Chapter 1
Literature Review

Enzymes-The Catalytic Machinery of Life

Life depends on a complex network of chemical reactions catalyzed by specific enzymes, and any modification of the enzyme pattern may have drastic consequences for a living organism. The mechanisms by which enzymes catalyze chemical reactions are in itself one of the most fascinating fields of scientific investigation being pursued at the present time. Thus, enzymes, as catalysts, have received increasing attention from many scientific fields, especially biochemistry, physical chemistry, microbiology, genetics, botany and agriculture, pharmacology and toxicology, medicine, and chemical engineering.

In general, our understanding of the mechanisms and physiological functions of enzymes is still quite limited. This is probably due to the complex structure and biological and physical instability of these macromolecules. Biochemical studies on enzymes provide the basis for understanding how enzymes function and how the catalytic sites react with substrates. Enzymes are key components in every metabolic pathway that occurs in the cell. Thus, investigating enzymes provides valuable information about the catalytic mechanism and function in metabolism. Catalysis is intimately related to the molecular interactions that take place between a substrate and a specific part of the enzyme molecule. Today, spectroscopic methods, x-ray crystallography, and more recently, multidimensional NMR methods, provide a wealth of structural insights on which theories of enzyme mechanisms can be built. However, fundamental questions still remain regarding the detailed mechanisms of enzyme activity and its relationship to enzyme structure.

ß-glucosidases (ß-D-glucoside glucohydrolases; EC 3.2.1.21) occur ubiquitously in plants, fungi, mammals and microorganisms (Woodward and Wiseman, 1982). ß-glucosidases have been the subject of much recent research due to the key role these enzymes play in biological processes and potential biotechnological applications. Among them, plant ß-glucosidases play role in defense against pests (Bell. 1981; Niemeyer, 1988; Poulton 1990), phytohormone activation (Brzobohaty et al., 1990; Matsuzaki et al., 1986, Schliemann, 1984; Smith and van Staden, 1978; Wiese and Grambow, 1986), lignification (Dharmawardhana, 1995), and cell wall catabolism (Leah et al, 1995; Simos et al., 1994).

ß-glycosidases have been classified into one of the 57 families of glycosidases (Henrisat and Romeu, 1995; Henrissat 1996). In Henrissat's classification, ß-glucosidases are classified as Family 1 glycosidases based on amino acid sequence similarity and substrate specificity. A remarkable feature of the enzymes belonging to this family is a wide range of substrate preferences despite high sequence homology. These enzymes display broad

substrate specificity with respect to the aglycone portion of the substrate. Many studies have provided insight into the substrate specificity of ß-glucosidases isolated from different organisms.

Microbial ß-Glucosidases

Microbial ß-glucosidases have been the subject of extensive research. One of the earliest bacterial ß-glucosidases was purified from *Agrobacterium fecalis.* The purified enzyme was characterized and the corresponding gene was identified and expressed in *E. coli* (Day and Withers, 1986; Wakarchuk et al., 1986). The *Agrobacterium* enzyme is a dimer of 50 kD monomers and shows high specificity for cellobiose. The enzyme has been well-characterized with respect to the residues at the catalytic center and the mechanism of catalysis (Trimbur et al, 1992).

The cellulolytic fungal ß-glucosidases have also been the subject of numerous investigations by various research groups. The fungal enzymes are used in several biotechnological processes, including development of novel carbohydrate foods, alcohol-based fuels and other commercial products from cellulose. Particularly, glucose production can be achieved from the most abundant biological macromolecule, cellulose, by the extracellular enzyme complex (cellulase) that is derived from various fungal species such as *Trichoderma.* The cellulase complex isolated from *Trichoderma reseei* comprises at least three different enzymes that together hydrolyze cellulose to oligosaccharides and glucose (Fowler, 1993). Of these, the endoglucanases and cellobiohydrolases synergistically hydrolyze cellulose into small cellooligosaccharides, mainly cellobiose. Subsequently, cellobiose is hydrolyzed to glucose by ß-glucosidase. The ß-glucosidase gene *bgl1* from *Trichoderma reseei* was cloned and sequenced by Barnet et al. (1991). Later, Fowler (1993) studied ß-glucosidase from the null strain of *Trichoderma reesei* to explore its role in the cellulase enzyme system. The hydrolysis of cellulose and induction of other cellulolytic enzyme components led Fowler to the conclusion that extracellular ß-glucosidase is required for the induction of the other cellulase enzymes.

Another application of microbial ß-glucosidases is in the flavor improvement industry. ß-glucosidases are key enzymes in the release of aromatic compounds from glucosidic precursors present in fruits and fermentating products (Gueguen et al., 1996). Indeed, many natural flavor compounds, such as monoterpenols, C-13 norisoprenoids, and skimate-derived

compounds, accumulate in fruits as flavorless precursors linked to mono- or diglycosides and require liberation by enzymatic or acidic hydrolysis (Vasserot et al., 1995; Winterhalter et al., 1997). Microbial ß-glucosidases can also be used to improve the organoleptic properties of citrus fruit juices, in which the bitterness is in part due to a glucosidic compound, naringin (4',5,7-trihydroxyflavanone-7-rhamnoglucoside), that is hydrolyzed by a α-rhamnosidase and a ß-glucosidase (Roitner et al., 1984). Monoterpenols in grapes (e.g., linalol, geraniol, nerol, citronelol, a-terpineol and linalol oxide) are linked to diglucosides, which contribute to the flavor of wine (Gunata et al., 1985). The enzymatic hydrolysis of these compounds requires a sequential reaction, which produce monoglucosides. Subsequently, monoglucosides are hydrolyzed by the action of ß-glucosidases. Endogeneous ß-glucosidases from grape are not sufficient to process the hydrolysis of monoterpenyl-glucosides. The grape enzymes display limited activity towards these glucosides and a large fraction of the aromatic compounds remains unprocessed in mature fruit. The addition of glucose-tolerant exogenous ß-glucosidase isolated from fungi (e.g. *Aspergillus oryzae*) was shown to improve the hydrolysis of glucoconjugated aromatic compounds and enhance wine quality (Riou et al., 1998). In conclusion, microbial ß-glucosidases have been well-characterized and many of them have been used in biotechnological applications.

Mammalian ß-Glucosidases

Two different ß-glucosidases have been characterized in mammalian systems: cytosolic and lysosomal ß-glucosidases (glucocerobrosidase) (Beutler, 1992). Cytosolic ß-glucosidases are mainly present in the liver, kidney and intestines (Gopolan et al., 1992). Gopolan et al. (1992) suggested that cytosolic ß-glucosidases play a key role in detoxification of plant ß-glucosides. This was based on the finding that the enzyme displays a broad specificity towards aglycone moieties of mono and disaccharide substrates such as L-picein, salicin, arbutin, amygdalin, prunasin, visine and linamarin that are found in plants consumed by animals (Gopolan et al., 1992). Additional evidence is that cytosolic ß-glucosidases are present in significant quantities in the liver and intestine, which are special organs containing detoxification enzymes.

