The Aglycone Specificity-determining Sites Are Different in 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA)-glucosidase (Maize β-Glucosidase) and Dhurrinase (Sorghum β-Glucosidase)*

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Muzaffer Cicek‡, David Blanchard‡, David R. Bevan§, and Asim Esen‡¶

From the ‡Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0406 and §Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0308

The maize β-glucosidase isozyme Glu1 hydrolyzes a broad spectrum of substrates in addition to its natural substrate DIMBOAGlc (2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one), whereas the sorghum β-glucosidase isozyme Dhr1 hydrolyzes exclusively its natural substrate dhurrin (p-hydroxy-(S)-mandelonitrile-β-D-glucose). To study the mechanism of substrate specificity further, eight chimeric β-glucosidases were constructed by replacing peptide sequences within the C-terminal region of Glu1 with the homologous peptide 477ENGCERTMKR486 of Dhr1. The maize β-glucosidase isozyme Dhr1 was also cloned and sequenced in our laboratory (14); it shares ~70% sequence identity with each of the two maize isozymes. The catalytically active form of both maize and sorghum β-glucosidases is a 120-kDa homodimer or its multimers. The primary structures of both enzymes contain the peptide motifs TFNEP and ITENG, which are shown to be highly conserved and make up the catalytic site in all family 1 β-glucosidases (15–17). Furthermore, the three-dimensional structures of six family 1 β-glucosidases (white clover linamarase, white mustard myrosinase, Lactococcus lactis 6-phospho-β-galactosidase, Bacillus polymyxa β-glucosidase, Sulfolobus sulfataricus β-glucosidase, and Thermoplasma aggregans β-glucosidase) have recently been solved using crystals of the enzyme-glycosyl complexes (18–23). In these four cases, the residues of the TFNEP and (I/V)TENG motifs or their equivalents were found to form part of a pocket or crater-shaped active site (24). The two catalytic glutamic acids in β-β-glucosidases (i.e. the nucleophile and the acid-base catalyst) and one glutamic acid (i.e. the nucleophile) and a glutamine (the water activator) in myrosinases were positioned within the active site at appropriate distances (2.5–3Å) on opposite sides of the glycosidic bond.

There are two fundamental questions about β-glucosidase-catalyzed reactions. 1) How do β-glucosidases catalyze the hydrolysis of the β-glycosidic bond between two glycone residues (e.g. cellobiose and other β-linked oligosaccharides) or that between glucose and an aryl or alkyl aglycone (e.g. many naturally occurring substrates in plants)? 2) What determines substrate specificity, including the site and mechanism of aglycone binding? Much progress has been made in understanding the mechanism of catalysis and defining the roles of specific amino acids involved in catalysis within the active site. However, there is virtually no information as to how β-glucosidases recognize their substrates and interact with them, specifically the aglycone moiety, which is the basis of tremendous diversity in natural substrates and is responsible for subtle substrate specificity differences among β-glucosidases. The maize β-glu-

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¶ To whom correspondence should be addressed. Tel.: 540-231-5894; Fax: 540-231-9307; E-mail: aevatan@vt.edu.

1 The abbreviations used are: DIMBOA-glucosidase, 2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucosidase; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; PCR, polymerase chain reaction; chim, chimera; PAGE, polyacrylamide gel electrophoresis; 4MUGlc, 4-methylumbelliferyl-β-D-glucoside; 6BNGlc, 6-bromo-2-naphthyl-β-D-glucoside; NPGlc, nitrophenyl-β-D-glucoside.

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Muzaffer Cicek‡, David Blanchard‡, David R. Bevan§, and Asim Esen‡¶

From the ‡Department of Biology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0406 and §Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061-0308
cosidase isozyme Glu1 and its sorghum homologue Dhr1 provide an ideal model system to address questions related to substrate specificity, because these enzymes represent extremes in substrate specificity. Although Dhr1 hydrolyzes only its natural substrate dhurrin, Glu1 hydrolyzes a broad spectrum of artificial and natural substrates in addition to its natural substrate DIMBOAGlc, but it does not hydrolyze dhurrin (Fig. 1).

The first attempt to investigate substrate specificity using chimeric β-glucosidases was made by Singh and Hayashi (25), who exchanged the C-terminal 58-amino acid-long domain of a prokaryotic β-glucosidase from *Cellobiirio gilvus* with the C-terminal 60-amino acid-long domain of *Agrobacterium tumefaciens* β-glucosidase. They showed that the resulting chimeric enzyme exhibited the substrate specificity of *A. tumefaciens* β-glucosidase. Hoa and Hayashi2 also show that the deletion of 70 amino acids from the C-terminal region of *C. gilvus* β-glucosidase leads to complete loss of activity. These investigators concluded that the C-terminal region of the enzyme played a major role in determining substrate specificity and catalytic activity.

The importance of specific enzyme domains in substrate specificity and catalytic efficiency was also shown in a number of other enzyme chimeras produced from two enzymes that differ with respect to substrate specificity. For example, it was shown that exchanging a small N-terminal portion between two rice α-amylase isozymes (Amy A and Amy 3D) resulted in a chimeric enzyme (Amy A/3D) that shows high activity on both soluble starch and oligosaccharides, whereas parental enzymes have high activity only on either soluble starch (Amy A) or oligosaccharides (Amy 3D) (26).

The purpose of the studies described in this paper was to gain insight into the mechanism of substrate (aglycone) recognition and binding in β-glucosidases using two plant enzymes, the maize isozyme Glu1 and sorghum isozyme Dhr1, as model systems. To this end, we have constructed eight chimeric enzymes by reciprocal domain-swapping. Target domains were selected based on amino acid sequence comparisons and analysis of modeled three-dimensional structures. We demonstrate that the maize Glu1 isozyme gains the ability to hydrolyze dhurrin when the C-terminal 47-amino acid-long region or specific subdomains within this region are replaced by corresponding Dhr1 region, whereas the reciprocal replacement has no effect on the substrate specificity of the sorghum Dhr1 isozyme, except a 12-fold reduction in activity.

