A Novel Method, and its Applications, to Study Alcohol Oxidase from Auxiliary Activity

Family 5 Based on Direct Monitoring of Enzymatic Reactions

by

Fan Xia

B.Sc., The University of British Columbia, 2015

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Chemistry)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

January 2019

© Fan Xia, 2019
The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

A Novel Method, and its Applications, to Study Alcohol Oxidase from Auxiliary Activity Family 5 Based on Direct Monitoring of Enzymatic Reactions

submitted by Fan Xia in partial fulfillment of the requirements for the degree of Master of Science in Chemistry

Examining Committee:

Prof. Harry Brumer, Chemistry and Michael Smith Laboratory

Co-supervisor
Prof. Jason Hein, Chemistry

Co-supervisor
Prof. Martin Tanner, Chemistry

Supervisory Committee Member
Prof. Russ Algar, Chemistry

Supervisory Committee Member
Prof. Elliott Burnell, Chemistry

Committee Chair
Abstract

Auxiliary Activity Family 5 (AA5) carbohydrate-active enzymes are mononuclear copper radical oxidases (CROs) capable of oxidizing a variety of alcohols to their corresponding aldehydes without organic cofactors. Classically, AA5 characterization is performed using a horseradish peroxidase (HRP) coupled assay using ABTS (2,2’-azino-bis(3-ethylbenthiazoline-6-sulphonic acid) as a colorimetric indicator. However, this HRP-ABTS coupled assay indirectly monitors reaction progress – it measures a small portion of reactant conversion and provides no information about the extent of oxidation (percentage conversion) or potential product inhibition. Therefore, we developed an alternative approach to assess alcohol oxidation by AA5 enzymes that allows direct monitoring of reaction progress by incorporating tandem reaction progress analysis. This new approach collects time-resolved information about active chemical species and facilitates the interrogation and optimization of the system. CgrAlcOx, an AA5 oxidase from the phytopathogenic fungal species Colletotrichum graminicola, was previously characterized and displayed activity on a wide variety of aromatic and aliphatic alcohols. Reaction monitoring using the HRP-ABTS assay in this previous study was used as a benchmark to develop our new approach. We tested different instruments and identified high performance liquid chromatography coupled with an ultraviolet (UV) detector as the best way to directly monitor, on-line, the depletion of alcohols and the formation of aldehydes in alcohol oxidation catalyzed by CgrAlcOx. Reaction conditions were optimized, and the kinetics of aromatic alcohol oxidation were determined to support the applicability of this approach for monitoring enzymatic reactions. This new approach also allowed the detailed study of CgrAlcOx, including the screening of new substrates and the detection of product inhibition; previous assays did not permit the latter. Lastly, this new approach...
was combined with the HRP-ABTS coupled assay in a complementary way to overcome the inability of the previous assay to characterize reaction inhibitors. This allowed the discovery of new inhibitors of CgrAlcOx, such as benzylamine and benzyl mercaptan. In addition, using this combinatorial approach, butanethiol was identified as both an inhibitor and a substrate towards CgrAlcOx.
Lay Summary

Auxiliary Activity Family 5 (AA5) carbohydrate-active enzymes are mononuclear copper radical oxidases (CROs) capable of oxidizing a variety of alcohols to their corresponding aldehydes without organic cofactors. However, the activity assay widely used to characterize CROs is not adequate in providing enough information for the industrial utilization of AA5 proteins. Therefore, we developed a different approach to assess alcohol oxidation by AA5 enzymes that allows direct monitoring of reaction progress by incorporating tandem reaction progress analysis. CgrAlcOx, an AA5 oxidase from the phytopathogenic fungus Colletotrichum graminicola, was previously characterized and was used in the development of this new technique. This new approach was utilized to directly monitor, on-line, alcohol oxidation catalyzed by CgrAlcOx. It permitted us to determine percentage conversion, reaction kinetics, and product inhibition; to undertake reaction optimization; and to carry out new substrate and inhibitor screening.
Preface

The work present in this thesis is an original and unpublished work by the author F. Xia. The project was designed by my supervisors, Dr. Harry Brumer and co-supervisor, Dr. Jason Hein. All the experiments were performed by the author with different collaborators listed below.

The use of HPLC and ReactIR was through collaboration with Dr. Jason Hein’s lab. The $^1$H-NMR experiments for kinetics were performed through help of Mark Okon, NMR facility manager, in Dr. Lawrence McIntosh’s lab, but the results were analyzed by the author. The data analysis was done with collaboration with my supervisor.
# Table of Contents

Abstract .................................................................................................................................................. iii

Lay Summary ........................................................................................................................................ v

Preface .................................................................................................................................................... vi

Table of Contents ................................................................................................................................... vii

List of Tables ........................................................................................................................................ xi

List of Figures ......................................................................................................................................... xii

List of Symbols and Abbreviations ....................................................................................................... xviii

Acknowledgements ................................................................................................................................. xxi

Dedication ............................................................................................................................................... xxii

Chapter 1: ............................................................................................................................................... 1

1.1 Introduction ....................................................................................................................................... 1

1.2 Thesis objectives ................................................................................................................................ 17

Chapter 2: New Assay Development with Cerium Oxide Nanoparticle ............................................. 18

2.1 Introduction of CeO$_2$ Nanoparticle as a Colorimetric Assay and its Capability in Monitoring Enzymatic Reaction .................................................................................................................. 18

2.2 Development of CeO$_2$ Nanoparticle Assay to Detect Enzymatic Activity ..................................... 20

Chapter 3: Assay Development based on Tandem Reaction Progress Analysis .................................... 25

3.1 Introduction ....................................................................................................................................... 25

3.2 ReactIR Approach by using DST Conduit Model and Flow Cell .................................................... 25

3.3 $^1$H-NMR Approach .......................................................................................................................... 32

3.4 Monitoring CgrAlcOx Oxidation with HPLC-UV ............................................................................. 37
3.5 Adaptation of an HPLC Method to Determine Stereochemistry of Alcohol Oxidation 40
3.6 Summary of Assays Development ................................................................. 43

Chapter 4: Using HPLC-UV Approach for Enzymatic Reaction Analysis ............... 44

4.1 Optimization of CgrAlcOx Oxidation for Kinetics ....................................... 44
   4.1.1 Optimizing CgrAlcOx Oxidation using Catalase as H$_2$O$_2$ Scavenger Enzyme .... 44
      4.1.1.1 Depletion of Substrate, and Stability of CgrAlcOx Effect on Reaction Conversion Rate ................................................................. 44
      4.1.1.2 Test of Product Inhibition by Benzaldehyde on CgrAlcOx ....................... 46
      4.1.1.3 Converting Reaction Progress Curves to a Michaelis-Menten Plot ............ 47
      4.1.1.4 Michaelis-Menten Plots using Catalase as the Only Hydrogen Peroxide Scavenger Enzyme .................................................................... 49
   4.1.2 Optimizing CgrAlcOx Oxidation Reaction Testing Both HRP and Catalase as H$_2$O$_2$ Scavenger Enzymes and Delivery Means of O$_2$ ........................................ 50
      4.1.2.1 CgrAlcOx Oxidation Reaction Testing Both HRP and Catalase as H$_2$O$_2$ Scavenger Enzymes .................................................................. 50
      4.1.2.2 Test of Best Means to Deliver O$_2$ into Solution .................................... 54
   4.1.3 Optimized Kinetics by HPLC ..................................................................... 56

4.2 Study of CgrAlcOx Activation using HPLC-UV ............................................... 58

Chapter 5: Applications of HPLC-UV Approach in Studying New Substrates and Inhibitors .............................................................................................................. 62

5.1 Introduction ........................................................................................................ 62
5.2 Competition Experiments for Potential Substrate Screening ............................ 63
5.3 HPLC-UV Approach used for Product Inhibition Study .................................... 65
Chapter 6: Conclusion and Future Work ......................................................... 88

6.1 Conclusion ......................................................................................... 88

6.2 Future Work ...................................................................................... 90

Bibliography ............................................................................................. 92

Appendices ............................................................................................... 98

Appendix A CgrAlcOx Production and Activity Determination ....................... 97

A.1 CgrAlcOx Production and Optimization ................................................ 97

A.2 Purification and Deglycosylation of CgrAlcOx ...................................... 97

A.3 CgrAlcOx Activity Determination using HRP-ABTS Assay .................... 99

Appendix B Methods Session ..................................................................... 101

B.1 General .............................................................................................. 101

B.2 Enzymatic Test with CeO₂ Nanoparticle ............................................. 102

B.3 ReactIR Reactions: DST Series AgX Fibre Conduit Model .................... 102

B.4 ReactIR Reactions: DS Micro Flow Cell Model ................................... 103

B.5 ¹H NMR Test for Kinetics .................................................................... 103
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.6</td>
<td>General preparation procedure for reactions in reaction progress analysis ..........104</td>
</tr>
<tr>
<td>B.7</td>
<td>Procedure for HPLC Analysis ........................................................................104</td>
</tr>
<tr>
<td>B.8</td>
<td>Calibration Curves Generated by HPLC ...............................................................106</td>
</tr>
<tr>
<td>B.9</td>
<td>Glycerol Oxidation and Analysis on HPLC-UV .....................................................106</td>
</tr>
<tr>
<td>B.10</td>
<td>Enzymatic Reaction Bubbling with O(_2) ................................................................107</td>
</tr>
<tr>
<td>B.11</td>
<td>Substrate/inhibition Screening Experiment ........................................................108</td>
</tr>
<tr>
<td>B.12</td>
<td>Irreversible Inhibition Study ..............................................................................108</td>
</tr>
<tr>
<td>B.13</td>
<td>Duration of Inhibition Experiments using HPLC-UV ..............................................109</td>
</tr>
<tr>
<td>B.14</td>
<td>(^1)H NMR Test for Product and Reaction Analysis ............................................110</td>
</tr>
</tbody>
</table>

Appendix C Calibration Curves for HPLC Analysis .................................................111

Appendix D \(^1\)H-NMR Spectra ..............................................................................112

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.1</td>
<td>Glyceraldehyde DNP Hydrazone MS and (^1)H-NMR .............................................112</td>
</tr>
<tr>
<td>D.2</td>
<td>2-phenyl-1-propanol and 2-phenylpropanal .........................................................113</td>
</tr>
<tr>
<td>D.3</td>
<td>2-phenylethanol and phenylacetaldehyde ...............................................................114</td>
</tr>
<tr>
<td>D.4</td>
<td>3-phenyl-1-propanol and 3-phenylpropionaldehyde ...............................................115</td>
</tr>
<tr>
<td>D.5</td>
<td>3-benzyloxy-1-propanol and 3-benzyloxypropionaldehyde .....................................115</td>
</tr>
<tr>
<td>D.6</td>
<td>2-(benzyloxy)ethanol and benzyloxyacetaldehyde ..................................................116</td>
</tr>
</tbody>
</table>
List of Tables

Table 1.1 Summary of apparent parameter changes with each type of reversible inhibition ......16
Table 4.1 Summary of kinetic parameters obtained from Michalis-Menten plots by using 11.5
μM catalase only as H₂O₂ scavenger enzyme.................................................................50
Table 4.2 Summary of the kinetic parameters obtained from Michaelis-Menten Kinetic plots
obtained using HPLC-UV to monitor the depletion of the starting material or the formation of
product................................................................................................................................57
Table 5.1 Amine and thiol candidate compounds for screening .............................................62
Table 5.2 Summary of percentage conversion of substrates observed through ¹H NMR........74
Table 5.3 18.7 mM BnNH₂ was incubated in different conditions to determine percentage
conversion to benzaldehyde.....................................................................................................76
Table A.1 Different ratio of BMGY and BMMY media in CgrAlcOx production...............97
Table A.2 Reproduced activity of CgrAlcOx with HRP-ABTS assay and compare to the previous
reported values .......................................................................................................................99
**List of Figures**

Figure 1.1-1 Mechanism of alcohol oxidation by the AA5_2 family of carbohydrate-active enzymes reproduced from reference 18 .................................................................5

Figure 1.1-2 The underlying basis of the HRP-ABTS assay ..................................................6

Figure 1.1-3 A mechanism of irreversible enzyme inhibition ...............................................12

Figure 1.1-4 A mechanism of competitive enzyme inhibition ..............................................13

Figure 1.1-5 A mechanism for uncompetitive enzyme inhibition .......................................14

Figure 1.1-6 A mechanism for mixed enzyme inhibition .....................................................14

Figure 1.1-7 Theoretical representation of Michaelis-Menten kinetics for different types of reversible inhibition ...............................................................................................16

Figure 2.1-1 Mechanism of CeO$_2$ nanoparticles, showing catalase mimetic activity ............19

Figure 2.2-1 Linear response of CeO$_2$ nanoparticles towards H$_2$O$_2$ monitored by UV-vis spectrometer ........................................................................................................20

Figure 2.2-2 Ten μL of CeO$_2$ nanoparticles were dissolved in water (-----) or 50 mM sodium phosphate at pH 8.0 (-----). The solution was continuously monitored by UV-vis spectrometry at 465 nm for 2 minutes. ........................................................................................................21

Figure 2.2-3 CeO$_2$ nanoparticles react with H$_2$O$_2$ in solution to produce a peak, and subsequent relaxation, in absorbance .............................................................................................22

Figure 2.2-4 Comparison of the sensitivity of the CeO$_2$ assay and HRP-ABTS assay ..........24

Figure 3.2-1 The reaction monitored by ReacIR with two modules: A. with DST series AgX fibre conduit model; B. DS micro flow cell model ........................................................................26
Figure 3.2-2 A. Spectra of 100 mM benzyl alcohol oxidation catalyzed by CgrAlcOx, with the buffer, catalase, and HRP spectra subtracted; B. Magnified view of the spectra from 1400 to 1900 cm⁻¹; C. Change in intensity of the spectra monitored at 1705 cm⁻¹.................................................27

Figure 3.2-3 A. Spectrum of 28 mM benzaldehyde in water; B. Magnified view of the spectrum from 1400 to 1900 cm⁻¹; C. Change in intensity of the spectrum monitored at 1705 cm⁻¹...........28

Figure 3.2-4 A. The spectrum of 28 mM benzaldehyde in ethanol; B. Magnified view of the spectrum from 1600 to 1900 cm⁻¹; C. Change in the intensity of the spectrum monitored at 1705 cm⁻¹.................................................................29

Figure 3.2-5 A. Spectrum of 100 mM benzaldehyde in ethanol; B. Magnified view of the spectrum from 1600 to 1900 cm⁻¹; C. Change in the intensity of the spectrum monitored at 1705 cm⁻¹.................................................................30

Figure 3.2-6 A. Spectrum of 100 mM benzaldehyde in water; B. Magnified view of the spectrum from 1400 to 1900 cm⁻¹; C. Change in the intensity of the spectrum monitored at 1705 cm⁻¹.....31

Figure 3.3-1 The ¹H-NMR spectrum of each component in the alcohol oxidation reaction added in series........................................................................................................................................34

Figure 3.3-2 Overlay of the spectra of the CgrAlcOx oxidation reaction monitored by ¹H NMR........................................................................................................................................35

Figure 3.3-3 A. Kinetics of CinOH oxidation using the ABTS assay; B. Spectrum of 0.2 mM CinOH oxidation compared to 2 mM CinOH oxidation measured by ¹H NMR.........................36

Figure 3.4-1 Effect of catalase on substrate conversion..........................................................38

Figure 3.4-2 Reaction curves generated by monitoring the depletion of alcohol and the formation of aldehyde in a 0.5 mM benzyl alcohol oxidation reaction ..................................................39

Figure 3.5-1 Stereochemistry determination of glycerol oxidized by CgrAlcOx ..................42
Figure 4.1-1 A CgrAloCx spiking experiment showing a slowing of reaction rate in response to depletion of substrate ........................................................................................................................................44

Figure 4.1-2 Substrate spiking experiment: the decline in reaction rate was not caused by the denaturation of CgrAloCx ......................................................................................................................................45

Figure 4.1-3 Overlay of reaction curves to test for potential product inhibition caused by benzaldehyde. ........................................................................................................................................47

Figure 4.1-4 Schematic representation of the method for converting reaction progress curves into Michaelis-Menten kinetic plots ......................................................................................................................................48

Figure 4.1-5 Michaelis-Menten kinetic plots using 11.5 μM catalase to scavenge H2O2 in alcohol oxidation of: A. benzyl alcohol; B. cinnamyl alcohol. ......................................................................................................................................49

Figure 4.1-6 Two mechanisms by which HRP breaks down H2O2 (reproduced from ref 60) .....51

Figure 4.1-7 Mechanism by which catalase breaks down H2O2 ........................................................................................................................................52

Figure 4.1-8 Reaction curves generated using either catalase (■) or HRP (●) as H2O2 scavenger enzymes........................................................................................................................................52

Figure 4.1-9 Both catalase and HRP are required to maximize CgrAloCx activity ..................53

Figure 4.1-10 Molecular oxygen in the reaction mixture is necessary to boost CgrAloCx activity. ........................................................................................................................................54

Figure 4.1-11 A. Air bubbles created when the reaction was bubbled with O2 gas. B. Test of O2 diffusion in the reaction and test of the best way to introduce O2 into solution .........................55

Figure 4.1-12 Michaelis-Menten Kinetics of benzyl alcohol obtained by monitoring: A. Depletion of benzyl alcohol, or B. Formation of benzaldehyde .................................................................58

Figure 4.1-13 Michaelis-Menten Kinetics of cinnamyl alcohol by monitoring: A. Depletion of cinnamyl alcohol, or B. Formation of cinnamyl aldehyde .................................................................58
Figure 4.2-1 HPLC was used to study the activation of $CgrAlcOx$. ..........................................................60

Figure 5.2-1 Competition experiment between BnOH and CinOH. ...............................................................64

Figure 5.3-1 Competition experiment between BnOH and BuOH. .................................................................66

Figure 5.3-2 Test of butyraldehyde as a product inhibitor. .................................................................................67

Figure 5.3-3 Study of irreversible inhibition by butyraldehyde. .................................................................68

Figure 5.3-4 Concentration-dependent inhibition of $CgrAlcOx$ by butyraldehyde. .................................69

Figure 5.3-5 Study of reversible inhibition by butyraldehyde showing the Michaelis-Menten kinetics of different concentrations of butyraldehyde (graph) and the parameters that were obtained (table) ..........................................................................................................................70

Figure 5.3-6 A. The HRP-ABTS assay for 1 mM benzyl alcohol oxidation containing different concentrations of butyraldehyde: no butyraldehyde (---); 0.28 mM (---); 1.4 mM (---); 2.8 mM (---); 4.2 mM (---); 5.6 mM (---); 8.4 mM (---). B. Slopes extracted from the curves in panel A from 0 to 0.5 minutes (——) and from 3.5 to 4 minutes (—); all slopes were converted into s$^{-1}$ and were plotted against the concentration of butyraldehyde. ............71

Figure 5.3-7 Reaction progress curves generated by different instruments to study butyraldehyde product inhibition........................................................................................................................................72

Figure 5.4-1 Reaction curve of 1 mM BnOH oxidation by $CgrAlcOx$ with different concentrations of BnNH$_2$. ........................................................................................................................................75

Figure 5.4-2 $^1$H NMR analysis of BnNH$_2$ conversion to benzaldehyde. .........................................................77

Figure 5.5-1 Competition experiments between 1 mM BnOH and different concentrations of BuNH$_2$, indicating that the inhibition was concentration-dependent ..................................................79

