Mutational and Structural Analysis of Aglycone Specificity in Maize and Sorghum β-Glucosidases*

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Plant β-glucosidases display varying substrate specificities. The maize β-glucosidase isozyme Glu1 (ZmGlu1) hydrolyzes a broad spectrum of substrates in addition to its natural substrate DIMBOA-Glc (2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one), whereas the sorghum β-glucosidase isozyme Dhrl (SbDhrl) hydrolyzes exclusively its natural substrate dhurrin (p-hydroxy-γ-(S)-mandelonitrile-β-D-glucoside). Structural data from cocrystals of enzyme-substrate and enzyme-aglycone complexes have shown that five amino acid residues (Phe196, Phe305, Thr378, Phe466, and Ala467) are located in the aglycone-binding site of ZmGlu1 and form the basis of aglycone recognition and binding, hence substrate specificity. To study the mechanism of substrate specificity further, mutant β-glucosidases were generated by replacing Phe196 by a valine had the most drastic effect on activity, because the capacity of this enzyme to hydrolyze β-glucosides was almost completely abolished. The analysis of this mutation was completed by a structural study of the double mutant ZmGlu1-ET196V in complex with a natural substrate. The structure reveals that the single mutation F198V causes a cascade of conformational changes, which are unpredictably by standard molecular modeling techniques. Some other mutations led to drastic effects: replacing Asp361 by an asparagine decreases the catalytic efficiency of this simple mutant by 75% although replacing Tyr373 by a phenylalanine increase its efficiency by 300% and also provides a new substrate specificity by hydrolyzing dhurrin.

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) occur in all living organisms (bacteria, archaea, and eukarya) and play key roles in a variety of fundamental biological processes. In bacteria and fungi, the cellulyotic β-glucosidases are

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natural substrates and is responsible for subtle substrate specificity differences among β-glucosidases. Recently, using the sorghum β-glucosidase Dhr1 and maize β-glucosidase Glu1 chimeras, Cicek et al. (18) have determined that the aglycone (i.e. substrate) specificity-determining sites are different in ZmGlu1 and SbDhr1. These two enzymes provide an ideal model system to address questions related to substrate specificity, because they represent extremes in substrate specificity. SbDhr1 hydrolyzes only its natural substrate dhurrin, whereas ZmGlu1 hydrolyzes a broad spectrum of artificial and natural substrates, including its natural substrate DIMBOA-Glc, but it does not hydrolyze dhurrin. Cicek et al. (18) showed that specificity for dhurrin hydrolysis resides mostly in a C-terminal octapeptide (462SSGYTERF469) of SbDhr1 where SbDhr1 and ZmGlu1 sequences differ from each other by four amino acid substitutions, although specificity for DIMBOA-Glc hydrolysis is not within the homolog of the aforementioned octapeptide in ZmGlu1, nor within the extreme 47-amino acid long C-terminal domain of ZmGlu1. The recently resolved crystal structures of ZmGlu1 and SbDhr1, respectively, demonstrated that the aglycone moiety of the substrate is sandwiched between Trp378 on one side and Phe198, Phe205, and Phe466 on the other. The major mechanism of aglycone recognition and binding appears to be aromatic stacking and other electrostatic interactions between aromatic aglycons and the above mentioned amino acids. Moreover, several others amino acids (e.g. Pro455, Met463, Ala467, and Tyr473) were identified to be potentially involved in substrate specificity (11).

The purpose of the study described in this paper was to investigate further the mechanism of substrate (aglycone) recognition and binding in β-glucosidases using the maize isozyme ZmGlu1 and the sorghum isozyme SbDhr1 as model systems. To this end, we have produced site-directed mutants of ZmGlu1 and SbDhr1 by employing a combination of domain-swapping between ZmGlu1 and SbDhr1 and used in a previously published study (18). The targeted residues were chosen based on their localization in the active site aglycone binding pocket, as derived from the structural data, and on amino acid sequence comparisons between ZmGlu1 and SbDhr1. The two catalytic glutamates were indicated by a yellow background.

**EXPERIMENTAL PROCEDURES**

**General Methods**—Manipulation of DNA and Escherichia coli were performed using standard procedures (19). DNA synthesis by the PCR was performed with the high fidelity Pfu Turbo DNA polymerase (Stratagene). DNA was purified from gel with the QiaQuick gel extraction kit (Qiagen). All restriction enzymes were from Stratagene and T4 DNA ligase from Promega. All constructs were sequenced for confirmation in the VBI Core Laboratory Facility (Blacksburg, VA).

