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Production, Purification, and Characterization of Recombinant AAD-12 Expressed in *Pseudomonas fluorescens* for Submission on Supporting Regulatory Toxicology and Eco-toxicology Study

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Six 20 L scale fermentations were carried out and subjected to large scale purification for AAD-12 (Aryloxyalkanoate Dioxygenase) at the Dow San Diego facility. One fermentation tank with induction conditions at 32 °C showed low AAD-12 expression in the soluble fraction (3-4 g/L) and was difficult to capture and purify by column chromatography. Subsequent tank fermentations were performed with induction conditions at 25 °C and had constant higher levels of soluble AAD-12 expression (9-13 g/L). The AAD-12 protein could be purified from those bulk materials to homogeneity in two column steps using anion exchange and hydrophobic interaction chromatography. The pilot scale purification was then carried out in two batches where batch EP466-026 yielded 19 g of purified AAD-12, and batch EP466-028 combined yielded 47 g of AAD-12. Final purified products were lyophilized for long-term storage and shipped to DAS for additional characterization by SDS-PAGE, native gel, MALDI-TOF mass spectrometry, total amino acid analysis, and enzymatic activity determination. The purified batches of AAD-12 were comparable in quality and both retained full activity to degrade 2,4-D with an apparent V_{max} of 3.7-4.9 $\mu\text{mol/min/mg}$ of protein, and a K_m for 2,4-D of 130-140 μM . The products were released to DAS Regulatory Lab for conducting anticipated toxicology and eco-toxicology studies to support DAS AAD-12 soybean de-regulation.

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INTRODUCTION

In order to supply enough protein samples to conduct toxicity and eco-toxicity test, supporting a stage phase 1C activity for a DAS transgenic soybean registration processes, a large scale (in the range of 50-100 grams) production of AAD-12 was conducted in collaboration between DAS Indy and Dow San Diego sites. A previous study was released, which detailed molecular cloning, expression and small scale protein purification studies on the target protein AAD-12 (Lin *et al.*, 2007). This report summarizes the process data, and protein analysis on the large production lots from both sites, as an additional supplement for Regulatory submission document.

MATERIALS AND METHODS

Bacterial Strain

The *P. fluorescens* strain DC579 is derived from host MB324 strain by transformation with pMYC1803 expression vector carrying a synthetic *AAD-12* gene, details on molecular cloning, protein expression, and small scale purification study were described previously (Lin *et al.* 2007).

P. fluorescens Fermentation and Sampling

The seeds for the fermentor cultures were each generated by inoculating a shake flask containing 600 ml of PS20 medium, a chemically defined medium supplemented with yeast extract and glycerol, with 1 ml frozen culture glycerol stock ($OD_{600} = 4-5$). After 16 to 24 hours of incubation with shaking at 32 °C, the shake flask culture was then aseptically transferred to a 20 L fermentor containing DGMp2.2 medium. Dissolved oxygen was maintained at a positive level in the liquid culture by regulating the sparged air flow and the agitation rates. The pH was controlled at the desired set-point of 6.5 through the addition of aqueous ammonia. Temperature was initially controlled at 32 °C. The fed-batch high density fermentation process was divided into an initial growth phase of approximately 24 hours, and a gene expression phase, in which 0.3 mM IPTG was added to initiate recombinant gene expression and the temperature control set-point was adjusted to 25 °C (except one initial comparative experiment for testing 32 °C). The expression phase of the fermentation was then allowed to proceed for 18-24 hours. During this phase, samples were withdrawn from the fermentor to determine cell density, the level of target gene expression, and other parameters. For SDS-PAGE and Western blot analyses, aliquots of 0.1 ml of whole fermentation broth were centrifuged to separate the cell free broth from the cells, which were then separated and frozen for subsequent analysis. At the final time point of 24 hour (18 hour only for the last four runs, adjusted for optimal AAD-12 expression) post-induction, larger samples were harvested by centrifugation in 1 L bottles (750 ml broth each) at $10,000 \times g$ for 90 min. The cell pellets and cell free broths were separated and frozen at -80 °C for further downstream processing. The remaining culture was rendered non-viable *in-situ* either through a holding period at low pH solution or by treatment with heat steam.

SDS-PAGE and Western Blot Analysis of Fermentation Samples

Frozen cell pellets derived from 100 μ l broth were resuspended in chilled water to an equivalent dilution of 5-fold of the original fermentation broths and a 200 μ l sample was sonicated for 10 minutes (Branson Ultrasonics). The lysates were centrifuged at $16,000 \times g$ for 20 minutes at 4 °C and the supernatants were removed (as soluble fraction). The pellets were then resuspended in 200 μ l of phosphate buffered saline (PBS, pH 7.2). Further dilutions of samples of an equivalent of up to 80-fold of the original fermentation broth were performed in PBS. Cell free broths from the fermentor were diluted in PBS to an equivalent of 4-fold of the original fermentation broths. Samples were prepared for SDS-PAGE using a Criterion 12% Bis-Tris gel (Bio-Rad cat# 345-0118) with MES buffer under non-reducing condition. Gels were stained with Coomassie Blue, densitometry was performed using the Personal Densitometer SI (Molecular Devices) with ImageQuant software.

For Western blot, soluble and insoluble fractions were separated by SDS-PAGE as described above, with the exception that only 10 μ l was loaded per lane, and transferred onto nitrocellulose (Bio-Rad cat# 162-0232) using $1 \times$ Tris-Glycine-SDS transfer buffer (Invitrogen cat# NP0006) for 1 hour at 100 V. After transfer, the blot was washed briefly in $1 \times$ PBS and then blocked in 1% casein in PBS Blocker (Pierce cat#37528) at 4 °C. The blocker was poured off and fresh solution was added containing a 1:5,000 dilution of rabbit anti-AAD12 sera. The blots were incubated 4 hours or longer at room temperature. The antibody/diluent solution was then poured off and the blots washed in $1 \times$ PBST (Sigma cat#P-3563) with vigorous shaking for 5 minutes. The PBST was changed and washing was repeated twice. After washing, diluent containing a 1:5,000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate was added to blots. The blots were incubated for 2 hours at room temperature. The blots were then washed as described above. For development, the blots were removed from the PBST solution and immersed in prepared solution using the 1-Step NBT/BCIP (Pierce cat#34042). The blots were incubated with gentle shaking for 10 minutes and then removed from the solution and allowed to dry on paper. The blots were immediately photographed using an Alpha Innotech FluorImager. Densitometry was performed using the ImageQuant software.

Lysate Preparation

All buffers for downstream processing steps were prepared with deionized water ($< 18 \text{ m}\Omega\text{-cm}$) using a Millipore Super-Q System (Model 2FSQ115P4). All tubing used for downstream processing steps meets FDA and USP criteria (available from PharMed, Westlake, OH, catalog # 06485-36 and catalog # 06485-73). Frozen cell paste was thawed and resuspended in lysis buffer [20 mM Tris-HCl, pH 8.0 (JT Baker, cat# 4109-06) containing 1 mM EDTA (ethylenediaminetetraacetic acid, JT Baker, cat# 899401) and 1 mM AEBSF (Sigma, cat# INHIB-1)], at a ratio of 1 g wet cell paste per 4 mL lysis buffer (i.e., 20% w/v solids). Cells were lysed by two passages through a Microfluidizer (model 110Y, Microfluidics Corporation, Newton, MA) at 15,000 psi. Cell debris and unbroken cells were removed by centrifugation for 30 min at 15,000 rpm at 4 °C using Beckman JA-10 model, 8.1000 rotor, and 1 L polypropylene bottles (type 366752) with liners [(JLA-8/9 1K), Beckman-Coulter, Inc., Fullerton, CA].

The extract was passed through Cuno Biocap 1000 30ZA and 60ZA depth filters (CUNO, Inc., Menden, CT, cat#s BC1000A30ZA, and BC1000A60ZA, respectively) at 1.2 L/min. The filtrate

was diluted with deionized milli-Q water at 1:2 ratio, passed through a 10" Sartorius SARTOBRAN P, 0.2 micron filter (Sartorius North America, Inc., product number 5231307HQ00-V) at 1 L/min, and collected in a 50 L sterile bag (Stedim Biosystems, Inc., Concord, CA, Flexboy Assembly 50L, cat# 952554) and stored at 4 °C for further processing. The filtrate conductivity was < 5 mS/cm.

Chromatography

All pilot-scale chromatography steps were performed using a BioProcess Engineering System (Pharmacia model 1226, 6 mm skid) with UNICORN 5.0 control software. All steps were performed at 4 °C inside a cold-room.

Anion exchange chromatography. A BPG 200 column (GE Healthcare, part# 56-1168-57) containing 8 L Q Sepharose FF (GE Healthcare, cat# 17-0510-01) media was flowed packed (20 cm diameter; 25 cm bed height) and qualified for use with *HETP* values of $N/m > 4,000$ and peak symmetry values of $A_s 1.0 \pm 0.2$, respectively. The flow velocity was adjusted to $64 \text{ cm} \cdot \text{h}^{-1}$ to maintain a 24 min residence time on the column. The column was equilibrated with buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) and the protein loading was approximately 17 g AAD-12 per liter of resin. After loading, the column was washed with 5 CV of equilibration buffer A, and then further developed by stepping to 32.5% buffer B (20 mM Tris-HCl, 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. Column fractions were collected in polypropylene containers as 1 L aliquot. The column was cleaned in place (CIP) with 2 CV of 20 mM Tris, pH 8.0, 2 M NaCl and sanitized in place with 2 CV of 1 N NaOH (JT Baker, cat# 3728-07). The column was stored in 0.1 N NaOH. Fractions were analyzed for the presence of AAD-12 by SDS-CGE/PAGE and/or RP-HPLC. Fractions containing AAD-12 with > 80% purity were pooled and held at 4 °C.

Hydrophobic interaction chromatography. A BPG 200 column containing 5 L Phenyl Sepharose FF (GE Healthcare, cat# 17-5095-01) media was flowed packed (packed column volume in 20 cm diameter and 20 cm bed height) and qualified for use with *HETP* values of $N/m > 4,000$ and peak symmetry values of $A_s 1.0 \pm 0.2$, respectively. The flow velocity was adjusted to $200 \text{ cm} \cdot \text{h}^{-1}$ to maintain a 4.8 min residence time on the column. The sample was diluted with equilibration buffer A (20 mM Tris-HCl, pH 8.5 containing 1M NaCl) resulting in a concentration of 3 g/L of AAD-12, and a column load of approximately 20 g AAD-12 per liter of resin. The column was washed with 3 CV of buffer A after sample loading. The column was then developed with an 8 CV linear gradient from 10% to 100% buffer B (20 mM Tris-HCl, pH8.0), followed by a hold at 100% B for 12 CV. Column fractions were collected in polypropylene containers. The column was washed with 2 CV of water and 2 CV of 30% v/v isopropanol (EMD, catalog number PX1834-1). The column was stored in 0.1 N NaOH. Fractions were analyzed for the presence of AAD-12 by SDS-CGE/PAGE and/or RP-HPLC. Fractions containing AAD-12 with > 95% purity were pooled and held at 4 °C.

Buffer Exchange

Buffer exchange was performed using a tangential flow filtration system (Millipore Proflux M12 Tangential Flow Filtration) equipped with an ultrafiltration membrane (Millipore Pellicon 2 membrane, part number P2C005C01, 5 kDa MWCO, 1.5 m² area). The Phenyl Sepharose eluate

pool was concentrated to ~10 g/L AAD-12 and exchanged with 3 diavolumes of final buffer (10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM dithiothreitol (DTT, MP Biochemicals, Inc., cat# 100597)) at 4 to 8 °C. During diafiltration, the trans-membrane pressure was maintained at about 15 psi resulting in a permeate flux of 12 L/m²-h. After buffer exchange, trehalose (Sigma, cat# T-9531) was added to 1% w/v and the material passed through a 0.2 µm vacuum cup filter (Nalgene, cat# 567-0020). The material was then poured into stainless steel lyophilization trays (~1 L per 50 × 25 × 4 cm tray) and frozen at -80 °C.

Lyophilization

Lyophilization was performed using a Virtis Genesis 25ES freeze dryer. Trays containing frozen AAD-12 with temperature monitoring thermocouples were placed under vacuum (< 250 mTorr) and subjected to the following temperature ramp: 12 hours at -25 °C; 12 hours at -10 °C; 12 hours at 0 °C; 12 hours at 10 °C; and 12 hours at 25 °C. The material was scraped from trays into 500 ml plastic bottles (VWR, product number 15900-198), weighed, and placed at 4 °C.