Figure 5.5-2 Study of irreversible inhibition by BuNH$_2$.................................................................................80
Figure 5.5-3 Study of reversible inhibition by BuNH₂ using the HRP-ABTS assay showing the Michaelis-Menten kinetics of different concentrations of BuNH₂ (graph) and the parameters that were obtained (table).................................................................81

Figure 5.5-4 A. Competition experiment between 1 mM BnOH and 1 mM BnSH. B. Study of irreversible inhibition by BnSH. Black curve (■■■): no incubation; Red curve (●●): incubation with 3 mM BnSH for 7 minutes; Pink curve (●●): incubation with 5 mM BnSH for 10 minutes; Green curve (●): incubation with 3 mM BnSH for 26 minutes; Blue curve (▲): incubation with 5 mM BnSH for 23 minutes.................................................................82

Figure 5.5-5 Study of irreversible inhibition by BnSH using the HRP-ABTS assay ........................................83

Figure 5.5-6 Competition studies between BnOH and BuSH .................................................................85

Figure 5.5-7 ¹H-NMR analysis of BuSH oxidation .............................................................................86

Figure 5.5-8 Study of irreversible inhibition by BuSH against CgrAlcOx with the HRP-ABTS assay ........................................................................................................................................87

Figure A.1 SDS-PAGE gel of A. deglycosylated protein: band at 57 kDa is CgrAlcOx, and band located at 35kDa is the PN-Gase F. B. Purified CgrAlcOx ..................................................................................98

Figure A.2 Michaelis-Menten plots using HRP-ABTS assay with substrates A. n-butanol, B. cinnamyl alcohol and C. benzyl alcohol ...........................................................................................................100

Figure C.1 Calibration curve of A. cinnamyl alcohol, B. cinnamyl aldehyde. .................................111

Figure C.2 Calibration curve of A. benzyl alcohol, B. benzaldehyde. ................................................111

Figure D.1-1 ESI-MS of glyceraldehyde DNP hydrazone after purification .................................112

Figure D.1-2 ¹H-NMR spectrum of glyceraldehyde DNP hydrazone after purification ..............112

Figure D.2 ¹H-NMR spectrum of oxidation of 2-phenyl-1-propanol after quick extraction of deuterated chloroform. .................................................................................................113
Figure D.3 $^1$H-NMR spectrum of oxidation of 2-phenylethanol after quick extraction of deuterated chloroform. .................................................................................................................. 114

Figure D.4 $^1$H-NMR spectrum of oxidation of 3-phenyl-1-propanol after quick extraction of deuterated chloroform. .................................................................................................................. 115

Figure D.5 $^1$H-NMR spectrum of oxidation of 3-phenyl-1-propanol after quick extraction of deuterated chloroform. .................................................................................................................. 116

Figure D.6 $^1$H-NMR spectrum of oxidation of 3-phenyl-1-propanol after quick extraction of deuterated chloroform. .................................................................................................................. 117
## List of Symbols and Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Auxiliary Activity Family</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-azino-bis(3-ethylbenthiazoline-6-sulphonic acid</td>
</tr>
<tr>
<td>ABTS&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>Oxidized ABTS</td>
</tr>
<tr>
<td>AgX</td>
<td>Silver halide</td>
</tr>
<tr>
<td>BMGY</td>
<td>Pichia cell growth media</td>
</tr>
<tr>
<td>BMMY</td>
<td>Protein inducing media</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Bu</td>
<td>butyl</td>
</tr>
<tr>
<td>CAZymes</td>
<td>Carbohydrate-Active EnZymes</td>
</tr>
<tr>
<td>CBM</td>
<td>Carbohydrate binding module</td>
</tr>
<tr>
<td>CE</td>
<td>Carbohydrate Esterases</td>
</tr>
<tr>
<td>Cgl</td>
<td>Colletotrichum gloeosporioides</td>
</tr>
<tr>
<td>Cgr</td>
<td>Colletotrichum graminicola</td>
</tr>
<tr>
<td>CgrAlcOx</td>
<td>Colletotrichum graminicola alcohol oxidase</td>
</tr>
<tr>
<td>Cin</td>
<td>cinnamyl</td>
</tr>
<tr>
<td>cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>wavenumber</td>
</tr>
<tr>
<td>CRO</td>
<td>Copper radical oxidases</td>
</tr>
<tr>
<td>D-GAP</td>
<td>D-glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>E</td>
<td>Enzyme</td>
</tr>
<tr>
<td>EI</td>
<td>Enzyme-inhibitor complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>ES</td>
<td>Enzyme-substrate complex</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ET</td>
<td>Electron transfer</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FgrGalOx</td>
<td><em>Fusarium Graminearum</em> galactose oxidase</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GH</td>
<td>Glycoside hydrolase</td>
</tr>
<tr>
<td>GHG</td>
<td>Greenhouse gas</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>HAT</td>
<td>Hydrogen atom transfer</td>
</tr>
<tr>
<td>HisTrp column</td>
<td>Nickel affinity column</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>I</td>
<td>Inhibitor</td>
</tr>
<tr>
<td>$k_{app}$</td>
<td>Apparent first-order rate constant for irreversible inhibition</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>Unimolecular rate constant</td>
</tr>
<tr>
<td>$K_{ic}$</td>
<td>Competition inhibition constant</td>
</tr>
<tr>
<td>$K_{iu}$</td>
<td>Dissociation constant of ESI complex</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>$K_{m,app}$</td>
<td>Apparent Michaelis constant</td>
</tr>
<tr>
<td>L-GAP</td>
<td>L-glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>LPMO</td>
<td>Lytic polysaccharide monooxygenases</td>
</tr>
</tbody>
</table>
MeCN  Acetonitrile
NADH  Reduced nicotinamide adenine dinucleotide
NADPH Reduced nicotinamide adenine dinucleotide phosphate
Phe  phenylalanine
PL  Polysaccharide lyases
PNGase F Peptide:N-glycosidase F
PT  Proton transfer
PTFE polytetrafluoroethylene
\textit{rac-} racemic
ReactIR Real-time, in situ mid-infrared-based system
\textit{R}_f  Retention factor
rpm  Revolutions per minute
\textit{R}_2  Ratio of absorbance due to hemin to absorbance due to protein
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide
SEC  Size exclusion chromatography
3D  Three-dimension
Trp  tryptophan
\textit{V}  \( k_2[E] \)
\textit{V}_{app}  Apparent \textit{V}
W  tryptophan
Acknowledgements

I am very grateful that I have encountered so many nice people. Firstly, I would like to show my greatest appreciation to my supervisor, Dr. Harry Brumer. Thank you for accepting me into the group, and providing me with valuable guidance, help, and support whenever I need. It was a great pleasure to work with and learn from you!

Also, I would like to thank Dr. Shaheen Shojania for helping me settle in the lab and providing a lot of help during my study. I would like to thank all current and past Brumer lab members, thank you all for being so awesome. It is a great pleasure to know you all.

I would like to thank my collaborators, Dr. Jason Hein and Dr. Lawrence McIntosh for letting me get access to their instruments. Thanks to Ryan Chung from Hein’s lab, and Mark Okon from McIntosh’s lab for providing me with huge amount of help and patience while I was using their instruments.

Thanks to all staff in Chemistry Department, and in Michael Smith Laboratory for all of your help.
Dedication

To my lovely wife, Yan Ma, and my dearest parents, Deli Xia and Jing Jiang for their generous care and support! I love you all!
Chapter 1:

1.1 Introduction

The demand for fossil energy has increased dramatically in the modern world because of an upsurge in industrialization and motorization\(^1\). This drastic increase in demand for this unsustainable energy source has increased greenhouse gas (GHG) emissions, which has led to global warming and climate change, both of which negatively impact the global economy\(^2\)–\(^4\). To address this alarming issue, 196 members of the United Nations Framework Convention on Climate Change (UNFCCC) unanimously adopted the Paris climate agreement at the 21st conference of parties of the UNFCCC in December 2015. Canada has committed to a 30% reduction in GHG emissions by 2050. Canada, with the third-largest forest in the world, holds one of the possible solutions to mitigate climate change\(^5\). Forests have a high carbon storing capacity; not only do they absorb large amounts of CO\(_2\) during plant growth, but they also provide a large source of biomass feedstock that can be biorefined into materials, chemicals, fuels, and energy to replace fossil fuel-intensive products\(^5\),\(^6\).

Biomass is an important and versatile renewable feedstock for the biofuel and chemical industry\(^7\). In plants, the absorption of water and carbon dioxide enables the biosynthesis of vital primary and secondary metabolites through photosynthesis\(^7\). Primary metabolites result in the production of several important polymers, including carbohydrates and lignin. The most abundant polymer in plants is cellulose, which is found in complex with hemicelluloses and lignin to form lignocellulose. This complex can be converted into biofuels through depolymerization that is mediated by a variety of enzymes derived from microorganisms that specialize in the degradation of plant material\(^7\)–\(^9\). Secondary metabolites in plants include gums, resins, waxes, terpenes, and steroids,
among others. Although present at low levels in plants, these secondary metabolites can be used to produce high-value chemicals, such as flavouring agents for food, pharmaceuticals, and cosmeceuticals\textsuperscript{10}. In an effort to fully utilize primary and secondary metabolites within plant biomass, microorganisms that can degrade their complex components have been mined intensively to build up an arsenal of new and improved natural biocatalysts for a wide range of applications\textsuperscript{11–13}.

Interestingly, biocatalysts are not only used in biomass degradation; they are also widely used in organic synthesis because they offer several advantages over traditional synthetic routes. First, biocatalysts are more environmentally friendly due to their biodegradable properties and low toxicity compared to common heavy metal catalysts. Second, they are capable of catalyzing reactions in a remarkably regioselective and stereoselective manner. Finally, biocatalysis shortens synthetic routes, reduces reaction costs by minimizing the use of protecting groups, and runs reactions in milder conditions. Commercially, oxidation reactions have used a variety of enzymatic processes in which one third of the process uses bioredox catalysis because of its high selectivity\textsuperscript{13}.

Alcohol oxidation is a fundamental reaction in organic chemistry and is important in a variety of biotechnological applications\textsuperscript{13–15}. There are four major classes of oxidoreductase enzymes: dehydrogenases, oxidases, oxygenases, and peroxidases, all of which can oxidize a range of substrates such as alcohols and certain simple sugars. However, these enzymes have a few drawbacks. For example, most of them require organic cofactors, including nicotinamide cofactors such as NADH or NADPH or flavin cofactors such as FAD or FMN\textsuperscript{14}. Unfortunately, these cofactors are expensive, which can inflate the cost of using these enzymes in industry. Also, the
activity of some of these enzymes is difficult to control, resulting in over-oxidized carboxylic acid products rather than more desirable aldehydes. This limits their use as biocatalysts.

In contrast, copper radical oxidases (CRO) are a family of enzymes that do not require organic cofactors. The CRO family’s name stems from the fact that these enzymes require an inorganic copper metal ion as an oxidation center, and a molecular oxygen as a co-substrate to regenerate the biocatalyst during substrate conversion. This class of enzymes catalyzes reactions in a highly chemo-selective and stereoselective manner.

There is a wide variety of enzymes in the Carbohydrate-Active EnZymes (CAZy) database, in which enzymes are classified based on their amino acid sequence similarities, putative folding, and function or predicted functions. There are many different classes of enzymes, such as glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE), glycosyltransferases (GT), and carbohydrate binding modules (CBM). However, the newly discovered members of family CBM33 and family GH61, which are lytic polysaccharide monooxygenases (LPMO), has led to a new class of enzymes – the “Auxiliary Activity” (AA) family. It is believed that the role of AA redox enzymes is to help GH, PL, and CE enzymes gain access to carbohydrates that are encrusted in the plant cell wall. Currently, there are 15 families in the AA enzyme class: eight families of lignolytic enzymes and five families of lytic polysaccharide monooxygenases.

The Auxiliary Activity Family 5 (AA5) enzymes are copper radical oxidases that are categorized into two subfamilies: glyoxal oxidases (AA5_1), which oxidize a range of aldehydes to their
corresponding carboxylic acids$^{16}$, and galactose oxidases (AA5_2), which oxidize the C6 hydroxyl group of the monosaccharide galactose to its corresponding aldehyde$^{17}$. Even though there are over 500 publicly available AA5 sequences in the CAZy database, only three members from the AA5_1 subfamily and seven from the AA5_2 subfamily have been fully characterized.

Recently, our lab investigated two enzymes belonging to the AA5_2 subfamily from the fungal pathogens *Colletotrichum graminicola* (*Cgr*) and *Colletotrichum gloeosporioides* (*Cgl*). These enzymes were expected to have similar activity towards substrates of the archetypal *F. Graminearum* galactose oxidase (*FgrGalOx*) (i.e., galactose), but their specific activity was found to be 50-fold higher for glycerol over galactose. Therefore, the activity of these enzymes towards a wide variety of alcohols has been tested. Both proteins were found to have very high activity towards aliphatic alcohols containing four to seven carbons in their backbone chain and conjugated primary alcohols (e.g., benzyl alcohol and cinnamyl alcohol). 1-butanol was used as a benchmark substrate to determine their pH-rate profile and temperature stability$^{18}$. This new finding extends the biochemical and industrial applications of the AA5 family of enzymes, particularly in the fragrance and food industries. Historically, aldehyde compounds have been produced under harsh conditions involving expensive transition metal catalysts that have fairly poor selectivity$^{19}$. In comparison, these newly discovered enzymes are highly selective and can be utilized during organic synthesis.

The mechanism of AA5_2 oxidases is summarized in Figure 1.1. The active site consists of a unique cysteine-tyrosine cross-linked moiety, and a deprotonated tyrosine side chain with two histidine residues that coordinate the mononuclear copper center. The formation of cysteine-
tyrosine cross-linked moiety in the active site is intrinsic and autocatalytically by the coordinated Cu center\textsuperscript{20,21}. These residues are strictly conserved at the active site of the AA5_2 subfamily of proteins. For CgrAlcOx, the amino acids coordinating the copper center are the same as described. However, CgrAlcOx and FgrGalOx differ in their secondary shell: in CgrAlcOx, the tryptophan residue (Trp290) of FgrGalOx is replaced with phenylalanine (Phe138)\textsuperscript{18}. This Trp290 residue in FgrGalOx is important for stabilizing the tyrosine radical at the active site. However, it is known that the catalytic activity of GalOx is not affected by substitution of Trp290 with Phe in FgrGalOx, which implies that Phe in the secondary shell can also stabilize the tyrosine radical\textsuperscript{22}.

![Mechanism of alcohol oxidation by the AA5_2 family of carbohydrate-active enzymes reproduced from reference 18](image)

AA5_2 oxidases catalyze oxidation through a two-step reaction (as shown in Figure 1.1-1). The first half of the reaction consists of substrate oxidation involving proton transfer (PT) to the deprotonated tyrosine, a hydrogen atom transfer (HAT) to the cross-linked cysteine tyrosine residue, and an electron transfer (ET) to the copper center leading to the reduction of Cu\textsuperscript{2+} to Cu\textsuperscript{1+}. 
Once the substrate is converted into the corresponding aldehyde, molecular oxygen binds to the protein and initiates the second half of the reaction. Regeneration of the enzyme occurs through the same process as the first half of the reaction but in reverse (Figure 1.1-1 B). At the same time, the molecular oxygen is reduced to hydrogen peroxide.

Because hydrogen peroxide is produced as a by-product during each round of substrate conversion, researchers have utilized this property to design a coupled assay to monitor the activity of the oxidases in AA5_2 subfamily. This coupled assay requires a peroxidase, often horseradish peroxidase (HRP), and a colorimetric electron donor, specifically 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). HRP captures and breaks H₂O₂ down into water with the facilitation of ABTS as an electron donor, which generates an ABTS radical. This radical generation changes the color of the assay solution from green to blue, which can be monitored by UV-vis spectroscopy at 414 nm (see Figure 1.1-2). This assay is a continuous assay that estimates the initial rate of the reaction.

The kinetics measured using the HRP-ABTS assay are based on initial rates of the reaction. An enzymatic reaction with one substrate can be written as follows:
\[
\frac{[E] + [S]}{k_1} \xrightarrow{k_2} \frac{[ES]}{k_1} \xrightarrow{k_2} \frac{[E] + [P]}{k_1}
\]

where \([E]\) is the concentration of enzyme, \([S]\) is the concentration of substrate, \([ES]\) is the enzyme-substrate complex, and \([P]\) is the concentration of product. The rate equation of the reaction can be written as:

\[
v = \frac{d[E]}{dt} = k_1 ([E] - [ES])[S] - k_{-1}[ES] - k_2[ES]
\]  
(1)

The steady state approximation states that the \([ES]\) complex is constant. Therefore,

\[
\frac{d[ES]}{dt} = k_1 ([E] - [ES])[S] - k_{-1}[ES] - k_2[ES] = 0
\]  
(2)

After rearrangement, the equation leads to the expression of the steady-state value of \([ES]\):

\[
[ES] = \frac{k_1[E][S]}{(k_{-1} + k_2 + k_1[S])}
\]  
(3)

By substituting the expression of \([ES]\) into the reaction rate equation:

\[
v = \frac{k_2[E][S]}{k_{-1} + k_2 + [S]}
\]  
(4)

This is the Michaelis-Menten equation, a fundamental equation of enzymatic kinetics. The Michaelis constant, \(K_m\), is defined as \(\frac{k_{-1}+k_2}{k_1}\). If \([S]\) is sufficiently large, the denominator of the equation is dominated by \([S]\), and \(K_m\) is negligible. The rate of reaction can be simplified to \(v = k_2[E][S] = V[S]\), where \(V\) is a constant that equals \(k_2[E]\). \(k_2\) can be replaced by the catalytic constant \(k_{cat}\), which is defined as the number of molecules of substrate that one molecule of enzyme can convert into product in one unit of time. Michaelis and Menten showed that the behavior of enzymes could be studied much more simply using initial rates of reaction.
The kinetics of the AA5_2 subfamily of enzymes is more complicated, because it follows a typical ping-pong mechanism:

\[
[E] + [S_1] \xrightarrow{k_1} [ES_1] \xrightarrow{k_2} [E] + [P_1] + [S_2] \xrightarrow{k_3} [ES_2] \xrightarrow{k_4} [E] + [P_2]
\]

According to Cornish-Bowden, the Michaelis-Menten equation with two substrates follows initial-rate measurements and can be modified as

\[
v = \frac{V[S_1][S_2]}{[S_1][S_2] + K_{m1}[S_2] + K_{m2}[S_1] + K_{m1}K_{m2}}
\]

(5)

If the concentration of one of the substrates, such as \([S_2]\), is large enough to saturate the enzyme, the parameters associated with that substrate are negligible because the other substrate (i.e., \(S_1\)) becomes rate-limiting\(^{23}\). Therefore, in this case, the Michaelis-Menten equation can be modified as

\[
v = \frac{V[S_1]}{K_{m1} + [S_1]},
\]

which is the same as the Michaelis-Menten equation (4) derived with only one substrate.

Despite being widely used to characterize oxidases from the AA5 family, the HRP-ABTS initial rate assay has some limitations. First, HRP is expensive (costing approximately $120/100 mg), possibly creating a financial burden for research and industrial labs wanting to study the activities of enzymes from the AA5_2 family. In addition, HRP must be carefully kept on ice so that it does not lose its activity during assays. For these reasons, it is important to identify an alternative to the HRP-ABTS assay that is efficient, reliable, cheaper, and easier to perform.

Secondly, the HRP-ABTS coupled assay monitors the reaction indirectly by monitoring the color change of the ABTS compound. This approach does not provide any information about percentage
conversion or by-product formation. Normally, thin layer chromatography is used to identify whether any potential by-product is formed. Flash chromatography is used to isolate product from the reaction mixture and obtain percentage conversion. $^1$H-NMR is used to analyze and determine the identity of the purified compounds. These approaches are tedious and time consuming.

