Determination of the Binding Site and the Key Amino Acids on Maize β-Glucosidase Isozyme Glu1 Involved in Binding to β-Glucosidase Aggregating Factor (BGAF)

Hyun Young Yu

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Committee Chair
Asim Esen

Committee Members
David R. Bevan
Richard A. Walker
Zhaomin Yang
Farooqahmed S. Kittur

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ABSTRACT

β-Glucosidase zymograms of certain maize genotypes (nulls) do not show any activity bands after electrophoresis. We have shown that a chimeric lectin called β-glucosidase aggregating factor (BGAF) is responsible for the absence of β-glucosidase activity bands on zymograms. BGAF specifically binds to maize β-glucosidase isozymes Glu1 and Glu2 and forms large, insoluble complexes. Furthermore, we have previously shown that the N-terminal (Glu^{50}-Val^{145}) and the C-terminal (Phe^{466}-Ala^{512}) regions contain residues that make up the BGAF binding site on maize Glu1. However, sequence comparison between sorghum β-glucosidases (dhurrinases, Dhr1 and Dhr2), to which BGAF does not bind, and maize β-glucosidases, and an examination of the 3-D structure of Glu1 suggested that the BGAF binding site on Glu1 is much smaller than predicted previously. To define more precisely the BGAF binding site, we constructed additional chimeric β-glucosidases. The results showed that a region spanning 11 amino acids (Ile^{72}-Thr^{82}) on Glu1 is essential and sufficient for BGAF binding, whereas the extreme N-terminal region Ser^{1}-Thr^{29}, together with C-terminal region Phe^{466}-Ala^{512}, affects the size of Glu1-BGAF complexes. To determine the importance of each region for binding, we determined the dissociation constants (K_d) of chimeric β-glucosidase-BGAF interactions. The results demonstrated that the extreme N-terminal and C-terminal regions are important but not essential for binding. To confirm the importance of Ile^{72}-Thr^{82} on Glu1 for BGAF binding, we constructed chimeric Dhr2 (C-11, Dhr2 whose Val^{72}-Glu^{82} region was replaced with the Ile^{72}-Thr^{82} region of Glu1). C-11 binds to BGAF, indicating that the Ile^{72}-Thr^{82} region is indeed a major interaction site on Glu1 involved in BGAF binding. We also constructed mutant β-glucosidases to identify and define the contribution of individual amino acids in the

above three regions to BGAF binding. In the N-terminal region (Ile^{72}-Thr^{82}), critical region for BGAF binding, Glu1 mutants K81E and T82Y failed to bind BGAF in the gel-shift assay and their frontal affinity chromatography (FAC) profiles were essentially similar to that of sorghum β-glucosidase (dhurrinase 2, Dhr2), a non-binder, indicating that these two amino acids within Ile^{72}-Thr^{82} region are essential for BGAF binding. In the extreme N-terminal (Ser^{1}-Thr^{29}) and C-terminal (Phe^{466}-Ala^{512}) regions, N481E [substitution of asparagine-481 with glutamic acid (as in Dhr)] showed lower affinity for BGAF, whereas none of the single amino acid substitutions in the Ser^{1}-Thr^{29} region showed any effect on BGAF binding indicating that these regions play a minor role. To further confirm the importance of lysine-81 and threonine-82 for BGAF binding, we produced a number of Dhr2 mutants, and the results showed that all four unique amino acids (isoleucine-72, asparagine-75, lysine-81, and threonine-82) of Glu1 in the peptide span Ile^{72}-Thr^{82} are required to impart BGAF binding ability to Dhr2. The sequence comparison among plant β-glucosidases supports the hypothesis that BGAF binding is specific to maize β-glucosidases because only maize β-glucosidases have threonine at position 82.

DEDICATION

I dedicate this work to my entire family for all their love and support over the years. To my parent, Tae Shick Yu and Tae Sook Nam and my wife, Jee Won Lee and my son, Alex (Jae Min) Yu, and to my sisters, Ju-Kyung Yu and Hyun-Su Yu.

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1.1 General Aspects of β-Glucosidases

Plants, unlike animals, cannot flee or hide from their predators or harsh environments. Through the course of evolution, plants have developed defense systems against herbivores and other pests by collectively synthesizing natural chemical compounds and adapted to different types of abiotic environmental stresses such as drought, salt and temperature. Plant defense compounds are grouped into preformed defense compounds (phytoanticipins), which work as the first chemical barrier against herbivores or pathogens and defense compounds that are synthesized in response to herbivore or pathogen attack (phytoalexins) (Vanetten et al. 1994).

β-Glucosidases belong to the glycoside hydrolase family 1 (β-D-glucoside glucohydrolase, EC 3.2.1.21) and hydrolyze aryl and alkyl-β-D-glucosides as well as glucosides with a carbohydrate moiety (Reese 1977). They are found widely in all three (Archaea, Eubacteria, and Eukarya) domains of living organisms (Woodward and Wiseman 1982). In plants, substrates (defense compounds) for β-glucosidase are stored in a non-toxic glucosylated form to chemically stabilize and increase their solubility to make them suitable for storage in the vacuole (Jones and Vogt 2001). After cell disruption by herbivore or pathogen attack, the substrates in the vacuole (Kesselmeier and Urban 1983; Oba et al. 1981; Saunders and Conn 1978; Saunders et al. 1977) come in contact with β-glucosidases stored in plastids (Esen and Stetler 1993; Nisius 1988; Thayer and Conn 1981), cell wall or intracellular protein bodies (Frehner and Conn 1987; Kakes 1985; Oba et al. 1981; Poulton and Li 1994), and the substrates are bio-activated via hydrolysis by the enzymes. This two-component system (β-glucosidases and substrates) provides immediate chemical defense against herbivore or pathogen attack in plants (Morant et al. 2008) .

The physiological functions of plant β-glucosidases are reasonably well established. The key function of these enzymes is defense against pathogens and herbivores (Hughes et al. 1992; Phillips and Streit 1996; Poulton 1990). Although the main function of β-glucosidases in plants

is defense, but they also have a diverse array of other physiological roles in such processes as lignification (Dharmawardhana et al. 1995; Escamilla-Trevi et al. 2006; Hosel et al. 1978), floral development (Koes et al. 1994), activation and degradation of phytohormones (Kleczkowski and Schell 1995; Kristoffersen et al. 2000; Lee et al. 2006). To match this functional diversity, a typical plant (e.g., Arabidopsis, rice, poplar, grape, and others) produces more than 40 β-glucosidase isoforms encoded by a multigene family whose members are subject to temporal and spatial regulation. β-Glucosidases also have significance for biotechnological applications, e. g., aroma and flavor improvement in tea (Mizutani et al. 2002) and wine (Fia et al. 2005) and cellulose degradation (Van Remsburg et al. 1998) for biofuel production.

Microbial β-glucosidases have been well characterized and used for biotechnological applications. For example, microbial β-glucosidase is used in the flavor improvement industry because it releases aromatic compounds from glucosidic precursors present in fruits and fermentation products (Gueguen et al. 1996). Endogenous β-glucosidases in grape are not sufficient to hydrolyze monoterpenyl-glucosides and display limited activity for these glucosides, so the majority of the aromatic compounds remains unprocessed in mature fruit. However, addition of exogenous β-glucosidase from fungi (*Aspergillus oryzae*) was shown to improve the hydrolysis of glucoconjugated aromatic compounds and wine quality (Riou et al. 1998).

One of the well-characterized microbial β-glucosidases was identified and cloned from *Agrobacterium tumefaciens*. The β-glucosidase in *Agrobacterium tumefaciens* is involved in crown gall disease in conifers. In this disease, the enzyme hydrolyzes coniferin to form coniferyl alcohol and leads to a virulence cascade (Castle et al. 1992; Morris and Morris 1990). In mammals, two different β-glucosidases, cytosolic and lysosomal, have been characterized. Cytosolic β-glucosidase in the guinea pig liver catalyzes the hydrolysis of various phenolic (e.g. arbutin and salicin) and cyanogenic plant glucosides (e.g. prunasin). The plant glucosides (e.g., amygdalin, prunasin, and vicine) are transported across the small intestine of the guinea pig without being hydrolyzed, and thus cytosolic β-glucosidase appears to be functioning in biotransformation of toxic plant glucosides (Gopalan et al. 1992). In contrast, lysosomal β-glucosidase (also known as glucocerebrosidase) cleaves the substrate glucosylceramide, producing ceramide and glucose and is present in most tissues and cells with various levels of

catalytic activity. Glucocerebrosidase in humans plays a role in hydrolysis of glucosylceramide. However, some people have an inherited deficiency of the enzyme, which leads to accumulation of glucocerebroside, which in turn leads to enlargement of the liver and spleen and lesions in the bone (Beutler 1992; Sawkar et al. 2002).

1.2 The Defense Function of β-Glucosidases in Plant

The major function of β-glucosidase in plants is defense against pests. Especially, cyanogenic glucosides and benzoxazinoid glucosides are key glucosides in plants, and the mechanism of action of these glucoside-β-glucosidase systems are reasonably well understood.

Cyanogenic glucosides are synthesized from valine, isoleucine, leucine, phenylalanine, tyrosine and 2-cyclopentenyl-glycine, a non-protein amino acid (Morant et al. 2008). In sorghum, tyrosine-derived dhurrin (*p*-hydroxy-(*S*)-mandelonitrile-β-D-glucoside) is produced at up to 30% of dry weight in leaves of etiolated seedlings, and the entire dhurrin biosynthetic pathway is well elucidated (Halkier and Moller 1989; Saunders et al. 1977). Dhurrin is synthesized from amino acids, via the concerted action of two cytochromes P450, CYP79A1 and CYP71E1, and a UDPG-glucosyltransferase, UGT85B1 (Bak et al. 1998; Halkier and Moller 1990; Halkier et al. 1995; Hansen et al. 2003; Jones et al. 1999; Kahn et al. 1997; Sibbesen et al. 1994, 1995; Thorsoe et al. 2005). The biosynthetic enzymes are organized into a multienzyme complex, a metabolon, to aid channeling and avoid secretion of toxic intermediates (Jorgensen et al. 2005; Nielsen et al. 2008).

Cyanogenic β-glucosidases are found in a wide variety of cyanogenic plants such as sorghum, cassava, white clover, rubber tree (*Heves brasiliensis*), black cherry (*Prunus serotina*), and flax (*Linum ussitatissimum*) (Fan and Conn 1985; Hosel et al. 1987; Kuroki and Poulton 1986, 1987; Mkpong et al. 1990; Pocsi et al. 1989; Selmar et al. 1987). Typically, the cyanogenic glucoside and its corresponding β-glucosidase are compartmentalized. For example, in sorghum, the cyanogenic glucoside dhurrin is stored in the epidermal cell layer whereas the β-glucosidases (dhurrinases) are stored in the chloroplasts of the bundle sheath cells (Kojima et al. 1979; Thayer and Conn 1981). Upon tissue disruption, dhurrin is hydrolyzed by dhurrinases, yielding an

unstable aglycone, which is enzymatically (by α-hydroxynitrilase) degraded to *p*-hydroxybenzaldehyde and toxic HCN (Poulton 1990). HCN causes the inactivation of the mitochondrial cytochrome oxidase and blocks cellular respiration (Nelson 2006), suggesting an important natural pesticide function for dhurrin (Morant et al. 2008). In addition to defense function in plants, cyanogenic glucosides also have been proposed to play a role as nitrogen storage compounds (Sanchez-Perez et al. 2008; Selmar et al. 1988)

The benzoxazinoids (also called hydroxamic acids) DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one) and DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) are well known plant defense compounds found in Poaceae (monocots) and a number of eudicotyledenous plant species (Baumeler et al. 2000; Niemeyer 1988). The genes and enzymes involved in DIBOAGlc and DIMBOAGlc pathway have been isolated and characterized in maize (Frey et al. 1997; Frey et al. 2003; Jonczyk et al. 2008; von Rad et al. 2001) and wheat (Nomura et al. 2002). The biosynthetic pathway branches off from that of tryptophan at the point of indole, synthesized from indole-3-glycerol phosphate by a tryptophan synthase α-homologue denoted as Bx1 (Morant et al. 2008). Five genes, denoted as *Bx1* through *Bx5*, are required for biosynthesis of DIBOA in maize. Of these, *Bx2* through *Bx5* encode cytochrome P450-dependent monooxygenases that catalyze four consecutive hydroxylation reactions to convert indole-3-glycerol phosphate into DIBOA (Frey et al. 1997). The conversion of DIBOA to DIMBOA requires two additional enzymes, 2-oxoglutarate-dependent dioxygenase and *O*-methyltransferase, denoted as Bx6 and Bx7, respectively (Frey et al. 2003; Jonczyk et al. 2008), and glucosylation by glucosyltransferase (Bx8 and Bx9), which produces non-toxic BxGlcs, such as DIBOAGlc and DIMBOAGlc (von Rad et al. 2001). The biosynthetic pathway for DIBOA in maize and rye proceeds via identical enzymatic steps (Glawischnig et al. 1999), suggesting that biosynthetic pathway for DIBOA is identical in maize, wheat, and rye (Morant et al. 2008).

The β-glucosidases involved in the bio-activation of DIBOAGlc and DIMBOAGlc have been isolated and characterized from maize (Cicek and Esen 1999; Esen 1992), rye (Nikus et al. 2003; Sue et al. 2000b) and wheat (Sue et al. 2000a). β-Glucosidases in these plants differ with respect to substrate specificity. For example, the predominant substrate for rye β-glucosidases is DIBOAGlc, whereas it is DIMBOAGlc for maize and wheat β-glucosidases (Niemeyer 1988). In

maize, the major benzoxazinoid glucoside is DIMBOAGlc, whose aglycone DIMBOA is the primary defense chemical against the European corn borer (*Ostrinia nubilais*) and aphids. Maize mutants deficient in DIMBOA biosynthesis are extremely susceptible to pests such as the European corn borer (Frey et al. 1997). DIMBOAGlc in maize is produced up to 1% of the dry weight in young plant parts, and the substrate DIMBOAGlc and the β-glucosidases that hydrolyze it are physically separated in that the former is in the vacuole and the latter is in the plastid. After tissue disruption by insects, fungal or bacterial attacks, the enzyme and substrate come into contact and then a toxic aglycone, DIMBOA, is released from DIMBOAGlc by hydrolysis by the enzyme (Kahler and Wehrhahn 1986; Massardo et al. 1994). DIMBOA is highly effective in conferring resistance to phloem feeding aphids.

1.3 Reaction Mechanism of β-Glucosidases

Glycosidases are enzymes that are responsible for the transfer of glycosyl moieties from a donor sugar to an acceptor (e.g. water) and the result being hydrolysis (Ly and Withers 1999). Glycosidases are divided into two classes based on the mechanism, inverting and retaining (Koshland 1953). There are significant similarities between the active sites of the two classes, consistent with their stabilization of similar transition states. Both enzyme classes have a pair of carboxylic acids in the active site that play important roles in the mechanism. However, the two carboxylic acids are separated by different distances from each other in both cases; 0.95 nm for inverting glycosidases and 0.55 nm for retaining glycosidases (Ly and Withers 1999).

The inverting glycosidases use a direct displacement mechanism whereas the retaining glycosidases use a two-step double displacement mechanism. In the direct displacement mechanism used by the inverting glycosidases, two carboxylic acids at the active site are correctly located such that one provides general base catalytic assistance to the attack of water, whereas the other provides general-acid-catalytic assistance to cleavage of the glycosidic bond. Reaction progresses through an oxocarbenium ion-like transition state. The distance between two carboxylic acids is approximately 0.95 nm and this distance is presumably ideal to allow the water and the substrate to bind simultaneously (Ly and Withers 1999). The two-step double

displacement mechanism used by the retaining glycosidases involves first the formation and then hydrolysis of a covalent glycosyl-enzyme intermediate, both steps proceeding through oxocarbenium ion-like transition states. The two carboxylic acids in the active site have different roles in this mechanism. One carboxyl group serves as the nucleophile, attacking at the sugar anomeric center to form a covalently linked glycosyl-enzyme intermediate, whereas the other carboxyl group acts as a general acid/base catalyst, protonating the glycosidic oxygen in the first step (general-acid catalysis) and deprotonating the water in the second step (general-base catalysis). The narrow distance between two residues (~0.55 nm) compared with that in a direct displacement mechanism (~0.95 nm) is therefore consistent with the requirement for direct attack of the nucleophile (Ly and Withers 1999). This mechanism closely resembles the double displacement mechanism used for lysozyme (Clarke et al. 1993; Kempton and Withers 1992).

All family 1 β-glycoside hydrolases including β-glucosidases hydrolyze the glycosidic bond between the anomeric carbon (C1 of the glucose) and the glucosidic oxygen by the double displacement mechanism (retaining) (Davies and Henrissat 1995). The primary structures of maize and sorghum β-glucosidases have the highly conserved peptide motifs TFNEP and I/VTENG, which make up the catalytic site in all family 1 β-glycosidases (Henrissat 1991; Trimbur et al. 1992). The hydrolysis of the substrate involves two steps (glycosylation and deglycosylation) and requires an acid-base catalyst and a nucleophile, which are glutamic acids E191 and E406 in the maize β-glucosidase isozyme Glu1, and both occur in the two conserved motifs TFNEP and I/VTENG, respectively (Czjzek et al. 2000). The acidic group (E191 in Glu1) in the active site donates a proton to the glycosidic oxygen, and a nucleophilic group (E406 in Glu1) facilitates the bond breaking by attacking the anomeric carbon (C1 of the glucose). The hydrolysis of the β-glycosidic bond releases the aglycone DIMBOA from DIMBOAGlc. After aglycone departure, a water molecule provides a proton to the base catalyst and the OH group to the covalent bond between the glycone and β-glucosidase, releasing the glycone and regenerating the nucleophilic glutamic acid (Cottaz et al. 1996; Moracci et al. 1996; Wang et al. 1995; Withers et al. 1990).

1.4 β-Glucosidase Aggregating Factor (BGAF)

In certain maize genotypes (null-lines), a β-glucosidase activity band is not detected on zymograms (Stuber et al. 1977); therefore these genotypes (null-lines) are expected to be homozygous for a null allele at the *glu1* locus. However, biochemical and immunological studies showed that the null genotypes have β-glucosidase activity when assayed in solution and the immunoblotting data showed that they have a 60-KDa polypeptide that specifically reacted with anti-β-glucosidase antibody (Esen and Cokmus 1990). The β-glucosidase is not detected on zymograms because a protein called β-glucosidase aggregating factor (BGAF) specifically interacts with the enzyme and produces high molecular weight complexes (> 1.5 x 10^6 Da) that fail to enter the gel (Esen and Blanchard 2000).

BGAF (ZmBGAF) is a homodimer of 32 KDa monomers, and it consists of two domains: the disease response or "dirigent" domain at the N-terminus (1-151 amino acids) and the jacalin-related lectin (JRL) domain at the C-terminus (152-306 amino acids). The MALDI-TOF mass spectrum of purified native BGAF showed the predicted polypeptide of 306 amino acids (Kittur et al. 2007). BGAF is a lectin because it agglutinates sheep and rabbit erythrocytes, and it binds lactosyl agarose affinity matrix. Studies on the inhibition of hemagglutination indicate that the most potent inhibitors among the monosaccharides tested were Gal, followed by GalN, Man, GalNAc, and N-acetylneuraminic acid. Among di- and oligosaccharides, *N*-acetyllactosamine, lactose, raffinose, and stachyose were good inhibitors. Among glycoproteins, the most potent inhibitors of hemagglutination were asialofetuin, ovalbumin, and porcine stomach mucin (PSM), which showed more than 6000 times inhibition than free Gal. The sugar binding site and the Glu1 binding site on BGAF do not overlap and are thus expected to be located separately, because the Glu1–BGAF complex can still bind sugars and the interaction between Glu1 and BGAF is not inhibited by the presence of sugar (up to 125 mM concentration) (Kittur et al. 2007).

Of the two domains (dirigent and JRL) of BGAF, the JRL domain, not the dirigent domain, is involved in interaction with Glu1, but it alone does not form precipitable aggregates with Glu1 even at high concentration of Glu1 (~1000 nM). Based on pull-down assays, BGAF forms precipitable aggregates with Glu1. This result is also supported by gel-shift assays of

chimeric BGAF (sbdirigent-ZmJRL). The sorghum BGAF homolog (SbBGAF) does not interact with maize Glu1. Kittur et al. constructed a chimeric BGAF, which included the dirigent domain of SbBGAF (1-153) and the JRL domain of ZmBGAF (152-306). The gel-shift assay data showed that this chimeric BGAF binds to Glu1 in the absence of the maize BGAF dirigent domain (Kittur et al. 2007). It appears that to form large aggregates of Glu1-BGAF complex, part of the dirigent domain fused with the JRL domain is required because a truncated BGAF (Δ 1-138, the N-terminal region (1-138 amino acids of BGAF was removed) that contains 14 amino acids from the predicted extreme C-terminal region of the dirigent domain binds Glu1 and forms precipitable complexes (Kittur et al. 2007). Thus, although the dirigent domain of BGAF is not directly involved in interaction with Glu1, it is required for aggregation of the Glu1-BGAF complex.

Recently, the Glu1 binding site on BGAF has been defined by Kittur et al. They constructed chimeric BGAFs to determine the Glu1 binding site on BGAF, and the results of gel-shift assays showed that Gly^{164}-Gly^{219} in the JRL domain is involved in Glu1 binding. The chimeric BGAF, C-7 (164-219 of ZmBGAF was replaced with the corresponding region of SbBGAF) failed to bind Glu1, whereas C-8 (164-219 of SbBGAF was exchanged with the corresponding site of ZmBGAF) bound Glu1. The Glu1 binding site of BGAF does not overlap with the two sugar binding motifs of BGAF (Kittur et al., unpublished data).

The sorghum lectin (SbBGAF), a homolog of maize BGAF, was identified and its complete sequence determined. The native and recombinant forms of sorghum lectin were purified and expressed in *Escherichia coli*, respectively. The native protein is a 32 KDa monomer and shares high sequence identity (67%) with maize BGAF. It also has the same modular structure (a dirigent domain at the N-terminus and a JRL domain at the C-terminus). SbBGAF is a GalNAc-specific lectin and binds to Mannosyl- and GalNAc-agarose lectin affinity columns (Kittur et al., 2009).

Although SbBGAF has many features in common with ZmBGAF such as high sequence identity and the same modular structure, it does not bind β-glucosidases. Based on the results of gel-shift assays, SbBGAF does not interact with the maize β-glucosidase isozyme Glu1 to which ZmBGAF binds. Interestingly, SbBGAF also does not bind with the sorghum β-glucosidase

(dhurrinase) isozyme Dhr2 (Dhr1 not tested). These results clearly suggest that SbBGAF does not participate in protein-protein interactions (Kittur et al., 2009). This difference provides an ideal model system to address questions related to β-glucosidase binding specificity and mapping the β-glucosidase binding site on ZmBGAF.

1.5 Differences between Maize and Sorghum β-Glucosidases

Maize β-glucosidase isozymes Glu1 and Glu2 have been purified and characterized with respect to substrate specificity and other biochemical properties (Esen 1992; Esen and Cokmus 1990). At least 34 members of glycosyl hydrolase family 1 β-glucosidase genes have been reported in rice (Opassiri et al. 2006), so it is expected that at least equal numbers of homologous genes are present in maize (Coneva et al. 2007). The full-length cDNAs of *glu1* and *glu2* encode 566 and 563 amino acid-long precursor proteins, respectively. Glu1 and Glu2 contain 54 and 51 amino acid-long transit peptides for plastid targeting, respectively (Bandaranayake and Esen 1996; Brzobohaty et al. 1993). Glu1 and Glu2 hydrolyze the natural substrate DIMBOAGlc and a vast array of artificial substrates (e.g., *p*-nitrophenyl-β-D-glucoside (*p*NPGlc), 4-methylumbelliferyl-β-D-glucoside (4-MUGlc), and others). Of these, *p*NPGlc and 4-MUGlc are used for frontal affinity chromatography (FAC) and gel-shift assays, respectively in this study. Although Glu1 and Glu2 have similar substrates specificity and share 90% sequence identity with each other, the spatial regulation of their genes is different. Glu1 is expressed in all plant parts, whereas Glu2 is expressed at low level and predominately in leaves starting at day 6 after germination (Esen and Blanchard 2000).

Sorghum β-glucosidase (dhurrinases) isozymes Dhr1 and Dhr2 were purified from sorghum seedlings (Hosel et al. 1987), and their cDNAs also have been cloned and characterized (Cicek and Esen 1995). The cDNAs of *dhr1* and *dhr2* with a full-length coding sequence encode 565 and 571 amino acid-long precursor proteins, respectively. The Dhr1 and Dhr2 precursor proteins contain 51 and 54 amino acid transit peptides for plastid targeting, respectively (Cicek and Esen 1998). Although Dhr1 and Dhr2 share 75% sequence identity, they exhibit differences regarding localization and substrate specificity. Dhr1 is expressed in shoots of seedlings in the

darkness whereas Dhr2 occurs in the green shoots and leaves of young seedlings in the light (Cicek and Esen 1998; Hosel et al. 1987). Based on Unigene database, Dhr1 and Dhr2 are expressed in whole plant, but Dhr1 is mainly expressed in shoot and root whereas Dhr2 in shoot, leaf, and panicle (http://www.ncbi.nlm.nih.gov/unigene). In the case of substrate specificity, Dhr1 hydrolyzes only the natural substrate dhurrin with high efficiency, whereas Dhr2 hydrolyzes the natural substrate dhurrin with high efficiency and shows weak activity on certain artificial substrates, including *p*NPGlc and 4-MUGlc (Cicek and Esen 1998).

