g L^{-1} with buffer C (20 mM Tris-HCl pH 8.0 containing 1 M NaCl), applied to a phenyl Sepharose FF column (GE Healthcare), and washed with 3 CV of buffer C. The column was then developed with an 8 CV linear gradient from 10% to 100% buffer D (20 mM Tris-HCl pH 8.0), followed by a hold at 100% D for 12 CV. Fractions were collected and analyzed for the presence of AAD-12 by SDS-PAGE. Fractions containing AAD-12 with >95% purity were pooled and buffer exchanged into 10 mM Tris-HCl pH 8.0, 0.1 M NaCl, and 2 mM dithiothreitol. After buffer exchange, trehalose was added to 1% (w/v), and the material passed through a 0.2 μm vacuum cup filter (Nalgene). The material was then frozen at −80 °C, lyophilized, and stored with desiccant packs at 4 °C.

Lateral Flow Test Strip Assay, SDS-PAGE, and Western Blot of Plant Material. To confirm the presence/absence of the AAD-12 protein in the pooled tissues, approximately 15 mg of the lyophilized tissue was weighed in a 1.5 mL microfuge tube and tested by the lateral flow test strip assay as described by the manufacturer's instructions (American Bionostica, Inc.). The soluble proteins were solubilized by adding 0.5 mL of extraction buffer, with one drop of additive, and grinding with a disposable pestle. The tubes were capped and further mixed by shaking the samples for ~10 s. The resulting supernatants were clarified by centrifuging the samples for 5 min at 20000*g*. The test strips were then incubated in the samples for 10 min. After the assay was complete, the strips were removed and allowed to air-dry, and the results were recorded.

SDS-PAGE and Western blot analysis of extracts from the transgenic events DAS-68416-4 and DAS-444Ø6-6 and nontransgenic Maverick soybean were performed using precast gels (Bio-Rad). Extracts were prepared by geno-grinding (Spex) ~75 mg of tissue with steel ball bearings in phosphate-buffered saline-Tween 20 (PBST) buffer for 3 min in a chilled Teflon microfuge tube holder. The supernatants were clarified by centrifugation for 5 min at 20000*g*, and

160 μL of each extract was mixed with 40 μL of 5× Laemmli sample buffer (LSB). The samples were then heated for 5 min at ∼95 °C. After a brief centrifugation (2 min at 20000*g*), 40 μL of the supernatant was loaded directly on the gel. The microbe-derived AAD-12 and bovine serum albumin (BSA) proteins were diluted with 2× LSB to a concentration similar to that of the soybean-derived AAD-12 protein. After electrophoresis, the gel was cut in half, and one-half was stained with Thermo Scientific GelCode Blue total protein stain. The remaining half of the gel was electroblotted to a nitrocellulose membrane (Bio-Rad) and probed with an AAD-12-specific polyclonal rabbit antibody (lot #DAS F1197-167-2, 4.3 mg mL^{-1}). A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase (Thermo Scientific) was used as the secondary antibody. Chemiluminescent substrate (GE Healthcare, ECL Plus) was used for development and visualization of the immunoreactive protein bands. The membranes were exposed to CL-XPosure detection film (Thermo Scientific) for various time points and subsequently developed with a film developer.

Detection of Post-translational Glycosylation. The purified soybean-derived AAD-12 protein, *Pf* AAD-12, soybean trypsin inhibitor, BSA, and horseradish peroxidase were diluted with LSB, heated at ∼95 °C for 5 min, and centrifuged at 20000*g* for 2 min. The resulting supernatants were applied to a Bio-Rad criterion gel and electrophoresed as described earlier. After electrophoresis, the gel was cut in half, and one-half was stained with GelCode Blue stain for total protein according to the manufacturer's protocol. The remaining half of the gel was stained with a GelCode glycoprotein staining kit (Thermo Scientific) according to the manufacturer's protocol to visualize the glycoproteins.

Peptide Mass Fingerprinting and Sequence Analysis of AAD-12. Purified soybean- and *Pf*-derived proteins were digested in-solution or in-gel. Proteins were reduced, alkylated, proteolytically digested, and subsequently analyzed by MALDI-TOF/TOF and LC-ESI-Q-TOF mass spectrometry. For in-solution digests (microbe-derived protein), AAD-12 protein samples were solubilized in 6 M guanidine hydrochloride in 400 mM ammonium bicarbonate buffer (pH 7.8) and reduced with dithiothreitol for 30 min at 65 °C. The denatured and reduced protein samples were then alkylated with iodoacetamide for 2 h in the dark at ambient temperature. The denatured, reduced, and alkylated samples were buffer exchanged into 50 mM ammonium bicarbonate buffer (pH 7.5) and digested with a combination of various endoproteases for 15 h at 37 °C. For in-gel digests (plant-derived protein), samples were analyzed using SDS-PAGE, and the band corresponding to AAD-12 protein was excised and destained. Samples were reduced, alkylated, and digested in a manner similar to that described above (see Supporting Information for endoproteases used).