Thirdly, HRP has been shown to increase the activity of canonical galactose oxidase, which also belongs to AA5_2 family, although the mechanism of activation is not clear$^{24}$. The ABTS assay is dependent on HRP, so it is impossible to determine which species – either HRP or the intermediate generated in the reaction – is activating the AA5_2 proteins. To study this phenomenon, a more direct approach to monitor the enzymatic reaction is needed.

Moreover, when enzyme kinetics are obtained using the HRP-ABTS assay, it is necessary to determine which conditions generate a progress curve that is straight during the period of measurement. It is ideal to set the reaction with less than 1% of the substrate converted so that the progress curve is indistinguishable from a straight line$^{23}$. However, strictly speaking, this is not possible because the rate of reaction will decrease as substrates are consumed and products are accumulated$^{23}$. In an actual experimental setting, due to enzyme behaviour or the detection limit of instruments, it is inherently difficult to identify the period of the measurement in which the progress curve is linear. It is highly biased to choose the portion of the curve that represents the initial rate.

In addition, the HRP-ABTS initial rate assay is not suitable for studying slow inhibition. Because a small amount of enzyme is used to achieve low percentage conversion of the reaction, only a
small portion of the reaction is monitored in the assay. If an inhibitor is slowly inhibiting the enzyme, enzyme activity changes dramatically much later in the reaction. Because the initial rate assay monitors the beginning of the reaction, it cannot capture this type of slow inhibition.

The alcohol substrate scope of CgrAlcOx was well defined in a previous study\textsuperscript{18}, and several industrial partners have showed great interest in studying this newly discovered biocatalyst in detail. One area of interest is to explore more substrates for this enzyme, especially towards amine compounds. If CgrAlcOx can oxidase amine to imine, this would provide some insight into how change of a hetero atom on the substrate could affect the oxidation catalyzed by CgrAlcOx.

Another area of interest is to explore inhibitors of CgrAlcOx. Wood decomposing fungi, mostly found in Ascomycota and Basidiomycota, have received much attention because of their potential in the production of biofuels and other bioprocesses\textsuperscript{25}. Brown rot and white rot are the two broad categories of wood decay chemistries. Enzyme-generated oxidizing species, such as the hydroxyl radical produced through the Fenton reaction ($\text{H}_2\text{O}_2 + \text{Fe}^{2+} + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{Fe}^{3+} + \cdot \text{OH}$), is utilized by brown rot fungi in depolymerizing cellulose by breaking the $\beta$1-4 linkages\textsuperscript{26–29}. In comparison, in white rot fungi, lignin peroxidase, manganese peroxidase, and versatile peroxidase use $\text{H}_2\text{O}_2$ as a co-substrate to break down lignin\textsuperscript{30,31}. Because there is no known $\text{H}_2\text{O}_2$-transport mechanism, $\text{H}_2\text{O}_2$ must be generated extracellularly by secreted enzymes\textsuperscript{32}. Galactose oxidase, which belongs to the AA5_2 subfamily, was found to be specifically expressed by Fusarium species, and may facilitate the metabolism of cellulose and lignin\textsuperscript{33,34}. It was also found that deletion of the glyoxal oxidase gene significantly reduced the pathogenic properties of Ustilago maydis\textsuperscript{35}. Also, knockout of the sucrose non-fermenting protein kinase 1 gene in Fusarium virguliforme prevented
expression of the gene-encoding galactose oxidase. This may have rendered this species incapable of colonizing soybean roots early in the infection process. These examples indicate that inhibiting extracellular H₂O₂-producing enzymes from the AA5 family may provide novel fungicides for agricultural applications. Also, because glyoxal and galactose oxidases share a highly conserved active site, screening for inhibitors of CgrAlcOx may also provide insight into the design of inhibitors for other oxidases in the AA5 family. Amine and thiol derivative compounds are promising inhibitor candidates. According to the Hard and Soft Acids and Bases principle, Cu⁺ is a soft acid while Cu²⁺ is borderline; therefore, copper binding sites are expected to be dominated by amino acids containing side chains with soft or borderline ligands, such as histidine, cysteine, and methionine. Indeed, these amino acid residues are the predominant ligands of cuproproteins, providing side chains containing nitrogen and sulfur atoms a greater chance of binding to the copper center. In agreement with this idea, it was speculated that amine and thiol derivative compounds would have a greater affinity for binding to the copper center of CgrAlcOx, preventing substrate binding and leading to reduced enzyme activity.

Substances that decrease the rate of enzyme-catalyzed reactions are inhibitors. There are two types of inhibitors, irreversible and reversible. If the effect of inhibition caused by a substance cannot be reversed by removing the inhibitor through dialysis or dilution, this substance is an irreversible inhibitor or catalytic poison. This type of inhibitor binds with free enzyme to form an enzyme-inhibitor complex, mediated by the affinity between the inhibitor and a particular site on the enzyme. The enzyme then takes on an inactive conformation (E') that is unable to turn over substrate.
It is assumed that inactivation of enzyme E proceeds via an intermediate EI complex. The rate of inactivation can be calculated as

\[- \frac{dE}{dt} = k_2[E][I] = \frac{k_2[E][I]}{K_i + [I]} \quad (6)\]

where \(K_i = (k_{-1} + k_2)/k_2\). If the inhibitor concentration is present in sufficient excess to be constant, and active enzyme concentration decreases as inactivation progresses, the loss of enzyme activity occurs via a pseudo-first-order process. The apparent first-order rate constant is \(k_{\text{app}} = k_2[I]/(K_i + [I])\); it is an apparent value because the value of this parameter varies when it is measured in the presence of an inhibitor. The equation to calculate \(k_{\text{app}}\) is in the same form as the Michaelis-Menten equation, so \(K_i\) and \(k_2\) can be estimated by taking measurements at different concentrations of inhibitor.

If a substance forms a dynamic complex with an enzyme that causes the enzyme to be less effective than the free enzyme, this substance is classified as a reversible inhibitor\(^{23}\). Reversible inhibitors can be subcategorized according to their effect on Michaelis-Menten parameters. The most common type of reversible inhibitors are competitive inhibitors, in which the substrate and inhibitor compete for the active site. The mechanism may be presented in the general terms:
Figure 1.1-4 A mechanism of competitive enzyme inhibition

The competitive inhibition constant is defined as $K_{ic} = [E][I]/[EI]$. The defining equation of competitive inhibition is

$$v = \frac{V[S]}{K_m \left( 1 + \frac{[I]}{K_{ic}} \right) + [S]} \quad (7)$$

where $V_{app}$ and $K_{m,app}$ are the apparent values of $V$ and $K_m$:

$$V_{app} = V$$

$$K_{m,app} = K_m \left( 1 + \frac{[I]}{K_{ic}} \right) \quad (8)$$

This equation indicates that $V$ is not affected by competitive inhibition, whereas $K_m$ is changed by a factor of $(1 + \frac{[I]}{K_{ic}})$.

Another type of reversible inhibition is uncompetitive inhibition, in which the inhibitor binds exclusively to the enzyme-substrate complex:
Figure 1.1-5 A mechanism for uncompetitive enzyme inhibition

$K_{iu}$ represents the dissociation constant of the ESI complex. In uncompetitive inhibition, $V_{app}$ decreases, whereas $V_{app}/K_{m,app}$ is not affected because $K_{m,app}$ also decreases by the same factor:

$$V_{app} = \frac{V}{1 + [I]/K_{iu}} \quad (9)$$

$$K_{m,app} = \frac{K_m}{1 + [I]/K_{iu}} \quad (10)$$

$$\frac{V_{app}}{K_{m,app}} = \frac{V}{K_m} \quad (11)$$

Mixed inhibition is a more complicated type of reversible inhibition. The inhibitor binds to a site other than the active site of the enzyme, to either the enzyme or enzyme-substrate complex. The simplest mechanism for this type of inhibition is as follows:

Figure 1.1-6 A mechanism for mixed enzyme inhibition
The inhibitor binds to the free enzyme to produce an EI complex with the dissociation constant $K_{ic}$, or to the enzyme-substrate complex to give an ESI complex with the dissociation constant $K_{iu}$. It is also possible for the EI to bind with substrate to generate an ESI complex, so the binding steps must be at equilibrium. Both $V_{app}$ and $K_{m,app}$ are altered by mixed inhibition:

$$V_{app} = \frac{V}{1 + [I]/K_{iu}}$$  \hspace{1cm} (12)

$$K_{m,app} = \frac{K_m(1 + [I]/K_{ic})}{1 + [I]/K_{iu}}$$  \hspace{1cm} (13)

Noncompetitive inhibition is a special type of mixed inhibition, in which the $K_{m,app}$ of the enzyme is not affected by the concentration of the inhibitor. Table 1.1 and Figure 1.1-7 summarize how $K_{m,app}$ and $V_{app}$ change with each type of reversible inhibition.
Table 1.1 Summary of apparent parameter changes with each type of reversible inhibition

<table>
<thead>
<tr>
<th>Types of reversible inhibition</th>
<th>$V_{app}$</th>
<th>$K_{m,app}$</th>
<th>$V_{app}/K_{m,app}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Competitive</td>
<td>$V$</td>
<td>$K_m(1 + [I]/K_{ic})$</td>
<td>$V/K_m(1 + [I]/K_{ic})$</td>
</tr>
<tr>
<td>Uncompetitive</td>
<td>$V/(1 + [I]/K_{iu})$</td>
<td>$K_m/(1 + [I]/K_{iu})$</td>
<td>$V/K_m$</td>
</tr>
<tr>
<td>Mixed</td>
<td>$V/(1 + [I]/K_{iu})$</td>
<td>$K_m(1 + [I]/K_{ic})/(1 + [I]/K_{iu})$</td>
<td>$V/K_m(1 + [I]/K_{ic})$</td>
</tr>
<tr>
<td>Non-competitive (special case of mixed inhibition)</td>
<td>$V/(1 + [I]/K_{iu})$</td>
<td>$K_m$</td>
<td>$V/K_m(1 + [I]/K_{ic})$</td>
</tr>
</tbody>
</table>

Figure 1.1-7 Theoretical representation of Michaelis-Menten kinetics for different types of reversible inhibition.
1.2 Thesis objectives

In this thesis, there were three main objectives: (1) To develop an alternative assay for measuring alcohol oxidation that overcomes the limitations of existing assays that are based on measurement of initial rate kinetics, i.e., the HRP-ABTS assay. An assay based on the ability of CeO$_2$ nanoparticles to scavenge H$_2$O$_2$ was tested as a replacement to the currently used initial rate assay. (2) To test the ability of different instruments to monitor the enzymatic reaction. This objective was based on tandem reaction progress analysis, a new technique that was developed by Dr. Jason Hein to study the reaction mechanism, to gather selectivity information, and to optimize reaction conditions. (3) To use the newly developed assay to optimize the enzymatic reaction, to determine enzyme activity, and to study CgrAlcOx in detail. (4) To use the newly developed assay and the HRP-ABTS assay in a complementary way to identify new substrates and inhibitors of CgrAlcOx.
Chapter 2: New Assay Development with Cerium Oxide Nanoparticles

2.1 A New, CeO₂ Nanoparticle-Based Colorimetric Assay that can be Used to Monitor an Enzymatic Reaction

Cerium oxide (CeO₂) nanoparticles have attracted much attention due to their various engineering and biological applications, such as solid-oxide fuel cells, catalytic materials, and potential pharmacological agents. Cerium can occur in either a +3 or +4 state due to its partially filled subshells of electrons, 4f and 5d. In its oxide form, the +4 state of cerium is preferred due to its stable electron configuration of xenon. Each cerium atom is surrounded by eight oxygen anions, and every oxygen atom occupies a tetrahedral position. However, a significant portion of cerium exists in the +3 state, and the proportion of Ce³⁺ and Ce⁴⁺ is a function of particle size. Quick changes in the oxidation state of cerium between Ce³⁺ and Ce⁴⁺ gives CeO₂ nanoparticles a dual function as oxidation and reduction catalysts. Recently, multi-enzyme mimetic properties have been identified.

CeO₂ nanoparticles have higher catalase mimetic activity when more cerium is in the +4 state. The mechanism is proposed to involve two half reactions, in which the oxidative half reaction breaks down H₂O₂ with the release of oxygen gas and protons, and the reductive half reaction breaks down another H₂O₂ molecule to restore the oxidation state of the cerium and to release water (Figure 2.1-1). Because the nanoparticles also have superoxide dismutase mimetic properties, it is postulated that anti-oxidative efficiency depends on the coordination of the two enzyme-like activities – the H₂O₂ decomposition rate is equal to or greater than its generation rate. In addition, CeO₂ nanoparticles also exhibit peroxidase-like activity, which is believed to proceed similarly to a Fenton-like reaction. It has been proposed that nanoparticles could be a potential
replacement for unstable and expensive natural peroxidase\textsuperscript{48,49}. In fact, it has been shown that when HRP was immobilized in CeO$_2$ nanocubes on a glass carbon electrode, the resulting CeO$_2$ nanocubes showed increase in electron transfer to the glass carbon electrode, and also the immobilized HRP had higher enzymatic activity towards H$_2$O$_2$\textsuperscript{52}.

\begin{center}
\textbf{Figure 2.1-1 Mechanism of CeO$_2$ nanoparticles, showing catalase mimetic activity}\textsuperscript{42}
\end{center}

There are some advantages to use CeO$_2$ nanoparticles in place of natural peroxidase. First, not only do the nanoparticles have multi-enzyme mimetic properties, they also change color upon oxidation state changes, which can be used for activity detection. Andreescu \textit{et al} have demonstrated that glucose oxidase and CeO$_2$ nanoparticles can be immobilized on filter paper, and that this modified paper can be utilized to quantify glucose oxidase activity based on the intensity of the color change in presence of glucose\textsuperscript{50}. Thus, there is a great interest in using this approach as a new colorimetric probe for enzymatic activity detection. Second, CeO$_2$ nanoparticles (at $120/25$ g) are much cheaper than HRP, which reduces the experimental cost of enzyme characterization. Third, CeO$_2$ nanoparticles are much easier to handle compared to natural peroxidases because they can be kept
at room temperature. In contrast, natural peroxidases such as HRP must be kept on ice to prevent loss of enzyme activity during the assay period. However, the use of nanoparticles as a colorimetric assay in aqueous solution has not been reported. In the current work, the suitability of using CeO₂ nanoparticles in enzymatic activity determination in solution was tested and compared with the traditional HRP-ABTS coupled assay.

2.2 Development of the CeO₂ Nanoparticle Assay to Detect Enzymatic Activity

CeO₂ nanoparticles change color from yellow to orange upon reacting with H₂O₂, and the extent of this color change in response to different concentrations of H₂O₂ was monitored by a UV-vis spectrometer at 465 nm. A linear response was obtained, and the slope of the standard curve was calculated as 128 ± 4 M⁻¹cm⁻¹ (Figure 2.2-1).

![Figure 2.2-1 Linear response of CeO₂ nanoparticles towards H₂O₂ monitored by UV-vis spectrometer](image)

However, when the reaction was monitored continuously using a UV-vis spectrometer at 465 nm, the observed behaviour of the nanoparticles in aqueous solution indicated that the nanoparticles might not permit the accurate measurement of reaction activity. First, the baseline was not stable;
instead, it continuously decreased. We tested the reaction using different compositions of solution (e.g., water or 50 mM sodium phosphate at pH 8.0), but these conditions did not stabilize the baseline (Figure 2.2-2). This phenomenon likely explains the large error bars on the standard curve in Figure 2.2-1.

![Absorbance vs Time](image)

**Figure 2.2-2** Ten μL of CeO2 nanoparticles were dissolved in water (black) or 50 mM sodium phosphate at pH 8.0 (red). The solution was continuously monitored by UV-vis spectrometry at 465 nm for 2 minutes.

Second, to examine how the nanoparticles reacted with H2O2 in real time, H2O2 was added directly into a solution containing nanoparticles, and the reaction was continuously monitored using a UV-vis spectrometer (Figure 2.2-3). An initial spike in absorbance was observed, followed by a sharp decline in absorbance after the maximum was reached. As seen in Figure 2.1-1, the nanoparticle scavenges H2O2 through two half reactions with quick changes in the oxidation states of Ce. Once all Ce4+ on the surface of nanoparticles were reduced to Ce3+ by H2O2, the maximum absorbance was achieved. Then, the Ce3+ was oxidized back to Ce4+ by the unreacted H2O2 with emission of two water molecules. Following the first half reaction, the amount of unreacted H2O2 was less than Ce3+ on the surface of the nanoparticle, thus suggesting only a small portion of Ce3+ was oxidized. This is the reason why the absorbance does not reduce to the baseline.
In addition, the sensitivity of nanoparticles to H$_2$O$_2$ was significantly lower than ABTS. It was assumed that the reaction between nanoparticles and H$_2$O$_2$ occurs at a 1:1 stoichiometric ratio, making the slope obtained from the standard curve in Figure 2.2-1 comparable to the extinction coefficient of ABTS for sensitivity evaluation. The slope of the CeO$_2$ standard curve was calculated to be 128 M$^{-1}$cm$^{-1}$, and the extinction coefficient of ABTS is 3.6 x 10$^4$ M$^{-1}$cm$^{-1}$. The 283-fold difference between these two values indicates that CeO$_2$ nanoparticles are not as sensitive as ABTS when used as a colorimetric probe.

To determine the ability of CeO$_2$ nanoparticles to detect enzymatic activity, different reaction conditions were tested (Figure 2.2-4). When a reaction was set up containing 0.841 nM CgrAlcOx, nanoparticles, and 20 mM benzyl alcohol, no activity was observed even though each component was varied and tested. When HRP was added to the assay together with 8.41 nM of CgrAlcOx
which was 10-fold higher than the amount of alcohol oxidase used in the HRP-ABTS assay), a very low activity (0.0244 Abs/min) was observed; this activity was 13.2% of the rate obtained from the HRP-ABTS coupled assay that used 0.841 nM of CgrAlcOx. The reaction curves were converted into ‘molecules that were converted by one enzyme in every minute’ using the slope obtained from the standard curve in Figure 2.2-1 and the concentration of CgrAlcOx (Figure 2.2-4B). The activity observed in the nanoparticle-HRP experiment required a rate of converting of $2.27 \times 10^5$ molecules per min. In comparison, the activity observed in the HRP-ABTS coupled assay, which was 7-fold higher in rate with 10-fold less loading of oxidase, required $3.05 \times 10^3$ molecules per min. For the nanoparticle assay to achieve the same magnitude of activity as measured using the ABTS assay, the loading of enzyme must be increased by at least another 7-fold. However, because of their low colorimetric sensitivity, nanoparticles are not useful for monitoring enzymatic activity. Varying amounts of HRP and nanoparticles were used in the reactions, but none of the conditions tested were able to change the rate of reaction significantly.
In conclusion, there were many drawbacks to use CeO₂ nanoparticles as a colorimetric probe in aqueous solution. For example, the CeO₂ nanoparticles produced an unstable baseline and a quick reduction in absorption after H₂O₂ was fully consumed. The most important drawback was that CeO₂ nanoparticles were much less sensitive in detecting enzymatic activity compared with the HRP-ABTS coupled assay.
Chapter 3: Assay Development based on Tandem Reaction Progress Analysis

3.1 Introduction

Tandem reaction progress analysis is a method that can be used to directly measure enzymatic reactions—it uses one or more instruments, such as ReactIR or HPLC, to sample a reaction continuously without quenching steps while gathering online information regarding reaction progress. The collected information can be used to optimize reaction conditions, to validate kinetic data, and to gain mechanistic and selectivity information. It can be used to monitor both starting materials and final products, as well as some other reactive intermediates or potential by-products. Hein et al. have successfully developed this technique to monitor the dysprosium (III) triflate-catalyzed aza-Piancatelli rearrangement of 2-furylcarbinols in order to gather information about an unseen intermediate and about the intrinsic chemoselectivity of the reaction. If this new technique can be applied to alcohol oxidation by CgrAlcOx, it may compensate for the limitations of the HRP-ABTS coupled assay. Because this technique needs to use instruments in a tandem setting to monitor the reaction, different instruments were tested on their own during initial development for their ability to monitor enzymatic reactions. Benzyl alcohol and cinnamyl alcohol, which can be oxidized by CgrAlcOx to benzaldehyde and cinnamyl aldehyde, have been used as benchmark substrates in developing assays based on tandem reaction progress analysis.