Under normal conditions, the substrate glucosylceramide (glucocerebroside) is cleaved to ceramide and glucose by lysosomal glucocerobrosidase (EC 3.2.1.45) (Beutler, 1992). This enzyme is membrane associated and weakly glycosylated. Lysosomal ß-glucosidase is

present in most tissues and cell types with various levels of catalytic activity. A deficiency of the lysosomal ß-glucosidase in humans results in a condition known as Gaucher disease. The enzyme has 497 amino acids and contains 4 N-linked glycans. Seven different mutations in human ß-glucosidase have been shown to result in inactive enzymes. A landmark experiment with human ß-glucosidase was performed by Ohhassi et al. (1992). These investigators were able to transduce and express the human ß-glucosidase (glucocerobrosidase) gene in mouse hematopoietic stem cells using a recombinant retroviral vector. This accomplishment points out the feasibility of using gene therapy for treating Gaucher disease. Three phenotypes of Gaucher's disease are differentiated on the basis of clinical severity, age of onset and neuronal involvement: type 1 (adult form), type 2 (infantile form) and type 3 (juvenile form) (Beutler and Grabowski, 1995). It has been reported that lysosomal ß-glucosidase is active not only on the natural lipid substrate glycosylceramide, but also on a number of artificial substrates such as 4MUG (4-methylumbellifery-ß-D-glucopyronaside) and *p*NPG (para-nitrophenyl-ß-D-glucopyronaside). The enzyme shows optimum activity around pH 4.5-5.0 and it is irreversibly inhibited by conduritol ß-epoxide (Grabowski et al., 1985). It is also activated by phosphatidylserine in combination with the sphingolipid activator protein 2 (SAP-2) (Basu et al., 1984).

The existence of a second ß-glucosidase, which is a non-lysosomal tightly membrane-bound glucocerebrosidase, has been reported (Van Weely et al., 1993). Like the lysosomal enzyme, non-lysosomal glucocerebrosidase hydrolyzes glucosylceramide and 4MUG, but it differs from the lysosomal enzyme in several ways. Particularly, this enzyme is strongly membrane-bound, and is not localized in the lysosome. Additionally it shows a more neutral optimum pH and is not inhibited by conduritol ß-epoxide. It has also been reported that it is not deficient in Gaucher disease patients (Van Weely et al., 1993).

Plant ß-Glucosidases

In plants, ß-glucosidases play an important role in defense against some pathogens and herbivores by releasing hydroxamic acids, coumarins, thiocyanates, terpenes, and cyanide from the corresponding glucosides (Fenwick et al., 1983; Niemeyer, 1988; Hruska, 1988; Jones, 1988; Poulton, 1990 and Oxtoby, 1991). Plant ß-glucosidases also function in the hydrolysis of conjugated phytohormones (i.e., glucosides of giberellins, auxins, abscisic acid, and cytokinins) (Nowachi et al., 1980; Schliemann, 1984; Wiese and Grambow, 1986;

Brzobohaty et al., 1993).

One of the best understood functions of ß-glucosidases in plants is the hydrolysis of the cyanogenic glucosides (cyanogenesis). In this case, the enzyme and substrate occur in different subcellular compartments and come into contact with each other only after tissue disruption by pathogens or herbivores. As a result, the cyanogenic glucoside is hydrolyzed, releasing toxic hydrogen cyanide (HCN) (Conn, 1981; Jones, 1988; Poulton, 1988 and Oxtoby, 1991).

In most higher plants, cyanogenesis is the catabolism of the cyanogenic glucosides. Many economically-important crops, including sorghum, cassava, lima beans and cherries accumulate cyanogenic glucosides. Hydrolysis of these compounds produces the respiratory poison HCN in a 2-step process (Conn, 1981; Poulton, 1993). First, the catabolism of the cyanogenic glucosides by ß-glucosidase results in the accumulation of glucose and a hydroxynitrile. Second, hydroxynitrile is subsequently broken down to ketone and HCN by a hydroxynitrile lyase (Fig 1).

In white clover (*Trifolium repens* L), two related cyanogenic glucosides, linamarin and lotaustralin are the substrate of linamarase (ß-glucosidase), which is a glycosylated protein having high-mannose-type N-asparagin-linked oligosaccharides. The hydrolysis of these substrates results in products, glucose and HCN (Hughes, 1993). Linamarases are localized the cell walls of the epidermal cells of leaves (Kakes, 1985; Kakes, 1993). Another cyanogenic linamarase from cassava has been studied in detail by various researchers (Hughes, 1982; Mkpong et al., 1990; Nok et al., 1990; Yeoh et al., 1992; Hughes, 1993). The Cassava plant is a staple food in most African countries. It is a highly cyanogenic plant and causes acute cyanide poisoning in humans (Poulton, 1989).

Cyanide poisoning from high-cyanogenic casava plant is typically associated with insufficient consumption of Cys and Met in diet. Reduced sulfur-containing compounds are the substrate for the detoxification of cyanide catalyzed by the enzyme ß-cyanoalanine synthase (White and et al., 1998). Hughes (1993) studied the localization and expression of cyanogenic ß-glucosidase in cassava and showed that linamarase in cassava was synthesized in the latex vessels of young leaves. Then they are transported to the latex vessels (Pancoro and Hughes, 1992) of the petiole and possibly to the vessels of the stem and roots.

Another well-characterized dicot ß-glucosidase (myrosinase) occurs in the plant family *Brassicaceae.* Myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) catalyses the hydrolysis of secondary compounds, glucosinolates (thioglucosides), which consist of a glucose residue with a sulfur- and nitrogen-containing side chain. Glucosinolates are found

