CHAPTER 3

Identification of BGAF and Mapping of BGAF Binding Domains on Maize (*Zea mays* L.) β-glucosidase.

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β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) occur ubiquitously in prokaryotes and eukaryotes. They belong to the glycosyl hydrolase family 1 (Henrissat, 1996) and catalyze the hydrolysis of aryl and alkyl β-D-glucosides as well as glucosides with a carbohydrate moiety such as cellobiose and other β-linked oligosaccharides (Reese, 1977). The occurrence and activity of maize β-glucosidase is correlated with growth and certain desirable traits (Kahler and Wherhahn, 1986). For example, castanospermin, a general glucosidase inhibitor, inhibits the growth of maize seedlings as much as 50% and the formation of secondary roots completely (Nagahashi et al., 1990). β-glucosidases from different grasses, including maize, are implicated in phytohormone activation, the release of indole acetic acid (IAA) from its glucoconjugates (Wiese and Grambow, 1986; Campos et al. 1993). Maize β-glucosidase is also implicated in the activation of cytokinins during germination (Smith and van Staden, 1978). The major function of maize β-glucosidase however, appears to be in the defense of young plant parts against pathogens and herbivores by releasing toxic aglycone (i.e., hydroxamic acid) from their glucosides. Hydroxamic acids, derivatives of 1,4-benzoxazin-3-one, are considered to be the major defense compounds in maize, wheat, rye, and barley (Niemeyer, 1988). The predominant hydroxamic acid in maize is 2-glucopyranosyl 4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOAGlc), whose aglycone DIMBOA is the primary defense chemical against aphids and the European corn borer (*Ostrinia nubilalis*). The aglycone DIMBOA is present as a glucoconjugate (DIMBOAGlc) in intact tissues of maize. It has been shown that DIMBOA inhibits the electron transport system and phosphorylation reactions in bovine mitochondria (Niemeyer et al., 1986) and spinach chloroplasts (Queirolo et al., 1983). Additionally, several studies have shown a high correlation between DIMBOA content of maize genotypes and the level of resistance to or inhibitory affect on insects and pathogens (Beck et al., 1961; BeMiller and Pappelis, 1965; Corcucera et al., 1978; Klun et al., 1967; Long et al., 1975; Massardo et al., 1994; Niemeyer, 1991, Reid et al., 1991; Sahi et al., 1990; Xie et al., 1991).

Molecular recognition is a key component in virtually all biochemical processes, many of which involve protein-protein interactions. In certain maize genotypes (nulls), β-glucosidase occurs as part of large insoluble quaternary aggregates (Esen and Cokmus, 1990). The β-glucosidase zymograms of such genotypes are devoid of enzyme bands (Stuber et al., 1977).

These genotypes were originally thought to be homozygous for a null allele at the *glu1* gene. However, biochemical and immunological data from our laboratory established that the so-called null genotypes have β-glucosidase activity when assayed in solution, and have a 60 kD polypeptide reacting specifically with anti-β-glucosidase sera on immunoblots (Esen and Cokmus, 1990). The enzyme is not detected on zymograms because it occurs as large quaternary structures (>1.5 X 10^6 D), which fail to enter the gel. After dissociation of these structures by SDS, the enzyme can be detected on gels (Esen and Blanchard, 2000). We have also shown that the null phenotype is due to a β-glucosidase aggregating factor (BGAF), which specifically interacts with the enzyme forming high molecular weight heterocomplexes (Esen and Blanchard, 2000). There are other examples of β-glucosidase aggregation and β-glucosidase binding proteins in various plants. β-glucosidases from flax and oat occur in high molecular mass forms ranging from 245 to 1,200 kD (Nisius, 1988; Fields and Gerhardt, 1994; Gus-Mayer et al., 1994). Additionally, Falk and Rask (1995) reported two myrosinase (β-thioglucosidase)-binding proteins (50 and 52 kD) from rapeseed.

We have identified BGAF as a protein belonging to the small heat-shock protein family (sHsp) and BGAF is solely responsible for β-glucosidase aggregation and insolubility, and thus, the apparent null phenotype. Heat-shock proteins (Hsp) are a diverse group of proteins that are produced in response to a variety of abiotic stresses, a number of which are now known to function as molecular chaperones (Georgppoulos and Welch, 1993; Hendrick and Hartl, 1993; Boston et al., 1996; Hartl, 1996). During high-temperature stress, it is believed that molecular chaperones function to prevent irreversible protein denaturation that would otherwise be detrimental to the cell (Parsell and Lindquist, 1993). Another group of Hsps that are not well characterized are the small heat-shock proteins (sHsps), which have monomeric molecular masses ranging from 15 to 40 kD, but are known to form oligomeric complexes (Lee and Vierling, 2000). According to Waters et al. (1996) sHsps are the most diverse and abundant Hsps that are synthesized by plants. Vierling (1991) reported that higher plants have at least 20 sHsps and some plant species may have as many as 40 different sHsps. In contrast, most other organisms have one or only a few small heat-shock proteins. There are 4 major Hsps in *Drosophila*, 3 in mammals including αA- and αB-crystallin, 1 in yeast, and 1 in *Saccharomyces*

cerevisiae (Arrigo and Landry 1994). The divergence of the plant sHsps occurred after the split of the plant and animal lineages. This suggests that the tremendous diversity of sHsps in plants may reflect adaptations to stresses unique to plants.

The objective of the present study is to elucidate the mechanism of the β-glucosidase-BGAF interaction. To identify the nature of the binding site(s) on β-glucosidase involved in the recognition and binding of BGAF we utilized homolog-scanning (Wells, 1991). In maize, cDNAs corresponding to a β-glucosidase gene (*glu1)* have been cloned and sequenced (Brzobohaty et al., 1993; A. Esen and M. Shahid, direct submission GenBank accession no. U25157). The cDNA corresponding to another β-glucosidase gene (*glu2)* has also been isolated and sequenced (Bandaranayake and Esen, 1996). The putative protein product of this cDNA, Glu2, shows 90% sequence identity with Glu1. Additionally, a β-glucosidase gene (*dhr1*) from sorghum has been cloned and sequenced (Cicek and Esen, 1998). Despite their high sequence identity, the maize and sorghum enzymes are functionally different with respect to BGAF binding. BGAF binds tightly to both maize isozymes (Glu1 and Glu2), but does not bind to their sorghum homolog Dhr1, which shares 70% sequence identity with Glu1 and Glu2. Therefore, they provide an excellent system to study functional differences at nonconserved residues and elucidate the mechanism of enzyme aggregation and insolubility. In this study we have generated a series of β-glucosidase chimeras to identify BGAF binding domains. By examining the behavior of these chimeras in binding assays we demonstrate that BGAF binding is conformation dependent and highly specific. Additionally, we have identified two disparate polypeptide segments in the primary structure of the maize β-glucosidase isozyme Glu1 that forms a BGAF binding site in the tertiary structure of the enzyme.

EXPERIMENTAL PROCEDURES

Cloning of parental β-glucosidases-We previously reported the cloning and expression of cDNAs encoding Glu1 and Glu2 in *E. coli* (Cicek and Esen, 1999). The cloning and expression of the cDNA encoding Dhr1 was essentially the same. The cDNA encoding the mature sorghum β-glucosidase (dhurrinase-1) protein was amplified by the polymerase chain reaction (PCR) using oligonucleotide primers (sense, 5' GCCACGA*GCTAGC*ACGATAAGCAGTGAG 3' and antisense, 5' AGCAG*CTCGAG*TTCTACTTAATTAGTTAAGC 3') containing *Nhe*I (5' –end) and *Xho*I (3' –end) restriction enzyme sites, respectively. The thermostable DNA polymerase, *Pfu*, was used in PCR and the PCR product was blunt end ligated into the *Sma*I site of pBluescript® II SK (+/-). The construct was used to transform the *E. coli* strain DH10B and the presumptive positive (white) colonies were screened by PCR. Vector-specific primers (T3 and T7), flanking the cloning site were used to determine if white colonies had an insert of expected size (Gussow and Clackson, 1989). Recombinant colonies were isolated and grown for plasmid isolation. After large-scale plasmid isolation, the inserts were cut with *Nhe*I and *Xho*I, gel purified and cloned into the expression vector pET-21a that had been double-digested with *Nhe*I and *Xho*I and gel purified. The *E. coli* host strain pLys S [F- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm*] was transformed with the recombinant plasmid construct and used in expression studies. Recombinant colonies containing the cDNA insert were identified using vector-specific (T7 promoter and T7 terminator) primers.