**Mutagenesis of β-Glucosidases**—The criteria for choosing the targeted residues were their localization in the active site aglycone binding pocket of the ZmGlu1 protein and amino acid sequence comparisons between ZmGlu1 and SbDhr1. The double mutant ZmGlu1-E191D,F198V, F198V was produced and overexpressed for the purpose of the structural analysis in complex with the natural substrate. For that reason, it was necessary to additionally mutate the acid base residue, to be sure to obtain a completely inactive enzyme. Mutated cDNAs were constructed by the PCR-mediated overlap extension using a pair of complementary primers (sense: 5'-ATGCT- and antisense: 5'-TAATACGACTCACTATAGGG-3') and T_{prom} (sense) (5'-ATGGC-AGTATTGCTGCGG-3') and T_{antisense} (antisense) (5'-ATGGCT-AGTATTGCTGCGG-3'). The oligonucleotides used in this study are listed in Table I. The DNA templates used in this study were PET21a-ZmGlu1, pET21a-Chim36, and pET21a-Chim21, which were previously used for expression in E. coli and subsequent purification in our laboratory (11). The T_{prom} and T_{antisense} PCR products were gel purified and combined in the second PCR step using T_{prom} and T_{antisense} oligonucleotides to obtain the full-length mutated cDNA. The resulting PCR product was gel purified.
Table I

Synthetic oligonucleotides used to generate mutant maize (ZmGlu1) and sorghum (ShDhr1) β-glucosidases

| Oligonucleotide sequence 5’-3’ | Mutation introduced |
|-------------------------------|---------------------|
| CTATGACTTCCGTTCTCAGCCGAAC   | ZmGlu1-F198V         |
| GGGTTCACCAAGGCTTGGGCTCCTG    | ZmGlu1-F198V,F205L   |
| AGGCCTGCGTTACGTATGAGTTA      | ZmGlu1-D261N         |
| GCTATCTGGCCTGTGGCATACGCG     | ZmGlu1-M263F         |
| GGGAATGCACTGGATCATACTGATG    | ZmGlu1-P377A         |
| CCGAACGTGTGAGCTATGGCTAGG     | ShDhr1-V196F,L203F   |
| CTGATCATTGTCACCAGGTTCTAGG    | ShDhr1-A375P         |
| CGGACGGTATCCGAGCTGTC         | ShDhr1-F469Y         |

purified, digested with NheI and XhoI restriction enzymes and cloned into the expression plasmid pET28a (Novagen). This plasmid allows the fusion of His-tagged residue extension at the N-terminal part of the mutated β-glucosidases, allowing purification on a Ni²⁺ column (see below).

Expression and Purification of Recombinant β-Glucosidases by Ni²⁺ Column Chromatography—Wild-type, chimeric and mutated β-glucosidases were produced in E. coli BL21(DE3) (pLysS) (FompT hadSP rfg, gal dcm) (Stratagene) under the control of the T7 RNA polymerase promoter in the expression plasmid pET28a (Novagen). Cells were grown at 37 °C until absorbance at 600 nm reached 0.5, and recombinant protein expression was induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 22 °C. Cell lysis was performed using 10 ml of extraction buffer (100 mM Tris-HCl, 50 mM NaCl, pH 8.0, 0.2 mM phenylmethylsulfonfluryl fluoride, 200 units of DNase I) for 1 g of cell pellet using a French Press (3 × 15,000 psi), and the extract was centrifuged at 30,000 × g for 20 min. The cell debris pellet was extracted 2 more times with 5 ml of extraction buffer. All protein-containing supernatant were pooled, applied on the His-Bind²⁺ (Ni²⁺ resin) column and purified as recommended by Novagen. Ammonium sulfate was added to purified protein to a final concentration of 1.0 M and the solution was centrifuged at 16,500 × g for 15 min. The supernatant was applied on HitTrap Phenyl-HP (Amersham Biosciences) column and the protein was further purified by fast protein liquid chromatography using a 0.8 to 0.2 M ammonium sulfate (pH 7.0) gradient. Purified β-glucosidase preparations were checked for purity by SDS-PAGE on a standard 10% polyacrylamide gel under reducing conditions (19) and their protein concentration was determined using the Bio-Rad protein assay (Bio-Rad) and bovine serum albumin as standard.