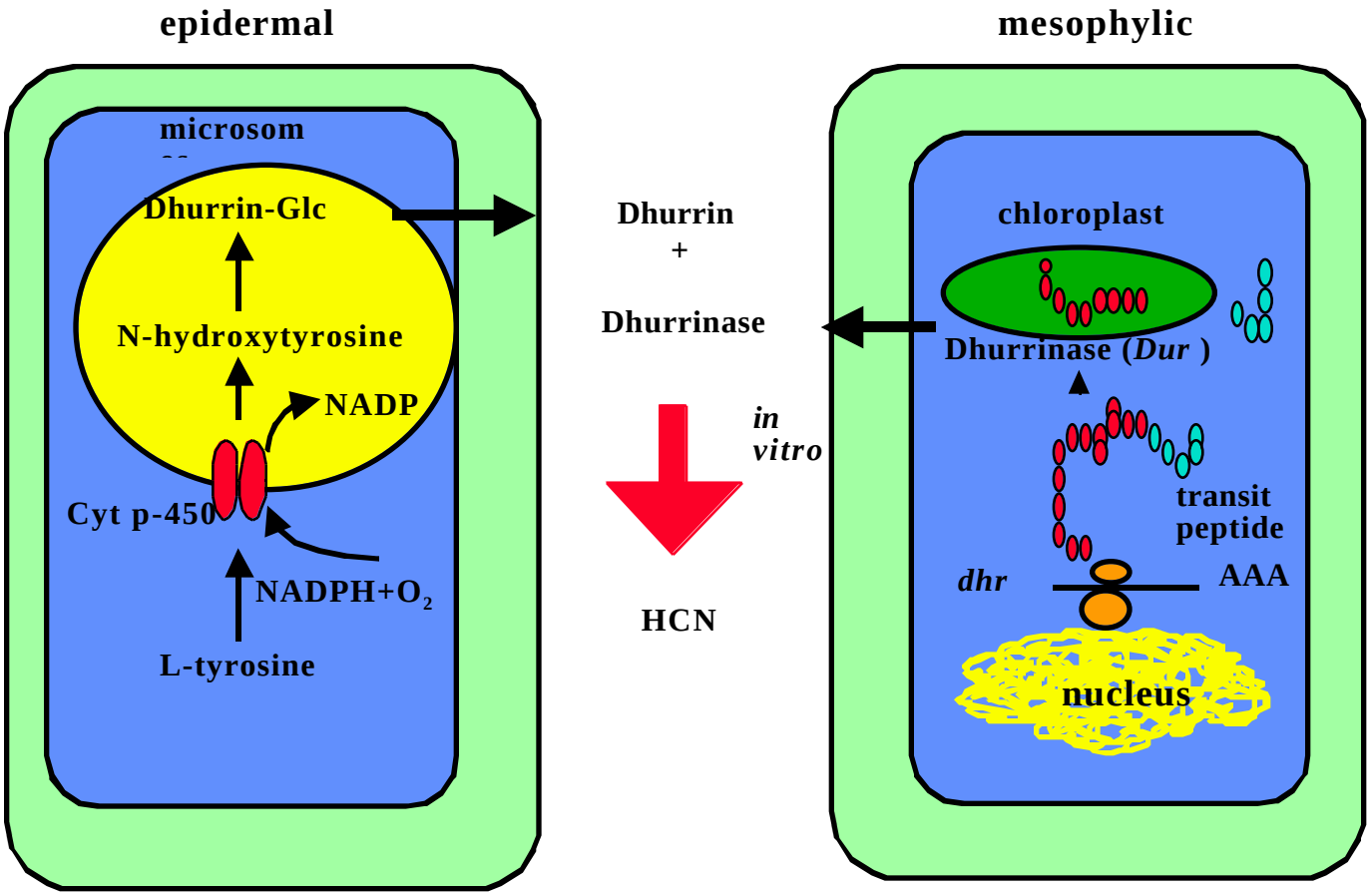

Fig. 1. Cyanogenesis in sorghum (*S. bicolor* (L.) Moench)

mainly, but not exclusively, in crucifers, including oilseed rape (*Brassica napus*) (Bennett and et al., 1995). In oilseed rape there are three structural classes of glucosinolates, derived from phenylalanine (aromatic), methionine (aliphatic/alkenyl) and tryptophan (indolyl). Glucosinolates are stored in the vacuoles of plants and when the tissue is disrupted they are converted by myrosinase to toxic compounds such as isothiocyanates, thiocyanates, and nitriles. The resulting byproducts of the reaction cause thyroid and liver disease in animals and humans who consume glucosinolate-rich foods such as rape (*Brassica napus*). This enzyme-substrate system is implicated as a defense system against herbivorous insects, slugs, and fungal pathogens as well as being involved in host-plant recognition by specialized insect pests of oilseed rape (Fenwick et al., 1983).

Myrosinase has been found in leaves, stems, and roots of glucosinolate-containing plants. It was originally thought that the enzyme was localized in particular idioblasts (myrosin cells) of parenchymatous tissue. However, recent studies have revealed that myrosinase and its substrate, sinigrin, are subcellularly localized in aleuron cells (Kell et al., 1998). The same investigators localized the myrosinase to myrosin cells.

A variety of forms of myrosinase have been identified in a large number of plants. These isozymes may display different properties with respect to substrate specificity. In general, myrosinases are activated by ascorbic acid although the extent of this activation is variable. Ohtsuru and Hata (1979) have studied the interaction of myrosinase with ascorbic acid in detail. They concluded that ascorbic acid is not itself involved in catalysis, but changes the conformation of the active site. West et al. (1977) also performed a similar study. These investigators worked on properties of myrosinase in leafy tissue of cabbage. They observed that myrosinase was activated by ascorbic acid. Myrosinase from *Raphanus sativus* was isolated and characterized with its physiological substrate sinigrin in the presence of ascorbic acid (Shikata et al., 1999). Ascorbic acid was found to activate sinigrin hydrolysis linearly.

Recent studies showed that the glucosinolates and myrosinase-generated byproducts can be exploited as potential targets for pharmaceutical applications. Epidemiological and pharmacological studies have shown that consuming vegetables containing glucosinolates can reduce colorectal cancer development. Some glucosinolates and myrosinase-generated isothiocyanates-derived products have been shown to exhibit antiproliferative activity in colorectal cancer (Leoni et al., 1997). Shapiro et al., (1998) found that many isothiocyanates inhibit the neoplastic effect of various carcinogens at a number of organ sites. Therefore, these compounds are attracting attention as potential chemoprotectors against cancer.

Cyanogenic ß-glucosides are known as defense compounds against some pathogens

and herbivores after being hydrolyzed by ß-glucosidases (Jones, 1988; Poulton, 1990; Kakes, 1993). Most cyanogenic glucosides are derived from five hydrophobic amino acids (tyrosine, phenylalanine, valine, leucine and isoleucine). In sorghum, the major natural substrate of ß-glucosidase is the cyanogenic ß-glucoside dhurrin (ß-D-glucopyranosyl-oxy-(S)-p-hydroxymandelonitrile). The pathway of dhurrin biosynthesis is elucidated in sorghum seedlings (Halkier et al.1989). In sorghum seedlings, dhurrin is derived from L-tyrosine (Halkier et al., 1989). In the graminae, L-tyrosine is synthesized via shikimate, which is a key substance in the synthesis of plant secondary compounds including lignin, flavonoids, anthocyanins, cumarins and phenolic acids (Conn, 1994). The shikimate pathway is also known as the common aromatic biosynthetic pathway. In this metabolic sequence, primary metabolites, phospho pyruvate and erythrose-4-phosphate, are converted to chorismate, which is a precursor for the aromatic amino acids Phe, Tyr, and Trp (Herrmann, 1995). Recently, Sibbesen et al. (1995) found that cytochrome P-450, a hemethiolate enzyme, catalyzes the committed step (N-hydroxylation of L-tyrosine to N-hydroxytyrosine) in the biosynthesis of dhurrin in *Sorghum bicolor* (L.) Moench. The shoots of young sorghum plants contain large amounts (up to 75 mMol/g fresh wt) of the cyanogenic glucoside dhurrin. Enzymatic hydrolysis of dhurrin occurs after tissue disruption in sorghum (Conn, 1994).

Hosel et al. (1987) purified two dhurrinases (Dhurrinase 1 and Dhurrinase 2) from sorghum seedling. Dhurrinase 1 occurs in shoots of seedlings grown in darkness; in contrast, dhurrinase 2 occurs in the green shoots and leaves of young seedlings grown in the light (Hosel et al., 1987; Cicek and Esen, 1998). Sorghum and maize ß-glucosidases share a number of characteristics such as subunit molecular weight (60 kD), acidic pH (6.2 and 5.8 respectively), pI (5.5) (Hosel et al., 1987; Esen, 1992) and plastid localization (Kojima et al., 1979; Thayer and Conn, 1981; Esen and Stetler, 1993). However, the enzymes exhibit subtle differences with respect to substrate specificity.

