The Comparative Analysis of Wild-Type and *F242y/F242a* Mutant Porcine D- Amino Acid Oxidase

Scott Carlaw[1,2], Baofeng Jia[1,2], Nick Medeiros[1,2], Donovan Ramkishun[1,2], & Peter Simovic[1,2]

[1]Biochemistry Student Level III, McMaster University, Hamilton, Ontario, Canada
[2]Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada
Direct Correspondence To: carlawse@mcmaster.ca

Abstract
D-amino acid oxidase (DAO) is a flavoenzyme that utilizes D-amino acids from the peptidoglycan layer of bacterial cell walls to generate hydrogen peroxide, a known antibacterial agent. The objective of this study is to further explore the currently unknown potential of DAO as an antibacterial agent by examining its activity through active site mutations. In this study, *F242Y* and *F242A* porcine DAO mutants were created by site directed mutagenesis (SDM) and splicing by overlap extension (SOE) PCR respectively. Spectrophotometric assays were utilized to determine the effectiveness of wild type DAO in producing hydrogen peroxide with D-alanine, D- serine and L-alanine. It was determined that D- alanine, the natural substrate of DAO, contained the greatest hydrogen peroxide producing capability. D-serine also produced hydrogen peroxide but not to the extent of D-alanine, while L-alanine demonstrated no production. By utilizing the activity of wild type DAO as a baseline, we hope to create potent DAO mutants that are effective against a greater variety of peptidoglycan D-amino acids. Future implications of this research include the development of DAO injections or orally administered drugs that can combat currently resistant infectious diseases.

Abbreviations
DAO: D-amino acid oxidase protein, SOE-PCR: Splicing by overlap extension polymerase chain reaction, SDM: Site-directed mutagenesis, FAD: Flavin adenine dinucleotide, WT: Wild-type.

Introduction

D-amino acid oxidase (DAO) is an antibacterial agent commonly found in the peroxisomes of kidney, liver, and brain cells[1]. It is also located on the outer surface of neutrophils and catalyzes the oxidative deamination of free but essential D-amino acids from the peptidoglycan layer of bacterial cell walls[1]. DAO exhibits the highest affinity toward hydrophobic amino acids such as D-alanine and D-proline compared to more acidic or basic residues[1]. The oxidative deamination of D-amino acids by DAO occurs in a two-step process. First, a hydride equivalent is transferred from a D-amino acid to a co-factor molecule of Flavin Adenine Dinucleotide (FAD) in a deamination reaction to produce an α-keto acid and ammonia[2]. The reduced FAD is oxidized by oxygen gas resulting in the production of hydrogen peroxide, a reactive oxygen species[2]. Hydrogen peroxide produces free radicals that damage proteins, lipids, and DNA in bacterial cells, which ultimately leads to cell death[3]. DAO is thus utilized by human immune cells as a method to limit the survival of catalase-negative bacteria[3].

Although DAO is known to be involved in many catalytic processes, its biological role in mammals remains unclear[1]. DAO can potentially kill catalase-negative resistant bacteria by oxidizing free D-amino acids surrounding the peptidoglycan layer to produce hydrogen peroxide[1]. In our study, we further explore the currently unknown potential of DAO as an antibacterial agent by examining its activity through active site mutations at phenylalanine 242 (*F242*) of porcine DAO due to its close proximity to the D-amino acid substrate and FAD. Phenylalanine was hypothesized to have a significant role in the enzyme's catalytic ability, as it is a large amino acid and thus would encompass a larger volume of space in the active site. By changing the size of the active site, D-amino acids' ability to enter the active site would be altered. *F242Y* and *F242A* were investigated and it was predicted that the *F242Y* mutation would decrease the bactericidal effect because of the altered hydrophobicity of the active site, which would deny entrance of uncharged D-amino acids. On other hand, the *F242A* mutation would increase this effect due to the increased active site size while maintaining hydrophobicity thus allowing bigger amino acids to enter the cavity.

In this study, the mutant *F242Y DAO* gene was created through site directed mutagenesis (SDM) while the *F242A* mutant was created through splicing by overlap extension polymerase chain reaction (SOE- PCR). A spectrophotometric assay conducted on purified wt-DAO demonstrated that D- alanine, the natural substrate of DAO, produced the most hydrogen peroxide while D-serine, another known substrate of DAO, produced hydrogen peroxide but not to the extent of D- alanine. Furthermore, L-alanine was not able to produce hydrogen peroxide

due to DAO's inability to deaminate L-amino acids. These results are consistent with current findings and experiments with DAO[1]. Future work on this study will include the confirmation of the *F242A* mutant through sequencing. This will be followed by the expression and purification of both mutants for the spectrophotometric and bactericidal assays to determine their respective antibacterial activities.