Endotoxin Assay

Bacterial endotoxin (LPS) levels were determined by gel-clot LAL method (USP 85) and performed by MoBio Laboratories, Inc. (Carlsbad, CA). Serial two-fold and ten-fold dilution schemes in pyrogen-free water were conducted for each sample, with an end point dilution at 1:16 and 1:10⁻⁸, respectively. Prior to dilution, all samples were boiled for 15 min to denature proteins in the sample matrix. Control Endotoxin Standard (CES) and Limulus Amebocyte Lysate (LAL) used were obtained from Associates of Cape Cod, Inc. (East Falmouth, MA, product number E0005 and product number G5006, respectively; lysate sensitivity was 0.06 EU/mL). LAL-free water from Bio Whittaker, Inc. (product number W50-100) was used. The assay was conducted at 36 to 38 °C in a heat block, with an incubation time of 60 min (± 2 min). A true assay end point was defined as the last clotting dilution followed by two consecutive negative dilutions.

Reversed-Phase HPLC Analysis

Separations were carried out on an Agilent 1100 Series liquid chromatography system (Agilent Technologies, Inc., Palo Alto, CA) equipped with an autosampler, quaternary pump, and diode array detection modules. Mobile phase reagents were of analytical grade or best available. Acetonitrile used was HPLC grade (cat# AH015, Burdick and Jackson, Muskegon, MI). Trifluoroacetic acid (TFA) was obtained from Pierce (cat# 28904). Deionized water was obtained using a Milli-Q system (Millipore, Bedford, MA) and filtered prior to use with 0.2 µm nylon filters (Nalgene, cat# 567-0020). Mobile phase A contained 0.1 % TFA in water (v/v); solvent B contained 0.1 % TFA in Acetonitrile (v/v). Samples were diluted 1:10 in reducing sample buffer [6.0 M guanidine hydrochloride (Sigma, cat# G4505), 400 mM dithiothreitol (Invitrogen, cat# 15508-013) in 85 mM Tris, pH 7.5 (Trizma Pre-Set Crystals pH 7.5, Sigma, cat# T-7818)], and 10 µl injected onto a Zorbax 300SB-CN (Agilent, Part No. 880995) column (300 Å pore, 5 µm particle size, 4.6 × 250 mm) equipped with a Zorbax 300SB-CN guard column. The flow rate was 1 ml/min and UV detection was set at 215 and 280 nm wavelengths. Mobile phase was programmed to run a linear gradient from 35% to 50% mobile phase B over 30 min. The column was operated at 50 °C.

Protein Quantification

The protein concentration was obtained by using several methods during the processing. Bradford assay was used to determine total protein content with a Bio-Rad Protein assay kit (cat# 500-0006). Capillary gel electrophoresis (CGE) using Caliper Protein LabChip 90 was applied to analyze in-process samples (Caliper Life Sciences, Protein Express Labchip kit, part# 760301) followed manufactures' guidelines. RP-HPLC for semi- and purified sample analysis as described above. For final production lot, total amino acid analysis (AAA) was used to determine the protein concentration. Hydrolyzed samples were analyzed in Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA) using amino acid calibration standards purchased through Agilent (cat# PN5061-3330).

SDS-PAGE and Native Gel Analysis of Purified Samples

SDS-PAGE and native-PAGE gel analysis were applied to final purified samples. SDS-PAGE condition was essentially identical as described before, except that a 12% NuPAGE gel from Invitrogen (cat# NP0323BOX) was used to replace Bio-Rad Criterion gel for smaller set of samples. For native gel analysis, 1 µg of protein sample was applied to 4-20% Tris-Glycine gel (Invitrogen cat# EC26502BOX) with 1 × Tris-Glycine running buffer and electrophoresed at 100 V for 2 hours. Native protein size makers were purchased from Amersham (cat# 17-04450-01) as molecular weight standards. Gel was stained with Coomassie Blue for protein visualization.

MALDI TOF Mass Spectrometry

Purified AAD-12 proteins were submitted to DAS Proteomics Lab for MALDI-TOF-MS analysis. The suspected AAD-12 protein band from an SDS-PAGE gel (approximately 1 µg) was excised and treated in 25% acetonitrile/12.5 mM ammonium bicarbonate, and dried in a Speed-Vac centrifuge tube. The eluted protein was then digested with 12.5 ng/µl of Trypsin (Promega, cat# V5111) at 37 °C overnight. The reaction was stopped by adding 10% formic acid, and peptides were purified with a C18 Zip Tip (Millipore, cat# 2TC18S096). Mass spectral analyses were performed on a Voyager Biospectrometer (PerSeptive Biosystems, model DE STR). A sample of 1 µl of the digested protein was mixed with 1 µl of Maldi matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA). One microliter of the mixture was spotted onto the Maldi sample plate and mass spectra were collected in the positive ion reflector mode. The data were input into the analysis program PAWS (Proteometrics Inc.) to search for the peptide identity as compared to the AAD-12 sequence.

AAD-12 Enzyme Activity Assay

Enzyme activity was determined using a colorimetric assay that detecting the product phenol (Hotopp *et al.*, 2003). Lyophilized AAD-12 protein was reconstituted in water at a concentration approximately 4 mg/ml (protein) based on total amino acid analysis results and dry power weight. Assays were performed in 96-well ELISA plates with a total volume of 0.16 ml containing 20 mM MOPS, pH 6.8, 200 µM NH₄FeSO₄, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, and the appropriate substrate (added from a 100 mM 2,4-D stock made up in DMSO). AAD-12 protein was added at serial-diluted concentrations in triplicate. Reactions were initiated by addition of α-ketoglutarate at time zero. After 5 minutes of incubation at room temperature, the reaction was terminated by addition of 30 µl of a 1:1:1 mix of 50 mM Na EDTA

pH 10 buffer (borate buffer) (3.09 g boric acid + 3.73 g KCl + 44 ml 1 N KOH) and 2% 4-aminoantipyrine. Then 10 μ l 0.8 % potassium ferricyanide was added and after 5 min, the absorbance at 510 nm was read in a spectrophotometric reader (SpectraMax 190, Beckman Coulter) using SoftMax Pro software (Molecular Devices).

RESULTS AND DISCUSSION

Growth and Expression in 20 L Fermentations

Growth of strain DC579 was evaluated at the 20L fermentation scale and two induction temperatures were tested for AAD-12 expression at San Diego site. One tank (060124G) was performed using the standard 32 °C induction temperature and the second tank (060124F) was performed at 25 °C for strain DC579 after receiving from DAS. Previous work conducted in shake flasks suggested that among many parameters surveyed, including various culture media, inducer concentrations, induction lengths, and induction temperatures, the single most critical one affecting AAD-12 expression is the choice of using lower induction temperatures (20-25 °C) (see Lin *et al.*, 2007). At elevated growth temperatures (28-32 °C), most of AAD-12 was accumulated in an insoluble and inactive form. Growth of DC579 in the high cell density fed-batch fermentation process from both culturing temperatures was typical. Both of the cultures reached the cell density targeted for induction of 160 to 170 absorbance units (AU) measured at 575nm (OD) at 20 to 22 hr elapsed fermentation time. During the expression phase, the cell densities were maintained at OD's ranging from 180 to 270 AU.

As shown in figure 1, AAD-12 expression was clearly detectable on SDS-PAGE in the soluble fractions and in the insoluble fractions from six hours post-induction onwards. AAD-12 bands in the cell free broth fractions were barely detectable. The strong signals of the bands co-migrating with the AAD-12 standard on the Western blot suggested that the identity of the protein as AAD-12 overproduced in the soluble fractions, as well as the insoluble and cell free broth fractions (Figure 2). According to estimation by SDS-CGE, AAD-12 accumulation peaked at approximately at 9 to 13 g/L in the fermentations set at 25 °C during induction (from total five tanks fermentation), whereas one fermentation set at 32 °C only peaked at approximately 3.5 g/L.

Aliquot samples from the first two tanks were further evaluated for their activity on degrading 2,4-D, a preferred substrate for the AAD-12 enzyme. The samples were processed by sonication and fractionation, similar as described before, upon receiving shipment from SD site. Only soluble materials were subjected to further analysis and enzymatic assay (Figures 3 and 4). By activity determination, the sample from 25 °C induction tank has higher activity, peaked at 18 hrs time point, and then decreased by about 15% at 24 hrs time point. By comparison, samples from 32 °C induction tank contained flat 4-8 fold less activity, peaked earlier at 12 hrs and then slightly decreased over time by the 18 and 24 hrs points.

Taken together, the data suggested that strain DC579 with Tank G (060124G) induction conditions was suitable for large scale purification processing. In preparation for purification campaigns, four additional 20 L fermentations were performed using these optimal parameters for producing AAD-12. All tanks were harvested at 18 hrs post-induction. The yield estimation for AAD-12 was measured by densitometry analysis of the SDS-PAGE (Figure 5). The AAD-12 expression at 25 °C induction ranged from 9 to 13 g/L in fermentation, compared to only approximately 3 g/L at the 32 °C induction fermentation. All of the cultures reached the cell density targeted for induction of 160 to 170 absorbance units (AU) measured at 575nm (OD) at

20 to 22 hr elapsed fermentation time. During the expression phase, the cell densities were maintained at OD's ranging from 180 to 270 AU (Figure 6).

AAD-12 Purification

A general AAD-12 protein purification method was previously developed at the DAS Indy site, which can process approximately 0.5 kg of cell paste and recover 4-5 grams of final purified materials (Lin *et al.*, 2007). The method applied two sequential column separations involving anion exchange (IEX) to capture and hydrophobic interaction chromatography (HIC) to improve yield of the AAD-12 protein with $\geq 99\%$ purity (Figure 7). This process was further optimized and adjusted to process multiple kilograms of cell paste per run at a larger scale. This was achieved by scaling down the process to 20-ml columns (20 mL bed volume) and optimizing processing times to achieve the same residence time and resolution at skid chromatography (small pilot) scale.

In the protein primary recovery stage, the use of the complete protein inhibitor cocktails from the previous protocol was eliminated. Instead, only AEBSF, a key serine protease inhibitor, in combination of EDTA, was utilized in the sample preparation for cost savings. This approach seemed to work well to prevent AAD-12 degradation in the upstream processes. Figure 8 shows the protein profile after primary recovery of the soluble fraction from one of the production batch, as can be seen, no visible AAD-12 degradation was observed.

It is worthy to note that during the AAD-12 chromatography development, that AAD-12 from extracts derived from the 060124G tank (i.e., the 32 °C induced tank), failed to efficiently bind to the capture column using the developed column parameters. The binding capacity was approximately 1-2 g AAD-12/L resin and the subsequent chromatography resolution and purity was very poor. Further analysis and scouting with AAD-12 tank material derived from 060124F (i.e., the 25 °C induced tank) appeared to bind at a much higher capacity (~12 g/L resin) and eluted with higher purity. This observation suggested that AAD-12 from the higher temperature induction fermentation was in the form of a large soluble aggregate (in comparison to the lower temperature induction), however no further analysis was performed to characterize this form of AAD-12. All further purification development was performed with AAD-12 derived from fermentations with 25 °C induction conditions.

After optimization, two larger scale chromatography columns were packed, a 7 L Q-Sepharose FF column, and a 5.5 L Phenyl Sepharose FF column, respectively, were used for the larger scale process. One challenge was that the flow rate set at a small scale was too slow for the pumps on the skid (for process scale). A linear gradient from 0-6% buffer B could not be achieved. Therefore, process flow rate was manually increased, and a step elution gradient was also utilized. Another challenge was the use of a linear gradient which had caused additional difficulties and sample fraction pooling, it required more analytical effort and could result in some pooling errors. Therefore more step gradients at pilot scale operation were recommended and used in the next rounds of separation.

Protein purity after the two-step column separation was achieved close or above 90% from intermediate to large scale purification. As shown in the SDS-PAGE analysis in Figure 13, when

overloaded, the only other visible contaminant protein was an approximately 20 kDa unknown sample, AAD-12 was the dominant band with apparent molecular weight of 31 kDa. The endotoxin (LPS) level was also tested from several scouting runs and the final preparation, in all cases the reading was below 0.02 EU/ μ g sample. This level of LPS/protein was shown acceptable via an IP injection study in mice (Carney *et al.*, 2005). The measured LPS level on batch EP466-026 was 0.006 EU/ μ g.

AAD-12 from batch EP466-026 was buffer exchanged and lyophilized from 2.3 L solution containing 10 mM Tris (2.9 g), 100 mM NaCl (13.3 g), 2 mM DTT (0.7 g) and 1% Trehalose (23 g). The protein concentration measured by total amino acid hydrolysis was 9.08 mg/ml. Freeze-drying produced a final 58 g of dried fine powder. By calculation, approximately 39.9 g was the non-protein solids (salts and additives as stabilizer), and approximately 18-20 g (or about 1/3) was AAD-12 protein in this sample. More specifically, this batch contains 19.4 g of AAD-12 by the mass calculation. A small portion (< 2-3%) of this batch was also removed from the bulk and used in our biochemistry group to study its broad herbicidal activities across DAS herbicide collections before releasing to the Regulatory Lab. The column processes and protein analysis for batch EP466-026 are included in figures 9, 10, and 13.