Enzyme Assay—Kinetic parameters, $K_{cat}$ and $V_{max}$ ($V_{max}$/ $K_{cat}$) for the wild-type enzymes (ZmGlu1 and ShDhr1), chimeric constructs (chimeras 16 and 21), and mutated β-glucosidases were determined by varying the substrate concentration from 0.078 to 25 μM in citrate-phosphate buffer (pH 5.8) for the artificial substrates NPGlc and pNPGlc and from 0.039 to 2.5 μM for 4MU Glc. For the substrates dhurrin, the range of substrate concentration was from 0.01 to 2.5 μM. The reaction mixture was incubated at 37 °C for 15 min and the absorbance was read at 410 nm. Then, the glucose release was determined by the peroxidase/glucose-oxidase coupled reaction (22) by adding 100 μl of peroxidase/glucose-oxidase enzymes and 50 μl of ABTS (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). The reaction mixture was incubated at 37 °C for 15 min, and the absorbance was read in a microtiter plate reader at 410 nm. Because dhurrin is not resistant to the enzyme–heat inactivation step, dhurrinase activity was monitored by a mixture of 25 μl of substrate, 25 μl of enzyme solution, 100 μl of peroxidase/glucose-oxidase enzymes, and 50 μl of ABTS in a microtiter plate. The absorbance was read at 410 nm after incubation at 37 °C for 1 h.

Crystal Structure Determination of ZmGlu1-E191D,F198V in Complex with DIMBOA-Glucoside—The crystals were grown under similar conditions as the native enzyme (15). Two μl of the protein solution was mixed with 2 μl of reservoir containing 0.1 M Hepes (pH 7.5), 22% PEG 4000, and 5% isopropl alcohol. The crystals belong to space group P2₁ and have unit cell parameter $a = 58.1\text{ Å}, b = 114.1\text{ Å},$ and $c = 80.1\text{ Å}$, $β = 93.8°$. The ZmGlu1-E191D,F198V-DIMBOA-Gluc complex was obtained in soaking experiments, in which 92 μl of the crystallization solution was mixed with 5 μl of glycerol (the cryo-protectant) and 3 μl of ligand (10 mM) solution. Subsequently, 45.5 μl of this mixture was supplemented with 2.5 μl of glycerol and 2 μl of ligand solution. The crystals were soaked in these two solutions for 1 h and then frozen in a stream of N₂ at 100 K. The crystals diffraction to a resolution of 1.9 Å. Data were collected at the synchrotron ESRF (Grenoble, France) on beam-line ID14-EH4. The data set was treated with DENZO (23) and scaled with SCALeu, part of the CCP4 package (24). The statistics on the data collection are given in Table I.

The crystals were grown under similar conditions as the new unit cell with respect to the β-glucosidase by molecular replacement using AMoRe (25) and the native maize β-glucosidase (Protein Data Bank accession code 1e1e) as the model. Two solutions were obtained, which correspond to the two molecules in the asymmetric unit related by a non-crystallographic 2-fold symmetry, with a correlation coefficient of 70% and an $R$ factor of 31.1% in the resolution range of 15.0 to 4.2 Å. The complex was analyzed with SIGMAA (24) weighted $F_{obs} - F_{calc}$ maps calculated with model phases. The final $R$ and $R_{work}$ factors for the complexed ZmGlu1-E191D,F198V were 18.4 and 22.1%, respectively.

A model of the ZmGlu1-E191D,F198V mutant structure was produced with the homology modeling program MODELLER (26) starting with the crystal structure of ZmGlu1-E191D (Protein Data Bank code 1E4L) and introducing the single mutation F198V into the sequence to be modeled (data not shown).

RESULTS

N-terminal His-tagged Glu1 Is Fully Active in Vitro—Because our goal was the characterization of a large number of β-glucosidases, we chose to use polyhistidine-tagged β-glucosidases to speed up protein purification. Thus we first had to demonstrate that the addition of such a His tag does not affect β-glucosidase stability and activity in vitro. For this, we chose the ZmGlu1 β-glucosidase, and cloned its cDNA into an expression plasmid, pET28a, using the NheI/XhoI restriction sites to fuse a polyhistidine extension at the N-terminal end. The recombinant protein was then produced in E. coli and purified on a Ni²⁺ column. The in vitro activity of the His₆-tagged and the native ZmGlu1 β-glucosidases were tested on the artificial substrates NPGlc and pNPGlc. No significant differences in stability and kinetic parameters were observed between the two forms (data not shown).

Substrate Specificity and Kinetics of Wild-type Enzymes and Site-directed Mutants on Artificial Substrates—The catalytic activity of wild-type and mutant enzymes was assayed toward three artificial substrates (onNPGlc, pNPGlc, and 4MU Glc) in solution and the kinetic parameters ($K_{cat}$, $k_{cat}$, and $k_{cat}/K_{m}$) were determined. The data are summarized in Table III.