In maize, two ß-glucosidases, homologs of dhurrinases in sorghum, were characterized with respect to stability and substrate specificity. These enzymes hydrolyze a broad spectrum of aryl ß-glucosides, in addition to the abundant physiological substrate DIMBOA-glucoside (2-O-ß-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one) (Babcock and Esen, 1994). On the other hand, dhurrinase 1 only hydrolyzes dhurrin, having little or no activity towards the natural and artificial substrates that maize and many others ß-glucosidases readily hydrolyze.

The cDNAs for sorghum dhurrinase 1 and dhurrinase 2 have been cloned and characterized by Cicek and Esen (1995) and Vichithpan and et al. (unpublished data),

respectively. The full-length *dhr1* and *dhr2* cDNAs each code for 565 and 571 amino acid precursor proteins, respectively. The Dhr1 and Dhr2 precursor proteins have 51 and 54 amino-acid plastid targeting transit peptides, respectively, and share 75% amino acid identity. These enzymes are expressed in different organs; Dhr1 is expressed in the root tip, mesocotyl, node and coleoptile, while Dhr2 is expressed in nodes and leaves (Cicek and Esen, 1998). The two isozymes also differ from each other with respect to substrate specificity in that Dhr1 hydrolyzes only the natural substrate dhurrin with high catalytic efficiency. On the other hand, Dhr2 hydrolyzes dhurrin as well as the artificial substrates *p*NPG, *o*NPG and 4MUG. The Dhr1 and Dhr2 cDNAs were subcloned into an expression vector and expressed in *E.coli*. The resulting expressed proteins were catalytically active towards natural substrates and artificial substrates (Cicek and Esen 1999; Cicek et al., unpublished data, Vichithpan et al., unpublished data).

ß-glucosidases (Glu1 and Glu2) from maize have also been cloned and characterized with respect to substrate specificity and physiological function (Esen and Cokmus, 1991; Esen 1992; Shahid and Esen, unpublished). Cuevas et al. (1992) showed that maize ß-glucosidase cleaves hydroxamic acid glucosides (HxGlc), particularly 2-O-ß-*D*--glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOAGlc). The aglycone DIMBOA is present as a glucoconjugate (DIMBOAGlc) in intact tissues of maize, rye and wheat. DIMBOA is a potent, toxic aglycone that has been shown to inhibit the electron transport system and phosphorylation reactions in bovine mitochondria (Niemeyer et al., 1986) and spinach chloroplasts (Querolo et al., 1983). It has also been shown that HxGlc content of some Gramineae (e.g., maize and wheat) increases when the plant is subjected to pathogen attack (Gutierrez et al., 1988; Niemeyer et al., 1989; Thackray et al., 1988). The work in our laboratory has demonstrated that one (Glu2) of these enzymes is expressed exclusively in leaves starting at day 6 to 7 while the other (Glu1) is expressed in all plant parts (Shahid and Esen, unpublished). The cDNAs corresponding to these enzymes were cloned, sequenced and shown to have 88% sequence identity.

The purification and characterization of the maize ß-glucosidase isozyme Glu1 have been reported (Esen, 1992). The enzyme is an autodimer made up of 60 kD monomers; it has the highest catalytic activity at pH 5.8 and at 50^{o}C; and its pI is 5.2. The enzyme is an abundant protein in maize seedling parts, as well as in actively growing portions of the mature plant. Substrate specificity studies have shown that maize ß-glucosidase (Glu1) readily hydrolyzes a variety of aryl ß-glucosides, thus showing broad specificity for the aglycone moiety of the substrate, but it does not hydrolyze glycosyl glucosides like cellobiose and

gentiobiose (Babcock and Esen, 1994). In this respect, it is similar to rubber tree and white clover ß-glucosidases (Pocsi et al., 1989; Selmar et al., 1987). The same study also showed that maize ß-glucosidase was inhibited by various aglycones and ß-glucosides. However, monosaccharide glycones (e.g., glucose and fructose) were poor inhibitors of maize ß-glucosidase (Babcock and Esen, 1994).

cDNAs for maize ß-glucosidase Glu1 have been cloned and characterized by Brzobohaty et al. (1993) and Esen and Shahid (1995, direct submission). Similarly, a cDNA encoding another maize ß-glucosidase (i.e., Glu2) has been sequenced (Bandaranayake and Esen, 1996) and the corresponding enzyme was purified and characterized in our laboratory (Shahid et al., unpublished data). The full-length *glu1* and *glu2* cDNAs each code for a 566 and 563 amino acid-long precursor protein, respectively. The Glu1 and Glu2 precursor proteins have 54 and 51-amino acid-long transit peptides, respectively, for plastid targeting. Thus, both mature proteins are 512-amino acids long. Even though there is a 90% sequence identity between the Glu1 and Glu2 isozymes, they have different spatial and temporal regulation in that Glu1 is expressed in all seedling parts whereas Glu2 is expressed in green leaves. The two isozymes also differ from each other with respect to substrate specificity in that the Glu1 isoform hydrolyzes various artificial aryl ß-glucosides with high catalytic efficiency while the Glu2 isoform hydrolyzes the same substrates poorly or not all.

In conclusion, plant ß-glucosidases are known to function in chemical defense of young plant parts against pests by catalyzing the hydrolysis of toxic glucosides. It is also known that all ß-glucosidases isolated from grasses (e.g. maize, sorghum, oat, rice, ginger and indigo) are localized in the plastid (Thayer and Conn, 1981; Esen and Stetler, 1993; Inoue et al., 1996). In contrast, all ß-glucosidases isolated from dicots, including *Trifolium repens* (Kakes, 1985), black cherry (Poulton and Li, 1994) and *Brassica napus* (Thangstad et al., 1991; Hoglund et al., 1992), are localized in the cell wall or vacuole (protein bodies). In addition, monocot ß-glucosidases are not glycosylated while dicot ß-glucosidases are glycoproteins. Based on the evidence, which shows significant differences between monocot and dicot ß-glucosidases, the crucial question is how monocot and dicot ß-glucosidases have evolved and acquired differences in terms of physiological substrates, posttranslational modification, targeting, and function.