3.2 The ReactIR Approach to Tandem Reaction Progress Analysis using a DST Conduit Model or Flow Cell Model

CgrAlcOx can oxidize aliphatic and aromatic alcohols to their corresponding aldehydes, changing the functional group from a hydroxyl to a carbonyl. In an IR spectrum, hydroxyl stretch appears as a broad peak located around 3500 cm\(^{-1}\), and the carbonyl group of an aldehyde appears as a
sharp and intense peak located around 1600 cm\(^{-1}\). Therefore, infrared spectroscopy is a suitable instrument to monitor changes in functional groups in reactions catalyzed by alcohol oxidases.

Figure 3.2-1 The reaction monitored by ReactIR with two modules: A. DST series AgX fibre conduit model; B. DS micro flow cell model.

Two models of Mettler-Toledo ReactIR were tested (Figure 3.2-1). The DST series AgX fibre conduit model was equipped with a Di-Comp (diamond) probe connected by an AgX (silver halide) fiber. This model allowed maximum flexibility of usage in a lab set with a wide range of vessels. The probe was inserted into a 20 mL PTFE-lined septum fitted reaction vial. Alternatively, the DS micro flow cell consisted of an optical window, and the reaction was set up in a 15 mL falcon tube; the content of the reaction was continuously recorded at the optical window by circulating it into the flow cell at 1 mL/min with an isocratic pump. The spectra obtained by the two models of ReactIR are 3D surfaces, where the x-axis is the wavenumber (cm\(^{-1}\)), the y-axis is the intensity of the peak with an arbitrary unit (A.U.), and the z-axis is time (min). Components of the reaction
were added in sequence so that the spectrum of each component was recorded. Once CgLcOx was injected, the reaction was monitored for one hour from 800 to 3600 cm\(^{-1}\).

**Figure 3.2-2** A. Spectra of 100 mM benzyl alcohol oxidation catalyzed by CgLcOx, with the buffer, catalase, and HRP spectra subtracted; B. Magnified view of the spectra from 1400 to 1900 cm\(^{-1}\); C. Change in intensity of the spectra monitored at 1705 cm\(^{-1}\).

To confirm that ReactIR was capable of monitoring alcohol oxidation, a reaction containing 28 mM BnOH, 35.0 μM HRP, 2.4 μM catalase and 9.47 nM CgLcOx was set up and monitored (Figure 3.2-2). Each component was added to the reaction mixture in sequential order, and the spectrum was recorded after each addition. CgLcOx was the last component added to the mixture to initiate the reaction, and oxidation was continuously recorded for 2 hours. Spectra recorded after
the addition of each reaction component were subtracted from the total spectrum; therefore, theoretically, only the spectrum of functional groups that changed in the reaction remained (i.e., that of benzaldehyde). However, when the spectra were magnified at 1705 cm\(^{-1}\), where the carbonyl peak was expected to be, we observed that the peak decreased throughout the entire run. We hypothesized that water and proteins might be interfering with the detection of benzaldehyde formation. Therefore, additional experiments were conducted to identify which component was interfering with the reaction.

Figure 3.2-3  A. Spectrum of 28 mM benzaldehyde in water; B. Magnified view of the spectrum from 1400 to 1900 cm\(^{-1}\); C. Change in intensity of the spectrum monitored at 1705 cm\(^{-1}\).
Because the reaction was carried out in water, the hydroxyl stretch of the solvent overlapped with benzyl alcohol. Therefore, it was necessary to identify the location of the carbonyl stretch from benzaldehyde, and to determine the limit of detection of this instrument. Benzaldehyde was dissolved in water to make a 28 mM solution (the highest solubility of this compound in water), and the spectra were recorded by first circulating pure water into the flow cell, followed by circulation of the 28 mM benzaldehyde solution (Figure 3.2-3). The collected spectra were zoomed to the typical carbonyl region of 1600-1800 cm\(^{-1}\), but a broad peak was observed that corresponded to bending of the hydroxyl group of water. For water, two peaks are typically observed in the IR spectrum – OH stretch located at 3415 cm\(^{-1}\), and HOH bending located at 1850 cm\(^{-1}\). We speculated that the aldehyde peak was buried under this broad bending peak of water. Therefore, we used an alternative solvent, ethanol, to prevent interference in the region of 1600-1800 cm\(^{-1}\).

Figure 3.2-4 A. The spectrum of 28 mM benzaldehyde in ethanol; B. Magnified view of the spectrum from 1600 to 1900 cm\(^{-1}\); C. Change in the intensity of the spectrum monitored at 1705 cm\(^{-1}\).
28 mM benzaldehyde in ethanol was circulated into the flow cell and was monitored for 30 minutes after flowing pure ethanol solution for 7 minutes (Figure 3.2-4). A peak located at 1705 cm\(^{-1}\), corresponding to the carbonyl stretch of the aldehyde, was observed when the two solutions were switched. By selecting this peak, the spectrum change at this wavelength was captured (Figure 3.2-4C). The instrumental response to benzaldehyde was calculated from the graph to be 0.00042 A.U./mM.

![Graph of spectra](image)

**Figure 3.2-5** A. Spectrum of 100 mM benzaldehyde in ethanol; B. Magnified view of the spectrum from 1600 to 1900 cm\(^{-1}\); C. Change in the intensity of the spectrum monitored at 1705 cm\(^{-1}\).
To verify that the peak at 1705 cm$^{-1}$ was the carbonyl peak of benzaldehyde, the concentration of benzaldehyde was increased to 100 mM; peak intensity increased at this wavelength as benzaldehyde concentration increased, confirming that the peak at 1705 cm$^{-1}$ was due to the carbonyl stretch of benzaldehyde (Figure 3.2-5). The instrumental response to benzaldehyde was calculated, once again, to be 0.00043 A.U./mM.

Figure 3.2-6 A. Spectrum of 100 mM benzaldehyde in water; B. Magnified view of the spectrum from 1400 to 1900 cm$^{-1}$; C. Change in the intensity of the spectrum monitored at 1705 cm$^{-1}$.

To validate the hypothesis that the carbonyl of benzaldehyde was embedded under the water-bending peak, we performed another experiment in which we monitored 100 mM benzaldehyde in water (Figure 3.2-6). Because the highest solubility of benzyl aldehyde in water occurs at 28
mM, this 100 mM benzaldehyde solution was heterogeneous. The experiment was performed by circulating water into the flow cell for 10 minutes, followed by the 100 mM benzaldehyde solution. A shoulder peak was observed on the bending peak of water, confirming that the carbonyl peak was indeed embedded under this peak. Because of the significant interference from solvent and other proteins in the solution, we concluded that ReactIR was not the ideal technique for monitoring alcohol oxidation by CgrAlcOx.

3.3 $^1$H-NMR Approach

Proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy is normally used to analyze the structure of a product, and the proton of the aldehyde peak is uniquely located from 9-11 ppm without interference from other functional groups in the spectrum. Thus, NMR spectroscopy was used as an alternative approach to analyze the enzymatic reaction. However, several issues needed to be addressed to verify the suitability of this technique to directly monitor the reaction. First, water – the solvent used in the reaction – will suppress other signals because of its abundance relative to other components, so it is necessary to suppress the water signal. Although it is possible to carry out the reaction in pure heavy water, because CgrAlcOx is purified in normal water, it is first necessary to exchange normal water with D$_2$O by dissolving the enzyme in D$_2$O and lyophilizing it at least 2-3 times to get rid of water residue. This process is tedious and time consuming, and more importantly, it may affect the stability and activity of the enzyme. Second, there are many components in the reaction mixture, such as HRP, catalase, and CgrAlcOx, so it is possible that these components might cause interference. Third, to achieve our goals, this technique must be capable of measuring enzyme kinetics in addition to monitoring the enzymatic reaction. The NMR spectrometer must be able to detect a change in the concentration of a
compound as small as 0.01 mM. This level of resolution and sensitivity requires the use of a high magnetic field spectrometer. This part of the project was done in collaboration with Dr. Lawrence McIntosh, whose group specializes in studying proteins using the water suppression technique with a wide variety of high resolution spectrometers.

To determine if proteins interfere with the spectra of substrates or products of the reaction, a reaction involving 2 mM CinOH oxidation was carried out using conditions that were optimized using the HPLC approach (described in Section 4.1). The components of the reaction were added in series so that the spectra resulting from different solution compositions could be compared (Figure 3.3-1). The spectrum of 2 mM CinOH in 50 mM sodium phosphate buffer was not well resolved and noisy. Theoretically, a 10 mg sample of compound should provide a decent signal; however, in this experiment, the mass of CinOH used was 100-fold lower, at 0.13 mg. In addition, when HRP and catalase were added to the NMR tube containing the CinOH oxidation reaction, the baseline of the spectrum changed dramatically – a variety of broad peaks were observed due to the slow tumbling of proteins in the reaction.
The cinnamyl aldehyde peak, located at 9.58 ppm, appeared 10 minutes after the addition of CgrAlcOx. This was coincident with the disappearance of the aromatic peaks belonging to cinnamyl alcohol. Collectively, our observations demonstrated that the NMR approach was capable of monitoring 2 mM CinOH oxidation. Therefore, another experiment – to monitor the enzymatic reaction with a time course – was set up to show that this technique can be used to monitor the reaction online.
CgrAlcOx was added to the reaction last to initiate a 2 mM CinOH oxidation, the time was noted, and the spectrum was recorded every 7 minutes (Figure 3.3-2). As the reaction progressed, the aldehyde peak and the peaks in the aromatic region belonging to aldehyde increased, while peaks belonging to the alcohol decreased. These findings showed that an NMR spectrometer can be used to continuously monitor the enzymatic reaction and to gather information online. However, the concentration of substrate used in this experiment saturated the enzyme (see Figure 3.3-3A). To
validate if this technique could be used in kinetic experiments, a much lower concentration of substrate was tested to identify the detection limit of this instrument.

Figure 3.3-3 A. Kinetics of CinOH oxidation using the ABTS assay; B. Spectrum of 0.2 mM CinOH oxidation compared to 2 mM CinOH oxidation measured by $^1$H NMR.

A 0.2 mM cinnamyl alcohol oxidation was set up, and the reaction spectrum was recorded for one hour upon addition of CgrAlcOx (Figure 3.3-3). In the resulting spectrum, only broad peaks of proteins were observed; no cinnamyl alcohol or cinnamyl aldehyde peaks were observed. Theoretically, both cinnamyl aldehyde and alcohol should be observed in the spectrum, but the resulting spectrum indicated that the concentrations of the substrate or product were too low to be detected. Therefore, NMR spectroscopy can be utilized to monitor alcohol oxidation with CgrAlcOx at high substrate concentrations, but it cannot be used to perform kinetics due to the poor detection limit of the instrument.
3.4 Monitoring CgrAlcOx Oxidation with HPLC-UV

Protein interference and low instrumental detection limit are known to be problematic when monitoring enzymatic alcohol oxidation and performing reaction kinetics using the ReactIR and NMR approaches. High pressure liquid chromatography (HPLC) coupled to a UV detector was chosen to overcome these challenges. This approach offers two main advantages. First, this technique produces less protein interference because the sample that is injected must first pass through a column in which the desired analytes, such as aromatic alcohols and aldehydes, are separated from proteins. Second, the instrumental detection limit is proportional to the injection volume of the sample. Tests have been done to show that this technique can detect substrates or products at concentrations as low as 0.001 mM, suggesting that it can be used to overcome the inadequate detection limits that are associated with the ReactIR and NMR approaches.

To determine if HPLC-UV could be used to monitor the reaction continuously online, different reaction conditions were tested. Because HPLC was coupled to a UV detector, aromatic substrates such as benzyl alcohol and cinnamyl alcohol were studied. After the reaction was sampled by the instrument, the peak areas that were obtained were integrated and plotted against time. The collected peak areas were then converted to concentrations of compounds using calibration curves (Appendix C).

Because H₂O₂ is a by-product of this reaction, it was necessary to determine if it was necessary to use an H₂O₂ scavenger enzyme (e.g., catalase) in the reaction. Benzyl alcohol oxidations, with or without catalase and in the presence of CgrAlcOx, were analyzed. As shown in Figure 3.4-1, during the two-hour acquisition period, CgrAlcOx without catalase did actively convert benzyl
alcohol to benzaldehyde; however, the H₂O₂ that was produced inhibited the enzyme and slowed the speed of conversion. In comparison, the reaction containing catalase was much more efficient in converting alcohol into aldehyde, presumably because catalase broke down H₂O₂ into oxygen and water, and therefore prevented H₂O₂ from inhibiting enzyme activity. These results indicate that catalase is required for CgrAlcOx to work efficiently.

Figure 3.4-1 Effect of catalase on substrate conversion. Red curve (●●●): 1 mM BnOH with CgrAlcOx alone; Black curve (■■■): 1 mM BnOH + CgrAlcOx + catalase.
Figure 3.4-2 Reaction curves generated by monitoring the depletion of alcohol and the formation of aldehyde in a 0.5 mM benzyl alcohol oxidation reaction.

It was then necessary to optimize reaction conditions in the presence of catalase. We found that a reaction containing 11.5 μM catalase proceeded at the highest conversion rate. Using this optimized condition, the reaction curves for benzaldehyde formation and benzyl alcohol depletion were generated by monitoring the analytical wavelengths of these compounds (Figure 3.4-2). The yield of the reaction was 100%; all benzyl alcohol was converted to benzaldehyde without any observed by-product. The highly symmetric shape of the reaction curves suggested that the reaction proceeded at a 1:1 stoichiometry. In summary, the HPLC approach was able to directly monitor the enzymatic reaction by measuring changes in the starting material and product. This new approach was subsequently used to analyze the stereochemistry of glyceraldehyde formed in the reaction catalyzed by CgrAlcOx.
3.5 Adaptation of an HPLC Method to Determine the Stereochemistry of Alcohol Oxidation

The newly developed HPLC-UV approach can also be used to analyze the enantiomeric ratio of glyceraldehyde that is formed in CgrAlcOx-catalyzed glycerol oxidation. Because this molecule is not UV-active, this analysis was achieved using an existing procedure in which a chromophore compound – 2,4-dinitrophenyl hydrazine – was used to trap glyceraldehyde through the formation of a Schiff base (Scheme 1). The racemic mixture was then analyzed by HPLC to determine the stereoisomeric ratio.\(^{55}\)

\[
\begin{align*}
\text{HO-} & \text{OH} & \xrightarrow{\text{CgrAlcOx}} & \text{HO-} & \text{OH} \\
\text{HO-} & \text{O} & \xrightarrow{\text{2,4-dinitrophenyl hydrazine}} & \text{NH}_{2}^{-} & \text{N}^{-} & \text{NH}
\end{align*}
\]

Scheme 1: Trapping of glyceraldehyde by 2,4-dinitrophenyl hydrazine through formation of Schiff base.

This analysis is particularly useful in the biodiesel industry because glycerol is produced in large quantities as a by-product; in general, 10 kg of glycerol is generated for every 100 kg of biodiesel produced.\(^{56}\) This by-product is unrefined raw material that cannot be used in the food, pharmaceutical, or cosmetic industries, and the process of purifying this by-product is also very expensive. Therefore, it is of great interest to understand how glycerol can be used in a way that is economical and environmentally friendly.
To address this issue, various methods of utilizing unrefined glycerol have been developed, one of them being to convert glycerol into value-added products\textsuperscript{57-59}. Glyceraldehyde, an oxidation product of glycerol, is an important compound that can be utilized in pharmaceutical applications. For example, the phosphorylated metabolite L-glyceraldhedye-3-phosphate (L-GAP) has a bactericidal effect on \textit{Escherichia coli}, whereas D-GAP does not\textsuperscript{60}. Thus, there is great interest in exploring the formation of enantiopure glyceraldehyde using biocatalysts. \textit{CgrAlcOx} showed a high specificity towards glycerol during protein characterization, making it of interest to explore whether it can oxidize glycerol in a regioselective manner. Luckily, the newly developed HPLC approach can be used to analyze the enantiomeric ratio of glyceraldehyde formed during \textit{CgrAlcOx}-catalyzed glycerol oxidation.

A solution of 0.445 mmole glycerol was incubated with a high concentration of \textit{CgrAlcOx} (0.0378 mM) to maximize the oxidation of glycerol to glyceraldehyde, and an excess of 2,4-dinitrophenyl hydrazine was used to maximize the conversion of aldehyde to glyceraldehyde hydrazone. The reaction mixture was purified using flash chromatography, and the identity of glyceraldehyde hydrazone was confirmed by MS and \textsuperscript{1}H-NMR (see Appendix D.1).

The mixture of \textit{rac}-glyceraldehyde hydrazone was separated on a chiral column, and the enantiomeric ratio of D- and L-enantiomers was analyzed by HPLC-UV. Although the reaction mixture was purified, and major peaks were identified using MS and NMR, there was an unidentified peak at 0.9 minutes in the HPLC-UV chromatogram (Figure 3.5-1C). The UV-vis spectrum of this peak was different from glyceraldehyde hydrazone, and because it was well-separated from D- and L-glyceraldehyde hydrazone, it did not interfere with quantification of the
ratio of the two stereoisomers. We found that CgrAlcOx oxidized glycerol to 90% D- 
glyceraldehyde and 10% L-glyceraldehyde. Interestingly, our lab has found that canonical 
galactose oxidase-catalyzed glycerol oxidation produces 80% L-glyceraldehyde and 20% D- 
glyceraldehyde, which is in stark contrast to the oxidation catalyzed by CgrAlcOx (Maria 
Cleveland, PhD candidate, unpublished result). This difference was not explored further in this 
thesis due to time constraints.

![Figure 3.5-1 Stereochemistry determination of glycerol oxidized by CgrAlcOx. A. 
Chromatogram of L-glyceraldehyde-hydrazone; B. Chromatogram of D/L-glyceraldehyde-
hydrazone; C. Glyceraldehyde-hydrazone composition after CgrAlcOx oxidation of 
glycerol and linking onto the UV chromophore.](image-url)
3.6 Summary of Assay Development

Different instruments were tested during the development of an approach to directly monitor Cg rA lcO x-catalyzed alcohol oxidation. The ReactIR approach was not suitable to monitor the enzymatic reaction because of high solvent interference and the low detection limit of the instrument. The $^1$H-NMR approach was useful for analyzing the enzymatic reaction at high substrate concentrations, but the low instrumental detection limit made this technique incapable of performing kinetic analyses. In contrast, the HPLC approach was found to be most suitable for monitoring the enzymatic reaction directly via direct measurement of the depletion of starting material and the formation of product; using these data, reaction progress curves could be generated. These curves revealed that the reaction proceeded through a 1:1 stoichiometric ratio with 100% conversion of substrate to product. HPLC analysis was also useful for identifying the isomeric ratio of glyceraldehyde, which is the product of Cg rA lcO x-catalyzed glycerol oxidation. The remainder of this thesis will explore the capabilities of this new technique in enzymatic analysis.
Chapter 4: Enzymatic Reaction Analysis by HPLC-UV

4.1 Optimization of CgrAlcOx Oxidation for Kinetic Analysis

4.1.1 Optimizing CgrAlcOx Oxidation using Catalase as an H₂O₂ Scavenger Enzyme

We found that HPLC-UV was the most suitable instrument for monitoring CgrAlcOx-catalyzed alcohol oxidation (Section 3.4). The reaction curve suggested that this reaction does not proceed in a linear fashion, but instead proceeds exponentially following pseudo 1st order kinetics. However, it was important to confirm that the change of conversion rate was not caused by enzyme denaturation or product inhibition.