Chimeric β-glucosidase construction-The construction and the expression of chimeric cDNAs were done using the wild type parental plasmids as templates (Glu1, Glu2, and Dhr1 above). The initial constructs tested for BGAF binding (C-2, C-4) were selected randomly and the results led to the eventual production of twelve chimeras used in this study. Chimeric cDNAs were constructed by using the PCR based technique of overlap extension (Cicek and Esen, 1999; Horton et al., 1990). The chimeric molecules are diagrammed in Fig. 2. The internal oligonucleotides used to assemble chimeric β-glucosidases are given as follows: **C-2** (sense) 5' GGCTACTTCGCGTGGTCTCT 3' and antisense 5'AGAGACCACGCGAAGTAGCC 3'; **C-4**

sense same as C-2 sense, and C-4 antisense same as C-2 antisense; **C-15** (sense) 5' GCC-AAGTGGTTGAGGAGTTCAA 3' and antisense 5' TTGAACTCCTGCAACCACT-TGGC 3'; **C-16** (sense) 5' GCCAGGTGGTTGAAAGAGTTCAA 3' and antisense 5''TTGAACTCTTT-CAACCACCTGGC 3'; **C-21** (sense) 5' GGCATYGTCTACGTCGAYCGC 3' and antisense 5' GCRRTCGACGTAGACRATGCC 3' (chimera 16 was the template in PCR); **C-22** (sense) 5' GGCATYGTCTACGTCGAYCGC 3' (chimera 16 was the template in PCR) and antisense 5' GCRRTCGACGTAGACRATGCC 3'; **C-19** (sense) 5''TGCCCCRGGRCGRTGCTCAC-CKGG 3' and antisense 5' CCMGGTGAGCAYC-GYCCYGGGGCA 3'; **C-18** (sense) 5' GGC-TACTTCGCGTGGTCTCT 3' and antisense 5' AGAGACCACGCGAAGTAGCC 3'; **C-5** (sense) 5' TGCCGCCACTTCAGCGTACCA 3' and antisense 5' TGGTACGCTGAAGTGGC-GGCAC 3'; **C-6** (sense) same as C-5 sense, and antisense same as C-5 antisense; **C-26** (sense) 5' TATGTAACAATTT-TCCACTGGGA 3' and antisense 5' TCCCAGTGGAAAATTGTTA-CATA 3'.

Expression of β-glucosidases and chimeras-Fresh *E. coli* pLys S containing each of the recombinant plasmid constructs were grown at 37°C in 25 mL of LB medium supplemented with 50 µg/ml and 38 µg/ml of ampicillin and chloamphenicol, respectively. Expression was induced by adding isopropylthiogalactoside (IPTG) to a final concentration of 0.6 mM when the A_{600} reached 0.5 to 0.7 and cultures were incubated at 25°C for an additional 4 h to permit for accumulation of the expressed proteins. At the end of the induction period, the cells were recovered by centrifugation at 2700g for 10 min, washed with fresh LB, repelleted, and stored at -70°C until needed for protein isolation.

For protein extraction, the pellet was thawed and suspended in an optimized extraction buffer (100 mM Tris-HCl/50 mM NaCl, pH 8.0) containing 40 µg/mL DNase I, 0.2 mM PMSF, 2 mM $MnCl_2$, 2 mM $MgCl_2$, and incubated at room temperature for 30 min. The extraction buffer volume (milliliters) to cell pellet weight (grams) ratio was 10:1 in all extractions. The soluble protein fraction was recovered by centrifugation at 20,000g for 10 min and subjected to electrophoretic analysis by SDS-PAGE and for enzyme activity assays by spectrophotometry and

native PAGE. Constructs that directed the expression of recombinant proteins of the expected size and activity were used for large-scale induction of protein expression.

For the purification of the wild type β-glucosidase and chimeras (C-2, C-4, C-15, C-16, C-21, and C-22), the inductions were performed as above except that the culture volume was increased to 800 ml in replicates of four. Soluble enzyme was isolated as described above and then re-precipitated from these crude cell extracts with a 35 to 65% ammonium sulfate (AS) cut. The precipitate was dissolved in 50 mM Na-acetate buffer, pH 5 and centrifuged at 20,000g for 30 min. The supernatant was adjusted to a final concentration of 0.5 M AS, centrifuged and then applied to a ToyoPearl-butyl 650M (TasoHaas, Montgomeryville, PA) hydrophobic interaction chromatography column. The column was washed with 0.5 M AS in buffer and eluted with five bed volumes of a 0.5 M to 0.1 M AS linear downward gradient. The resulting fractions were assayed for β-glucosidase activity using the artificial substrate para-nitrophenyl-β-D-glucoside (*p*NPGlc) or the natural substrates DIMBOAGlc or dhurrin (para-hydroxy-S-mandelonitrile-β-D-glucoside). The fractions with activity were checked for purity by SDS-PAGE, pooled and chromatographed on a ToyoPearl-phenyl 650M column as described above.

BGAF binding assays-BGAF was isolated in its free form from the pellet fraction obtained after 3-d-old etiolated H95 shoot homogenates had been extracted four times with 50 mM sodium acetate (pH 5) buffer containing 30% AS (these conditions keep BGAF insoluble, but removes β-glucosidase). The fourth pellet containing predominantly free BGAF was extracted with 50 mM sodium acetate buffer (pH 5). The extract was centrifuged at 20,000g for 15 minutes. To concentrate BGAF, soluble proteins were precipitated with 40% AS and resuspended in a minimum volume (2 mL) of sodium acetate buffer. The concentrate was applied to a Sephacryl 200 HR gel filtration column (90 x 1.6cm) and 3 mL fractions were collected. BGAF containing fractions were detected by ELISA, then pooled, and concentrated 10-fold on a 10 K cut-off spin column (Gelman Sciences). The interaction between BGAF and β-glucosidase was measured in a binding assay by mixing purified BGAF at 10-fold molar excess with purified β-glucosidases or their chimeras. In the case of chimeras C-5, C-6, C-18, C-19, and C-26, crude expression extracts rather than purified enzymes were used as β-glucosidase source in binding assays. The

enzyme-BGAF mixes were incubated on ice for 1-2 h with occasional mixing. The reaction mixes were then electrophoresed on 8% native gels, and the gels were equilibrated with two changes of 50 mM citrate 100 mM phosphate buffer, pH 5.8 for 5 min each after electrophoresis. β-glucosidase activity was detected by incubating the equilibrated gels in a 1mM final concentration of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucoside (MUG) for 5-10 min. β-glucosidase activity zones (bands) were visualized under UV light and documented using an AlphaImagerä 2000 documentation and analysis system (Alpha Innotech Corporation, San Leandro, CA). To determine if BGAF bound to Dhr1 a different gel-shift assay was used for detection since Dhr1 does not hydrolyze MUG or any other substrate besides its natural substrate, dhurrin. The BGAF-Dhr1 binding assay was essentially the same as described above, except that mobility shifts of BGAF were analyzed by immunoblotting using anti-BGAF serum as probe rather than enzymatic activity. The assay was performed by initially incubating purified BGAF in 2-fold incremental concentrations ranging from 20 to 2.5 ug/mL with a fixed volume of a Dhr1 expression extract, and then mixtures were electrophoresed on 8% native gels. BGAF incubated and immunoblotted by itself and BGAF incubated with Glu1 served as neagative and positive controls, respectively, in BGAF-Dhr1 binding assays.

Mapping BGAF binding domains by molecular modeling-A model of the 3D structure of Glu1 and Dhr1 were generated by homology modeling, using the Modeller4 program (Sali and Blundell, 1993). The models were based on the coordinates of the known 3D structures of linamarase, the cyanogenic β-glucosidase from white clover (Barrett et al., 1995; PDB code: 1cbg), and myrosinase from white mustard (Burmeister et al., 1997; PDB code: 1myr). Five models of Glu1 were generated in Modeller4. The models were sufficiently similar; then an average structure for Glu1 prepared by coordinate averaging. The Leap module of AMBER4.1 (Pearlman et al., 1995) was used to add hydrogen atoms to the models, and bad contacts in the models were eliminated using energy minimization with the Sander module of AMBER. For energy minimization, 100 cycles of steepest decent minimization of hydrogen atom positions were done first, after which 600 steps of steepest descent minimization of all atoms were

performed (molecular modeling studies were performed by Dr. Muzaffer Cicek and Dr. David R. Bevin in the Biochemistry department).

Isolation, cloning, and sequencing of the BGAF cDNA-Free BGAF was extracted with 50 mM sodium acetate buffer, pH 5 as described above. The BGAF containing fractions obtained after Sephacryl HR-200 gel filtration were identified by ELISA, and pooled. Ammonium sulfate was added to the pooled fractions to a final concentration of 0.8 M and applied to a ToyoPearl-butyl 650M hydrophobic interaction chromatography column equilibrated with 50 mM sodium actetate, pH 5 containing 0.8 M AS. After the development of the column with a manual step gradient using 0.1 M increments from 0.8 M to 0.1 M AS, BGAF containing fractions were determined by ELISA and pooled for concentration on a 10 K cut-off spin column (Gelman Sciences). The purity of BGAF was checked by SDS-PAGE, and approximately 250 ρmoles of BGAF were submitted to Commonwealth Biotechnology (Richmond, VA) for N-terminal sequencing. The N-terminal sequence was determined to be: [V, ?] [I, E] [G, P] [N, L] YAPIGIGATV. Therefore, the peptide APIGIGAT was used to design two (sense) degenerate primers (BGAF-6, CCNATHGGNATHGGNGCNAC; BGAF-7, GCNCCNATHGGNATH-GGNGC) (Fig. 6.). Messenger RNA was isolated from total RNA from etiolated 2-d-old H95 shoots using oligo-dT coated magnetic beads according to the vendors protocol (Dynal). An Oligo-dT-primer (RT-3, 5' TTTTTTTTTTTTTTTTTC 3') was used for first strand cDNA synthesis with AMV-reverse transcriptase (Promega). To amplify BGAF, the degenerate primers (BGAF-6, BGAF-7) were individually paired with RT-3 in separate PCR reactions. The 1 kb PCR product was reamplified with Pfu Turbo DNA polymerase, gel purified, and blunt-end cloned into the *SmaI* site of pBluescript® II SK (+/-) for sequencing in both directions (Virginia Tech DNA sequencing facility).