Results

SOE-PCR and Ligation

Both of the first cycle reactions was done with the inclusion of a temperature gradient to cover a wider range of annealing temperatures in case of incorrect annealing temperatures. PCR was successful for the *F242Y* and the outside forward, inside reverse reaction was successful for the *F242A* mutation (Figure 1). Bands found at 750 bp for the AB reaction and 250 bp for CD suggested success. The CA+D reaction was unsuccessful due to the band found at 1000 bp. The CA+D reaction and the full overlap of F242Y was tested once more and yielded successful bands found at 750 bp and 250 bp for the CA+D reaction (Figure 2). The second round of SOE PCR was successful based on the bands found at 1000 bp at each temperature for the *F242A* and *F242Y* mutants (Figure 3). Transformation of pET26b-*dao* into *E. coli* DH5α was unsuccessful since there were no colonies present on the LB-Agar plate supplemented with ampicillin.

Site Directed Mutagenesis and DNA Cloning

Transformation into XL10-Gold ultracompetent cells was successful only for the *F242Y* mutant. The mutation was verified by sequencing the purified SDM product. F242Y pET3a-dao was successfully transformed into *E. coli* BL21 (DE3) as suggested by colonies present on the LB-Agar plate with ampicillin.

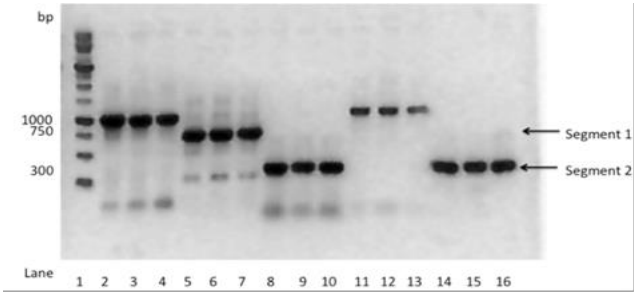

Figure 1. *Successful SOE PCR Amplification of Both Segments for F242Y and Amplification of Segment 2 for F242A.* GelRed was added to the agarose gel to help visualize bands underneath the UV Transilluminator. Full length of the DAO gene is 1026 base pairs and the segments generated from the first round of SOE PCR are 726 and 300 base pairs. Lanes of the gel are as followed: Lane 1-DNA Ladder, 2-WT DAO control at 52ºC 3-54.7ºC 4- 57ºC 5- A+BY reaction at 52ºC, 6- 54.7ºC, 7- 57ºC 8-CY+D reaction at 52ºC 9- 54.7ºC 10- 57ºC 11- A+BA reaction at 52ºC 12- 54.7ºC 13- 57ºC 14- CA+D reaction at 52ºC 15-54.7ºC 16- 57ºC

WT-DAO Expression and Characterization

SDS-PAGE of the samples taken from the Ni-NTA purification as well as cell lysate and the cell pellet was performed. Single bands at approximately 39 kDa were present within each elution fraction (Figure 4). The concentration of first wt-DAO elution fraction was obtained using the NanoDrop spectrophotometric method. An absorbance of 0.8 was observed and the extinction coefficient for DAO is 75 860, yielding a concentration of 1.54 mg/mL using the Beer-Lambert law.

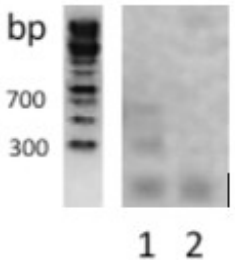

Figure 2. *Successful Amplification of F242A SOE PCR Segments.* 1% agarose gel stained with GelRed was used to separate PCR products. The first lane shows the first round of SOE PCR of the F242A mutant. The bands found at 726 and 300bp showed amplification of segment 1 and 2. Unsuccessful overlap of segments 1 and 2 of the F242Y mutation is shown in the 2nd lane.

Spectrophotometric Assay

Peroxide producing capability of wt-DAO was measured for either D-alanine, D-Serine or L-Alanine through a spectrophotometric technique. With the presence of horseradish peroxidase and o-dianisidine the absorbance at 460 nm is directly proportional to the production of H_2O_2 from the oxidative deamination of D- amino acids by DAO[4]. D-Alanine produced the highest absorbance that correlated to the largest amount of peroxide produced, while L-Alanine produced the least (Figure 5).