For MALDI-TOF/TOF analysis, samples were desalted using ZipTip$_{C18}$ and eluted via step gradient with an increasing concentration of acetonitrile in water supplemented with 0.1% trifluoroacetic acid. Samples were mixed with an α-cyano-4-hydroxycinnamic acid matrix, deposited onto the MALDI target, and air-dried. MALDI-TOF/TOF MS and MS/MS spectra were acquired using an AB Sciex 4800 MALDI-TOF/TOF mass spectrometer.

For LC-ESI-Q-TOF analysis, a 10 μL volume of sample was injected on a Waters Acquity UPLC system equipped with an Acquity BEH C_{18} column (1.7 μm, 150 mm × 2.1 mm) heated to 50 °C and equilibrated in 5% B (solvent A = 0.1% formic acid in water; solvent B = 0.1% formic acid in acetonitrile). The sample was fractionated by employing a gradient of 5−40% B over 58 min with a 100 μL min^{-1} flow rate and was directly subjected to ESI-Q-TOF MS using a Waters Q-Tof Micro mass spectrometer. Data were acquired in ESI positive mode with leucine-enkephalin directly infused as lock mass with lock spray sampling at 7 s (MS) and 10 s (MS/MS) intervals. The acquired spectra were processed manually.

AAD-12 Colorimetric Activity Assay. A colorimetric assay was used to assess the activity of both the soybean- and *Pf*-derived enzymes. The method was adapted to 96-well plate format, and reactions were performed in a total of 150 μL as previously described.[9] Assays contained 100 mM MOPS pH 7.0, 200 μM $(NH_4)_2Fe^{II}(SO_4)_2$, 200 μM sodium ascorbate, the appropriate substrate (in DMSO), and 0.1−1 μM AAD-12. All reactions were initiated by addition of α-ketoglutarate to a final concentration of 1 mM. After the appropriate incubation time, the reaction was terminated by addition of 10 μL of 100 mM sodium EDTA. Phenol products were detected by the addition of 15 μL of pH 10 buffer (3.09 g of boric acid + 3.73 g of KCl + 44 mL of 1 N KOH), 1.5 μL of 2% 4-aminoantipyrine, and 1.5 μL of 8% potassium ferricyanide. After 3 min, the absorbance at 510 nm was recorded in a SpectraMax 190 microplate reader. Control reactions were performed excluding enzyme to account for background absorbances. Kinetic determinations were performed as described except *S*-dichlorprop was varied from 0 to 1 mM.

***P. fluorescens* AAD-12/Succinyl-CoA Synthetase-Coupled (SCS-Coupled) Activity Assay.** Enzyme assays monitoring succinate production were carried out in 96-well plates similar to those described in the literature.[10] Assays contained 100 mM MOPS pH 7.0, 1 mM PEP, 0.4 mM NADH, 0.4 mM coenzyme A, 0.4 mM ATP, 1 mM α-ketoglutarate, 0.1 mM $MgCl_2$, 0.2 mM $(NH_4)_2Fe^{II}(SO_4)_2$, and 0.2 mM sodium ascorbate. The coupling enzymes SCS (5 μM) and pyruvate kinase (6−10 units)/lactate dehydrogenase (9−14 units), along with *Pf*-derived AAD-12 (1−10 μM), were added separately in a final volume of 200 μL. The reactions were initiated by the addition of α-ketoglutarate, and activity was measured in a SpectraMax 190 plate reader monitoring the decrease in absorbance at 340 nm. The linear portion of the reaction progress curve was used to calculate the rate of NADH oxidation (ε_{340} = 6220 M^{-1} cm^{-1}). Water or dimethylsulfoxide (DMSO) replaced substrate in control reactions to account for background oxidation of NADH. Kinetic parameters for *Pf* AAD-12 were determined for nonphenoxy acids at substrate concentrations between 4 and 8000 μM, while *S*-dichlorprop was varied between 2 and 2000 μM. The data were fitted to either the Michaelis−Menten equation (eq 1) or substrate inhibition (eq 2) equation where applicable.

