ABSTRACT

Expression, Purification and Catalytic Turnover of Mn-Quercetin Dioxygenase on Various Substrates

Donald F. Gray, II

Director: Patrick J. Farmer Ph.D.

Genetically transformed *E. coli* cell line was cultured to express Mn-QDO. The product was collected for use in the catalytic turnover rate studies conducted, as well as for future EPR analysis. One ~250 μL purified and quantified batch of QDO was obtained, and determined to have a QDO concentration of ~229 μM. Another unpurified batch, which showed high catalytic activity, was used for preliminary catalytic activity. The activity was studied on the native substrate quercetin and was shown to have an aerobic catalytic turnover rate, or oxygenase activity, of 3.5×10^{-4} Au/s. Nitroxygenase activity is measured using a similar analysis of an iosolectronic anaerobic reaction using HNO. The nitroxygenase activity on quercetin was 2.9×10^{-4} Au/s. The oxygenase and nitroxygenase activity on another flavonol, myricetin was determined to be 1.1×10^{-4} Au/s and 4.3×10^{-4} Au/s, respectively. An activity study toward HOPTO in buffers of pH from 5 to 8 was performed. The lower pHs seemed to correspond to the fastest rates of activity.

APPROVED BY DIRECTOR OF HONORS THESIS:

_____

Dr. Patrick J. Farmer, Department of Chemistry

APPROVED BY HONORS PROGRAM

_____

Dr. Andrew Wisely, Director

Date: _____

EXPRESSION, PURIFICATION AND CATALYTIC TURNOVER OF MN-QUERCETIN DIOXYGENASE ON VARIOUS SUBSTRATES

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Donald F. Gray, II

Waco, Texas

December 2012

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

I would like to thank Baylor University and the Chemistry/Biochemistry Department for allowing me use of laboratory resources. I greatly appreciate Dr. Patrick J. Farmer's for taking me on and giving me a project when I needed a lab to work in. Along with Dr. Farmer I wish to thank Long Dao, Dr. Ravi Kumar and Tara Clover for their guidance and direction during this process.

DEDICATION

Dedicated to mother, Virginia L. Sill, for her love and support

CHAPTER ONE

Introduction

Oxygenases

Most atmospheric oxygen consumption is from respiration by living organisms; the balance is used in metabolic synthesis of certain biological molecules and in the conversion of certain toxins to waste for excresion.[1] Oxygenases, first identified by both Osamu Hayaishi and Howard S. Mason in 1955,[2,3] are responsible for catalyzing these reactions. Oxygenases are a class of enzymes that fall under oxidase and oxidoreductase.[4] They are separated into monooxgygenases or dioxygenases depending on whether one or two of the oxygen atoms from the dioxygen molecules are incorporated in the product. A majority of oxygenases possess metal cofactors at the active site. The native forms of these enzymes have Fe, Cu or other metal ions in the active site.[5]

There are two classes of nonheme dioxygenases. One class binds oxygen directly to the metal center, while in the other class oxygen is bound to the substrate.[6] Catechol 1,2-dioxygenase cleaves the carbon-carbon bond between the hydroxyl groups, this forms the decarboxylated *cis,cis*-muconic acid.[7] Dioxygenases include catechol dioxygenase, cysteine dioxygenase, lipoxygenases, tryptophan oxygenase, and flavonol 2,3-dioxygenase, also known as quertecin dioxygenase (QDO).[8,9]

My research is on QDO. The substrate of this enzyme is a flavonoid, quercetin. The polyphenolic hydrocarbon is found in numerous plants and some fungi, where it functions as antioxidant and antimicrobial agent. Soil microorganisms use QDO along

with oxygen in a catabolic process to utilize quercetin as a source of carbon. The reaction involves the C-C cleaving, the insertion of both oxygen atoms, and the release of carbon monoxide. This oxidative decarbonylation produces the corresponding depside of quercetin,[9] as shown in Figure 1.1.