4.1.1.1 Substrate Depletion and the Effect of CgrAlcOx Stability on Reaction Rate

![Graph showing enzyme activity over time](image)

Figure 4.1-1 A CgrAlcOx spiking experiment showing a slowing of reaction rate in response to depletion of substrate
A 0.5 mM benzyl alcohol oxidation was initiated by the addition of 0.227 μM CgrAlcOx to the reaction mixture, followed by supplementation with an additional 0.227 μM CgrAlcOx at 60 minutes (Figure 4.1-1). The CgrAlcOx supplementation caused the reaction rate to double from 5.26 μM/min to 12.25 μM/min. Recall that this enzymatic reaction follows a ping-pong mechanism:

\[
[E] + [S_1] \xrightarrow{k_1} [ES_1] \xrightarrow{k_2} [E] + [P_1] + [S_2] \xrightarrow{k_3} [ES_2] \xrightarrow{k_4} [E] + [P_2]
\]

For the substrate to be converted into product, it first must bind with the enzyme to form an ES₁ complex. As substrate concentration ([S₁]) decreased, the frequency with which CgrAlcOx bound to its substrate also declined, which discouraged ES₁ complex formation and led to a decrease in enzymatic activity. Supplementation with additional CgrAlcOx increased the frequency of encounters between enzyme and substrate, thereby restoring the initial reaction velocity.

Figure 4.1-2 Substrate spiking experiment: the decline in reaction rate was not caused by the denaturation of CgrAlcOx
An additional 0.5 mM benzyl alcohol reaction was initiated with 0.227μM CgrAlcOx, and benzyl alcohol was added to the reaction mixture at 65 minutes to restore the concentration of the starting material (Figure 4.1-2). The rates of conversion were almost identical before (5.25 μM/min) and after (5.23 μM/min) addition of benzyl alcohol, which indicated that the decline in reaction rate was not caused by denaturation of the enzyme. Collectively, these two experiments support the view that the decline in reaction rate can be attributed to the depletion of substrate.

### 4.1.1.2 Test of Benzaldehyde Product Inhibition on CgrAlcOx

To test if benzaldehyde – product of benzyl alcohol oxidation – caused product inhibition, three benzyl alcohol oxidation reaction conditions were compared. Reactions contained: (1) 0.5 mM benzyl alcohol; (2) 0.3 mM benzyl alcohol + 0.2 mM benzaldehyde; or (3) 0.3 mM benzyl alcohol (Figure 4.1-3). If benzaldehyde was causing product inhibition, we expected that the reaction curves of the 0.3 mM benzyl alcohol + 0.2 mM benzaldehyde reaction, and the 0.5 mM benzyl alcohol reaction, would deviate from the 0.3 mM benzyl alcohol reaction curve. However, we found that all three reaction curves were almost identical. This indicated that benzaldehyde was not causing product inhibition.
The results in Sections 4.1.1.1 and 4.1.1.2 showed that the decline in reaction rate was caused exclusively by substrate depletion, and not by enzyme denaturation or product inhibition. This confirmed that \textit{CgrAlcOx} catalyzes alcohol oxidation following typical pseudo first-order reaction kinetics.

### 4.1.1.3 Conversion of Reaction Progress Curves to Michaelis-Menten Plots

Figure 4.1-4 summarizes the method used to convert reaction progress curves into Michaelis-Menten kinetic plots. Cinnamyl alcohol oxidation with fully optimized condition were used as an example. The Michaelis-Menten plots can be obtained by following the formation of the aldehyde or the depletion of the alcohol.
Figure 4.1-4 Schematic representation of the method for converting reaction progress curves into Michaelis-Menten kinetic plots

The progress curve for the formation of cinnamyl aldehyde was obtained using HPLC by integrating the peak areas, adding a time-course, and converting back to a concentration. The curve was fitted with an exponential function, and this function was then differentiated to get a function of derivatives. Using this derivative function, the reaction rate at any time point on the progress curve could be calculated. By taking $t = 0$ minutes, the initial rate of the 0.5 mM BnOH reaction
was calculated and could be used to generate a kinetic plot. To obtain a Michaelis-Menten kinetic plot, data was obtained from 7-9 reactions containing different concentrations of alcohol (Figure 4.1-4C). Each reaction was converted in the same manner as shown in Figure 4.1-4A and B.

4.1.1.4 Michaelis-Menten Plots using Catalase to Scavenge Hydrogen Peroxide

Because the amount of catalase used in the assay had been optimized (described in Section 3.4), full kinetics were performed using benzyl alcohol and cinnamyl alcohol. The activities that were obtained were about 100-fold lower than those obtained from the HRP-ABTS coupled assay (see Appendix A.3), which indicated that some components in the initial velocity assay were required for the alcohol oxidase to attain its highest conversion rate. The components required for the full activation of CgrAlcOx were explored in Section 4.1.2 and 4.2.

![Figure 4.1-5](image)

**Figure 4.1-5** Michaelis-Menten kinetic plots using 11.5 μM catalase to scavenge H$_2$O$_2$ in alcohol oxidation of: A. benzyl alcohol; B. cinnamyl alcohol.
Table 4.1 Summary of kinetic parameters obtained from Michalis-Menten plots by using 11.5 μM catalase only as H$_2$O$_2$ scavenger enzyme.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benzyl alcohol</td>
<td>2.18 ± 0.02</td>
<td>0.107 ± 0.009</td>
<td>2.04 x 10$^4$</td>
<td>94 ± 1</td>
<td>0.69 ± 0.04</td>
<td>1.4 x 10$^5$</td>
</tr>
<tr>
<td>cinnamyl alcohol</td>
<td>1.35 ± 0.06</td>
<td>0.154 ± 0.036</td>
<td>8.80 x 10$^3$</td>
<td>93 ± 1</td>
<td>0.060 ± 0.003</td>
<td>1.6 x 10$^6$</td>
</tr>
</tbody>
</table>

4.1.2 Optimization of the CgrAlcOx Oxidation Reaction using HRP and Catalase as H$_2$O$_2$ Scavengers and the Means of O$_2$ Delivery

4.1.2.1 Analysis of HRP and Catalase as H$_2$O$_2$ Scavenger Enzymes in the CgrAlcOx Oxidation Reaction

Oxidase activity can be affected by many factors. First, peroxidases such as HRP are known to activate galactose oxidases, although the mechanism and the components involved are not known$^{61,62}$. Second, CgrAlcOx requires molecular oxygen as a second substrate to regenerate the active site; however, the solubility of oxygen in water is 0.258 mM, which would limit the speed of the reaction. In fact, it has been shown that galactose oxidase is severely oxygen-limited at the beginning of the reaction$^{62}$. In addition, as discussed in Section 1.1, the CgrAlcOx catalyzed reaction follows a Ping-Pong mechanism. Therefore, if the reaction follows Michaelis-Menten
kinetics, O₂, as the second substrate, must saturate the enzyme during the period of acquisition. Thus, it is necessary to test whether the amount of O₂ in the reaction mixture is sufficient to saturate the enzyme. Finally, if the amount of O₂ does in fact limit the rate of the reaction, it is necessary to determine the best way to deliver it into the solution – either by maximizing O₂ diffusion through proper mixing, or by another means that is easily applied.

Figure 4.1-6 Two mechanisms by which HRP breaks down H₂O₂ (reproduced from ref 60).

HRP breaks down H₂O₂ via two mechanisms (Figure 4.1-6): (1) HRP acts as a peroxidase when it is coupled with an electron donor compound such as ABTS or phenol. As a peroxidase, HRP breaks down H₂O₂ by taking an electron from ABTS or phenol to generate water and a radical species. (2) HRP can also act as a catalase in the absence of an electron donor, breaking down two molecules of H₂O₂ into water (Figure 4.1-7); however, this scavenger property is not as efficient as catalase⁶³,⁶⁴. Because of its low scavenger properties, catalase was first tested for its ability to scavenge H₂O₂ in an enzymatic reaction in Section 4.1.1.
To determine the effect of hydrogen peroxide scavenger enzymes on the rate of benzyl alcohol oxidation, HRP and catalase were tested independently (Figure 4.1-8). The reaction containing HRP was slightly faster at the beginning of the reaction, but it slowed down significantly after 5 minutes due to the limited capacity of HRP to break down H$_2$O$_2$ without an electron donor. In contrast, the reaction containing catalase was slightly slower at the beginning of the reaction but was faster later in the reaction. However, in both cases, enzyme activity was significantly lower than that obtained from the ABTS assay. This set of reactions was performed three times, and the behaviours of the reactions were almost the same each time.

**Figure 4.1-7** Mechanism by which catalase breaks down H$_2$O$_2$.$^{64}$

\[
\text{Enz (Por-Fe}^{\text{III}}\text{)} + \text{H}_2\text{O}_2 \rightarrow \text{Cpd I (Por-}^{+}\text{-Fe}^{\text{IV}}\text{)} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{Cpd I (Por-}^{+}\text{-Fe}^{\text{IV}}\text{)} + \text{H}_2\text{O}_2 \rightarrow \text{Enz (Por-Fe}^{\text{III}}\text{)} + \text{H}_2\text{O} + \text{O}_2 \quad (2)
\]

**Figure 4.1-8** Reaction curves generated using either catalase (■) or HRP (●) as H$_2$O$_2$ scavenger enzymes.
When both HRP and catalase were used in the reaction, the rate of conversion was much faster. In these experiments, the reaction was started with CgrAlcOx alone, and the scavenger enzymes were added in series after 10 or 28 minutes (Figure 4.1-9). For comparison, another reaction was prepared in which both scavenger enzymes and CgrAlcOx were present in the reaction. The reaction proceeded at a slow rate if only one of the scavenger enzymes was present, but the conversion rate was 24-fold higher when both scavenger enzymes were present. This significant increase in activity suggested that the two scavenger enzymes were working in synergy, and it was hypothesized that some intermediates generated when HRP is coupled with catalase are involved in activating CgrAlcOx (see Section 4.2).

Figure 4.1-9 Both catalase and HRP are required to maximize CgrAlcOx activity. Blue curve (△—△): the reaction was started with HRP and catalase; Black curve (■—■): the reaction was started with CgrAlcOx, catalase was added at 10 minutes, and HRP was added at 28 minutes; Red curve (●—●): the reaction was started with CgrAlcOx, HRP was added at 10 minutes, and catalase was added at 28 minutes.
4.1.2.2 Test of the Best Method to Deliver O₂ into Solution

![Diagram showing the effect of mixing on enzymatic activity](image)

**Figure 4.1-10** Molecular oxygen in the reaction mixture is necessary to boost *CgrAlcOx* activity. Red curve (---): the reaction was mixed using a P1000 micropipette; Black curve (-----): the reaction sat still in a HPLC tray.

To determine if the amount of O₂ in solution affected enzymatic activity, a P1000 micropipette was used to mix the reaction during the entire acquisition period to facilitate the diffusion of air (Figure 4.1-10). The reaction was faster beyond the conversion of 0.3 mM of substrate when the reaction was properly mixed. This was due to the depletion of O₂ in solution, resulting in the inability of *CgrAlcOx* to regenerate the active site (because the solubility of O₂ was 0.3 mM). However, because the motion of mixing created a huge amount of air bubbles, the progress curve that was obtained was not as smooth as that obtained without mixing.

Because oxygen was verified to be a necessary reaction component, we sought to determine the best way to deliver oxygen into solution (Figure 4.1-11). Thus, the best way to deliver oxygen into
solution was researched (Figure 4.1-11). It has been shown that bubbling with pure O$_2$ gas does saturate the reaction with enough co-substrate$^{62}$. Therefore, an alcohol oxidation reaction was set up in which O$_2$ gas was bubbled into solution during the acquisition period. It was observed that if the reaction was bubbled with pure O$_2$ gas, the rate of reaction was much higher than when the reaction was mixed with a micropipette (although a 70% conversion was also observed; Figure 4.1-11, blue and purple curves). This incomplete conversion was likely due to the formation of air bubbles, which formed hydrophobic layers that significantly reduced protein stability, leading to protein denaturation$^{62}$. Thus, an alternative way to deliver O$_2$ into the reaction solution was explored.

Figure 4.1-11 A. Air bubbles created when the reaction was bubbled with O$_2$ gas. B. Test of O$_2$ diffusion in the reaction and test of the best way to introduce O$_2$ into solution.

Because catalase breaks down H$_2$O$_2$ into O$_2$ and water, H$_2$O$_2$ could, in theory, be used to deliver O$_2$ into the reaction mixture. Furthermore, H$_2$O$_2$ is a liquid, so the O$_2$ that is generated is much
easier to diffuse into the solution to saturate the reaction mixture. We confirmed that H₂O₂ in the presence of catalase is a more efficient way to deliver O₂ than bubbling the reaction with O₂ gas; the former approach allowed the reaction to reach complete conversion. More importantly, supplementation with higher amounts of H₂O₂ improved the rate of conversion beyond that achieved by bubbling the reaction with gas. Therefore, adding H₂O₂ and catalase together into the reaction appears to be a better way to introduce O₂ into the reaction mixture.

Since the $K_{m}$ of galactose oxidase towards oxygen was reported to be $\geq 3$ mM, it implied that the co-substrate was not enough to saturate oxidases in any kinetic assays. Reaction supplemented with H₂O₂ in the presence of catalase was shown to be the best way to deliver O₂ into aqueous solution. However, since there was no way to test the amount of O₂ in the solution, this new method could not guarantee that saturating conditions of the co-substrate were being achieved during the enzymatic reaction. In the recent published literature, similar catalase mediated delivery of O₂ to alcohol oxidation catalyzed by galactose oxidase were reported, and it was verified as a better means of comparison to microreactors and bioreactors. The reaction was supplemented with H₂O₂ four times (total of 300 mM) to ensure the highest percentage of conversion of the substrate at multiple positions on the newly developed multipoint injection flow reactor. More research was needed to modify the current method, and to ensure the saturation of co-substrate in the entire reaction acquisition period of the HPLC approach.

4.1.3 Optimized Kinetics by HPLC

This new assay requires the use of many different components, such as catalase, HRP, and H₂O₂. To utilize this assay for kinetics, reaction conditions must be optimized to maximize the catalytic
efficiency of CgrAlcOx. The optimal conditions were identified to be: 2.4 μM catalase, 17.5 μM HRP, 9.47 nM CgrAlcOx, and 15 mM H₂O₂. Kinetics were performed using these optimal conditions with two different substrates. Because HPLC was coupled with a UV detector, only UV-active compounds (CinOH and BnOH) could be monitored. Michaelis-Menten plots were obtained by following the formation of the aldehyde or the depletion of the alcohol. The results were similar to those obtained by HRP-ABTS assay (Appendix A.3). Therefore, this new technique is capable of monitoring CgrAlcOx-mediated alcohol oxidation and can be used to characterize enzyme activity.

Table 4.2 Summary of the kinetic parameters obtained from Michaelis-Menten Kinetic plots obtained using HPLC-UV to monitor the depletion of the starting material or the formation of product.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Depletion of alcohol</th>
<th>Formation of aldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (mM)</td>
</tr>
<tr>
<td>BnOH</td>
<td>101 ± 1</td>
<td>0.686 ± 0.025</td>
</tr>
<tr>
<td>CinOH</td>
<td>79.5 ± 0.1</td>
<td>0.00989 ± 0.00188</td>
</tr>
</tbody>
</table>
Figure 4.1-12 Michaelis-Menten Kinetics of benzyl alcohol obtained by monitoring: A. Depletion of benzyl alcohol, or B. Formation of benzaldehyde.

Figure 4.1-13 Michaelis-Menten Kinetics of cinnamyl alcohol by monitoring: A. Depletion of cinnamyl alcohol, or B. Formation of cinnamyl aldehyde.

4.2 Study of \textit{CgrAlcOx} Activation using HPLC-UV

Figure 4.1-9 shows that the activity of \textit{CgrAlcOx} was maximized when both HRP and catalase were used in the reaction. When HRP was used as the only H$_2$O$_2$ scavenger enzyme, it did not affect the activity of \textit{CgrAlcOx}, which indicated that HRP was probably not activating the alcohol
oxidase. It has been hypothesized that an intermediate generated in the reaction mixture activates galactose oxidase, which also belongs to the AA5_2 family of enzymes. Because the HPLC-UV approach was capable of monitoring the reaction directly through analysis of the reaction’s starting material or product, this approach was used to study the components activating CgrAlcOx.

Because the activity of CgrAlcOx obtained from the HRP-ABTS assay was the same as that obtained in reactions containing the two scavenger enzymes, a 1 mM BnOH oxidation containing HRP and ABTS was analyzed by monitoring the formation of benzaldehyde by HPLC (Figure 4.2-1A). Although the reaction was inhibited after 10 minutes, the rate of conversion at the beginning of the reaction was as fast as the reaction containing catalase and HRP. This observation indicated that maximal activation was achieved only if HRP was coupled with an electron donor or catalase. Thus, it was hypothesized that CgrAlcOx would be activated if another electron donor compound was used in place of ABTS.
Figure 4.2-1 HPLC was used to study the activation of CgrAlcOx. A. Benzyl alcohol oxidation catalyzed by 18.9 nM CgrAlcOx containing different components: Black curve (■): 1 mM benzyl alcohol oxidation with 1.3 μM HRP, 0.587 μM catalase, and 6 mM H₂O₂; Red curve (●): 1 mM benzyl alcohol reaction with 1.3 μM HRP and 1 mM ABTS; Blue curve (▲): 1 mM benzyl alcohol with 1.3 μM HRP, 0.587 μM catalase, and 1 mM ABTS. B. Benzyl alcohol oxidation catalyzed by 9.47 nM CgrAlcOx containing different components: Black curve (■): 1 mM benzyl alcohol oxidation 17.5 μM HRP, 2.4 μM catalase, and 15 mM H₂O₂; Blue curve (▲): 1 mM benzyl alcohol oxidation with 17.5 μM HRP and 1.5 mM pyruvic acid; Dark blue curve (●): 1 mM benzyl alcohol with 17.5 μM HRP; Green curve (◆): 1 mM benzyl alcohol with 2.4 μM catalase; Pink curve (▲): 1 mM benzyl alcohol oxidation without any H₂O₂ scavenger enzyme.