Expression of BGAF cDNA-The BGAF cDNA sequence was utilized to design flanking oligonucleotides for expression and to contain an *NdeI* site at the 5' end of the cDNA and an *XhoI* site at the 3' end. The primer sequences are as follows with restriction sites underlined in italics: BGAF-13 (sense), 5’ *CATATG*GCTAGCGTCATAGACAACAAGGCGCCG 3’; BGAF-

14 (antisense), 5’ CTAT*CTCGAG*TCACAGGGATCGCACGTAAACG 3’. The primers were used to amplify the BGAF cDNA from the previous pBluescript clone using Pfu Turbo Taq DNA polymerase. The PCR product was subsequently ligated into the *SmaI* site in pBluescript® II SK (+/-). The construct was used to transform the *E. coli* strain DH10B and the presumptive positive (white) colonies were screened by PCR. Vector-specific primers (T3 and T7), flanking the cloning site were used to determine if white colonies had an insert of expected size (Gussow and Clackson, 1989). Recombinant colonies were isolated and grown for plasmid isolation. After large-scale plasmid isolation, the inserts were excised with *Nde*I and *Xho*I, gel purified, and cloned into the expression vector pET-21a that had been double-digested with *Nde*I and *Xho*I and gel purified. The *E. coli* host strain pLys S [F- *ompT hsdS*$_B$ (r_B-m_B-) *gal dcm*] was transformed with the recombinant plasmid construct and used in expression studies. Recombinant colonies containing the cDNA insert were identified using vector-specific (T7 promoter and T7 terminator) primers. Expression of BGAF from the presumed BGAF cDNA was corroborated by functional binding assays as described previously except crude BGAF expression extracts were used to incubate with purified rGlu1. Additionally, BGAF expression extracts were electrophoresed on 12% (w/v) SDS gels according to the method of Laemmli (1970) using a Mini-Protean II cell (BioRad Laboratories, Hercules, CA). After elecrophoresis, the gels were soaked in 1 X blotting buffer (25 mM Tris-125 mM glycine/0.25% (w/v) SDS/20% (v/v) MeOH) and electroblotted onto a BioTraceä PVDF membrane for immunostaining. The membranes were incubated overnight with anti-BGAF serum, which was produced in rabbit. The primary antibody (rabbit anti-BGAF IgG) binding was detected with peroxidase conjugated goat-anti-rabbit IgG (secondary antibody) using the chromogenic substrate 4-chloro-1-naphthol. The clone generated from the BGAF-13 primer resulted in the expression of a truncated 28 kD protein, about 5 kD smaller compared to the wild type BGAF protein isolated from the maize inbred H95, which is 32-33 kD (Fig. 6A, lanes 3 and 4). Therefore, the 5' end of the BGAF cDNA was obtained by overlapping its 5' end (327 bp) with the highest match (97%; 316/327 matches when excluding residues assigned N from the analysis) EST in the maize EST database (Genbank T70648). The identity of the EST clone was confirmed by reamplification of the original H95 cDNA using a primer (BGAF-15; 5' *CATATG*GCTAGCATGGCCAGCCTCC-

AAGTGACTCC 3') designed to start with the first methionine residue in the 5' end of the EST clone along with a BGAF specific 3' end primer. The resulting clone was sequenced for confirmation and expressed in *E.coli* to produce putative full-length mature BGAF polypeptide.

RESULTS

Sequence comparisons of Glu1 and Glu2 share 90% amino acid sequence identity and Dhr1 shares ~70% amino acid sequence identity with both Glu1 and Glu2 (Fig. 1). However, there are 154 amino acid substitutions that are unique to both Glu1 and Glu2; they are different from those occupying the homologous sites in Dhr1. The lack of interaction between Dhr1 and BGAF is thought to be due to the amino acid substitutions that separate Dhr1 from Glu1 and Glu2 in the BGAF binding domain. These substitutions in Dhr1 are thought to be at sites that are either directly involved in the BGAF-β-glucosidase interaction or indirectly affect the topology of β-glucosidase such that BGAF cannot recognize the binding site. To examine the molecular basis of the BGAF-β-glucosidase interaction and define the location of the putative binding sites, chimeric β-glucosidases were constructed (Fig. 2). Fusion sites in chimeras were chosen at residues that are identical or equivalent among Glu1, Glu2, and Dhr1 to minimize nonspecific effects on chimeric protein structure. All wild type and chimeric enzymes were expressed without tags to eliminate the possibility of nonspecific affects on protein structure, activity, and stability.

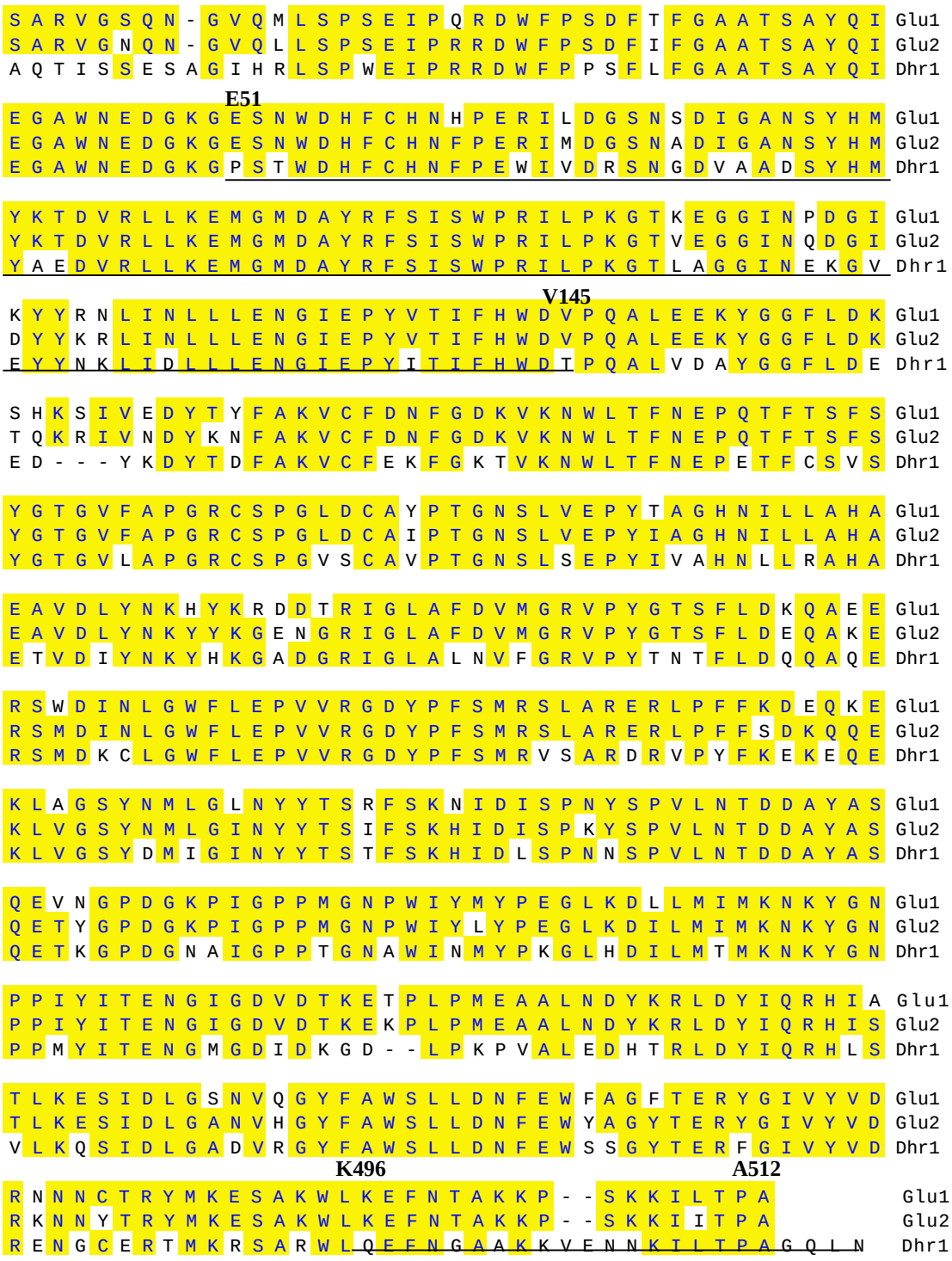

Fig. 1. Deduced amino acid sequence identity among mature maize (Glu1 and Glu2), and sorghum (Dhr1) β-glucosidases. The deduced amino acid sequences were aligned using the clustal multiple alignment program (MEGALIGN) of the DNASTAR software package. Sequences against gray background indicate regions of amino acid identity among Glu1, Glu2, and Dhr1. The two domains (Domain K496-A512 and E51-V145) that contribute to the recognition and binding of BGAF are underlined. Both domains form a contiguous surface patch in the putative tertiary structure of Glu1 (see Fig. 5.).