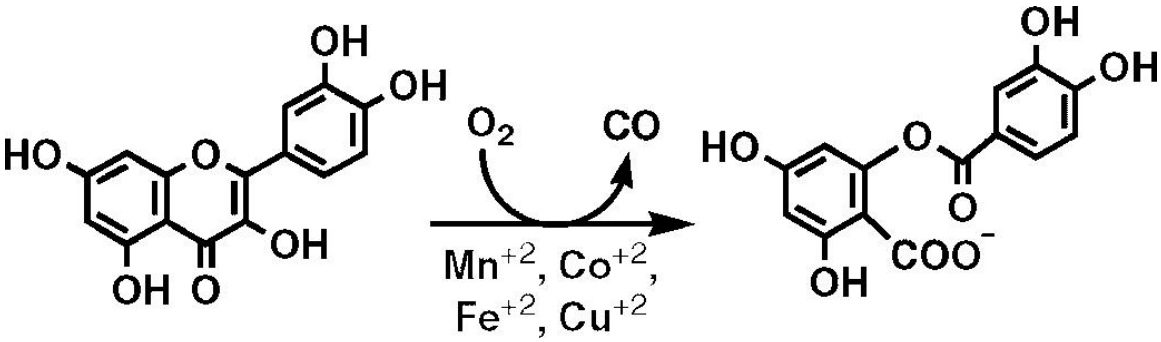

Figure 1.1. Reaction of quercetin to its corresponding depside as catalyzed by QDO.[11]

The QDO of interest is from *Bacillus subtilis*, the first known example of bacterial QDO.[11] The 3-D represintation of the enzymes active site is shown in Figure 1.2. It is a homodimeric glycoprotein, with each subunit possessing a Cu(II) ion. The protein has a mass of approximately 100 kDa. The active site is approximately 10 Å within the proteins surface, but is accessible by solution. Two conformations of the active site have been observed in a seven to three ratio. Each involves ligation with three histidine residues and a water molecule, hydrogen bonded to a glutamine. The major conformation of the Cu has a distorted tetrahedral structural configuration. The Cu of the minor species also has a bond with a glutamate residue, which leads to a trigonal bipyramidal structure. To form the enzyme substrate complex, the deprotonated oxygen of the hydroxyl group at the C3 position replaces the water in the copper coordination.[11] Both subunits belong to the superfamily cupin, characterized by a β-barrel tertiary

structure. Two conserved motifs comprise the cupin domain separated by a loop of approximately 20 residues. The two motifs have been shown to ligate multiple divalent metals. QDO has been speculated to contain one Fe cofactor per sub unit;[12] however recent studies indicate that Mn is more likely to be the native cofactor.[11] Histidine residues 62, 64, and 103 and Glu 69 form the coordination sphere of the N-terminal motif, and His 234, 236, and 275 and Glu 241 of the C-terminal motif. Analysis of QDO from *B. subtilis* has shown that of the various substitutions of metal cofactor Mn(II) has been shown to be the most active.[11]

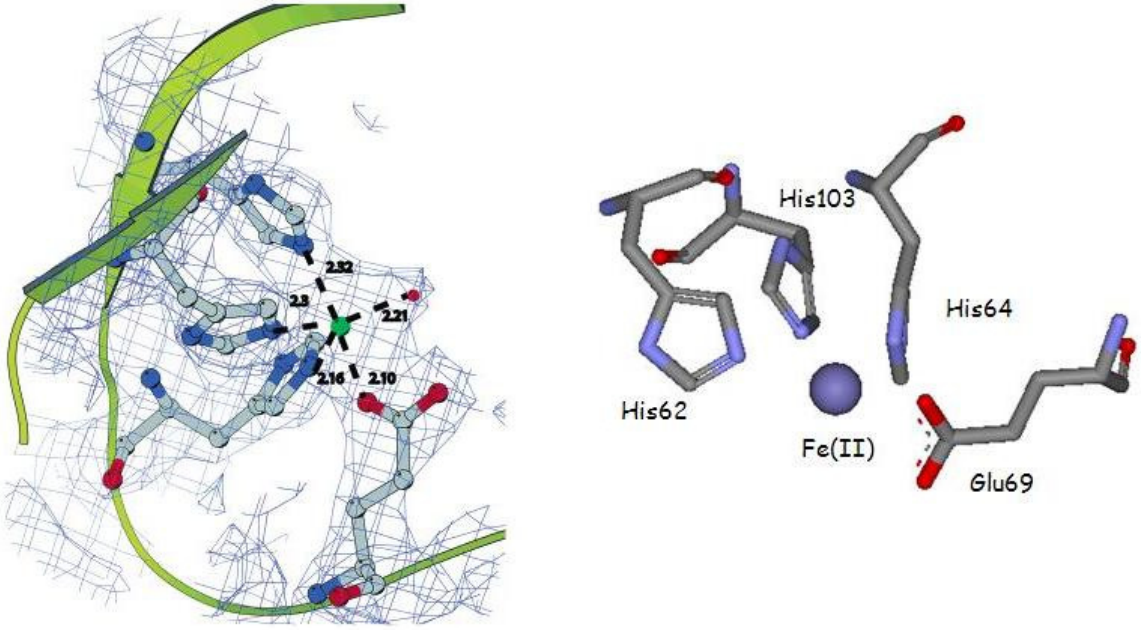

Figure 1.2. Active site of QDO from *B. Subtilis*.[12]