The Mechanism of Catalysis

All family 1 ß-glucosidases share a general mechanism for the hydrolysis of the ß-glycosidic linkage between an anomeric carbon and glycosidic oxygen. There are two steriochemically-different hydrolytic mechanisms proposed for cleavage of the beta linkage between the glycone and aglycone parts of a ß-glucoside. These mechanisms differ with respect to retention and inversion at the anomeric center of the reduced sugar residue. The ß-glucosidases, together with most cellulases and xylanases, are known to hydrolyze the substrate while retaining the anomeric configuration of the sugar moiety (Sinnot, 1990; Clarke et al., 1993). The retaining mechanism involves acidic catalysis that protonates the substrate and leads to formation of a transition state oxocarbonium ion. The orientation of the catalytic group is thought to be complementary to anomeric configuration of sugar moiety. The acidic site (i.e. the carboxyl group) interacts with the site where glycosidic oxygen is found (Legler, 1990). At the end of the cleavage, the anomeric carbon of the monosaccharide reacts with a water molecule to generate ß-D-glucopyranose. This mechanism closely resembles the double displacement mechanism proposed for lysozyme (Koshland, 1953; Kempton and Withers, 1992; Clarke et. al., 1993) (Fig. 2). The inverting mechanism of the anomeric configuration is based on single displacement by a nucleophilic water molecule (Koshland, 1953). The difference between retention and inversion of the anomeric configuration is that only one transition state occurs in the inversion step of the single displacement mechanism. The final product in the inverting mechanism is α-D-glucopyranoside (Sinnot, 1990). Withers and Street (1989) proposed the double displacement mechanism for ß-glucosidase catalysis based on Nuclear Magnetic Resonance (NMR) studies. They substituted the hydroxyl group at C-2 of the sugar by electronegative fluorine to destabilize the adjacent positive charge at the transition state. This resulted in decreased rates of glycosyl-enzyme formation and hydrolysis. They also used a reactive leaving group, dinitrophenolate, incorporated into the aglycone group so that the reaction could be followed spectrometrically. This incorporation brought about considerable acceleration of glycosyl-enzyme formation without affecting the rate of glycosyl enzyme hydrolysis. In contrast, the 2-glycoside-fluorine resulted in retardation of deglycosylation step of the hydrolysis. The glycosyl enzyme intermediate was trapped easily in a transition state, thereby allowing the mechanism of ß-glucosidase catalysis to be examined. Withers and Street (1989) also suggested that a covalent glycosyl enzyme intermediate formed during the formation of oxocarbonium ion in the transition state.

The formation of the enzyme-substrate complex in ß-glucosidase-catalyzed reactions is

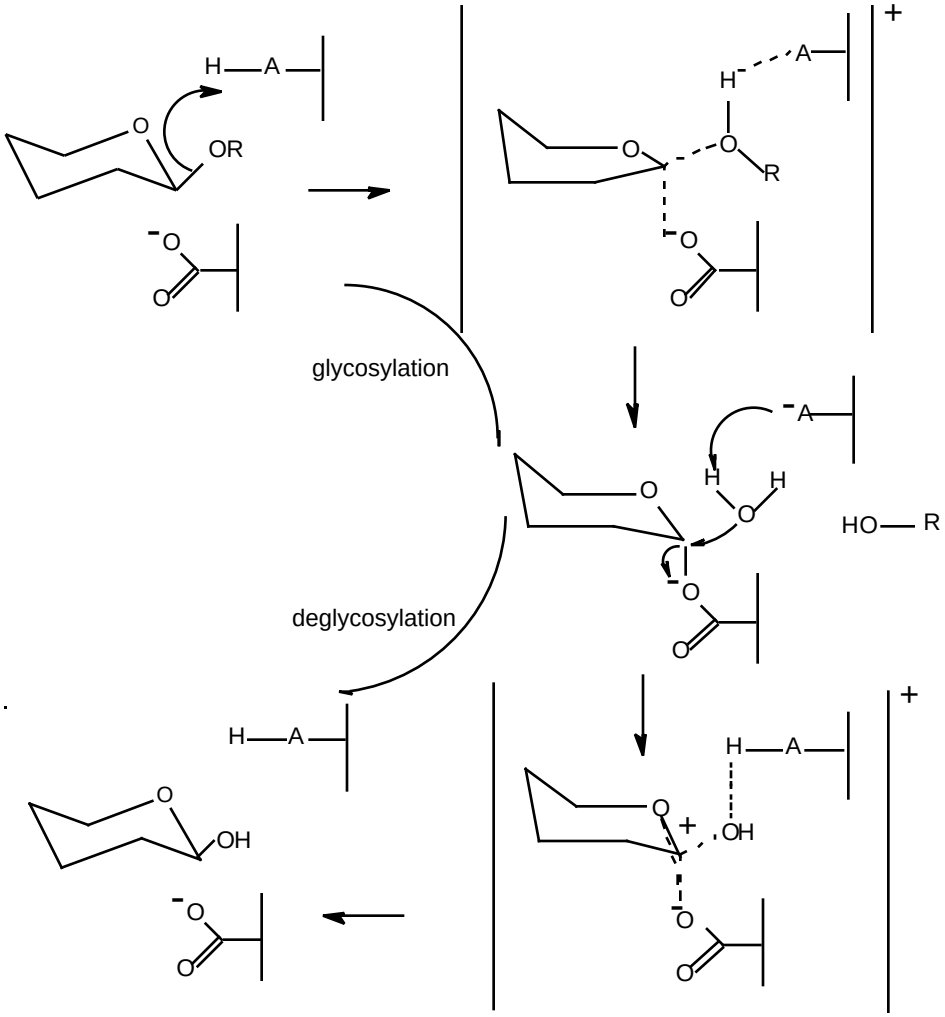

Fig. 2. Proposed mechanism for hydrolysis of ß-glycosidic bond by the “retaining” ß-glycosidases (Wang and Withers, 1995).

not well understood. Two pathways are proposed for the transition state of ß-glucosidase in the double displacement mechanism: endocyclic and exocyclic pathways. Both pathways require two amino acid residues in the active site, one serving as a proton donor and the other as a nucleophile. In the case of the exocyclic pathway, the carboxylic acid of the amino acid protonates the exocyclic oxygen (the glycosidic oxygen) of the substrate to make it a good leaving group. This gives rise to an unfavorable electronic geometry. The alternative pathway, endocyclic, involves the protonation of the endocyclic oxygen in the formation of the enzyme-substrate complex (Clarke et al., 1993). However, Withers and Street (1988) have proposed that these pathways may not be mutually exclusive and that ß-glycosidases may act via either the endo or exocyclic pathway depending on the substrate. After the transition state has been reached, ß-glucosidases hydrolyze the substrate while the configuration of the transition state is maintained.

In retaining and inverting mechanisms, at least two carboxyl groups are thought to participate in the catalysis of glycosyl hydrolases. In inverting enzymes, these residues show an average distance of 9.3 Å. However, in retaining enzymes, these two residues are separated, on average, by 5.0 Å. In the retaining mechanism, the acidic group (glutamic acid) in the active site donates a proton to glycosidic oxygen and a nucleophilic group facilitates the bond breaking by attacking to C1 of glucose. Of these, the acid-base catalyst glutamic acid is in the motif TFNEP while the nucleophilic glutamic acid is in the motif I/VTENG (Fig. 3). The hydrolysis of the ß-glycosidic bond releases the aglycone part. Next, a water molecule provides a proton to a base catalyst (glutamic acid) and the OH^- group to the covalent bond between the glycone and the enzyme, releasing the glycone and regenerating the nucleophilic glutamic acid.

The active-site nucleophile has been identified using 2'-4'-dinitrophenyl-2-deoxy-2-fluoro-ß-D-glucopyronaside, which is a mechanism-based inhibitor of the enzyme (Withers et al., 1990; Lawson et al., 1996). As mentioned above, these investigators used 2-deoxy-2-fluoro glucosides with a reactive leaving group (dinitrophenolate) to trap the transition stage of ß-glucosidase. They identified the sequence motif, ITENGA or its variant YITENGA, as being part of the active center. They also discovered that the glutamic acid residue of this sequence was attached to the sugar moiety. In another study, it was shown that glutamic acid 274 was in the consensus region ITELD in the active site of *Cellulomonas fimi* exoglucanase. The ITELD region is functionally similar to the YITENGA region of ß-glucosidases and ß-galactosidases (Tull and Withers, 1991).

