Mechanism of Substrate Specificity and Catalysis in Retaining β-Glucosidases
From Maize and Sorghum

By
Muzaffer Cicek

Dissertation submitted to the faculty of the
Virginia Polytechnic Institute and State University
In Partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
In
Biology
(Cellular and Molecular Biology)

Asim Esen, Chair
Eric Beers
Charles L. Rutherford
Brenda Winkel-Shirley
Richard Walker

September 7th, 1999
Blacksburg, Virginia
Copyright 1999, Muzaffer Cicek
Mechanism of Substrate Specificity and Catalysis in Retaining β-Glucosidases from Maize and Sorghum

Muzaffer Cicek

(ABSTRACT)

β-glucosidases catalyze the hydrolysis of aryl and alkyl β-D-glucosides as well as glucosides with a carbohydrate moiety. The maize β-glucosidase isozymes Glu1 and Glu2 hydrolyze a broad spectrum of substrates in addition to its natural substrate DIMBOAGlc, while the sorghum β-glucosidase Dhr1 (dhurrinase-1) hydrolyzes exclusively its natural substrate dhurrin. For the expression of mature β-glucosidase isozymes Glu1 and Glu2 of maize and Dhr1 of sorghum, complementary DNAs were amplified by PCR and cloned into the expression vector pET-21a. Recombinant Glu1, Glu2 and Dhr1 enzymes were found to display activity towards the physiological substrates DIMBOAGlc and dhurrin, respectively, at levels similar to their native counterparts. It has been a subject of the subsequent studies by our lab and others to investigate what determines the aglycone specificity in β-glucosidases, and how β-glucosidases catalyze the hydrolysis of β-glycosidic bond between sugar and aglycone moieties. Molecular modeling techniques allowed to predict the substrate binding sites in Glu1 and Dhr1. Based on structural analysis of Glu1 and Dhr1, chimeric β-glucosidases (Glu1/Dhr1) were constructed by shuffling the C-terminal amino acids of Glu1 with the homologous region of Dhr1 to study the mechanism of substrate specificity. The resulting chimeric enzymes were characterized with respect to substrate specificity as well as kinetic, immunological, and electrophoretic properties. Shuffling a small portion of the C-terminal region altered the substrate specificity and improved by 2-4 fold the catalytic efficiency on other substrates in the chimeric β-glucosidases. These experiments showed that one or more of the 10 amino acid substitutions in the 30 amino acid long Dhr1 subdomain, 462SSGYTERFGIVYVDRENGCERTMKRSARWL491, plays a key role in dhurrin recognition and hydrolysis. To further investigate dhurrin recognition within this peptide region, two chimeric enzymes containing 462SSGYTERF469 and 466FAGTERY473 Dhr1 peptides, respectively, were generated. The kinetic parameters indicated that Dhr1 peptide,
462SSGYTERF469, alone is sufficient to convert Glu1 to Dhr1 substrate specificity when it replaces the homologous peptide, 466FAGFTERY473, of Glu1.

Maize β-glucosidases share high sequence similarities with Family 1 O-glucosidases. Therefore, these proteins are classified as retaining glycosyl hydrolases whose active site contains two glutamic acids (E) as the key catalytic residues, one as a general acid/base catalyst (E191) and the other as a nucleophile (E406). To confirm the identity and function of the acid/base catalyst E191, we have changed this residue to isosteric glutamine (Q) and aspartic acid (D) in both Glu1 and Glu2 isozymes by site-directed mutagenesis. The resulting mutant proteins were purified and their kinetic parameters (K_m, k_cat and k_i) were determined. The replacement of the acid/base catalyst E191 in the active site of maize β-glucosidase by Q and D resulted in inactivation of the enzyme. The kinetic analysis of the E191Q mutants showed that catalytic activity was reduced 200- and 110-fold towards ortho- and para-nitrophenyl-β-D-glucosides, respectively, when compared with the wild type enzyme. The E191D mutants showed no detectable activity towards any of the substrates tested. The back mutation of the E191Q mutants of the Glu1 and Glu2 isozymes to wild type restored full catalytic activity in both cases. These data indicate that E191 in maize β-glucosidases functions as an acid/base catalyst, and its function in catalysis cannot be performed by an isosteric residue such as glutamine or by a carboxyl group on a shorter side chain such as in aspartic acid.
Dedication

I dedicate this dissertation to my wife Mine and my son Hakan
Acknowledgements

I would especially like to express my sincere gratitude and appreciation to Dr. Esen for giving me the opportunity to work in his laboratory. His valuable advice allowed me to gain more knowledge. I am truly grateful for the support, encouragement, guidance, and friendship he has provided. I like to thank the members of my Graduate Advisory Committee, Dr. Brenda Winkel-Shirley, Dr. Charles L. Rutherford, Dr. Richard Walker and Dr. Eric Beers for their valuable and helpful suggestions through my research program.

Special thanks to Dr. David Bevan, Department of Biochemistry, for his guidance and help in computer modeling of monocot β-glucosidases.

Many thanks to the Department of Biology, in particular to Dr Joe Cowles, for supporting me monetarily during my years in Graduate School. I thank to Sue Rasmussen for all her assistance.

My research was enhanced and made pleasurable by the inspiration, warmth, friendship and good humor of my laboratory mates Sukanda Vichitphan and David Blanchard. Their great friendship and help will never be forgotten.