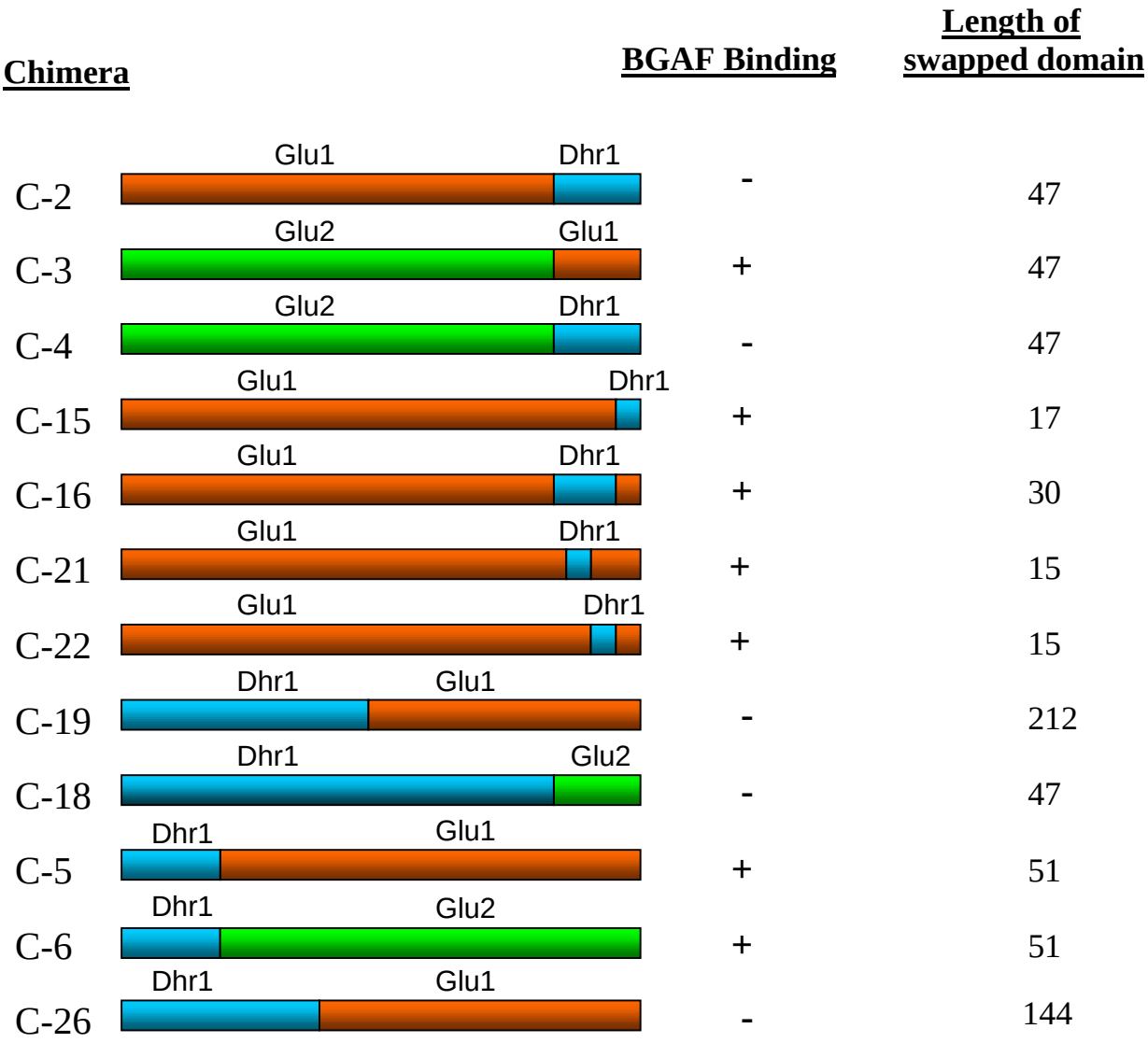

Fig. 2. Schematic representation of the 12 chimeric β-glucosidase constructs used to identify BGAF binding domains in the maize β-glucosidase isozymes Glu1 and Glu2. All chimeras were expressed in soluble, active form, and screened for BGAF binding. The results of binding assays are summarized under the column "BGAF binding" where (+) and (-), respectively, indicate positive and negative tests for binding. The lengths of the swapped domains are indicated to the right of each chimera.

BGAF Binding Assays-The interaction between BGAF and β-glucosidase is so specific and reminiscent of an antigen-antibody interaction that binding assays could be performed with both purified and unpurified ligands with no effect on assay sensitivity and specificity. Consequently, crude β-glucosidase or BGAF preparations such as bacterial cell lysates or plant tissue extracts were used as ligand source in most assays. Binding assays to test BGAF binding to Dhr1 by immunoblotting shows that BGAF does not bind to rDhr1 (Fig. 3, lower right panel) where the results were similar to those obtained with negative control BGAF by itself (Fig. 3, upper left panel) and BGAF plus *E. coli* lysate (Fig. 3, upper right panel). In contrast, the amount of BGAF detectable by immunoblotting decreased as the amount of BGAF reacting with the positive tester β-glucosidase (Glu1) increased and was unable to enter the gel (Fig. 3, lower left panel). Additionally, rDhr1 did not bind BGAF in a co-precipitation assays (data not shown) performed as described previously (Esen and Blanchard, 2000).

When we tested intact Glu1 and Glu2 and a Glu2/Glu1 chimera (C-3) for BGAF binding, the sensitive gel-shift assay used to detect binding yielded positive results as evident from the formation of BGAF-β-glucosidase complexes with reduced electrophoretic mobility on native PAGE gels. When stained for β-glucosidase activity using the fluorogenic substrate MUG, such gels clearly showed that both rGlu1 and rGlu2 and their chimera (C-3) bind to BGAF with high affinity as evident from shifting of β-glucosidase activity zones (bands) towards the cathodic end of the gel (Fig. 4, lanes 4, 6, and 19). The binding assays also demonstrate that when the C-terminal 47 amino acids from either Glu1 or Glu2 are replaced with the corresponding C-terminal 53 amino acids from Dhr1 (as is the case with C-2 and C-4, respectively) BGAF binding activity is almost completely lost (Fig. 4, lanes 8 and 10). However, when the C-terminal 17 amino acids of Glu1 is replaced by the C-terminal 23 amino acids of Dhr1, which yielded C-16, BGAF binding activity was mostly restored (Fig. 4, lane 12). Not surprisingly, replacing the extreme C-terminal 17 amino acids of Glu1 with its 23 amino acid long homologue (C-15) showed BGAF binding activity (Fig.4 , lane 14). The binding data from chimeras C-21 and C-22 show that BGAF binding becomes tighter when the disruptive region (from Dhr1) spanning amino acids G453-L495 is bisected (Fig. 4, lanes 16 and 18). In the case of Dhr1/Glu1 or Dhr1/Glu2 chimeras, in which the C-terminal Glu1 or Glu2 domains were fused with the N-

terminal Dhr1 domains varying from 144 (C-26) and 212 (C-19) to 461-amino-acids-long (C-18, data not shown), no BGAF binding to any of these three chimeras was observed (Fig. 4, lane 26 and 28).

The binding assays also show that both chimeras C-5 and C-6 have BGAF binding activity. Of these, C-5 is a Dhr1/Glu1 chimera and C-6 is a Dhr1/Glu2 chimera in which the extreme N-terminal 51 amino acid long segments of Glu1 and Glu2 were replaced with their Dhr1 homologue, indicating that this segment is clearly not involved in BGAF binding (Fig. 4, lanes 22 and 24). The domain spanning amino acids E51-P212 was further bisected through the construction of chimera C-26 in which the N-terminal amino acids S1-D144 of Glu1 were replaced with its Dhr1 homologue. Interestingly, C-26 did not have any BGAF binding activity (Fig. 4, lane 28), which establishes that the region spanning amino acids E51-V145 must contain the other domain(s) of Glu1 or Glu2 that is involved in forming the BGAF binding site on the surface of β-glucosidase.

Mapping BGAF Binding Domains-Our structural analysis was based on the modeled 3D structure of Glu1 generated by homology modeling as described above. The modeled 3D structure of Glu1 is in excellent agreement with the Glu1 structure now resolved by x-ray crystallography. Recombinant Glu1 was expressed and purified in our laboratory and in collaboration with Bernard Henrissat's crystallography group in Marseille, France, the crystal structure was solved (Czjzek et al., in preparation). Our initial finding that the C-terminal 17 amino acids of Glu1 and Glu2 (C-16) were essential, but not sufficient, for BGAF binding led to the search of the location of this domain on the surface region of the modeled Glu1 tertiary structure. To identify the other domain(s) which is (are) involved in BGAF binding, we scanned structural elements and amino acids, which are located on the surface in the direct vicinity of the C-terminal 17 amino acids. Analysis of the modeled 3D structure of Glu1 indicates that the N-terminal region maps proximally to the C-terminus in the tertiary structure (Fig. 5). On this basis, chimeras C-5, C-6, and C-26 were tested for BGAF binding activity. Chimeras C-5 and C-6 had binding activity, while C-26 did not. Collectively, the binding results of C-5, C-6, C-26, and C-19 indicate that the region between amino acids E51-V145 in the N-terminal half of the

primary structure must contain the other determinant(s) that is (are) involved in BGAF binding. Analysis of the modeled 3D structure of Glu1 confirms that the structural domains contained within the region spanning amino acids E51-V145 are located on the surface proximal to the C-terminal 17 amino acids. Both the binding data and the structural data corroborate the postulate that binding requires the formation of a site by bringing together two disparate regions of the primary structure in the tertiary structure through folding.