Maize and sorghum β-glucosidases share some characteristics including subunit sequence identity (~70%), molecular weight (~ 60KDa), acidic pH (5.8 for maize β-glucosidases and 6.2 for sorghum β-glucosidases), pI (~5.5) and localization of the enzymes (in plastid) (Esen 1992; Esen and Stetler 1993; Hosel et al. 1987; Kojima et al. 1979; Thayer and Conn 1981). However, the enzymes show considerable differences with respect to substrate specificity and BGAF binding. Maize β-glucosidase isozymes, Glu1 and Glu2 hydrolyze their natural substrate DIMBOAGlc but they cannot hydrolyze sorghum natural substrate dhurrin. The sorghum β-glucosidase isozymes Dhr1 and Dhr2 also hydrolyze their natural substrate, dhurrin, but not DIMBOAGlc, the natural substrate for maize β-glucosidases (Cicek et al. 2000). However, interestingly, the hydrolysis of DIMBOAGlc by maize β-glucosidases is inhibited by dhurrin and the hydrolysis of dhurrin by sorghum β-glucosidases is inhibited by DIMBOAGlc. So, dhurrin is a potent competitive inhibitor of Glu1 and Glu2, as is DIMBOAGlc for Dhr1 and Dhr2 (Cicek et al. 2000). As to BGAF binding, both maize β-glucosidase isozymes, Glu1 and Glu2, bind BGAF with high affinity, but sorghum β-glucosidases Dhr1 and Dhr2 do not bind BGAF despite their high sequence identity (~70%) (Blanchard et al. 2001). This difference provides an ideal model system to address questions related to BGAF binding specificity and mapping the BGAF binding site on maize β-glucosidases.

1.6 The Mechanism of β-Glucosidase and β-Glucosidase Aggregating Factor (BGAF) Interaction

The characteristics of the β-glucosidase-BGAF interaction system were shown by Esen and Blanchard, using two maize inbreds, H95 (null type) and K55 (wild type) (Esen and Blanchard 2000). A high concentration of BGAF causing aggregation of Glu1 was found in H95 whereas a low concentration of BGAF was detected in K55 (Esen and Blanchard 2000). The interaction between β-glucosidase and BGAF is inhibited by SDS (Sodium Dodecyl Sulfate). The electrophoretic data (Native-PAGE) indicated that H95 extracts containing 0.5% (w/v) SDS cause the dissociation of β-glucosidase-BGAF aggregates, with zymograms showing unreacted β-glucosidase (dimeric form) in the resolving gel as it occurs in wild type genotype (K55). This result suggests that the aggregation between β-glucosidase and BGAF is formed and stabilized by non-covalent interactions such as hydrophobic interactions (Esen and Blanchard 2000). The interaction between Glu1 and BGAF is also affected by pH and the optimum pH for β-glucosidase-BGAF interaction is pH 5 (Esen and Blanchard 2000).

The mapping of BGAF binding sites on maize β-glucosidase isozyme Glu1 was previously done by Blanchard et al. (Blanchard et al. 2001). To determine the regions contributing to the BGAF binding site on maize β-glucosidases, these authors constructed chimeric β-glucosidases by domain swapping between the maize β-glucosidase isozymes Glu1 and Glu2 (binder) and the sorghum β-glucosidase isozyme Dhr1 (non-binder). The interaction between chimeric enzymes and BGAF provided data to broadly define the BGAF binding regions on Glu1 by gel-shift assay (Figure 1.1). Chimera C-2 (Phe^{466}-Ala^{512} on Glu1 was replaced with the corresponding residues on Dhr1) showed no interaction with BGAF in the presence of a 10-fold molar excess of BGAF, suggesting that the C-terminal region (Phe^{466}-Ala^{512}) of Glu1 is one of the key regions for BGAF binding. C-26 (Ser^{1}-Asn^{127} on Glu1 was replaced with the corresponding region on Dhr1) failed to interact with BGAF, whereas C-5 (Ser^{1}-Thr^{29} of Glu1 was exchanged with its homolog in Dhr1) showed BGAF binding although this chimera yielded two discrete complexes (bands) instead of a smear, suggesting that the N-terminal region (Ser^{1}-Thr^{29}) of Glu1 is also involved in BGAF binding. Additional chimeric

enzymes showed that an N-terminal region (Glu^{50}-Val^{145}) together with a C-terminal region (Phe^{466}-Ala^{512}) on Glu1 is involved in BGAF interaction (Blanchard et al. 2001). Analysis of the 3D-structure of Glu1 (Figure 1.2) suggested that Ile^{72}-Thr^{82} within the N-terminal region Glu^{50}-Val^{145} of Glu1 is likely to make a major contribution to BGAF binding, because the unique amino acids compared to Dhr1 or Dhr2 in this region are within a surface patch and close to those from another key region contributing to the BGAF binding site, C-terminal region (Phe^{466}-Ala^{512}). In Ile^{72}-Thr^{82}, the unique amino acids on Glu1 that differ from those in Dhr1 and Dhr2 are Ile^{72}, Asn^{75}, Lys^{81}, and Thr^{82}, so we expected that one or more among these four amino acids are actually involved in BGAF interaction (Blanchard et al. 2001). The BGAF binding site is away from the active site (Figure 1.2, *arrowhead*) and the interaction does not affect enzyme activity and kinetic parameters (Blanchard et al. 2001; Cicek et al. 2000), suggesting that BGAF binding does not block the active site or cause significant conformational change in the enzyme. In addition, BGAF showed no activity toward inactive, denatured Glu1, indicating that the tertiary structure of the properly folded β-glucosidase is required to form a functional binding site (Blanchard et al. 2001). The nature of the binding between β-glucosidase and BGAF is specific and non-covalent, like that of an antigen-antibody interaction (Esen and Blanchard 2000)

1.7 The Physiological Significance of the β-Glucosidase-β-Glucosidase Aggregating Factor (BGAF) Interaction

The physiological role of BGAFs and the β-glucosidase-BGAF interaction is not known at this time. However, the intriguing possibilities of roles for BGAF can be surmised from the literature on stand-alone dirigent or JRL proteins and BGAF-like proteins (Kittur et al. 2007).

Dirigent is induced in plants during response to insect and pathogen attack (Culley et al. 1995), and it catalyzes the regio- and stereospecific coupling of biomolecular phenoxy radicals, which are important for lignin and lignin biosynthesis (Davin et al. 1997). Lignans, their derivatives, play an important role in plant defenses, and their constitutive deposition significantly helps confer durability and resistance to the heartwoods against wood-rotting fungi

(Gang et al. 1999). Therefore, dirigent proteins are expected to be involved in plant defense systems. Similarly, JRL is also expected to play a role in plant defense (Chisholm et al. 2001; Gorlach et al. 1996; Williams et al. 2002). The long-distance movement of tobacco etch virus (TEV) in *Arabidopsis thaliana* is restricted by the function of at least three genes, RTM1 (restricted TEV movement 1), RTM2, and RTM3. Especially, the protein product of RTM1 has JRLs like BGAF (Chisholm et al. 2001). Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) protected wheat systemically against powdery mildew infection by the induction of a number of wheat chemically induced (WCI) genes, which include a gene encoding a BGAF-like protein (Gorlach et al. 1996).

Non-catalytic proteins capable of binding enzymes and forming large complexes in plants have been described in the literature (Eriksson et al. 2002). The best examples of these proteins are myrosinase binding proteins (MBPs) (Eriksson et al. 2002) and PBP1 (PYK10-binding protein 1) (Nagano et al. 2005). MBPs are found in *Brassica napus* and other members of the family *Brassicaceae* (Eriksson et al. 2002). They have one or more JRL domains (Taipalensuu et al. 1997) and their binding to myrosinases does not seem to affect enzyme activity (Chen and Halkier 1999; Eriksson et al. 2002). Myrosinase and MBPs are localized separately in developing seeds. Myrosinase is present in the myrosin cells, whereas MBP is localized in all cells in the mature embryo, except myrosin cells, epidermis, and provascular tissue, but co-localized in later growth stages (Andreasson et al. 2001; Eriksson et al. 2002). PBP1 is a β-glucosidase binding protein in *Arabidopsis thaliana* and also has a jacalin-like lectin domain (Nagano et al. 2005). PBP1 and PYK10 (a β-glucosidase) are localized in cytosol and ER bodies, respectively, and the two proteins encounter each other and interact when tissue is damaged (Nagano et al. 2005). PBP1 interacts with inactive PYK10 (soluble form) but not active PYK10 (insoluble form), and is believed to play a role in activation of PYK10 (Nagano et al. 2005). The physiological functions of these β-glucosidase binding proteins (BGAF, MBPs, and PBP1) are not well understood. It has been speculated that they function in plant defense systems because they and their ligand (β-glucosidases) are initially localized separately, but encounter each other and interact upon tissue damage due to herbivory and pathogen infection (Chisholm et al. 2001; Gorlach et al. 1996; Williams et al. 2002).

Recently, groundbreaking studies on the role of a BGAF-like protein from wheat (HFR1) in plant defense have been reported by Subramanyam *et al.*(Subramanyam et al. 2008). Like BGAF, HFR1 has an N-terminal dirigent and a C-terminal JRL domain as well as lectin activity. A high level of HFR1 is accumulated at the site of larva feeding, and it deters the Hessian fly larva from feeding on resistant plants (Subramanyam et al. 2008). It is conceivable that the function of BGAF is similar to that of HFR1 in that it deters the European corn borer, a major pest of maize. Thus, one of the foci of future research on BGAF should be determination of its role in defense against the European corn borer and other maize pests.

1.8 Project Goal

As mentioned above, the β-glucosidase binding partner, BGAF (β-glucosidase aggregating factor), is a chimeric lectin (Kittur et al. 2007) and it specifically binds to the maize β-glucosidase isozymes Glu1 and Glu2 but not the sorghum β-glucosidase isozymes Dhr1 and Dhr2 although they have 70% sequence identity (Blanchard et al. 2001; Esen and Blanchard 2000). This result gave us an ideal system to study β-glucosidase-BGAF complex formation and its mechanism. The basic biochemical characteristics of BGAF including the sugar specificity have been studied in our laboratory for a number of years now. The BGAF like proteins, myrosinase binding protein (MBP) in *Brassica napus* and PBP1 (PYK10-binding protein 1) in *Arabidopsis thaliana* have also been studied well by other laboratories for many years (Eriksson et al. 2002; Nagano et al. 2005). However, the function of those proteins (BGAF and BGAF-like proteins) as well as the interaction of β-glucosidase and β-glucosidase binding proteins including their binding sites is not well studied. If we know the binding site for these proteins, these results will give us important insights into the interaction between these proteins and their function. With these considerations in mind, my dissertation research had the following specific objectives: 1) Determination of BGAF binding sites on the maize β-glucosidase isozyme, Glu1 by gel-shift, pull-down, and FAC assay. 2) Identification of the key amino acids on Glu1 involved in BGAF interaction by gel-shift and FAC assay. 3) Determination of biochemical properties of β-glucosidase and BGAF complex.

The first objective of the work presented in this dissertation is to determine the BGAF binding site of Glu1. Previously, the BGAF binding regions on Glu1 were broadly determined by Blanchard et al., (Blanchard et al. 2001). However, the previous results showed two wide regions (Glu^{50}-Val^{145} and Phe^{466}-Ala^{512}) of Glu1 for BGAF binding and we expect that not all amino acids in these regions are involved in the binding. Based on the sequence comparison between maize and sorghum β-glucosidases and 3D-structures of Glu1, there are many amino acid identities in the above-mentioned regions between Glu1 and dhurrinases and some amino acids in these regions are located on the surface of Glu1. Therefore, we constructed additional chimeric β-glucosidases and used them in gel-shift assays to determine whether or not they bind BGAF. However, the gel-shift assay does not provide quantitative data for the interaction between chimeric β-glucosidase and BGAF. Therefore, we used frontal affinity chromatography (FAC) to determine the dissociation constant (K_d) values for the interaction between these chimeric β-glucosidases and BGAF. To perform FAC, we immobilized BGAF on a lactosyl agarose column and applied wild type and chimeric β-glucosidase to the column. The K_d values were obtained and used to assess the relative contribution of each region in Glu1 to interaction with BGAF. We also studied other factors such as optimum pH and salt concentration required for Glu1-BGAF interaction by FAC, as well as the stoichiometry of Glu1-BGAF complex formation by gel-shift assay.

The second main objective of this study was to identify amino acids in Glu1 that are essential and important for BGAF binding. Based on the results of the studies pertaining to the first objective, one can narrow down the regions (Ser^{1}-Thr^{29}, Ile^{72}-Thr^{82}, and Asn^{481}-Thr^{500}) in Glu1 that form the BGAF binding site and then generate mutant enzymes by introducing amino acid substitutions at positions within those regions that differ between maize and sorghum β-glucosidases. The experiments for identifying key amino acids for BGAF binding used the same methods (i.e., gel-shift and FAC assay) as those for chimeric β-glucosidases. We also constructed a number of single Dhr2 mutants and finally made a Dhr2 mutant by introducing amino acid substitutions sequentially that allowing to bind BGAF although the affinity in this case was lower than that of wild type Glu1. After we identified the key amino acids for BGAF binding in Glu1, we compared the sequence of maize β-glucosidase isozymes, Glu1 and Glu2

with those of other plant β-glucosidases including sorghum β-glucosidase. This sequence comparison strongly supported the specificity of maize β-glucosidase-BGAF interaction.

The third objective of this work was to determine biochemical properties of the β-glucosidase and BGAF complex. Based on the previous result, the optimum pH for Glu1-BGAF interaction is pH 5. We determined the effect of pH and ionic strength by FAC and the stoichiometry of Glu1-BGAF complex by gel-shift assay. The stoichiometry of Glu1-BGAF complex strongly supported the liner chain model for Glu1-BGAF interaction proposed previously.

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Figure 1.1: Sequence alignment of the maize β-glucosidase isozymes Glu1 and Glu2 and their sorghum homologs Dhr1 and Dhr2. The two regions (Ile[72]-Thr[82] and Phe[466]-Ala[512]) of Glu1 expected in BGAF-binding are shown in ***Gray*** background and the amino acids that map to the predicted BGAF-binding site or affect binding indirectly in these regions are shown in ***Black*** background.

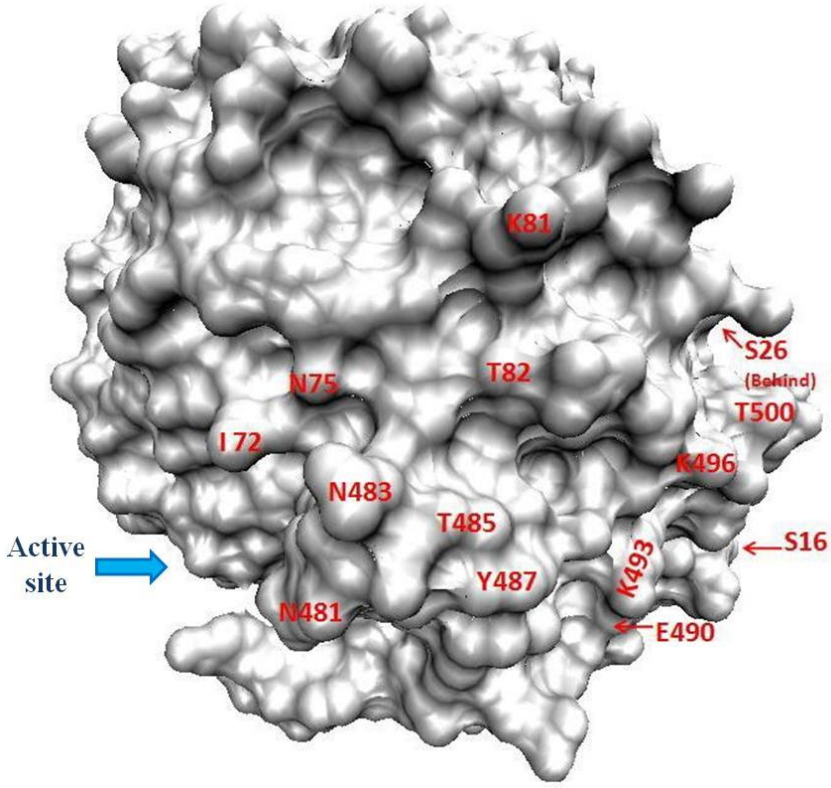

Figure 1.2: Three dimensional structure of maize β-glucosidase isozyme Glu1 (PDB entry 1E1E). The amino acids that are postulated to be critical for BGAF binding are labeled in ***Red***. The arrowhead on the left side is the active site of β-glucosidase, which is away from the BGAF binding site. Visual Molecular Dynamics (VMD) was used to generate these images (surface mode).

Chapter 2

2.1 Title Page

The N-terminal region (Ile^{72}-Thr^{82}) on maize β-glucosidase isozyme Glu1 is essential for binding β-glucosidase aggregating factor (BGAF), and its extreme N- and C-terminal regions are required for the formation of large complexes with BGAF.

Hyun Young Yu[a], Farooqahmed S. Kittur[a], David R. Bevan[b] and Asim Esen[a*]

[a]Department of Biological Sciences and [b]Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA

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2.2 Abstract

β-Glucosidases Glu1 and Glu2 in maize specifically interact with a lectin called β-glucosidase aggregating factor (BGAF). We have shown that the N-terminal (Glu^{50}-Val^{145}) and the C-terminal (Phe^{466}-Ala^{512}) regions of maize Glu1 are involved in binding to BGAF. Sequence comparison between sorghum β-glucosidases (dhurrinases, non-binder) and maize β-glucosidases, and the 3D-structure of Glu1 suggested that the BGAF binding site on Glu1 is much smaller than predicted previously. To define more precisely the BGAF binding site, we constructed additional chimeric β-glucosidases. The results showed that a region spanning 11 amino acids (Ile^{72}-Thr^{82}) on Glu1 is essential and sufficient for BGAF binding, whereas the extreme N-terminal region Ser^{1}-Thr^{29}, together with C-terminal region Phe^{466}-Ala^{512}, affects the size of Glu1-BGAF complexes. The dissociation constants (K_d) of chimeric β-glucosidase-BGAF interactions also demonstrated that the extreme N-terminal and C-terminal regions are important but not essential for binding. To confirm the importance of Ile^{72}-Thr^{82} on Glu1 for BGAF binding, we constructed a chimeric sorghum β-glucosidase, Dhr2 (C-11, Dhr2 whose Val^{72}-Glu^{82} region was replaced with the Ile^{72}-Thr^{82} region of Glu1). Based on the gel-shift assay, chimeric Dhr2, C-11 interact with BGAF, supporting that the Ile^{72}-Thr^{82} region is indeed a major interaction site on Glu1 for BGAF binding.

2.3 Introduction

Family 1 β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) hydrolyze aryl and alkyl-β-D-glucosides as well as glucosides with a carbohydrate moiety (Reese, 1977). They are found widely in all three (Archaea, Eubacteria, and Eukarya) domains of living organisms. They play important functions in many biological processes in plants, such as degradation of cellulose (Shewale, 1982), lignification (Hosel et al., 1978), floral development (Koes et al., 1994), defense against pathogens and herbivores (Hughes et al., 1992; Phillips and Streit, 1996; Poulton, 1990), and releasing active phytohormones from their inactive glucoconjugates (Brzobohaty et al., 1993). In maize, two β-glucosidase isozymes (Glu1 and Glu2) have been identified and their cDNAs cloned (Bandaranayake and Esen, 1996). The major function of these two enzymes is defense against pests by releasing a toxic aglycone from the abundant natural β-glucoside 2-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOAGlc), which occurs mostly in young seedling parts of maize at millimolar concentrations. The aglycone of DIMBOAGlc is DIMBOA, and it is the major defense compound in maize against aphids, the European corn borer and other pests (Kahler and Wehrhahn, 1986; Massardo et al., 1994).

In certain maize genotypes (nulls), β-glucosidase activity is not detected on zymograms (Stuber et al., 1977) because a protein called β-glucosidase aggregating factor (BGAF) specifically interacts with the enzyme and produces high molecular weight complexes (> 1.5 x 10^6 Da) that fail to enter the gel (Esen and Blanchard, 2000). BGAF is a chimeric protein consisting of an N-terminal dirigent (disease response) domain and a C-terminal jacalin-related lectin (JRL) domain. Recently, we demonstrated that BGAF is a lectin and its JRL domain is responsible for β-glucosidase aggregation (Kittur et al., 2007). Furthermore, we showed that the sugar and the β-glucosidase binding sites are located in the JRL domain, but they do not overlap with each other nor do they interfere with each other's ligand binding (Kittur et al., 2007). The enzymatic activity of Glu1 and Glu2 is unaffected by BGAF binding, suggesting that interaction with BGAF neither blocks the active site nor changes the conformation of the enzymes (Blanchard et al., 2001).

Maize β-glucosidase isozymes (Glu1 and Glu2) share 90% sequence identity. Similarly, sorghum β-glucosidase isozymes (Dhr1 and Dhr2) share 70% sequence identity with each other and with maize β-glucosidases. However, maize and sorghum β-glucosidases have significant differences with respect to substrate specificity and BGAF binding activity. Maize β-glucosidases hydrolyze the natural substrate DIMBOAGlc, as well as many artificial substrates such as *p*-nitrophenyl-β-D-glucoside (*p*NPGlc) and 4-methylumbelliferyl-β-D-glucoside (4-MUGlc) (Cicek et al., 2000). In contrast, sorghum β-glucosidases (Dhr1 and Dhr2) exhibit strict specificity for their natural substrate dhurrin, except that Dhr2 shows measurable activity toward the artificial substrates *p*NPGlc and 4-MUGlc (Cicek et al., 2000; Cicek and Esen, 1998). Maize and sorghum β-glucosidases are also strikingly different with respect to BGAF binding in that both maize β-glucosidases bind to BGAF with high affinity, whereas sorghum β-glucosidases do not bind to BGAF (Blanchard et al., 2001). These differences between maize and sorghum β-glucosidases provide an ideal system to study the β-glucosidase-BGAF interaction and its mechanism.

The regions contributing to BGAF binding on the maize β-glucosidase isozyme Glu1 were mapped previously by Blanchard et al. (Blanchard et al., 2001). The results of these studies suggested that an N-terminal region (Glu^{50}-Val^{145}) and a C-terminal region (Phe^{466}-Ala^{512}) are involved in forming the BGAF binding site. However, sequence comparisons between maize Glu1 and Glu2 (binders) and sorghum β-glucosidase isozymes Dhr1 and Dhr2 (non-binders) show that the two regions implicated in BGAF binding are long and have many shared amino acid identities (Figure 2.1). We postulate that BGAF binding regions on maize Glu1 and Glu2 are actually shorter than those predicted by previous results. The aim of the present study was to investigate further the interaction between BGAF and β-glucosidase and define more precisely the contribution of each region on the maize β-glucosidase isozyme Glu1 to the BGAF binding site. To this end, we constructed a number of chimeric β-glucosidases based on the 3D-structure of Glu1 and determined dissociation constants (K_d) of chimeric β-glucosidases and BGAF interaction by frontal affinity chromatography (FAC). The results of gel-shift assays, pull-down assays and FAC showed that the actual number of amino acids forming the BGAF binding site is

much smaller than suggested by previous results and there are one key and two auxiliary regions on Glu1 that affect BGAF binding. A key region is essential for BGAF binding, whereas the two auxiliary sites are important for polymerization or aggregation of the Glu1-BGAF complexes.

2.4 Results

2.4.1. Cloning, expression and purification of wild type and chimeric β-glucosidases and BGAF

Wild type and chimeric β-glucosidases (see Figure 2.2) were expressed at high levels (4.5-6.0 mg enzyme /1000 mL culture volume) in the *E. coli* BL 21 CodonPlus strain except the chimeric enzyme C-2, which was expressed at a low level (0.7-1.0 mg enzyme /1000 mL culture volume) (Supplement Figure 2.1. *lane 6*). Although C-2 was expressed at a low level, it had the same enzyme specific activity as wild type Glu1 (data not shown). Therefore, we made no further efforts to improve its yield nor did we determine the cause of its lower expression. The degree of purity of recombinant β-glucosidases was 95% or better as judged by SDS-PAGE profiles (Supplement Figure 2.1, *lanes 2-15*). Recombinant BGAF yielded a near homogenous preparation in one step (Supplement Figure 2.1, *lane 1*) after affinity purification on a lactosyl-agarose column.