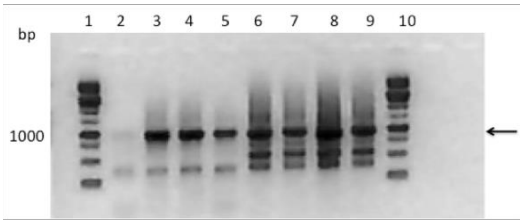

Figure 3. *Amplification of F242A and F242Y SOE PCR Mutants.* 1% Agarose gel stained with GelRed was used to separate PCR products. The arrow at 1000bp verifies the full overlap of DAO, which has a gene size of 1026 bp. Lane 1 and 10 is the 1kb DNA ladder, 2- F242A at 53°C, 3- 55°C, 4- 57°C, 5- 60°C, 6- F242Y at 53°C, 7- 55°C, 8- 57°C, 9- 60°C.

Bactericidal Assay

The bactericidal potential of wt-DAO was measured with D-alanine, D-serine, L- alanine, D-phenylalanine, and D-glutamate. Colonies found on the plate were too numerous to count for each dilution, so bactericidal activity of DAO could not be determined.

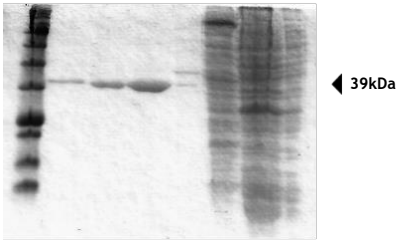

Figure 4. *Wt-DAO protein was expressed and purified from E.coli BL21 (DE3) using Ni-NTA column chromatography.* Purification fraction samples from Ni-NTA chromatography were mixed with Laemmli Sample Buffer and loaded into a 12.5% polyacrylamide gel. The gel was then stained overnight using Coomassie stain and visualized. Lane 1 contained the BLUeye Protein Ladder. 2- wt-DAO elution fraction 3. 3- elution fraction 2. 4- elution fraction 1. 5- wash fraction. 6- flow through. 7- *E.coli* cell lysates. band at 39kDa, as indicated by the arrow correspond to DAO protein.

Discussion

The aim of this study was to elucidate the antibiotic potential of DAO through the development of novel active site mutations. DAO derives its function from its structure which is a result of its amino acid sequence. Alterations to the sequence, especially within the active site, affect the structure and therefore its function. Utilizing functional based assays to comparatively analyze wt-DAO and mutants, the extent of the effects could be quantified.

Our experiment began with the cloning of DAO genes and creation of mutants. The first round of SOE PCR was successful as indicated by the presence of bands at approximately 750 and 250 bps corresponding to segments 1 and 2 of the gene respectively. Successful final round of SOE was indicated by presence of ~1000bp bands that correspond to annealing of our segments resulting in our full gene. All temperature gradients yielded identical results, which confirmed that annealing temperature was not an issue in our unsuccessful attempts. Incorrect primer use was the reason for bands at
~1000 bp in lanes 11-13 (Figure 1). For our final attempt of SOE, we used extra template DNA to ensure there was enough template DNA. This led to the presence of extra bands on our gel that was likely the result of non-specific annealing between homologous segments
(Figure 3). Since *Taq* polymerase catalyzes
DNA at the rate of 1kb/min, a one minute elongation time may not have been sufficient for it to polymerize some gene or gene segments. This may also be a source of error in our failed SOE attempts (Figure 2). Troubleshooting was performed by adding 2 uL of each gene fragment on a temperature gradient of 52°C,
54.7°C, 57°C and 60°C for each overlap reaction.

The SDS-PAGE performed for the Ni- NTA purification fractions indicate that wt-DAO was successfully expressed and purified. The single bands observed in the three elution fractions at approximately 39 kDa correspond to the molecular weight of porcine DAO in the literature[5]. The first elution fraction contained the greatest amount of protein, which was expected since most of the protein was displaced from the resin with the first addition of the elution buffer. In future studies, a western blot could be utilized to identify DAO protein with greater certainty instead of solely basing its identity on molecular weight.

The spectrophotometric assay was utilized as an indirect method to determine the antibacterial activity of wt-DAO. The absorbance at 460 nm is directly proportional to the production of H_2O_2 from the oxidative deamination of D-amino acids by DAO. As H_2O_2 is produced, it is converted to a fluorophore in the presence of horseradish peroxidase and o-dianisidine[4]. The absorbance for D-alanine increased more rapidly compared to D-serine and L-alanine and indicates that D-alanine is the preferred substrate for wt-DAO. D-serine exhibited a relatively lower rate of reaction compared to D-alanine as indicated by a smaller slope in the absorbance graph (Figure 5). This was expected since D-serine contains a hydroxyl group that can disrupt hydrophobic interactions and result in a decreased binding affinity for the DAO active site[2]. L-alanine did not exhibit any significant activity in the presence of DAO. L-amino acids cannot effectively transport their electrons to the cofactor FAD thus inhibiting the first step of oxidative deamination[2]. In future studies, additional amino acids, such as D-glutamate and D-phenylalanine, could be utilized to determine their affinities for the DAO active site. D-glutamate is of particular interest since it is located in bacterial cell walls and could be a target of DAO mutants.