Flavonoids

Flavonoids, a class of plant metabolites, have been theorized, with some supportive evidence, that number and location of hydroxyl groups on flavonoids may play a role in substrates specificity.[11] Activity of QDO towards quercetin, myricetin, and HOPTO will be studied.

HNO

Nitrosyl hydride (HNO) has been shown to form a stable adduct with Fe containing oxygen binding globulins, and will be investigated as a dioxygen analogue with the native and metal substituted QDO. HNO is the reduced and protonated form of nitric oxide (NO).[13] HNO rapidly dimerizes to N_2O and H_2O, Equation 1.1, and therefore has a limited lifetime in solution.[14] Above pH 12 the triplet deprotonated state $^3NO^-$ exists, at lower pH the singlet state ^1HNO dominates. NO^- is isoelectronic with dioxygen. Because of its limited lifetime in solution, HNO is generated from precursors that decompose to release HNO, such as Angeli's Salt (AS), Equation 1.2. HNO has been shown to form stable adducts in both myoglobin[15] and hemoglobin.[14] HNO can replace the dioxygen in the enzymatic reaction, incorporating both the oxygen and the nitrogen in the product. Mn-QDO has also been shown to mimic its oxygenase activity on HNO, with simultaneous release of CO. This is referred to as its nitroxygenase activity.[8]

Equation 1.1. $2HNO \rightarrow [H_2N_2O_2] \rightarrow N_2O_{(g)}$ and H_2O

Equation 1.2. $HN_2O_3^- \leftrightarrows HNO + NO_2^-$

Studying the activity of oxygenases under various conditions can provide insight into the mechanisms by which they work. Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE, and kinetic spectrophotometry were used to identify and study the activity of the enzyme. Purified samples were also collected for an EPR analysis, but it was not a sufficient amount by the time of this publication.

CHAPTER TWO

Procedure

Summary

Escherichia coli cells were transformed with a Mn-QDO plasmid obtained from Francisco Lab. A small starter culture was prepared from colonies from an agar plate coated in kanamycin to allow only for the growth of transformed cells. Several large cultures were prepared from the starter culture. The cells were harvested by centrifugation, and lysed using a French press. The lysed cells were centrifuged and the supernatant was purified using anion exchange chromatography. The final purification involved running the product through size exclusion liquid chromatography, using a HiLoad 26/60 Superdex 200 prep grade column.

The kinetic activities of samples of QDO were assayed with native activity for quercetin and other flavinoids. One battery of tests was performed in the presence of dioxygen, and another anaerobic in the presence of HNO.

Transformation

Competent BL21DE3 *Escherichia coli* cells were purchased from Lucigen, and *B. subtilis* QDO plasmid, pQUER4, was obtained from Francisco Lab. The pQUER4 is the expression vector for yxaG expression in pET-30a(+). After thawing for 20 minutes on ice, 1 μL of plasmid was added to 40 uL of competent cells, and left on ice for an additional 20 minutes. The cells were heat shocked for 1 minute at 42 °C. A volume of 0.5 mL of LB media was added to cells. The cells were incubated in a shaker at 205 rpm

for 1 hour at 37 °C. The cells were centrifuged and resuspended in 100 uL of LB media. The cells were then plated on LB agar plates containing 50 μg/mL kanamycin. The cells were incubated overnight at 37 °C.[16]

Growth Media

Some M9 salt was first prepared by dissolving into 800 ml of deionized water 128 g Na_2HPO_4-$7H_2O$, 30 g KH_2PO_4, 5 g NaCl, and 10 g NH_4Cl. The pH was adjusted to 7.2 ± 0.2. This was diluted to 1 L with deionized water, and autoclaved to sterelize. A volume of 900 mL of water was autoclaved in a Fernbach flask. To this 100 mL of M9 salt, 4 g of glucose, and 2 mL of 1M $MgSO_4$.[17]