Another batch (EP466-028) was subsequently processed. The endotoxin level for this batch was also very low, reported to be 0.00007 - 0.0001 EU/ μ g. The sample was lyophilized from 5.6 L of solution in 10 mM Tris (1.21 g/L), 0.1 M NaCl (5.84 g/L), 2 mM DTT (0.31 g/L), and 1% Trehalose (10 g/L). The sample was divided into 2 parts of roughly 2.3 L each and subjected for lyophilization process. Due to technical problems with the freeze-dryer, there was some ice still remaining in batch #2, therefore the sample was resolubilized in pure water and sent to lyophilization for another round. This later batch was designated as EP466-028B, and the first half was then designated as EP466-028A. The dry weight of the powder for EP466-028A was 70 g, and was 60 g for EP466-028B. They were released directly to our Regulatory Lab. The column processes and protein analysis for batch EP466-028 are recorded in figures 11, 12, and 13.

The final product was analyzed by total amino acid hydrolysis (AAA) for most accurate quantification. Total AAA results confirmed that the percentage of protein in batch EP466-026 is 33.6%. And batches EP-466-028A and EP466-028B each had identical 36.7% protein content in the lyophilized material. Tables 1-3 summarized the AAA results for each released product (EP466-026, EP466-028A, and EP466-028B). AAA results confirm amino acid content consistent with the predicted gene translation product.

Aliquots from each released batch of about 10-20 mg in dry weight were removed from the bottled bulk sample, and used for further comparison study with AAD-12 reference standard. Those include gel analysis (Fig. 14), maldi PMF analysis (Fig. 15), and activity determination (Fig. 16).

In summary, biochemical analyses for all three batches of AAD-12 received from SD group (EP466-026, EP466-028A, and EP466-028B) were identical to the reference standard in terms of protein purity and enzymatic activity. Therefore the protein batches met the requirements specified for toxicology studies. Because the samples were in lyophilized form, they were stored

conveniently in 4 °C in cold room or kept in the refrigerator. Long-term protein stability has not yet been investigated, although previous work from smaller-scale processing demonstrated that lyophilized samples were stable over a year (Lin *et al.*, 2007). The materials described here were released and submitted to Regulatory Lab.

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TABLE I. Total Amino Acid Analysis of Batch EP466-026.

AA Protein Residue Recovery Calculator
Version 1.8 - SG 1/11/2005
Protein: AAD12

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	699	768682	17470	46.6	105.8%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	280	249151	14656	15.1	88.8%
E GLU	Y	129	11	11	1419	1419	419	202938	18449	12.3	111.8%
F PHE	Y	147	8	8	1176	1176	130	143083	17885	8.7	108.4%
G GLY	N	57	25	0	1425	0	312	0	0	0	
H HIS	Y	137	16	16	2192	2192	223	245151	15322	14.9	92.8%
I ILE	Y	113	11	11	1243	1243	167	183922	16720	11.1	101.3%
K LYS	N	128	6	0	768	0	27	0	0	0	
L LEU	Y	113	24	24	2712	2712	386	424056	17669	25.7	107.0%
M MET	Y	131	10	10	1310	1310	127	139352	13935	8.4	84.4%
N ASN	Y	114	4	4	456	456		58624	14656	3.6	88.6%
P PRO	N	97	14	0	1358	0					
Q GLN	Y	128	14	14	1792	1792		258285	18449	15.6	111.8%
R ARG	Y	156	18	18	2808	2808	286	314154	17453	19.0	105.7%
S SER	N	87	13	0	1131	0	153	0	0	0	
T THR	Y	101	18	18	1818	1818	228	250438	13913	15.2	84.3%
V VAL	N	99	24	0	2376	0	300	0	0	0	
W TRP	N	186	8	0	1488	0					
Y TYR	Y	163	5	5	815	815	82	90012	18002	5.5	109.1%

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	1309	1440010	32727	46.8	106.4%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	526	468045	27532	15.2	89.5%
E GLU	Y	129	11	11	1419	1419	787	380988	34635	12.4	112.6%
F PHE	Y	147	8	8	1176	1176	241	265466	33183	8.6	107.9%
G GLY	N	57	25	0	1425	0	625	0	0	0	
H HIS	Y	137	16	16	2192	2192	414	454991	28437	14.8	92.5%
I ILE	Y	113	11	11	1243	1243	304	334315	30392	10.9	98.8%
K LYS	N	128	6	0	768	0	48	0	0	0	
L LEU	Y	113	24	24	2712	2712	708	779038	32460	25.3	105.5%
M MET	Y	131	10	10	1310	1310	236	259279	25928	8.4	84.3%
N ASN	Y	114	4	4	456	456		110128	27532	3.6	89.5%
P PRO	N	97	14	0	1358	0					
Q GLN	Y	128	14	14	1792	1792		484894	34635	15.8	112.6%
R ARG	Y	156	18	18	2808	2808	529	581842	32325	18.9	105.1%
S SER	N	87	13	0	1131	0	286	0	0	0	
T THR	Y	101	18	18	1818	1818	430	473131	26285	15.4	85.5%
V VAL	N	99	24	0	2376	0	553	0	0	0	
W TRP	N	186	8	0	1488	0					
Y TYR	Y	163	5	5	815	815	153	168646	33729	5.5	109.7%

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	1396	1536072	34911	46.7	106.1%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	561	499726	29396	15.2	89.3%
E GLU	Y	129	11	11	1419	1419	841	407020	37002	12.4	112.5%
F PHE	Y	147	8	8	1176	1176	260	285736	35717	8.7	108.5%
G GLY	N	57	25	0	1425	0	666	0	0	0	
H HIS	Y	137	16	16	2192	2192	437	481166	30073	14.6	91.4%
I ILE	Y	113	11	11	1243	1243	326	358380	32580	10.9	99.0%
K LYS	N	128	6	0	768	0	47	0	0	0	
L LEU	Y	113	24	24	2712	2712	763	839356	34973	25.5	106.3%
M MET	Y	131	10	10	1310	1310	254	278868	27887	8.5	84.8%
N ASN	Y	114	4	4	456	456		117583	29396	3.6	89.3%
P PRO	N	97	14	0	1358	0					
Q GLN	Y	128	14	14	1792	1792		518025	37002	15.7	112.5%
R ARG	Y	156	18	18	2808	2808	566	622833	34602	18.9	105.2%
S SER	N	87	13	0	1131	0	305	0	0	0	
T THR	Y	101	18	18	1818	1818	461	507567	28198	15.4	85.7%
V VAL	N	99	24	0	2376	0	593	0	0	0	
W TRP	N	186	8	0	1488	0					
Y TYR	Y	163	5	5	815	815	164	180084	36017	5.5	109.5%

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	1576
Theoretical (ug/mL)	1433
average protein (ug/mL)	475
% Protein	33.2%
dalton	31675
residues	actual 20 used 13
average pmol protein	16506
maximum residue recovery	111.8%
minimum residue recovery	84.3%
% residue coverage	68.3%
% RSD ave. protein	10.6%

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	2882
Theoretical (ug/mL)	2620
average protein (ug/mL)	886
% Protein	33.8%
dalton	31675
residues	actual 20 used 13
average pmol protein	30754
maximum residue recovery	112.6%
minimum residue recovery	84.3%
% residue coverage	68.3%
% RSD ave. protein	10.4%

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	3070
Theoretical (ug/mL)	2791
average protein (ug/mL)	947
% Protein	33.9%
dalton	31675
residues	actual 20 used 13
average pmol protein	32904
maximum residue recovery	112.5%
minimum residue recovery	84.8%
% residue coverage	68.3%
% RSD ave. protein	10.5%

Average protein content: 33.6%

TABLE II. Total Amino Acid Analysis of Batch EP466-028A.

AA Protein Residue Recovery Calculator

Version 1.8 - SG 1/11/2005

Protein: AAD12 466-028A Rep 1

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	1891	2079656	47265	44.6	101.4%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	908	808820	47578	17.4	102.1%
E GLU	N	129	11	0	1419	0	1164	0	0	0	
F PHE	Y	147	8	8	1176	1176	340	373655	46707	8.0	100.2%
G GLY	Y	57	25	25	1425	1425	1070	1177421	47097	25.3	101.0%
H HIS	N	137	16	0	2192	0	610	0	0	0	
I ILE	N	113	11	0	1243	0	431	0	0	0	
K LYS	N	128	6	0	768	0	200	0	0	0	
L LEU	Y	113	24	24	2712	2712	1029	1132399	47183	24.3	101.2%
M MET	N	131	10	0	1310	0	134	0	0	0	
N ASN	Y	114	4	4	456	456		190311	47578	4.1	102.1%
P PRO	N	97	14	0	1358	0					
Q GLN	N	128	14	0	1792	0		0	0	0	
R ARG	N	156	18	0	2808	0	719	0	0	0	
S SER	Y	87	13	13	1131	1131	530	582469	44805	12.5	96.1%
T THR	Y	101	18	18	1818	1818	731	803702	44650	17.2	95.8%
V VAL	N	99	24	0	2376	0	875	0	0	0	
W TRP	N	186	8	0	1488	0					
Y TYR	N	163	5	0	815	0	174	0	0	0	

Protein: AAD12 466-028A Rep 2

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	1587	1745210	39664	44.6	101.3%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	764	680032	40002	17.4	102.2%
E GLU	N	129	11	0	1419	0	978	0	0	0	
F PHE	Y	147	8	8	1176	1176	285	313937	39242	8.0	100.2%
G GLY	Y	57	25	25	1425	1425	901	991392	39656	25.3	101.3%
H HIS	N	137	16	0	2192	0	507	0	0	0	
I ILE	N	113	11	0	1243	0	363	0	0	0	
K LYS	N	128	6	0	768	0	166	0	0	0	
L LEU	Y	113	24	24	2712	2712	864	950214	39592	24.3	101.1%
M MET	N	131	10	0	1310	0	107	0	0	0	
N ASN	Y	114	4	4	456	456		160008	40002	4.1	102.2%
P PRO	N	97	14	0	1358	0					
Q GLN	N	128	14	0	1792	0		0	0	0	
R ARG	N	156	18	0	2808	0	606	0	0	0	
S SER	Y	87	13	13	1131	1131	444	488914	37609	12.5	96.1%
T THR	Y	101	18	18	1818	1818	613	674114	37451	17.2	95.7%
V VAL	N	99	24	0	2376	0	733	0	0	0	
W TRP	N	186	8	0	1488	0					
Y TYR	N	163	5	0	815	0	149	0	0	0	

Protein: AAD12 466-028A Rep 3

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	3091	3399731	77267	44.5	101.0%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	1489	1325588	77976	17.3	102.0%
E GLU	N	129	11	0	1419	0	1910	0	0	0	
F PHE	Y	147	8	8	1176	1176	557	612474	76559	8.0	100.1%
G GLY	Y	57	25	25	1425	1425	1768	1944390	77776	25.4	101.7%
H HIS	N	137	16	0	2192	0	997	0	0	0	
I ILE	N	113	11	0	1243	0	706	0	0	0	
K LYS	N	128	6	0	768	0	341	0	0	0	
L LEU	Y	113	24	24	2712	2712	1688	1856553	77356	24.3	101.2%
M MET	N	131	10	0	1310	0	308	0	0	0	
N ASN	Y	114	4	4	456	456		311903	77976	4.1	102.0%
P PRO	N	97	14	0	1358	0					
Q GLN	N	128	14	0	1792	0	1166	0	0	0	
R ARG	N	156	18	0	2808	0		0	0	0	
S SER	Y	87	13	13	1131	1131	868	954784	73445	12.5	96.0%
T THR	Y	101	18	18	1818	1818	1201	1321090	73394	17.3	96.0%
V VAL	N	99	24	0	2376	0	1438	0	0	0	
W TRP	N	186	8	0	1488	0					
Y TYR	N	163	5	0	815	0	270	0	0	0	

Average protein content: 36.7%.

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	4018
Theoretical (ug/mL)	3653
average protein (ug/mL)	1342
% Protein	36.7%
dalton	31675
residues	actual 20 used 8
average pmol protein	46608
maximum residue recovery	102.1%
minimum residue recovery	95.8%
% residue coverage	52.2%
% RSD ave. protein	2.6%

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	3344
Theoretical (ug/mL)	3040
average protein (ug/mL)	1127
% Protein	37.1%
dalton	31675
residues	actual 20 used 8
average pmol protein	39152
maximum residue recovery	102.2%
minimum residue recovery	95.7%
% residue coverage	52.2%
% RSD ave. protein	2.6%

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	6694
Theoretical (ug/mL)	6085
average protein (ug/mL)	2202
% Protein	36.2%
dalton	31675
residues	actual 20 used 8
average pmol protein	76469
maximum residue recovery	102.0%
minimum residue recovery	96.0%
% residue coverage	52.2%
% RSD ave. protein	2.5%

TABLE III. Total Amino Acid Analysis of Batch EP466-028B.