2.4.2. Demonstration of the interaction between β-Glucosidase and BGAF

The interaction between β-glucosidase and BGAF was detected by two assays: gel-shift and pull-down. In the case of the gel-shift assay, when a wild type or chimeric enzyme interacted with BGAF, its electrophoretic mobility in native-PAGE gels was reduced (shifted up towards the cathodic end) and zymograms showed a smeared or broad zone of enzyme activity extending from the sample well in the stacking gel to the upper part of the resolving gel (Figure 2.3, *lanes, 2, 8, 10, 12, 14, 16, 18, 20, 24, and 26*). In addition to indicating the binding of BGAF to β-glucosidase, the gel-shift assay provided a measure of the size range within a population of β-glucosidase-BGAF complexes. However, when BGAF did not interact with β-glucosidase,

electrophoretic mobility of the enzyme did not change relative to the sample that was not incubated with BGAF (Figure 2.3).

In the pull-down assay, when a wild type or chimeric enzyme forms a large precipitable complex with BGAF, the remaining β-glucosidases activity in the supernatant was reduced compare with the control (no BGAF) (Figure 2.4A, Glu1-BGAF). However, when β-glucosidase did not form a large precipitable complex with BGAF, the remaining β-glucosidase activity in the supernatant did not change relative to the control (no BGAF) (Figure 2.4A, C-3-BGAF). The positive control (wild type Glu1 incubated with BGAF) showed maximum pull-down at the molar ratio of ~1.5:1 (Glu1: BGAF). At this molar ratio, about 90% of β-glucosidase activity precipitated upon centrifugation (Figure 2.4A).

Our earlier domain swapping experiments between maize and sorghum β-glucosidases indicated that the N-terminal region Glu^{50}-Val^{145} in the maize β-glucosidase isozyme Glu1 was one of the two regions essential and sufficient for BGAF binding (Blanchard et al., 2001). However, based on sequence comparisons between maize and sorghum β-glucosidases (Figure 2.1) and the three-dimensional structure of the maize β-glucosidase isozyme Glu1 (PDB entry 1E1E), we postulated that an 11 amino acid long peptide (Ile^{72}-Thr^{82}) in Glu1 is important for recognition and binding by BGAF. To test this hypothesis, the 11 amino acid long peptide of Glu1 (Ile^{72}-Thr^{82}) was exchanged with the corresponding peptide of Dhr1 (Figure 2.2, C-2). As seen in Figure 2.3 (*lane 6*), C-2 failed to bind to BGAF even at a saturating concentration of BGAF, indicating the importance of the 11 amino acid long peptide Ile^{72}-Thr^{82} in Glu1 for BGAF binding. To confirm further that this region is indeed important for BGAF binding, it was used to replace the corresponding position in Dhr2 (C-11). As expected, C-11 bound to BGAF, as evident from retardation of its mobility in the gel-shift assay (Figure 2.3, *lane 26*). Interestingly, it yielded a single shifted band rather than a smear. The pull-down assay also showed no evidence of precipitable complexes of C-11 with BGAF in that all of the β-glucosidase activity remained in the supernatant after incubation with BGAF and centrifugation (Figure 2.4C).

In our previous studies (Blanchard et al., 2001), the equivalent of chimera C-3, in which the C-terminal 47 amino acids of Glu1 were replaced with the corresponding C-terminal 53 amino acids of Dhr1, lost the ability to bind BGAF, suggesting that the C-terminal region

(Phe^{466}-Ala^{512}) of maize β-glucosidase is also critical for BGAF binding. In the present study, when we incubated C-3 (150 nM) with a saturating concentration of BGAF (>7,800 nM), the position of C-3 shifted toward the cathodic end and appeared as one discrete, major band (top band) and one minor band (bottom band, arrow head in Figure 2.3) instead of a smear (Figure 2.3, *lane 8*). The minor band of faster electrophoretic mobility migrates to a position consistent with free C-3, whereas the major band is of a size suggestive of complex comprising one C-3 molecule and one or two BGAF molecules. In the pull-down assay, no precipitable complexes of C-3 were observed even in the presence of saturating concentrations of BGAF (Figure 2.4A). To investigate further the role of the C-terminal region of Glu1 in BGAF binding, we constructed additional Glu1 chimeras (C-4, C-5, C-6, and C-7; see Figure 2.2) by swapping C-terminal regions Phe^{466}-Arg^{480} (C-4), Asn^{481}-Thr^{500} (C-5), Asn^{481}-Ala^{512} (C-6), and Phe^{466}-Thr^{500} (C-7) with corresponding regions from Dhr1. We examined their ability to bind and form insoluble complexes with BGAF by gel-shift and pull-down assays. In gel-shift assays, chimeras C-4, C-5, and C-7 showed a smear, indicating formation of large complexes (Figure 2.3, *lanes, 10, 12, and 16*, respectively), whereas C-6 showed one discrete band (Figure 2.3, *lane 14*) similar to C-3 (Figure 2.3, *lane 8*). Pull-down assays also showed that C-4, C-5, and C-7 could form large, precipitable complexes, whereas C-6 forms only a soluble complex (Figure 2.4A), which is consistent with the results of the gel-shift assay.

We previously showed that the extreme N-terminal region (Ser^{1}-Thr^{29}) of Glu1 was also involved in BGAF binding (Blanchard et al., 2001). When we swapped this region of Glu1 with the corresponding region from Dhr1, the resulting chimera (C-5) interacted with BGAF and produced two distinct β-glucosidase-BGAF bands instead of a smeared zone (Blanchard et al., 2001). In the present study, we reconstructed the same chimera but with a His-tag (named C-8 in the present study, see Figure 2.2) to aid in the purification process. When we incubated C-8 (150 nM) with a saturating concentration of BGAF (7,800 nM), the bottom band shifted up and yielded one single band (Figure 2.3, *lane 18*), indicating a smaller Glu1-BGAF complex. The pull-down assay showed no evidence of formation of precipitable complexes; all of the β-glucosidase activity remained in the supernatant after centrifugation (Figure 2.4B).

To confirm further the regions making up the BGAF binding site on the maize β-

glucosidase isozyme Glu1, we swapped both the N- (Val^{72}-Glu^{82}) and C- terminal (Ser^{466}-Ala^{519}) regions of Dhr2 with those of Glu1 (C-10, Figure 2.2). Dhr2 does not interact with BGAF (Figure 2.3, *lanes 21 and 22*), but surprisingly, the Dhr2 chimera C-10 (see Figure 2.2) not only interacted with BGAF but also formed precipitable complexes with it. The gel-shift pattern of C-10 showed a smeared zone (Figure 2.3, *lane 24*), and the pull-down assay confirmed the formation of large insoluble complexes of C-10 at the same BGAF concentration as the wild type Glu1 (Figure 2.4C). A Dhr2 chimera constructed by swapping only the C-terminal (Ser^{466}-Ala^{519}) region of Dhr2 with the corresponding region (Phe^{466}-Ala^{512}) of Glu1 (C-12) failed to bind to BGAF (Figure 2.3, *lane 28*). It should be emphasized again that the swapping of the N-terminal region (Val^{72}-Glu^{82}) of Dhr2 with the corresponding region (Ile^{72}-Thr^{82}) of Glu1 (chimera C-11) conferred BGAF binding ability to Dhr2, but not the ability to form precipitable complexes with BGAF even at saturating concentrations (Figure 2.4C, pull-down assay).

2.4.3. Determination of dissociation constant (K_d) values of wild type and chimeric β-glucosidases by frontal affinity chromatography (FAC)

The gel shift and pull-down data do not allow one to define quantitatively the contribution of individual regions in Glu1 to BGAF binding. Therefore, we employed FAC to obtain a quantitative measurement of affinity of wild type Glu1 and chimeric β-glucosidases for BGAF. As part of determining K_d values, we investigated the effect of pH and ionic strength on the Glu1-BGAF interaction because the dissociation constant usually changes depending on ionic strength, pH and temperature of the reaction medium (Grynkiewicz et al., 1985; Lattanzio Jr and Bartschat, 1991). The pH of the buffer had a dramatic effect on the Glu1-BGAF interaction. High affinity binding occurred at pH 5.0 (K_d = 0.7 nM), whereas K_d values of 76, 99, and 82 nM were obtained at pH 6, 7, and 8, respectively (Supplement Figure 2.2A). Lowest affinity was observed at pH 4 and pH 9, which yielded dissociation constants of 290 and 300 nM, respectively. We observed only a moderate effect of ionic strength on Glu1 affinity for BGAF (Supplement Figure 2.2B). Although the lowest K_d value was obtained for Glu1 in sodium acetate buffer (pH 5.0), PBS (pH 7.0) instead of acetate buffer was used for FAC studies because some of the chimeric β-

glucosidases precipitated in sodium acetate buffer (pH 5.0) during storage and analysis. Using these conditions, the K_d value for wild type Glu1 at a B_t value of 6.45 x 10^{-9} M was 119 nM (Figure 2.5). Another series of experiments with Glu1 at a B_t of 2.08 x 10^{-9} M yielded a K_d of 103 nM (data not shown). Thus, the average K_d value for Glu1 was estimated to be 111 ± 11 nM (Figure 2.2).

FAC analysis of Glu1 chimeras constructed by swapping the C-terminal region in Glu1 with the corresponding region from Dhr1 (C-3, C-4, C-5, C-6, and C-7) showed that they elute at lower elution volumes than wild type Glu1, indicating altered affinities for BGAF (Figure 2.2 and Supplement Figure 2.3A and B). Notably, the affinity of C-3 for BGAF is higher (K_d = 256 nM) than that of C-6 (K_d= 372 nM), even though C-3 contains a slightly larger segment from Dhr1 (466-512) than does C-6 (481-512). An analogous situation is observed when comparing C-7 (residues 466-500 of Dhr1; K_d= 280 nM) with C-5 (residues 481-500 of Dhr1; K_d= 347 nM).

Exchanging the Ile^{72}-Thr^{82} region alone (C-2) or both Ile^{72}-Thr^{82} and Phe^{466}-Ala^{512} regions (C-1) of Glu1 with the corresponding regions of Dhr1 completely abolished binding to BGAF (see the FAC profile of C-2 and C-1 in Figure 2.6A). Both chimeras elute from the FAC column with Dhr2, a non-binder. Chimera C-8, which was constructed by swapping the extreme N-terminal Ser^{1}-Thr^{29} region of Glu1 with the corresponding region of Dhr1 eluted earlier than wild type Glu1 (Supplement Figure 2.3C), suggesting lower affinity (K_d =197 nM) for BGAF than the wild type enzyme (see Figure 2.2). Interestingly, when both the extreme N-terminal (Ser^{1}-Thr^{29}) and C-terminal (Phe^{466}-Ala^{512}) regions of Glu1 are swapped with the corresponding regions in Dhr1, the K_d (547 nM) of the resulting chimera (C-9) was 5-fold higher than wild type Glu1 (Figure 2.2). Although the gel-shift assay data suggested interaction of Dhr2 chimera C-10 and C-11 with BGAF, we could not determine a K_d for them because their elution profiles were complex (Figure 2.6B).

2.4.4. Determination of stoichiometry of Glu1-BGAF complexes

The stoichiometry of the Glu1-BGAF complex was determined indirectly by the gel-shift assay. When 400 nM of Glu1 was mixed with increasing amounts of BGAF (1 – 1,000 nM), 400

nM of BGAF was the minimum required to titrate all of the Glu1 (Figure 2.7A, *lane 6*). At less than 400 nM of BGAF, there was free, unreacted Glu1 band on the gel (Figure 2.7A, *lanes 2–5*). In the reverse experiment, when a constant amount of BGAF (400 nM) was incubated with increasing concentrations of Glu1 (1 – 1,000 nM), 300 nM of Glu1 was the minimum for complete Glu1-BGAF interaction (Figure 2.7B, *lane 6*). Only a minor amount of unreacted Glu1 band was visible on the gel when 400 nM of Glu1 was incubated with 400 nM of BGAF (Figure 2.7B, *lane 7*).

2.5 Discussion

The Glu1-BGAF interaction is an exquisite case of protein-protein interaction involving two proteins, a chimeric lectin (BGAF) and a β-glucosidase (Glu1 or Glu2) of maize, both of which have been implicated in plant defense responses. Interaction of BGAF-like protein with β-glucosidase has been reported from other plants and the best examples are myrosinase binding proteins (MBPs) from *Brassica napus* (Eriksson et al., 2002) and PBP1 (PYK10-binding protein 1) from *Arabidopsis thaliana* (Nagano et al., 2005). However, the physiological function of these protein-protein interactions and the information about binding sites on two ligands (BGAF-like protein and β-glucosidase) are lacking. Thus, our research has provided new insights into understanding the interaction between BGAF-like proteins (lectin) and β-glucosidases.

Previously, we had identified broadly that the BGAF binding site was a surface patch on Glu1, and it was formed by bringing together parts of an N-terminal (Glu^{50}-Asn^{127}) and a C-terminal (Phe^{466}-Ala^{512}) peptide span (Blanchard et al., 2001). In the current study, we delineated further the actual BGAF binding site on Glu1. In addition, we are also currently studying the Glu1 binding site on BGAF and have found a region (Gly^{164}-Gly^{219}) on its JRL domain that is involved in binding to Glu1 (Kittur et al., unpublished data).

Sequence alignment and comparison data show that the N-terminal polypeptide span Glu^{50}-Asn^{127} contains 4 amino acid substitutions (Ile^{72}, Asn^{75}, Lys^{81}, and Thr^{82}) whereas the C-terminal (Phe^{466}-Ala^{512}) peptide span contains 6 amino acid substitutions (Asn^{481}, Asn^{483}, Thr^{485}, Tyr^{487}, Glu^{490}, and Thr^{500}) that separate the maize β-glucosidase isozymes Glu1 and Glu2 (binders) from

the sorghum β-glucosidase isozymes Dhr1 and Dhr2 (non-binders) in the predicted BGAF binding site. This suggested that the residues that contribute to the BGAF binding site within the Glu^{50}-Asn^{127} region reside in the peptide span Ile^{72}-Thr^{82}. Similarly, within the C-terminal Phe^{466}-Ala^{512} region, the key residues should be in the peptide span Asn^{481}-Thr^{500}. Of the four amino acid substitutions (Ile^{72}, Asn^{75}, Lys^{81}, and Thr^{82} in Glu1 vs. Val^{73}, Asp^{76}, Ala^{82}, and Glu^{83} in Dhr1 and Val^{72}, Asp^{75}, Glu^{81}, and Glu^{82} in Dhr2, Figure 2.1), Lys^{81} in Glu1 is likely to be a critical one because it protrudes from the surface of Glu1 (Figure 2.8B) and may fit in a depression or form a salt bridge with a glutamic acid or aspartic acid on the surface of BGAF. Equally critical might be Thr^{82}, which occurs only in Glu1 and Glu2 among hundreds of family 1 β-glucosidases in the GenBank database. As for the six amino acid substitutions (Asn^{481}, Asn^{483}, Thr^{485}, Tyr^{487}, Glu^{490}, and Thr^{500}) in the C-terminal region peptide span Asn^{481}-Thr^{500} that differentiate Glu1 from Dhr1 and Dhr2, they all have side chains that can potentially form hydrogen bonds (Glu^{490} can also form salt bridge) with one or more amino acids on the Glu1 binding surface of BGAF.

The importance of the above-mentioned regions of Glu1 in BGAF binding was confirmed by interaction of Glu1 or Dhr2 chimeras with BGAF. Gel-shift assay and FAC results obtained with these chimeric enzymes clearly showed that C-1 and C-2 do not bind BGAF (Figure 2.3, *lanes 4 and 6* and Figure 2.6A, respectively), confirming that the Glu1 peptide Ile^{72}-Thr^{82} is required for BGAF recognition and binding. The fact that C-11 produced a single discrete band of slower mobility in the gel after incubation with BGAF (Figure 2.3, *lane 26*) provides strong evidence that one or more of the four amino acids (Ile^{72}, Asn^{75}, Lys^{81}, and Thr^{82}) in the Ile^{72}-Thr^{82} region swapped from Glu1 are essential and sufficient for BGAF binding. The synthetic peptide (CSNSDIGANSYHMYKTDVRLL: underlined sequence is corresponding to Ile^{72}-Thr^{82} in Glu1) failed to competitively inhibit the Glu1-BGAF interaction in a gel-shift assay (data not shown). This observation suggests that the key residues in Ile^{72}-Thr^{82} recognize and bind BGAF only when they are in the context of a correctly folded protein, which is consistent with the previous report that BGAF does not bind to catalytically inactive, denatured Glu1 (Blanchard et al., 2001). However, additional regions (e.g., Phe^{466}-Ala^{512}) in Glu1 are required to form large precipitable complexes with BGAF. We could not determine the K_d of the complex between C-11

and BGAF because the FAC profile of C-11 showed a complex pattern (Figure 2.6B). This pattern may indicate that the affinity of C-11 for BGAF is considerably lower than that of wild type Glu1. It is conceivable that C-11 rapidly binds and dissociates from the BGAF-immobilized lactosyl-agarose column, yielding an unsteady elution profile (Figure 2.6B).

Although the N-terminal region (Ile^{72}-Thr^{82}) of Glu1 is sufficient and essential for binding to BGAF, it produces a soluble β-glucosidase-BGAF complex of discrete size (Figure 2.3, *lane 26*), suggesting that the formation of large, stable and mostly insoluble complexes with BGAF requires contribution of the C-terminal region of Glu1. In view of these results, we addressed the issue of what particular peptide span in the C-terminal Phe^{466}-Ala^{512} region of Glu1 was important for enhancing affinity and promoting larger complex formation (insoluble complex) by dividing this region into segments and replacing each with their Dhr1 homolog. The results from these experiments showed that the C-terminal Phe^{466}-Arg^{480} region is not involved in interaction with BGAF because C-4 (a Glu1 whose Phe^{466}-Arg^{480} is replaced with its Dhr1 homolog) showed a smear (Figure 2.3, *lane 10*), indicating formation of large insoluble complexes. Within this region, Glu1 differs from Dhr1 by only three amino acid substitutions (Phe466Ser, Ala467Ser, and Tyr473Phe), and these substitutions are in the aglycone binding site of the Glu1 active site (Figure 2.8) (Cicek et al., 2000), which is away from the predicted BGAF binding site.

The peptide span Asn^{481}-Thr^{500} within Phe^{466}-Ala^{512} of Glu1 is likely the region that enhances the affinity of Glu1 for BGAF binding because C-5 shows reduced affinity (K_d= 347 nM, see Figure 2.2) and it also requires higher concentrations of BGAF to produce large precipitable complexes (Figure 2.4A). Asn^{481}-Thr^{500} differs from its homolog in Dhr1 by six amino acid substitutions (Asn481Glu, Asn483Gly, Thr485Glu, Tyr487Thr, Glu490Arg, and Thr500Ala). These residues are in and near the predicted binding site (Figure 2.8A), and thus one or more of these amino acids affects the affinity of Glu1 for BGAF and formation of larger and more stable complexes. The extreme C-terminal region (Ala^{501}-Ala^{512}) does not appear to have a direct involvement in interaction with BGAF because the dissociation constants for C-3 and C-7 (see Figure 2.2) are similar (256 and 280 nM, respectively). Likewise, C-5 and C-6 (see Figure 2.2) also have very similar K_d values (347 and 372 nM, respectively). The difference between these chimeric enzymes is at the extreme C-terminal region (Ala^{501}-Ala^{512}). However, C-5

produces precipitable complexes with BGAF in the pull-down assay (Figure 2.4A) and a smear in the gel-shift assay (Figure 2.3), but C-6 produces no precipitable complex (Figure 2.4A) and shows one discrete band instead of a smeared zone (Figure 2.3). These data suggest that the extreme C-terminal region (Ala^{501}-Ala^{512}) of Glu1 does not affect the affinity of Glu1 for BGAF but is involved in formation of large, precipitable complexes with BGAF. Dhr2 chimeras constructed by swapping the C-terminal region of Glu1 also support the importance of the C-terminal region (Phe^{466}-Ala^{512}) of Glu1 for formation of larger Glu1-BGAF complexes. For example, C-11 interacts with BGAF, but the FAC profile of C-11 was irregular (Figure 2.6B), but still indicative of lower affinity of C-11 for BGAF. However, when we exchanged both the N- and C-terminal regions of Dhr2 with those of Glu1 (Figure 2.2, C-10), the FAC profile showed a more stable elution profile than that of C-11 (Figure 2.6B).

The extreme N-terminal region of Glu1 also affects β-glucosidase aggregation in a manner similar to the C-terminal region (Phe^{466}-Ala^{512}). In the present study, C-8 showed only one band with BGAF (at 52-fold molar excess) (Figure 2.3, *lane 18*), suggesting that this chimera forms a discrete sized complex with BGAF. Thus both the extreme N-terminal (Ser^{1}-Thr^{29}) and extreme C-terminal regions (Ala^{501}-Ala^{512}) of Glu1 contribute to the formation of large, insoluble Glu1-BGAF complexes, because exchanging these regions with their Dhr1 counterparts inhibited the formation of precipitable complexes (pull-down data) and smeared zones (gel-shift assay), which represent large aggregates. FAC data also suggest that the C-terminal region contributes relatively more to aggregation than the extreme N-terminal region because the K_d for C-8 (197 nM) is lower than that for C-3 (256 nM). Chimera C-9 had a much higher K_d (547nM) when compared to those of C-3, C-8 and wild type, showing cumulative negative effects of Dhr1 extreme N-terminal and C-terminal regions on β-glucosidase aggregation by BGAF. As alluded to above, the pull-down assay results are consistent with those of the gel shift assay (Figure 2.3), both showing that C-3, C-8, and C-9 chimeras produced small, discrete complexes instead of insoluble high molecular weight complexes (Figure 2.4A and B). In contrast, Dhr2 chimera C-10 formed large, precipitable complex with BGAF as was observed for Glu1-BGAF in pull-down assay (Figure 2.4C). The fact that C-10 forms large complexes (smeared zones) in gel shift assay but C-11 does not (the difference between the two is the presence of the C-terminus of Glu1 in

C-10) confirms further the importance of the Glu1 C-terminal region for aggregation of Glu1 by BGAF (Figure 2.3, *lane 24* and Figure 2.4C, respectively).

As for the stoichiometry of the interaction, we previously showed that BGAF is a homodimer and each monomer has one β-glucosidase binding site (in the JRL domain) (Kittur et al., 2007). Consequently, one BGAF dimer can interact with one or two β-glucosidase dimers, and these two products should appear as discrete bands in the gel-shift assay. The gel-shift assay data (Figure 2.7A and B) however, showed a retarded activity zone when Glu1 and BGAF are at an equimolar ratio (1:1 ratio) (Figure 2.7A, *lane 6* and Figure 2.7B, *lane 6*). The most plausible explanation for this result is that one Glu1 dimer interacts with one BGAF dimer. Since both proteins are dimers and have two binding sites for each other, they form a linear chain, consistent with a model that we described recently (Kittur et al., 2007).

Our data show that the N-terminal region (Ile^{72}-Thr^{82}) of Glu1 is the primary determinant of the interaction, while the extreme N-terminal (Ser^{1}-Thr^{29}) and the C-terminal (Asn^{481}-Ala^{512}) regions contribute less, but enhance the affinity for BGAF. Protein-protein interaction similar to Glu1-BGAF has been reported for human proteins TRADD (TNF receptor-associated death domain) and TRAF2 (TNF receptor-associated factor 2) (Park et al., 2000). Region I (Tyr^{16}, Phe^{18}, His^{65} and Ser^{67}) on TRADD is the primary energetic determinant for TRAF2 interaction, whereas region II (Gln^{143}, Asp^{145}, Arg^{146}, and Leu^{147}) on TRADD has minimal effect on binding to TRAF2. Region II is thought to provide long range attraction forces that facilitate interaction (Park et al., 2000). Region I and II on TRADD are located side by side in the 3D-structure, a situation similar to that in Glu1. In the Glu1-BGAF interaction, contact residues of Glu1 recognizing BGAF and initiating binding are likely to be located in Ile^{72}-Thr^{82} and Asn^{481}-Thr^{500} whereas those enhancing affinity and promoting formation of large aggregates are in Ser^{1}-Thr^{29} and Ala^{501}-Ala^{512}). Moreover, the soluble and insoluble complexes do not differ from each other with respect to enzyme activity, because the active site of Glu1 is away from its BGAF binding site (Figure 2.8). However, in another BGAF-like protein-β-glucosidase interaction, the polymerization of the complex is important for activation of the enzyme. In *Arabidopsis thaliana*, binding of PBP1 (β-glucosidase binding protein) to PYK10 (β-glucosidase) plays an important role in activation of the enzyme by aggregation (Nagano et al., 2005). In this case, the

insoluble state of PYK10 is active, whereas its soluble state (not bound to PBP1) is inactive. Thus, PBP1 only interacts with soluble, inactive PYK10, and the interaction causes aggregation and activation of PYK10 (Nagano et al., 2005). At this time, it is not known how and where PBP1 binds PYK10 and promotes aggregation and activation. In contrast, in the case of myrosinase binding proteins (MBPs) in *Brassica napus*, there is no difference in myrosinase activity in the presence or the absence of MBPs, as is the case with BGAF-β-glucosidase interaction (Eriksson et al., 2002).

