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Determination of β-glucosidase aggregating factor (BGAF) binding and polymerization regions on the maize β-glucosidase isozyme Glu1

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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, which do not bind to BGAF) 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). 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.

1. Introduction

Glycosyl hydrolase family 1 (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 the three (Archaea, Eubacteria, and Eukarya) domains of living organisms. They play or has been proposed to have 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 rice, more than 34 members of the glycosyl hydrolase family 1 genes are present (Opassiri et al., 2006); thus an equal or greater number of the glycosyl hydrolase family 1 genes and their protein products are expected to be present in maize. Among them, two β-glucosidase isozymes, Glu1 and Glu2, have been studied and their cDNAs have been cloned (Bandaranayake and Esen, 1996). These two isozymes are each dimers of 58 KD monomers and their major function is believed to be 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 (>1.5×10^6 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

Abbreviations: 4-MUGlc, 4-methylumbelliferyl-β-D-glucoside; BGAF, β-glucosidase aggregating factor; Dhr1, sorghum β-glucosidase isozyme Dhr1; Dhr2, sorghum β-glucosidase isozyme Dhr2; DIMBOAGlc, 2-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; FAC, frontal affinity chromatography; Glu1, maize β-glucosidase isozyme Glu1; IPTG, isopropyl β-D-1-thiogalactopyranoside; JRL, jacalin-related lectin; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; *p*NPGlc, *p*-nitrophenyl-β-D-glucoside; HPLC, high performance liquid chromatography.

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(a dimer of 32 KD monomers) 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 (Cicek and Esen, 1999) share 90% sequence identity. Similarly, sorghum β-glucosidase isozymes Dhr1 and Dhr2 (dimers of 57- and 62-KD monomers, respectively) (Hosel et al., 1987) 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 towards 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. (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, which bind to BGAF, and sorghum β-glucosidase isozymes Dhr1 and Dhr2, which do not bind to BGAF, show that the two regions implicated in BGAF binding are too long or broad and have many shared amino acid identities (Fig. 1). We postulate that BGAF-binding regions on maize Glu1 and Glu2 are actually shorter than those predicted by previous studies. Therefore, 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.

2. Results

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

Wild type and chimeric β-glucosidases (see Fig. 2) were expressed at high levels (4.5–6.0 mg enzyme/1000 mL culture volume) in the *Escherichia coli* BL 21 CodonPlus RIL strain except for the chimeric enzyme C-2, which was expressed at a low level (0.7–1.0 mg enzyme/1000 mL culture volume) (Supplement Fig. 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 Fig. 1, *lanes 2–15*). Recombinant BGAF yielded a near homogenous preparation in one step (Supplement Fig. 1, *lane 1*) after affinity purification on a lactosyl-agarose column.

2.2. β-Glucosidase–BGAF-binding assay

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 (Fig. 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 (Fig. 3). In the pull-down assay, the positive control (wild type Glu1 incubated with BGAF) showed a 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 (Fig. 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 (Fig. 1) and the three-

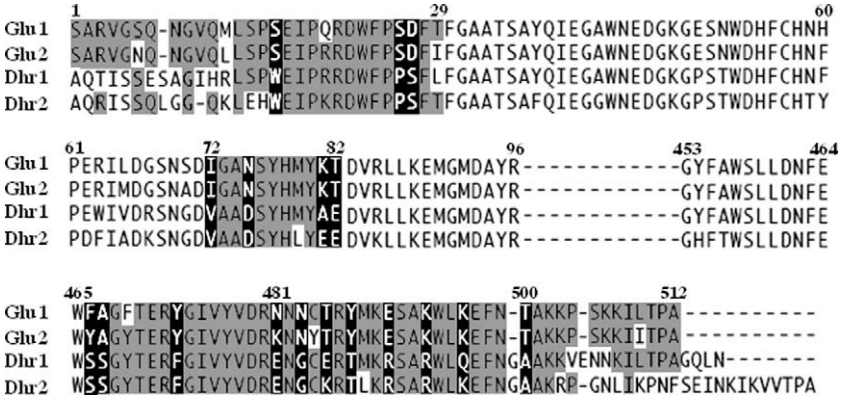

Fig. 1. Sequence alignment of the N-terminal (1–96) and C-terminal (453–512) regions of maize (Glu1 and Glu2) and sorghum (Dhr1 and Dhr2) β-glucosidases. The three regions (Ser^{1}–Thr^{29}, Ile^{72}–Thr^{82}, and Phe^{466}–Ala^{512}) making up the BGAF-binding site in Glu1 are in *Gray* background.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