AA Protein Residue Recovery Calculator

Version 1.8 - SG 1/11/2005

Protein: AAD12 466-028B Rep 1

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	1549	1703899	38725	44.6	101.4%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	747	665063	39121	17.4	102.5%
E GLU	N	129	11	0	1419	0	952	0	0	0	0
F PHE	Y	147	8	8	1176	1176	277	304684	38085	8.0	99.8%
G GLY	Y	57	25	25	1425	1425	878	965591	38624	25.3	101.2%
H HIS	N	137	16	0	2192	0	489	0	0	0	0
I ILE	N	113	11	0	1243	0	351	0	0	0	0
K LYS	N	128	6	0	768	0	156	0	0	0	0
L LEU	Y	113	24	24	2712	2712	840	924307	38513	24.2	100.9%
M MET	N	131	10	0	1310	0	60	0	0	0	0
N ASN	Y	114	4	4	456	456		156485	39121	4.1	102.5%
P PRO	N	97	14	0	1358	0					
Q GLN	N	128	14	0	1792	0		0	0	0	0
R ARG	N	156	18	0	2808	0	588	0	0	0	0
S SER	Y	87	13	13	1131	1131	433	476846	36680	12.5	96.1%
T THR	Y	101	18	18	1818	1818	598	657757	36542	17.2	95.7%
V VAL	N	99	24	0	2376	0	712	0	0	0	0
W TRP	N	186	8	0	1488	0					
Y TYR	N	163	5	0	815	0	139	0	0	0	0

Protein: AAD12 466-028B Rep 2

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	1656	1821360	41395	44.6	101.4%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	795	708060	41651	17.3	102.0%
E GLU	N	129	11	0	1419	0	1021	0	0	0	0
F PHE	Y	147	8	8	1176	1176	297	327105	40888	8.0	100.2%
G GLY	Y	57	25	25	1425	1425	940	1033815	41353	25.3	101.3%
H HIS	N	137	16	0	2192	0	526	0	0	0	0
I ILE	N	113	11	0	1243	0	378	0	0	0	0
K LYS	N	128	6	0	768	0	174	0	0	0	0
L LEU	Y	113	24	24	2712	2712	901	990648	41277	24.3	101.1%
M MET	N	131	10	0	1310	0	90	0	0	0	0
N ASN	Y	114	4	4	456	456		166602	41651	4.1	102.0%
P PRO	N	97	14	0	1358	0					
Q GLN	N	128	14	0	1792	0		0	0	0	0
R ARG	N	156	18	0	2808	0	631	0	0	0	0
S SER	Y	87	13	13	1131	1131	463	509833	39218	12.5	96.1%
T THR	Y	101	18	18	1818	1818	640	703912	39106	17.2	95.8%
V VAL	N	99	24	0	2376	0	769	0	0	0	0
W TRP	N	186	8	0	1488	0					
Y TYR	N	163	5	0	815	0	151	0	0	0	0

Protein: AAD12 466-028B Rep 3

Protein Analysis Profile							Analysis				
Name (symbol)	Use Residue in Analysis (Y/N)	Residue FW	residues	residues analyzed	AA Residue Mass	AA Selected Mass	pmol/L residue	residue pmol in solution	average pmol protein	residue calculated	residue recovery (%)
A ALA	Y	71	44	44	3124	3124	1635	1797991	40863	44.7	101.5%
C CYS	N	103	3	0	309	0					
D ASP	Y	115	17	17	1955	1955	785	698814	41107	17.4	102.1%
E GLU	N	129	11	0	1419	0	1008	0	0	0	0
F PHE	Y	147	8	8	1176	1176	293	322639	40330	8.0	100.2%
G GLY	Y	57	25	25	1425	1425	927	1019521	40781	25.3	101.3%
H HIS	N	137	16	0	2192	0	516	0	0	0	0
I ILE	N	113	11	0	1243	0	373	0	0	0	0
K LYS	N	128	6	0	768	0	167	0	0	0	0
L LEU	Y	113	24	24	2712	2712	888	972777	40720	24.3	101.1%
M MET	N	131	10	0	1310	0	28	0	0	0	0
N ASN	Y	114	4	4	456	456		164427	41107	4.1	102.1%
P PRO	N	97	14	0	1358	0					
Q GLN	N	128	14	0	1792	0		0	0	0	0
R ARG	N	156	18	0	2808	0	623	0	0	0	0
S SER	Y	87	13	13	1131	1131	456	501767	38597	12.5	95.9%
T THR	Y	101	18	18	1818	1818	631	694137	38563	17.2	95.8%
V VAL	N	99	24	0	2376	0	761	0	0	0	0
W TRP	N	186	8	0	1488	0					
Y TYR	N	163	5	0	815	0	148	0	0	0	0

Average protein content: 36.7%.

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	3298
Theoretical (ug/mL)	2998
average protein (ug/mL)	1099
% Protein	36.7%
dalton	31675
residues	actual 20 used 8
average pmol protein	38177
maximum residue recovery	102.5%
minimum residue recovery	95.7%
% residue coverage	52.2%
% RSD ave. protein	2.7%

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	3522
Theoretical (ug/mL)	3202
average protein (ug/mL)	1175
% Protein	36.7%
dalton	31675
residues	actual 20 used 8
average pmol protein	40817
maximum residue recovery	102.0%
minimum residue recovery	95.8%
% residue coverage	52.2%
% RSD ave. protein	2.6%

Protein Sample	
Protein Solution (uL)	1100
protein (ug)	3476
Theoretical (ug/mL)	3160
average protein (ug/mL)	1159
% Protein	36.7%
dalton	31675
residues	actual 20 used 8
average pmol protein	40258
maximum residue recovery	102.1%
minimum residue recovery	95.8%
% residue coverage	52.2%
% RSD ave. protein	2.6%

FIGURES

Figure 1. SDS-PAGE Analysis of Two 20 L Fermentations for Strain DC579 at Two Induction Temperatures

Soluble, insoluble, and cell free broth (supe) samples from two tanks conducted at 25 °C and 32 °C, respectively, were analyzed by SDS-PAGE. The time course samples are denoted by 0, 6, 12, 18, and 24 hr post-induction. The molecular weight markers (MW) are in kDa. The AAD-12 bands are indicated by the arrows.

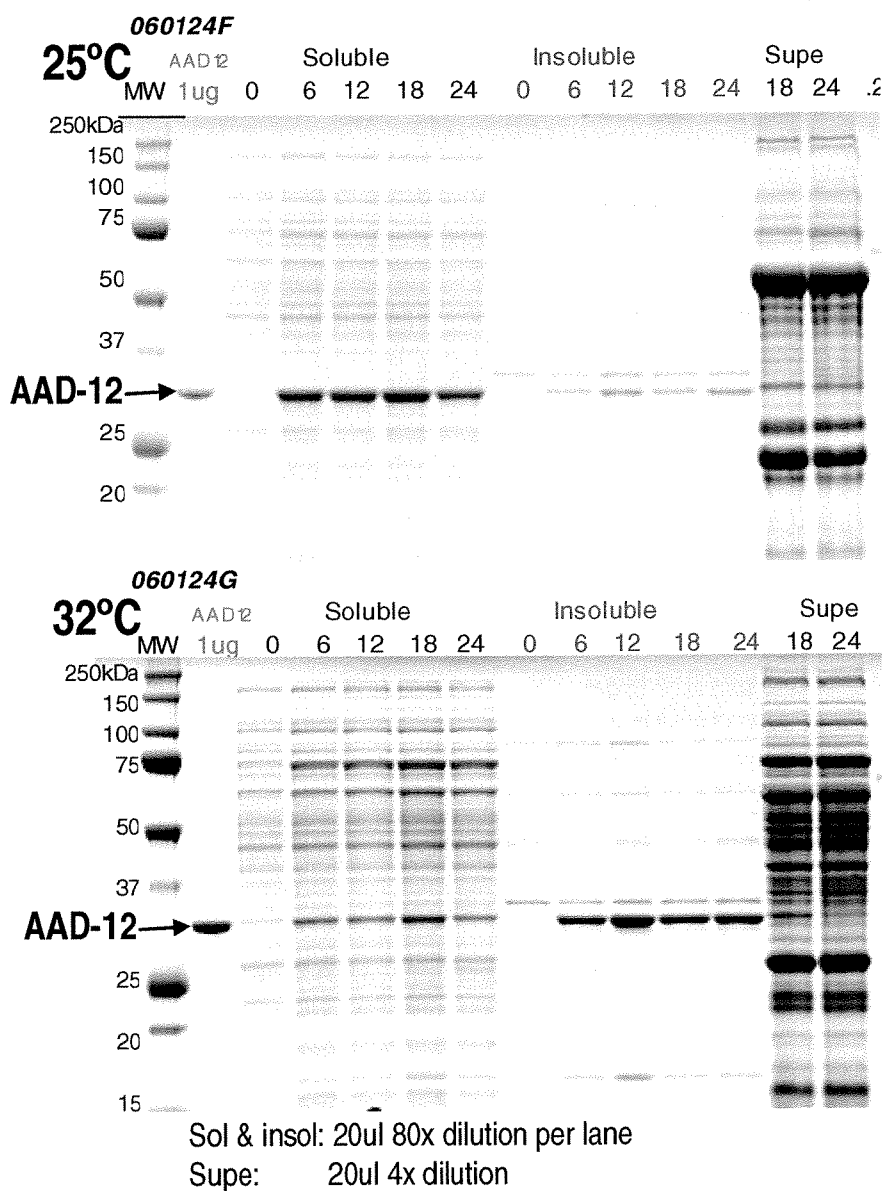


Figure 2. Western Blot Analysis of AAD-12 Expression in 20 L Fermentations

Soluble, insoluble, and cell free broth (supe) samples from two 20 L tanks, F and G, respectively, were analyzed by SDS-PAGE and Western blot. The time course samples are denoted by 0, 6, 12, 18, and 24 hr post-induction. The molecular weight markers (MW) are in kDa. The AAD-12 bands are indicated by the arrow.

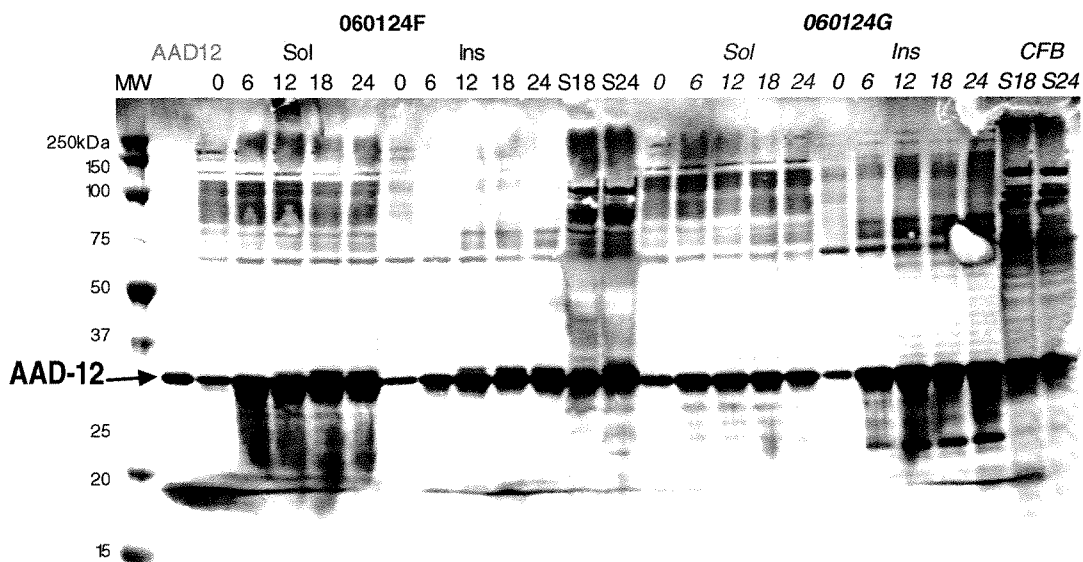


Figure 3. Soluble AAD-12 Protein Comparison by SDS-PAGE

Only soluble fractions from aliquots collected in the two tanks (060124F and 060124G) were analyzed by SDS-PAGE. The time course samples are denoted by 12, 18, and 24 hrs post-induction. Control is the AAD-12 reference standard. AAD-12 protein band is indicated by the arrow.