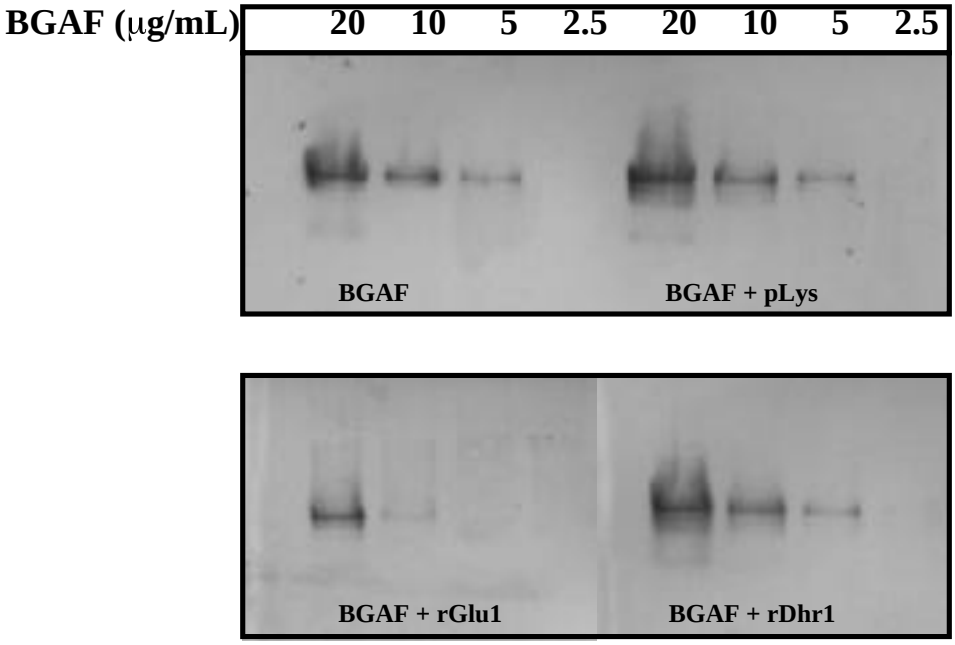

Fig. 3. BGAF binding assay for sorghum β-glucosidase isozyme Dhr1 by immunoblot analysis. Both immunoblots above were performed by mixing purified BGAF in 2-fold decreasing increments with pLys (cell line used for expression), rGlu1, and rDhr1 . The mixes were electrophoresed on 8% native gels and electroblotted onto PVDF membranes. Detection was performed sequentially using rabbit anti-BGAF serum, goat-anti-rabbit IgG peroxidase conjugate, and the chromogenic peroxidase substrate 4-chloro-1-naphthol + H_2O_2. Purified BGAF (upper left Panel) and the mixture of BGAF and pLys cell lysate from bacterium transformed with nonrecombinant plasmid (upper right panel) served as negative controls. The BGAF-rGlu1 mixes (lower left panel) served as a positive control, indicating the removal of BGAF in lanes corresponding to 5 to 20 μg/mL BGAF. The BGAF-rDhr1 mixes (lower right panel) shows that BGAF does not bind Dhr1. Note that the BGAF-rDhr1 panel is essentially identical with those of negative controls (BGAF alone and BGAF + pLys S cell lysate).

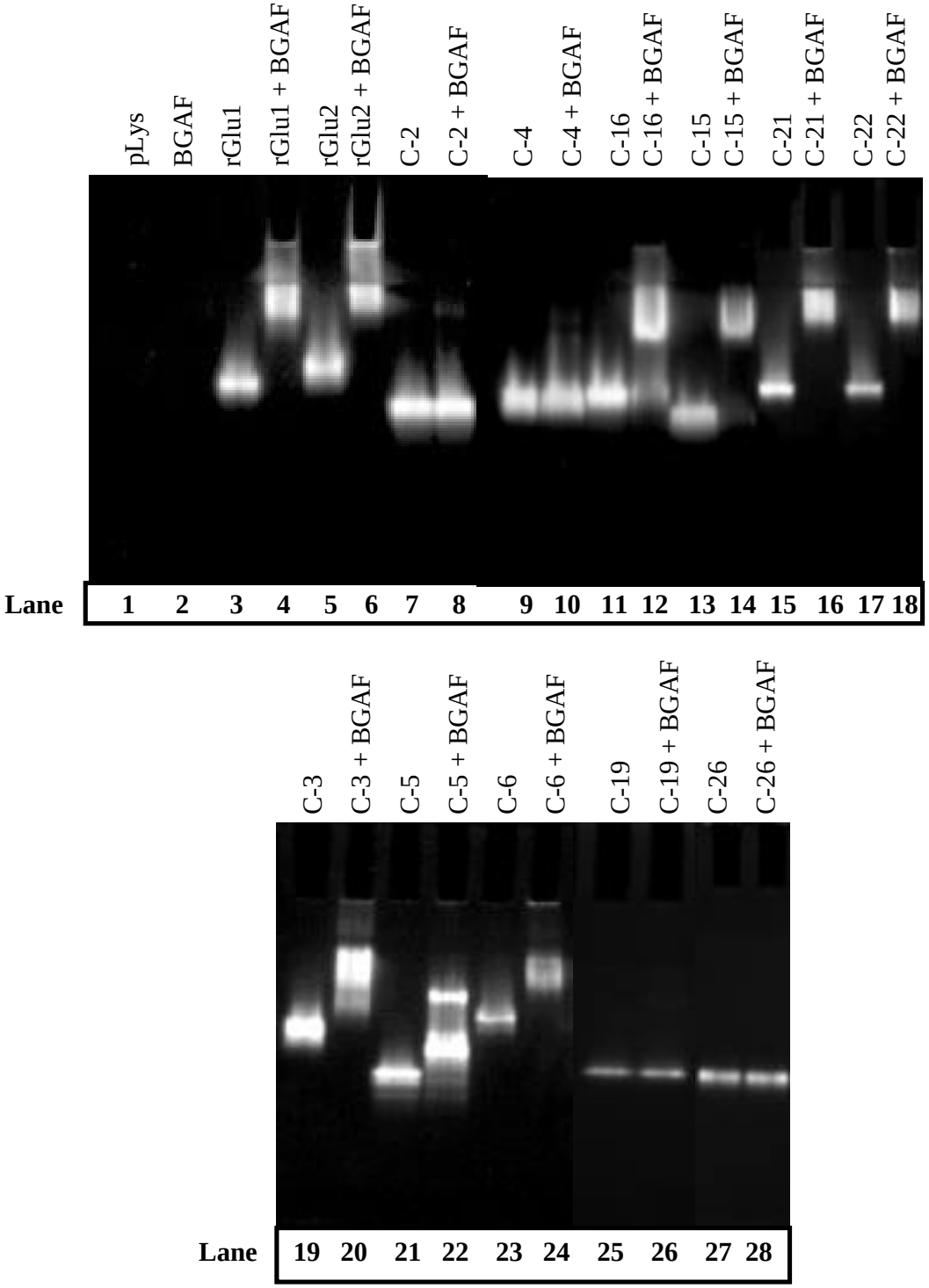

Fig-4. Gel-shift assay for the detection of BGAF binding by retarding activity zones on native gels. Purified BGAF (37 ρmoles; lane2) was incubated with approximately 3 ρmoles of purified rGlu1, rGlu2, C-2, C-4, C-16, C-15, C-21, and C-22. In the case of chimeras C-3, C-5, C-6, C-19, and C-26 crude expression extracts were used to mix with purified BGAF (37 ρmoles). Note that β-glucosidase activity zones (bands and smearing) are retarded in a region extending from the top of the resolving gel to the sample well in the stacking gel when BGAF binds wild type β-glucosidases (lanes 4 and 6) and their chimeras (lanes 12, 14, 16, 18, 20, 22, and 24).