The physiological role of β-glucosidase-binding protein interactions described here is not well understood at this time. We speculate that these interactions have a key function in plant defense responses. The BGAF-β-glucosidase, MBP-Myrosinase and PBP1-PYK10 interactions have two things in common. First, these lectins and their ligands (β-glucosidases or myrosinases) are localized separately and encounter each other when compartments are disrupted by pathogenesis, herbivory or other processes (Andreasson et al., 2001; Nagano et al., 2005). Second, the end products (DIMBOA and thiocyanates, isothiocynates, nitrils and oxazolidithione in the case of maize β-glucosidases and myrosinase, respectively) of reactions that the enzymes catalyze are toxic to insects (Haramoto and Gallandt, 2007; Kahler and Wehrhahn, 1986; Massardo et al., 1994; Tripathi and Mishra, 2007). There is good reason to believe that these lectins deter the insect larvae from feeding by lodging the ligands (enzymes) in the oral cavity or the midgut (by binding to glycoproteins) and causing a localized burst of toxic chemicals in these cavities. In fact, the protein product of the *Hfr-1* gene, a BGAF homolog from wheat, has been shown to deter Hessian fly larvae from feeding on resistant plants by binding to sensory receptors (Subramanyam et al., 2008). Recently, we identified five additional genes in maize encoding a family of BGAF-like proteins (unpublished data). Furthermore, we discovered that the product of *bgaf2* gene is also a β-glucosidases aggregating factor (unpublished data). It is now becoming clear that there are at least two chimeric lectins in maize that specifically interact with β-glucosidases. Since they do not affect enzyme activity, we believe they help the plant to launch a powerful defense response to attack by pests.

2.6 Conclusions

We have shown that the N-terminal region (Ile^{72}-Thr^{82}) on Glu1 is essential and critical for BGAF interaction and this result is supported by Dhr2 chimera C-11 (Dhr2 whose Val^{72}-Glu^{82} region was replaced with the Ile^{72}-Thr^{82} region of Glu1), which can bind to BGAF. In the C-terminal region, Asn^{481}-Thr^{500} on Glu1 enhances the affinity of Glu1 for BGAF binding, but it not critical for BGAF binding judged by FAC. The extreme N-terminal (Ser^{1}-Thr^{29}) region together with the extreme C-terminal (Ala^{501}-Ala^{512}) region are essential to produce stable and larger Glu1-BGAF complexes, which account for β-glucosidase aggregation and insolubility in the so-called null lines of maize. The pH of the buffer had a dramatic effect on the Glu1-BGAF interaction (the highest affinity was at pH 5.0) whereas the ionic strength had only a moderate effect on the interaction. The stoichiometry of Glu1-BGAF interaction is a 1:1 ratio, and this result supports a linear chain model for the Glu1-BGAF complex because both proteins are dimers and have two binding sites.

2.7 Experimental

2.7.1. Materials

The following materials were purchased as indicated: T4 DNA ligase (Invitrogen, Carlsbad, CA); *pfu* DNA polymerase and plasmid vector pBluescript I SK^+ (Stratagene, La Jolla, CA); restriction endonucleases (New England Biolab Inc., Ipswich, MA); Gel-extraction kit (QIAGEN, Valencia, CA); Bradford assay reagent (Bio-Rad, Hercules, CA); RT-PCR kit (Sigma-Aldrich, St. Louis, MO); expression vectors pET21a and pET28a (Novagen, Madison, WI); Lactosyl-agarose lectin affinity matrix (EY Laboratories, San Mateo, CA); Nickel-Sepharose™ 6 Fast Flow (GE Healthcare Biosciences Corp., Piscataway, NJ); 4-methylumbelliferyl-β-D-glucoside (4-MUGlc), *p*-nitrophenyl-β-D-glucoside (*p*NPGlc), and isopropyl β-D-1-thiogalactopyranoside (IPTG) (Research Products International Corp. Mount Prospect, IL)

2.7.2. Cloning and expression of chimeric β-glucosidase cDNAs

Cloning and expression of cDNAs encoding Glu1 and Dhr1 was as described by Cicek and Esen (Cicek and Esen, 1999). We constructed and used Dhr2 chimeras instead of Dhr1 chimeras because Dhr2 hydrolyzes the artificial substrates *p*NPGlc and 4-MUGlc, the two substrates we used to measure and detect β-glucosidase activity after FAC and gel-shift experiments, respectively, whereas Dhr1 does not. Complementary DNA encoding the mature sorghum β-glucosidase (Dhr2) was amplified by the polymerase chain reaction (PCR) using gene-specific primers (183 and 19, see Supplement Table 1), which incorporated NheI and HindIII sites into the 5' and 3' ends of PCR products, respectively. Chimeric β-glucosidase cDNAs (proteins diagrammed in Figure 2.2) were constructed by overlap extension PCR using wild type β-glucosidase cDNA as template (oligonucleotides used for chimeric β-glucosidase cDNA construction are listed in Supplement Table 1). The cDNA of wild type Glu1, Dhr2 and chimeric β-glucosidases were blunt end ligated to the SmaI site in pBluescript SK^+. After confirming the sequence of cloned cDNAs, inserts were isolated by digesting the above constructs with NheI and HindIII or XhoI, gel-purified and then ligated to the pET28a expression vector, which had been digested with the same set of restriction endonucleases. The expression construct was transformed into *E. coli* BL21 CodonPlus competent cells. Wild type Glu1, Dhr2, and chimeric β-glucosidase expressions were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.6 mM final concentration) at room temperature overnight. Cells were collected by centrifugation, suspended in extraction buffer (100 mM Tris-HCl, pH 8, containing 50 mM NaCl, 0.05% SDS), and lysed using a French press.

2.7.3. Purification of wild type and chimeric β-glucosidases

Wild type and chimeric β-glucosidases (all with a hexahistidine tag at the N-terminus) were purified by Ni^{2+}-chelation chromatography. In a typical experiment, imidazole (final concentration of 40 mM) was added to 30 ml of *E. coli* lysate containing recombinant proteins, incubated at 4°C for 10 min and applied to the column. After binding, the column was washed

with binding buffer (20 mM sodium phosphate buffer, pH 7.4 containing 500 mM NaCl and 40 mM imidazole), and bound protein was eluted with elution buffer (20 mM sodium phosphate buffer, pH 7.4 containing 500 mM NaCl and 500 mM imidazole). Fractions containing a high concentration of protein were combined and dialyzed against two changes of PBS, pH 7.0, overnight and kept at - 20°C until use.

2.7.4. Cloning and expression of BGAF cDNA in E. coli and purification of recombinant BGAF (rBGAF)

The cloning, expression and purification of BGAF cDNA in *E. coli* was described by Kittur et al. (Kittur et al., 2007). As mentioned before (Kittur et al., 2007), the expressed full-length BGAF contained an unexpected C-terminal His-tag. To obtain full-length BGAF with no extra amino acids, we re-cloned it by reverse transcriptase PCR (RT-PCR) using poly A^+ mRNA isolated from H95 seedling shoots on oligo-dT-coated magnetic beads (Invitrogen, Carlsbad, CA). The first strand of the BGAF cDNA was synthesized by RT-PCR using oligo-dT as primer and then the BGAF cDNA with full-length coding sequence was amplified using the primer pair 71 and 72 (see Supplement Table 1). The PCR product was blunt end-ligated to the SmaI site in pBluescript SK^+ and transformed into *E. coli* DH10B cells. Colonies containing recombinant plasmid were identified by colony PCR, and plasmids were isolated and sequenced at the VBI (Virginia Bioinformatics Institute, Virginia Tech) Core Facility. After confirming the sequence of the cDNA, it was excised, ligated to pET21a and transformed into *E. coli* BL21 CodonPlus cells for expression. For induction of protein production, IPTG was added to 500 ml of LB medium at 0.6 mM final concentration and the cells were incubated at room temperature overnight. *E. coli* lysate containing rBGAF was applied to a lactosyl-agarose column in PBS, pH 7.0, and the column was washed with the same buffer. Bound rBGAF was eluted with 100 mM lactose in PBS, pH 7.0. Fractions containing high concentration of protein were pooled and dialyzed against PBS, pH 7.0 overnight. The purity and size of rBGAF was checked by SDS-PAGE.

2.7.5 Protein determination and SDS-PAGE

Estimates of protein concentration were made with Bradford assay (Bradford, 1976) using BSA as standard (0-1.0 mg/ml concentration range). The micro assay format in 96-well flat bottom microtiter plates was employed as described by the vendor (Bio-Rad Laboratories, Hercules, USA). After 20 min of incubation of the dye and protein mixture, the absorbance was read at 595 nm on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA). Protein concentrations were also measured spectrophotometrically, using $\varepsilon_{280nm}^{1\% cm}$=18.7, 17.1 and 14.9 for Glu1, Dhr2, and BGAF, respectively (The extinction coefficients were calculated by a web-based tool: http://ca.expasy.org/tools/protparam.html). BGAF concentration values obtained by the Bradford assay were multiplied by a factor of 2.6 to correct for the underestimation of BGAF concentration by the Bradford method. Purified rBGAF was used in all assays unless otherwise stated. SDS-PAGE was performed according to the method of Laemmli (Laemmli, 1970), using minigels (1.0 mm thick, 1.5 cm stacking gel and 6.5 cm separating gel). SDS-PAGE was performed through a 10% gel in the presence of 2-mercaptoethanol. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250.

2.7.6. β-Glucosidase gel-shift assay

Wild type Glu1 and chimeras (C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, each 150 nM) were mixed with 7,800 nM of BGAF (52-fold molar excess) in 100 µl total volume in PBS, pH 7.0, and incubated at room temperature for 2 hrs. In the case of wild type Dhr2 and Dhr2 chimeras (C-10, C-11, and C-12), 500 nM of enzyme was incubated with 26,000 nM of BGAF. Following incubation, 25 µl of the reaction mixture was mixed with 5 µl of native sample buffer and electrophoresed through 8% native gel at 15 milliamps for 2 hrs. After electrophoresis, the gel was soaked in 50 mM citrate-100mM phosphate buffer, pH 5.8, for equilibration to the pH optimum for β-glucosidase activity. The gel was incubated with a 1 mM solution of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucoside (4-MUGlc) at room temperature for 10 min, and β-glucosidase activity zones were visualized under UV light. Glu1 (binder) and Dhr2 (non-binder) were used as positive and negative controls, respectively. When a wild type or

a chimeric enzyme interacts with BGAF and forms a stable complex, the mobility of the complex through native gel is retarded (shifts towards the cathodic end).

2.7.7. β-Glucosidase pull-down assay

Formation of large, insoluble β-glucosidase-BGAF complexes were detected by pull-down assay as described (Kittur et al., 2007). Briefly, a fixed concentration of wild type or a chimeric β-glucosidase (50 nM) was mixed with increasing concentrations of BGAF (0 – 1,040 nM) in PBS, pH 7.0, and incubated at room temperature for 2 hrs. After incubation, the reaction mixtures were centrifuged at 20,000g at 4 ℃ for 10 min, and the β-glucosidase activity remaining in the supernatant was determined using *p*NPGlc as substrate (Cicek et al., 2000) and expressed as percent activity with respect to control reaction (enzyme only) incubated without BGAF.

2.7.8. Frontal affinity chromatography (FAC) on BGAF-lactosyl agarose

The frontal affinity chromatography method for determining dissociation constants (K_d) was performed as described by Ohyama et al. (Ohyama et al., 1985). To prepare a BGAF-immobilized affinity column, a 10 cm long glass column was packed with 6 ml of lactosyl agarose. After washing extensively with PBS, pH 7.0, purified BGAF was applied to the column (BGAF is a lectin and it binds to the lactosyl agarose gel (Kittur et al., 2007)). Unbound BGAF was removed by washing with the same buffer, and the lactosyl-agarose matrix with immobilized BGAF was moved to a vial and kept at 4 °C until use. For FAC, 200 μl of BGAF-immobilized lactosyl agarose gel was packed in a column (4.5 x 70 mm; bed volume, 1 ml). FAC analysis was performed at room temperature. Wild type or chimeric β-glucosidases (150 to 250 nM) in PBS, pH 7.0 (30 ml) were applied to the column through a loop connected to a pump at a flow rate of 0.25 ml/min. Elution of the front was determined by monitoring β-glucosidase activity, which was measured as described (Cicek et al., 2000). For determination of void volume (V_0), Dhr2, which does not bind to BGAF, was used.

2.7.9. Determination of dissociation constant (K_d) using FAC data

The basic equation for determination of K_d has been described by Ohyama et al. (Ohyama et al., 1985).

$$V = \frac{B_t}{K_d + [A]_0} + V_0$$

Where A denotes the analyte, and $[A]_0$ is the concentration of the analyte (i.e., wild type and chimeric β-glucosidases) applied to the column. B_t is total concentration of ligand (i.e., BGAF) in the column that interacts with analyte. V and V_0 are the elution volume of the front for binder (e.g. Glu1) and the elution volume of the front for non-binder (e.g. Dhr2) (void volume), respectively, and K_d is the dissociation constant. If K_d, B_t, and V_0 are constant, V will change depending on $[A]_0$, and the above equation can be rearranged as follows, so that the plot of $1/[A]_0(V-V_0)$ versus $1/[A]_0$ will be linear.

$$\frac{1}{[A]_0\,(V - V_0)} = \frac{K_d}{B_t} \cdot \frac{1}{[A]_0} + \frac{1}{B_t}$$

The values of K_d and B_t are determined from the intercept on the abscissa and the ordinate of the plot, respectively. Once B_t has been determined, K_d for other enzymes can be calculated by putting the V value for each enzyme into the equation below.

$$K_d = \frac{B_t}{V - V_0} - [A]_0$$

2.7.10. Effect of pH and ionic strength on β-glucosidase-BGAF interaction

To determine the effect of pH on Glu1-BGAF interaction, 250 nM of wild type Glu1 was prepared in the following buffers: 50 mM sodium citrate, pH 4.0; 50 mM sodium acetate, pH 5.0; 2-(N-morpholino)-ethanesulfonic acid (MES), pH 6.0; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0; 50 mM Tris-HCl, pH 8.0; 2-(cyclohexylamino)

ethanesulfonic acid (CHES), pH 9.0. The BGAF immobilized lactosyl-agarose column was equilibrated with the above buffers before applying Glu1 (30 ml), and FAC analysis was performed as described above. To investigate the effect of ionic strength on Glu1-BGAF interaction, NaCl was added to solutions of Glu1 (250 nM in HEPES buffer, pH 7.0) to final concentrations of 0, 50, 140, 350, and 700 mM. FAC analysis was performed and K_d values were computed as described above.

2.7.11. Determination of stoichiometry of Glu1-BGAF complexes

The basic procedure for determination of stoichiometry by gel-shift assay was described by Weeks *et al.* (Weeks et al., 1990) and Wang *et al.* (Wang et al., 1995). A constant amount of Glu1 (400 nM) was mixed with increasing concentrations of BGAF (0 – 1000 nM), or a constant concentration of BGAF (400 nM) was mixed with increasing concentrations of Glu1 (0 – 1000 nM) in a total volume of 100 µl in PBS, pH 7.0, and incubated at room temperature for 2 hrs. The reaction mixtures were then subjected to gel-shift analysis as described before to determine the minimum concentration of Glu1 or BGAF required to completely titrate BGAF or Glu1. From this, the stoichiometry of BGAF-Glu1 interaction was computed.

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2.9 Figures

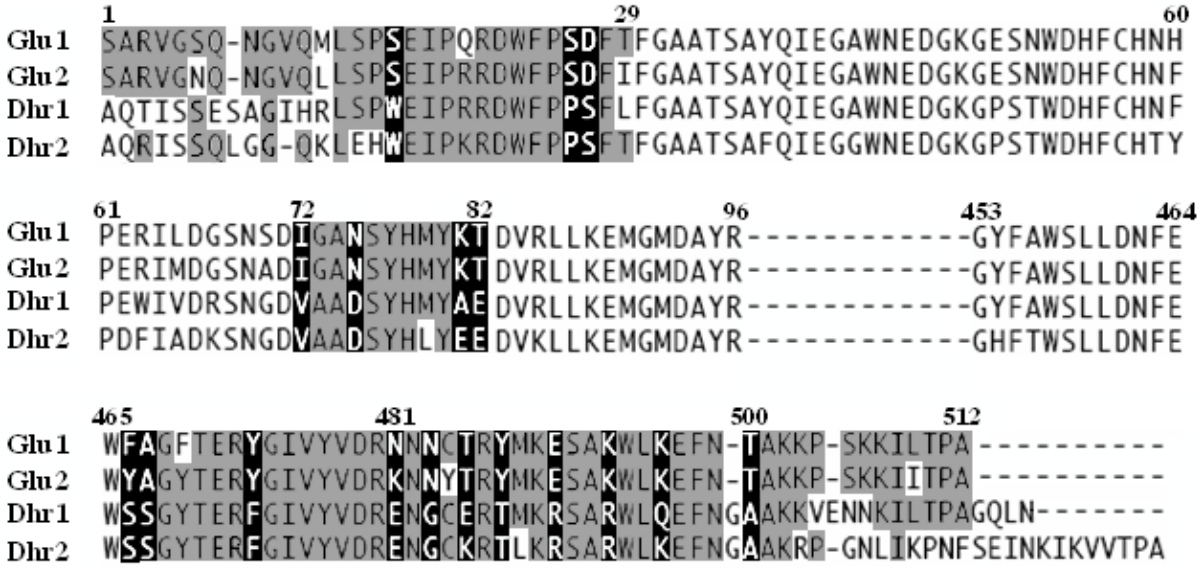


Figure 2.1: Sequence alignment of the N-terminal (1-96) and C-terminal (453-512) regions of maize (Glu1 and Glu2) and sorghum (Dhr1 and Dhr2) β-glucosidases. The three regions (Ser[1]-Thr[29], Ile[72]-Thr[82], and Phe[466]-Ala[512]) making up the BGAF-binding site in Glu1 are in *Gray* background. In these regions, the residues highlighted in *Black* background are postulated to be involved in BGAF binding.

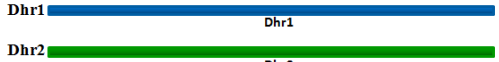
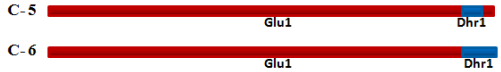
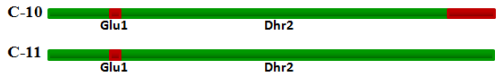
Chimeric β-Glucosidases	Swapped regions	BGAF binding (Gel-shift assay) Binding	Comments	Pull-down assay	Role in BGAF binding	Affinity (K_d) (nM)
 Glu1		+	smear	++		111 ± 11
 Dhr1		–		–		NB[a]
 Dhr2		–		–		NB
C-1 	Ile^{72}-Thr^{82}, Phe^{466}-Ala^{512}	–		–	+	NB
C-2 	Ile^{72}-Thr^{82}	–		–	+	NB
C-3 	Phe^{466}-Ala^{512}	+	discrete band	–	–	256 ± 37
C-4 	Phe^{466}-Arg^{480}	+	smear	+++	–	182 ± 10
C-5 	Asn^{481}-Thr^{500}	+	smear	+	–	347 ± 48
C-6 	Asn^{481}-Ala^{512}	+	discrete band	–	–	372 ± 22
C-7 	Phe^{466}-Thr^{500}	+	smear	+++	–	280 ± 15
C-8 	Ser^{1}-Thr^{29}	+	discrete band	–	–	197 ± 0.9
C-9 	Ser^{1}-Thr^{29}, Phe^{466}-Ala^{512}	+	discrete band	–	–	547 ± 42
C-10 	Val^{72}-Glu^{82}, Ser^{460}-Ala^{519}	+	smear	++	+	ND[b]
C-11 	Val^{72}-Glu^{82}	+	discrete band	–	+	ND
C-12 	Ser^{460}-Ala^{519}	–		–	–	NB

Figure 2.2: Line diagrams of wild type and chimeric β-glucosidases constructed to determine the BGAF binding site in Glu1. The peptide segments swapped among Glu1, Dhr1 and Dhr2 to generate chimeric enzymes are identified under the column *Swapped regions*. The results of BGAF binding assays (+ for binding and – for not binding) are given under the column *BGAF binding (gel-shift assay)*. The results of pull-down assays (presence (+) or absence (–) of insoluble complexes; +, ++, +++: intensity) are described under the column *Pull-down assay*. The role for each swapped region for BGAF binding is summarized under the column *Role in BGAF binding* (+ for essential and – for not essential). The dissociation constant (K_d) is given under the column *Affinity* to show the importance of swapped regions and is the average of 2 measurements ± SD. (NB[a], no binding; ND[b], not determined).

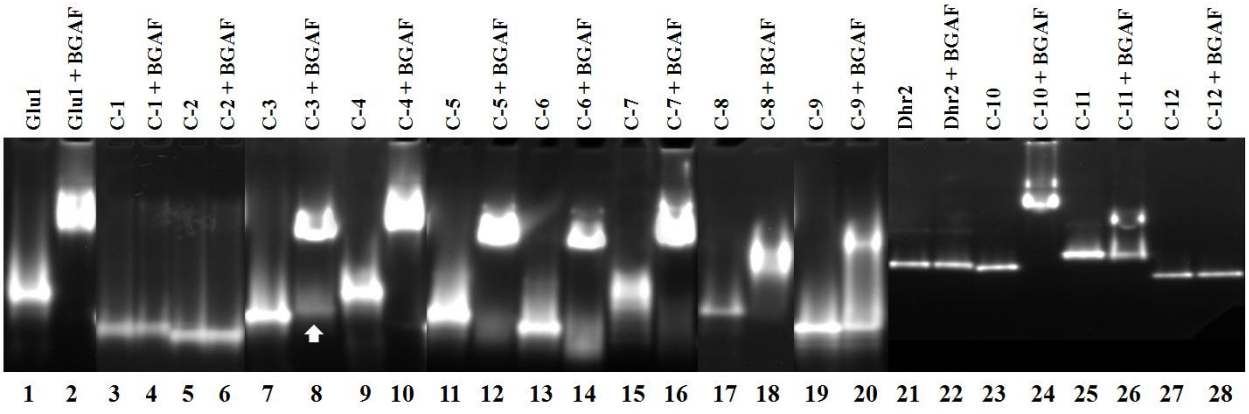


Figure 2.3: Gel-shift assay to detect binding of wild type and chimeric β-glucosidases to BGAF. Activity zones with retarded mobility (smearing and discrete bands) in lanes containing enzyme + BGAF correspond to the Glu1-BGAF complexes. Note that the arrowhead in *lane 8* is indicating the position of unreacted C-3.

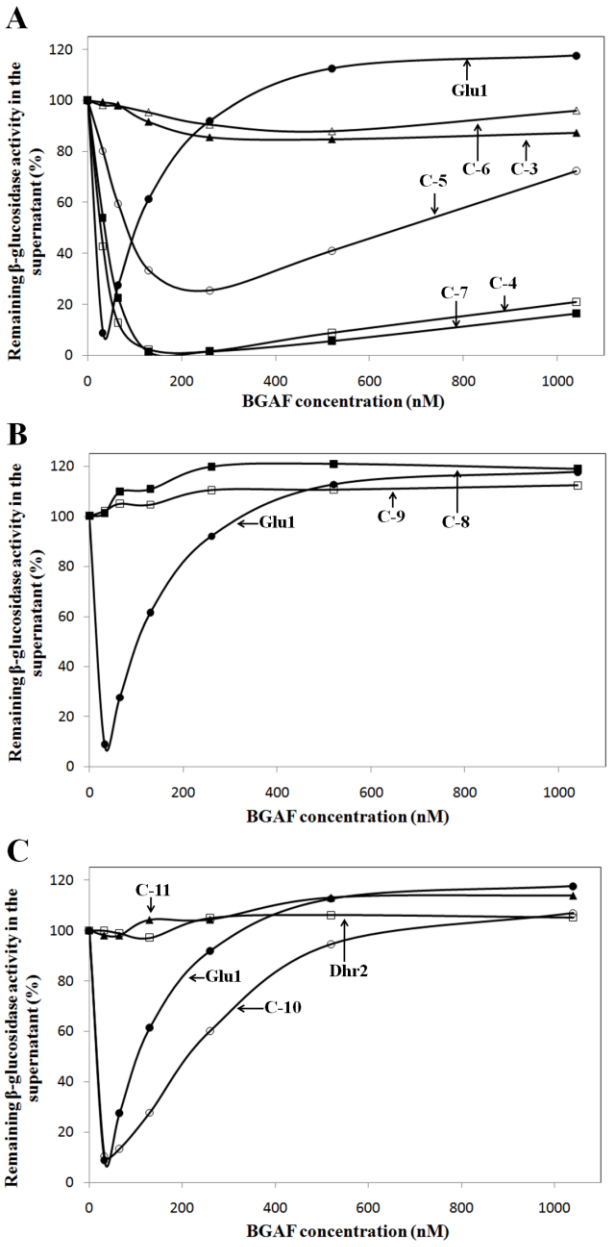


Figure 2.4: Pull-down assay to detect aggregation of chimeric β-glucosidases by BGAF. (A) Precipitation of Glu1 chimeras (see Figure 2.2 for details) by BGAF; Glu1 (●), C-3 (▲), C-4 (□), C-5 (○), C-6 (Δ), and C-7 (■). (B) Precipitation of Glu1 chimeras by BGAF; Glu1 (●), C-8 (■), and C-9 (□). (C) Aggregation of Dhr2 chimera by BGAF; Glu1 (●), Dhr2 (□), C-10 (○), and C-11 (▲).