Pyruvic acid was used to replace ABTS because it has been reported to be an H₂O₂ scavenger and an electron donor \(^{67,68}\). As shown in Figure 4.2-1B, when HRP and pyruvic acid were present in the reaction, the activity of CgrAlcOx was elevated. When HRP was present in the reaction without pyruvic acid, or when only catalase was used in the reaction, the activity of CgrAlcOx was minimal. These findings indicated that the alcohol oxidase was activated when HRP was coupled with an electron donor compound – either ABTS or pyruvic acid. Because previous results showed that CgrAlcOx was not activated by HRP alone, we hypothesized that activation must be caused by
certain intermediates that were generated in the reaction; we did not, however, confirm the identity of the intermediates.
Chapter 5: Applications of HPLC-UV Approach in Studying New Substrates and Inhibitors

5.1 Introduction

The previous chapters demonstrated that the HPLC-UV approach can be used to directly monitor CgrAlcOx-mediated alcohol oxidation, and can also be used to optimize reaction conditions and to perform kinetics. Therefore, there was great interest in further developing this technique, or combining it with the HRP-ABTS assay, to use in new applications.

One potential application of this technique, as mentioned in the introduction, is the screening of new substrates or inhibitors for CgrAlcOx, especially amine and thiol compounds. Because 1-butanol was used as the benchmark substrate when this enzyme was characterized using the HRP-ABTS assay, and benzyl alcohol was used to develop the HPLC-UV approach, we started by screening amine and thiol compounds that resembled the structure of 1-butanol and benzyl alcohol, as summarized in Table 5-1.

Table 5.1 Amine and thiol candidate compounds for screening

<table>
<thead>
<tr>
<th>Amine Compounds</th>
<th>Thiol Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylamine</td>
<td>1-butanethiol</td>
</tr>
<tr>
<td>Benzyamine</td>
<td>Benzyl mercaptan</td>
</tr>
</tbody>
</table>

The HPLC-UV approach can also be used to replace the HRP-ABTS assay in some studies where the initial rate assay is not applicable. 1-butanol ($k_{cat} = 96 \pm 1 \text{ s}^{-1}$) was identified as one of the best substrates for CgrAlcOx, but it was not fully converted into butyraldehyde. In contrast, aromatic
alcohols – such as cinnamyl alcohol – had similar enzymatic specificity and were completely oxidized by CgrAlcOx into their corresponding aldehydes. We suspected that the aliphatic aldehydes were inhibiting the enzyme through product inhibition. We attempted to resolve this issue using the initial rate assay, but our findings were not conclusive. In addition, the HRP-ABTS assay was not suitable for screening activity against some of the amine compounds because the ABTS precipitated. Because the initial rate assay was not useful in these cases, the newly developed HPLC-UV approach was used as an alternative.

Because the HPLC was coupled to a UV detector, only UV-active compounds could be monitored directly using this approach. To utilize this new approach to study substrates or inhibitors that were not UV-active, competition experiments were used. In these experiments, two different compounds were set up in the reaction; one compound was a benchmark substrate (such as benzyl alcohol), while the other compound was a potential substrate or inhibitor (the non-benchmark compound). If the non-benchmark compound was UV-active, the progress curves of both compounds were generated and analyzed. If the compound was not UV-active, only the benzaldehyde progress curve was monitored. In this case, the effect that the non-benchmark compound had on the reaction curve of the benchmark substrate was used to predict whether the compound was a substrate or an inhibitor.

### 5.2 Competition Experiments for the Screening of Potential Substrates

Competition experiments were used to screen for new substrates. To demonstrate that this approach can be used for this purpose, a reaction was set up with 0.5 mM benzyl alcohol and 0.5 mM cinnamyl alcohol (Figure 5.2-1). Because both compounds are UV-active, starting materials
and products were monitored. It was observed that cinnamyl alcohol oxidation occurred more rapidly than benzyl alcohol oxidation; for benzyl alcohol oxidation, there was a significant lag phase. This was because cinnamyl alcohol had a similar $k_{\text{cat}}$ value, but a much lower $K_m$ value, compared to benzyl alcohol. Because both substrates were converted into their corresponding aldehydes without any by-product at the end of the reaction, both compounds were substrates to $Cgr\text{AlcOx}$. This proof of concept experiment demonstrated that competition experiments, based on the conversion of substrates, can be used to screen for potential substrates. It also showed that competition experiments can be used to gather qualitative information, such as $K_m$ values relative to benzyl alcohol, because the difference in $K_m$ value affected the shape of the reaction curve.

![Figure 5.2-1 Competition experiment between BnOH and CinOH. Black Curve (—): cinnamyl alcohol; Blue curve (—△): benzyl alcohol; Pink curve (—■): benaldehyde; Red curve (—●): cinnamyl aldehyde.](image)

*Reaction Condition*

0.5 mM BnOH + 0.5 mM CinOH

$Cgr\text{AlcOx}$: 0.227 μM

Catalase: 1.17 μM
5.3 The HPLC-UV Approach Used to Study Product Inhibition

5.3.1 Steps Involved in Inhibition Studies

Four steps are typically used to screen for inhibitors. First, a competition experiment is set up with benzyl alcohol and the target compound. Based on the effect of the compound on the reaction curve of the benzaldehyde, the target compound can be identified as an inhibitor or a substrate. If the compound is identified as an inhibitor, an irreversible inhibition study is done by incubating $Cgr$AlcOx with different concentrations of target compound for different durations. The incubated protein is diluted 1000-fold and is used to convert benzyl alcohol to benzaldehyde. If the activity of $Cgr$AlcOx is decreased after incubation, the compound is assumed to be an irreversible inhibitor. In this case, a full irreversible inhibition study is performed to derive inhibition constants. If the compound does not appear to be an irreversible inhibitor, the compound is subjected to reversible inhibition studies which aim to determine if inhibition is dependent on the concentration of the target compound. This is followed by a full reversible inhibition study to verify the type of reversible inhibition and to extract inhibition constants.

5.3.2 Study of Product Inhibition by Butyraldehyde

As mentioned in Section 5.1, $Cgr$AlcOx was not able to fully convert aliphatic alcohols, regardless of initial enzyme loading. We suspected that the aliphatic aldehyde (the oxidation product of alcohol oxidation) was causing product inhibition. A competition experiment was used to study this hypothesis in which a reaction containing 0.5 mM benzyl alcohol and 0.5 mM 1-butanol (Figure 5.3-1) was set up. Because 1-butanol was not UV active, progress curves of benzyl alcohol and benzaldehyde were generated and analyzed. The reaction curves for benzaldehyde formation in the presence of 1-butanol deviated from those containing only 0.5 mM BnOH, indicating that
some component generated in the competition reaction was inhibiting $CgrAlcOx$. Because benzaldehyde and butyraldehyde were formed in the competition experiment, and benzaldehyde was previously confirmed not to be an inhibitor (Section 4.1.1.2), we examined the possibility that butyraldehyde was inhibiting the reaction.

Figure 5.3-1 Competition experiment between BnOH and BuOH. Black curve (---): 0.5 mM BnOH oxidation reaction; Red curve (---): 0.5 mM BnOH and 0.5 mM BuOH competition reaction.

A 0.5 mM BnOH oxidation was initiated by adding $CgrAlcOx$, and at 13 minutes, the reaction was supplemented with 0.5 mM butyraldehyde (Figure 5.3-2). The supplementation slowed down benzaldehyde formation dramatically and stopped the reaction within 10 minutes. This result confirmed that butyraldehyde was causing product inhibition. Therefore, follow-up studies were performed to identify the type of inhibition caused by butyraldehyde.
We first tested the possibility that butyraldehyde is an irreversible inhibitor (Figure 5.3-3). *CgrAlcOx* was incubated with different concentrations of butyraldehyde for different durations. After incubation, *CgrAlcOx* was diluted 1000-fold so that the concentration of butyraldehyde in the reaction mixture was negligible; this diluted enzyme was then used to convert 1 mM benzyl alcohol. The reaction curves of incubated enzymes were almost identical to the progress curve for non-incubated *CgrAlcOx*. Therefore, butyraldehyde was not an irreversible inhibitor.
Figure 5.3-3 Study of irreversible inhibition by butyraldehyde. CgrAlcOx was incubated with either 0.5 mM butyraldehyde for 1.83 minutes (-----), 8.5 minutes (-------), and 26.87 minutes (------), or 0.9 mM butyraldehyde for 3.53 minutes (-----), and 26.1 minutes (--------). After incubation, CgrAlcOx was recovered by diluting 1000-fold and used to convert 1 mM benzyl alcohol. The generated reaction curves were compared to BnOH oxidation reaction catalyzed by non-incubated CgrAlcOx (-----).

To further explore the inhibitory behaviour, competition reactions were prepared using 1 mM benzyl alcohol and varied amounts of butyraldehyde (from 0.1 mM to 2 mM). The reaction curves showed that the percentage conversions were lower as inhibitor concentration was increased, indicating that butyraldehyde was causing a concentration-dependent inhibition of CgrAlcOx activity.
A full reversible inhibition study was performed using butyraldehyde, which showed that the $K_m$ of BnOH increased and the $k_{\text{cat}}$ decreased as the concentration of butyraldehyde was increased. As discussed in the introduction, when $V_{\text{app}}$ decreases by a factor of $(1 + [I]/K_{iu})$ and $K_{m,\text{app}}$ increases by a factor of $(1 + [I]/K_{ic})$, it suggests mixed inhibition (Figure 5.3-5). However, even though this butyraldehyde inhibition experiment was repeated multiple times, consistent large errors were propagated through the calculations of $K_{ic}$ ($0.694 \pm 0.106$ mM) and $K_{iu}$ ($3.66 \pm 1.50$ mM) preventing a model of inhibition from confidently being assigned.
It was curious that butyraldehyde was identified as an inhibitor, but benzaldehyde was not. One speculation was that the inhibition was a result of the presence of hydrated aldehydes, where the germinal diol was occupying the active site without being turned over. Theoretically, the equilibrium constants of aliphatic aldehyde hydration, such as propanal, was predicted about 376-fold higher than benzaldehyde in aqueous solution\textsuperscript{69}, which indicated that the hydration of aliphatic aldehydes were more common than benzaldehyde. However, because the binding site of butyraldehyde on \textit{CgrAlcOx} was not investigated in this thesis, more research is needed.
Based on our findings, the HPLC-UV approach appears to be more suitable than the initial rate assay for studying the inhibition. Because benzaldehyde formation was not stopped instantaneously after the addition of inhibitor, we hypothesized that butyraldehyde causes slow inhibition (Figure 5.3-2). Moreover, Figure 5.3-4 shows that there was approximately 10% conversion observed when the reaction was set up with two times more butyraldehyde than BnOH. The HRP-ABTS assay was used to validate this hypothesis.

Figure 5.3-6 A. The HRP-ABTS assay for 1 mM benzyl alcohol oxidation containing different concentrations of butyraldehyde: no butyraldehyde (---); 0.28 mM (-----); 1.4 mM (-----); 2.8 mM (-----); 4.2 mM (-----); 5.6 mM (-----); 8.4 mM (-----). B. Slopes extracted from the curves in panel A from 0 to 0.5 minutes (—■—) and from 3.5 to 4 minutes (—●—); all slopes were converted into s^{-1} and were plotted against the concentration of butyraldehyde.

The reactions were prepared with same amount of BnOH (20 mM) and different amounts of butyraldehyde (Figure 5.3-6). The reaction progress curves generated by HRP-ABTS assay were combined after being monitored by UV-vis spectrometry for 4 minutes. The reaction plateaued faster when the reaction contained higher amounts of butyraldehyde. When the slopes of the progress curves extracted at the beginning of the reaction (before 0.5 minutes) were plotted against
the concentration of butyraldehyde, reaction velocity decreased by 10% at 8.4 mM butyraldehyde. If the slopes were extracted at the end of the reaction (between 3.5 and 4 minutes), reaction velocity dropped significantly (by 90%). This validated the hypothesis that butyraldehyde was causing slow inhibition.

![Figure 5.3-7 Reaction progress curves generated by different instruments to study butyraldehyde product inhibition: A. Trace from the HRP-ABTS assay for a 1 mM benzyl alcohol oxidation reaction containing different concentrations of butyraldehyde: no butyraldehyde (—); 0.28 mM (—); 1.4 mM (—); 2.8 mM (—); 4.2 mM (—); 5.6 mM (—); 8.4 mM (—); 8.4 mM (—). B. Reaction curves generated by HPLC-UV for 1 mM BnOH oxidation with different concentration of butyraldehyde: no butyraldehyde (—); 0.65 mM (—); 3.70 mM (—); 6.00 mM (—).](image)

Comparison of the reaction progress curves generated by HRP-ABTS assay and HPLC-UV revealed that HPLC-UV was more suitable for studying the slow inhibition caused by butyraldehyde; inhibition was obvious in the reaction containing 0.648 mM butyraldehyde. The HPLC-UV approach monitors reactions over a longer period of time, whereas the initial rate assay, as its name suggests, monitors the initial portion of the reaction when the fastest conversion rate is observed. Use of the initial rate assay to study slow inhibition requires that a region in which there is primarily slow inhibition be chosen for analysis, which creates a high level of bias. In
contrast, the reaction curves obtained by HPLC-UV accurately represent the behaviours of the reaction and do not require subjective interpretation, thereby reducing errors in rate determination.

5.3.3 Other Substrates May Exhibit Product Inhibition

Because CgrAlcOx can completely oxidize aromatic alcohols such as benzyl alcohol and cinnamyl alcohol, we hypothesized that if the hydroxyl group was moved farther away from the aromatic ring or conjugated system, the aldehyde that was produced would cause product inhibition. Thus, five substrates were chosen to test this hypothesis (see Table 5-4 for selection of substrates). The reactions were prepared using 15 mM substrate, 7.45 μM CgrAlcOx, 1.3 mM catalase, and 5.2 mM HRP. By comparison, only 74.5 nM CgrAlcOx was needed to fully oxidize benzyl alcohol and cinnamyl alcohol. The percentage conversion was estimated using the ratio of the integrated aldehyde proton peak and the benzylic proton peak in the $^1$H-NMR spectrum. The results are summarized in Table 5.4 and the $^1$H spectra of the reactions and the assignment of the proton peaks are listed in Appendices D.2-D.6.

Among the substrates listed in Table 5-4, 2-benzyloxy ethanol underwent the highest conversion, at 43%. This trend implied that each reaction was inhibited by the formation of its corresponding aldehyde. However, the type of inhibition was not explored further in this thesis.
Table 5.2 Summary of percentage conversion of substrates observed through $^1$H NMR

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Percentage Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-phenylethanol</td>
<td><img src="image" alt="Structure" /></td>
<td>5.2%</td>
</tr>
<tr>
<td>3-phenyl-1-propanol</td>
<td><img src="image" alt="Structure" /></td>
<td>26%</td>
</tr>
<tr>
<td>2-phenyl-1-propanol</td>
<td><img src="image" alt="Structure" /></td>
<td>11%</td>
</tr>
<tr>
<td>3-benzylloxy-1-propanol</td>
<td><img src="image" alt="Structure" /></td>
<td>13%</td>
</tr>
<tr>
<td>2-benzylloxy-ethanol</td>
<td><img src="image" alt="Structure" /></td>
<td>43%</td>
</tr>
</tbody>
</table>

The proton on the carbon connected to the hydroxyl group of benzyl or cinnamyl alcohol was activated by the conjugated system, allowing these two aromatic alcohols to be easily oxidized by CgrAlcOx. This was also observed for trans,trans-2,4-Hexadien-1-ol, which had a higher $k_{cat}/K_m$ than cinnamyl alcohol; it was completely converted to its corresponding aldehyde$^{18}$. Therefore, substrates do not appear to exhibit product inhibition towards CgrAlcOx when the hydroxyl group is connected to the carbon next to a conjugated system. However, when the carbon connected to the hydroxyl group is further away from the conjugated system (e.g., in the substrates tested in Table 5-4), the percentage conversion by the enzyme is significantly lower. The size of the substrate also seems to affect the conversion of the substrate; cinnamyl alcohol was a good substrate for CgrAlcOx, and 3-phenyl-1-propanol and 2-benzylloxy-ethanol, which are almost the same size as cinnamyl alcohol, underwent slightly better conversion compared to other substrates.

Conversion rate was significantly reduced in molecules that were smaller or larger in size.
5.4 Alternative Substrate Screening: Benzylamine

Because ABTS reacts with benzylamine and precipitates when used at a concentration of 5 mM, the initial rate assay was not suitable for studying benzylamine. Because the HPLC approach directly monitors the reaction, it was a better approach to study benzylamine. If CgrAlcOx were to oxidize BnNH$_2$ into imine, it would be readily hydrolyzed by a water molecule to make benzaldehyde (scheme 2). BnNH$_2$ can be studied by directly monitoring the rate of formation of benzaldehyde in a competition experiment between BnOH and BnNH$_2$. If the reaction curve of benzaldehyde reached beyond 100% conversion, it was a substrate; otherwise, it was an inhibitor.

Scheme 2: Benzylamine is oxidized by CgrAlcOx and the resulting imine hydrolyzed by water

![Scheme 2: Benzylamine oxidation](image)

Figure 5.4-1 Reaction curve of 1 mM BnOH oxidation by CgrAlcOx with different concentrations of BnNH$_2$: Black curve (---): no BnNH$_2$; Red curve (---): 3.73 mM BnNH$_2$; Blue curve (---): 18.7 mM BnNH$_2$; Pink curve (---): 37.3 mM BnNH$_2$.

Reaction Condition
CgrAlcOx: 9.47 nM
Catalase: 2.40 μM
HRP: 17.5 μM
In competition experiments with BnOH and BnNH₂, the formation of benzaldehyde occurred at the same rate as the control; however, the reaction curve was shifted by a factor that corresponded to the amount of BnNH₂ solution that was added to the reaction mixture (Figure 5.4-2). These results indicated that extra benzaldehyde was already present before it was added to the alcohol oxidation reaction. To explore whether benzyamine was a substrate for CgrAlcOx, another approach was used. Reactions were set up with 18.7 mM BnNH₂ incubated in different conditions (Table 5-4) on a shaker for 16 hours, with reaction composition analyzed by HPLC-UV. Approximately 0.3% of BnNH₂ was converted to benzaldehyde in the presence of buffer or catalase and HRP. In contrast, when the reaction was incubated with catalase, HRP, and CgrAlcOx, the amount of BnNH₂ conversion to benzaldehyde increased to 0.971%. Because the percentage conversion of BnNH₂ was very low even after 16 hours of incubation, we concluded that BnNH₂ is a substrate, albeit a poor one, for CgrAlcOx. ¹H NMR analysis was used to confirm that benzaldehyde was the product of benzyamine oxidation catalyzed by CgrAlcOx (Figure 5.4-3).

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>[benzaldehyde] (mM)</th>
<th>Percentage conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>BnNH₂ + buffer</td>
<td>0.0717</td>
<td>0.383%</td>
</tr>
<tr>
<td>BnNH₂ + catalase + HRP</td>
<td>0.0613</td>
<td>0.328%</td>
</tr>
<tr>
<td>BnNH₂ + catalase + HRP + CgrAlcOx</td>
<td>0.1815</td>
<td>0.971%</td>
</tr>
</tbody>
</table>
It was hypothesized that if CgrAlcOx could oxidize benzylamine, the formed imine could readily hydrolyze into benzaldehyde. However, it was surprising that benzaldehyde was present when BnNH$_2$ was incubated with buffer alone or with H$_2$O$_2$ scavenger enzymes. This phenomenon was observed when the pH of the BnNH$_2$ solution was readjusted to 8.00 using NaH$_2$PO$_4$ after addition of the amine compound.

The low conversion of benzylamine could be resulted from protonation of the amine substrate in the reaction. Scrutton et al. suggested that the binding of deprotonated amine substrates in
mammalian monoamine oxidases were more preferred over their protonated form\textsuperscript{70}. It was also reported that the change in pH and buffer strength of the reaction would change the composition of amine oxidations final products catalyzed by laccases, a multi-copper oxidase\textsuperscript{71}. Due to the time constraint, pH dependence of amine oxidation catalyzed by \textit{CgrAlcOx} was not investigated in this thesis.