Stephen Withers and his colleagues have proven the identity and the function of the

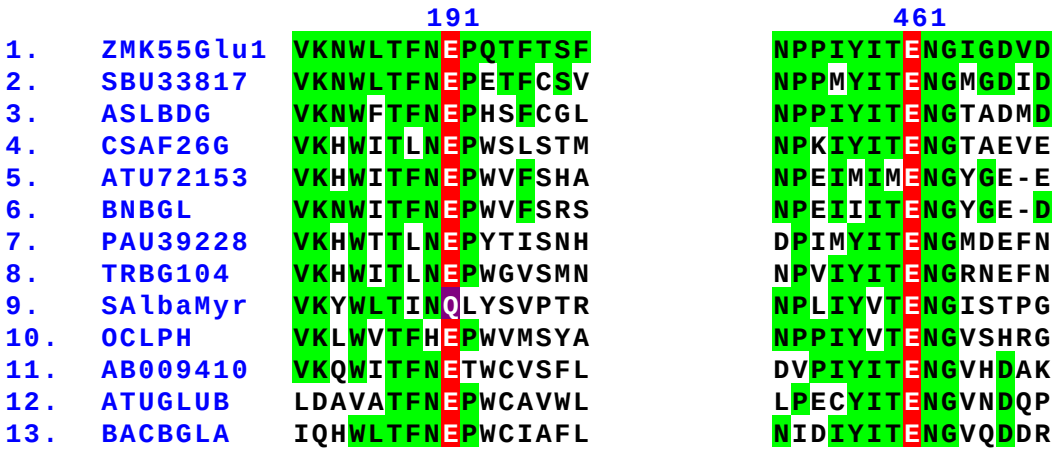

Fig.3. Sequence alignment in Family 1 ß-glycosidases. Only the acid/base catalyst and the nucleophile containing portions of each polypeptide are shown. Their Genbank codes are indicated in blue. 1, *Zea mays*; 2, *Sorghum bicolor*; 3, *Avena sativa*; 4, *Coctus speciosus*; 5, *Arabidopsis thaliana*; 6, *Brassica napus*; 7, *Prunus avium*; 8, *Trifolium repens*; 9, *Sinapsis alba* (myrosinase); 10, *Rabbit* (lactose pyrolozin hydrolase); 11, *Bacillus spp.*; 12, *Agrobacterium tumafaciens*; 13, *Bacillus polymyxa*.

nucleophilic glutamic acid, utilizing the mechanism-based inhibitor 2,4-dinitrophenyl 2-fluoro-ß-D-glucose (2,4-DNP2FGlc) (Street et al., 1990; Withers, et al., 1990) and site-directed mutagenesis (Trimbur et al., 1992; Trimbur et al., 1993). The 2,4-DNP2FGlc enhances the rate of the glycosylation reaction but decreases the rate of the deglycosylation reaction, resulting in trapping of the glycosyl-enzyme intermediate. The Glu358 was identified to be the nucleophile in *Agrobacterium* ß-glucosidase (Withers, et al., 1990). Such covalent glycosyl-enzyme intermediates were also trapped in myrosinase and *Sulfolobus solfataricus* ß-glycosidase and the nucleophilic glutamic acids were identified as Glu387 (Moracci et al., 1996) and Glu409, respectively (Cottaz et al., 1996). Another mechanism-based inhibitor, salicortin, was used with in *Agrobacterium faecalis* ß-glucosidase (Zhu et al., 1998). The aglycone of salicortin produced by enzymatic hydrolysis rapidly converts *o*-quinone methide, which is subsequently captured by nucleophiles at or near the active site of the enzyme. This gives rise to the formation of modified enzymes that have reduced activity and altered substrate specificity (Zhu et al., 1998). In one major study, 43 point mutations were introduced at 22 different sites around the YITENGA region, focusing on the active site nucleophile Glu358 (Withers et al., 1992; Trimbur et al., 1992). Furthermore, Trimbur et al. (1992) generated 9 different mutations at the Glu358 position, which decreases the activity to a minimum of 10,000-fold. In addition, the replacement of Gly360 also resulted in a reduction of the enzyme activity. These results indicated that all five residues around Glu358 were found to be important for catalysis. In retaining glycosidases, Hardy and Poteete (1991) proposed that the active-site nucleophile could be replaced with cystein in T4 lysozyme. Nearly wild-type activity was observed on mutating Asp20, a putative nucleophile, to a cysteine residue. A similar study was also done on *Agrobacterium* ß-glucosidase (Lawson et al., 1998). However, replacement of the catalytic nucleophile Glu358 in *Agrobacterium* ß-glucosidase by a cysteine residue yielded an enzyme with very low activity that followed the double displacement mechanism (Lawson et al., 1998).

The other key residue in the active site serves as a general acid/base catalyst, providing protonic assistance in the first step for the departure of the leaving group and presumably also serving as the general base by deprotonating the water as it attacks the glycosyl-enzyme intermediate. The most plausible candidates for the role of acid/base catalyst can therefore be identified by searching for conserved glutamic acid and aspartic acid residues through sequence alignments of related enzymes. Site-directed mutagenesis studies established that the acid-base catalyst glutamic acid in *Agrobacterium* ß-glucosidase was Glu170 in the TFNEP motif (Wang et al., 1995; Wang and Withers, 1995) and Glu206 in the TMNEP motif

in *S. solfataricus* ß-glycosidase (Moracci et al., 1996). These results indicate that the equivalents of Glu170 and Glu206 in other organisms would also be the acid-base catalyst.

The involvement of a carboxylic acid in acid catalysis by ß-glucosidases has been determined by site-directed mutagenesis experiments. For example, the importance of the amino acid glutamic acid for catalytic activity in chicken egg white lysozyme (CEWL) was confirmed by site-directed mutagenesis (Malcolm et al., 1989). Bacterio-phage T-7 lysozyme was also shown to have a catalytic site similar to that of the CEWL lysozyme, even though the two enzymes have different amino acid sequences. The positions of Glu11 and Asp 20 in the T-7 lysozyme correspond to Glu35 and Asp52 in the catalytic site of CEWL lysozyme. This suggests that the catalytic mechanisms are for these enzymes (Anand et al., 1988; Malcolm et al., 1989).

In the case of human ß-glucosidase, the active site interacts with the fatty acid acyl-sphingosyl and glycone moieties of glycosyl ceramide substrates. Dinur et al. (1986) have used Br[^{3}H], which is an active site-directed covalent inhibitor for affinity labeling. The results showed that the Asp443 is the nucleophile participating in catalysis of human ß-glucosidase.