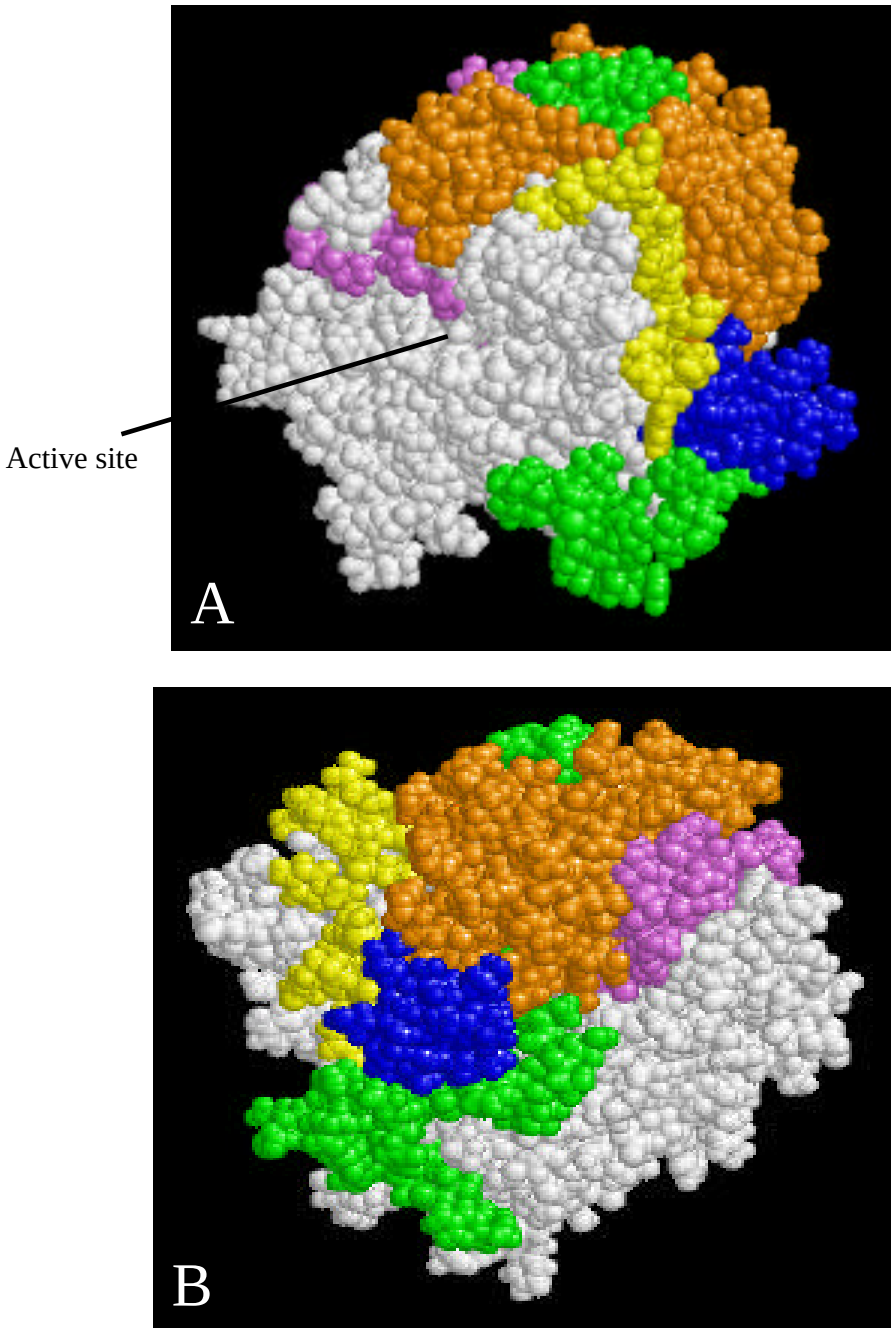

Fig. 5. 3-D structure of the modeled β-glucosidase viewed from the right-side (**A**) and left-side (**B**). The two regions involved in BGAF binding identified by binding assays are shown in blue (K496-A512) and orange (E51-V145). Note that both regions map to the same location on the surface of the enzyme and form one contiguous surface patch. Also note that the BGAF binding site (K496-A512; E51-V145) is away from and nearly perpendicular to the active site of the enzyme, consistent with our finding that BGAF binding has no effect on enzyme activity. The region spanning amino acids N481-L495 is shown in yellow. The regions shown in green (S1-G50) and violet (P146-L214) served as internal controls, since they directly surround regions that were shown to play a role in BGAF binding, yet themselves did not have any affect on BGAF binding (chimeras C-5, C-6, and C-26).

Isolation and identification of the BGAF cDNA-The cDNA encoding the BGAF protein was cloned and sequenced as described above and was previously reported (Blanchard and Esen, 2000; PGR 00-044; Genbank Accession no. AF232008)). The cloned putative BGAF cDNA was expressed in moderate yield in *E. coli* and resulted in a protein whose electrophoretic mobility was faster than BGAF isolated from plant tissue (Fig. 7A, lanes 3 and 4). Interestingly, the truncated BGAF protein was still able to bind and aggregate β-glucosidase (Fig. 7B, lane 4). Reamplification of the H95 cDNA using primers BGAF-15 (primer designed starting with the first methionine residue at the 5' end of the EST clone; Genbank T70648) and BGAF-14 resulted in a cDNA clone encoding a protein whose electrophoretic mobility and molecular size were identical to BGAF isolated from plant tissue (Fig. 7A, lanes 5-7). The deduced protein sequence of the truncated BGAF shows 96% (142/147 positives) amino acid identity with the deduced protein product of the EST sequence in the N-terminal region where they overlap. The putative BGAF cDNA is 1086-bp long and includes a 912-bp coding sequence, and a 174-bp 3' untranslated region (Blanchard and Esen, 2000; PGR 00-044; Genbank Accession no. AF232008). A BLAST search was performed on the maize EST database using the truncated BGAF cDNA. The BLAST search exclusively identified heat-shock protein EST's, that matched to putative BGAF with similarities ranging from 56% - 97%. Figure 6 shows the deduced primary structure of BGAF, which is 304 amino acids in length and contains two hexapeptide (WGGSGG) repeats that are separated by 42 amino acids. These two repeat domains are postulated to be involved in the recognition and binding of β-glucosidase.

Expression of the BGAF cDNA in *E. coli*-The expression of the BGAF cDNA was performed using two clones constructed using a primer designed from the N-terminal sequence (BGAF-13) as well as a primer designed to start with the first methionine residue in the 5’ end of the EST clone (BGAF-15). The western blot analysis of the protein products of the two BGAF cDNA’s showed the same immunoreactivity towards anti-BGAF serum as did BGAF extracted from maize shoots (Fig. 7, lanes 3-7). The BGAF cDNA clone, amplified using the 5'-end sense primer derived from the experimentally determined N-terminal sequence, encodes a polypeptide 38 amino acids shorter than that encoded by the near full-length cDNA amplified using the 5'-

end sense primer derived from the EST T70648 sequence. The absence of these 38 amino acids results in a truncated protein whose calculated molecular size is 27.5 kD and electrophoretic mobility is faster than BGAF isolated from maize (Fig. 7A, lanes 3, 4, and 7). In contrast, when the BGAF cDNA amplified using the 5' EST derived primer (BGAF-15) is expressed its immunoreactivity and electrophoretic mobility are identical to BGAF isolated from maize (Fig. 7A, lanes 5-7). The calculated molecular mass of the deduced protein is 31.8 kD, which is consistent with the molecular mass observed on SDS gels (Esen and Blanchard, 2000). More unequivocal evidence that the putative BGAF cDNA encodes BGAF was demonstrated by functional assays. Figure 7B shows a gel-shift assay for BGAF binding to β-glucosidase as described above. The gel clearly shows that BGAF expressed in *E. coli* binds β-glucosidase and retards its electrophoretic mobility in a manner identical to that obtained with native BGAF isolated from maize (Fig. 7B, lanes 4 and 6).

MVSLQVTPTSAFTEWNELKFEGLYLFHNYVGSGANQTR

VIDNK*APIGIGAT*VVNNWTVCDGPNAKLIARAQGLHIQ

AGNWVNSFSLVFVDQRFSGSPLEVTGIVVESGEWAIVGGT

GQFAMANGVISKKLHGKTSEGDIIQLTIHAFCPVLGATKR

SVTKVGPWGGSGGSPMDITAEPQRLKSITVATGIAVTSIA

FSYVDSAGQTQSAGRWGGSGGETEPVIQLGDSEVLTELS

GTIGNVDGLTVITSIKFVTSLKTYGPFGAWGNGSDTPFAIP

VQQGSAIVGFFARAGVYLDALGVYVRSL

Fig. 6. Primary structure of the putative BGAF polypeptide. The two hexapeptide repeat regions postulated to be involved in the recognition and binding of maize β-glucosidases are highlighted in red and underlined. The hydroplilicity plot (data not shown) predicts that both repeat domains reside on the surface of BGAF and thus, would be available for interaction with β-glucosidase. The N-terminal sequence determined by sequencing a purified BGAF preparation and used to derive primers from for RT-PCR are indicated by asterisks

DISCUSSION

The null β-glucosidase phenotype previously reported in maize is due to the specific interaction between maize β-glucosidase and BGAF (Esen and Blanchard, 2000). The specificity of the interaction is exemplified by the fact that β-glucosidases from fungi (*Trichoderma and Aspergillus*) and other plant sources (e.g., almond, black cherry, sorghum, rice, and oats) do not bind BGAF, despite high amino acid sequence identities up to 70% as is the case between maize and sorghum. We have cloned the cDNA encoding BGAF (Blanchard and Esen, 2000; PGR00-044; Genbank Accession no. AF232008) based on evidence from binding and immunological assays and have identified BGAF as a member of the sHsp family. The sHsp family includes an abundant and diverse group of proteins in plants, which are not well characterized. Studies of the sHsp complexes are important in elucidating the function of these ubiquitous proteins. The previous reports on protein interactions involving the sHsps suggest that these proteins cooperate with larger Hsps and act as molecular chaperones. Recently, a model for the molecular chaperone activity of sHsps has been proposed (Waters et al., 1996; Ehrnsperger et al., 1997; Lee et al., 1997; Veinger et al., 1998; Lee and Vierling, 2000). This model suggests that sHsps act to bind nonnative proteins, preventing their aggregation and maintaining them in a state competent for ATP-dependent refolding by other chaperones. The BGAF-β-glucosidase interaction represents a highly specific, novel sHsp interaction, which is reminiscent of antigen-antibody interactions. In this study we have provided insight into the mechanism of the BGAF-β-glucosidase interaction, by taking advantage of the high degree of amino acid sequence identity among Glu1, Glu2, and Dhr1 to create chimeras among them. The binding of BGAF to the chimeras was then examined by combined use of a highly sensitive functional gel-shift and β-glucosidase activity assay. Importantly for the interpretation of the results, all of the constructs were enzymatically active, confirming that all were expressed in soluble and catalytically active form.