The results obtained on Chim16 and Chim21 in this study are similar to those previously published (18) in that both chimeric enzymes show increased catalytic efficiency, as compared with the parental ZmGlu1, in pNPGlc hydrolysis (284 and 358% reported here, compared with 340 and 380% increase in the previous study (18)). However, the reported kinetic parameters of parental enzymes and chimeras are slightly different (i.e. for ZmGlu1: $K_{m}$ of 0.64 μM instead of 0.38 μM and $k_{cat}$ of 29.5 s⁻¹ instead of 24.2 s⁻¹) from those published by Ciek et al. (18), but similar to the one published of Zhouhar el. (27) ($K_{m}$ of 0.64 μM and $k_{cat}$ of 28 s⁻¹ reported for ZmGlu1).
First, we analyzed the effect of substitutions at the three phenylalanines forming one wall of the aglycone binding site of the slot-like active site on the activity and substrate specificity of mutant enzymes. Of the three single mutants, the F205L mutant shows no significant effect on pNPGlc hydrolysis other than doubling the $k_{cat}$. In contrast, the F198V and F466S single mutations had drastic effects on catalytic efficiency. For example, single mutant F466S exhibits a marked increase in relative efficiency in comparison to wild-type ZmGlu1 on all substrates tested (from 28 to 123%), because of increased substrate turnover (e.g. 4 times better for pNPGlc hydrolysis). As for the F198V mutant, the $k_{cat}$ of 4MUGlc hydrolysis is reduced by about 90% resulting in a 82% reduction in relative efficiency, whereas the hydrolysis of other artificial substrates is drastically reduced. For example, the F198V mutant is unable to hydrolyze pNPGlc and shows only a negligible activity toward oNPGlc (7% of the relative efficiency of the wild-type enzyme). Furthermore, adding the double F198V,F205L substitutions to Chim16 or Chim21 results in a 90% decrease in relative efficiency of ZmGlu1 on all substrates tested, but little effect on the $K_m$, whereas the M263F substitution had a slight effect on the $k_{cat}$. This last mutation has opposite effects depending on the substrate considered because the relative efficiency on pNPGlc and 4MUGlc hydrolysis is reduced by about 40 and 50% whereas it shows an increase of about 60% on oNPGlc hydrolysis (Table III). This last result has also been observed by Zouhar et al. (27) on pNPGlc with a 70% decrease in the turnover rate of this mutant as opposed to 62% decrease in our study. At last, the P377A mutation leads to changes (decreases) in both $k_{cat}$ and $K_m$ with no decrease in the overall relative efficiency. The reciprocal substitution A375F in SbDhr1 does not yield any significant or detectable change in the artificial substrate hydrolysis.

### Substrate Specificity and Kinetics of Wild-type Enzymes and Site-directed Mutants on the Natural Substrate Dhurrin

The catalytic efficiency of the mutants described above was also tested on dhurrin (Table IV), the natural substrate of the sorghum SbDhr1. Dhurrin competitively inhibits maize ZmGlu1 (19), and this enzyme hydrolyzes dhurrin with a lower catalytic efficiency only when a C-terminal octapeptide (FAGFTERY$^{477}$) is exchanged with the homologous octapeptide of SbDhr1 (466SSGYTERF$^{469}$). This peptide substitution leading to amino acid substitutions at four sites (bold) is found in Chim21 and Chim16 (with an extra 22 amino acids substitution, cf. Fig. 2) enables ZmGlu1 to hydrolyze dhurrin (18) with less than 10% of the relative efficiency of SbDhr1 (7.4% for Chim21 and 9.5% for Chim16). We investigated the role of three substitutions found in the above mentioned 8-amino acid long peptide fragment and located in Phe$^{466}$ and Ala$^{467}$ and near Tyr$^{473}$ in the active site by testing the activity of mutants F466S, F466S,A467S (double mutant), and Y473F. Whereas the first two mutations have little effect on dhurrin hydrolysis (about 3% of SbDhr1 relative efficiency in dhurrin hydrolysis),