In conclusion, the above studies suggest that the glutamic acid in the conserved ITENG region acts as a nucleophile in catalysis by all ß-glucosidases. Aspartic acid also has been suggested as a candidate in catalysis (Wang et al., 1995; Wang and Withers, 1995; Moracci et al., 1996). Moreover, when the nucleophile glutamate was replaced with the shorter aspartate, the activity of the enzyme was reduced 1000-2000 fold. In general, the steric requirement for the amino acid residue in the active site in such reactions is critical for catalysis even though the amino acid residues have similar chemical properties (Trimbur et al., 1992). Inhibitor studies also indicate that Glu358 plays a nucleophilic role in the active site of *Agrobacterium* ß-glucosidase (Withers et al., 1990; Trimbur et al., 1992).

Structure and Substrate Specificity of ß-glucosidases

The three dimensional structures of enzymes from several glycosyl hydrolase families have been determined, together with the mechanism of glycosidic bond hydrolysis. The active-site topologies were classified into three classes regardless of whether the enzyme is inverting or retaining (Davies and Henrissat 1995). These three topologies were classified as 1) pocket or crater, 2) cleft or groove and 3) tunnel. Other enzymes in the same group showed similar folding with the same catalytic residues. Family 1 of glycosyl hyrolases is

classified under pocket-crater topology. This topology is optimal for recognition of the non-reducing saccharide extremity found in monosaccharides such as ß-glucosides, ß-galactoside, sialic acid and neuramic acid. On the other hand, these enzymes are not very efficient on fibrous substrates such as native cellulose, which does not have free chain ends.

The three dimensional structures of four family 1 enzymes have been determined. These are a phospho-ß-galactosidase from *lactoccus lactis* (Wiesmann et al., 1995), a cyanogenic ß-glucosidase from *Trifolium repens* (Barret et al., 1995), a myrosinase from *Sinapsis alba* (Burmeister et al., 1997) and very recently a glucosidase from *Bacillus polymyxa* (Sanz-Aparicio et al., 1998) (Fig 4). These enzymes certainly show a common structural folding pattern providing insight into the molecular and mechanistic basis of substrate binding sites. The 3D structure data show that all four enzymes share the same 8(ß/α) barrel topology, and a crater-like active site. These enzymes also have the same catalytic mechanism belonging to the same glycosyl hydrolase family (Family 1). The general mechanism of all Family 1 O-ß-glucosidases is similar due to their sequence and folding similarity and shows the retention of the anomeric configuration via the double-displacement mechanism proposed first by Koshland (1953). Glycosyl hydrolases have been classified into five of the 57 families of glycosidases (Henrissat, 1996). In Henrissat's classification, ß-glucosidases were classified as Family 1 glycosidases based on amino acid sequence similarity and substrate specificity. In this classification, enzymes with different substrate specificities are found in the same family, indicating an evolutionary divergence to acquire new specificities as is found in Family 1.

ß-glucosidases display broad specificity with respect to both the aglycone and the glycone moieties of their substrates. In fact, ß-glucosidases from all sources have a similar specificity for the glycone (glucose) portion of the glucoside, however they vary with respect to aglycone specificity. The cyanogenic diglycoside (R)-amygdalin (the gentiobioside of (R)-mandelonitrile) that accumulates in black cherry seeds and other stone fruits contains a disaccharide as the glycone part of the substrate (Poulton, 1993).

The natural substrates of ß-glucosidases include steroid ß-glucosides and ß-glucosyl ceramides of mammals, cyanogenic ß-glucosides and glucosinolates of plant secondary metabolism, and oligosaccharide products released from digestion of the cellulose of plant cell-walls (Clarke et al., 1993). Artificial substrates such as benzyl, nitrophenyl and methylumbelliferyl-glycosides are frequently used during the purification and characterization of glycosidases. These substrates allow investigators to easily assay enzyme velocities of hydrolysis and substrate affinities. However, qualitative and quantitative enzymatic assays on

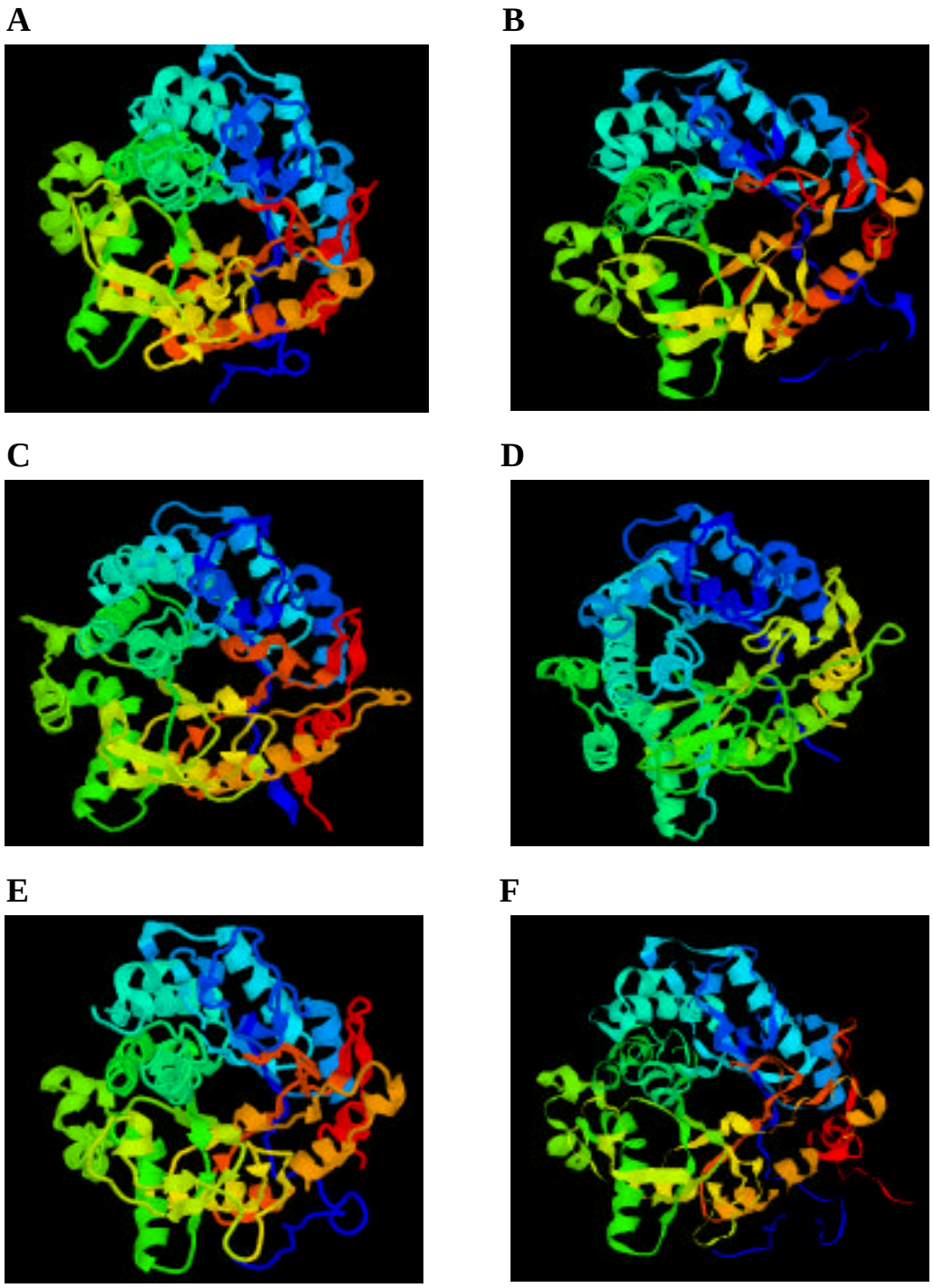

Fig. 4. Ribbon representation of white mustard myrosinase crystal structure (**A**), *Trifolium repens* linamarase (**B**), *Bacillus polymyxa* ß-glucosidase (**C**), *Lactococcus lactis* 6-phospho-ß-galactosidase (**D**) and maize ß-glucosidase and Sorghum dhurrinase 3-D structure (**E** and **F**, respectively) modeled according to the coordinates of myrosinase. Plant ß-glucosidases show overall structural similarity although their amino acid homologies showed only less than 50% sequence identity at the primary structure level.

the natural substrates are limited when compared to artificial substrates.