**EXPERIMENTAL PROCEDURES**

**Construction of Chimeric β-Glucosidases**—The first step toward understanding the basis of substrate specificity in maize and sorghum β-glucosidases was to construct cDNAs encoding chimeric enzymes by domain swapping. Since we had already cloned and expressed cDNAs encoding maize Glu1 and Glu2 isozymes as well as the sorghum Dhr1 isozyme in *Escherichia coli* (27), the construction and expression of chimeric cDNAs using these wild type parental templates were straightforward. The criteria for chimeric constructs was that the swapped region includes active amino acid and substitution positions within the C-terminal domain that map to or around the active center in the modeled three-dimensional structures of Glu1 and Dhr1. Chimeric cDNAs were constructed by the PCR-based recombination technique of overlap extension and the high fidelity thermostable Turbo® Ffu polymerase (Stratagene) as described (27, 28). Three of the primer pairs (P100-P101, P166-P167, and P168-P169, Table I) were from conserved regions, such that they will function on both glu1 and dhr1 cDNA templates. Sequences of oligonucleotide primers used in PCR are given in Table I. The corresponding peptides that were swapped are shown in Table I and Figs. 2, A and B, respectively. The constructs were made so as to encode chimeric enzymes Glu1/Dhr1 or Dhr1/Glu1, where the enzyme before the slash contributed the N-terminal region, and the one after the slash contributed the C-terminal region (Fig. 2B). As a first step in defining the domain-determining substrate specificity of Glu1 and Dhr1, chimeric 2 (abbreviated hereafter as chim 2) was constructed by replacing the extreme 47-amino acid-long C-terminal region (amino acids 466–512) of Glu1 with the corresponding 53-amino acid-long region (amino acids 462–514) of Dhr1. The 5′ portion of the chimeric cDNA was amplified on the glu1 template using the vector-specific primer T7 (sense) and gene-specific primer P101 (antisense), whereas the 3′ portion was amplified on the dhr1 template using the primers P100 (sense) and P101 (antisense) were derived from the region of cDNA encoding the peptide sequence GWFAWSL, which is invariant in Glu1 and Dhr1 (Fig. 2A). The two PCR fragments were gel-purified, denatured, mixed, annealed (by overlapping the complementary ends that contain primers P100 and P101 sequences) and extended to obtain full-length chimeric Glu1/Dhr1 cDNA sequence. The full-length chimeric cDNA was amplified by using the vector-specific primer pair of T7 (sense) and T7 antisense on the overlap extended template. The resulting PCR product was purified, digested with NheI and XhoI, and cloned into the expression plasmid pET21a. The construction of other chimeric cDNAs (chim 15, 16, 17, 21, 22, 23, and 29) followed the procedure described for chim 2, except the primer pairs used for PCR (Table I). Among the eight chimeric enzymes produced, the sizes of the swapped peptides varied from 8 to 53, all coming from the C-terminal fragment 462–514 of Dhr1 and 466–512 of Glu1 (Table I).

**Expression and Purification**—Wild type and chimeric β-glucosidases were produced in *E. coli* pLyS cells (*Fomp* hadSP *rT*-*mU*-*gal dcm*) under the control of the T7 RNA polymerase promoter in the expression plasmid pET-21a (Novagen) as described by Cieck and Esen (27). The cell lysis and protein extraction procedure was performed as described (27). For purification, β-glucosidase was precipitated from crude cell extract with a 50 to 65% ammonium sulfate (NH4)2SO4 cut. The precipitate was dissolved in 50 mM sodium acetate buffer, pH 5.0, and centrifuged at 18,000 × g for 30 min. The supernatant was adjusted to a final concentration of 0.5 M (NH4)2SO4 and centrifuged at 18,000 × g for 30 min. Then the supernatant was applied to a Toyopearl-buty1 650M (TosoHaas) hydrophobic interaction chromatography column (1.6 × 14 cm). The column was washed to baseline absorbance with 0.5 M (NH4)2SO4 in buffer and eluted with approximately 5 bed volumes of

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2 T. T. Hoa and K. Hayashi, unpublished results.
a reverse salt gradient of 0.5 m to 0.1 m (NH₄)₂SO₄ in 50 mm sodium acetate buffer, pH 5.0. The resulting fractions were assayed for β-glucosidase activity using the artificial substrate pNPglc or the natural substrates DIMBOAglc or dhurrin. The fractions with activity were pooled based on purity as judged by SDS-PAGE. The pooled fractions that had chimeras were not stable at pH 5.0 (acetate buffer). Again, the fractions were pooled based on activity by rechromatography on a TocoPearl-phenyl 650M column (Toso Haas) as described above. The resulting fractions were assayed for β-glucosidase activity.

Expression and Purification of Chimeric Enzymes—The maize Glu1 and sorghum Dhr1 isozymes and their eight chimeras (Fig. 2B) were generated in E. coli BL21 (DE3) pLyS cells. The purified parental Glu1, Dhr1, and their chimeras (Fig. 2B) were then used for kinetic analysis with special emphasis on substrate specificity.

Thin Layer Chromatography—The natural substrates dhurrin and DIMBOAglc were purified as described (27). The purified parental Glu1, Dhr1, and their chimeras (Fig. 2B) were then used for kinetic analysis with special emphasis on substrate specificity. A “minus enzyme control” was included in the assay.

Expression of DimBOAglc—The model of the three-dimensional structure of Glu1 and Dhr1 were generated by homology modeling using the Modeller4 program (33). The models were based on the known three-dimensional structures of linamarase, the cyanogenic β-glucosidase from white clover (Protein Data Bank code 1cbg), and myrosinase from white mustard (Protein Data Bank code 1myr). Five models each of Glu1 and Dhr1 were generated in Modeller4. The models were sufficiently similar to allow the generation of a “minus enzyme control” was included in the assay.