Starter Culture

Several colonies were picked from the plate and used to inoculate 10 mL of LB media containing 50 ug/mL of kanamycin. The media was allowed to shake overnight. A volume of 10 mL of the starter culture was used to inoculate 100 mL of M9 media containing 50 μg/mL of kanamycin.[18]

Large Culture

A volume of 70 mL of kanamycin was used to inoculate 7 L of M9 media. Fernbach flasks containing M9 media were shaken at 37 °C until an, optical density at 600 nm, OD_{600}, of between 0.4 and 0.8 AU was observed. The cells were induced with the addition of IPTG to a concentration of 0.75 mM and the addition of $MnCl_2$ to a concentration of 1 mM. To this 10 mL of a 20% glucose solution was added. The cells were again shaken at 205 rpm at 25 °C overnight.[18]

Harvesting

Cells were harvested by centrifugation at 5000 Gs for 15 minutes. Roughly 40 g of cell paste can be obtained from 7 L of M9 media. Cells were frozen at -30 °C until lysed.[19]

A buffer, 100 mL pH 7.5, for the purpose of lysing cells in the french press was prepared using 0.788 g tris-HCl, 35 μL βME and 0.017 g PMSF in DMSO.[20]

Lysis

The cells were allowed to thaw, and were resuspended in a lysis buffer, approximately 3 mL per every 4 g of paste. The cells were fully lysed using a french press. The cells were ultracentrifuged at 25000 rpm for 30 minutes at 4 °C. The supernatant was decanted and collected, and the debris was disposed of.[21]

The French press was prepared by applying lubricant to all rubber parts and assembly of the six parts. The plunger was inserted and the vessel was filled upside down. This was attached and the bottom was held, flipped over and installed into press. The pressure was raised to 500 psi and switched to medium. Attention was paid to any leaks. The pressure was raised to 1200 psi and switched to high. After 7 minutes the valve was opened slowly maintaining pressure above 1000 psi. The apparatus was switched to medium then to down, opening and closing the valve to get remaining paste. The pressure was reduced to atmospheric. This was repeated three times per sample.

Purification

The supernatant was added to a DEAE column equilibrated with 50 mM tris 100 mM NaCl buffer. The buffer was run through column until a stable A_{280} was reached, at which time 50 mM tris 600 mM NaCl was run through column. Fractions showing absorbance at 280 nm were collected and concentrated using ammonium sulfate precipitation. The concentrated proteins were run through a size exclusion column (Superdex 26/60) for at least 16 hours using the 50 mM tris 175 mM NaCl buffer.[16]

Ammonium Sulfate Precipitation

A volume of approximately 25 mL was weighed exactly into paired JA 25.50 centrifuge tubes along with 9.33 g of ammonium sulfate. The tubes were shaken gently at 4°C for one hour. The pairs of tubes were centrifuged at 15,000 g for 30 min at 4 °C. Each pellet was resuspended in 0.3 to 0.4 mL of 175 mM NaCl 50 mM Tris buffer.[22]

Expression

Cells grown with Mn cofactor were suspended in M9 media, and induced with 50 mg/L isopropyl-β-D-thiogalactopyranoside. The substitution metal was introduced by the addition of 1 mM of the respective metal chloride salts. The cells were harvested and the proteins were purified as before, except that the protein was eluted from a DEAE-Sephacel column with 125 followed by 175 mM NaCl in 50 mM TRIS buffer pH 7.5.[11]

Enzymatic Assay

Activity was assayed spectrophotometrically at 380 nm. The use of quercetin as the substrate in an aerobic environment was used in each case as a benchmark. The most pure and active samples were combined and concentrated.[16] Kinetic assays were performed on three different substrates aerobically and anaerobically using HNO.

HNO Precursors

Angeli's salt (AS) was purchased from Cayman Chemicals. AS decomposes to yield HNO.[10] An excess of the HNO precursor was required due to the rapid dimerization of HNO to N_2O.