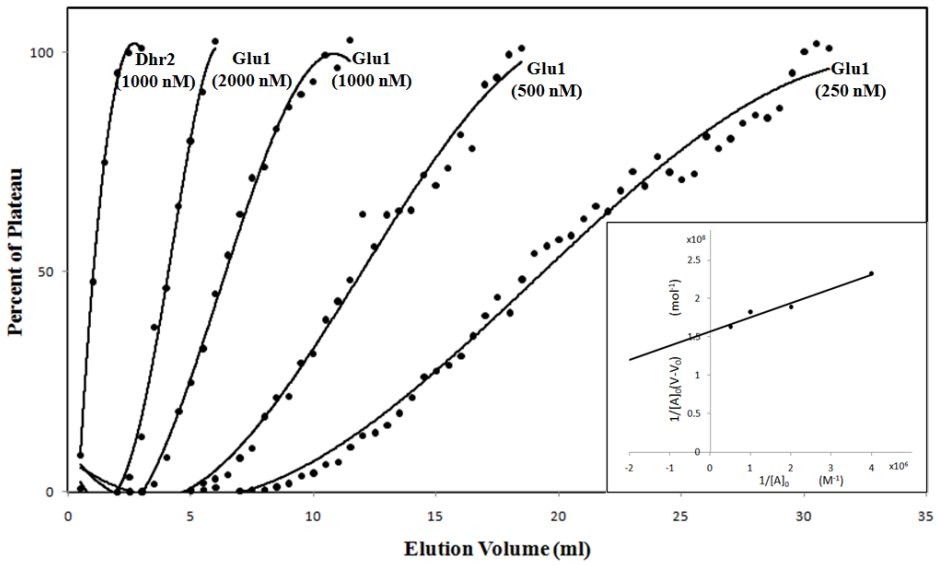


Figure 2.5: FAC profiles of wild type Glu1 on BGAF immobilized lactosyl-agarose column. Glu1 solutions of different concentrations (as noted in the Figure) in PBS, pH 7.0 were applied to the column. β-Glucosidase elution was monitored by measuring β-glucosidase activity as described previously (Cicek et al., 2000). The first graph on left is the elution profile of Dhr2 (1000 nM), which was used to determine the void volume (V_0). *Inset,* $1/[A]_0(V\text{-}V_0)$ *versus* $1/[A]_0$ plot. From plot of $1/[A]_0(V\text{-}V_0)$ vs. $1/[A]_0$, K_d value was computed as described in *Materials and Methods*.

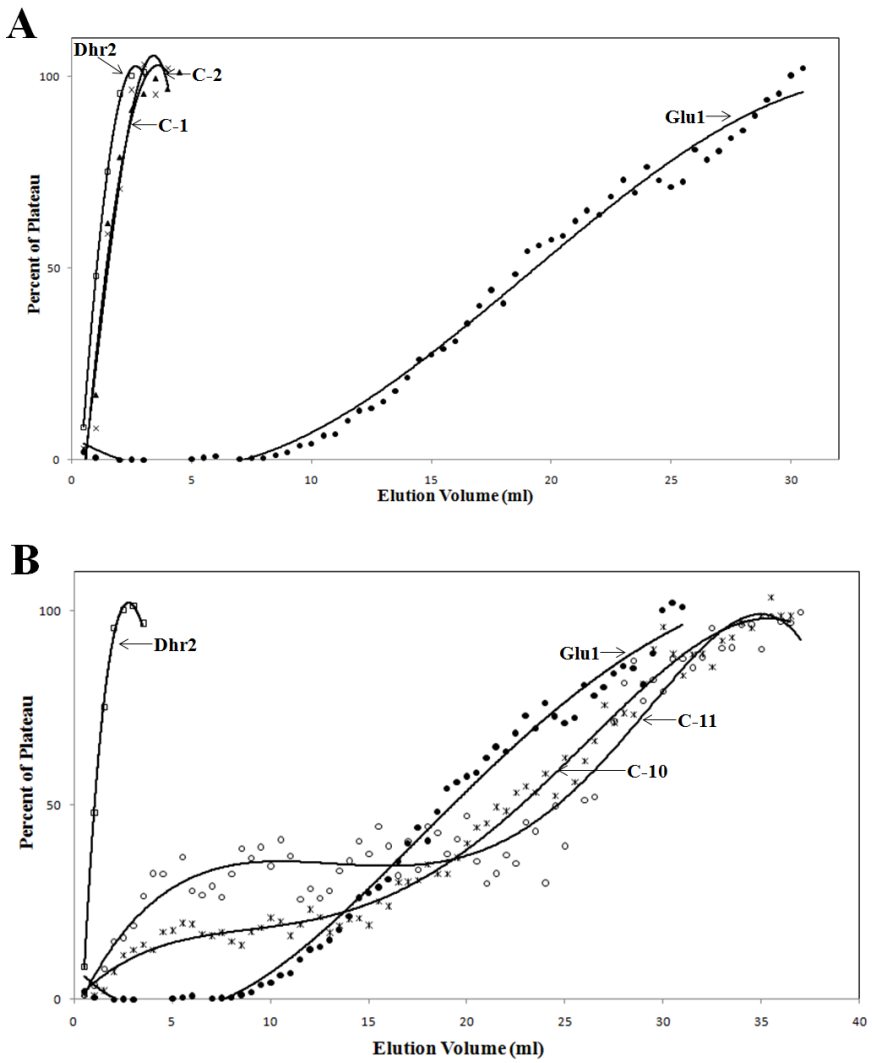


Figure 2.6: FAC profiles of wild type and chimeric β-glucosidases. (A) FAC profiles of Glu1 (●), Dhr2 (□), C-1 (▲), and C-2 (×). (B) FAC profiles of Glu1 (●), Dhr2 (□), C-10 (✳), and C-11 (○).

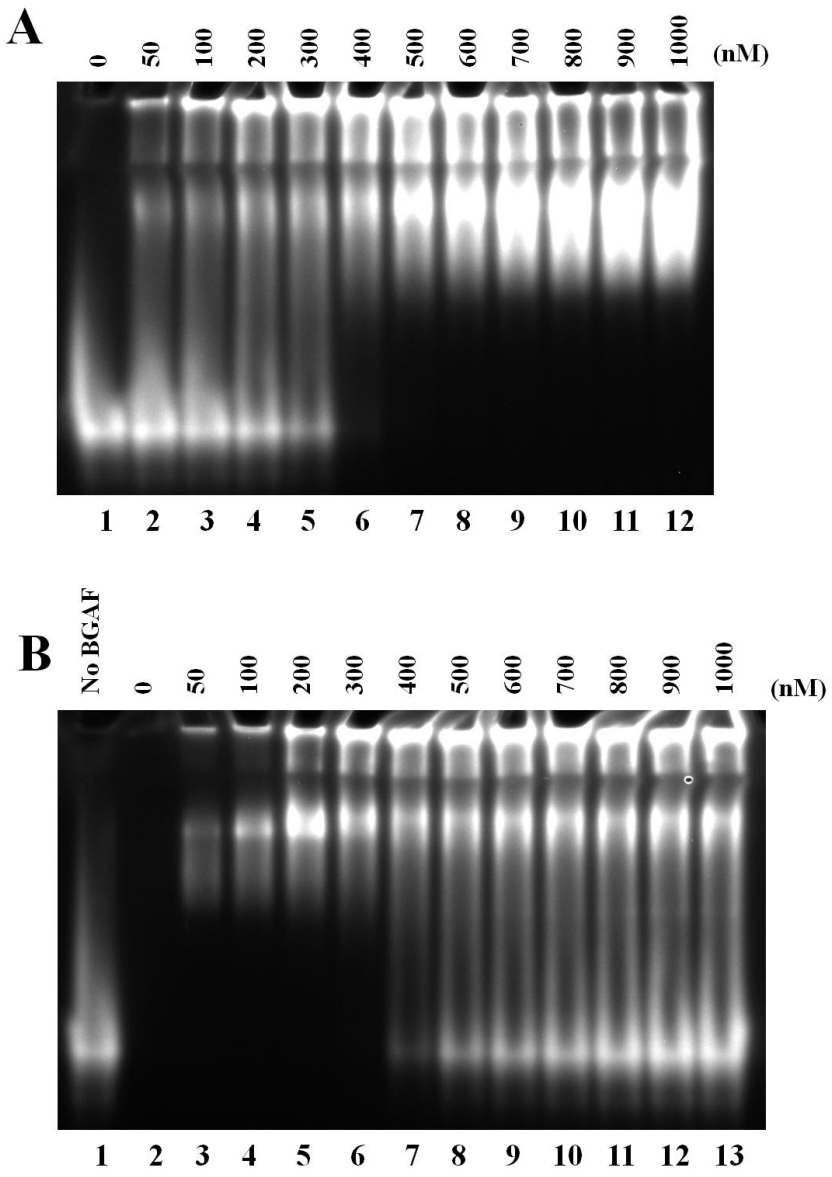


Figure 2.7: Determination of stoichiometry of Glu1-BGAF complexes by gel-shift assay. (A) Constant amount of Glu1 (400 nM) was incubated with increasing amounts of BGAF (0 – 1,000 nM, BGAF amount shown on top). (B) Constant amount of BGAF (400 nM, except *lane 1* (only Glu1)) was mixed with increasing amounts of Glu1 (0 – 1,000 nM, Glu1 amount shown above the picture).

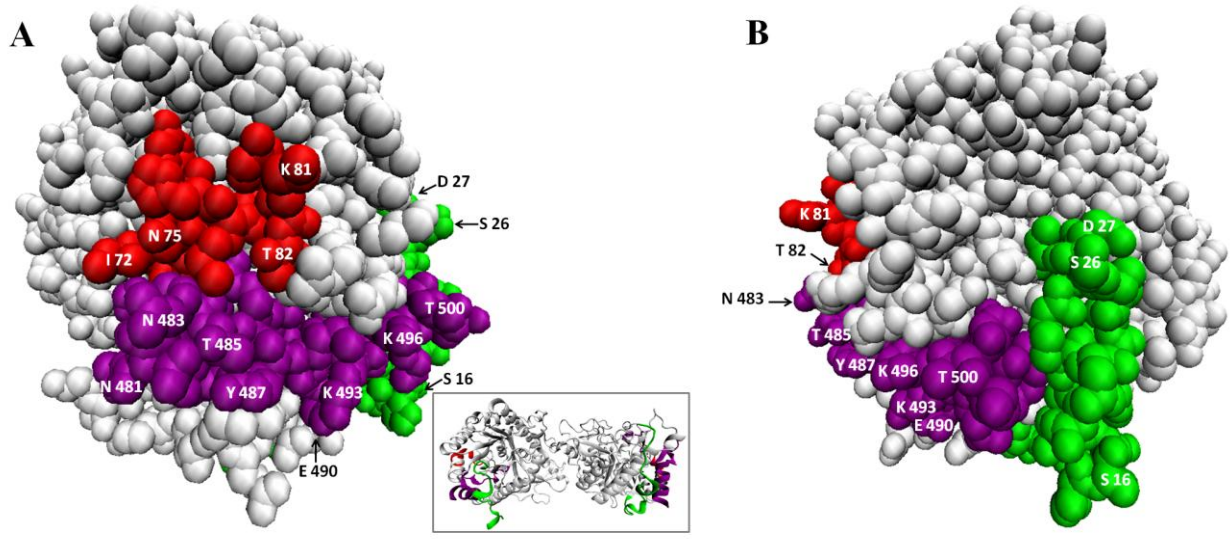
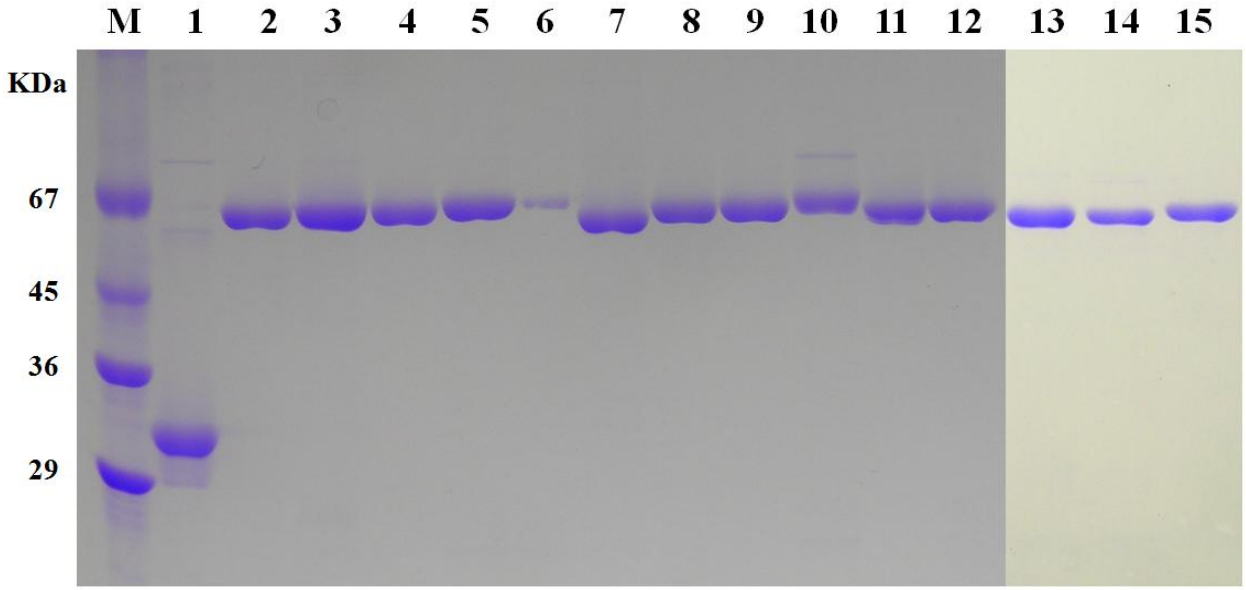
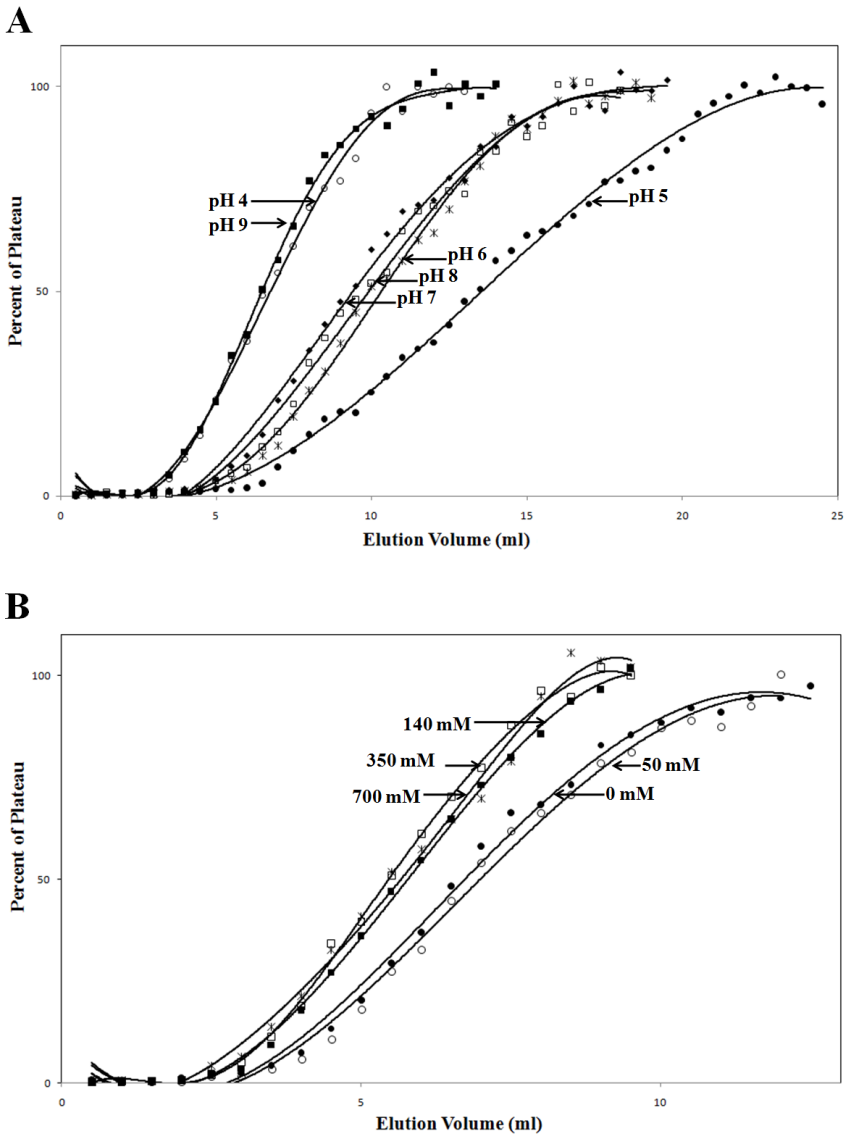


Figure 2.8: The crystal structure of maize Glu1 (PDB entry 1E1E) showing the region contributing to the BGAF binding site. The extreme N-terminal region (Ser^{1}-Thr^{29}), N-terminal region (Ile^{72}-Thr^{82}), and C-terminal region (Phe^{466}-Ala^{512}) are shown in *green*, *red* and *purple*, respectively. The amino acid residues that differ between Glu1 and Dhr1 or Dhr2 and are postulated to be involved in BGAF binding are identified by their position number. To create this view, Visual Molecular Dynamics (VMD) was used. (A) Front view of Glu1 (space-filling model) (*Inset*, dimeric form of Glu1 (NewCartoon model)). (B) Side view of Glu1 (space-filling model).

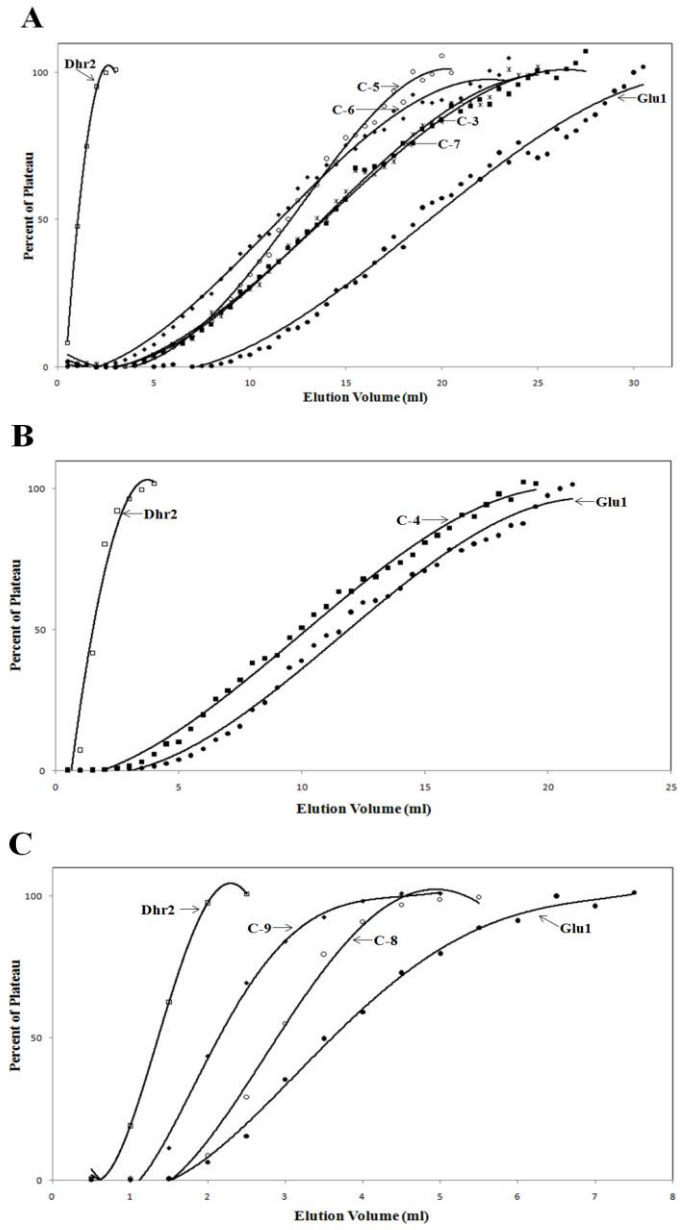
2.10 Supplement Figures and Table



Supplemental Figure 2.1: SDS-PAGE profiles of purified wild type and chimeric β-glucosidases. *Lane M*, Molecular weight markers; *lane 1*, BGAF; *lane 2*, wild type maize Glu1; *lane 3*, wild type sorghum Dhr2; *lane 4*, C-3; *lane 5*, C-1; *lane 6*, C-2; *lane 7*, C-10; *lane 8*, C-5; *lane 9*, C-6; *lane 10*, C-7; *lane 11*, C-11; *lane 12*, C-12; *lane 13*, C-8; *lane 14*, C-4; *lane 15*, C-9.



Supplemental Figure 2.2: Effect of pH (A) and ionic strength (B) on elution profiles (FAC profiles) of Glu1 from BGAF immobilized lactosyl agarose column. (A) Effect of pH; sodium citrate, pH 4.0 (○), NaAc, pH 5.0 (●), MES, pH 6.0 (✳), HEPES, pH 7.0 (♦), Tris-HCl, pH 8.0 (□), and CHES, pH 9.0 (■). (B) Effect of ionic strength; 0 mM NaCl (●), 50 mM NaCl (○), 140 mM NaCl (■), 350 mM NaCl (□), and 700 mM NaCl (✳).



Supplemental Figure 2.3: FAC profiles of wild type and chimeric β-glucosidases. (A) Elution profiles of wild type and chimeric β-glucosidases; Glu1 (●), Dhr2 (□), C-3 (■), C-5 (○), C-6 (♦), and C-7 (✳). (B) Elution profile of Glu1 (●), Dhr2 (□), and C-4 (■). (C) Elution profiles of Glu1 (●), Dhr2 (□), C-8 (○), and C-9 (♦). Dissociation constants (K_d) for wild type and chimeric β-glucosidase are summarized in Figure 2.2.

Supplemental Table 2.1: Oligonucleotide primer pairs (S, sense; A, antisense) used to construct wild type and chimeric β-glucosidase cDNAs

Construct	Sense[a]	Antisense[a]	Template used
WT Glu1	111	113	Glu1
WT Dhr1	123	124	Dhr1
WT Dhr2	183	19	Dhr2
BGAF	71	72	BGAF cDNA
Chimeric Glu1			
C-1	1 & 111	2 & 124	C-3
C-2	1 & 111	2 & 113	Glu1
C-3	100 & 111	101 & 124	Glu1 & Dhr1
C-4	35 & 111	36 & 113	Glu1
C-5	7, 9 & 111	8, 10 & 113	Glu1& Dhr1
C-6	7 & 111	8 & 124	Glu1 & Dhr1
C-7	9 & 111	10 & 113	Glu1 & Dhr1
C-8	110 & 123	62 & 113	Glu1 & Dhr1
C-9	123 & 109	57 & 124	C-3 & C-8
Chimeric Dhr2			
C-10	5, 109 & 183	6, 57 & 113	Dhr2 & Glu1
C-11	5 & 183	6 & 19	Dhr2 & Glu1
C-12	183 & 109	57 & 113	Dhr2 & Glu1

Primer ID #	Primer Sequence[b]
1	CAGACGTTGCAGCGGATTCGTATCATATGTACGCTGAGGACGTCAGATTG
2	CAATCTGACGTCCTCAGCGTACATATGATACGAATCCGCTGCAACGTCTG
5	CAATGGGGACATTGGAGCGAATTCGTACCATATGTACAAAACGGATGTAAA ATTGC
6	TTTTACATCCGTTTTGTACATATGGTACGAATTCGCTCCAATGTCCCCATTG
7	CTACGTCGACCGCGAGAATGGCTGCGAG
8	CAGCCATTCTCGCGGTCGACGTAGACAATG
9	GTTCAATGGAGCGAAAAAGCCCAGCAAG
10	CTGGGCTTTTTCGCTCCATTGAACTCCTGC
19	CTATAAGCTTTTAAGCTGGCGTAAGAATCTTC
35	CTTTGAATGGTCCAGCGGCTACACCGAGCGTTTCGGCATTGTCTAC
36	GTAGACAATGCCGAAACGCTCGGTGTAGCCGCTGGACCATTCAAAG
57	AAGTTGTCCAGGAGAGACCA
62	TGGTACGCTGAAGTGGCGGCAC
71	GAGAGCCATATGGCCAGCCTCCAAGTCAC
72	GTGAGACTCGAGTCACAGGGATCGCACGTAAAC
100	GGCTACTTCGCGTGGTCTCT
101	AGAGACCACGCGAAGTAGCC
109	TGGTCTCTGCTGGACAACTT
110	TRCCGCCRCTTCAGCGTACCA
111	ACTACAGCTAGCGCAAGAGTAGGCAGCCAAAAT
113	CTATCTCGAGTTAAGCTGGCGTAAGAATCTTC
123	GCCACGAGCTAGCACGATAAGCAGTGAG
124	AGCAGCTCGAGTTCTACTTAATTAGTTAAGC
183	TCACGAGCTAGCAGGATAAGCAGTCAG

[a]numbers in the sense and antisense columns represent primers used in PCR

[b]primer sequences are from 5' to 3'. Underlined bases indicate where the restriction sites (NheI/ NdeI and XhoI/HindIII at 5' and 3' ends, respectively) were introduced.