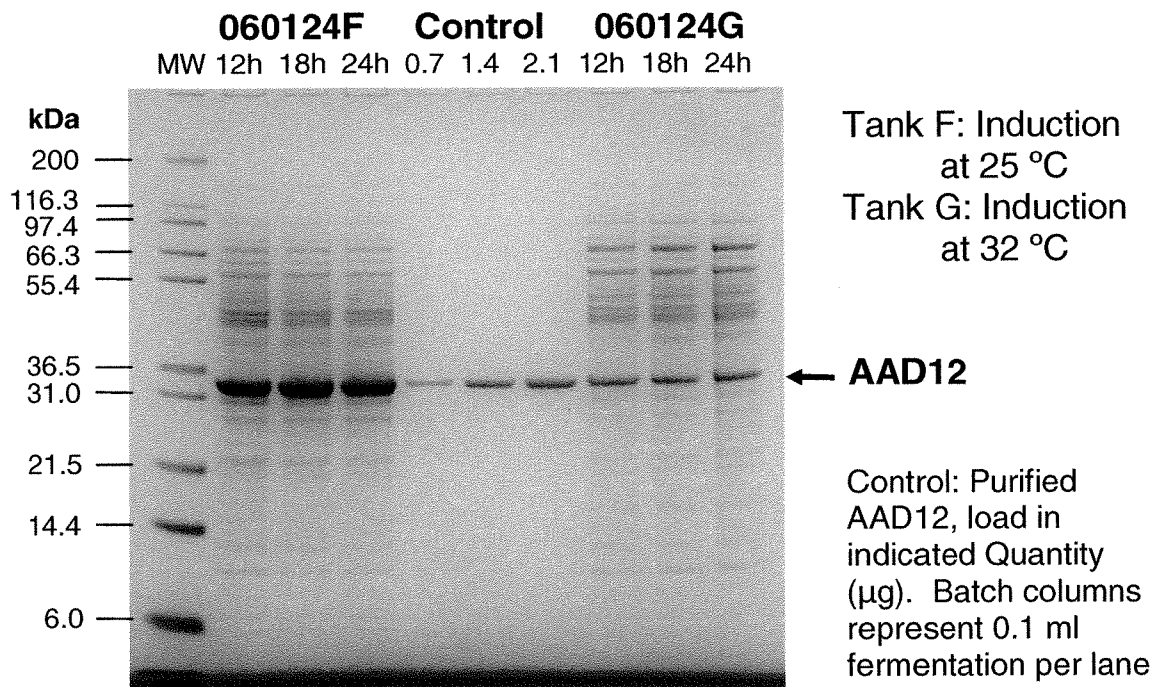
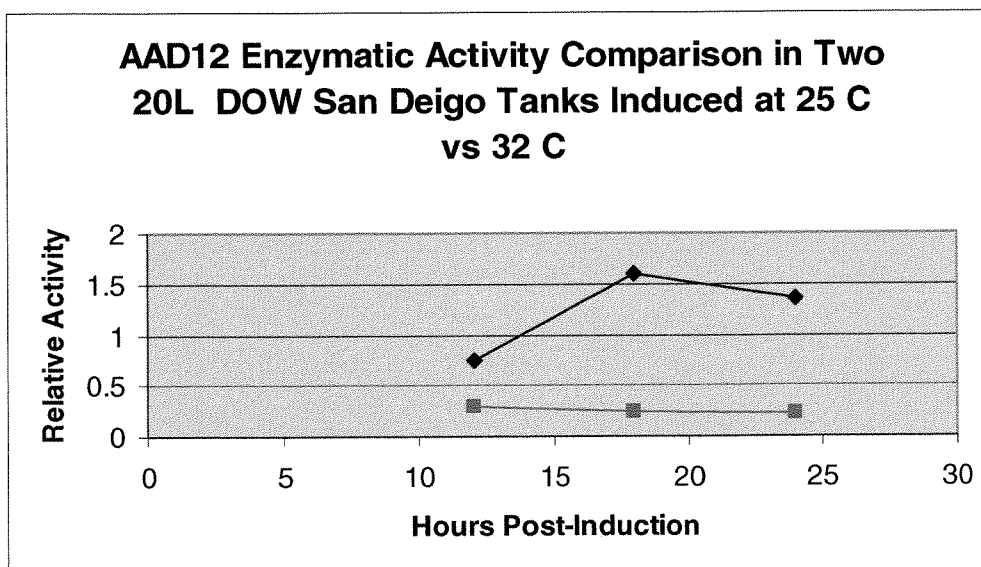


Figure 4. AAD-12 Enzymatic Activity Determination

Assay was performed as described in the Materials and Methods. Relative activity of each sample was plotted and compared for 2,4-D degradation.



- ◆ Cells induced at 25 °C
- Cells induced at 32 °C

Figure 5. Time course of AAD-12 Expression from Fermentation Processes

AAD-12 production estimated by SDS-PAGE densitometry. Various amounts BSA ranging from 0.1 μ g to 2 μ g was loaded in the same gels for generating calibration curve.

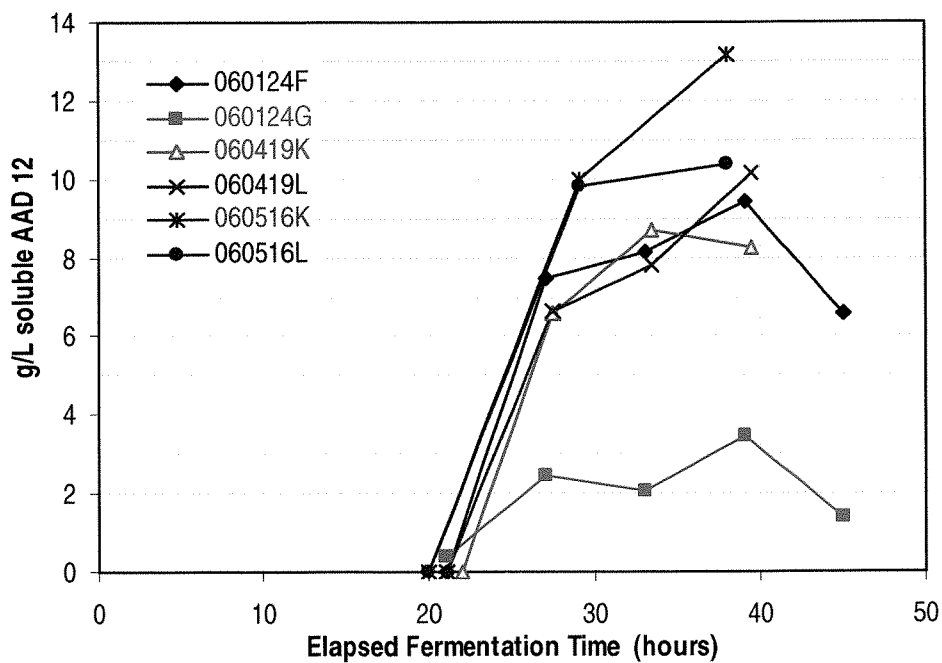


Figure 6. Growth of Strain DC579 in 20L Fermentors

Cell densities (OD_{575nm}) of 20 L fermentations of DC579 were plotted against time. The 060124G culture was induced at 32°C, whereas all the other cultures were induced at 25°C. Cell densities throughout the 24 hours induction phase ranged from 180 to 270.

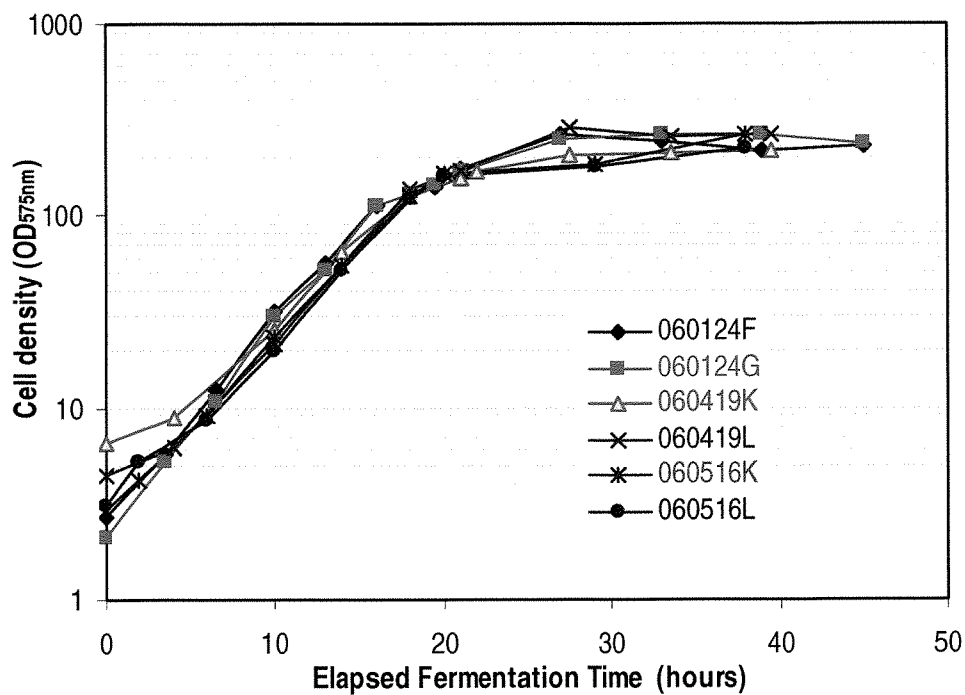


Figure 7. Schematic process scheme for recovery and purification of AAD-12

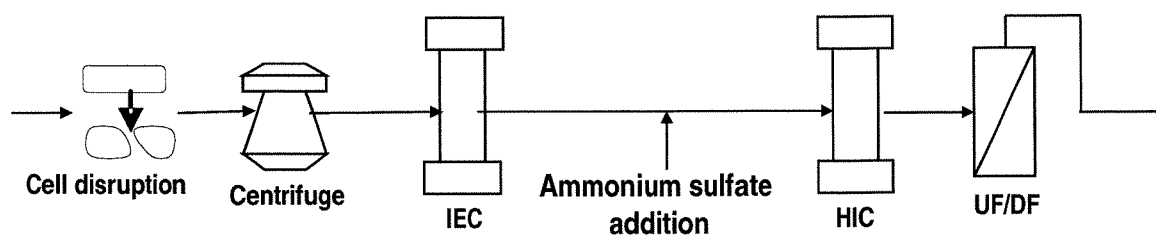


Figure 8. Primary Recovery of Process Batch EP466-026

SDS-PAGE analysis of primary recovery. Sample aliquot from each stage of the process was prepared and analyzed on a 12% NuPAGE (Invitrogen) gel. MW marker is Invitrogen's SeeBlue Plus, AAD-12 Std refers to purified AAD-12 standard. AAD-12 is shown as dominant band on the gel with an apparent molecular weight about 31 kDa.