The BGAF binding assays clearly show that replacement of the C-terminal 47 amino acids of Glu1 with the corresponding C-terminal region (53 amino-acid-long) of Dhr1 (C-2) results in a near complete loss of binding activity (Fig. 4, lane 8). It was also observed that restoring the C-terminal 17 amino acids of Glu1 onto C-2 (C-16) reconstituted most of the

binding activity lost in chimera C-2 (Fig. 4, lane 12). Given the results of chimeras C-2, C-4, and C-16 one would be predict that there was a single domain for BGAF binding, which spans the 30 amino acids from F466 to L495 in the C-terminal region. Consequently, one would also predict that chimera C-15 in which the extreme C-terminal 17 amino acids of Glu1 was replaced with the C-terminal 23 amino acids of Dhr1 would not bind BGAF, but C-15 had binding activity (Fig. 4, lane 14). However, the result is not surprising since most protein-protein interfaces are confined to a single surface patch that is usually composed of more than one stretch of polypeptide chain. Additionally, Stites (1997) reported that 17 amino acids per monomer typically contribute to the recognition and binding in protein-protein interactions. It is unlikely that all 17 amino acids in the C-terminal binding domain of Glu1 are accessible or involved in BGAF binding. The fact that typical binding domains are made up more than one stretch of polypeptide is also supported by the finding that chimeras C-18 (data not shown) and C-19 did not bind BGAF (Fig. 4, lane 26). This indicates that the BGAF binding domain of maize β-glucosidases is not solely formed by the C-terminus, and thus, it alone is insufficient to evoke BGAF binding. Additionally, BGAF showed no binding activity towards inactive denatured rGlu1 extracted from inclusion bodies (data not shown). Again, this information indicates that the tertiary structure of the correctly folded enzyme is essential to form a functional binding site to interact with BGAF, similar to antigen-antibody interactions.

The gel-shift assay (Fig. 4, lane 14) also indicates that chimera C-15 has a higher affinity for BGAF than C-16 (Fig 4, lane 12), suggesting that key amino acids that make up the binding site in the C-terminus are localized in the region spanning amino acids F466-L495. However, this is not likely since chimeras C-21 and C-22 bind BGAF with the same affinity (Fig. 4, lanes 16 and 18). In C-21 and C-22 the disruptive region spanning amino acids F466-L495 is bisected and consequently has no discernible affect on BGAF binding. A comparison of C-21, C-22, and C-16 suggests that the smaller domain swaps (C-21 and C-22) introduce fewer amino acid substitutions such that the tertiary structure of the enzyme closely resembles that of the wild type; hence BGAF binding is tighter. Additionally, the smaller domain swaps correctly position the determinant in the C-terminal 17 amino acids such that the topology of the BGAF

binding site is structurally intact. It is also plausible that both C-15 and C-16 contain certain amino acids that make up the BGAF binding site.

The finding that chimera C-19 did not bind BGAF suggested that the N-terminal 212 amino acids contain the other domain(s) that is (are) involved in BGAF binding. To identify the other domain(s) we searched surface patches in the direct vicinity of the C-terminal 17 amino acids in the tertiary structure. Analysis of the modeled 3D structure of Glu1 indicates that several domains contained within the N-terminal 212 amino acids are located on the surface proximal to the C-terminus in the tertiary structure (Fig. 5). On this basis, chimeras C-5, C-6, and C-26 were constructed to define more specifically the binding domain(s) in the N-terminal region. The binding assay showed that C-5 and C-6 had BGAF binding activity, while C-26 did not (Fig. 4, lanes 22, 24, and 28). Since the domains tested in chimeras C-5, C-6, and C-26 surround the disruptive domains in the tertiary structure of Glu1, they serve as internal controls, helping define the boundary of the binding site. This indicates that the region spanning E51-V145 contains the other determinants involved in BGAF binding. Analysis of the modeled 3D structure of rGlu1 confirms that structural domains contained within amino acids E51-V145 constitute a surface patch, which includes the C-terminal 17 amino acids (Fig. 5). Thus, the binding assays have identified two discontinuous segments that are brought together in the modeled β-glucosidase to form a functional BGAF binding site. The fact that two discontinous segments are brought together in the tertiary structure to form a typical binding site supports the view that the disruptive effects were caused by local structural changes, not any type of global misfolding.

BGAF isolation and identification-The BLAST search performed on the maize EST database using the BGAF cDNA exclusively identified heat-shock proteins, which matched with similarities ranging from 56%-97%. The highest 5'-end match in the EST database (Genbank T70648) was used to obtain the 5'-end of BGAF by overlapping the 5' EST (327 bp) with the 5' end of the truncated BGAF sequence. The identity of the EST clone was confirmed by reamplification of the H95 cDNA using a primer designed to start with the first methionine residue at the 5'-end of the EST clone (Genbank T70648) along with a BGAF specific 3'-end primer. The resulting clone was sequenced and the 5'-end of the deduced BGAF protein shows

96% (142/147 positives; 38 amino acids or 114 bp longer than our original overlap) amino acid sequence identity with the EST deduced protein where the two overlap. The few nucleotide mismatches that were found were attributed to sequencing errors from the EST clone since these clones are subjected to single pass sequencing, and thus, contain errors. In fact, 6 of the 18 nucleotide mismatches between the 5' BGAF sequence and the EST sequence in the overlap region (441 bp) were assigned N (any nucleotide) in the EST sequence due to sequencing errors. Interestingly, BGAF also shared significant amino acid identity (53% and 56%) with three heat-shock proteins (Genbank AF021258, U434396, and U43497) from barley that function in the systemic acquired resistance response. To a lesser extent BGAF shared amino acid identity (38%) with a similar protein (Genbank U32427; Ryals et. al, 1996) from wheat, which is also involved in systemic acquired resistance.

The fact that the experimentally determined N-terminal sequence (YAPIGIGATV) is actually an internal peptide is a quandary. The most plausible explanation for this observation is that BGAF was degraded by an endogenous protease and is supported by the routine finding of immunoreactive low molecular mass fragments on westerns probed with anti-BGAF serum. Additionally, leaves, which are rich in proteases were used as protein source for the extraction of BGAF during purification. Alternatively, this determined sequence really is the N-terminal sequence of the mature protein and it is post-translationally modified (e.g., glycosylated). If this is the case, the region between the N-terminal residue M and the experimentally determined N-terminal residue belongs to a signal or transit sequence. This interpretation does not contradict the similarity in electrophoretic mobility in SDS-PAGE between the presumptive recombinant BGAF and BGAF isolated from maize because both the recombinant precursor protein and the posttranslationally modified mature protein may have similar molecular weights.

The unambiguous identity of the putative BGAF cDNA was shown following its expression in *E. coli*. Western blots of lysates of *E. coli* in which the putative mature BGAF coding clone (5'-end obtained by overlapping with the EST) was expressed and probed with anti-BGAF serum showed an immunoreactive band whose electrophoretic mobility and molecular size were identical to BGAF isolated from plant extracts (Fig. 7A, lanes 5-7). Additionally, functional assays clearly showed the presence of BGAF in *E. coli* expression extracts, which

tested positive for β-glucosidase aggregating activity in gel-shift assays (Fig. 7B, lanes 5 and 6). Expression extracts of the truncated BGAF protein generated from the experimentally determined N-terminal sequence also showed β-glucosidase aggregating activity (Fig. 7B, lanes 3 and 4) as well as immunoreactivty towards anti-BGAF serum (Fig. 7A, lanes 3 and 4).
The most intriguing finding was that the primary structure of BGAF contains two hexapeptide repeats (WGGSGG) that are separated by 42 amino acids. The hydrophilicity plot predicts that both hexapeptide repeats reside on the surface of BGAF and thus, would be available for interaction with their binding site on maize β-glucosidases. Our previous finding that the BGAF-β-glucosidase aggregates are in excess of 1.5 X 10^6 Daltons (Esen and Blanchard, 2000) suggests that both BGAF and β-glucosidase must be bivalent. If BGAF were monovalent, it could only bind one β-glucosidase dimer, resulting in a quaternary association of ~ 180-190 kD, which is not observed. Additionally, the 180-190 kD aggregates would be soluble and would not sediment at 20,000g, which is also not observed (Esen and Blanchard, 2000). Densitometric analysis of BGAF and β-glucosidase monomer intensities after co-sedimentation and correcting for size differences suggests a stoichiometry of about 2 molecules of β-glucosidase (homodimer) to 1 molecule of BGAF (monomer), consistent with the bivalency of both molecules. Based on the binding assays, the finding of two hexapeptide repeats in the primary structure of BGAF, and the biochemical data of the BGAF-β-glucosidase association (Esen and Blanchard, 2000) we have generated a hypothetical model of how the BGAF-β-glucosidase aggregates exist in vitro or in vivo, or both (Fig. 8). The model predicts that the BGAF molecule binds as a divalent ligand to two β-glucosidase dimers, and serves as a linker between β-glucosidase dimers in a linear chain in which monomeric BGAF with two binding sites and β-glucosidase alternate. In fact, the β-glucosidase-BGAF interaction mechanism is reminiscent of antigen-antibody interactions and is characterized as having an equivalence point where optimal precipitation occurs (Esen and Blanchard, 2000). When soluble β-glucosidase and BGAF are present in the correct ratios, optimal precipitation occurs. Consistent with our model, it is predicted that only large insoluble β-glucosidase-BGAF complexes are formed in the region of equivalence. In the region of either β-glucosidase or BGAF excess only small complexes are formed. Thus, in β-glucosidase excess, most BGAF sites are combined with β-glucosidase, but few β-glucosidase molecules would have

both sites bound to BGAF, resulting in the premature termination of β-glucosidase-BGAF complexes that would remain soluble.