Most importantly, I would like to thank my beloved wife, Mine, whose endless love, assistance, understanding, and encouragement have made my Ph.D. education possible. “Thank you for having confidence in me”. Lastly, I would like to express my deep and sincere love for my parents, Makbule and Mirza Cicek, my sister Leyla Turuk and my brother Zafer Cicek, whose support and encouragement have made my education possible.
Table of Contents

Chapter 1. Literature Review ......................................................... 1
Enzymes-The Catalytic Machinery of Life ........................................... 2
Microbial ß-Glucosidases ........................................................... 3
Mammalian ß-Glucosidases ......................................................... 4
Plant ß-Glucosidases ............................................................... 5
The Mechanism of Catalysis ....................................................... 12
Structure and Substrate Specificity of ß-glucosidases ......................... 17
Project Goal ................................................................. 20
Literature Cited ............................................................... 23

Chapter 2. Expression of Soluble and Catalytically Active Plant (Monocot) ß-Glucosidases in E.coli
Title Page ................................................................. 36
Abstract ................................................................. 37
Introduction ................................................................. 38
Materials and Methods ...................................................... 39
Results ................................................................. 44
Discussion ................................................................. 47
Literature Cited ............................................................... 56

Chapter 3. Changing the specificity of DIMBOA-glucosidase (maize ß-glucosidase) to that of dhurrinase (sorghum ß-glucosidase) by domain swapping within the C-terminal region
Title Page ................................................................. 59
Abstract ................................................................. 60
Introduction ................................................................. 61
Material Methods ........................................................ 64
Results ................................................................. 69
List of Figures

Chapter 1.
Figure 1. Cyanogenesis in sorghum ......................................................... 7
Figure 2. Proposed mechanism for hydrolysis of glycosidic bond .............. 16
Figure 3. Sequence alignment in family 1 β-glucosidases ......................... 13
Figure 4. 3D ribbon representations of plant β-glucosidases....................... 19

Chapter 2.
Figure 1. Deduced amino acid sequence identity among mature maize (MzGlu1 and MzGlu2), sorghum (SorGlu) and oat (OatGlu) β-glucosidases ...................... 40
Figure 2. Diagram of maize glu1 and glu2 cDNA expression constructs .......... 45
Figure 3. SDS-PAGE profiles of cell lysates from E. coli .......................... 49
Figure 4. Native PAGE (6%) gel zymograms of native maize β-glucosidase isozymes and of their recombinant counterparts ........................................... 50
Figure 5. TLC chromatogram showing the activity and substrate specificity of two maize β-glucosidase isozymes ....................................................... 51

Chapter 3.
Figure 1. Hydrolysis of natural substrates by sorghum and maize β-glucosidases .... 63
Figure 2. Diagrammatic representation of structures of wild type parental Glu1 and Dhr1 and five Glu1/Dhr1 chimeric β-glucosidases ........................................... 65
Figure 3. SDS-PAGE profiles of parental β-glucosidases Glu1 and Dhr1 and their chimeric forms ................................................................. 71
Figure 4. TLC chromatograms showing the reaction products .................... 72
Figure 5. Active sites of modeled Glu1 and Dhr1 ..................................... 79

Chapter 4.
Figure 1. Proposed catalytic mechanism for a retaining β-glucosidase .......... 92
Figure 2. Sequence alignment in family 1 β-glycosidases ......................... 93
Figure 3. SDS-PAGE profiles of the total (T) and soluble (S) protein fractions from *E.coli*, and purified wild type glucosidase.

Figure 4. A zymogram developed with the fluorogenic substrate 4MUGlc (4-methylumbelliferyl-β-D-glucoside).

Figure 5. TLC chromatogram showing the activity of wild type Glu1, E191Q and E191Q→D mutants towards the physiological substrate DIMBOAGlc.

Figure 6. pH dependence of specific activity for native Glu1 versus E191Q.

Figure 7. Time dependent activity enhancement.

Figure 8. A modeled view of the active site of wild type, E191Q and E191D mutant β-glucosidases.
List of Tables

Chapter 2.
Table 1. Comparison of activities of the recombinant (rGlu1 and rGlu2) maize β-glucosidase isozymes with their native (MzGlu1 and MzGlu2) counterparts………………………… 48
Table 2. Comparison of kinetic parameters of the recombinant (rGlu1 and rGlu2) maize β-glucosidase isozymes with their native (MzGlu1 and MzGlu2) counterparts…………… 48

Chapter 3.
Table 1. Oligonucleotide primer pairs (S, sense; A, antisense) used to generate chimeric β-glucosidase cDNAs…………………………………………………………………… 68
Table 2. Comparison of the kinetic parameters of parental (Glu1 and Dhr1) and chimeric (Glu1/Dhr1) β-glucosidases………………………………………………………………… 75
Table 3. Comparison of the kinetic parameters of parental (Glu1 and Dhr1) and chimeric (Glu1/Dhr1) β-glucosidases for the natural substrates dhurrin and DIMBOAGlc…… 76

Chapter 4.
Table 1. Kinetic parameters of \(\text{para/or}t\text{o}\)-nitrophenyl-β-D-glycopyranosides with rGlu1 and rGlu1 E191Q enzymes……………………………………………………………………………… 104
Table 2. Inhibition of β-glucosidase activity by \(p\)-nitro-phenyl-thio-Glc at pH 5.8 105