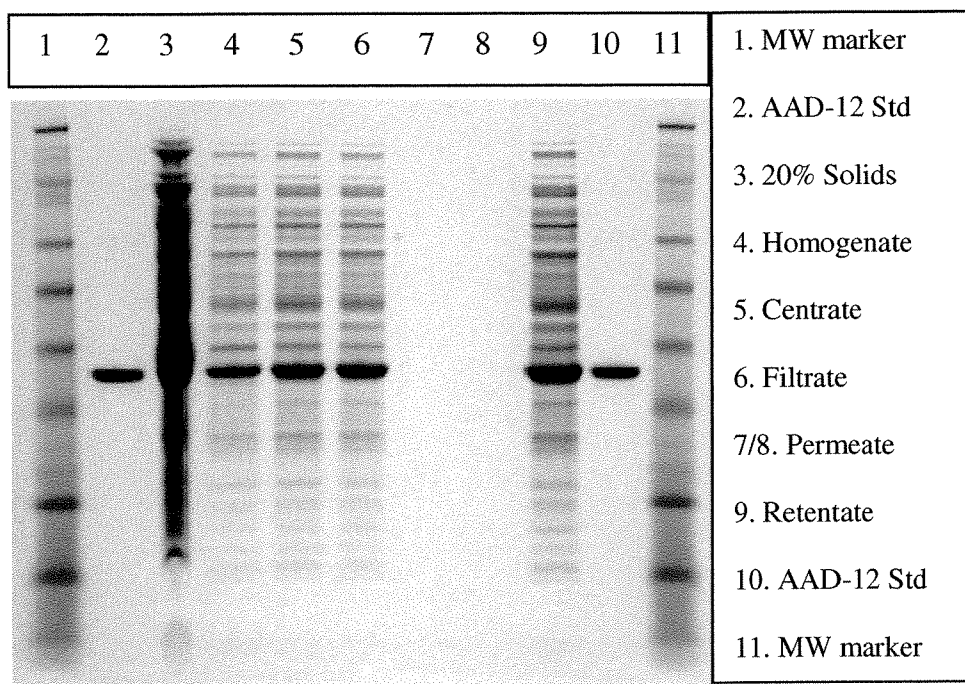
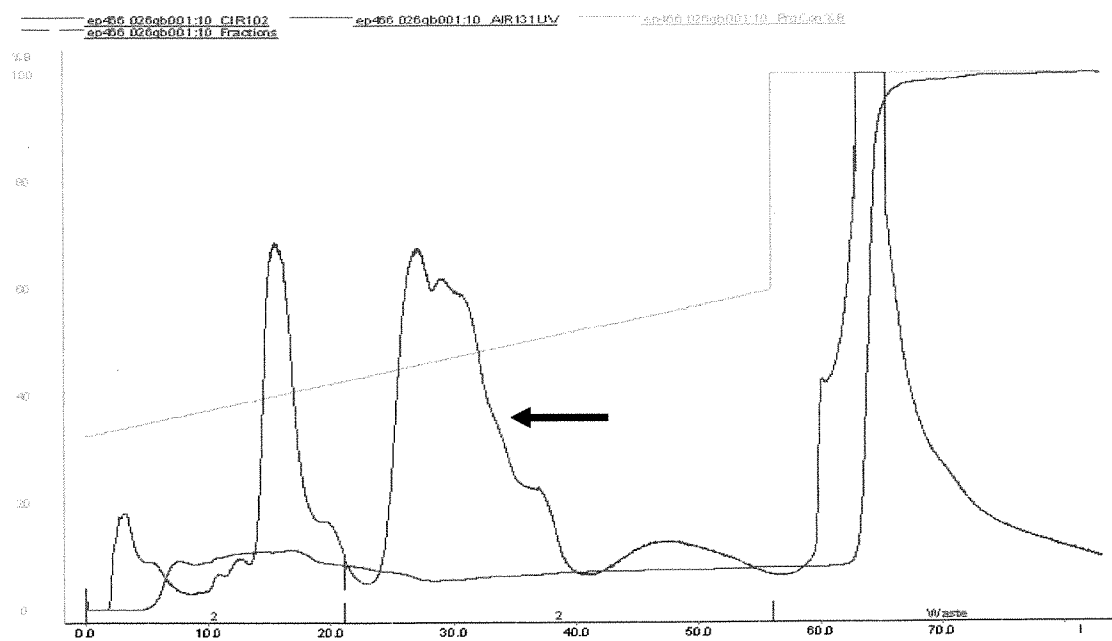


Figure 9. FFQ Chromatography and SDS-PAGE Analysis

Batch EP466-026 FFQ column separation and SDS-PAGE analysis. Main peak in the chromatography contains AAD-12 is indicated by the arrow. Aliquot of sample from different separations and elution fractions were loaded on SDS-gel. MW marker and size is shown on the left side of photo. The AAD-12 band is indicated by the arrow.

Chromatography Profile



SDS-PAGE Analysis

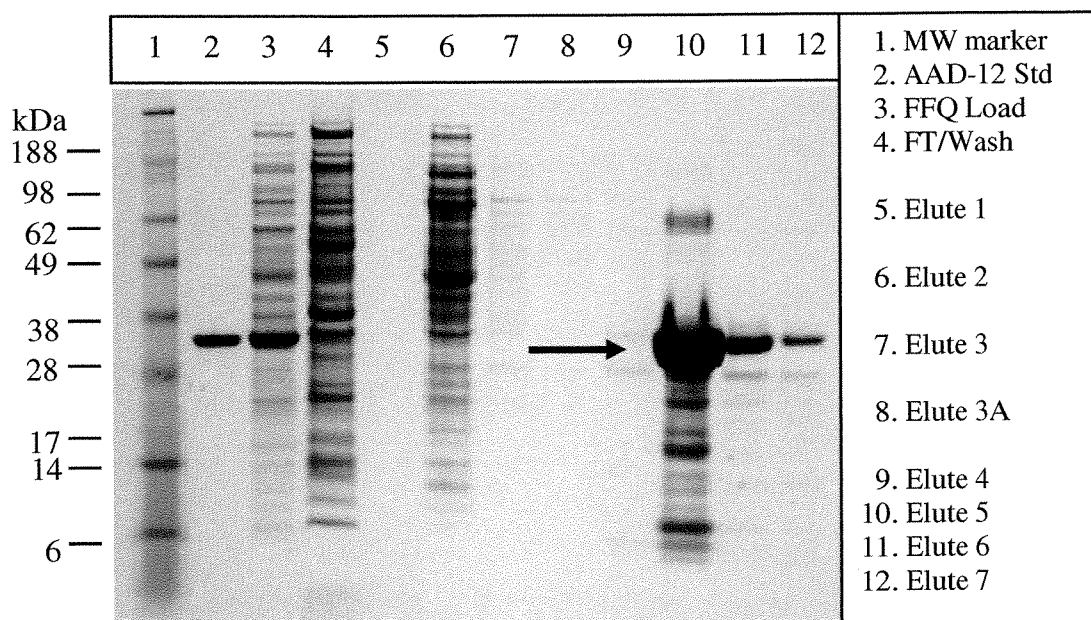
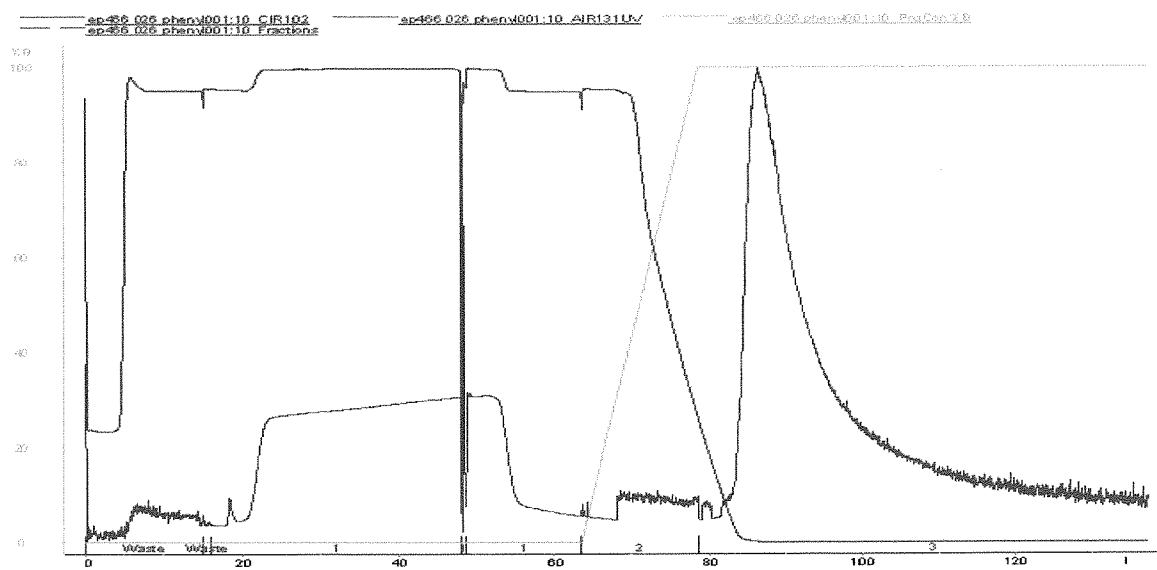


Figure 10. Phenyl Chromatography and SDS-PAGE Analysis

Batch EP466-026 HIC column separation and SDS-PAGE analysis. Only one main peak in the chromatography was observed and contained AAD-12. Aliquot of sample from different separations and elution fractions were loaded on SDS-gel. MW marker and size is shown on the left side of photo. AAD-12 is the dominant protein band with apparent MW about 31 kDa.

Chromatography Profile



SDS-PAGE Analysis

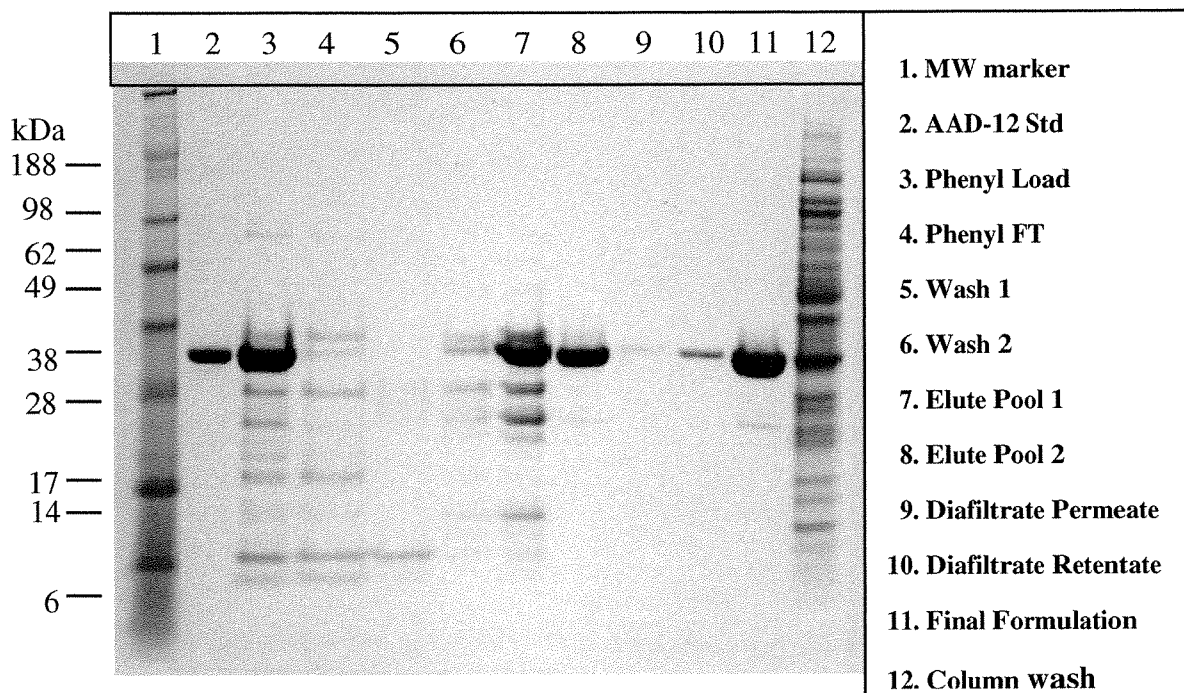
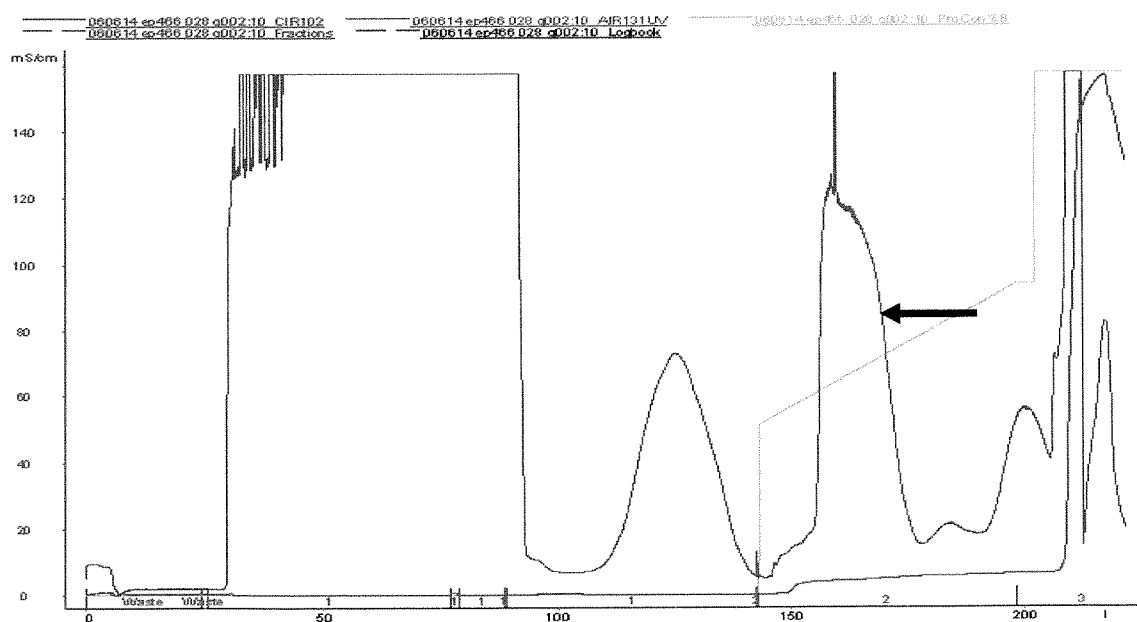


Figure 11. FFQ Chromatography and SDS-PAGE Analysis

Batch EP466-028 FFQ column separation and SDS-PAGE analysis. Main peak in the chromatography contains AAD-12 is indicated by the arrow. Aliquot of sample from different separations and elution fractions were loaded on SDS-gel. MW marker and size is shown on the left side of photo. AAD-12 band is indicated by the arrow.