Chapter 3

3.1 Title Page

Lysine-81 and Threonine-82 on Maize β-Glucosidase Isozyme Glu1 Are the Key Amino Acids Involved in β-Glucosidase Aggregating Factor Binding.

Hyun Young Yu[‡], Farooqahmed S. Kittur[‡], David R. Bevan[§], and Asim Esen[‡]*

From the [‡]Department of Biological Sciences and [§]Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0406

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Biochemistry. 48: 2924-2932

3.2 Abstract

In certain maize genotypes (nulls), β-glucosidase specifically interacts with a chimeric lectin called β-glucosidase aggregating factor (BGAF) resulting in high molecular weight complexes. Previously, we showed that three regions (S1-T29, E50-N127, and F466-A512) on the maize β-glucosidase isozyme Glu1 are involved in interaction and aggregation with BGAF. Recently, we found that the peptide span I72-T82 within E50-N127 is essential and sufficient for BGAF binding, whereas the S1-T29 and F466-A512 regions are required for formation of large complexes. To define the contribution of individual amino acids in the above three regions to BGAF binding, we constructed mutant β-glucosidases based on sequence differences between maize β-glucosidase and sorghum β-glucosidase (dhurrinase 2, Dhr2), which does not bind BGAF. Binding was evaluated by gel-shift assay and affinity by frontal affinity chromatography (FAC). In the gel-shift assay, Glu1 mutants K81E and T82Y failed to bind BGAF, and their FAC profiles were essentially similar to that of Dhr2, indicating that these two amino acids within the I72-T82 region are important for BGAF binding. Substitution of N481 with E (as in Dhr2) lowered affinity for BGAF, whereas none of the mutations in the S1-T29 region showed any effect on BGAF binding. To further confirm the importance of K81 and T82 for BGAF binding, we produced a number of Dhr2 mutants, and the results showed that all four amino acids (I72, N75, K81, and T82) that differ between Glu1 and Dhr2 in the peptide span I72-T82 are required to impart BGAF binding ability to Dhr2.

3.3 Introduction

Family 1 β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) are of common occurrence in all three domains (Archaea, Eubacteria, and Eukarya) of living organisms. They catalyze the hydrolysis of glycosidic bonds in aryl and alkyl β-D-glucosides and cellobiose or short gluco-oligosaccharides (*1*). The physiological functions of plant β-glucosidases are reasonably well established. The key function of these enzymes is defense against pathogens and herbivores (*2-4*). In maize, β-glucosidases play a key role in defense against pests by hydrolyzing natural β-glucosides. The main natural β-glucoside in maize is 2-*O*-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOAGlc), which is found mostly in young seedling parts. When DIMBOAGlc is hydrolyzed by the enzyme, a toxic aglycone, DIMBOA, is released, which is the major defense chemical in maize against aphids and the European corn borer (*5, 6*). Although the main function of β-glucosidases in plants is defense, they also have a diverse array of other physiological roles such as lignification (*7-9*), floral development (*10*), activation and degradation of phytohormones (*11-13*). To match this functional diversity, a typical plant (e.g., Arabidopsis, rice, poplar, grape, and others) produces more than 40 β-glucosidase isoforms encoded by a multigene family whose members are subject to temporal and spatial regulation. β-Glucosidases also have significance for biotechnological applications, e. g., aroma and flavor improvement in tea (*14*) and wine (*15*), and cellulose degradation (*16*) for biofuel production.

In some maize genotypes, β-glucosidases interact with a chimeric lectin called β-glucosidase aggregating factor (BGAF) and form high molecular weight complexes that are mostly insoluble aggregates (*17*). BGAF is comprised of an N-terminal dirigent (disease response) domain and a C-terminal jacalin-related lectin (JRL) domain. The JRL domain of BGAF is involved in binding to β-glucosidase, but the JRL domain alone fails to cause aggregation because it is a monomer, whereas BGAF is a dimer. To produce aggregating complexes with β-glucosidases, the dirigent domain is required, and thus the dirigent domain is indirectly involved in β-glucosidase interaction and aggregation. The JRL domain also has a sugar binding site, but the sugar and β-

glucosidase binding sites are separate (*18*). The physiological role of BGAF is not known at this time, but recently, the Hessian fly resistance (*Hfr-1*) gene product, a BGAF homolog from wheat, was shown to prevent Hessian fly larvae from feeding on resistant plants (*19*).

The maize β-glucosidase isozymes Glu1 and Glu2 have high sequence identity (~ 70%) with the sorghum β-glucosidase isozymes dhurrinase 1 and 2 (Dhr1 and Dhr2) (*20*). However, they differ with respect to substrate specificity and BGAF binding. Maize β-glucosidases hydrolyze a broad spectrum of natural and artificial substrates, including DIMBOAGlc, *p*-nitrophenyl-β-D-glucoside (*p*NPGlc), 4-methylumbelliferyl-β-D-glucoside (4-MUGlc) and others, but do not hydrolyze dhurrin, a natural substrate of sorghum β-glucosidases. In contrast, sorghum β-glucosidases exhibit narrow substrate specificity in that they hydrolyze their natural substrate dhurrin. Dhr2, but not Dhr1, shows weak activity on certain artificial substrates, including *p*NPGlc and 4-MUGlc. Although dhurrin is not hydrolyzed by Glu1 and Glu2, and DIMBOAGlc is not hydrolyzed by Dhr1 and Dhr2, dhurrin is a potent competitive inhibitor of Glu1 and Glu2 as is DIMBOAGlc of Dhr1 and Dhr2 (*20*). With respect to BGAF binding, maize β-glucosidases can bind BGAF with high affinity, but sorghum β-glucosidases do not (*21*). This difference provides an ideal model system to address questions related to BGAF binding specificity and mapping the BGAF binding site on maize β-glucosidases.

Previously, the BGAF-binding site on maize β-glucosidase was broadly mapped by Blanchard *et al.* (*21*). The results indicated that an N-terminal region (E50-N127) and a C-terminal region (F466-A512) of Glu1 play a key role in BGAF binding. Recently, we found that the N-terminal region I72-T82 on Glu1 is sufficient for BGAF binding, whereas the C-terminal region (F466-A512) plays a minor role in binding BGAF (in review). The BGAF binding site is away from the active site and the interaction does not affect enzyme activity (*21*, *22*), suggesting that BGAF binding does not block the active site or cause significant conformational change in the enzyme. Although we knew the general BGAF binding regions on Glu1, the contribution of individual amino acids within these regions responsible for BGAF binding was not defined (Figure 3.1).

We hypothesized that amino acid substitutions in peptide spans (S1-T29, I72-T82, and F466-A512) are responsible for differences in the BGAF-binding ability of maize and sorghum β-

glucosidases. If true, replacement of variant amino acids within these regions in Glu1 with the corresponding residues of Dhr1 or Dhr2 should abolish BGAF interaction, whereas their replacement in Dhr1 or Dhr2 with the corresponding amino acids of Glu1 may confer BGAF binding ability to Dhr1 and Dhr2. To identify and determine the contribution of individual amino acids on Glu1 to BGAF binding, we performed site directed mutagenesis (SDM) experiments and evaluated the interaction of mutant enzymes with BGAF by gel-shift assay and frontal affinity chromatography (FAC). Our gel-shift assay and FAC data showed that K81 and T82 are the most important amino acids in Glu1 for BGAF binding because their replacement with the corresponding amino acids from Dhr1 or Dhr2 resulted in complete abolishment of binding or sharply decreased affinity for BGAF. Furthermore, introducing K81 and T82 along with I72 and N75 of Glu1 in Dhr2 conferred upon it the ability to bind BGAF.

3.4 Experimental Procedures

Materials – *pfu* DNA polymerase and plasmid vector pBluescript I SK^+ were purchased from Stratagene (La Jolla, CA). All restriction endonucleases were from New England Biolab Inc. (Ipswich, MA). T4 DNA ligase was purchased from Invitrogen (Carlsbad, CA). The expression vectors pET21a and pET28a were purchased from Novagen (Madison, WI). The protein purification column, lactosyl-agarose affinity matrix and Nickel-Sepharose^{TM} 6 Fast Flow were purchased from EY Laboratories (San Mateo, CA) and GE Healthcare Biosciences Corp. (Piscataway, NJ), respectively. Artificial substrates, 4-methylumbelliferyl-β-D-glucoside (4-MUGlc) and *p*-nitrophenyl-β-D-glucoside (*p*NPGlc), and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from Research Products International Corp. (Mount Prospect, IL). Bradford assay reagent was purchased from Bio-Rad (Hercules, CA).

Cloning, expression and purification of mutant β-glucosidase – Cloning and expression methodology used for wild type, chimeric, and mutant β-glucosidases was as described by Cicek *et al.* (*23*). We constructed Dhr2 mutants instead of Dhr1 because Dhr2 hydrolyzes two artificial

substrates, *p*NPGlc and 4-MUGlc (used in FAC and gel-shift experiments, respectively), whereas Dhr1 does not. Mutant β-glucosidase cDNAs (Figure 3.2) were constructed by overlap extension PCR using wild type Glu1 or Dhr2 cDNA as template. The cDNAs of wild type Glu1, Dhr2 and mutant β-glucosidases were blunt end ligated to the SmaI digested pBluescript SK^+. The cloned cDNAs was sequenced at the VBI (Virginia Bioinformatics Institute, Virginia Tech) Core Facility, and inserts were isolated by digesting the above constructs with NheI and HindIII or XhoI, gel-purified and then ligated to the pET28a expression vector, which had been digested with the same set of restriction endonucleases. The expression construct was transformed into *E. coli* BL21 CodonPlus competent cells. Wild type Glu1, Dhr2, and mutant β-glucosidase expressions were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.6 mM final concentration) at room temperature for overnight. Cells were collected by centrifugation, suspended in extraction buffer (100 mM Tris-HCl, pH 8, containing 50 mM NaCl, 0.05% SDS), and lysed using a French press. Wild type and mutant β-glucosidases (all with His-tag at the N-terminus) were purified by Ni^{2+}-chelation chromatography. Fractions containing high concentration of protein were combined and dialyzed against two changes of PBS, pH 7.0, overnight. To ascertain proper folding of the mutant enzymes, the enzyme activities of all mutants were assayed and found to be similar to those of the wild type enzymes (data not shown).

Cloning and expression of BGAF cDNA in E. coli and purification of recombinant BGAF (rBGAF) – Cloning, expression and purification of recombinant BGAF in *E. coli* were as described by Kittur *et al*. (*18*). Recombinant BGAF was used for all experiments described in the present work.

Protein concentration determination – Protein concentration was determined with the Bradford assay (*24*) according to manufacturer's protocol (Bio-Rad (Hercules, CA)). Protein concentrations were also measured spectrophotometrically, using $\varepsilon_{280nm}^{1\%\,cm}$=18.7, 17.1 and 14.9 for Glu1, Dhr2, and BGAF, respectively (The extinction coefficients were computed by web-based tool: http://ca.expasy.org/tools/protparam.html). Because of underestimation of BGAF

concentration by the Bradford method, the protein concentration values obtained by Bradford assay were multiplied by a factor of 2.6 to correct for BGAF concentration.

β-Glucosidase gel-shift assay – Gel-shift assay for β-glucosidase-BGAF interaction was carried out as described by Blanchard *et al.* (*21*) and Kittur *et al.* (*18*). Briefly, wild type Glu1 and its N-terminal (I72-T82) region mutants (I72V, N75D, K81A, T82E, K81E, T82S, and T82Y, each 150 nM) were mixed with 7,800 nM of BGAF in 100 µl total volume in PBS, pH 7.0, and incubated at room temperature for 2 hrs. In the case of wild type Dhr2 and Dhr2 mutants (Dhr2 mutant #1 (E82T), Dhr2 mutant #2 (E81K/E82T), Dhr2 mutant #3 (V72I/D75N), Dhr2 mutant #4 (V72I/E81K/E82T), Dhr2 mutant #5 (D75N/E81K/E82T), and Dhr2 mutant #6 (V72I/D75N/E81K/E82T), see Figure 3.2), 500 nM of each mutant enzyme was incubated with 39,000 nM of BGAF. The extreme N-terminal (S1-T29) and C-terminal (F466-A512) region mutants (N481E, N483G, T485E, T485K, Y487T, E490R, T500GA (this construct was made by substituting T500 of Glu1 with GA of Dhr1/Dhr2), S16W, S26P, and D27S, each 150 nM) were mixed with 390 nM of BGAF. After 2-hr incubation, 25µl of the mixture was added to 5µl of native gel sample buffer and electrophoresed through an 8% native gel at 15 mA for 2 hrs. The native gel was first equilibrated with two changes of 50 mM citrate-100mM phosphate buffer, pH 5.8, then incubated with 1 mM solution of the fluorogenic substrate 4-MUGlc at room temperature for 10 min, and β-glucosidase activity zones were visualized under UV light (*21*). Wild type Glu1 and Dhr2 were used as positive and negative controls, respectively.

Frontal affinity chromatography (FAC) and determination of dissociation constant (K_d) – Frontal affinity chromatography for determining dissociation constants (K_d) was as described by Ohyama *et al.* (*25*), with slight modification. Briefly, 6 ml of lactosyl agarose gel was packed in a 10 cm long glass column and the column was washed with PBS, pH 7.0 for equilibration. Purified BGAF was applied to the column (BGAF is a chimeric lectin and binds to lactosyl agarose column (*18*)) and washed with the same buffer to remove unbound BGAF. BGAF-bound lactosyl agarose was transferred to a vial and kept at 4℃ until use. For FAC, 200 µl of BGAF-immobilized lactosyl agarose gel was packed in a column (4.5 x 70 mm; bed volume, 1 ml).

Wild type or mutant β-glucosidases (30 ml of 250 nM solution) in PBS, pH 7.0, were applied to the column at a flow rate of 0.25 ml/min at room temperature. Elution of the front was monitored by measuring β-glucosidase activity in the eluate as described by Cicek *et al.* (*22*). Wild type Dhr2 (non-binder) was used for determination of the void volume (V_0).

Determination of dissociation constant (K_d) using FAC data – The equation for determination of K_d has been described by Ohyama *et al.* (*25*).

$$V = \frac{B_t}{K_d + [A]_0} + V_0$$

The analyte is denoted as A and the concentration of the analyte applied to the column is referred to as $[A]_0$ (i.e., Glu1, Dhr2, and mutant β-glucosidases). Total concentration of ligand in the column is denoted as B_t (i.e., BGAF). The elution volume (for binder) and the void volume (for non-binder) of the front refer to V and V_0, respectively, and K_d is the dissociation constant. If K_d, B_t, and V_0 are constant, V will change by $[A]_0$, and the equation can be modified as follows, so that the plot of $1/[A]_0(V-V_0)$ versus $1/[A]_0$ will be linear.

$$\frac{1}{[A]_0\,(V - V_0)} = \frac{K_d}{B_t} \cdot \frac{1}{[A]_0} + \frac{1}{B_t}$$

The values of K_d and B_t can be determined from calculation and once B_t is known, K_d for other enzymes is calculated by placing the V value for other enzymes into the equation below.

$$K_d = \frac{B_t}{V - V_0} - [A]_0$$

3.5 Results and Discussion

Cloning, expression and purification of mutant β-glucosidase – Previously, we showed that an N-terminal region (E50-N127), together with a C-terminal region (F466-A512), of Glu1 is involved in binding to BGAF (*21*). However, recently we found that the I72-T82 peptide span in the N-terminal region (E50-N127) of Glu1 is sufficient for BGAF binding, and the C-terminal region (F466-A512) plays a minor role in interaction with BGAF (in review). In addition, Blanchard *et al.* showed that chimera 5 (C-5), constructed by replacing S1-T29 of Glu1 with the corresponding region from Dhr1, showed two distinct bands instead of a smear (*21*), so we expected that the extreme N-terminal region (S1-T29) of Glu1 is also involved in BGAF interaction. Therefore, based on the sequence comparison between maize and sorghum β-glucosidases (Figure 3.1), we generated mutant Glu1 and Dhr2 enzymes by SDM to determine which specific amino acids within the above regions are involved in BGAF binding, as well as their relative contribution to the interaction. We added a His.tag to the N-terminus of recombinant mutant β-glucosidases to speed up purification. All mutant β-glucosidases were expressed in high yield (1.0 – 5.0 mg enzyme /1000 ml culture volume) in *E. coli* BL21 CodonPlus cells, and the level of purity achieved was 95% or better based on SDS-PAGE profiles (data not shown). The expression level and purity of BGAFs were comparable to those of recombinant mutant β-glucosidases.

Analysis of Glu1 mutants by gel-shift assay and frontal affinity chromatography (FAC) – The interaction between β-glucosidase and BGAF was detected and measured by the gel-shift assay and FAC. In the gel-shift assay, when wild type or mutant β-glucosidases interact with BGAF, their electrophoretic mobility is reduced, producing either a smeared zone or discrete band of enzyme activity (Figure 3.3, *lanes, 2, 6, 8, 10, 16, and 20*). In contrast, the electrophoretic mobility of wild type Dhr2 or certain mutant maize β-glucosidases (i.e., Glu1 mutants), which do not bind to BGAF, remains unaltered (Figure 3.3, *lanes 4, 12, 14, and 18*) even in the presence of greater than 50-fold molar excess of BGAF. However, the gel-shift assay does not yield

quantitative data to evaluate the contribution of individual amino acids within the predicted BGAF binding site on Glu1. Therefore, we also used FAC to determine K_d values for interaction of mutant β-glucosidases with BGAF, and the data are summarized in Table 1.

The N-terminal peptide span I72-T82 of Glu1 is sufficient for BGAF binding. The sequence alignment indicates that maize β-glucosidases differ from their dhurrinase homologs by four amino acid substitutions within this 11-amino acid long region (Figure 3.1). Therefore, we produced mutants of Glu1 in which these 4 amino acids are substituted with corresponding residues from the dhurrinase counterparts. The gel-shift assays showed that two of the single mutants (I72V and N75D) produced smeared zones of reduced mobility after incubation with BGAF (Figure 3.3, *lanes 6, and 8*, respectively), suggesting that neither I72 nor N75 is essential and sufficient for BGAF binding. However, the FAC data showed that the I72V had about 2.5-fold reduction in affinity (K_d = 277 nM) and N75D had slight increase in affinity (K_d =79 nM) (Table 1), although these changes in affinity were not evident in the more qualitative gel-shift assays. These K_d value changes suggest that both I72 and N75 are involved in BGAF interaction but their relative contribution is not critical for BGAF binding.

The K81A single mutant also interacted with BGAF, yielding two discrete bands (Figure 3.3, *lane 10*). The top band is a K81A-BGAF complex, whereas the bottom band is free, unreacted K81A mutant (Figure 3.3, *lane 10*). The gel shift data indicate that the K81A mutant interacts with BGAF, but its affinity for BGAF is lower than that of the wild type Glu1. This observation is consistent with the FAC data in that the K_d for the K81A mutant (231 nM) is higher than that for wild type Glu1 (Table 1). When we mutated K81 to E (as in Dhr2), the mutant showed no detectable interaction with BGAF in the gel-shift assay (Figure 3.3, *lane 14*) and no binding in FAC (Figure 3.4A, Table 1). In the K to E substitution, a longer, positively charged side chain is replaced with a smaller, negatively charged side chain, which could disrupt the Glu1-BGAF interaction. These results illustrate the critical importance of K81 to Glu1-BGAF binding. Substitution of T82 to the corresponding residue (E) in Dhr1 and Dhr2 abolished binding to BGAF even when the BGAF concentration was 52-fold higher than that of mutant (Figure 3.3, *lane 12*). However, the T82E mutant yielded a minor retarded band that was visible above the free, unreacted T82E band when the BGAF concentration was in about a 65-fold molar

excess (Figure 3.3, *lane 20, arrow head*), indicating that the T82E mutation drastically reduced the affinity of this mutant enzyme to BGAF when compared to wild type Glu1. These data are consistent with the FAC data in that the T82E mutant had about a three-fold lower affinity (K_d =317 nM) than the wild type Glu1 for BGAF. When examining other mutants at position 82, the T82S mutant bound to BGAF in the gel-shift assay, whereas the T82Y mutant did not bind at all (Figure 3.3, *lanes 16, and 18, respectively*), which is consistent with the FAC results in which the K_d of the T82S mutant was comparable to that of Glu1 (76 nM vs. 111 nM) and the T82Y mutant showed no detectable binding (Figure 3.4A, Table 1). These results demonstrate the importance of the role of T82 in the binding of Glu1 to BGAF. Interestingly, the T82S mutant was as effective as wild type Glu1 in binding to BGAF, suggesting the importance of a hydroxyl group in the interaction. However, T82Y did not bind to BGAF, probably due to the much bulkier side chain.

The extreme N- (S1-T29) and C-terminal (F466-A512) regions of Glu1 also play a role in BGAF interaction and formation of large, insoluble complexes. In the C-terminal region, the peptide span A501-A512 could not be resolved in the 3D structure of Glu1 (Figure 3.5). Thus, the role of the region A501-A512 in binding of Glu1 to BGAF is not yet defined, but this region apparently does not mask the residues involved in the binding interaction (Figure 3.5). The peptide span F466-R480 in Glu1 is not likely involved in BGAF interaction and aggregation because it is in the aglycone binding site of the Glu1 active site (Figure 3.5) (*22*), which is away from the key BGAF binding site (I72-T82) on Glu1. Thus we mutated the amino acids in the C-terminal peptide span N481-T500 of Glu1 that are different between Glu1 and Dhr1/Dhr2 and tested the mutants for BGAF binding by gel-shift assay. Each of these single Glu1 mutants (N481E, N483G, T485K, Y487T, E490R, and T500GA) except T485E appeared to have a high affinity for BGAF based on the gel-shift assay, producing patterns similar to that of wild type Glu1 (Figure 3.6). In the case of T485E, a low concentration of BGAF (390 nM) was not sufficient for full interaction with T485E (Figure 3.6, *lane 8*). However, it produced a smeared gel-shift pattern similar to that of wild type Glu1 when the BGAF concentration was raised to 7,800 nM and above, which is at least a 52-fold molar excess (data not shown). Interestingly, most of these mutants differed in affinity by no more than a factor of about 2 based on K_d values derived from

FAC, including the T485E mutant. We believe the discrepancies between the gel-shift and FAC data result from the qualitative nature of the gel-shift assays. Of particular note is the greatly enhanced affinity of the T500GA mutant (K_d = 4 nM) for BGAF. Based on the 3D structure of Glu1 (Figure 3.5B), T500 in Glu1 is located at a distance (16.2 Å) from the key contact residues (K81 and T82). The reason for the enhanced affinity is not obvious from the available data, but the results suggest that a hydrophobic contact in the region of residue 500 in Glu1 may be important. In general, the results from the gel-shift assays and FAC experiments suggest that the residues in the peptide span N481-T500 of Glu1 contribute to, but are not essential for, binding to BGAF.

For the extreme N-terminal region S1-T29, we produced three single mutants of Glu1 (S16W, S26P, and D27S) by changing the amino acids at positions 16, 26 and 27 to their Dhr1/Dhr2 counterparts. Although these amino acids are not in the predicted BGAF-binding site, they are chosen because they represent nonequivalent and non-conservative substitutions between Glu1 and Dhr1/Dhr2. Gel-shift assay results indicated no discernable differences between the three single mutants (S16W, S26P, and D27S) and wild type Glu1 in BGAF binding because they all produced a smeared enzyme activity as did wild type Glu1 (Figure 3.6, *lanes 18, 20, and 22*), suggesting that these three sites individually do not play a role in the Glu1-BGAF interaction. In addition, the K_d values for the binding of these mutants to BGAF (Table 1) do not differ by more than about two-fold from that for the wild type Glu1, suggesting that these residues are not essential for the interaction.