Enzyme Assays—For activity assays in native PAGE gels, the purified parental and chimeric enzymes were electrophoresed into 6% alkaline gels to obtain zymograms using the fluorogenic substrate 4MUGlc and the chromogenic substrate 6BNGlc as described (29, 30).

TABLE I

| Oligonucleotide primers | Chimeric Glu1/Dhr | β-Glucosidase-1 peptide sequence swapped (Glu1-specific amino acids shown in bold) |
|-------------------------|------------------|----------------------------------------------------------------------------------|
| 100-GCC TAC TTC GCG TGG TCT CTS | 17 | **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ |
| 101-AGA GAC CAC GCG AAG TAG CCA | 2 | **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ |
| 166-GCC AAG TGG TTG AG GAG TTC AA(S) | 23 | **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ |
| 167-TTG AAC TCT TGC AAC CAC CTG GCA | 39 | **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ **[S]**GFTERYGVYVRNNTMCKMYSKWLKEMTAP**[K]**SILTPA**[G]**KQ |

Expression levels were about 10% of the total E. coli protein, and solubility was close to 30% (data not shown) when cultures were grown at 37 °C and induced at room temperature. Since the expressed proteins did not contain affinity tags, they were purified by a simple two-step conventional procedure, differential solubility (35–60% (NH₄)₂SO₄ cut) followed by hydrophobic
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Fig. 2. A, alignment of the sequences of maize β-glucosidase (Glu1) and sorghum dhurrinase (Dhr1). The regions of sequence identity are shown in yellow background. The downward arrow indicates the boundary of the swapped C-terminal region in chim 2 where variant amino acids between Glu1 and Dhr1 are shown in red background. The bold-faced peptide motifs TFNEP and ITENG are highly conserved in all family 1 β-glycosidases; they contain the two key catalytic glutamic acids and also form the glycone-binding site within the active site. The invariant GYFAWSL peptide was the junction site from which oligonucleotides for PCR were derived to construct chim 2.

B, diagrammatic representation of wild type parental Glu1 and Dhr1 isozymes and their five Glu1/Dhr1 and three Dhr1/Glu1 chimeras generated by domain-swapping. Sequences of swapped domains and their variant sites are given below the diagram of each chimeric enzyme. The lengths of exchanged domains ranged from 8 (chim 21) to 53 (chim 2), and they were all expressed in E. coli and characterized with respect to substrate specificity. The sites at which Glu1 and Dhr1 sequences differ within the extreme C-terminal domain are highlighted in red.
interaction chromatography. This procedure yielded essentially homogenous protein in all cases, as evident from SDS-PAGE profiles (Fig. 3A). Moreover, the native PAGE electrophoretic mobilities of two chimeras (chim 21 and 22) containing the shortest segments from Dhr1 were identical to that of Glu1 (Fig. 3, B–C, lanes 1, 4, and 7), whereas those of three other chimeras (chim 2, 15, and 16) containing longer Dhr1 segments were faster than that of Glu1 (Fig. 3, B–C, lanes 3, 4, 5). The electrophoretic mobilities of Dhr1 and Dhr1/Glu1 chimeras (chim 17, 23, and 39) are not known because they are not active on the substrates used for zymogram development (Fig. 3, B–C, lanes 2, 8, 9, and 10).

**Substrate Specificity and Kinetics of Glu1 and Glu1/Dhr1 Chimeras**—The catalytic activity of the parental enzymes and five chimeras toward natural (DIMBOAGlc and dhurrin) and artificial substrates (pNPGlc, oNPGlc, 4MUGlc, and 6BNGlc) was assayed in solution and in activity gels. The substrate specificity data showed that Glu1 had activity toward both its natural substrate DIMBOAGlc and each of the four artificial substrates (Table II and III; Fig. 3, B–C). Similarly, all five Glu1/Dhr1 chimeras had activity on all of these five substrates tested (Tables II and III; Fig. 3, B–C). Thus, replacement of either a large (chim 2, 15, and chim 16) or a small (chim 21 and chim 22) portion of the C terminus of Glu1 with the homologous portion of Dhr1 altered and broadened the substrate specificity in Glu1/Dhr1 chimeras. In other words, each of the Glu1/Dhr1 chimera and the extreme 23-amino acid-long C-terminal region (amino acids 492–514) from Dhr1, and none of these residues mapped to the catalytic site of the modeled parental enzyme Dhr1. The Km for dhurrin is 0.25 m M, which is about one-third that determined by Hösel et al. (35). Chim 15 had the highest Km (0.51 mm) for dhurrin followed by chim 2 and 22 and the lowest specificity constants (Table III). The Km for Dhr1 for dhurrin was 0.051 mm, which was about one-third that for Glu1 and chim 15 was clearly evident in specificity coefficients (kcat/Km) and relative efficiencies (Table II). Moreover, Glu1 and Glu1/Dhr1 chimeras had about 4-fold higher kcat values for oNPGlc than for pNPGlc. Chimo 22 had the lowest Km and highest relative efficiency for oNPGlc among all five Glu1/Dhr1 chimeras. Although all Glu1/Dhr1 chimeras hydrolyzed the natural substrates dhurrin and DIMBOAGlc, there were significant differences among them with respect to kinetic parameters (Tables II and III). The Km for Dhr1 for dhurrin was 0.051 mm, which was about one-third that determined by Hösel et al. (35). Chimo 15 had the highest Km (0.51 mm) for dhurrin followed by chim 2 and 22 and the lowest specificity constants (Table III). Two chimeras (chim 16 and 21) had the lowest Km values among the five for dhurrin (0.1 mm), which were twice the value of that for the parental enzyme Dhr1. The Km and kcat values of chimeras (except chim 16) for DIMBOAGlc hydrolysis were closer to those of the parental Glu1. However, chim 16 stood out among the five with lowest specificity coefficient (kcat/Km) and relative efficiency (32%), whereas others varied from 65 to 88% when compared with that of Glu1 (Table III). Thus, in all cases, acquiring the ability to hydrolyze dhurrin was accompanied by lowered catalytic efficiency toward DIMBOAGlc. It should be noted that chimeras 15 and 16 were obtained by splitting the 53-amino acid-long Dhr1 domain at the C terminus of chim 2 into two subdomains to further define the basis of specificity for dhurrin hydrolysis. Chimo 15 contained the extreme 23-amino acid-long C-terminal region (amino acids 492–514) from Dhr1, and none of these residues mapped to the catalytic site of the modeled parental enzymes. Instead, this region resides on the surface of the tertiary structures of Glu1 and Dhr1. Kinetic data indicate that the 23-amino acid-long peptide from the extreme C-terminal region of Dhr1 still has an effect on chim 15 for dhurrin specificity, although its catalytic efficiency coefficient (kcat/Km) for dhurrin was 57-fold and 19-fold, respectively, lower than that of Dhr1 and chim 21 (Table III). Km and kcat values for chim 15 remained similar to those of the parental enzyme Glu1 for...
three Dhr1/Glu1 chimeras hydrolyze dhurrin only. Enzymes Glu1 and Dhr1 hydrolyze their natural substrate DIMBOAGlc and dhurrin, respectively, as do all five Glu1/Dhr1 chimeras, whereas all parental 2 or their chimeras, and the minus (−) denotes incubation of the substrate without any enzyme source (negative control). Note that the parental enzymes Glu1 and Dhr1 hydrolyze their natural substrate DIMBOAGlc and dhurrin, respectively, as do all five Glu1/Dhr1 chimeras, whereas all three Dhr1/Glu1 chimeras hydrolyze dhurrin only.