Chromatography Profile



SDS-PAGE Analysis

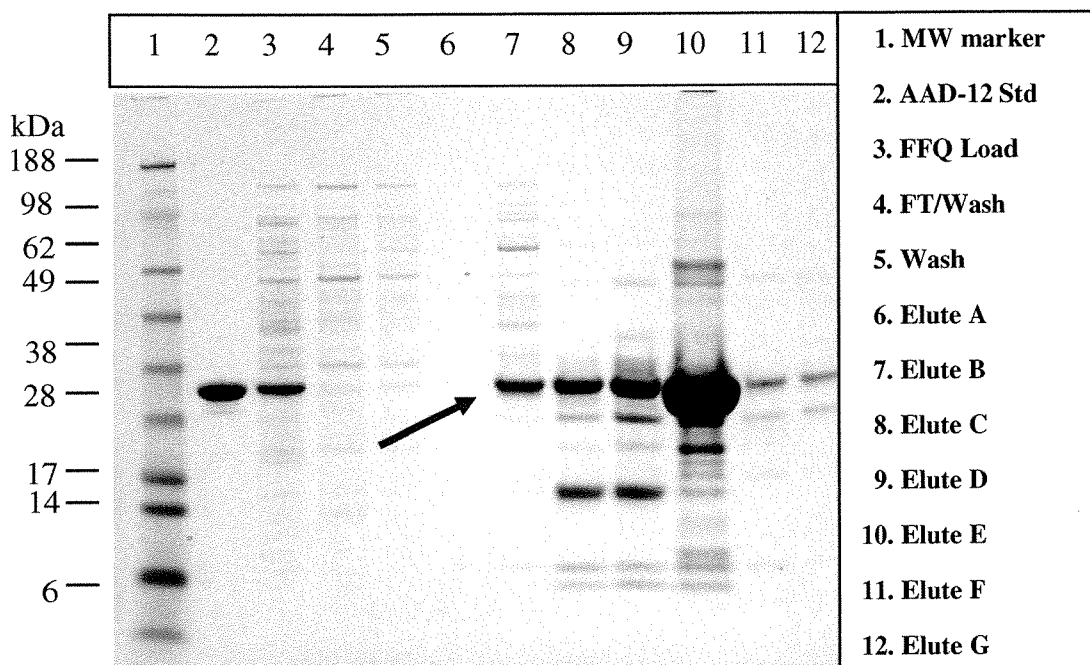
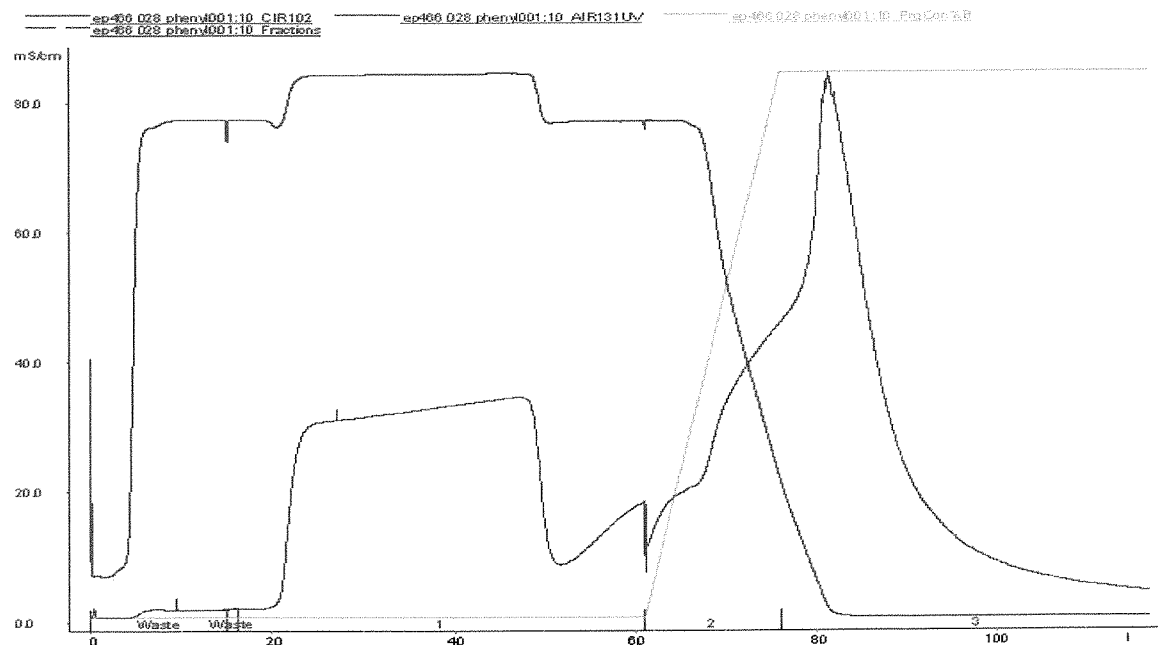


Figure 12. Phenyl Chromatography and SDS-PAGE Analysis

Batch EP466-028 HIC column separation and SDS-PAGE analysis. Only one main peak in the chromatography was observed and contained AAD-12. Aliquot of sample from different separations and elution fractions were loaded on SDS-gel. MW marker and size is shown on the left side of photo. AAD-12 is the dominant protein band with apparent MW about 31 kDa.

Chromatography Profile



SDS-PAGE Analysis

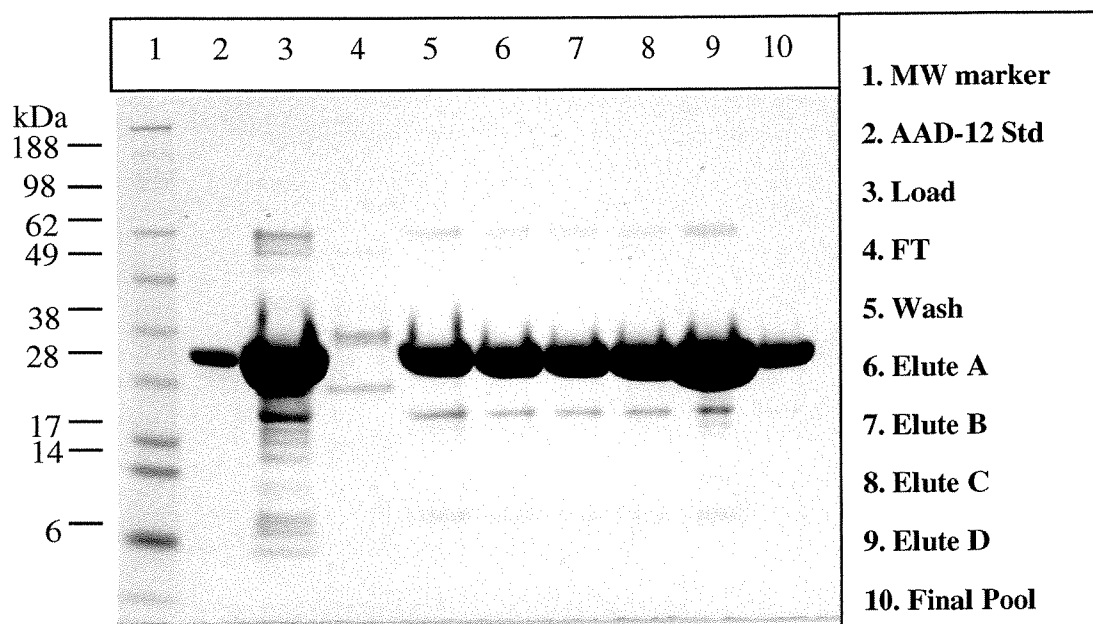
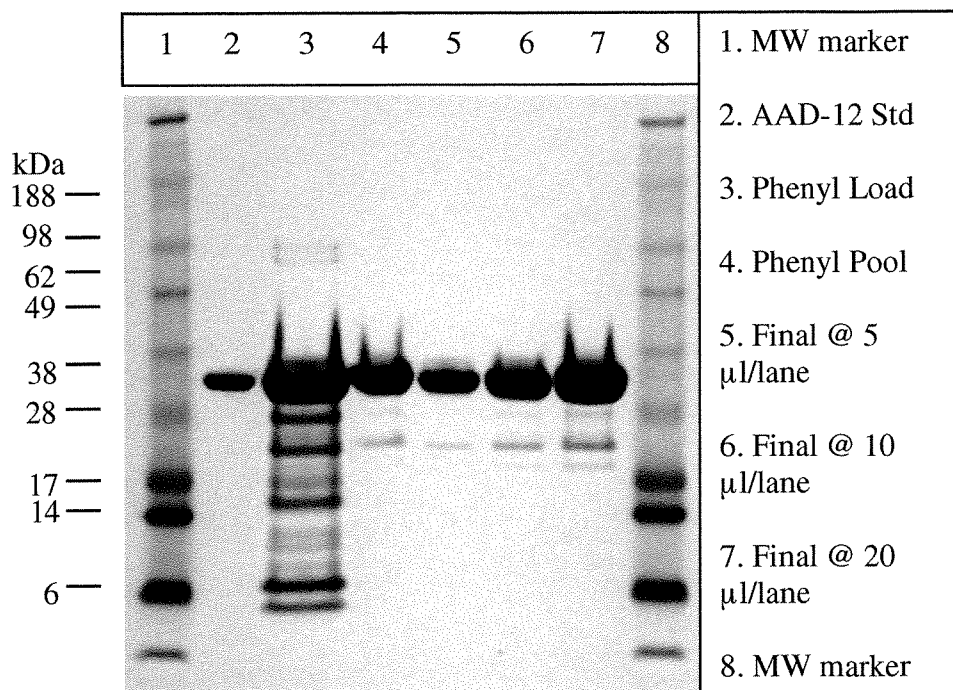


Figure 13. Final Diafiltration SDS-PAGE Analysis

Gel analysis on a 12% SDS-PAGE. Aliquot with indicated volumes or approximately 5 μg of protein were analyzed, and compared to the reference AAD-12 standard.

Batch EP466-026



Batch EP466-028

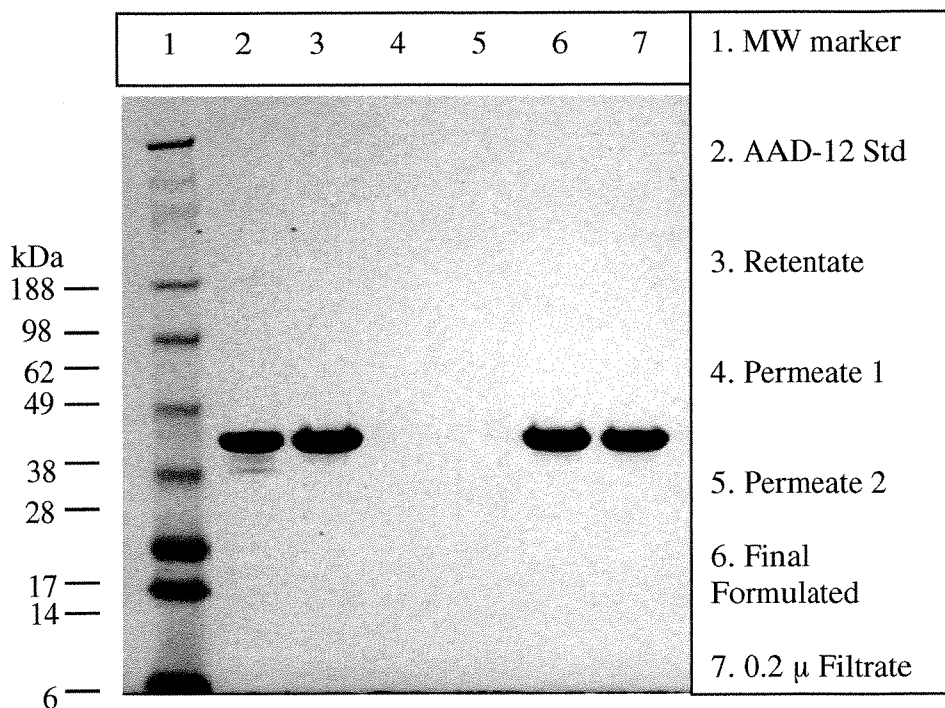
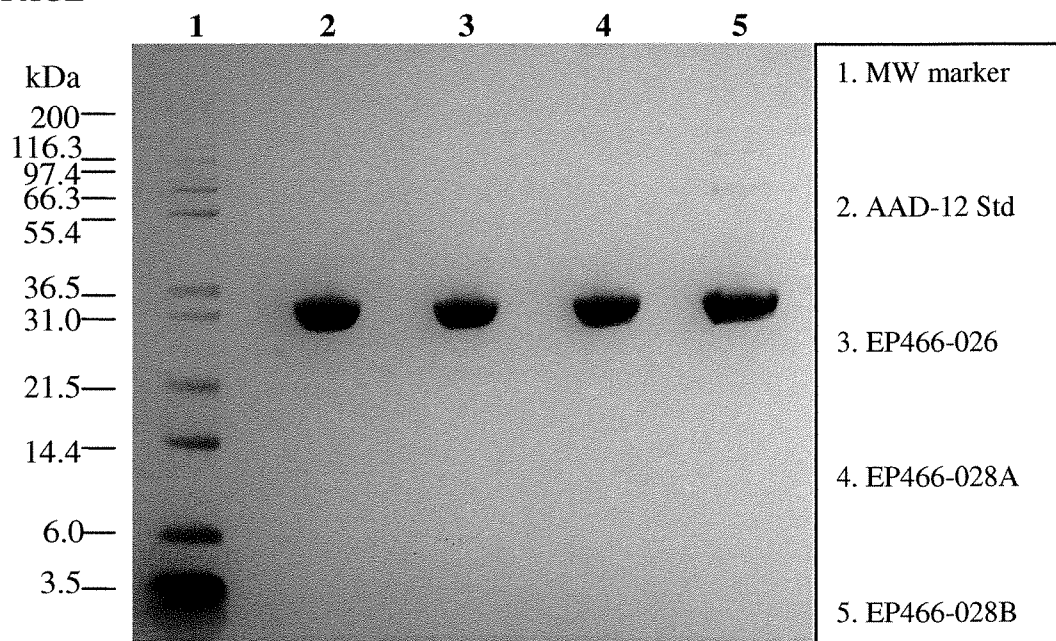