CHAPTER THREE

Results and Discussion

Expression and Purification

Over 20 batches of QDO were expressed, however only three proved to be viable, two of which were used in this thesis. Enzyme 1 was not fully purified and therefore not quantified. Enzyme 2 was purified and was ~250 μL of ~229 μM QDO. The concentration was calculated from the absorbance at 280 nm of a 100X dilution, 0.14082, and the molar extinction coefficient 61455 cm^{-1} M^{-1} [23] using Beer's law. All of the catalytic turnover rate studies were done using Enzyme 2. The FPLC chromatograph is pictured in Figure 3.1. Figure 3.2 shows an SDS-PAGE gel of the third sample in the fourth well. The fifth and sixth wells were from other fractions of the same FPLC sample. The first well is the ladder or marker. The mark in the fourth well corresponds approximately to 50 kDa in mass. This was expected since QDO is an approximately 100 kDa dimer, and only monomers show up in SDS-PAGE.

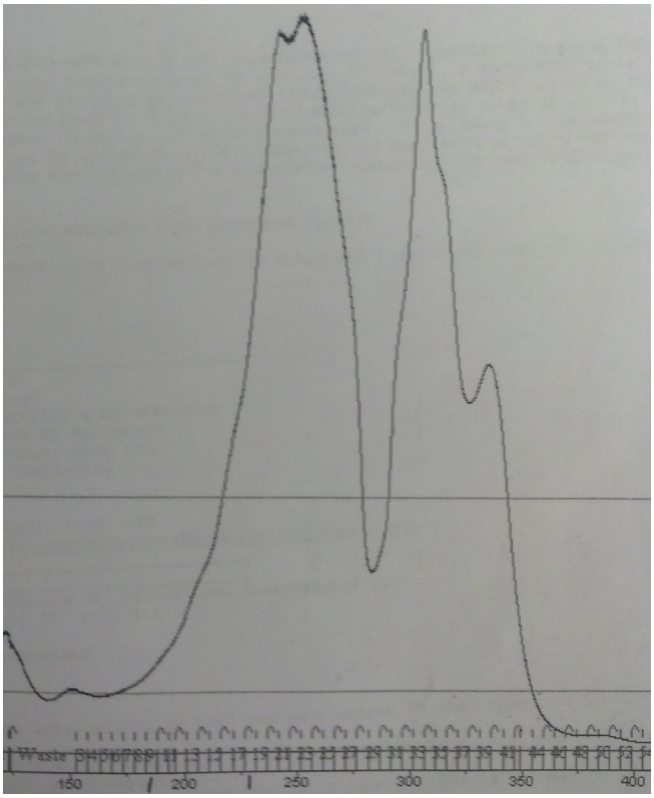

Figure 3.1. FPLC Chromatograph. Fractions 9 through 18 contained QDO.

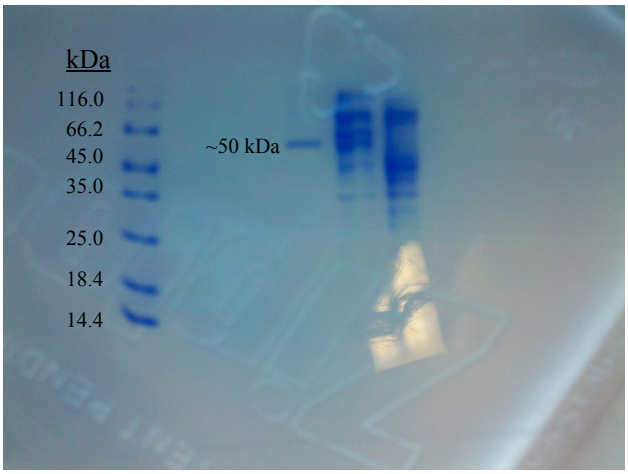

Figure 3.2. SDS-PAGE gel. Lane 1 is the marker. Lane 5 contains one line at approximately 50 kDa indicating QDO. Lanes 6 and 7 are from other fractions and indicate a mixture of proteins.

Kinetics Data

Each kinetics assay was performed on a UV-Vis spectrophotometer for a period of one hour at one-minute intervals. Figure 3.3 shows the data from Enzyme 2, quercetin, and O_2. The reaction using Enzyme 2, myricetin and HNO is shown in Figure 3.4. The first image is the complete spectral data focused in on the wavelength of maximum absorbance, λ_{max}, for the myricetin. The second shows a graph of the absorbance over time for λ_{max}.