### TABLE II
Comparison of the kinetic parameters of parental (Glu1 and Dhr1) and chimeric (Glu1/Dhr1) β-glucosidases

| Enzyme | $K_m$ (mM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹/mM) | Relative efficiency |
|--------|------------|----------------|------------------------|-------------------|
| Glu1   | 0.38 ± 0.01| 24.16 ± 0.12   | 63.57                  | 100               |
| Dhr1   | NA         | NA             | NA                     | NA                |
| Chim 2 | 0.39 ± 0.01| 107.91 ± 1.03  | 276.69                 | 435               |
| Chim 15| 0.35 ± 0.01| 34.26 ± 0.21   | 97.88                  | 153               |
| Chim 21| 0.36 ± 0.01| 87.24 ± 6.48   | 242.55                 | 381               |
| Chim 22| 0.35 ± 0.07| 68.90 ± 2.8    | 196.42                 | 308               |
| Chim 17| NA         | NA             | NA                     | NA                |
| Chim 23| NA         | NA             | NA                     | NA                |
| Chim 29| NA         | NA             | NA                     | NA                |

* NA, no activity detected (the amount of activity measured was not significantly different from background-level activities measured with negative controls containing no enzyme).

### TABLE III
Comparison of the kinetic parameters of parental (Glu1 and Dhr1) and chimeric (Glu1/Dhr1) β-glucosidases for the natural substrates dhurrin and DIMBOAGlc

| Enzyme | $K_m$ (mM) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (s⁻¹/mM) | Relative efficiency |
|--------|------------|----------------|------------------------|-------------------|
| Glu1   | 0.05 ± 0.01| 77.70 ± 2.81   | 1494.2                 | 100               |
| Dhr1   | NA         | NA             | NA                     | NA                |
| Chim 2 | 0.36 ± 0.02| 22.70 ± 0.96   | 62.4                   | 4.0               |
| Chim 15| 0.51 ± 0.02| 13.46 ± 1.13   | 26.1                   | 1.7               |
| Chim 16| 0.10 ± 0.00| 31.83 ± 3.18   | 303.1                  | 20.8              |
| Chim 21| 0.09 ± 0.00| 46.02 ± 4.86   | 494.8                  | 33.1              |
| Chim 22| 0.18 ± 0.04| 27.17 ± 1.65   | 150.9                  | 10.0              |
| Chim 17| 0.15 ± 0.03| 18.68 ± 0.85   | 124.3                  | 8.3               |
| Chim 23| 0.04 ± 0.01| 53.96 ± 2.49   | 1349.8                 | 90.3              |
| Chim 39| 0.12 ± 0.03| 23.41 ± 1.29   | 195.0                  | 13.0              |

* NA, no activity detected (the amount of activity measured was not significantly different from background level activities measured with negative controls containing no enzyme).

### FIG. 4
TLC chromatograms showing the reaction products after incubation of the physiological substrates DIMBOAGlc (A) and dhurrin (B) with parental β-glucosidases Glu1 and Dhr1 (lanes 2 and 3), Glu1/Dhr1 chimeras (chim 2, 15, 16, 21 and 22 (lanes 4–8)), and Dhr1/Glu1 chimeras (lanes 9–12) expressed in E. coli. The plus (+) denotes incubation of the substrate with parental Glu1 and Dhr1 or their chimeras, and the minus (−) denotes incubation of the substrate without any enzyme source (negative control). Note that the parental enzymes Glu1 and Dhr1 hydrolyze their natural substrate DIMBOAGlc and dhurrin, respectively, as do all five Glu1/Dhr1 chimeras, whereas all three Dhr1/Glu1 chimeras hydrolyze dhurrin only.

pNPGlc, oNPGlc, and DIMBOAGlc. Chim 16 contained a 30-amino acid-long internal peptide (amino acids 462–490) from Dhr1. It had a $K_m$ value similar to that of the parental enzyme Glu1 but showed nearly a 4-fold increase in $k_{cat}$ for pNPGlc hydrolysis and a 3-fold increase in $k_{cat}$ for oNPGlc hydrolysis, similar to chim 2. The kinetic values ($K_m$ and $k_{cat}$) of chim 16 for DIMBOAGlc were similar to those of chim 2, whereas its $K_m$ value for dhurrin was about one-third that for chim 2. Its $k_{cat}$ value for dhurrin increased nearly 1.5-fold, and the catalytic efficiency increased nearly 5-fold when compared with chim 2.