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$v = \frac{V_{max}[S]}{K_m + \left(\frac{[S]^2}{K_i}\right) + [S]} \quad (2)$$

Fourier Transform/Mass Spectrometric (FT/MS) Analysis of *P. fluorescens*-Expressed AAD-12 Reactions. Enzymatic assays were performed as described above except that reactions were allowed to proceed for 45−60 min. Protein was removed from the sample using an Amicon Ultra centrifugal filtration device equipped with a 10 kDa molecular weight cutoff membrane. Mass spectral data were acquired for all samples on a Thermo LTQ-FT Ultra (San Jose, CA, USA). For high-resolution MS (HRMS) analyses, data were acquired in the FT cell at a nominal resolution of 12 500 in wide single-ion-monitoring with a scan range of m/z 100−450. Collisionally induced dissociation spectra were acquired in the ion trap at 35% normalized collision energy, 30 ms activation time, and an activation Q of 0.250. Chromatographic separation was accomplished using a Thermo Accela liquid chromatography system with 10 μL injections of sample onto a 2.1 × 50 mm, 1.9 μm Hypersil Gold (Thermo, San Jose, CA, USA) C_{18} column and eluted into the mass spectrometer using a 12 or 23 min gradient depending on the substrate analyzed. The 12 min gradient ranged from 90% buffer A (0.1% formic acid in water) to 90% buffer B (0.1% formic acid in acetonitrile), and the 23 min gradient ranged from 95% buffer A to 95% buffer B (flow rate = 0.25 mL min^{-1}). Absorption spectra from 200 to 600 nm were also acquired by using the photodiode array.

LC/TOF MS Analysis of *P. fluorescens*-Expressed AAD-12 Activity. Enzymatic assays were performed as described above, except that the SCS-coupling reagents were omitted and reactions were allowed to proceed for 120 min. The protein was removed from the sample using an Amicon Ultra centrifugal filtration device equipped with a 10 kDa molecular weight cutoff membrane. Mass spectral data were acquired in negative ion mode for all samples on an Agilent G1969A accurate-mass ESI/time-of-flight LC/MS (ESI/TOF) with a scan range of m/z 100−1000. Chromatographic separation was

accomplished using an Agilent 1200 liquid chromatography (LC) system. After removal of AAD-12, 10 μL of the flow through was injected onto an Agilent Eclipse XDB-C_{18} column (4.6 × 150 mm, 5.0 μm) and eluted into the mass spectrometer using a 21 min gradient (reaction samples were injected in triplicate). The flow rate was 1 mL min^{-1} and was split into the mass spectrometer at 0.5 mL min^{-1}. Buffer A consisted of 0.1% formic acid in water, and buffer B contained 0.1% formic acid in acetonitrile. Separation of the phenylpropanoid and flavonoid metabolites was achieved via a linear gradient from 5% buffer A to 95% buffer B. UV−visible chromatograms were obtained using a variable-wavelength detector set at 220 nm. Control assays were routinely performed using *S*-dichlorprop to verify AAD-12 activity under various conditions. Additionally, assays lacking α-ketoglutarate and AAD-12 were included to demonstrate that the presence of oxygenated products was due to the activity of AAD-12.

Quantitative Mass Spectrometric Analysis of Reactions with *trans*-2,4-Dichlorocinnamate and *trans*-Cinnamate. For *trans*-2,4-dichlorocinnamate, 47.5 μL of the reaction mixture was removed at various time points and quenched with 2.5 μL of 20% formic acid. Quenched reactions and calibration standards were diluted 10-fold in 0.1% acetic acid in water, mixed, and analyzed directly without further processing. For *trans*-cinnamate, 90 μL of the reaction mixture was removed and quenched with 10 μL of 100 mM EDTA pH 7.4. Protein was subsequently removed by filtering through an Amicon Ultra centrifugal filtration device (10 kDa MWCO membrane). Quenched reactions and calibration standards were diluted 20-fold in water, mixed, and analyzed directly without further processing. *trans*-2,4-Dichlorocinnamate and *trans*-cinnamate reactions were analyzed independently.

Matrix-matched, 12-member calibration standard curves were generated for each analyte ranging from 0.25 to 500 μM. Samples were injected on an Agilent 1290 UPLC system equipped with a BEH C_{18} column (1.7 μm, 50 mm × 2.1 mm) equilibrated in 3% buffer B (buffer A = 0.1% acetic acid in water; buffer B = 0.1% acetic acid in acetonitrile). The samples were fractionated by holding at 3% B for 30 s, then employing a gradient of 3−55% B over 3.5 min at 1.0 mL min^{-1} flow rate with a column temperature of 50 °C. UPLC eluent was directly introduced to an AB Sciex QTRAP 5500 linear ion trap quadrupole LC/MS/MS mass spectrometer without flow splitting. Data were acquired using a multiple-reaction-monitoring (MRM) scan type with previously identified and optimized transitions for the analytes monitored (Table S2 in the Supporting Information). Each sample (reactions and calibration standards) was analyzed in triplicate, and averages were reported. The calibration standards were plotted as area (counts) vs concentration (μM), and the curve was fit to linear regression with no weighting (R^2 > 0.995). Epoxide levels in reactions were determined from referencing the standard curve. The MRM data were integrated and analyzed using AB Sciex Analyst Software v 1.5.1 (Build 5218).