Analysis of Dhr2 mutants by gel-shift assay and FAC – To confirm the importance of the two key amino acids (i.e., K81 and T82 of Glu1) in the peptide span I72-T82 in Glu1 for BGAF binding, we produced a number of Dhr2 mutants by SDM (Figure 3.2). Initially, we postulated that if K81 and T82 are essential for BGAF binding, mutating the corresponding amino acids of Dhr2 (i.e., E81 and E82) to K and T should confer BGAF binding ability to Dhr2. The results showed that neither the single mutant E82T nor the double mutant (E81K/E82T) gained the ability to bind to BGAF even in the presence of an 80-fold molar excess of BGAF (39,000 nM) (Figure 3.7, *lanes, 6 and 8*, respectively). Moreover, no evidence of binding was observed in the

FAC assay. Another Dhr2 double mutant (V72I/D75N) involving two additional amino acids that differentiate Glu1 from Dhr2 in the peptide span I72-T82 also failed to bind to BGAF (Figure 3.7, *lane 10*), and binding was not observed in the FAC assay. This finding was not surprising because the V72I/D75N Dhr2 mutant lacks the two key amino acids (K81 and T82) of Glu1 for BGAF binding. Based on these results, we concluded that K81 and T82 are not sufficient to confer BGAF binding ability to Dhr2 and thus additional amino acids (i.e., I72 and N75) from Glu1 are required. Consequently, we introduced additional amino acid substitutions into the E81K/E82T Dhr2 double mutant in order to confer to it BGAF-binding ability. Neither triple mutants V72I/E81K/E82T or D75N/E81K/E82T showed any BGAF binding activity based on gel-shift assays (Figure 3.7, *lanes, 12 and 14*) or FAC. Finally, we produced a quadruple mutant of Dhr2, V72I/D75N/E81K/E82T, and this mutant showed interaction with BGAF (Figure 3.7, *lane 16*). However, the quadruple mutant had lower affinity for BGAF than wild type Glu1, as is evident from a large amount of the unreacted Dhr2 quadruple mutant enzyme with 78-fold molar excess BGAF (Figure 3.7, *lane 16, arrow head*). This result suggested that although four amino acids (I72, N75, K81, and T82) on Glu1 are sufficient for BGAF binding, other regions (extreme N-terminal (S1-T29) and C-terminal (F466-A512) regions) on Glu1 are also important to enhance Glu1 affinity to BGAF. However, we could not obtain K_d data for the quadruple Dhr2 mutant (Dhr2 mutant #6 (V72I/D75N/E81K/E82T)) because of its irregular FAC elution pattern (Figure 3.4B). This elution pattern may be due to rapid binding and dissociation of the mutant enzyme from the column. Nevertheless, it binds to the column and elutes after Dhr2, indicating that four unique Glu1 amino acids (I72, N75, K81, and T82) are required to confer to Dhr2 BGAF-binding ability.

Superimposition of the structure of Glu1 and Dhr1 – Superimposing the structure of Glu1 and Dhr1 allows us to speculate as to the nature of the Glu1-BGAF interaction (Figure 3.8). Although we showed that BGAF is a chimeric lectin consisting of two domains (*18*), we do not know the location and nature of the β-glucosidase binding site on the JRL domain. Studies to map the site using SDM and domain swapping are currently in progress. Because our data show that the K81E mutant abolishes BGAF binding and the T82E, N481E, and T485E mutations

reduce affinity (K_d = 317, 232, and 177 nM, respectively), it is likely that these mutations disrupt and modify ionic and hydrogen bonding interactions between β-glucosidase and BGAF in their respective binding sites. We expect that the actual β-glucosidase binding site on BGAF has one or more acidic amino acids (D or E) and these acidic amino acids play a key role in the β-glucosidase-BGAF interaction.

Sequence comparison of plant β-glucosidases – When we aligned and compared sequences of β-glucosidases from other plants with the maize β-glucosidase isozymes Glu1 and Glu2, we noted that only maize β-glucosidases have threonine at position 82 within the N-terminal peptide span I72-T82 (Figure 3.9). This position is occupied by a highly conserved glutamic acid in other plant β-glucosidases. Position 81, which is occupied by lysine in Glu1, is less conserved, in that other plant β-glucosidases have either lysine or glutamic acid at this position. As for I72 and N75, both of these amino acids are important but not to the extent that K81 and T82 are; they are essential for BGAF binding of the Dhr2 quadruple mutant (Figure 3.7, *lane 16*). Based on the sequence alignment of plant β-glucosidases, there are no other plant β-glucosidases that have all four of the amino acids (I72, N75, K81, and T82) that are directly involved in BGAF binding. Thus, the sequence alignment results also support the hypothesis that the β-glucosidase-BGAF interaction is specific to the maize β-glucosidase isozymes, Glu1 and Glu2.

Recently, three more maize β-glucosidases genes, *Zmdhr1*, *Zmdhr2*, and *Zmdhr3* whose predicted amino acid sequences are highly similar to dhurrinases, and are highly expressed in immature leaves, were reported by Coneva *et al.* (*26*). They aligned the predicted amino acid sequence of these gene products with Glu1, Glu2, and Dhr1 and found that the predicted proteins are more closely related by sequence to sorghum dhurrinase than maize β-glucosidase isozymes, Glu1 and Glu2 (*26*). The most interesting difference between these three *Zmdhr* gene products and Glu1 and Glu2 relates to the BGAF binding site (I72-T82) on maize β-glucosidases. The three key amino acids (I72, K81, and T82) for BGAF binding in Glu1 and Glu2 are substituted by V, R, and E, respectively, in putative Zmdhr1, Zmdhr2 and Zmdhr3 proteins (Figure 3.10). Therefore, we predict that all three putative Zmdhr proteins will not interact with BGAF.

The physiological function of BGAF – Interaction of other BGAF-like proteins with β-glucosidases has been reported in the literature. The best examples of these proteins are myrosinase binding proteins (MBPs) (*27*) and PBP1 (PYK10-binding protein 1) (*28*). MBPs are found in *Brassica napus* and other members of the family Brassicaceae (*27*). They have one or more jacalin-related lectin (JRL) domains (*29*) and their binding to myrosinases does not seem to affect enzyme activity (*27, 30*). Myrosinase and MBPs are localized separately in developing seeds. Myrosinase is present in the myrosin cells, whereas MBP is localized in all cells in the mature embryo, except myrosin cells, epidermis, and provascular tissue, but co-localized in later growth stages (*27, 31*). PBP1 is a β-glucosidase binding protein in *Arabidopsis thaliana* and also has a jacalin-like lectin domain (*28*). PBP1 and PYK10 (a β-glucosidase) are localized in the cytosol and ER bodies, respectively, and the two proteins encounter each other and interact when tissue is damaged (*28*). PBP1 interacts with inactive PYK10 (soluble form) but not active PYK10 (insoluble form), and is believed to play a role in activation of PYK10 (*28*). The physiological functions of these β-glucosidase binding proteins (BGAF, MBPs, and PBP1) are not well understood. It has been speculated that they function in plant defense systems because they and β-glucosidase are initially localized separately, but encounter each other and interact upon tissue damage due to herbivory and pathogen infection (*32-34*). Recently, groundbreaking studies on the role of a BGAF-like protein from wheat (HFR1) in plant defense have been reported by Subramanyam *et al.* (*19*). Both BGAF and HFR1 have an N-terminal dirigent and a C-terminal JRL domain as well as lectin activity. A high level of HFR1 is accumulated at the site of larva feeding, and it deterred the Hessian fly larva from feeding on resistant plants (*19*). It is conceivable that the function of BGAF is similar to that of HFR1 in that it deters the European corn borer, a major pest of maize. Thus, one of the foci of future research on BGAF should be determination of its role in defense against the European corn borer and other maize pests.

3.6 References

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3.7 Tables and Figures

Table 3.1: Frontal affinity chromatography (FAC) was used to determine the apparent dissociation constants ($K_{d(app)}$) of wild and mutant β-glucosidases.

β-glucosidase	BGAF Concentration (B_t)	Dissociation constant ($K_{d(app)}$)
	nmol	*nM*
Glu1	6.45	111 ± 11.3
Dhr2	6.45	[a]NB
I72V Glu1	2.08	277 ± 3.4
N75D Glu1	2.08	79 ± 0.7
K81A Glu1	2.08	231 ± 1.5
K81E Glu1	2.08	NB
T82E Glu1	4.87	317 ± 20.4
T82S Glu1	2.08	76 ± 1.4
T82Y Glu1	2.08	NB
N481E Glu1	4.87	232 ± 9.9
N483G Glu1	4.87	152 ± 1.8
T485E Glu1	4.87	177 ± 8.9
T485K Glu1	4.87	144 ± 1
Y487T Glu1	4.87	179 ± 1.1
E490R Glu1	4.87	145 ± 1
T500GA Glu1	4.87	4 ± 1.7
S16W Glu1	4.87	97 ± 5.1
S26P Glu1	4.87	54 ± 5.5
D27S Glu1	4.87	156 ± 7.7
Dhr2 Mutant #1	4.87	NB
Dhr2 Mutant #2	4.87	NB
Dhr2 Mutant #3	4.87	NB
Dhr2 Mutant #4	4.87	NB
Dhr2 Mutant #5	4.87	NB
Dhr2 Mutant #6	3.59	[b]ND

The BGAF concentration on a lactosyl agarose column was determined from the elution profiles of Glu1. All of the values reported are an average of 2 measurements. $[A]_0$ = 250 nM. [a]NB: not binding. [b]ND: not determined

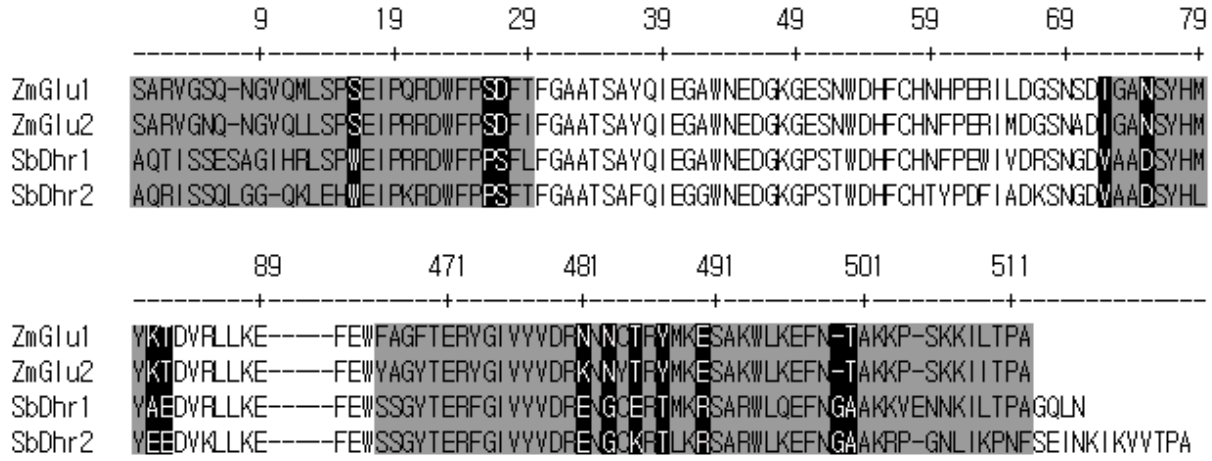

Figure 3.1: Sequence alignment of the maize β-glucosidase isozymes Glu1 and Glu2 and their sorghum homologs Dhr1 and Dhr2. The three regions (S1-T29, I72-T82, and F466-A512) of Glu1 involved directly and indirectly in BGAF-binding are shown in ***Gray*** background and the amino acids that map to the predicted BGAF-binding site or affect binding indirectly in these regions are shown in ***Black*** background.

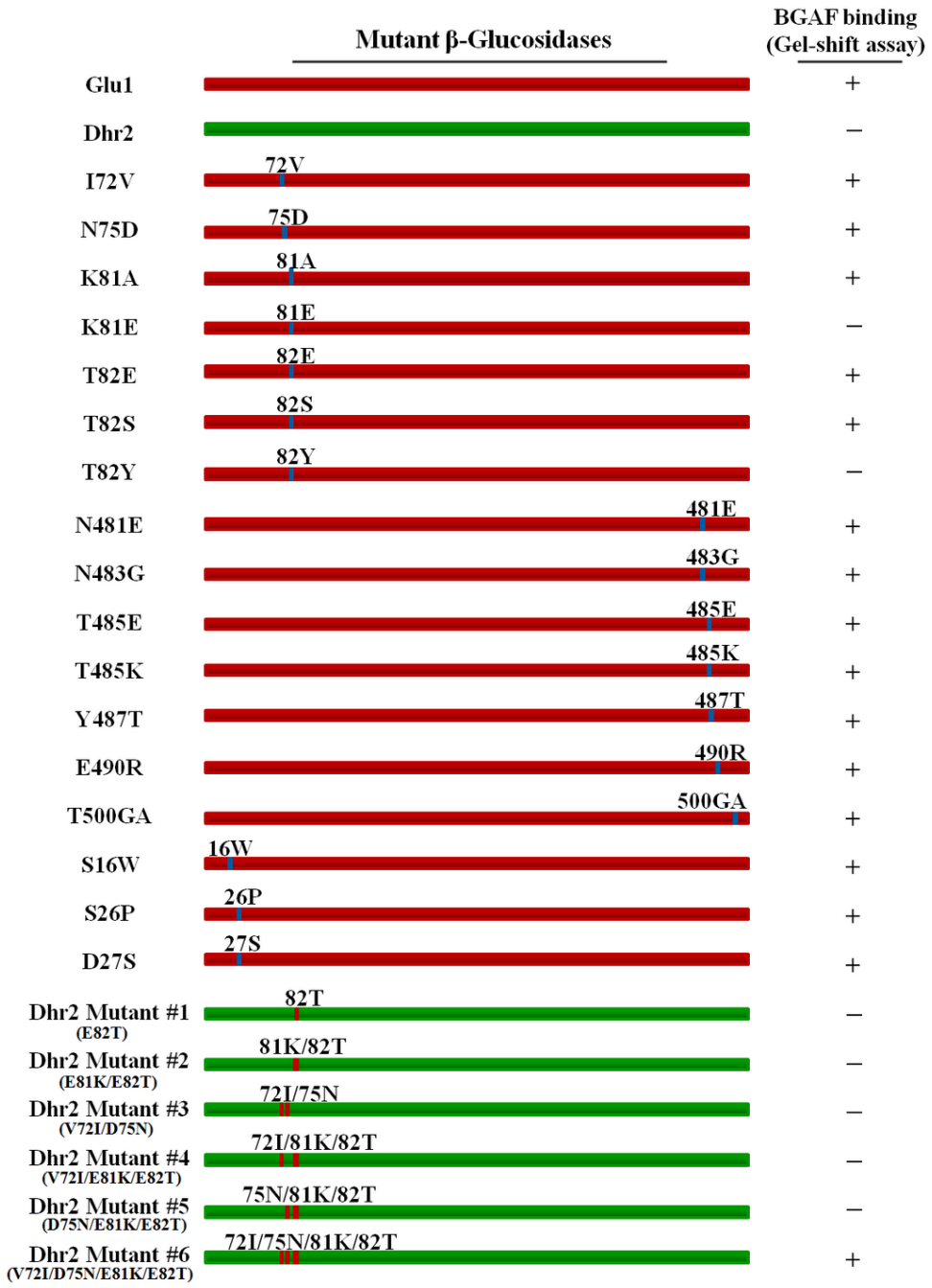

Figure 3.2: Line diagrams of wild type and mutant β-glucosidases showing the positions of key amino acids that are altered by site-directed mutagenesis to study their role in the Glu1-BGAF interaction. The results of gel-shift assay (+ for binding and – for not binding) are summarized under "BGAF binding (Gel-shift assay)".

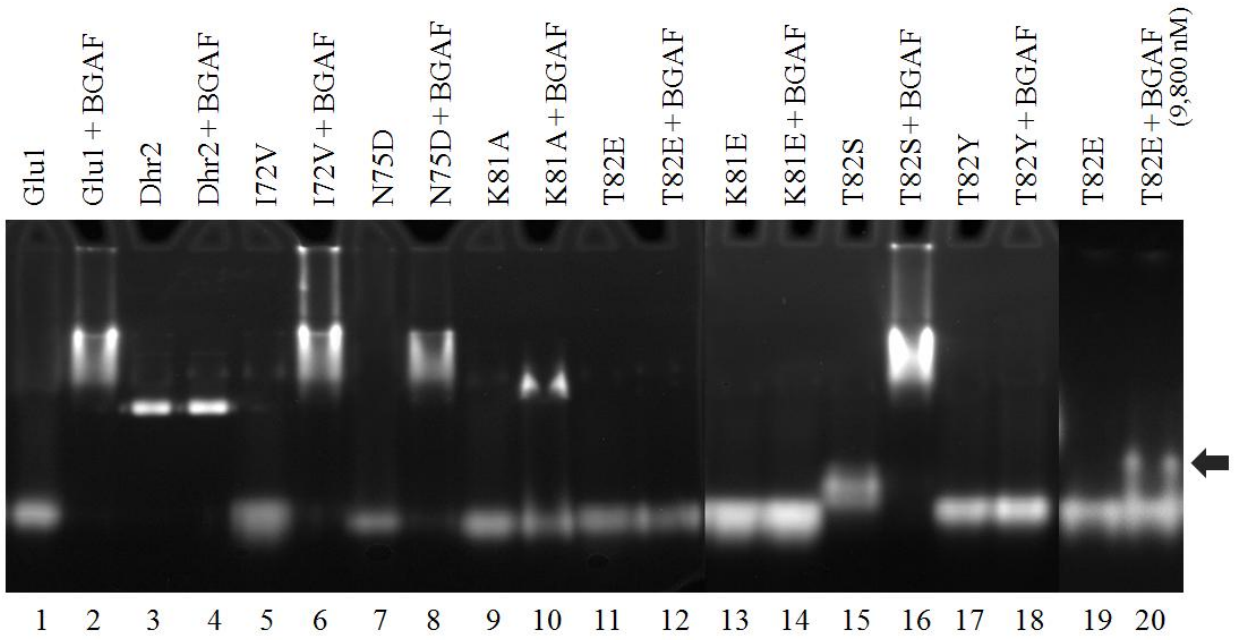

Figure 3.3: Gel-shift assay to detect the interaction of wild type and mutant Glu1 (mutated in the N-terminal I72-T82 region) with BGAF. The smeared or discrete zone of activity with reduced electrophoretic mobility in lanes containing enzymes + BGAF indicate binding of enzymes to BGAF. Note that the Glu1 mutants K81E, T82E, and T82Y failed to bind BGAF, showing that K81 and T82 are essential for the binding of Glu1 to BGAF at a BGAF concentration of 7800 nM. In the case of T82E, at high molar excess of BGAF (9,800 nM) over T82E, a small amount of T82E-BGAF complex is detected (*lane 20, upper band (arrow head)*).

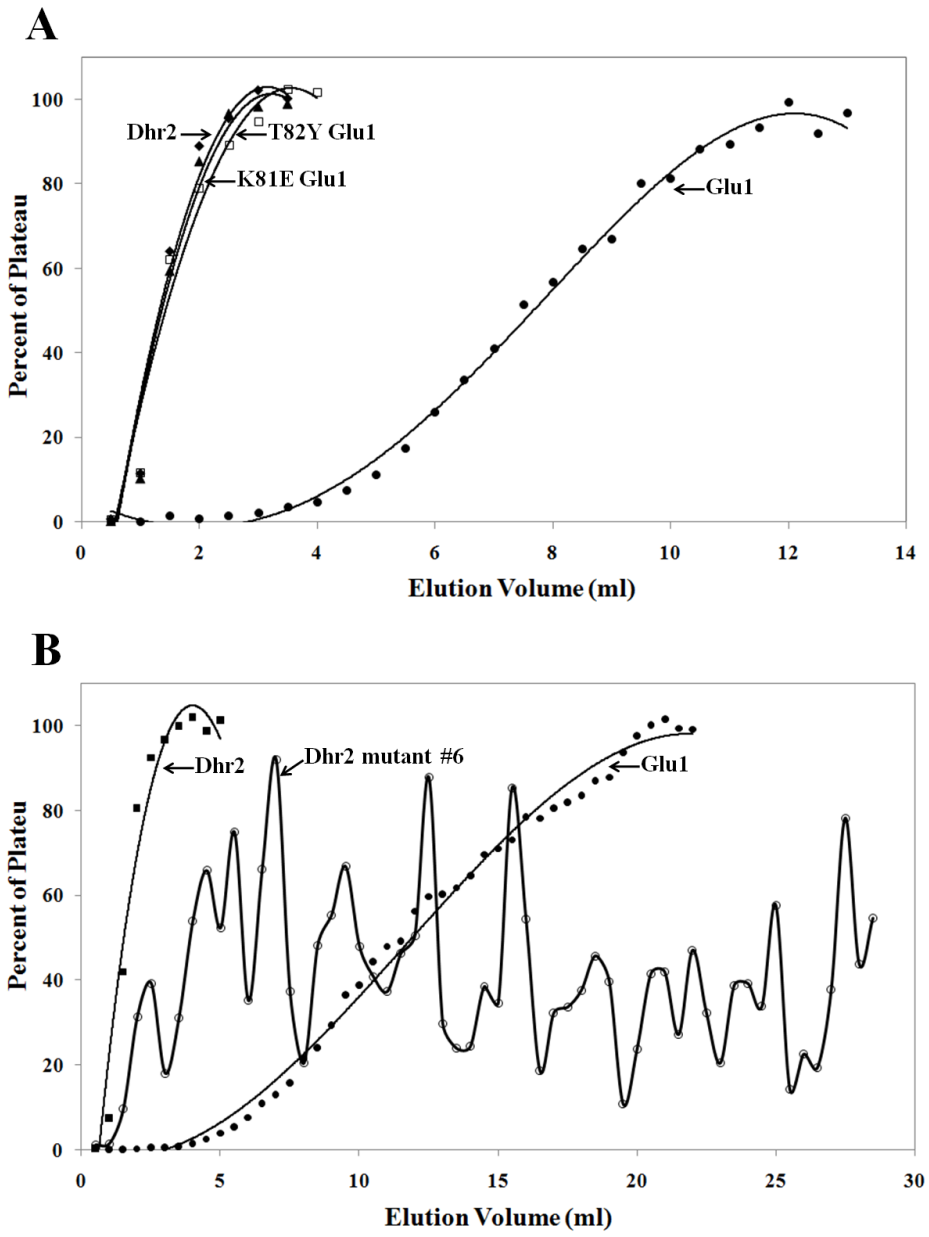

Figure 3.4: FAC profiles of wild type and mutant β-glucosidases. (A) FAC profiles of Glu1 (●), Dhr2 (♦), K81E (▲), and T82Y (□). Note that the elution volume of K81E and T82Y is the same as that of Dhr2 (non-binder). (B) FAC profiles of Glu1 (●), Dhr2 (■), and Dhr2 mutant #6 (○). Note that the shape of the FAC profile from the Dhr2 quadruple mutant is strikingly different from that of wild-type Glu1, and we are not able to calculate a K_d value for its interaction with BGAF.

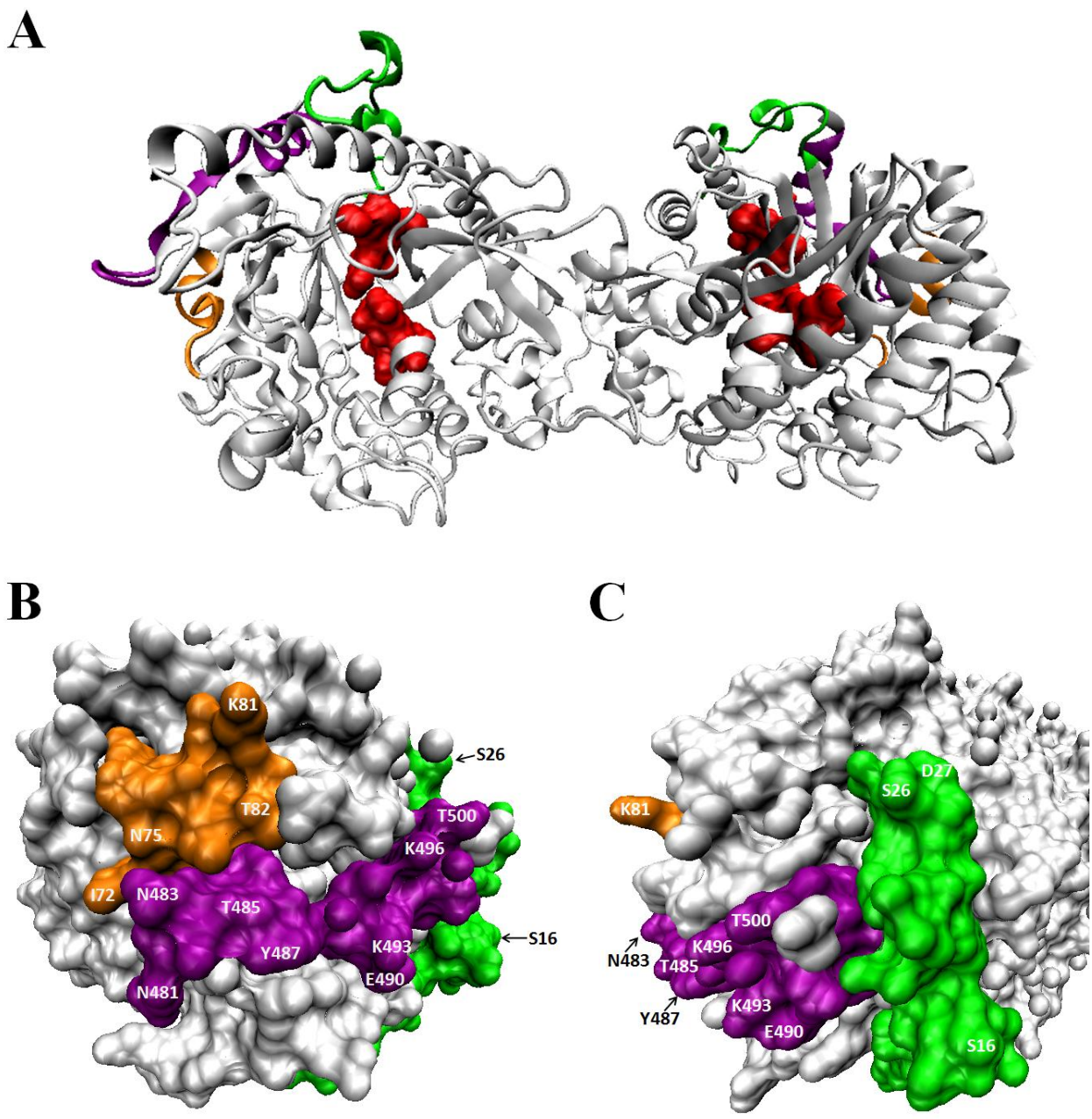

Figure 3.5: Three dimensional structure of maize β-glucosidase isozyme Glu1 (PDB entry 1E1E). The three region making up the BGAF binding site on Glu1, extreme N-terminal (Ser^{1}-Thr^{29}), N-terminal (Ile^{72}-Thr^{82}), and C-terminal region (Asn^{481}-Thr^{500}) are shown in *green*, *orange* and *purple*, respectively. (A) Structure of Glu1dimer showing the BGAF binding and the active site (shown in *red*, the two β-strands shown in red corresponds to ^{188}TFNEP^{192} and

^{404}ITENG^{408} motifs, which form the active site in Glu1). (B, C) The amino acids that are postulated to be critical for BGAF binding are labeled. (B) Front view of Glu1. (C) Side view of Glu1. Visual Molecular Dynamics (VMD) was used to generate these images (Newcartoon mode (A) and surface mode (B, C)).