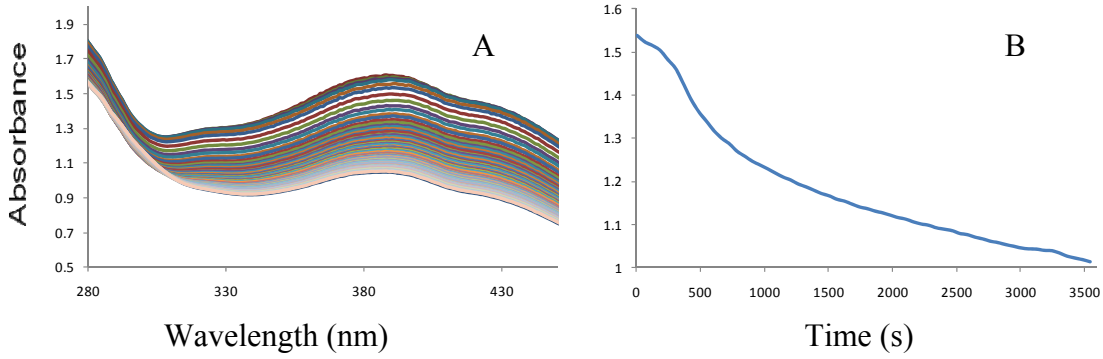

Figure 3.3. Time course UV-Vis spectra of reaction of O_2 with QDO (Enzyme 2) and quercetin in pH 7.0 phosphate buffer (A) absorbance versus wavelength (B) absorbance versus time.

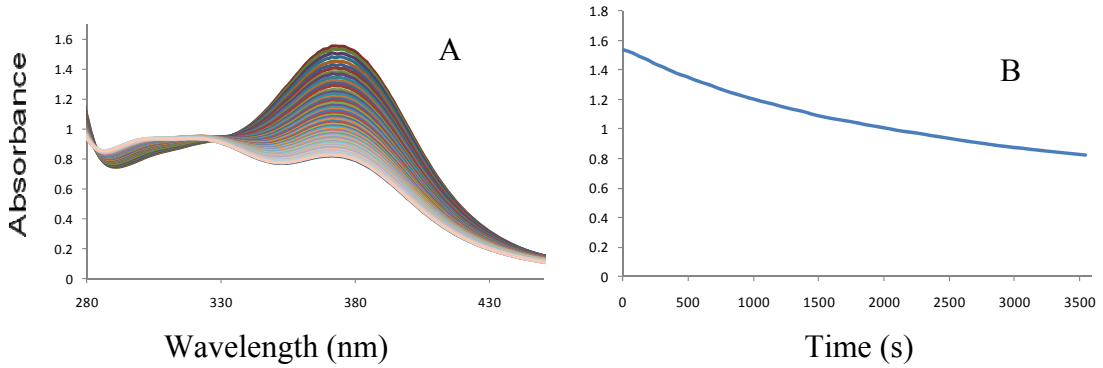

Figure 3.4. Time course UV-Vis spectra of reaction of AS (HNO) with QDO (Enzyme 2) and myricetin in pH 7.0 phosphate buffer (A) absorbance versus wavelength (B) absorbance versus time.

The rate of loss of myricetin (79 μM) on a reaction with Mn-QDO (1.3 μM) and AS (670 μM) was used for to calculate the catalytic turnover rate of 2.2 x 10^3 M^{-1} s^{-1}. The rate of loss of substrate in the reaction with O_2 and HNO for Enzyme 2 as calculated by the spectrophotometer software is shown in Table 3.1.

Table 3.1. Initial catalytic turnover rates.

Substrate	Dioxygen (Au/s)	HNO (Au/s)
Quercetin	3.5 x 10^{-4}	2.9 x 10^{-4}
Myricetin	1.1 x 10^{-4}	4.3 x 10^{-4}

HOPTO

Another similar study was performed using HOPTO, 4.4'-dithiobis(1,2-dimethylpyridinium-3-oxide). The results with no enzyme at pH 7 as well as with enzyme 1 at various pHs is shown in Figure 3.12. The lower pHs seemed to correspond to the fastest rates of activity.