To narrow down the basis of dhurrin hydrolysis specificity further, chim 21 and 22 were generated by splitting the Dhr1 domain of chim 16 into two parts. Thus, chim 21 and 22, respectively, contained the Dhr1 peptides 462SSGTYTERF469 and 477ENGERTMKE486, the remainder of the chimeric enzyme being contributed by Glu1. Based on the modeled structures, the 462SSGTYTERF469 peptide motif of Dhr1 and its Glu1 homologue 466FAGFTERY473 are predicted to be involved in substrate recognition and binding. Furthermore, peptide 465PAGFTERY478 from Glu1 and 462SSGTYTERF469 from Dhr1 are predicted to make up part of the active site cleft with changes in side chain size and orientation due to the variant
residues (Fig. 5). Chim 21 hydrolyzed dhurrin with a catalytic efficiency one-third of that of Dhr1 but nearly 8 times that of chim 2. Its activity (e.g. efficiency coefficient $k_{cat}/K_m$) toward DIMBOAGlc was similar to that of chim 2 but lower than that of Glu1 (Table III). Thus, the transfer of a total of four amino acid substitutions (F466S, A467S, F469Y, and Y473F, numbering based on Glu1 sequence) from Dhr1 to Glu1 enhanced Glu1 to hydrolyze dhurrin without substantially affecting its activity toward DIMBOAGlc and other substrates. Chim 22 also showed activity toward dhurrin. However, its catalytic efficiency was about 3-fold less than that of chim 21. In this case, a total of five substitutions (N481E, N483G, T485E, Y487T, and E490R) in the Dhr1 peptide $^{47\text{ENOCERTMKR}}$486 referred to Glu1 the ability to hydrolyze dhurrin with no change in specificity for other substrates that are hydrolyzed by Glu1, including DIMBOAGlc.

**Substrate Specificity and Kinetics of Dhr1 and Dhr1/Glu1 Chimeras**—In contrast to Glu1 and Glu/Dhr1 chimeras, Dhr1 had strict specificity towards its own natural substrate dhurrin (14, 35), as did three Dhr1/Glu1 chimeras (chim 17, 23, and 39), showing no detectable catalytic activity toward pNPGluc, oNPGluc, 4MUGluc, 6BNGluc, and DIMBOAGlc (Fig. 3, B–C, lanes 8–10, Fig. 4A, lanes 10–12, and Table II). When substrate specificities of parental and chimeric enzymes were compared using 4MUGluc and 6BNGluc in zymogram assays on native PAGE gels, Dhr1 and three Dhr1/Glu1 chimeras did not hydrolyze either of these substrates (Fig. 3, B–C, lanes 2, 8, 9, and 10). These results are in agreement with those from TLC analysis in which only the natural substrates DIMBOAGluc and dhurrin had been used. As expected, Dhr1 had no detectable activity on DIMBOAGluc even after 6 h of incubation (Fig. 4A, lane 3), nor did any of the three chimeras (Fig. 4A, lanes 10–12).

The kinetic parameters ($K_m$, $k_{cat}$, and $k_{cat}/K_m$) of both Dhr1 and three Dhr/Glu1 chimeras were determined, and the data are summarized in Tables II and III. Although these three chimeras hydrolyzed only dhurrin among all substrates tested and, thus, behaved like the parental enzyme Dhr1, their catalytic efficiencies differed considerably from each other and Dhr1 in that there was a negative relationship between activity and the length of the Glu1 domain replacing the C-terminal region of Dhr1. For example, chim 23 had a slightly lower $K_m$ (0.04 versus 0.05 mM) than Dhr1, and its other kinetic parameters and relative catalytic efficiency (~90%) were similar to those of Dhr1. This chimera had the shortest Glu1 domain, the extreme 16-amino acid-long C-terminal (amino acids $^{492–508}$), where Dhr1 and Glu1 differ by 4 amino acid substitutions, as well as a 2- and a 4-amino acid-long addition, making the Glu1 segment 6 amino acids shorter than its Dhr1 homolog. In contrast, chim 17 and 39, although both hydrolyzed dhurrin, had higher $K_m$ values and drastically reduced catalytic efficiencies, 12- and 7-fold, respectively, when compared with the wild type Dhr1 (Table III). Of these, chim 17 (reciprocal of chim 2) had the longest Glu1 domain (47 amino acids long, amino acids $^{466–512}$) and the poorest kinetic parameters and lowest activity toward dhurrin. Similarly, chim 39, which had a 31-amino acid-long internal C-terminal Glu1 domain (amino acids $^{462–492}$), had only slightly better kinetic parameters than chim 17.

**Inhibition Studies**—In view of the fact that dhurrin is not hydrolyzed by Glu1 and DIMBOAGlc is not hydrolyzed by Dhr1, the inhibitory effects of dhurrin on Glu1 and DIMBOAGlc on Dhr1 were studied. The results showed that dhurrin is a competitive ground state inhibitor for Glu1 (or DIMBOAGlc glucosidase) having a $K_i$ of 0.076 mM. DIMBOAGlc is also a potent competitive inhibitor for Dhr1, with a $K_i$ of 0.009 mM. The $K_i$ value for dhurrin using pNPGluc as substrate is in agreement with the data found in previous work (36). The inhibitory effect of DIMBOAGlc on Dhr1 has not been reported previously. Moreover, the three Dhr1/Glu1 chimeras, which do not hydrolyze DIMBOAGlc, were all inhibited by it, as was Dhr1. The DIMBOAGlc concentration causing 50% inhibition of dhurrin hydrolysis by these three chimeras was in the range of from 0.003 to 0.01 mM and was lower than that of Dhr1 (0.026 mM).