RESULTS AND DISCUSSION

During the development of AAD-12-expressing soybean, two events were selected (events DAS-68416-4 and DAS-444Ø6-6) for protein characterization, and the partially purified soybean-derived AAD-12 proteins were compared with the *Pf*-derived protein. Biophysical and biochemical data were used to investigate if the plant- and microbe-derived proteins were equivalent. Additionally, we assessed the *in vitro* substrate specificity of *Pf* AAD-12 toward a variety of xenobiotic and plant endogenous compounds.

Characterization of AAD-12 from Transgenic Soybean Events and from *P. fluorescens*. The presence of the AAD-12 protein in fresh leaf tissue (T4 or T5 generation) from events DAS-444Ø6-6 and DAS-68416-4 was confirmed using commercially available lateral flow test strips. The strips, capable of detecting between 1 and 10 ppb of AAD-12, easily discriminated between the transgenic events and nontransgenic Maverick tissue. All of the transgenic plants tested were positive for the AAD-12 protein, while none of the controls contained detectable amounts of immunoreactive protein. This result was also confirmed by the Western blot analysis using polyclonal antibodies specific to the AAD-12 protein (Figure 1).

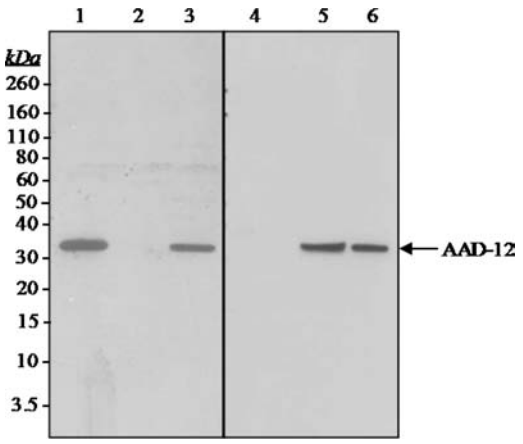

Figure 1. Western blot of AAD-12 proteins expressed in transgenic soybeans and recombinant *Pf*. Lanes 1 and 6, *Pf* AAD-12 (1 ng); lanes 2 and 4, nontransgenic leaf extract; lane 3, event DAS-444Ø6-6 leaf extract; lane 5, event DAS-68416-4 leaf extract.

To characterize the soybean-derived AAD-12 protein, the enzyme was partially purified from transgenic leaf or root tissue using hydrophobic interaction chromatography followed by immunoaffinity chromatography. The protein was examined by SDS-PAGE, which demonstrated that the final concentrated fractions contained the AAD-12 protein at an approximate molecular weight of ∼32 kDa (Figure 2). Once partially purified, the biochemical and physicochemical properties of soybean-derived AAD-12 were compared with those of the *Pf*-derived protein.

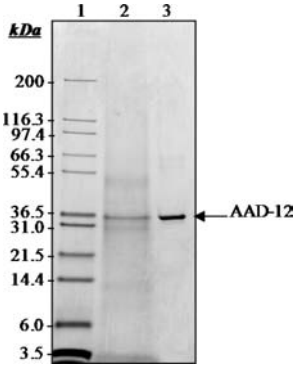

Figure 2. SDS-PAGE of AAD-12 proteins isolated from transgenic soybean and *Pf*. Lane 1, Invitrogen molecular weight markers; lane 2, event DAS-68416-4-derived AAD-12; lane 3, *Pf*-derived AAD-12 (1 μg).

The AAD-12 protein was overexpressed and purified from *P. fluorescens*. Large-scale fermentation was carried out to provide sufficient quantity of protein for toxicological and safety assessment studies. Active AAD-12 was expressed in a soluble form. Protein purification was carried out using conventional ion-exchange chromatography and hydrophobic interaction chromatography, which resulted in the recovery of purified

AAD-12 (Figure 2, lane 3). This large quantity of purified protein was used in characterization and safety studies.