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**Table II**

Data quality statistics and refinement parameters for the crystal structure of the mutant ZmGlu1-E191D,F198V in complex with DIMBOA-glucoside

| ZmGlu1-E191D,F198V in complex with DIMBOA-Glc |
|-----------------------------------------------|
| Data collection statistics                     |
| $R_c$        | 0.07                      |
| Completeness | 90 %                      |
| $I/\sigma(I)$ | 7.3                       |
| Multiplicity | 3.6                       |
| Refinement parameters                           |
| Resolution range (Å)                            | 27.1–1.9                  |
| $\alpha$-Cutoff (%) (no. of reflections)        | None                      |
| $R_{	ext{work}}$ factor (%) (5% of reflections) | 18.4 (67417)              |
| $R_{	ext{free}}$ factor (%) (5% of reflections) | 22.1 (3519)               |
| No. of non-H protein atoms                       | 7931                      |
| No. of hetero-atoms                              | 52                        |
| No. of solvent atoms                             | 488                       |
| R.M.S. deviation from ideal geometry             | 0.008                     |
| Bond angles (°)                                  | 1.23                      |
| Torsion angles (°)                               | 5.7                       |
| Average B factors (Å$^2$) (all atoms)            | 16.4                      |
| Main chain atoms; chain A (chain B)              | 22.4 (22.3)               |
| Side chain atoms; chain A (chain B)              | 23.0 (23.1)               |
| Water molecules; mol A (molB)                    | 21.2 (19.7)               |
| Ligand                                          | 38.1 (40.9)               |
| Luzzati coordination error (Å)                   | 0.20                      |
| Ramachandran outliers                           | None                      |
| Residues in most favorable regions               | 92.8                      |
Aglycone Specificity of Maize and Sorghum β-Glucosidases

Comparison of kinetics parameters of parental (ZmGlu1 and SbDhr1) β-glucosidases and their mutants in assays with artificial substrates

The relative efficiency is normalized from $k_{cat}/K_m$ values.

| Enzyme | $K_m$ | $h_{cat}$ | Relative efficiency | $k_{cat}$ | $h_{cat}$ | Relative efficiency | $k_{cat}$ | $h_{cat}$ | Relative efficiency |
|--------|------|----------|---------------------|----------|----------|---------------------|----------|----------|---------------------|
|        | $\text{mM}$ | $s^{-1}$ | %                  | $\text{mM}$ | $s^{-1}$ | %                  | $\text{mM}$ | $s^{-1}$ | %                  |
| WT     | 1.65 ± 0.12 | 121.5 ± 5.2 | 100 | 0.64 ± 0.09 | 29.5 ± 1.4 | 100 | 0.37 ± 0.03 | 30.3 ± 2.4 | 100 |
| F198V  | 1.22 ± 0.15 | 6.6 ± 2.1 | 7 | 0 | 0 | 0 | 0.23 ± 0.10 | 3.4 ± 0.8 | 18 |
| F205L  | 1.18 ± 0.21 | 137.3 ± 4.7 | 100 | 0.625 ± 0.08 | 57.3 ± 3.7 | 199 | 0.395 ± 0.04 | 34.5 ± 2.1 | 106 |
| D261N  | 1.70 ± 0.17 | 36.4 ± 5.1 | 29 | 0.90 ± 0.1 | 497 ± 0.9 | 12 | 0.42 ± 0.05 | 17.2 ± 2.1 | 50 |
| M263F  | 1.21 ± 0.09 | 143.8 ± 6.9 | 162 | 0.41 ± 0.07 | 11.1 ± 1.5 | 62 | 0.43 ± 0.04 | 17.8 ± 3.1 | 51 |
| P377A  | 1.24 ± 0.10 | 74.4 ± 4.6 | 82 | 0.45 ± 0.06 | 108.5 ± 1.1 | 53 | 0.36 ± 0.03 | 16.8 ± 2.4 | 126 |
| F466S  | 1.88 ± 0.07 | 177.1 ± 9.1 | 128 | 1.15 ± 0.11 | 118.5 ± 15.3 | 223 | 0.76 ± 0.05 | 87.2 ± 9.2 | 140 |
| F466S,F198V,F205L | 1.87 ± 0.03 | 172.4 ± 5.3 | 125 | 1.06 ± 0.04 | 100.7 ± 8.1 | 206 | 0.49 ± 0.1 | 97.9 ± 4.8 | 244 |
| F473F  | 1.85 ± 0.08 | 353.2 ± 21.1 | 259 | 1.04 ± 0.07 | 163.8 ± 2.6 | 349 | 0.55 ± 0.05 | 63.4 ± 4.1 | 141 |
| F198V,F205L,P377A | 3.76 ± 0.27 | 19.1 ± 5 | 7 | 0.94 ± 0.12 | 0.67 ± 0.15 | 2 | 0.33 ± 0.05 | 1.7 ± 0.3 | 6 |
| Ch21   | 1.17 ± 0.21 | 172.7 ± 5.1 | 200 | 1.18 ± 0.09 | 152.43 ± 3.4 | 294 | 0.49 ± 0.04 | 93.8 ± 6.3 | 235 |
| Ch16   | 1.62 ± 0.12 | 202.8 ± 6.2 | 62 | 1.31 ± 0.12 | 213.1 ± 6.2 | 358 | 0.56 ± 0.07 | 111 ± 5.4 | 242 |
| Ch16-F198V,F205L | 6.07 ± 0.34 | 93.3 ± 5.6 | 21 | 2.29 ± 0.12 | 9.31 ± 1.7 | 9 | 1.86 ± 0.16 | 15.2 ± 2.6 | 10 |
| Ch16-F198V,F205L,P377A | 5.51 ± 0.38 | 123.1 ± 8.1 | 30 | 2.67 ± 0.15 | 6.5 ± 1.1 | 5 | 1.35 ± 0.15 | 13.2 ± 3.4 | 12 |
| Dhr1   | 3.83 ± 0.41 | 13.5 ± 3.1 | 5 | NA | NA | NA | NA | NA | NA |
| Dhr1-V198F,L203F | 2.81 ± 0.35 | 10.1 ± 2.8 | 5 | NA | NA | NA | NA | NA | NA |
| Dhr1-A375F | 2.72 ± 0.31 | 11.4 ± 1.4 | 6 | NA | NA | NA | NA | NA | NA |
| Dhr1-A469Y | 5.51 ± 0.65 | 9.2 ± 2.6 | 2 | NA | NA | NA | NA | NA | NA |