**DISCUSSION**

In this study we have designed and produced chimeric enzymes from two naturally occurring enzymes, creating a novel enzymatic function as well as improved catalytic efficiency on certain substrates. Our model system was comprised of two $\beta$-glucosidases, Glu1 and Dhr1, each with a strict specificity for its own physiological substrate, although they share 79% sequence identity and contain identical catalytic amino acids and glycone recognition and binding motifs (TFNPEF and ITENG). Based on the modeled three-dimensional structures of Glu1 and Dhr1, we have successfully combined two different substrate specificities in a single chimeric enzyme by replacing the C-terminal domain of Glu1 with the homologous domain from Dhr1. This strategy added novel substrate specificity (e.g. dhurrin hydrolysis) to the maize Glu1 isozyme and a 2–4-fold improvement in its catalytic efficiency on other substrates (e.g. nitrophenyl $\beta$-glucosides, Table II).

The purpose of producing five Glu1/Dhr1 and three Dhr/Glu1 chimeras was to examine whether the selected Glu1 residues (Table II) were significant to enable Glu1 to hydrolyze dhurrin in Glu1/Dhr1 chimeras and are thought to be required for dhurrin hydrolysis. Note that the side chain of Phe-466 is projecting into the aglycone-binding site, which may be responsible for the incorrect positioning of dhurrin for hydrolysis by Glu1. The catalytic glutamic acids of Glu1 (Glu-406 and Glu-191, the nucleophile and the acid-base catalyst shown in red, respectively) and Dhr1 (Glu-404 and Glu-189, the nucleophile and the acid-base catalyst shown in purple, respectively) are superimposed, suggesting that angles and distances with respect to glycosidic bonds are similar in the active site.
chimeric β-glucosidases was to delineate the regions of primary structure that contain key amino acids and sequence motifs determining substrate (or aglycone) specificity. Each of the five Glu1/Dhr1 chimeric enzymes (chim 2, 15, 16, 21, and 22) exhibited the combined substrate specificities of the parental enzymes and, on average, 2–4-fold higher catalytic efficiency on certain substrates than the parental enzyme Glu1. The basis of the broadened substrate specificity and improved catalytic efficiency is thought to reside in amino acid substitutions that occur in the 53-amino acid-long C-terminal domain of Dhr1 or its shorter fragments that were swapped with the homologous regions of Glu1. However, three Dhr1/Glu1 chimeras (chim 17, chim 23, and chim 39) hydrolyzed only dhurrin, but catalytic efficiency was drastically reduced in the case of chim 17 and 39 because the length of the exchanged Glu1 domain increased. Thus, Dhr1/Glu1 chimeras exhibited neither widened substrate specificity nor improved catalytic efficiency (Tables II and III). These results allow us to draw three conclusions. 1) The C-terminal domain of β-glucosidases includes residues that are necessary, but not sufficient, for aglycone recognition and binding. This is in agreement with the results of Singh and Hayashi (25); they showed a chimeric enzyme obtained by replacing the C-terminal domain of a C. vuliscis β-glucosidase with the homologous C-terminal domain of an A. tumefaciens β-glucosidase had the substrate specificity of the C-terminal region donor. The N-terminal region, at least the first 41 amino acids of Glu1, does not appear to have a discernable role in substrate specificity. We replaced amino acids 1–41 of Dhr1 with the corresponding N-terminal region of Glu1. The resulting Dhr1/Glu1 chimeric enzyme hydrolyzed only dhurrin as does Dhr1 (data not shown) and exhibited kinetic properties of Dhr1, suggesting that the N terminus of Glu1 and possibly of other β-glucosidases is not involved in substrate (i.e. aglycone) specificity. However, the N-terminal region is required for catalysis because it contains a universally conserved amino acid (Gln-38 in Glu1 and Gln-39 in Dhr1, Fig. 2), which is in the aglycone binding pocket of the active site (18–23). 2) The dhurrin hydrolysis determinants in sorghum β-glucosidase (Dhr1) are among 22 amino acid substitutions in the extreme 53-amino acid-long C-terminal domain of this enzyme that distinguish it from the homologous 47-amino acid-long C-terminal domain of Glu1. More specifically, one or a combination of the amino acids Ser-462–Ser-463, Tyr-465, and Phe-469 that reside in the peptide 462SSGYTERF469 are essential for dhurrin hydrolysis because this peptide alone confers the capability to hydrolyze dhurrin to Glu1, as is the case in chim 21 (Table III and Fig. 4B, lane 7). 3) In contrast, the DMB0AGlc hydrolysis determinants are not in the 47-amino acid-long C-terminal domain of Glu1, although this domain may contain residues that are involved in DMB0AGlc or DMBD binding.