Possible glycosylation of soybean- and microbe-derived AAD-12 was assessed by the GelCode glycoprotein staining kit from Thermo Scientific. The purified soybean-derived AAD-12 protein from events DAS-444Ø6-6 and DAS-68416-4 was subjected to gel electrophoresis simultaneously with a set of control and reference protein standards. Horseradish peroxidase, a glycoprotein, was loaded as a positive indicator for glycosylation. Nonglycoproteins, soybean trypsin inhibitor, and bovine serum albumin were employed as negative reference controls. SDS-PAGE gels stained for total protein demonstrated that the proteins migrated at the expected molecular weight and were readily detectable (Figure 3A; Figure S2A in

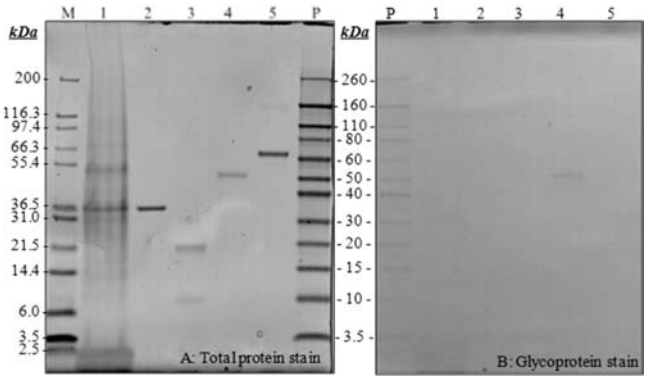

Figure 3. SDS-PAGE gels stained with (A) Coomassie blue and (B) glycoprotein stain. Lane M, Invitrogen molecular weight markers; lane 1, event DAS-68416-4-derived AAD-12; lane 2, *Pf*-derived AAD-12; lane 3, soybean trypsin inhibitor (negative control); lane 4, horseradish peroxidase (positive control); lane 5, bovine serum albumin (negative control); lane P, Novex prestained molecular weight markers.

the Supporting Information). The results of the gel stained for glycoproteins showed that neither the soybean- nor Pf-derived AAD-12 had detectable covalently linked carbohydrates, while the positive control reacted as expected (Figure 3B; Figure S2B in the Supporting Information). Additionally, the AAD-12 protein does not contain motifs that are typically required for glycosylation (Asn-Xxx-Ser/Thr).[11]

Mass spectrometry was also used to verify the amino acid sequence and any putative post-translational modifications of AAD-12 from soybean and *Pf*. Mass spectral analyses of proteolytic digests for soybean-derived AAD-12 protein from events DAS-444Ø6-6 and DAS-68416-4 resulted in peptide sequence coverage of 82.9% and 73.4%, respectively (Figures S3 and S4 in the Supporting Information). In addition, greater than 79% and 20% of the peptide sequence for DAS-444Ø6-6 and DAS-68416-4, respectively, was confirmed by MS/MS analysis, including the N- and C-terminal peptides. This analysis confirmed the microbe-derived AAD-12 protein amino acid sequence matched that of the soybean-derived protein (Figure S5 in the Supporting Information) at both the N- and C-terminus as well as a significant portion of the internal sequence. In the soybean-derived AAD-12 protein, two forms of the N-terminus were detected. The C-termini of the soybean- and microbe-derived AAD-12 proteins were indistinguishable and unmodified. A portion of the soybean-derived AAD-12 proteins was intact as predicted. In addition, a portion of the isolated proteins was missing the N-terminal methionine and the second amino acid, alanine, was acetylated. In eukaryotic (plant)-expressed proteins, this modification is observed in approximately 80−90% of the N-terminal residues.[12,13] This result implied that during or after translation in soybean and *P. fluorescens*, the N-terminal methionine was cleaved by a methionine aminopeptidase (MAP). MAPs cleave methionyl residues rapidly when the second residue on the protein is a small amino acid, such as Gly, Ala, Ser, Cys, Thr, Pro, or Val.[14] Also, it has been previously shown that proteins with serine and alanine at the N-termini are the most frequently acetylated.[15] The two co-translational processes, cleavage of an N-terminal methionine residue and N-terminal acetylation, are by far the most common modifications and occur on the vast majority of eukaryotic proteins.[15] However, examples demonstrating biological significance associated with N-terminal acetylation are rare.[16] Results of these analyses indicate that the amino acid sequence of the *Pf*-expressed protein is equivalent to that of the soybean-derived AAD-12 protein.