5.5 Combined Use of the HPLC-UV Approach and the HRP-ABTS Assay to Study Inhibition

Inhibition studies of \textit{CgrAlcOx} using the HPLC-UV approach exposed the limitations of the biochemical HRP-ABTS assay. However, tests of irreversible and reversible inhibition required experiments that were more than 12 hours in duration, which was problematic. The long duration of reversible or irreversible inhibition studies is necessary because these assays require the survey of numerous concentrations of both the substrate and inhibitor to acquire accurate data (Table B.13-1 and B.13-2 in Appendix B.13). Due to the nature of these inhibition studies and without the aid of an automated system, the operator must manually initiate each reaction by adding \textit{CgrAlcOx} following the termination of previous runs. The reaction is 30-40 minutes in duration, creating a large workload that may hinder the use of this technique efficiently. Therefore, the remainder of this thesis explored the combined use of the HPLC-UV approach and the HRP-ABTS assay to more efficiently study \textit{CgrAlcOx} inhibition. Theoretically, the HPLC-UV approach could be developed into an automated system that could be used to screen new substrates or inhibitors in an efficient way. Any identified inhibitor compounds were then characterized using the HRP-ABTS assay in order to shorten the duration of the characterization of this compound.
5.5.1 Studies of Butylamine Inhibition

Competition experiments were used to study the activity of CgrAlcOx towards BuNH$_2$. The results showed that BuNH$_2$ was an inhibitor; the more BuNH$_2$ that was present in the solution, the less benzaldehyde that was formed (Figure 5.5-1). In addition, BuNH$_2$ inhibition was concentration-dependent.

![Figure 5.5-1 Competition experiments between 1 mM BnOH and different concentrations of BuNH$_2$, indicating that the inhibition was concentration-dependent.](image)

Studies of irreversible inhibition by BuNH$_2$ were performed by incubating CgrAlcOx with different concentrations of butylamine for different durations (Figure 5.5-2). The reaction curves of incubated and non-incubated CgrAlcOx were almost identical, demonstrating that BuNH$_2$ was not an irreversible inhibitor.
Figure 5.5-2 Study of irreversible inhibition by BuNH$_2$. Black curve (---): no incubation; Blue curve (---): incubation with 3 mM BuNH$_2$ for 23 minutes; Red curve (---): incubation with 5 mM BuNH$_2$ for 11 minutes; Pink curve (---): incubation with 5 mM BuNH$_2$ for 27 minutes.

Unlike BnNH$_2$, which precipitated with ABTS, BuNH$_2$ was compatible with ABTS in the HRP-ABTS assay. Thus, a full reversible inhibition study of BuNH$_2$ was performed using this initial rate assay. The results showed that as the concentration of BuNH$_2$ increased, the $k_{\text{cat}}$ of BnOH decreased while the $K_m$ of BnOH was relatively unchanged (Figure 5.5-3). As discussed in the introduction, inhibition is non-competitive (a special case of mixed inhibition) when only $V_{\text{app}}$ changes. With $K_{\text{iu}} = 57.7 \pm 5.1$ mM, inhibition by BuNH$_2$ was non-competitive.
Because benzylamine was identified as a substrate for CgrAlcOx (Section 5.4), it was hypothesized that butylamine was inhibiting CgrAlcOx because butylamine was being converted into butyraldehyde, with butyraldehyde causing inhibition. An $^1$H-NMR analysis was done on a reaction containing 18.75 mM BuNH$_2$, 7.45 μM CgrAlcOx, 1.3 mM catalase, and 5.2 mM HRP. The reaction was incubated for over 20 hours, and the reaction contents were quickly extracted with deuterated chloroform. The extracted sample was analyzed by $^1$H-NMR and was compared
to the spectrum of BuNH$_2$. Only an amine compound was identified in the spectrum, indicating that BuNH$_2$ was not converted by CgrAlcOx. Based on these findings, we rejected the hypothesis that butyraldehyde causes inhibition, and instead attributed inhibition to the amine compound.

5.5.2 Study of Inhibition by Benzyl Mercaptan

Figure 5.5-4 A. Competition experiment between 1 mM BnOH and 1 mM BnSH. B. Study of irreversible inhibition by BnSH. Black curve (—■—): no incubation; Red curve (—○—): incubation with 3 mM BnSH for 7 minutes; Pink curve (—△—): incubation with 5 mM BnSH for 10 minutes; Green curve (—▲—): incubation with 3 mM BnSH for 26 minutes; Blue curve (—△—): incubation with 5 mM BnSH for 23 minutes.
The competition experiment between 1 mM benzyl alcohol and 1 mM BnSH indicated that benzyl mercaptan was an inhibitor; this conclusion was based on the observation that the conversion of benzyl alcohol was less than 5% when the reaction was sampled over an hour (Figure 5.5-4). An irreversible inhibition study showed that BnSH was an irreversible inhibitor because the activity of CgrAlcOx was lower when it was incubated with higher concentrations of BnSH or when it was incubated with BnSH for longer.

![Figure 5.5-5 Study of irreversible inhibition by BnSH using the HRP-ABTS assay. A. Decline in activity when the enzyme was incubated with different concentrations of BnSH. B. Plot of $K_{app}$ vs. concentration of BnSH.](image)
When $K_{\text{app}}$ was plotted against the concentration of BnSH, not every $K_{\text{app}}$ that was obtained aligned with the fitted curve, even though the experiment was repeated many times (Figure 5.5-5). Therefore, a 95% confidence interval for $K_{\text{app}}$ was plotted into which all the data fit; this brought statistical meaning to the data that was obtained. Irreversible inhibition constants were calculated as $K_i = 0.864 \pm 0.232$ mM and $k_2 = 0.0455 \pm 0.0040$ min$^{-1}$. As mentioned in the introduction in Chapter 1, according to the Hard and Soft Acids and Bases principle, thiol derivative compounds have a high affinity for binding to the copper center of a cupric protein. It was not surprising that benzyl mercaptan was identified as an irreversible inhibitor for $Cgr\text{AlcOx}$.

5.5.3 Study of Inhibition by 1-Butanethiol

Competition experiments between BuSH and BnOH revealed some interesting behaviours. In a reaction with 1 mM BnOH and 0.5 mM BuSH, there was no conversion of benzyl alcohol at the beginning of the reaction, but the rate of the reaction recovered after 45 minutes. The reaction rate was also inhibited later in the reaction, yielding a final conversion of approximately 70%. This behaviour appeared to be dependent on the concentration of BuSH, because higher initial concentrations of BuSH led to a longer lag phase and a lower final conversion of the reaction. This is the first time that this type of reaction behaviour (inhibition $\rightarrow$ rate recovery $\rightarrow$ inhibition again) has been observed with HPLC method.
BuSH has an unpleasant “skunk” odour. However, this odour disappeared over time when this compound was incubated with \(Cgr\text{AlcOx}\), indicating that BuSH might have been converted by the alcohol oxidase. Thus, BuSH was incubated with \(Cgr\text{AlcOx}\) in the presence of catalase and HRP, and the reaction contents were extracted with deuterated chloroform and then analyzed by \(^1\text{H}-\text{NMR}\). Comparison of the reaction spectrum with the spectrum for butyraldehyde suggested that BuSH was converted to butyraldehyde by the alcohol oxidase.
NMR analysis provided insight into the behaviour of the BuSH oxidation reaction that was observed by HPLC. There was no conversion of benzyl alcohol at the beginning of the reaction because CgrAlcOx was inhibited by BuSH. Because the inhibitor was slowly converted into butyraldehyde by the enzyme, the conversion rate recovered as the reaction progressed. However, as butyraldehyde – an inhibitor that causes slow inhibition – accumulated in the reaction, it inhibited CgrAlcOx (Section 5.3.2).

Further tests using the HRP-ABTS assay revealed that BuSH acted as an irreversible inhibitor when present at higher concentrations (Figure 5.5-8). At low concentrations, enzymatic activity recovered when CgrAlcOx was incubated with inhibitor for 10 minutes, and the enzyme was irreversibly inhibited after 10 minutes of incubation. This might be because CgrAlcOx converted...
a small amount of BuSH in the solution within 10 minutes. Incubation of the enzyme with a large amount of BuSH resulted in only irreversible inhibition because of the excess inhibitor in the solution. By combining HPLC-UV, the HRP-ABTS assay, and $^1$H-NMR analysis, we were able to confirm that BuSH was acting as both a substrate and an inhibitor towards CgrAlcOx.

![Graph](image)

**Figure 5.5-8 Study of irreversible inhibition by BuSH against CgrAlcOx with the HRP-ABTS assay**
Chapter 6: Conclusions and Future Work

6.1 Conclusions

To conclude, all three objectives mentioned in the introduction were successfully addressed in this thesis. To reduce the cost of enzyme characterization, CeO$_2$ nanoparticles were studied as an option to replace the HRP-ABTS assay. CeO$_2$ nanoparticles, however, were not found to be a suitable for this purpose. The results presented in Chapter 2 show that the sensitivity of the nanoparticle approach was much lower than the HRP-ABTS assay. The unstable baseline and relaxation of the nanoparticle would also increase the error associated with quantifying enzymatic activity.

To address the limitations of the initial rate assay, different instruments, such as ReactIR, NMR spectroscopy, and HPLC-UV – which are based on direct reaction monitoring – were studied in Chapter 3. Due to the significant interference caused by the solvent and proteins in the reaction, ReactIR was not a suitable technique for monitoring the enzymatic reaction. In addition, although the NMR approach was able to monitor the enzymatic reaction continuously at high concentrations of substrate, it was not suitable for performing kinetics because of its poor instrumental detection limit. In contrast, HPLC coupled with a UV detector was found to be the most applicable instrument for directly monitoring starting material and product in the enzymatic reaction. Information about the percentage conversion and the stoichiometric ratio of the reaction were extracted from the obtained reaction progress curves. In addition, this technique was also used to determine the stereochemistry of glyceraldehyde, which was the product of glycerol oxidation catalyzed by $Cgr$AlcOx.
The alcohol oxidase was studied in detail, and kinetics were performed using the HPLC approach in Chapter 4. Different tests were done to confirm that \textit{CgrAlcOx}-catalyzed alcohol oxidation proceeded through a pseudo first order reaction. In the process of optimizing reaction conditions, it was found that H$_2$O$_2$ was produced as a by-product in the reaction; therefore, scavenger proteins (catalase and HRP) were required to maximize the efficiency of the alcohol oxidase. This increase in activity was studied using the HPLC approach, which showed that \textit{CgrAlcOx} was activated by intermediates generated in a reaction containing either HRP and catalase, or HRP and an electron donor compound. Moreover, different means of introducing the second substrate, O$_2$, were investigated using the HPLC approach, and it was found that supplementing the reaction with H$_2$O$_2$ prevented conversion rate limitation by saturating the reaction with sufficient O$_2$. Kinetics studies of \textit{CgrAlcOx} were performed using optimized conditions, and the parameters obtained were almost identical to those obtained using the initial rate assay.

In Chapter 5, a new application of the HPLC approach – a competition experiment – was developed to characterize new substrates. A proof of concept competition reaction between cinnamyl alcohol and benzyl alcohol was performed to demonstrate that this application can be used as a more efficient method for screening new substrates. Benzylamine was identified as a substrate of \textit{CgrAlcOx}, but its conversion efficiency was poor. Moreover, competition experiments were used to address product inhibition caused by aliphatic aldehydes; the initial rate assay could not address this issue. It was found that butyraldehyde was an inhibitor that caused slow inhibition.

Despite its advantages, the HPLC approach has several pitfalls, such as the requirement for experiments of long duration when it is used in inhibition studies. This limitation was addressed
in Section 5.4, in which the HPLC approach, the HRP-ABTS assay, and $^1$H NMR analysis were combined to study different inhibitors. Butylamine was identified as a non-competitive inhibitor, and benzyl mercaptan was identified as an irreversible inhibitor. Interestingly, the combined use of all three techniques allowed butanethiol to be identified as both a substrate for and an inhibitor of CgrAlcOx.

6.2 Future Work

The development of the new HPLC-UV approach to directly monitor CgrAlcOx-catalyzed alcohol oxidation has opened a new window to the in-depth study of oxidases from the AA5 family. However, the development of this new technique is in its early stages, and much more work must be done. Tandem MS could be used to verify the identity of the compound being monitored by the UV detector in HPLC. Also, at this point, the HPLC approach can only be used to directly observe UV-active compounds. Although competition experiments using BnOH can be used to study non-UV-active compounds, more research is needed to determine the best way to directly analyze the reaction behaviours of aliphatic alcohols.

Moreover, butyraldehyde was identified as an inhibitor. However, this thesis did not verify the binding site for butyraldehyde on CgrAlcOx. Co-crystallization of CgrAlcOx with butyraldehyde will be useful for identifying binding sites, which may permit the design of better inhibitors for this enzyme.

Lastly, we verified that when HRP was coupled with catalase or an electron donor compound in the reaction, intermediates were generated that activated CgrAlcOx. However, we did not verify
the identity of these intermediates or the mechanism of activation. More research is required to solve this puzzle, which may eliminate the dependence of alcohol oxidase activation on HRP.
Bibliography


(43) Zhang, F.; Chen, C.-H.; Raitano, J. M.; Hanson, J. C.; Caliebe, W. A.; Khalid, S.; Chan, S.-W. Phase Stability in Ceria-Zirconia Binary Oxide Nanoparticles: The Effect of the Ce3+...


Appendices

Appendix A  CgrAlcOx Production and Activity Determination

A.1  CgrAlcOx Production and Optimization

CgrAlcOx has been produced by following previous reported protocol\(^\text{18}\). To increase the yield, different ratios of BMGY and BMMY media were tested, and it was found that the yield was slightly higher when 80% volume of BMMY compare to BMGY was used (Table 1.2-1). Aeration of the media during the production was very important in producing protein, so a beveled flask and a foam plug was used in the production (2.03 mg CgrAlcOx/250 mL BMMY media) rather than covering the flask with aluminum foil (0.293 mg CgrAlcOx/250 mL BMMY media).

Table A.1 Different ratio of BMGY and BMMY media in CgrAlcOx production

<table>
<thead>
<tr>
<th>BMGY (mL)</th>
<th>BMMY (mL)</th>
<th>Protein Yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>500</td>
<td>2.49</td>
</tr>
<tr>
<td>250</td>
<td>250</td>
<td>2.58</td>
</tr>
<tr>
<td>250</td>
<td>200</td>
<td>2.84</td>
</tr>
<tr>
<td>250</td>
<td>125</td>
<td>1.98</td>
</tr>
</tbody>
</table>

A.2  Purification and Deglycosylation of CgrAlcOx

CgrAlcOx was purified according to the previous reported protocol by using a nickel affinity chromatography (HisTrap Column) and size exclusion chromatography (SEC)\(^\text{18}\). Purity of CgrAlcOx was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE), and two close bands were observed at the desired molecular weight. It was suspected that both bands were desired protein with different degree of glycosylation, thus the deglycosylation of was performed on CgrAlcOx using Protein Deglycosylation Mix II kit.

The deglycosylation was performed using Protein Deglycosylation Mix II kit from Biolabs® Inc. 9 μL CgrAlcOx (1.32 mg/ml) was added with 1 μL of 10x glycoprotein denaturing buffer and this solution was incubated at 80 °C for 10 minutes. The heated sample was added with 2 μL of 10x GlucoBuffer 2, 2 μL 10% NP-40, 4 μL H₂O and 2 μL PN-Gase F to make a total volume of 20 μL, and this mixture was incubated at 37 °C for 2 hours. Then, 4.2 μL of incubated reaction mixture was added with 2 μL 6x gel loading dye and 5.78 μL H₂O. The sample containing dye was heated at 80 °C for 10 minutes. After the incubation, the mixture was loaded in to SDS-PAGE gel to check its composition. A lane containing only CgrAlcOx was used as a control. After deglycosylation, the two close bands became one located at around 63 kDa, and the band located at 35 kDa was the PN-Gase F. This result confirmed that the desired enzyme was produced, and it was pure.

![Figure 6.2 SDS-PAGE gel of A. Band at 57 kDa is deglycosylated CgrAlcOx, and band located at 35kDa is the PN-Gase F. B. Purified CgrAlcOx](image)

Figure 6.2 SDS-PAGE gel of A. Band at 57 kDa is deglycosylated CgrAlcOx, and band located at 35kDa is the PN-Gase F. B. Purified CgrAlcOx
A.3 *CgrAlcOx* Activity Determination using HRP-ABTS Assay

The activity of *CgrAlcOx* was tested by using HRP-ABTS coupled assay monitoring by a UV-vis spectrometer at 415 nm. The ABTS was acting as an electron donor in the process of HRP breaking down H$_2$O$_2$, and the generated radical species, ABTSox, was monitored at $\lambda_{max} = 414$ nm ($\epsilon = 36,000$ M$^{-1}$cm$^{-1}$). The assay was optimized containing 50 mM sodium phosphate at pH 8.0, 0.366 mM ABTS, 7.50 $\mu$M horseradish peroxidase, and 0.947 nM purified *CgrAlcOx*. The assay was performed at room temperature, and stock solutions, except for substrate solutions, were kept on ice. Three substrates were chosen to be tested the activity of this enzyme. n-butanol was chosen since it was the benchmark substrate when this enzyme was characterized. The enzyme has higher activity towards aromatic alcohols, so benzyl alcohol and cinnamyl alcohol were also selected. The obtained $k_{cat}/K_m$ of *CgrAlcOx* were almost consistent with the values reported previously which were summarized in Table A-2.

**Table A.2 Reproduced activity of *CgrAlcOx* with HRP-ABTS assay and compare to the previous reported values**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Produced</th>
<th>Previously reported$^{40}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>n-butanol</td>
<td>100 ± 2</td>
<td>1.23 ± 0.15</td>
</tr>
<tr>
<td>benzyl alcohol</td>
<td>126 ± 1</td>
<td>1.39 ± 0.08</td>
</tr>
<tr>
<td>cinnamyl alcohol</td>
<td>107 ± 1</td>
<td>0.0666 ± 0.0015</td>
</tr>
</tbody>
</table>
Figure 6.2 Michaelis-Menten plots using HRP-ABTS assay with substrates A. n-butanol, B. cinnamyl alcohol and C. benzyl alcohol.
Appendix B Methods Session

B.1 General

Chemicals and solvents were obtained from commercial sources (Aldrich, Acros, Fischer Scientific) and used as received. Catalase was purchased from Sigma Aldrich® as lyophilized powder, 2000-5000 units/mg. HRP was purchased from Bio Basic Canada Inc. as lyophilized powder Rz>3, 300 units/mg. Cerium (IV) oxide, 20% in H2O, nanoparticle dispersion, high pH was purchased from Alfa Aesar. TLC analysis was performed using a precoated silica gel 60 F254 plated. Silica gel for purification was from Silicycle with particle size of 230-400 mesh (40-63 μm). Low-resolution mass spectra (LR-MS) in electrospray ionization (ESI) mode were acquired using positive or negative ionization mode in H2O or MeCN on a Waters ZQ equipped with ESCI ion source spectrometer. Nuclear magnetic resonance spectra for product analysis were recorded in deuterated solvents. Proton nuclear magnetic resonance (1H-NMR) spectra were recorded using a 400 MHz Bruker NMR spectrometer using a standard pulse sequence. Chemical shifts for all spectra were reported in parts per million (ppm) relative to tetramethylsilane referenced to the residual solvent peak signal. NMR spectrometer used for kinetics was a 600 MHz Bruker NMR spectrometer in Prof. McIntosh’s lab.