*NA, no activity detected.

Fig. 2. Diagrammatic representation of wild-type parental ZmGlu1 and SbDhr1 isozymes and 2 chimeras generated by domain swapping, as well as 8 site-directed mutants. Sequences of the swapped regions are given on the top of the diagram in Chim16 and Chim21. The sites where ZmGlu1 and SbDhr1 differ within the swapped regions are highlighted in red, as are the amino acids exchanged by site-directed mutagenesis.

it is observed that the Y473F substitution alone leads to a significant dhurrin hydrolysis with a 10.3% relative efficiency as compared with that of SbDhr1. To confirm the role of Tyr473 in ZmGlu1 and its homologue in SbDhr1 (Phe469) in substrate specificity, we investigated the dhurrin hydrolysis of the SbDhr1-F469Y mutant. This single amino acid substitution (F469Y) results in a 75% decrease in relative efficiency of SbDhr1 in dhurrin hydrolysis, highlighting once more the critical role of this residue in substrate specificity. We also investigated the role of other amino acids located in or around the active site on dhurrin hydrolysis. None of the tested single amino acid substitutions on ZmGlu1 or on the chimeras led to a significant hydrolysis of dhurrin, for example, only F205L and M263F exhibit activity slightly higher than the background (1.4 and 2.2% respectively). However, some reciprocal substitutions made on SbDhr1, such as SbDhr1-V198F,L203F and SbDhr1-A375F led to a drastic decrease of dhurrin hydrolysis, mainly because of a decrease in the turnover rate (75% decrease in both mutants), implicating these 2 residues in dhurrin specificity. These results are interesting because the ZmGlu1-F198V,F205L double mutant and ZmGlu1-P377A single mutant are not able to hydrolyze dhurrin, suggesting that aglycone specificity of ZmGlu1 and SbDhr1 reside in different amino acids within the aglycone binding site of the active site.