ß-glucosidases demonstrate high specificity towards naturally-occurring substrates. Liebig and Wohler (1837) first described the enzymatic hydrolysis of the cyanogenic glycoside amygdalin by an enzyme mixture from almond known as emulsin. In fact, there are two enzymes involved in amygdalin hydrolysis. These glycosidases, amygdalin-hydrolase (AH) and prunasin-hydrolase (PH), catalyze the reaction stepwise by removing two glucose residues from amygdalin. Two homogenous forms of amygdalin hydrolase isoforms (AH I and AH II) occur in the cherry seed. AH I removes the terminal glucose of amygdalin and forms the monoglucoside prunasin. These two isozymes also hydrolyze *p*- and *o*-nitrophenyl-ß-D-glucosides (*p*NPG and *o*NPG) at somewhat reduced rates.

In plants, there are a vast number of aglycones, e.g., plant hormones, hydroxamic acids, flavanols, thiocyanates and mandelonitrils conjugated to glucose (Smitt and van Staden 1978; Cuevas et al 1982; Niemeyer 1988; Campos et al 1993; Conn 1993). Thus ß-glucosidases may have different functions depending on the aglycone moieties of their physiological substrates. The aglycone moieties of glucosides are implicated in a number of developmental and growth-related functions in plants (Selmar et al., 1987). Hosel and Conn (1982) have emphasized the importance of the aglycone in determining the specificity of ß-glycosidases. The most common function of the aglycones is their involvement in plant defense, as in the case of cyanogenic glucosides (Poulton, 1990). In these systems, a cyanogenic glucoside is hydrolyzed by a ß-glucosidase, releasing glucose and α-hydroxynitrile. The α-hydroxynitrile is either cleaved enzymatically by hydroxynitrile lyase, or spontaneously to hydrogen cyanide and benzaldehyde (Poulton, 1990).

Project Goals

ß-glucosidases have been the subject of much study by various researchers for many years. However, two fundamental questions about ß-glucosidase-catalyzed reactions remain debatable: (1) how do ß-glucosidases catalyze the hydrolysis of the ß-glycosidic bond between two glycone residues (e.g., cellobiose and other ß-linked oligosaccharides) or that between glucose and an aryl or alkyl aglycone (e.g., many naturally occurring substrates in plants)? (2) What determines substrate specificity, including the site and mechanism of aglycone binding? The first question relates directly to the mechanism of catalysis, while the second one relates to substrate specificity, binding and positioning. Much progress has been

made in understanding the mechanism of catalysis and defining the identity and roles of specific amino acid residues (within the active site) that are involved in catalysis. However, there is virtually no information as to how ß-glucosidases recognize and interact with substrates, specifically the aglycone moiety, which is the basis of tremendous diversity in natural ß-glucosidase substrates and is responsible for subtle substrate specificity differences among ß-glucosidases.

The first objective of the work presented here is to express the mature proteins coded by *glu1*, *glu2* and *dhr1* cDNAs in a bacterial expression system (*E. coli*) in order to obtain catalytically active enzymes. The *glu1*, *glu2* and *dhr1* had been previously cloned and sequenced in our laboratory. Recombinant protein expression was carried out using the pET21-a/pLys S expression system. Recombinant proteins were purified and the kinetic parameters were compared to the wild type counterparts isolated from maize seedlings. Furthermore, substrate specificity and their electrophoretic characterizations were performed by zymograms using chromogenic (6BNGlc) and flourogenic (4MUGlc) substrates. Determination of their substrate specificity towards the physiological substrate DIMBOAGlc and the artificial substrate *p*NPG were performed by thin layer chromatography (TLC). Large-scale isolations of the natural substrates DIMBOAGlc and dhurrin were also carried out by LH-20 gel filtration following methanol extraction of maize and sorghum shoots. The purity of the natural substrates was determined by TLC and NMR. Expression of the recombinant proteins and purification of the physiological substrates allowed the comparison of the substrate specificities of these enzymes. This opened a door to identifying aglycone-binding sites in the active center.

The second main objective of this research was to generate chimeric ß-glucosidases by domain swapping among Glu1, Glu2 and Dhr1 to identify key peptide regions responsible for substrate specificity. To address the question of what determines substrate specificity, including the site and mechanism of aglycone binding, which determine the aglycone binding of substrate, we constructed chimeric cDNAs between *glu1* and *dhr1*, *glu1* and *glu2*, *glu2* and *dhr1* and expressed them in *E. coli*. Since the three parental ß-glucosidases were catalytically active in the bacterial expression system, one would expect that the chimeric ß-glucosidases could be expressed in an active form in the same system. As stated earlier, Dhr1 has unique substrate specificity because it only hydrolyzes its own physiological substrate dhurrin. Glu1 hydrolyzes its physiological substrate DIMBOAGlc as well as artificial substrates *p*NPG, *o*NPG, 4MUG and 6BNG. Dhurrin is not hydrolyzed by Glu1 and DIMBOAGlc is not hydrolyzed by Dhr1. This is therefore an ideal system to attempt to engineer Glu1 to display

the substrate specificity of Dhr1 by swapping peptide regions, which mapped at or around the active site. Therefore, we characterized the existing chimeric ß-glucosidases with respect to substrate specificity and kinetics.

The third objective of this work is based on understanding the catalytic mechanism of maize ß-glucosidases (Glu1 and Glu2). The mechanism based-studies revealed that at least two carboxyl groups participate in catalysis. In other ß-glucosidases it has been shown that an acidic group (glutamic acid) donates a proton to a glycosidic oxygen and a nucleophilic group facilitates bond breaking by attacking to C1 of glucose. Of these, the proposed acid-base catalyst is the glutamic acid in the motif TFNEP, while the nucleophilic glutamic acid is in the motif I/VTENG. Mechanism-based inhibitors and site-directed mutagenesis techniques clearly showed that a glutamic acid in I/VTENG peptide motif acts as a nucleophile, however there is no concrete evidence reported on which amino acid is the acid-base catalyst responsible for proton donation. In the present research, E191 was replaced with isosteric Q and shorter D. Resulting mutant enzymes were purified, characterized and compared with wild type ß-glucosidases.

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