Comparison of Substrate Specificity and Kinetic Parameters of *P. fluorescens*-Derived and Soybean AAD-12. The substrate specificity of AAD-12 from soybean root extracts (event DAS-68416-4) and *Pf* was assessed by colorimetrically monitoring for phenol formation. The activities for the enriched soybean and purified *Pf* proteins were determined using a variety of phenoxyalkanoate substrates and AOPP graminicides (Figure 4). Both enzymes catalyzed

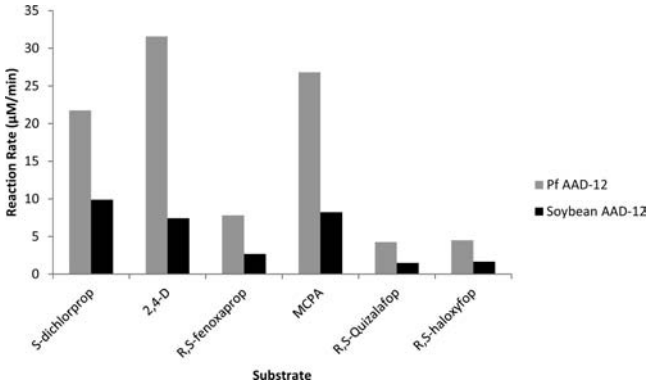

Figure 4. Substrate specificity of *Pf* AAD-12 (gray bars) and soybean AAD-12 (black bars). Reactions with contained 0.3 μM AAD-12 and 1 mM substrate (for AOPPs, 500 μM effective substrate concentration).

the degradation of the synthetic auxin herbicides 2,4-D, MCPA, and *S*-dichlorprop. The *S*-enantiospecificity was also demonstrated, as *R*-dichlorprop was not a substrate for either enzyme. The activity of the *Pf*-derived enzyme toward the AOPP herbicide had previously been established using *S*-cyhalofop.[1] Here, both enzymes demonstrated activity toward racemic mixtures of *R,S*-fenoxaprop, *R,S*-quizalofop, and *R,S*-haloxyfop (Figure 4). While the substrate specificity for both the *Pf*- and soybean-derived proteins was identical, the overall reaction rates for soybean AAD-12 were 35−50% of the purified *Pf* enzyme (>95% purity). The lowered reaction rates may be attributed to inaccuracies associated with determining the soybean AAD-12 concentration or soybean matrix interference, as AAD-12 represented only ∼1−2% of the total soluble protein.[5]

Both soybean and *Pf* enzymes displayed non-Michaelis−Menten kinetics using *S*-dichlorprop as a substrate (0−1 mM). Kinetic plots revealed that both enzymes experience substrate inhibition at *S*-dichlorprop concentrations over 200 μM;

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Table 1. Kinetic Parameters for *Pf* AAD-12[a]

substrate	K_m (μM)	V_{max} (μM/min)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_i (μM)	relative k_{cat}/K_m (M^{-1} s^{-1})
trans-cinnamate	629 ± 31.0	9.74 ± 0.14	0.032	51.5		0.019
trans-2-chlorocinnamate	322 ± 36.4	8.80 ± 0.25	0.029	90.9		0.034
trans-4-chlorocinnamate	47.4 ± 3.55	9.22 ± 0.10	0.031	647	9930 ± 467	0.242
trans-2,4-dichlorocinnamate	76.8 ± 5.11	17.2 ± 0.41	0.057	743	6610 ± 703	0.278
trans-3,4-dichlorocinnamate	12.1 ± 0.98	5.26 ± 0.11	0.018	1450	2020 ± 146	0.542
indole-3-acetic acid	3410 ± 170	16.9 ± 0.40	0.030	8.2		0.003
S-dichlorprop	21.7 ± 3.21	17.4 ± 1.00	5.80	267000	748 ± 122	100

[a]Kinetics were determined using the SCS-coupled assay.