We postulate that although Glu1 and Dhr1 differ by 151 amino acid substitutions, 5 deletions, and 7 additions at 514 positions (~30% sequence divergence), only a small number of these changes are relevant to the substrate specificity differences between them. Indeed only 4 (Ser-462–Ser-463, Tyr-465, and Phe-469) of the 22 variant amino acid sites in the extreme 47- to 53-amino acid-long C terminus map to or around the active site of the modeled enzymes (Fig. 5). Therefore, it is conceivable that more than 90% of the amino acid substitutions separating Glu1 and Dhr1 are adaptively and functionally neutral, which would be consistent with Kimura’s theory of neutral evolution (37). There are well documented examples in the literature supporting this postulate. For example, eubacterial and mitochondrial isocitrate dehydrogenases differ with respect to coenzyme specificity; the former is NADP-dependent, whereas the latter is NAD-dependent. Moreover, both enzymes have essentially the same tertiary structure, although they differ by 250 amino acid substitutions at 320 positions. Only 6 of these 250 amino acid substitutions determine coenzyme specificity, as shown elegantly by shifting NADP specificity to NAD specificity or vice versa by replacing these amino acids in the coenzyme binding pocket (38). Wilks et al. (39) provide even a more dramatic example in that they changed a lactate dehydrogenase to a malate dehydrogenase by replacing a single key amino acid although the two enzymes differed by 230 amino acid substitutions. Other examples of bringing about dramatic changes in substrate specificity and catalytic properties include changing the substrate specificity and double-bond positional specificity of an acyl-carrier protein desaturase by five amino acid replacements (40), increasing catalytic efficiency and broadening substrate specificity in a chimeric protease constructed by recombining the N-terminal domain of coagulation factor X with the C-terminal domain of trypsin (41), and introducing the active site of nonheme iron superoxide dismutase into E. coli thioredoxin and changing it to a superoxide dismutase (42).

The substrate specificity data from chim 2 provided the first clue to the fact that the C-terminal 53-amino acid-long domain of Dhr1 contains key determinants for dhurrin hydrolysis. However, these determinants alone did not change the specificity of a Glu1/Dhr1 chimera entirely to that of the C-terminal region donor Dhr1. Since there were 22 amino acid substitutions and two additions (a dipeptide and a tetrapeptide) in the 53-amino acid-long Dhr1 C-terminal domain that differentiate it from the homologous Glu1 domain (Fig. 2), it was not possible to identify specific amino acids or sequence motifs that are important for dhurrin hydrolysis. Consequently, this Dhr1 domain was split into two subdomains, which are represented in chim 15 (23-amino acid-long C-terminal subdomain) and chim 16 (30-amino acid-long N-terminal subdomain), to determine which subdomain was important for dhurrin hydrolysis. The substrate specificity and kinetic data from these two chimeras unequivocally showed that the 30-amino acid-long subdomain, which contains 10 amino acid substitutions, played a far greater role in dhurrin hydrolysis than the 23-amino acid-long subdomain. This is clearly evident in the fact that chim 16 hydrolyzes dhurrin 12 times better than chim 15 (Table III). These data are also consistent with the modeling data in that none of the 23 amino acids from the extreme C terminus of Dhr1 or Glu1 maps to and around the active site of the modeled three-dimensional structures of these enzymes. Therefore, the 8 substitutions and 4-amino acid-long addition that separate Glu1 from Dhr1 at the extreme C terminus has a rather small and probably indirect effect on substrate specificity.

The substrate specificity and kinetic data clearly suggested that one or more of the 10 amino acid substitutions in the 30-amino acid-long Dhr1 subdomain in chim 16 plays a key role in dhurrin hydrolysis. Again, to bring further clarity to the specific site(s) responsible for dhurrin hydrolysis, the 30-amino acid-long subdomain was divided into two segments after leaving out the invariant region GIVYVDR separating them. The resulting two Dhr1 peptides 462SSGYTERF469 and 477ENGCERTMKR486 were used to replace their homologues in Glu1 by domain swapping, which yielded chim 21 and 22, respectively (Table I and Fig. 2). The substrate specificity and kinetic data obtained with these two chimeras indicated that chim 21 hydrolyzed dhurrin and had the best kinetic properties, having the lowest $K_m$ and highest $k_{cat}$ and efficiency coefficient among five Glu1/Dhr1 chimeras (Table III). Thus, the Dhr1 peptide 462SSGYTERF469 alone is sufficient to enable Glu1 to hydrolyze dhurrin when it replaces the homologous peptide FAGFTERY473 of Glu1. Further support for the importance of pep-
Aglucose Specificity in Maize and Sorghum β-Glucosidases

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the enzyme-substrate complex to attain a transition state energetically favorable for hydrolysis. Thus, although substrate binding is the first essential step in hydrolysis, it is not sufficient for it unless the binding positions the β-glucosidic bond in the correct angle and distance with respect to the two catalytic glutamic acids. The above question also has bearing on the evolution and existence of two distinct β-glucoside biosynthesis pathways and chemical defense compounds in two closely related plant genera, Zea and Sorghum, which are thought to have diverged from a common ancestor 25–30 million years ago. For example, the dhurrin biosynthesis pathway starts with the amino acid tyrosine as the precursor and produces a cyanogenic β-glucoside (dhurrin) as the end product, whereas the DIMBOAGlc biosynthesis pathway starts with indole (a tryptophan analogue) as the precursor and produces a hydroxamic acid glucoside (DIMBOAGlc) as the end product. Which pathway did the common ancestor of sorghum and maize have and when, how, and why was another β-glucoside pathway and defense compound “invented” in one of the lineages are important questions for the evolutionary biologist to answer.

In conclusion, we were able to broaden the substrate specificity of the maize Glu1 isozyme (DIMBOAGlc) to hydrolyze the sorghum natural substrate dhurrin and improve its catalytic efficiency toward the artificial substrate pNPGLc 1.5–4.4-fold and pNPGlc 1.5–3.1-fold. This was accomplished by replacing a 47-amino acid-long C-terminal domain of Glu1 and its smaller segments with the homologous Dhr1 domain and its smaller segments. The shortest Dhr1 peptide to enable Glu1 to hydrolyze dhurrin was eight amino acids long, differing by four amino acid substitutions, three of which mapped to the active site of the modeled enzymes. Although all of the five Glu1/Dhr1 chimeric enzymes hydrolyzed both dhurrin and DIMBOAGlc, none of them either equaled or exceeded their parental enzymes in terms of catalytic efficiency for these natural substrates. However, with one exception (chim 16) they were better DIMBOA-glucosidases than dhurrinase, having 65 to 88% catalytic efficiency of Glu1. In general, DIMBOAGlc hydrolysis and dhurrin hydrolysis efficiencies were negatively correlated. In general, DIMBOAGlc hydrolysis and dhurrin hydrolysis efficiencies were negatively correlated. In general, DIMBOAGlc hydrolysis and dhurrin hydrolysis efficiencies were negatively correlated.