HPLC analysis were performed on Agilent 1260 under the following conditions:

1). Poroshell 120 EC-C18, 2.1 x 50 mm, 2.7-Micron Column; Temperature = 25 °C; Solvent A = water, 0.05% TFA; solvent B = MeCN, 0.05 % TFA; Flow rate = 0.649 mL/min; gradient elution was based on different compound. 200-375 nm variable wavelength, diode array detector was used for compound detection.
2). Chiralpak® IA-3, 2.1 x 150 mm, 2.7-Micron Column; Temperature = 25 °C; Solvent A = water, 0.05% TFA; solvent B = Acetonitrile, 0.05% TFA; Flow rate = 0.650 mL/min; MeCN: water 40: 60 for 4 min.

**B.2 Enzymatic Test with CeO$_2$ Nanoparticle**

5 μL CeO$_2$ nanoparticle in a 1 mL of 50 mM sodium phosphate at pH 8.0 gave the most sensitive signal without precipitation. The absorbance of the solution was monitored at 465 nm when H$_2$O$_2$ solution was added to the nanoparticle solution. It was assumed that one molecule of H$_2$O$_2$ reacted with one molecule of cerium at 4+ states when CeO$_2$ nanoparticle was used as a catalase. Thus, the standard curve of CeO$_2$ was obtained by plotting absorbance versus concentration of H$_2$O$_2$ and this curve was fitted with a linear function.

A reaction with same condition as initial velocity assay was used as a control. The test of nanoparticle reaction was monitored by UV-vis spectrometer with continuous reading mode at 465 nm for a period of 4 minutes. Different conditions of reaction were tested: 10 mM benzyl alcohol, 0.841 nM to 8.41 nM CgrAlcOx, 7.50 μM to 75.0 μM HRP and 5 μL CeO2 nanoparticle were used to test enzymatic activity.

**B.3 ReactIR Reactions: DST Series AgX Fibre Conduit Model**

The reaction was setup in a 20 mL PTFE-lined septum fitted glass vial with a stir bar. The reaction was mixed constantly at 300 rpm. The DST series AgX fibre conduit model is equipped with a Di-Comp (diamond) probe connected by an AgX (silver halide) fibre. The probe reached in the solution about 0.5 cm above the stir bar, and it was connected to the detector, in which the detector
was configured to monitor the reaction from 800 to 3600 cm\(^{-1}\). The component of the reaction, such as substrates, 2.4 \(\mu\)M catalase, 17.5 \(\mu\)M HRP, were added to the solution in sequence, and the reaction was allowed to reach equilibrium before next component was added. The spectrum of reaction was recorded after each component was added into the solution. Once 9.47 nM \(Cgr\)AlcOx was added to the solution, the reaction was continuously recorded for about an hour.

### B.4 ReactIR Reactions: DS Micro Flow Cell Model

The setup of the flow cell was slightly differently from the conduit model. The solution was continuously flowed into the flow cell that is directly connected to the detector. The reaction was set up in a 15 mL falcon tube, and an isocratic pump was used to circulate the solution into the flow cell at 1 mL/min. The method of recording spectra was as same as the conduit model.

### B.5 \(^1\)H NMR Test for Kinetics

The reaction was set up in a total volume of 500 \(\mu\)L that contains 10\% D\(_2\)O and 90\% 50 mM sodium phosphate at pH=8.0. 0.2 mM or 2 mM benzyl alcohol, 2.4 \(\mu\)M catalase, 17.5 \(\mu\)M HRP, 9.47 nM \(Cgr\)AlcOx, and 15 mM H\(_2\)O\(_2\) were used for the reaction. Bruker 600 MHz NMR spectrometer was used for high resolution and sensitivity, and a water suppression sequence was used to suppress water signal at 4.5 ppm. Each component was added to the reaction mixture in a sequential order, and the spectrum was recorded after each addition. \(Cgr\)AlcOx was added last to initiate reaction, and the time was recorded.
B.6 General preparation procedure for reactions in reaction progress analysis

The buffer was 50 mM sodium phosphate at pH = 8.0. The stock substrate solution was prepared by weighing the substrate on an analytical balance into a 100 mL volumetric flask and filled with buffer. Different concentrations of substrate solutions were prepared from the stock solution before the reaction started. Catalase (30 mg/mL; 2,000-5,000 unit/mg) and HRP (35 mg/mL; 300 unit/mg) stock solutions were prepared freshly on the day of experiment. HRP was kept on ice for the entire time, but catalase stock solution was wrapped by aluminum foil and was kept at room temperature. CgrAlcOx was split into aliquots (11 mg/ml to 36 mg/ml) after purification and was kept in -80 °C freezer. The aliquot was fast thawed over room temperature water on the day of experiment, then it was diluted down to approximately 0.5 mg/ml and was kept on ice. 15.40 mL H$_2$O$_2$ solution (Fischer, 30% w/v) was transferred by a serological pipette to a 100 mL volumetric flask and filled up with buffer. H$_2$O$_2$ solution was kept on ice at all time. Each delivery of 10 μL of this H$_2$O$_2$ solution to a 1 mL reaction mixture would give 15 mM H$_2$O$_2$. For kinetics, 2.4 μM catalase, 17.5 μM HRP, 9.47 nM CgrAlcOx, and 15 mM H$_2$O$_2$ were used in the reaction.

B.7 Procedure for HPLC Analysis

A sequence of sampling was created before the reaction was started. Depending on the nature of the experiment, it was set to sample the reaction between 10 and 40 times. The injection volume was dependent on the concentration of the compound in the reaction (below 0.1 mM: 5 μL; 0.1-1.5 mM: 3 μL; 1.5-2 mM: 2 μL; above 2 mM: 0.3 to 0.5 μL). The reaction was set up in a 1.8 mL clear HPLC vial with blue slit PTFE/Sil cap. CgrAlcOx was added to initiate the reaction, and the time of addition was recorded. 10 μL of H$_2$O$_2$ stock solution was added last to saturate the reaction.
with enough molecular oxygen. The HPLC vial was left in the sample tray during the entire acquisition.

Before analyzing reaction online, a method was created to separate substrate, oxidized product and potential by-product (acid form of substrate) on the EC-C18 column. The elution method was created as following:

A) For separation between benzyl alcohol, benzaldehyde, and benzoic acid:

starting conditions = 87.5% A, 20% B; 0.1-10 min, 100% B. Start from AcCN: 12.5%, gradient elution 0-100% in 3.5 minutes, and the stop time at 2 minutes with post time 1.5 minutes. UV detection: benzyl alcohol @ 210 nm; benzaldehyde @ 250 nm; benzoic acid @ 230nm. Benzyl alcohol eluted at 0.7 min, benzoic acid at 1.1 min, and benzaldehyde at 1.65 min.

B) For separation of cinnamyl alcohol, cinnamyl aldehyde, and cinnamic acid:

starting conditions = 87.5% A, 20% B; 0.1-10 min, 100% B. Start from AcCN: 21.5%, gradient elution 0-100% in 6 minutes, and the stop time at 2.5 minutes with post time 1.5 minutes. UV detection: cinnamyl alcohol @ 230nm; cinnamyl aldehyde @ 250 nm; cinnamic acid @ 270 nm. Cinnamyl aldehyde elutes at 2.5 min, cinnamic acid at 2.8 min, and cinnamyl aldehyde at 3.2 min.

The HPLC peak areas of both starting materials and products were collected, and the areas were converted to concentrations of compounds by using calibration curves. A time course was correlated with concentrations of compounds to track the change of the peak area – the reaction curves of compounds were obtained. This curve was fitted with an exponential function, $y = y_0 + \ldots$
\(Ae^{R_0x}\), in Origin\(^\circledast\). The derivative of this curve, \(y' = AR_0e^{R_0x}\), was calculated to obtain slope at a specific time on the reaction curve. By taking \(t = 0\) minute, the initial velocity of the reaction was calculated. This velocity was then used for Michaelis-Menten Kinetics plot for this particular concentration of the substrate.

For dosing experiments, a highly concentrated stock solution of the compound of interest was prepared, and only 10 \(\mu\)L of that stock solution was delivered to the reaction so that the volume of the reaction was not changing much.

### B.8 Calibration Curves Generated by HPLC

Three stock solutions of each compound were prepared by weighing the compounds into a 100 mL volumetric flask on analytical balance, and 50 mM sodium phosphate at pH = 8.0 was added to volumetric flask. The stock solution then was used to prepare nine different concentration solutions. Each concentration was prepared three times, and each solution was sampled with three different injection volumes (0.5-5 \(\mu\)L) on HPLC. The peak areas for each concentration were collected and averaged, and standard deviations of each concentration were also calculated. These parameters were used to plot a linear calibration curves.

### B.9 Glycerol Oxidation and Analysis on HPLC-UV

0.445 mmole glycerol was dissolved in 3 mL of 50 mM sodium phosphate at pH 8.0. 3.00 mM catalase, 1.75 mM HRP, and 37.8 \(\mu\)M CgrAlcOx, and 15 mM \(H_2O_2\) were added into the glycerol solution, and the reaction was shaken at 200 rpm on a platform shaker overnight. On the second day, the solution was centrifuged in a 30 mL 30 kDa Centricon\(^\circledast\) for 2 hours to separate reaction
mixture from proteins, and the filtrate was collected into a round bottom flask. 1.08 mmole 2,4-dinitrophenyl hydrazine was added into this flask, and the reaction mixture was incubated in the oil bath at 50 °C for 5-8 hours under continuous stirring. TLC plate was used to check formation of desired product (Rf = 0.45 100% EtOAc). The reaction mixture was vacuum filtered to get rid of excess non-dissolved 2,4-dinitrophenyl hydrazine, and the solvent of the filtrate was removed by using rotary evaporator. The crude was purified by flash chromatography with dry loading and elution of 9:1 EtOAc:Hexane. The purified compound was dried under vacuum pump overnight, and the identity of the compound was confirmed by using mass spectroscopy and 1H-NMR. The composition of the purified glyceraldehyde was analyzed on HPLC (10 μL injection, Chiracel® IA-3).

L-glyceraldehyde and D/L-glyceraldehyde were purchased from Sigma Aldrich®. These compounds were incubated with 2,4-dinitrophenyl hydrazine to make glyceraldehyde DNP hydrazones by using procedure listed above. These compounds were analyzed by HPLC, the obtained spectrum was used as standards, in which L-glyceraldehyde DNP hydrazone was eluted at 1.96 min, and D-glyceraldehyde DNP hydrazone was eluted at 1.56 min.

B.10 Enzymatic Reaction Bubbling with O₂

2.00 μM catalase, 15.0 μM HRP, 9.47 nM CgrAlcOx, and 1 mM benzyl alcohol were added into an 8 mL reaction in a 20 mL glass vial. The reaction was stirred on the stir plate at 300 rpm. An air balloon filled with pure O₂ gas was reached into the reaction mixture with a 22’ needle. Once the reaction was initiated with CgrAlcOx, it was continuously bubbled with O₂ gas. 500 μL of reaction mixture was taken from the glass vial and transferred to a clean 2 mL HPLC vial before
each injection in the HPLC. The reaction was continuously sampled by this method until there was no more conversion of substrate observed.

**B.11 Substrate/inhibition Screening Experiment**

The reaction condition was as same as kinetics. The solution was set to contain 1 mM BnOH and 1 mM of either a new substrate or an inhibitor. If this compound is an amine, the pH was adjusted by using 500 mM NaH₂PO₄ back to 8.00. The competition reaction was continuously sampled for 20 to 40 times, and the reaction curve of benzaldehyde was monitored.

If the compound is an inhibitor, a concentration-dependent experiment was performed containing 1 mM BnOH and different amounts of inhibitors. The reaction was continuously sampled for over 20 times, and the reaction curves of benzaldehyde were analyzed.

**B.12 Irreversible Inhibition Study**

*CgrAlcOx* stock was prepared at 5 mg/ml, and 10 μL of this stock solution was incubated with different amounts of inhibitors (1 to 5 mM) for different amount of time (up to 80 minutes). Once the incubation period is over, the alcohol oxidase was recovered by diluting the protein 1000-fold. This diluted protein was used to convert 10 mM benzyl alcohol by HRP-ABTS assay, and initial rates were obtained. Even though *CgrAlcOx* was recovered right after incubation with inhibitors, the activity was lowered than non-incubated enzyme. A normalization was done on the activity using non-incubated enzymatic activity by a calculated factor. This calculated factor was used to normalize all the activities after the enzyme was incubated with different concentration of inhibitors with different duration. These rates were collected and plotted against time of incubation.
The plot was fitted with an exponential decay function $y = y_0 + Ae^{-\frac{x}{\tau_1}}$, in which the $k_{app}$ can be calculated as $\frac{1}{\tau_1}$. $k_{app}$ was plotted against concentration of inhibitors, and the curve was fitted with a Michaelis-Menten Kinetics plot in Origin®, where obtained $V_{max}$ in the plot was $k_2$, and obtained $K_m$ was $K_i$ for the inhibitor.

**B.13 Duration of Inhibition Experiments using HPLC-UV**

Table B.13-1 Total duration of butyraldehyde reversible inhibition experiment. Duration of every reaction containing BnOH and butyraldehyde was 25 minutes.

<table>
<thead>
<tr>
<th>BnOH (mM)</th>
<th>0 mM [butyraldehyde]</th>
<th>0.65 mM [butyraldehyde]</th>
<th>3.74 mM [butyraldehyde]</th>
<th>7.51 mM [butyraldehyde]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>1</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Subtotal number of reactions: 6 8 8 8

Subtotal time (min): 150 200 200 200 750

Total duration of experiment (min): 750
Table B.13-2 Total duration of BnSH irreversible inhibition experiment. Total duration of experiment did not include incubation time between BnSH and CgrAlcOx.

<table>
<thead>
<tr>
<th>Duration of Incubation of CgrAlcOx with different amounts of BnSH (min)</th>
<th>Time required to sample reaction of 10 mM BnOH catalyzed by CgrAlcOx incubated with different amount of BnSH</th>
<th>Total Duration of experiment (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM BnSH (min)</td>
<td>1 mM BnSH (min)</td>
<td>3 mM BnSH (min)</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>20</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>50</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>75</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Subtotal</td>
<td>90</td>
<td>150</td>
</tr>
</tbody>
</table>

B.14 $^1$H NMR Test for Product and Reaction Analysis

The reaction was set with 10 mL of 50 mM sodium phosphate at pH=8.0, 10-20 mg chemical compound of interest. 9.37 nM -15.0 mM CgrAlcOx, 2.4 μM to 1.3 mM Catalase, 17.5 μM to 57.2 mM HRP, and 15 mM H$_2$O$_2$ were added to the reaction, and the reaction mixture was incubated on a rotatory shaker at 41 rpm for overnight. The reaction was extracted with 2 mL deuterium chloroform, and it was centrifuged at 4500 rpm for 30 seconds for better separation. The organic layer was collected and dried over anhydrous Na$_2$SO$_4$. The dried organic layer was transferred to NMR tube and analyzed using $^1$H-NMR.
Appendix C Calibration Curves for HPLC Analysis

Figure 6.2 Calibration curve of A. cinnamyl alcohol, B. cinnamyl aldehyde.

Figure 6.2 Calibration curve of A. benzyl alcohol, B. benaldehyde.
Appendix D $^1$H-NMR Spectra

D.1 Glyceraldehyde DNP Hydrazone MS and $^1$H-NMR

Figure 6.2-1 ESI-MS of glyceraldehyde DNP hydrazone after purification

Figure 6.2-2 $^1$H-NMR spectrum of glyceraldehyde DNP hydrazone after purification
$^1$H NMR (400 MHz, CD$_3$Cl): $\delta$= 11.2 (s, 1H, NH), 9.15 (s, 10H, H$^6$), 8.45 (d, 1H, H$^4$), 7.98 (d, 1H, H$^5$), 7.62 (d, 1H, H$^3$), 4.55 (t, 1H, H$^2$), 4.00 and 3.85 (dd, 2H, H$^1$).

D.2 2-phenyl-1-propanol and 2-phenylpropanal

$^1$H NMR (400 MHz, CD$_3$Cl): $\delta$ = 9.69 (d, 1H, H$^7*$), 7.51-7.18 (m, 10H, H$^{1-5}$ and H$^{11-5*}$), 3.72 (d, 2H, H$^7$), 3.65 (q, 1H, H$^{6*}$), 2.98 (h, 1H, H$^6$), 1.46 (d, 3H, H$^{8*}$), 1.27 (d, 3H, H$^8$).

![Figure 6.2 $^1$H-NMR spectrum of oxidation of 2-phenyl-1-propanol after quick extraction of deuterated chloroform.](image)
D.3  2-phenylethanol and phenylacetaldehyde

\[ \text{H NMR (400 MHz, CD}_3\text{Cl): } \delta = 10.0 (t, 1H, H^7), 8.20-7.10 (m, 10H, H^{1-5} \text{ and } H^{1*-5*}), 3.88 \text{ (t, 2H, H}^6), 3.69 \text{ (d, 1H, H}^6*), 2.88 \text{ (t, 2H, H}^7). \]

Figure 6.2 $^1$H-NMR spectrum of oxidation of 2-phenylethanol after quick extraction of deuterated chloroform.
D.4 3-phenyl-1-propanol and 3-phenylpropionaldehyde

\[ \text{\textsuperscript{1}H NMR (400 MHz, CD}_3\text{Cl): } \delta = 9.83 \text{ (t, 1H, H}^8\text{*)}, 7.35-7.15 \text{ (m, 10H, H}^{1-5}\text{ and H}^{1*-5*}\text{), 3.69 (t, 2H, H}^6\text{)}, 2.97 \text{ (t, 2H, H}^7\text{*)}, 2.79 \text{ (t, 2H, H}^6\text{*)}, 2.72 \text{ (t, 2H, H}^8\text{)}, 1.91 \text{ (p, 2H, H}^7\text{).} \]

Figure 6.2 \textsuperscript{1}H-NMR spectrum of oxidation of 3-phenyl-1-propanol after quick extraction of deuterated chloroform.

D.5 3-benzyloxy-1-propanol and 3-benzyloxypropionaldehyde
\(^1\)H NMR (400 MHz, CD\(_3\)Cl): \(\delta=9.80\) (t, 1H, H\(^9\)), 7.39-7.26 (m, 10H, H\(^1\)-\(^5\) and H\(^1\*)-\(^5\*)\), 4.50 (s, 4H, H\(^6\) and H\(^6\*)\), 3.82 (t, 2H, H\(^8\)), 3.78 (t, 2H, H\(^7\)), 2.79 (t, 2H, H\(^14\)), 3.67 (t, 2H, H\(^9\)), 2.70 (t, 2H, H\(^7\)), 1.88 (p, 2H, H\(^8\)).

Figure 6.2 \(^1\)H-NMR spectrum of oxidation of 3-phenyl-1-propanol after quick extraction of deuterated chloroform.

D.6 2-(benzyloxy)ethanol and benzyloxyacetaldehyde

\(^1\)H NMR (400 MHz, CD\(_3\)Cl): \(\delta=9.74\) (t, 1H, H\(^8\)), 7.54-7.20 (m, 10H, H\(^1\)-\(^5\) and H\(^1\*)-\(^5\*)\), 5.30 (t, 2H, H\(^7\)), 4.60 (s, 4H, H\(^6\) and H\(^6\*)\), 3.82 (t, 2H, H\(^7\)), 3.61 (t, 2H, H\(^8\)).
Figure 6.2 $^1$H-NMR spectrum of oxidation of 3-phenyl-1-propanol after quick extraction of deuterated chloroform.