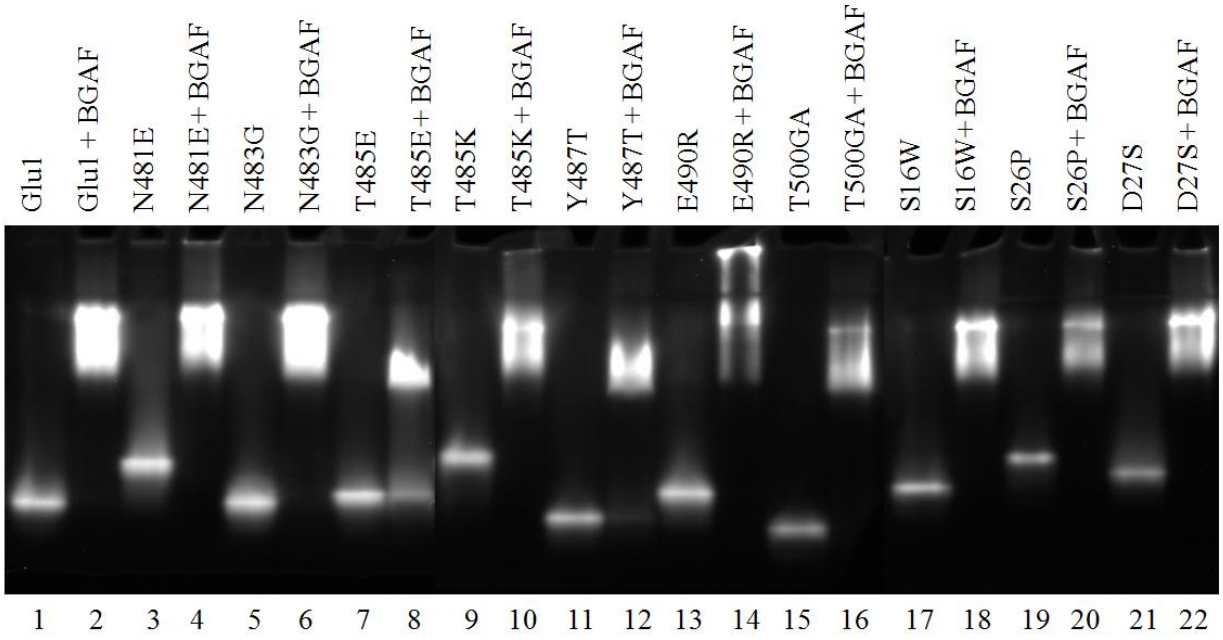

Figure 3.6: Gel-shift assay to detect the binding of wild type and mutant Glu1 (mutated in the extreme N-terminal S1-T29 and C-terminal F466-A512 region) to BGAF. The smeared or distinct zones of activity with reduced mobility in lanes containing enzyme + BGAF show the binding of enzymes to BGAF. Note that all single mutant β-glucosidases (refer to Figure 3.2) interact with BGAF.

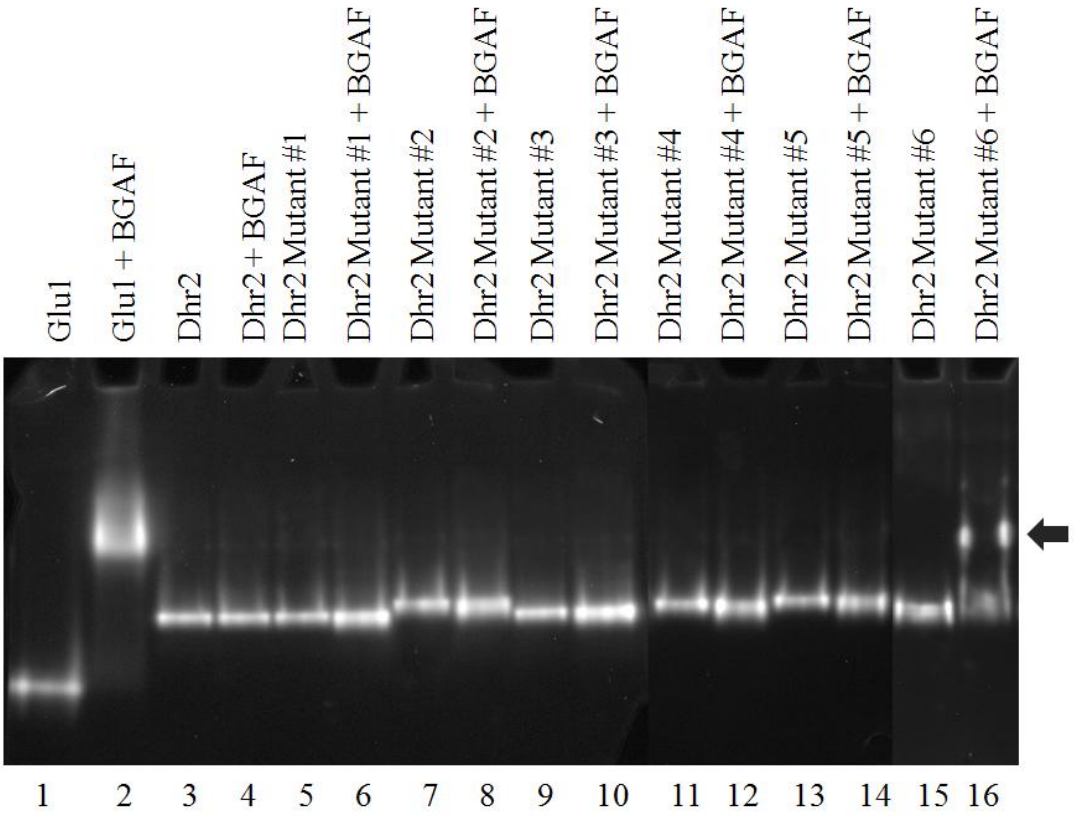

Figure 3.7: Gel-shift assay to detect the binding of wild type and Dhr2 mutants to BGAF. Activity zones with reduced mobility in lanes (e.g., 2 and 16) containing enzyme + BGAF indicate binding to BGAF. Among six Dhr2 mutants (see Figure 3.2), a Dhr2 quadruple mutant (V72I/D75N/E81K/E82T) was the only one that bound to BGAF (*lane 16, upper band (arrow head)*) albeit with low affinity as evident from the amount of unreacted (*lane 16, lower band*) mutant enzyme.

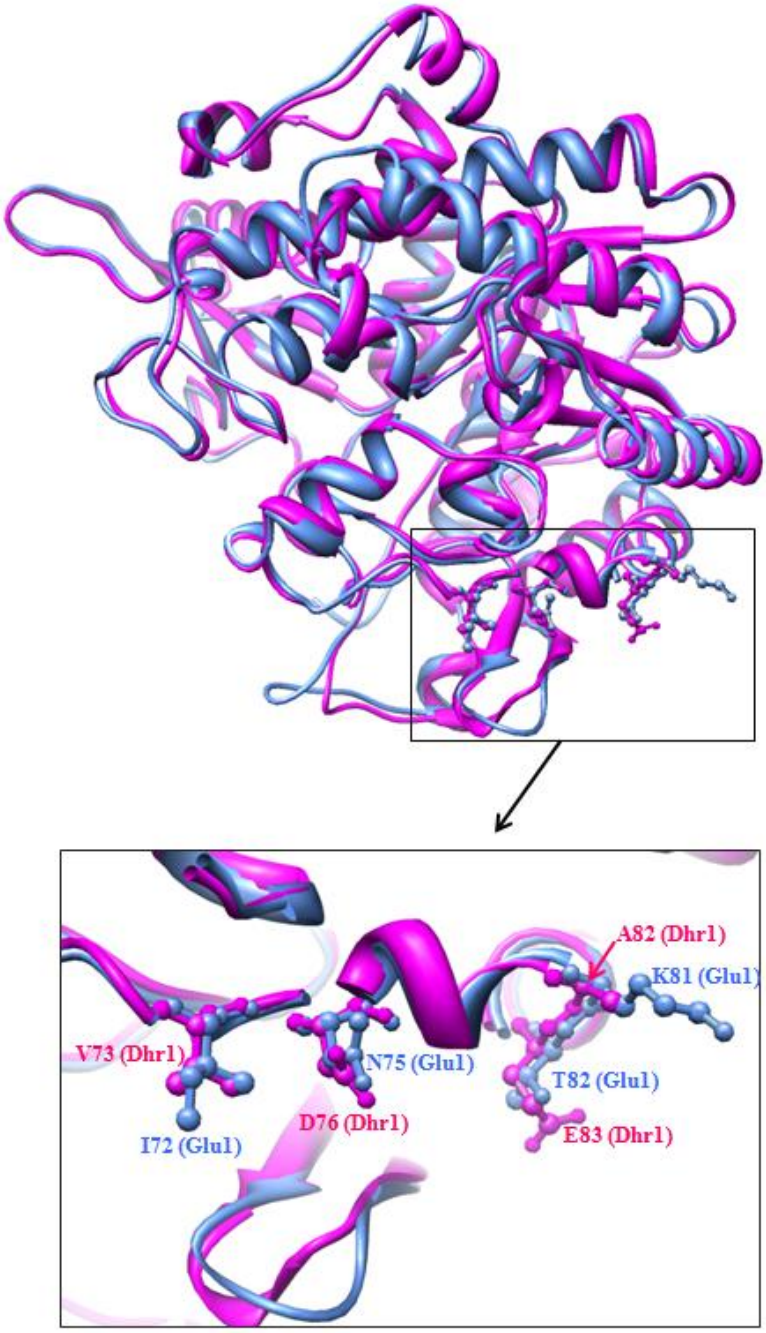

Figure 3.8: Superimposition of the structure of maize Glu1 (*Blue*, PBD entry 1E1E) and sorghum Dhr1 (*Red*, PDB entry 1V03). I72, N75, K81, and T82 of Glu1 are replaced by V73, D76, A82, and E83 in Dhr1, respectively, which account for the lack of BGAF binding to Dhr1. UCSFChimera was used to generate this image.

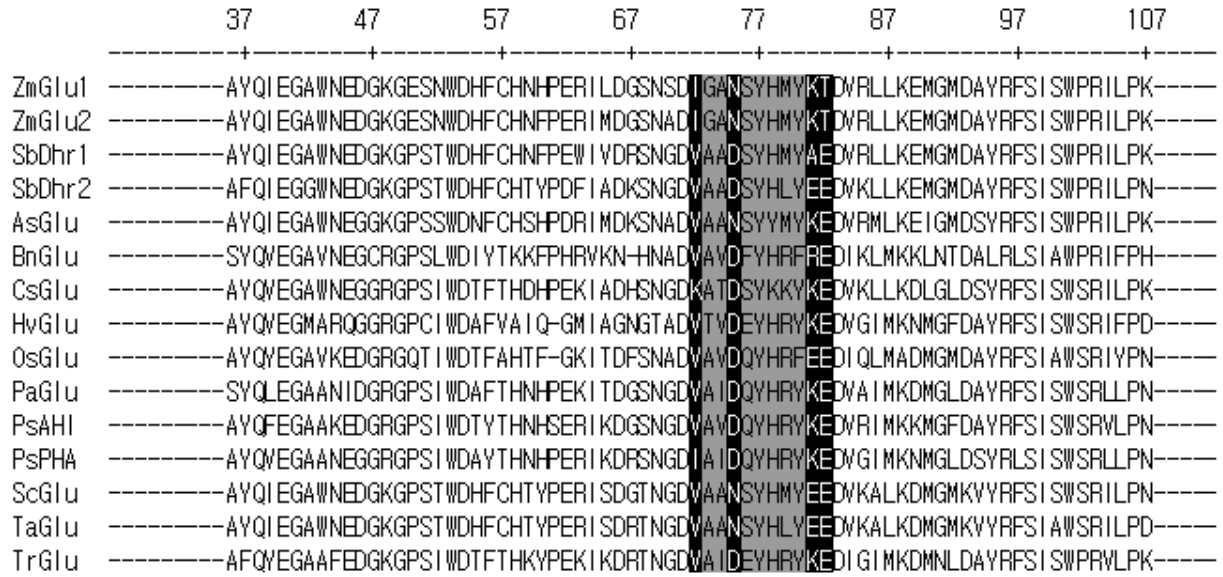

Figure 3.9: Sequence alignment of selected plant β-glucosidases. Residue numbering is based on the sequence of ZmGlu1. The BGAF-binding region I72-T82 is in ***Gray*** background and I72, N75, K81 and T82, the four amino acid residues that play a critical role in BGAF-binding are shown in ***Black*** background. Only maize β-glucosidases have threonine at position 82 and other plant β-glucosidases have a highly conserved glutamic acid at the same position. ZmGlu1, *Zea mays* β-glucosidase 1; ZmGlu2, *Zea mays* β-glucosidase 2; SbDhr1, *Sorghum bicolor* dhurrinase 1; SbDhr2, *Sorghum bicolor* dhurrinase 2; AsGlu, *Avena sativa* β-glucosidase; BnGlu, *Brassica napus* β-glucosidase; HvGlu, *Hordeum vulgare* β-glucosidase; OsGlu, *Oryza sativa* β-glucosidase; PaGlu, *Prunus avium* β-glucosidase; PsAHI, *Prunus serotina* amygdalin hydrolase isoform AH I; PsPHA, *Prunus serotina* prunasin hydrolase isoform PH A; ScGlu, *Secale cereale* β-glucosidase; TaGlu, *Triticum aestivum* β-glucosidase; TrGlu, *Trifolium repens* β-glucosidase.

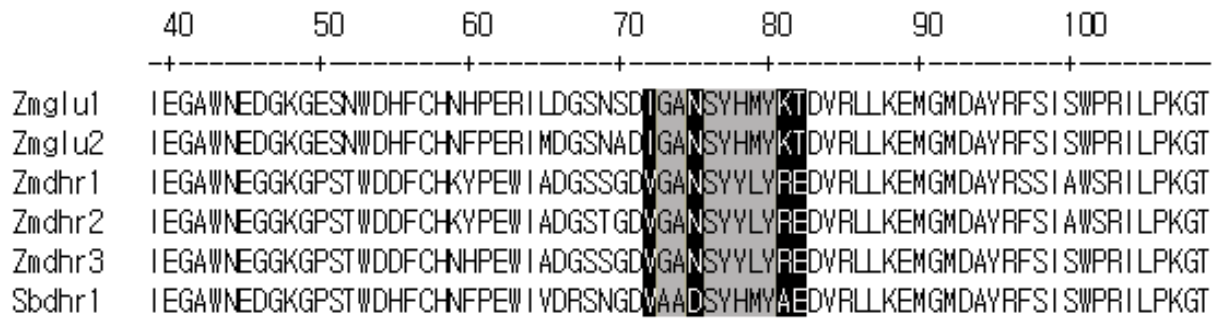

Figure 3.10: Sequence alignment of maize Glu1 and Glu2 with the predicted amino acid sequences of dhurrinase-like maize β-glucosidases and Dhr1. Residue numbering is based on the sequence of ZmGlu1. The BGAF binding site on β-glucosidases is shown in ***Gray*** background and the key amino acids within this region are shown in ***Black*** background.

Chapter 4: Conclusion

Previous studies in our laboratory showed that there are two regions [the N-terminal (Glu^{50}-Val^{145}) and the C-terminal (Phe^{466}-Ala^{512}) regions] in maize β-glucosidases that contribute to the BGAF binding site and the swapping of those regions in Glu1 with the corresponding sites in Dhr1 abolished BGAF binding. However, protein sequence comparisons between the maize β-glucosidase isozymes Glu1 and Glu2, which bind to BGAF, and the sorghum β-glucosidase isozymes Dhr1 and Dhr2, which do not bind to BGAF, show that the two regions implicated in BGAF binding are shorter than previously proposed and have many shared amino acid identities.

To narrow down BGAF binding sites in Glu1, we constructed additional chimeric β-glucosidases by swapping peptide spans between maize and sorghum β-glucosidases. To speed up chimeric enzyme purification, we added His-tag at the N-terminus and all chimeric enzymes were overexpressed in soluble and active form in *E. coli*.

We have shown that the Ile^{72}-Thr^{82} region of Glu1 is essential and sufficient for BGAF binding. Evidence supporting this conclusion is as follows: [1] The peptide span Ile^{72}-Thr^{82} within Glu^{50}-Val^{145} is essential and sufficient for BGAF binding. C-2 (Ile^{72}-Thr^{82} of Glu1 was swapped with that in Dhr1) abolished BGAF binding, whereas C-11 (Val^{72}-Glu^{82} of Dhr2 was exchanged with the corresponding region of Glu1) bound BGAF although the affinity of C-11 for BGAF was much lower than that of the wild type Glu1 as shown by gel-shift assay and FAC. [2] The role of the C-terminal (Phe^{466}-Ala^{512}) region is not critical for BGAF binding, because C-3 (Phe^{466}-Ala^{512} on Glu1 was swapped with the corresponding region on Dhr1) showed binding when 52-fold molar excess of BGAF was used in the gel-shift assay. The replacement of the C-terminal regions of Dhr2 by their Glu1 homologs does not confer on the resulting chimeric enzyme (C-12) the ability to bind BGAF. [3] The C-terminal (Phe^{466}-Ala^{512}) region of Glu1 is divided by three subregions (Phe^{466}-Arg^{480}, Asn^{481}-Thr^{500}, and Ala^{501}-Ala^{512}), and the importance of these subregions in BGAF binding was determined by gel-shift, pull-down assay and FAC (K_d). The C-terminal Phe^{466}-Arg^{480} region is not involved in interaction with BGAF, because C-4 (Glu1 whose Phe^{466}-Arg^{480} is replaced with its Dhr1 homolog) showed a smear, indicating

formation of large insoluble complexes and K_d for C-4 is similar to that for wild type Glu1. The peptide span Asn^{481}-Thr^{500} enhances the affinity of Glu1 for BGAF binding, but its role for BGAF binding is not critical compared with the N-terminal region Ile^{72}-Thr^{82}. C-5 (Asn^{481}-Thr^{500} on Glu1 is replaced with its Dhr1 homolog) shows reduced affinity and it also requires higher concentrations of BGAF to produce large precipitable complexes. The extreme C-terminal region (Ala^{501}-Ala^{512}), although not essential for binding, plays a role in the formation of large, insoluble Glu1-BGAF complexes. This is demonstrated by C-5, which produces precipitable complexes with BGAF, but C-6 (Asn^{481}-Ala^{512} on Glu1 was swapped with the corresponding region on Dhr1) produces no precipitable complex in the pull-down assay. [4] The extreme N-terminal region Ser^{1}-Thr^{29} is also not involved in BGAF interaction directly, but plays a role in the formation of precipitable complexes the same as the extreme C-terminus (Ala^{501}-Ala^{512}). C-8 (Ser^{1}-Thr^{29} on Glu1 is replaced with those in Dhr1) showed only one band with BGAF suggesting that this chimera forms a discrete sized complex with BGAF. [5] The N- (Ile^{72}-Thr^{82}) and C-terminal (Phe^{466}-Ala^{512}) regions act cooperatively as is the case with chimeric enzyme C-10 (Dhr2 with N- and C-terminal regions replaced with those of Glu1), which forms large, insoluble complexes, whereas C-11 (a chimeric enzyme C-10 without the Glu1 C-terminus) forms a soluble complex of discrete size with BGAF. [6] The effect of pH for Glu1-BGAF interaction is critical (the optimum pH for Glu1-BGAF interaction is pH 5.0), whereas the effect of salt on Glu1-BGAF binding is not. [7] The stoichiometry of the Glu1-BGAF complex, determined by gel-shift assay, suggested that one β-glucosidase dimer binds one BGAF dimer, producing linear chains, which is consistent with a model proposed by Kittur et al. (2007).

The most important question in the case of the Glu1-BGAF interaction is what are the key amino acids in the BGAF binding site of Glu1 that recognize and bind BGAF. In order to answer this question, we constructed mutant β-glucosidases within the three regions that form the predicted BGAF binding site. Like chimeric enzymes, we put a His tag at the N-terminus of mutant enzymes and used a Ni^{2+}-Sepharose column for purification.

Based on gel-shift assay and FAC data, two key amino acids, lysine-81 and threonine-82, in the N-terminal region peptide span Ile^{72}-Thr^{82}, play a critical role for BGAF interaction. This conclusion is supported by following results: [1] Lysine-81 and threonine-82 on Glu1 are key

amino acids for BGAF binding. The K81E and T82Y mutants showed no detectable interaction with BGAF in the gel-shift assay and no detectable binding in FAC. [2] Isoleucine-72 and asparagine-75 in the N-terminal region are involved in BGAF interaction but their relative contribution is not critical. I72V had about 2.5-fold reduction in affinity compared with wild type Glu1 and N75D showed a slight increase in affinity. [3] All of the four unique amino acids (isoleucine-72, asparagines-75, lysine-81, and theronine-82) of the N-terminal region are required for BGAF interaction to convert Dhr2 from a non-binder to a binder, because only the quadruple mutant V72I/D75N/E81K/E82T of Dhr2 showed binding to BGAF. [4] Any individual amino acid in the extreme N-terminal (Ser^{1}-Thr^{29}) and C-terminal (Asn^{481}-Thr^{500}) regions is not essential for the interaction, because the K_d values for mutants of Glu1 within those regions were similar or slightly higher than that for Glu1. [5] The β-glucosidase binding site of BGAF has one or more acidic amino acids (D or E) and these acidic amino acids play a key role in the β-glucosidase-BGAF interaction. Based on the FAC data, the K81E and T82E mutation abolishes or sharply decreases the affinity for BGAF binding, and N481E and T485E mutations reduce affinity. Therefore, it is likely that these mutations disrupt and modify ionic and hydrogen bonding interactions between β-glucosidase and BGAF in their respective binding sites.

When we aligned and compared sequences of β-glucosidases from other plants with those of the maize β-glucosidase isozymes Glu1 and Glu2, we noted that only maize β-glucosidases have threonine at position 82 within the N-terminal peptide span Ile^{72}-Thr^{82}. This position is occupied by a highly conserved glutamic acid in other plant β-glucosidases. Position 81, which is occupied by lysine in Glu1, is less conserved, in that other plant β-glucosidases have either lysine or glutamic acid in this position. Thus, the sequence alignment results provide additional support to the hypothesis that the β-glucosidase-BGAF interaction is specific to the maize β-glucosidase isozymes, Glu1 and Glu2.

As I mentioned in the introduction, BGAF is the only β-glucosidase binding protein whose binding site on β-glucosidase has been mapped and key residues within the binding site determined. This represents a fundamental contribution to understanding the mechanism by which Glu1 and BGAF interact. The determination of the β-glucosidase binding sites on BGAF

is currently in progress, and this result, when available, together with the results reported in this dissertation will provide a detailed and precise mechanism for the β-glucosidase and β-glucosidase-binding protein interaction.

Furthermore, the final and the most important question as regards the β-glucosidase-BGAF interaction is its physiological function, which now becomes amenable to answering in the near future. Recently, the role of a BGAF-like protein from wheat (HFR1) in plant defense has been reported. It is conceivable that the function of BGAF is similar to that of HFR1 in that it deters the European corn borer, a major pest of maize, and other maize pests. Recently, we identified five additional genes in maize encoding a family of BGAF-like proteins. Furthermore, we discovered that the product of *bgaf2* gene is also a β-glucosidases aggregating factor. It is now becoming clear that there are at least two chimeric lectins in maize that specifically interact with β-glucosidases. Since they do not affect enzyme activity, we believe they help the plant to launch a powerful defense response to attack by pests. Thus, one of the foci of future research on BGAF should be determination of its role in defense against the European corn borer and other maize pests.