Analysis of the Crystal Structure of ZmGlu1-E191D,F198V in Complex with DIMBOA-Glucoside—The structure at 1.9-Å resolution of ZmGlu1-E191D,F198V contains 980 residues, 493 water molecules, and two substrate molecules, one in the active site of each subunit of the homodimeric enzyme. The final $R$ and $R$-free factors are 18.4 and 22.1%, respectively. The final electron density map clearly defined all atoms of the residues located in the active site pocket (Fig. 3a) and the difference Fourier map revealed the location of a substrate molecule (Fig. 3b). Interestingly, the aglycone moiety was better defined by density in this structure than in the inactivated ZmGlu1-E191D, whereas the density, coming from the aglycone moiety is less visible here, indicating that the aglycone is possibly disordered. The overall structure of the mutant ZmGlu1-E191D-F198V is identical to the native structure (17) as well as to the simple mutant ZmGlu1-E191D (11). All forms of the enzymes have the classical (β/α) barrel fold where β-strands and α-helices within each β/α repeat are connected by loops at the top of the barrel. The quaternary structure of ZmGlu1-E191D,F198V is a 120-kDa homodimer, as is its parental wild-type enzyme (15). Three structural differences are encountered, besides the point mutation introducing a valine residue into the aglycone pocket instead of a phenylalanine, in that two residues undergo a conformational change and the position of one residue is affected in this structure with respect to the structure of the single mutant ZmGlu1-E191D. These are Glu464, which forms a hydrogen bond to the OH6 group of the glycone moiety, and Phe205 and Phe466 in the aglycone pocket (Fig. 3c). In contrary, no such changes were observed in the model created by homology modeling of the single mutant. These conformational changes lead to a completely different position of the substrate molecule in the active site pocket of
the double mutant ZmGlu1-F198V,E191D structure, when compared with the structure of the complex ZmGlu1-E191D,F198V. The map is a SIGMAA weighted 2Fobs - Fcalc synthesis contoured at 1σ, showing the well-defined density around the residues undergoing a positional shift in this mutant structure. a, the initial difference Fourier map (Fobs - Fcalc) located in the active site pocket, contoured at a 2σ level, showing the presence of a substrate molecule. b, structural superimposition of ZmGlu1-E191D (in blue) and ZmGlu1-E191D,F198V (the residues are color coded with standard atom-type colors), both in complex with DIMBOA-Glc (omitted from this figure). The F198V mutation (highlighted in blue) induces a positional change of Phe205, Phe466 takes a different rotameric conformation, and Glu464 in the glycone binding pocket is also displaced. c, the same structural superimposition as in b, viewed in a different orientation, illustrating the drastic change in the position of the DIMBOA-glucoside molecule in ZmGlu1-E191D,F198V (red substrate molecule and atom type colors for the residues) with respect to ZmGlu1-E191D (dark blue, substrate molecule, and blue, residues).

**DISCUSSION**

In this study, we have produced site-directed mutants of a maize (ZmGlu1) and a sorghum (SbDhr1) β-glucosidase and have determined the structure of the most affected mutant of ZmGlu1 in complex with the natural substrate. These two enzymes share 70% sequence identity and represent extremes in substrate specificity (18) in that the former hydrolyzes a wide spectrum of substrates in addition to its natural substrate DIMBOA-Glc, whereas the latter hydrolyzes its natural sub-

**TABLE IV**

Comparison of kinetics parameters of parental (ZmGlu1 and SbDhr1) β-glucosidases and their mutants in assays with the natural substrate dhurrin.

| Enzyme | Dhurrin | | | |
|---|---|---|---|---|
| | Km | kcat | Relative efficiency | |
| Dhr1 | 61 ± 10 | 14.3 ± 1.7 | 100 | |
| Dhr1-V196F,L203F | 65 ± 11 | 3.70 ± 0.19 | 24 | |
| Dhr1-A375P | 60 ± 16 | 3.6 ± 0.3 | 26 | |
| F198V | NA | NA | NA | |
| F205L | 31 ± 10 | 0.16 ± 0.05 | 2.2 | |
| D261N | NA | NA | NA | |
| M280F | 66 ± 21 | 0.21 ± 0.08 | 1.4 | |
| P377A | NA | NA | NA | |
| F466S | 65 ± 10 | 0.55 ± 0.09 | 3.6 | |
| F466SA467S | 92 ± 13 | 0.59 ± 0.05 | 2.7 | |
| Y473F | 47 ± 18 | 1.13 ± 0.15 | 10.3 | |
| F198VF205LP377A | 58 ± 13 | 0.70 ± 0.11 | 5.1 | |
| Ch21 | 76 ± 13 | 1.32 ± 0.24 | 7.4 | |
| Ch16 | 101 ± 10 | 2.23 ± 0.16 | 9.5 | |
| Ch21-F198VF205L | 70 ± 11 | 2.08 ± 0.21 | 12.8 | |
| Ch16-F198VF205L | 105 ± 18 | 2.43 ± 0.15 | 14 | |
| Glu1 | NA | NA | NA | |