In contrast, the bactericidal assay for wt-DAO was utilized as a direct method to determine its antibacterial activity. The assay produced inconclusive results as all serial dilutions produced too many colonies to count. It is hypothesized that D-alanine would have exhibited the fewest number of colonies while L- alanine would have exhibited approximately the same number as the control. Another 1:100 dilution in PBS would be required to obtain countable colonies in future experiments.

The utilization of DAO has great applications in today's society and presents enormous opportunity to combat one of the greatest problems affecting our generation – antibiotic resistance. The mutant *F242Y* and *F242A* genes can be utilized in future research in order to produce the mutant proteins so that their activity can be determined. This can help in understanding the key amino acids that facilitate its function, and the role of DAO as an antibiotic to combat known resistant bacteria. This unique approach in combating antibiotic resistance is promising, as it does not use synthetic drugs in which bacteria may confers resistance. Rather, it utilizes the radical damage which hydrogen peroxide produces to proteins, lipids and DNA. The main

results that were obtained from the study were the activity of the wt-DAO protein through the spectrophotometric assay. This provides a baseline measure of DAO activity which can be used for future comparisons to that of the mutant. This research can be applied to many different fields crossing the disciplines of biochemistry, molecular biology, genetics and medicine. It can further be used to novel ways to fight the threat of antibiotic resistance including potential DAO topical creams or orally administered drugs to kill bacteria.

Materials and Methods

Wild type pET3a-*dao* was kindly provided by the Dr. Coombes lab (McMaster University, Hamilton).

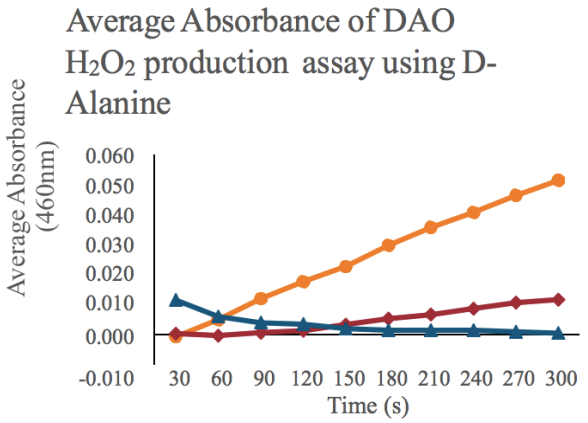

Figure 5. *Wt-DAO can successfully catalyze D-alanine and D-serine, but not L-Alanine*. Wt-DAO was added to a solution containing FAD, o-dianisidine, phosphate buffered saline and D-Alanine, D-Serine or L-Alanine. The absorbance of the reaction was measured at 460 nm using a spectrophotometer. DAO was able to metabolize D-Alanine and produce peroxide much faster than with D-serine. There was no positive change in absorbance in the reaction with L-alanine; DAO was not able to metabolize L-alanine.

F242Y and F242A mutagenesis and cloning

F242Y and *F242A* mutant DAO were created via SDM and SOE-PCR. All of the mutagenesis products were confirmed by sequencing. All primers (Table 1) used were synthesized at the McMaster MOBIX lab (Hamilton, ON). Two fragments of SOE-PCR was produced with wt- pET3a-*dao*, 100 µM of primer A, CA or CY for one segment and primer D, BA or BY for the second fragment. dNTP (FroggaBio), PCR buffer (FroggaBio), 2.5 units of i-Taq™ DNA polymerase (FroggaBio) and nuclease-free water (GeneDireX) were added for a total reaction volume of 50 µL. The Second cycle of SOE-PCR began with the previously amplified products, reagents and Primer A and D. The resultant products from both cycles were separated on a 1% agarose gel and purified using the PureLink® Quick Gel Extraction and PCR purification Combo Kit (Life Technologies) using the centrifuge method. The purified mutants, along with pET26b plasmid were digested with *NdeI* and *XhoI* in buffer O (Fermentas). The digested genes were separated on a 1% agarose gel and purified with PureLink® Quick Gel Extraction and PCR purification Combo Kit using the gel extraction method (Life Technologies). The digested mut- *dao* genes and pET26b were mixed with 0.5 units of T4 DNA ligase (Life technologies, Carlsbad, CA), T4 DNA ligase buffer (Life technologies), and nuclease-free water (GeneDireX) and incubated at room temperature overnight. The ligation reactions were added to two 40 µL of *E. coli* DH5α chemically competent cells (Life technologies,). The transformation was completed using the heat shock method (Sigma-Aldrich). Liquid LB broth (4.6 g of Tryptone, 2.3 g of Yeast, 4.6 g of NaCl; Sigma-Aldrich) was then added to each tube and placed in a cell shaker at 37°C for 30 minutes and spread onto LB plates supplemented with 100 µg/mL kanamycin (Sigma-Aldrich) and placed in 37°C incubator for 24 hours. One colony from the LB agar plate was inoculated into a liquid LB supplemented with kanamycin and grown overnight. F242A and F242Y pET26b-dao was isolated using alkaline lysis method with the PureLink® Quick Plasmid Miniprep Kit via centrifugation (Life Technologies).