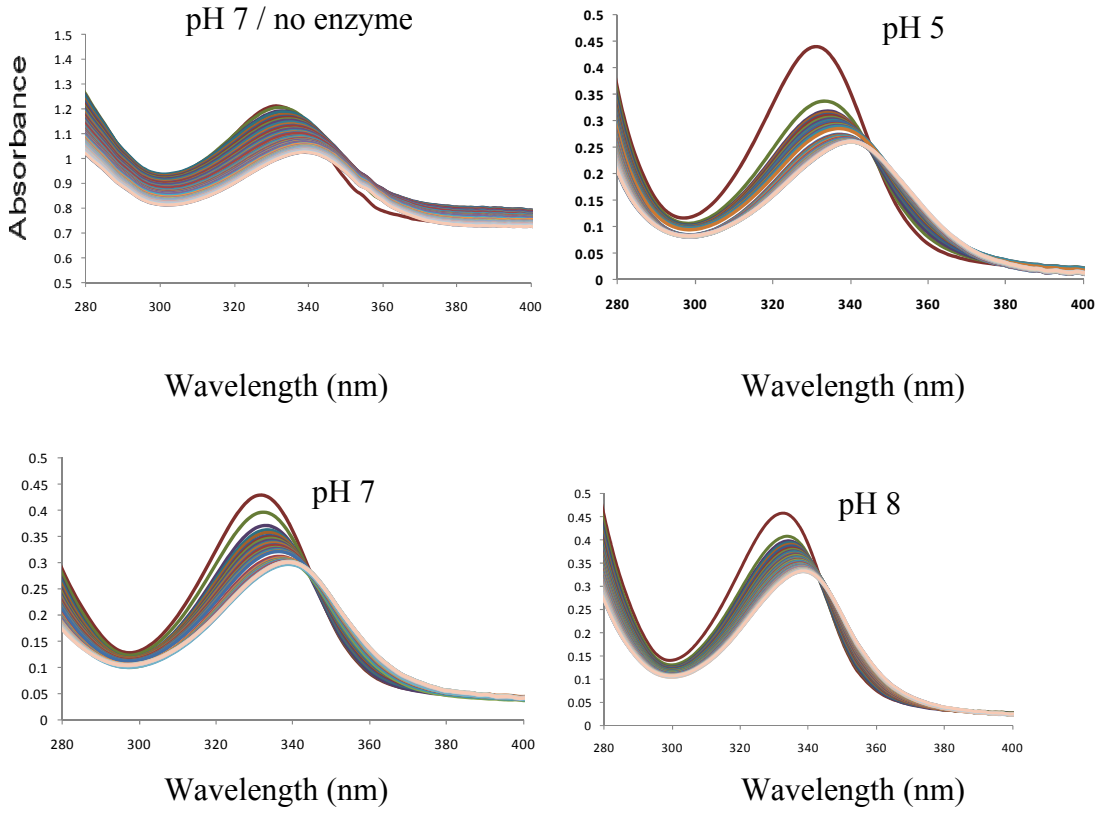

Figure 3.5. Time course UV-Vis spectra of reaction of AS (HNO) and HOPTO (A) with QDO (Enzyme 1) in pH 7.0 phosphate buffer (B) in pH 5.0 phosphate buffer (C) in pH 5.5 phosphate buffer (D) in pH 6.0 phosphate buffer (E) in pH 6.5 phosphate buffer (F) in pH 7.0 phosphate buffer (G) in pH 7.5 phosphate buffer (H) in pH 8.0 phosphate buffer absorbance versus wavelength.

CHAPTER FOUR

Conclusion and Future Work

Conclusions

The project failed to achieve many of the goals originally set, but was overall successful in that some Mn-QDO was purified and studied and the successful techniques derived can now be repeated to express and purify the large volumes of the enzyme required for future analysis. It can be concluded that the dioxygenase and nitroxygenase reactions occur at rates independent of one another. The turnover rate of quercetin is greater aerobically. The turnover rate of myricetin is greater anaerobically. Myricetin had the slowest rate observed aerobically but the highest rate anaerobically. It can be speculated at this point that the arrangements of the hydroxyl groups on the C ring also affects the turnover rate. Through looking at preliminary catalytic rate kinetic activity assays of other flavonols, it seems that the 2,3 double bond is necessary for the dioxygenase and nitroxygenase turnover activity of QDO.

The rate of the reaction involving HOPTO was faster in the conditions of lower pH and very slow in the absence of the QDO. This reaction not only showed a decrease in absorbance at λ_{max}, but a shift in the wavelength of maximum absorbance from ~ 330 to 340 nm.

Future Work

Currently, the process continues and Mn-QDO is being expressed and purified. Upon obtaining a 1 mL of 1 mM solution of QDO EPR, Electron Paramagnetic Resonance, analysis will be performed. EPR will allow for further visualization of the enzyme and possibly insight into the mechanisms of its activity. In addition Fe and Cu QDO will be produced and studied. Other flavonols to be studied include morin, catechin, luteolin, taxifolin, galangin, datiscetin, kampferol, and fisetin.