*NA, no activity detected.*
strate dhurrin only. The biochemical characterization of these mutants showed that some exhibit a novel substrate specificity by hydrolyzing dhurrin, whereas others exhibit improved or reduced catalytic efficiency on certain substrates, providing new and significant information on mechanism of substrate (i.e. aglycone) specificity. For example, it was found that a single amino acid substitution (Y473F) in ZmGlu1 enabled the hydrolysis of dhurrin, which is not hydrolyzed by wild-type ZmGlu1. The same mutant also improved catalytic efficiency toward such artificial substrates as pNPGlc. The level of hydrolysis is comparable with those of Chim16 and Chim21, indicating that this amino acid might be the critical residue in the ability of Chim16 and Chim21 to hydrolyze dhurrin. The higher $K_m$ found for these 2 chimeras (101 and 76 $\mu M$, respectively) compared with that of ZmGlu1-Y473F (47 $\mu M$) might be because of the other substitutions, namely A467S (with a $K_m$ of 92 $\mu M$). The improved catalytic efficiency provoked by the Y473F mutation may be explained by its role in stabilizing Trp in the native ZmGlu1 structure Trp473 is involved (via the phenolic OH group) in a hydrogen bond with the amide group of Trp478. The side chain of this latter residue therefore has no conformational freedom. Changing Trp473 to a phenylalanine removes this hydrogen bond and the side chain of Trp478 may be more flexible and therefore adapts better to the different orientations of the aglycones of artificial substrates and dhurrin. The influence of the flexibility of the Trp478 side chain on the binding of substrates is also demonstrated by the mutation of Pro477 by an alanine, introducing a higher flexibility to the loop carrying Trp478, which leads to a decrease of $K_m$ for artificial substrates of the enzyme ZmGlu1-P377A, and a lower $k_{cat}$ as a consequence of a bad positioning of the glycosidic bond.

In contrast, another single amino acid substitution (F198V) in the same enzyme resulted in an almost inactive $\beta$-glucosidase (Tables III and IV). The structural analysis shows that the presence of valine instead of phenylalanine at position 198 induces a rearrangement of three amino acids, namely Phe205, Phe206, and Glu264, which are all involved in either the glucose or aglycone binding pocket. This rearrangement leads to a different binding mode of the substrate and most probably is the reason for the loss of catalytic activity upon mutation. Our structural study of the F198V,E191D mutant clearly points to the limits of molecular modeling, because the cascade of rearrangements would have been difficult to predict by a model without taking into account very local and very precise energetic terms. The model calculated with a standard homology modeling algorithm did not reveal these conformational changes for the point mutation F198V.

It is interesting to note that, whereas the F198V substitution has a drastic effect on ZmGlu1 activity, the reverse substitution has no effect on SbDhr1 for the substrates hydrolyzed by the maize enzyme. It appears clear that more than one or two substitutions (the V198F,L203F has no effect) are required to change Sbdhr1 substrate specificity. Therefore it seems likely that other amino acids, whether located in the active site or not, are responsible for the strict specificity in Sbdhr1, whereas the corresponding residues have little or no role in ZmGlu1 substrate specificity. The structural analysis of Sbdhr1 and its inactive mutant in complex with dhurrin, which are in progress, will shed more light on this aspect. The mutation changing Asp251 to an asparagine mainly has an effect on the catalytic efficiency versus artificial substrates. In the structure of ZmGlu1, Asp251 is close to the catalytic acid/base Glu264 and displays no hydrogen bonds either with the glucose or the aglycone. Therefore, the loss of catalytic efficiency may be explained by a stabilizing role of Asp251 on the different protonation states of the acid/base catalyst. Interestingly, a recent mutational study of a human cytosolic $\beta$-glucosidase has also demonstrated that this position is critical for catalysis to take place in human cytosolic $\beta$-glucosidase, even though it is a phenylalanine (Phe225 in human cytosolic $\beta$-glucosidase) in that case (28). Apparently, this position plays an important role in maintaining the local environment of the acid/base catalytic glutamate optimal, counterbalancing the effects of the other surrounding residues.

The site-directed mutagenesis approach used here, whereas giving interesting information on the precise role of individual residues on substrate specificity, reached a limit in the goal of transforming ZmGlu1 into Sbdhr1 or vice versa. To attain such a transformation, it is also necessary to know the critical determining residues in Sbdhr1. However, from our study it appears most likely that more than a handful of residue substitutions are necessary for the inversion of specificity and that local influences of these substitutions are difficult to predict. Whereas site-directed mutagenesis has been shown to be very effective for removing chemically "essential" residues, it often led to altered catalytic mechanisms instead of abolished activity, because of the plasticity in enzyme active site (for review, see Refs. 29 and 30). Such "malleability" could be expected for non-chemically essential residues, as the ones involved in substrate specificity. To counteract the plurality of residues that might be involved in this specificity, a strategy using directed evolution, instead of site-directed mutagenesis, could reasonably be developed. This technique has been successful in changing a $\beta$-galactosidase in a $\beta$-fucosidase (31) or improving the low-temperature catalysis in the hyperthermostable Pyrococcus furiosus $\beta$-glucosidase CelB (32). For these reasons, we think that directed evolution between Sbdhr1 and ZmGlu1 will speed up the process of understanding the aglycone specificity in these enzymes, and $\beta$-glucosidases in general.

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Mutational and Structural Analysis of Aglycone Specificity in Maize and Sorghum \( \beta \)-Glucosidases

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