Site directed mutagenesis was conducted with pET3a-*dao*, 100 µM of primer E, F, G and H. The QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used for SDM.

Table 1. Collection of primers used for SOE-PCR and SDM, (5'-3')

Primer Name	Sequence
A,Outside forward	CATATGATGCGTGTGGTGGTGATTGG
BA, F242A inside reverse	CCCCACCTGGGCGGTGCCTCCAAGTGTC ACT GCCTGCAG
BY, F242Y inside reverse	CCCCACCTGGTAGGTGCCTCCAAGTGTC ACT GCCTGCAG
CA, F242A inside forward	GGAGGCACCGCCCAGGTGGGGAACTGG AAT GAGATAAATAATAT
CY, F242Y inside forward	GGAGGCACCTACCAGGTGGGGAACTGGAA TG AGATAAATAATAT
D, Outside reverse	CTCGAGTCAGAGGTGGGATGGTGGC
E, F242Y forward SDM	GTGACACTTGGAGGCACCTACCAGGTGGG GA ACTGGAAT
F, F242Y reverse SDM	CCCCACCTGGTAGGTGCCTCCAAGTGTC ACT GCCTGCAG
G, F242A forward SDM	GTGACACTTGGAGGCACCGCCCAGGTGGG GA ACTGGAAT
H, F242A reverse SDM	ATTCCAGTTCCCCACCTGGGCGGTGCCTC CA AGTGTCAC

DAO protein purification

E.coli BL21 (DE3) cells containing pET3a-*dao* plasmids were inoculated into liquid LB containing ampicillin and incubated at 37°C in the cell shaker. At 0.49 OD_{600}, 0.1 M IPTG (Sigma-Aldrich) solution

was added to the culture and incubated at 37°C for the next 3 hours. The cells were then pelleted and lysed using the BugBuster® Protein Extraction reagent (Novagen) containing 25 units/mL Benzonase Nuclease (Novagen) and protease inhibitor (Sigma-Aldrich). The supernatant was loaded into a nickel-nitrilotriacetic acid (Ni-NTA) bead column (Sigma-Aldrich) and eluted with an imidazole gradient of 10mM - 250mM. The supernatant was collected and stored at -20°C. Elution fractions were characterized on a 12.5% polyacrylamide gel via Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS- PAGE).

Spectrophotometric Assay for DAO Activity

5 mM D-amino acid (D-ala, D-ser, L- ala), 1X potassium phosphate buffer, 1.3 µg o- dianisidine, 0.33 µg horseradish peroxidase, 100 µM FAD and 0.308 mg of purified DAO, all obtained from Sigma-Aldrich, were added to a glass test tube in triplicates. Reaction's absorbance is measured at 460 nm every 30 seconds for 5 minutes with a spectrophotometer. The absorbance values were averaged and compiled into a graph using Microsoft Excel.

Bactericidal Functional Assay

E. coli DH5α was cultured in LB broth (5 g yeast extract, 10 g Tryptone, 10 g NaCl; Sigma-Aldrich) until an OD_{600} of 0.6 while shaken at 37°C at 200 rpm. A 1:100 dilution of the culture was added to a solution containing 0.5 mM amino acid, 200 µL of 1.54 mg/mL DAO and 10 µL of 10 µM FAD for a final volume of 200 µL. Each reaction was placed into a well on a 96 well plate, in triplicates, followed by a 10^{-6} serial dilution. 7 uL of each dilution was then pipetted onto LB agar plates and incubated at 37°C overnight. Colonies were counted the next day.

Acknowledgements

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