Figure 14. Release Analysis by SDS-PAGE and Native PAGE Gels

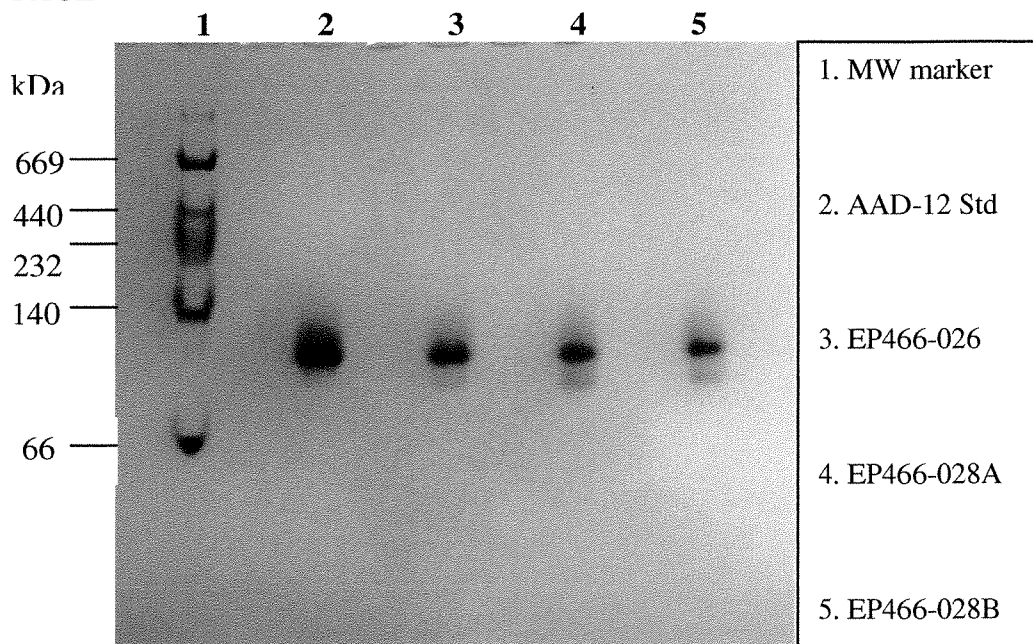
Upper panel: SDS-PAGE; lower panel: Native-PAGE. 10 μ g of sample was analyzed on each well, reference AAD-12 was used as standard. Molecular weight in kDa of protein size markers was labeled.

SDS-PAGE



Note: Denaturing gel shows AAD-12 monomeric form as 31 kDa protein.

Native-PAGE



Note: Native gel shows dimeric or/and trimeric form of AAD-12.

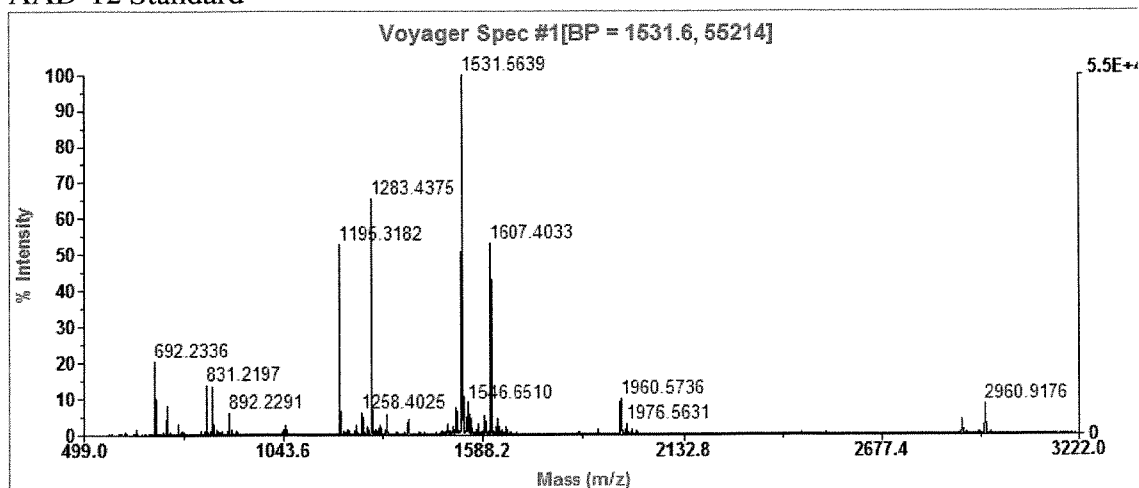
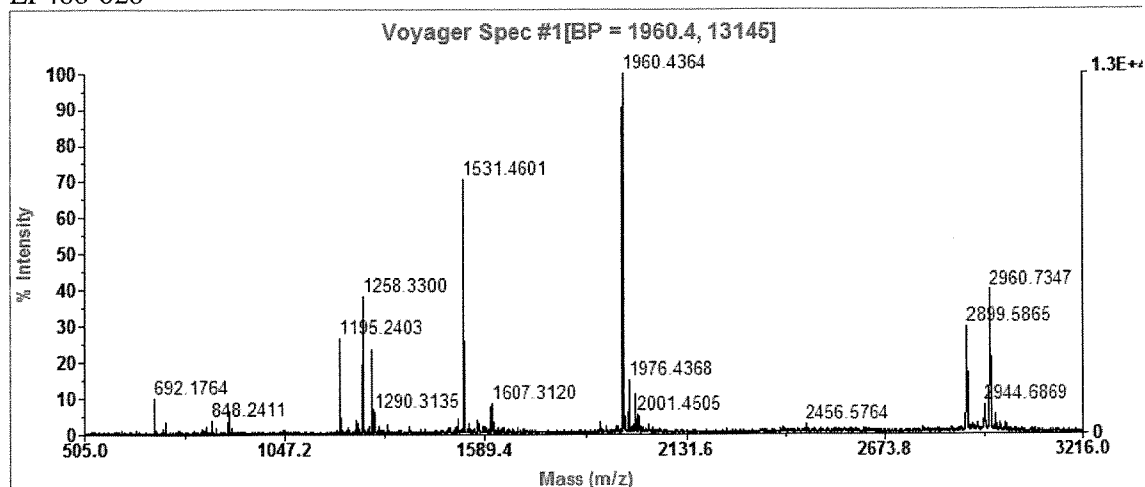
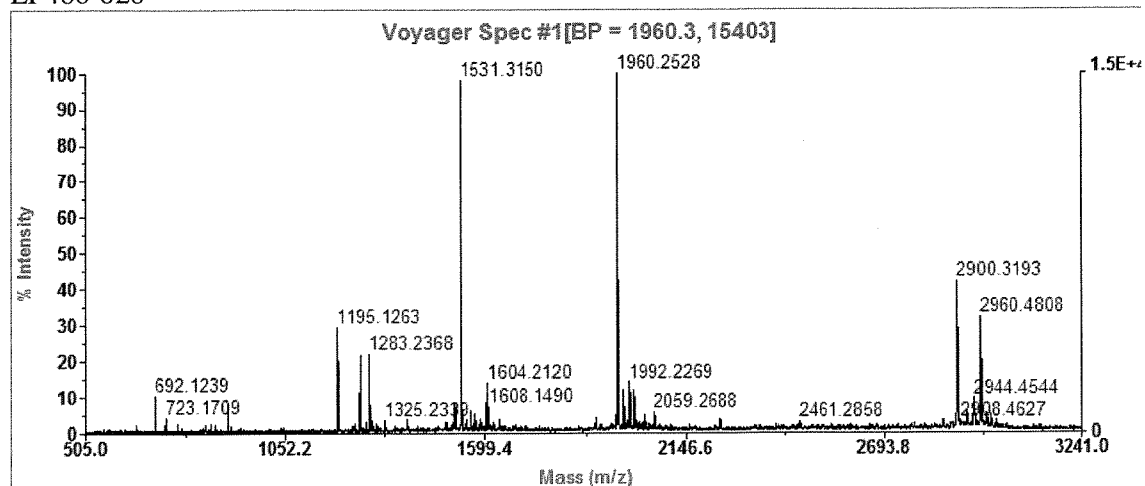
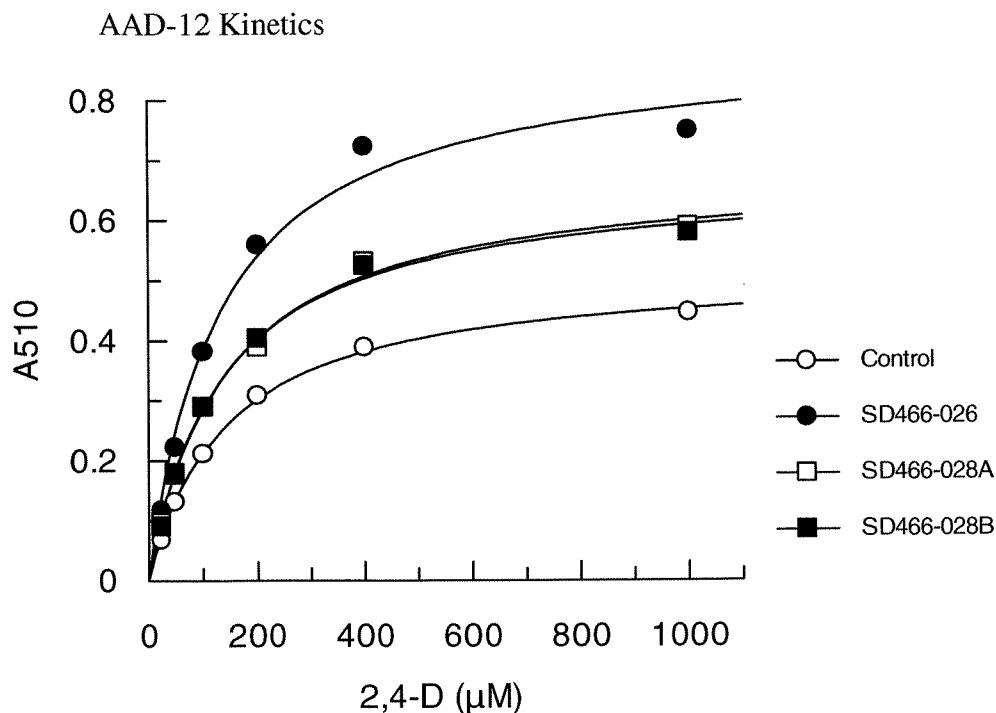
Figure 15. Maldi Analysis and Comparison of Purified AAD-12**AAD-12 Standard****EP466-026****EP466-028**

Figure 16. Enzyme Activity Determination and Kinetics on 2,4-D**Control**

Parameter	Value	Std. Error
Vmax	0.5179	0.0107
Km	143.5560	8.6799

SD466-028A

Parameter	Value	Std. Error
Vmax	0.6852	0.0212
Km	140.7846	12.8580

SD466-026

Parameter	Value	Std. Error
Vmax	0.8933	0.0500
Km	131.0482	22.1767

SD466-028B

Parameter	Value	Std. Error
Vmax	0.6733	0.0203
Km	135.1816	12.1846

Note: control was a reference AAD-12 sample prepared from previous purification and stored in PBS solution. Protein concentration from each every sample was determined by total AAA method