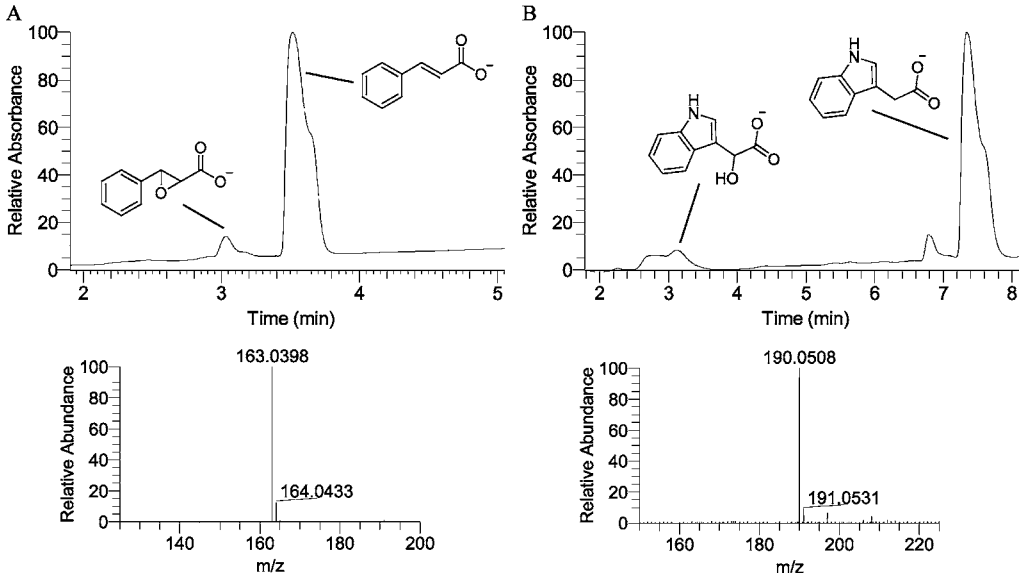

Figure 5. FTMS analysis of the *Pf* AAD-12 reactions using *trans*-cinnamate (A) and IAA (B) as substrates. Mass spectrum of 3-PGA (inset A) and IGA (inset B).

however, inhibition of *Pf* AAD-12 appears to be more pronounced than that of the enriched soybean enzyme (Figure S6 in the Supporting Information). Substrate inhibition using *S*-dichlorprop has been previously reported for SdpA.[6]

Activity of *P. fluorescens*-Derived AAD-12 toward Endogenous Plant Compounds. Activity assays were intended to determine if AAD-12 may impact important pathways when expressed in genetically modified plants. The activity of *Pf* AAD-12 was assessed using endogenous plant compounds as substrates. Relevant substrates were identified on the basis of structural similarity to herbicidal auxins, similar physiological function to known xenobiotic substrates, or presence within major primary/secondary metabolic pathways of plants. The endogenous compounds tested were separated into three groups: (1) the natural plant hormones indole-3-acetic acid (IAA), abscisic acid (ABA), and gibberellin (GA), (2) various phenylpropanoid and flavonoid secondary metabolites, (3) all 20 L-amino acids (Figures S7 and S8 in the Supporting Information). The activity toward plant endogenous compounds was initially examined by an *in vitro* enzyme-coupled system that detects succinate production (SCS-coupled assay). This system was used to guide high-resolution mass spectrometric analyses of oxidation products where applicable (either FT/MS or TOF). Together the data demonstrate that, of the endogenous compounds examined (Figure S8 in the Supporting Information), only *trans*-cinnamate and IAA were oxidized to a detectable extent and only when excessive amounts of enzyme were used. The SCS-coupled assay allowed for the determination of kinetic parameters using *trans*-cinnamic acid and IAA as substrates. The k_{cat} and K_m values for AAD-12 using *trans*-cinnamate were 0.032 s^{-1} and 629.3 μM, respectively (Table 1). The K_m was significantly elevated using IAA (~3.4 mM) and the k_{cat} was 0.03 s^{-1}. Mass spectrometry was used to identify the reaction products when *trans*-cinnamate and IAA were used as substrates. The retention times, UV−visible spectra, and MS^2 fragmentation patterns are consistent with the oxidation of *trans*-cinnamate to 3-phenylglycidate and IAA to indole-3-glycolic acid (IGA) (Figure 5).

Endogenous plant enzymes that efficiently utilize free *trans*-cinnamate have been described. Cinnamate-4-hydroxylase catalyzes the conversion of *trans*-cinnamate to *p*-coumarate during the biosynthesis of phenylpropanoids from L-phenylalanine. The enzyme from *Arabidopsis* has been characterized *in vitro* and has a K_m for *trans*-cinnamate of 0.5 μM and k_{cat}/K_m of 3.4×10^6 M^{-1} s^{-1}.[17] Additionally, the catalytic efficiency of UDP-glucose:cinnamate glucosyltransferase from strawberry has been reported.[18] The k_{cat}/K_m using *trans*-cinnamate was 0.42 μM^{-1} s^{-1}, which is ~8400-fold greater than that of *Pf* AAD-12. These kinetic parameters suggest that *trans*-cinnamate present *in planta* is efficiently directed into phenylpropanoid/flavonoid biosynthesis and that the presence of AAD-12 in transgenic crops would not likely impact the *trans*-cinnamate pool.

Indole-3-acetic acid (IAA) exists primarily as ester and amide conjugates *in planta*.[19] IAA amidosynthetase catalyzes the ATP-dependent amide bond formation between IAA and L-aspartic

acid. The enzyme from rice has a K_m of 123 μM for IAA and its k_{cat}/K_m is 2.75 × 10^3 M^{-1} s^{-1}.[20] A second enzyme, IAA glucosyltransferase, catalyzes the esterification of free IAA to glucose. The *Arabidopsis* enzyme has a K_m of 240 μM, while the enzyme from *Zea mays* has a K_m of 1.08 mM.[21,22] As with *trans*-cinnamate, the K_m and V_{max} using IAA were determined via the SCS-coupled assay (Table 1). A comparison of these kinetic parameters to endogenous plant enzymes suggests that *Pf* AAD-12 does not efficiently utilize the auxin.