REFERENCES
1. Bell, A. (1981) Annu. Rev. Plant Physiol. 32, 21–81
2. Conn, E. E. (1981) in Cyanogenic Glycosides. (Stumpf, P. K., and Conn, E. E., eds) pp. 479–500, Academic Press, Inc., New York
3. Niemeyer, H. M. (1988) Phytochemistry 27, 3349–3358
4. Poulton, J. E. (1990) Plant Physiol. (Sojka) 94, 401–405
5. Brzobohaty, B., Moore, I., Christofferson, P., Bako, L., Campos, N., Schell, J., and Palme, K. (1993) Science 262, 1051–1054
6. Matsuzaki, T., and Kowai, A. (1986) Agric. Biol. Chem. 50, 2193–2199
7. Schillemann, W. (1984) J. Plant Physiol. 116, 123–132
8. Smith, A. R., and van Staden, J. (1978) J. Exp. Bot. 29, 1067–1073
9. Wiese, G., and Gramkow, H. (1986) Phytochemistry 25, 2451–2455
10. Dharmawardhana, D. P., Ellis, B. E., and Carlson, J. E. (1995) Plant (Sojka) 97, 331–339
11. Leah, R., Kigel, J., Svendsen, I., and Mundy, J. (1995) J. Biol. Chem. 270, 1579–1579
12. Simos, G., Panagiotidou, C. A., Skoumbas, A., Choli, D., Ozouzinis, C., and Georgatous, G. J. (1994) Biochem. Biophys. Acts 1109, 52–58
13. Bandaranayake, H., and Essen, A. (1996) Plant Physiol. 110, 1048
14. Cieek, M., and Essen, A. (1998) Plant Physiol. 116, 1469–1478
15. Henriksat, B. (1991) Biochem. J. 280, 309–316
16. Trimbur, D. E., Warren, R. A. J., and Withers, S. G. (1992) J. Biol. Chem. 267, 10248–10211
17. Trimbur, D., Warren, R. A. J., and Withers, S. G. (1993) β-Glucosidases: Biochemistry and Molecular Biology (Essen, A., ed) pp. 42–55, ACS Symposium series 533, American Chemical Society, Washington, D. C.
18. Barrett, T., Suresh, C. G., Tulley, S. P., Dodson, E. J., and Hughes, M. A. (1995) Structure 3, 951–960
19. Bermejillo, M. P., Cortes, S., Driey, H., Iori, I., Palmieri, S., and Henriksat, B. (1997) Structure 5, 663–675
20. Sanz-Aparicio, J., Hormeso, J. A., Martinez-Ripoll, M., Lequerica, J. L., and Pulina, J. (1996) J. Mol. Biol. 275, 491–502
21. Wiesman, C. Best, G., Hengstenberg, W., and Schulz, G. E. (1995) Structure (Long.) 3, 961–968
22. Aguilar, C. F., Sanderson, I., Moretti, M., Ciaramella, M., Nucci, R., Rossi, M., and Porri, L. H. (1997) J. Mol. Biol. 271, 789–802
23. Chi, Y.-J., Martinez-Cruz, L. A., Swanson, R. V., Robertson, D. E., and Kim, S.-H. (1999) FEBS Lett. 455, 375–383
24. Davies, G., and Henriksat, B. (1995) Structure (Long.) 3, 853–859
25. Singh, A., and Hayashi, N. (1995) J. Biol. Chem. 270, 21928–21933
26. Terahashi, M., Hosono, M., and Katoh, S. (1997) Appl. Microbiol. Biotechnol. 47, 364–367
27. Cieek, M., and Essen, A. (1999) Biotechnol. Bioeng. 63, 392–400
28. Horten, R., Cai, M. Z., Ho, S. N., and Pease, L. R. (1990) Biotechniques 8, 528–535
29. Essen, A., and Cokus, C. (1990) Biochem. Genet. 28, 319–336
30. Martin, T., Wohner, R. V., Hummel, S., Willmitzer, L., and Frommer, G. (1992) in GUS Protocols, (Gallagher, S. R., ed) pp. 23–42, Academic Press, Inc., San Diego, CA
31. Raabo, E., and Terkildsen, T. C. (1960) J. Biol. Chem. 235, 779–815
32. Robyt, F. J., and White, B. J. (1990) Phytochemistry 27, 779–815
33. Barstow, D. A., Atkinson, T., Clarke, A. R., and Holbrook, J. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 2011–2015
34. Dean, A. M. (1998) Ann. Rev. Plant Physiol. 49, 26–37
35. Wilks, H. M., Hart, K. W., Feeney, R., Dunn, C. R., Muirhead, H., Chia, W. N., and Palme, K. (1993) J. Mol. Biol. 226, 541–554
36. Afshar, B., Wille, M., Palme, K., and Kollman, P. A. (1995) AMBER, Version 4.1, University of California, San Francisco
37. Hsieh, W., Toher, I., Eklund, S. H., and Conn, E. E. (1987) Arch. Biochem. Biophys. 252, 152–162
38. Babcock, G. W., and Essen, A. (1994) Plant. Sci. 120, 31–39
39. Kimura, M. (1983) The Neutral Theory of Molecular Evolution, Cambridge University Press, Cambridge, UK
40. Dean, A. M. (1998) Annu. Rev. Ecol. 29, 26–37
41. Hofner, K. P., Kopp, E., Kressel, G. B., Bode, W., Huber, R., and Engh, R. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9813–9818
42. Pinto, A. L., Hellinga, H. W., and Cardaropi, J. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5562–5567

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Muzaffer Cicek, David Blanchard, David R. Bevan and Asim Esen

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