REFERENCES

1 “Metal Ion Transport and Storage” Bertini, I.; Gray, H.B.; Lippard, S.J.; Valetine, J.S. Bioinorganic Chemistry, University Science Books, Mill Valley, CA **1994**, Chp. 4, 167-170.

2 Hayaishi, O.; Katagiri, M.; Rothberg, S. *J. Am. Chem. Soc.* **1955** 77 5450–5451.

3 Mason, H.S.; Fowlks, W.L.; Peterson, E. *J. Am. Chem. Soc.* **1955** 77 2914-2915.

4 Oxidoreductase. *MeSH*. http://www.nlm.nih.gov/cgi/mesh/2013/MB_cgi?mode=&index=9654&field=all&HM=&II=&PA=&form=&input= (accessed Nov 1, **2012**).

5 “Dioxygen Reactions” Bertini, I.; Gray, H.B.; Lippard, S.J.; Valetine, J.S. Bioinorganic Chemistry, University Science Books, Mill Valley, CA **1994**, Chp. 5, 253-254.

6 Kovaleva, E.G.; Lipscomb, J.D. *Nat. Chem. Biol.* **2008** 4 186–193.

7 Costas, M.; Mehn, M.P.; Jensen, M.P.; Que, L. Jr. *Chem. Rev*. **2004** 104 939–986.

8 Dioxygenase. *MeSH* http://www.nlm.nih.gov/cgi/mesh/2013/MB_cgi?mode=&index=22724&field=all&HM=&II=&PA=&form=&input= (accessed Nov 1, **2012**).

9 Pap, J.S.; Kaizer, J.; Speier, G. *Coor. Chem. Rev.* **2010** 254 781-783.

10 Kumar, M.R.; Zapata, A.; Ramirez, A.J.; Bowen, S.K.; Francisco, W.A.; Farmer, P.J. *PNAS*. **2011** 108.47 18926-18931.

11 Shaab, M.R.; Barney, B.M.; Francisco, W.A. *Biochem.* **2006** 45 1009.

12 Gopal, B.; Madan, L.L.; Betz, S.F.; Kossiakoff, A.A. *Biochem.* **2005** 44 193.

13 Shafirovich, V.; Lymar, S.V. *J. Am. Chem. Soc.* **2003** 125 6547–6552.

14 Kumar, M.R.; Pervitsky, D.; Chen, L.; Paulos, T.; Kundu, S.; Hargrove, M.S.; Rivera, E.J.; Diaz, A.; Colón, J.L.; Farmer, P.J. *Biochemistry*. **2009** 48 5018-5019.

15 Lin, R.; Farmer, P.J. *J. Am. Chem. Soc*. **2000** 122 2393-2394.

16 Barney, B.M.; Schaab, M.R.; LoBrutto, R.; Francisco, W.A. *J. Phys. lett. b.* **2003** 35 131-141.

17 "M9 Minimal Media" Sambrook, J.; Fritsch, E.F.; Maniatis, T. Molecular Cloning, *2nd ed*., Vol. 3, Cold Spring Harbor Laboratory Press, USA **1989** Appendix A, 3 A3.

18 Baev, M.V.; Baev, D.; Radek, A.J.; Campbell, J.W. *Appl. Microbiol Biotechnol.* **2006** 71(3) 310-316.

19 "Centrifugation in Biochemical Research" Boyer, R. Modern Experimental Biochemistry, *3rd ed*., Benjamin Cummings, San Francisco, CA **2000**, Chp. 7, 201.

20 Preparation of cell lysates from E.coli using a French Press. *EMBL.*. http://www.embl.de/pepcore/pepcore_services/protein_purification/extraction_clarification/cell_lysates_ecoli/french_press/index.html (accessed Oct 5, **2012**).

21 "Electron Transport and Oxidative Phosphorylation" Voet, D.; Voet, J.G.; Pratt, C. Fundamentals of Biochemistry, *3rd ed*., Wiley, USA **2008**, Chp. 18, 638.

22 Englard, S.; and Seifter, S. *Methods Enz.* **1990** 182 285-300.

23 Fittipuloli, M.; Steiner, R.A.; Matsushita M.; Dijkstra, B.W.; Groenen, E.J.; Huber, M.. *Biophys. J.* **2003** 85 (6) 4047-4054