Determination of Kinetic Parameters for *P. fluorescens*-derived AAD-12 Using *S*-Dichlorprop and *trans*-Cinnamates *via* the SCS-Coupled Assay. Previously, utilizing a colorimetric assay, AAD-12 kinetic measurements demonstrated that *S*-dichlorprop was utilized most efficiently followed by 2,4-D, 2-methyl-4-chlorophenoxyacetic acid (MCPA), and *S*-cyhalofop.[1] Using the SCS-coupled assay, the *Pf* AAD-12 kinetic parameters were re-evaluated with *S*-dichlorprop as a substrate (Table 1). The K_m and V_{max} were 21.7 μM and 17.42 μM min^{-1}, respectively. Additionally, a fit to eq 2 allowed for the determination of K_i, which was 748.3 μM.

Prior studies on alternative substrates for TfdA, another 2,4-D-degrading enzyme, reported the conversion of *trans*-olefins to epoxides for chloro-substituted and unsubstituted cinnamates.[23] Therefore, the substrate specificity of *Pf* AAD-12 toward *trans*-cinnamates was further expanded by determining the kinetic parameters for differentially substituted chloro-*trans*-cinnamates. The catalytic efficiencies demonstrated that the presence of chlorine atoms at positions 3,4- and 2,4- are significantly preferred over the unsubstituted phenyl (Table 1). The analyses also revealed that *Pf* AAD-12 displays substrate inhibition for all substrates except *trans*-cinnamate and *trans*-2-chlorocinnamate.

Uncoupled Succinate Production by *P. fluorescens*-Derived AAD-12. The SCS-coupled assay is limited to detection of the byproduct succinate. Under certain conditions succinate can be produced without oxidation of the primary substrate (i.e., the absence of the primary substrate or the presence of a poor substrate). This is referred to as "uncoupling" and has been demonstrated to occur among many α-ketoglutarate-dependent dioxygenases.[10,24] The drastic decrease in efficiencies using the series of *trans*-cinnamates led us to examine the extent to which uncoupled succinate production occurs. Consequently, quantitative mass spectrometry was employed to obviate detection of uncoupled succinate production by directly measuring oxygen addition to the primary substrate. To assess the extent of uncoupling catalyzed by *Pf* AAD-12, oxidized products were quantified in reactions using *trans*-2,4-dichlorocinnamate and *trans*-cinnamate as substrates. Initial rates for succinate production were simultaneously measured by the SCS-coupled assay and mass spectrometry at both 62.5 and 500 μM substrate. The rates of succinate production at 62.5 and 500 μM *trans*-2,4-dichlorocinnamate were 12.1 and 26.8 μM min^{-1}, respectively. The initial rates obtained via mass spectrometry were 3.52 and 4.91 μM min^{-1}, demonstrating that *Pf* AAD-12 catalyzes the production of succinate at approximately 0.18−0.29 the coupling efficiency when using *trans*-2,4-dichlorocinnamate as a substrate. Similar levels of uncoupling were obtained with *trans*-cinnamate (Table S2 in the Supporting Information). These results confirm that poor substrates can trigger uncoupled succinate production by *Pf* AAD-12 and suggest that the reported catalytic efficiencies determined for non-preferred substrates using the SCS-coupled assay are likely overestimated.

In conclusion, the results from this investigation support the functional and biological equivalency of soybean- and *Pf*-derived AAD-12 enzymes. This conclusion is supported by their similar immunoreactivity, physicochemical properties, and *in vitro* activity. Endogenous plant compounds were characterized as very poor substrates for *Pf* AAD-12 based on *in vitro* kinetic analyses, suggesting that the presence of the enzyme in transgenic soybean would not likely impact major plant metabolic pathways. These results are in agreement with the compositional equivalence previously reported between event DAS-68416-4 soybean and nontransgenic soybean (Maverick).[25] Collectively, these results support the use of the *Pf*-derived AAD-12 protein as a surrogate for the soybean-derived enzyme in safety studies.

■ ASSOCIATED CONTENT

Supporting Information

Protein sequence coverage maps for soybean- and *Pseudomonas fluorescens*-derived AAD-12 are provided. Additionally, structures of endogenous and xenobiotic compounds used in *in vitro* assays and substrate specificities are also provided. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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