It appears that binding of BGAF to β-glucosidase has no detectable effect on enzyme activity and kinetic parameters, suggesting that BGAF binding either does not sterically block the active site or does not change the conformation to affect enzyme activity. This suggestion is corroborated by the finding that the postulated BGAF binding site (K496-A512 and E51-V145) on β-glucosidase is essentially perpendicular to the active site (Fig. 5). One plausible function of BGAF and its interaction with β-glucosidase is that BGAF plays a protective role for β-glucosidase, shielding the enzyme from endogenous proteases or proteases in the salivary secretions of invading pests or pathogens. Additionally, the BGAF-β-glucosidase interaction keeps active β-glucosidase at the wound site, preventing the enzyme from diffusing to other parts of the plant where it has been shown to illicit deleterious affects/compounds (Hopke et al., 1994).

In conclusion we have unequivocally shown that BGAF is a sHsp that specifically interacts with β-glucosidase. Based on corroboratory binding and structural data we have identified two different regions in the primary structure of β-glucosidase, which form a BGAF binding site on the surface of the tertiary structure of the enzyme. We have also identified that the primary structure of BGAF contains two hexapeptide repeats, consistent with our finding one binding site per monomer of β-glucosidase (two per homodimer). The physiological relevance of the BGAF-β-glucosidase interaction remains to be elucidated. The finding that BGAF shares significant amino acid identity (52% and 56%) with three defense proteins (Genbank AF021258, U43496, and U43497) from barley involved in systemic acquired resistance coupled with the fact that β-glucosidase also plays a role in defense is suggestive that the interaction may have physiological relevance. Future studies will focus on precise identification of specific amino acids within the binding sites on BGAF and β-glucosidase and their roles using site-directed mutagenesis and X-ray crystallography

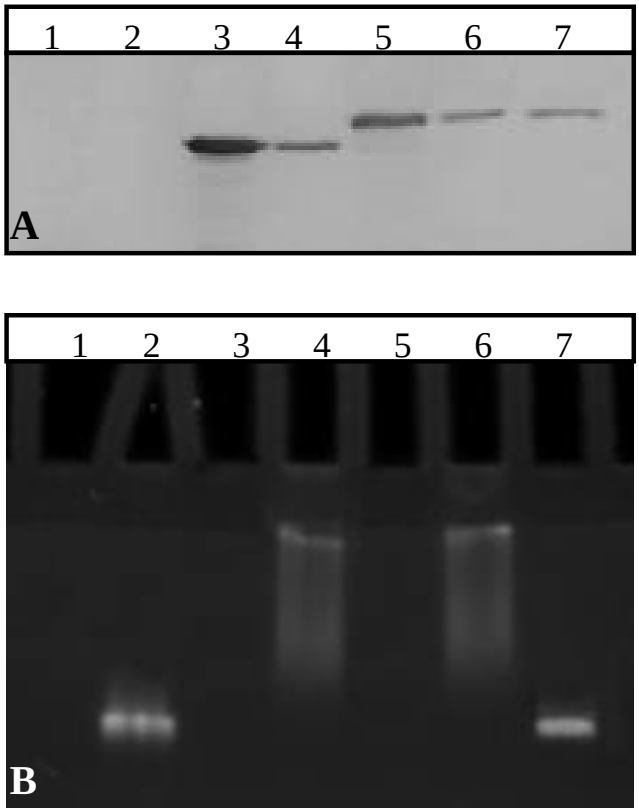

Fig. 7. Expression of the BGAF cDNA in *E. coli*. (**A**) Western blot of *E. coli* lysates probed with anti-BGAF serum. Lane 1, total protein from nonrecombinant pET21a containing *E. coli* pLys cells (negative control); 2, soluble protein from nonrecombinant pET21a containing *E. coli* pLys cells (negative control); 3, total protein from recombinant pET21a containing *E. coli* pLys cells in which the truncated BGAF cDNA is expressed; 4, same as lane 3 except soluble protein; 5, total protein from recombinant pET21a containing *E. coli* pLys cells in which the full-length mature protein coding BGAF cDNA is expressed; 6, same as lane 5 except soluble protein; 7, shoot extract from maize inbred H95 serving as a positive control. Note that putative full-length BGAF (lanes 5 and 6) produced in *E. coli* is the same size as that produced in the maize plant (lane 7). (**B**) BGAF binding to β-glucosidase detected on an 8% native-PAGE stained with 1mM MUG. Lane 1, pLys cells containing nonrecombinant pET21a (negative control); 2, rGlu1 (positive control); 3, expression extract from the truncated BGAF clone; 4, rGlu1 + expression extract from the truncated BGAF clone; 5, expression extract from the putative full-length (mature) BGAF clone; 6, same as 5, but mixed and incubated with rGlu1; 7, rGlu1 + lysate from pLys cells containing nonrecombinant pET21a (negative control). Note that only the lanes containing recombinant truncated (lane 4) and putative full-length BGAF show retarded β-glucosidase activity zones.

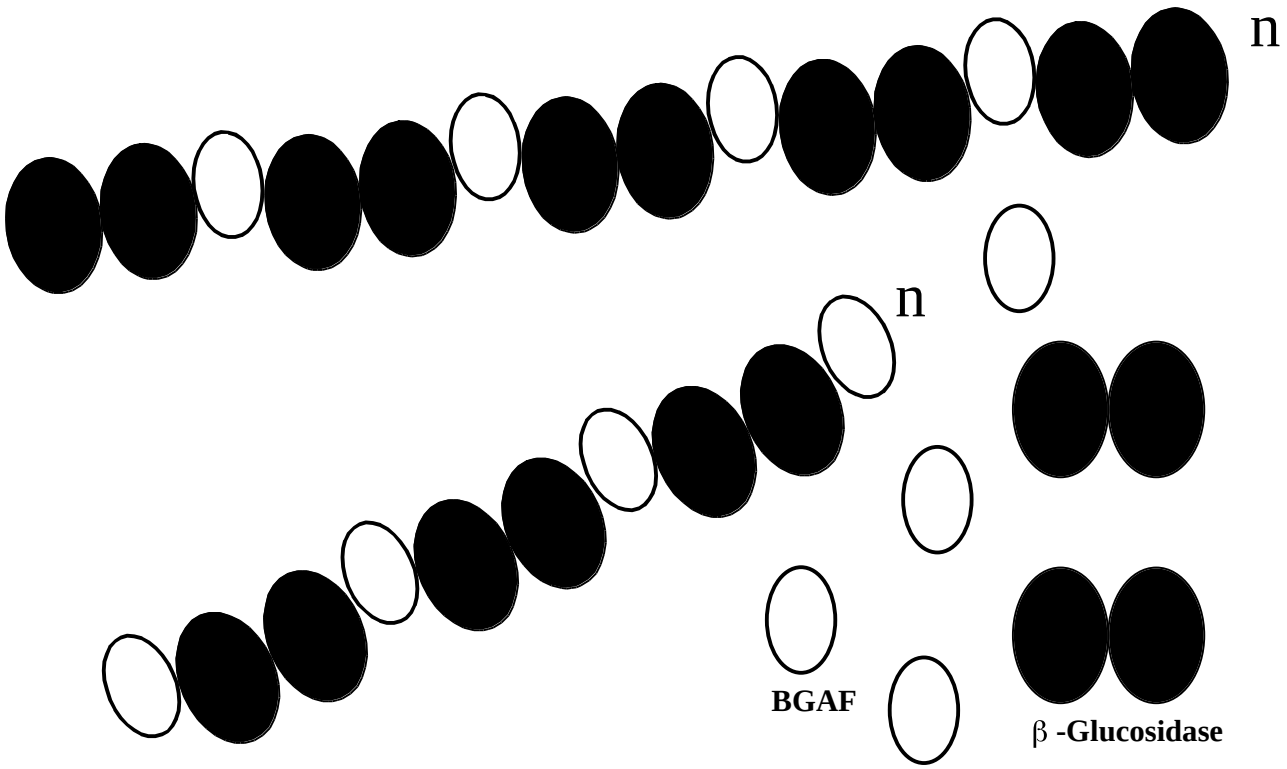

Fig. 8. Model of β-glucosidase-BGAF aggregates. The model is based on BGAF-β-glucosidase binding assays, which have identified one BGAF binding site per β-glucosidase monomer, two per homodimer. Additionally, BGAF in its uncomplexed form exists as a monomer and thus, must minimally be bivalent. The two hexapeptide repeats found in the primary structure of BGAF (Fig. 6; WGGSGG) support the model along with stoichiometric data, which shows a ratio of 2 molecules of β-glucosidase (dimer) per one molecule of BGAF.

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