Chapter 4

Glutamic acid 191 is the acid/base catalyst in the maize ß-glucosidase isozymes Glu1 and Glu2

Cicek, Muzaffer and Esen, Asim. *Dept. of Biology, Virginia Polytechnic Institute and State University, VA., 24061-406*

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ABSTRACT.

ß-Glucosidases (ß-D-glucoside glucohydrolase, EC 3.2.1.21) catalyze the hydrolysis of aryl and alkyl ß-D-glucosides as well as ß-linked oligosaccharides. In maize, ß-glucosidase occurs abundantly in young plant parts (e.g., root, mesocotyl, node, primordial leaves, coleoptile, silk, and ovule) is localized in plastids, and is encoded by two nuclear genes (*glu1* and *glu2)*. Maize ß-glucosidases share high sequence similarities with Family 1 O-glucosidases. Therefore, these enzymes are classified as retaining glycosyl hydrolases whose active site contains two glutamic acids (E) as the key catalytic residues, one as a general acid/base catalyst (E191) and the other as a nucleophile (E406). To confirm the identity and function of the acid/base catalyst E191, we have changed it to isosteric glutamine (Q) and aspartic acid (D) in both Glu1 and Glu2 isozymes by site-directed mutagenesis. Wild type and mutant enzymes were overproduced in *E. coli*, purified and characterized with respect to the catalytic properties. The kinetic analysis of E191Q mutants showed that its catalytic activity was reduced 200- and 110-fold towards ortho- and para-nitrophenyl-ß-D-glucosides, respectively, when compared with the wild type enzyme. The E191D mutants showed no detectable activity towards any of the substrates tested. The back mutation of the E191Q mutants to wild type restored full catalytic activity in both isozymes. These data suggest that E191 in Glu1 and Glu2 is the acid/base catalyst, and its function in catalysis cannot be performed by an isosteric residue such as glutamine or by a carboxyl group on a shorter side chain such as in aspartic acid. Modeling studies also supports the results of the site-directed mutagenesis studies.

INTRODUCTION

ß-glucosidase (ß-D-glucoside glucohydrolase, EC 3.2.1.21) is one of the oldest known enzymes. In 1837, Liebig and Wöhler reported on the emulsifying activity of a factor, isolated from almond meal, on the cyanogenic diglucoside amygdalin, which occurs abundantly in the meal (Liebig and Wohler, 1837). Plant ß-glucosidases have been implicated in a variety of key functions including defense against pests (1,2,3,4), phytohormone activation (5, 6,7,8), lignification (9) and cell wall degradation (10,11). Thus the ß-glucosidase-ß-glucoside system may have different functions in different plant species depending on the chemical structure of the aglycone moiety in substrates, organ, tissue and subcellular location of the enzyme and substrates.

Maize ß-glucosidase (DIMBOA-glucosidase) occurs in two different forms (isozymes), Glu1 and Glu2. Esen and Cokmus (12) and Esen (13) purified and partially characterized a ß-glucosidase (Glu1) from shoots of young maize seedlings. Cuevas et al. (14) partially purified two ß-glucosidases from 6-16 day-old maize leaves: each had specificity for hydroxamic acid glucosides, the glucoside of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in particular. Later work in our laboratory showed that one (Glu2) of these enzymes is expressed exclusively in leaves starting at day 6 to 7 while the other (Glu1) is expressed in all plant parts (Shahid and Esen, unpublished). The cDNAs corresponding to both isozymes were cloned, sequenced and shown to have 88% sequence identity (15). Maize ß-glucosidases have a pH optimum around 6, are made up of 58.4 (~60) kD monomers, and their catalytically active form is a 120 kD homodimer. The deduced primary structures of both isozymes contain peptide sequence motifs TFNEP and ITENG (Fig. 1), which are highly conserved and make up the catalytic site in all Family 1 ß-glycosidases (16,17). To date, the complete sequences of 13 plant ß-glucosidase cDNAs from 11 different species (white clover, cabbage, sweet cherry, black cherry, cassava, costus, maize, sorghum, barley, oats, rice, indigo, pine), representing 7 families and two classes, have been determined. Comparisons of plant ß-glucosidase nucleotide sequences indicate 40-90% identity. The molecular cloning of myrosinase (ß-O-glucosidase) cDNAs and genes from *Brassica* and *Sinapsis* spp. has also been reported (18,19,20). These enzymes show 35-40% sequence identity to ß-0-glucosidases.

ß-glucosidases hydrolyze the ß-glycosidic bond by a double displacement mechanism, which was originally proposed by Koshland (21) for lysozyme and further elaborated and developed by Sinnot (22) and S. Withers and colleagues (23,17) (Fig. 1).

All ß-glycosidases are “retaining” in that the anomeric configuration of the glycone is retained. Substrate hydrolysis involves two steps: **(1)** hydrolysis of the ß-glycosidic bond, glycosylation of the enzyme and release of the aglycone, and **(2)** deglycosylation of the enzyme and release of the glycone. The reaction requires participation of two carboxyl groups, in the side chain of glutamic acid (E), in all Family 1 ß-O-glycosidases. One of these catalytic Es serves as the nucleophile and the other as the acid-base catalyst. Of these, the acid-base catalyst E is in the motif TFNEP while the nucleophilic E is in the motif I/VTENG (Fig. 2), and the two motifs occur about 200-230 residues apart in the primary structure. In the glycosylation step, the nucleophilic E attacks at the anomeric carbon (C_1) of the substrate, forms a covalent glycosyl-enzyme intermediate with concomitant expulsion of the aglycone after protonation by the second catalytic E (the acid-base catalyst). In the deglycosylation step, the second catalytic E, now an anion and a base-catalyst, removes a proton from water, and the OH^- group performs nucleophilic attack on the covalent bond between the glycone and the enzyme, releasing the glycone and regenerating the nucleophilic E.

Stephen Withers and his associates in a series of elegant experiments have unequivocally proved the identity and the function of the nucleophilic E, utilizing the mechanism-based inhibitor 2,4-dinitrophenyl 2-fluoro-ß-D-glucose (2,4-DNP2FGlc) and site directed mutagenesis (24, 25). 2,4-DNP2FGlc enhances the rate of the glycosylation reaction but decreases the rate of the deglycosylation reaction, resulting in trapping of the glycosyl-enzyme intermediate. This group has identified E358 to be the nucleophile in *Agrobacterium* ß-glucosidase (26). Such covalent glycosyl-enzyme intermediates were also trapped and the nucleophilic E was identified as E387 in *Sulfolobus solfataricus* ß-glycosidase (27), and E409 in myrosinase (28). Similarly, site directed mutagenesis studies established that the acid-base catalyst glutamic acid in *Agrobacterium* ß-glucosidase was E170 in the motif TFNEP (29) and E206 in motif TMNEP in *S. solfataricus* ß-glycosidase (27). These results indicate that the equivalents of E170 and E206 in other organisms would also be the acid-base catalyst. Furthermore, the 3-D structures of four Family 1 ß-glucosidases (white clover linamarase, 6-phospho-ß-galactosidase, white mustard myrosinase, and *Bacillus polymyxa* ß-glucosidase, 6-phospho-ß-galactosidase) have recently been solved using crystals of enzyme-glycosyl complexes (30, 31,32,33). In these cases, the residues of the TFNEP and I/VTENG motifs or their equivalents were found to form a pocket or crater-shaped active site (34), interacting with the glycone. The two catalytic Es were positioned within the active site at appropriate distances (2.5-3Å) on opposite sides of the glycosidic bond.

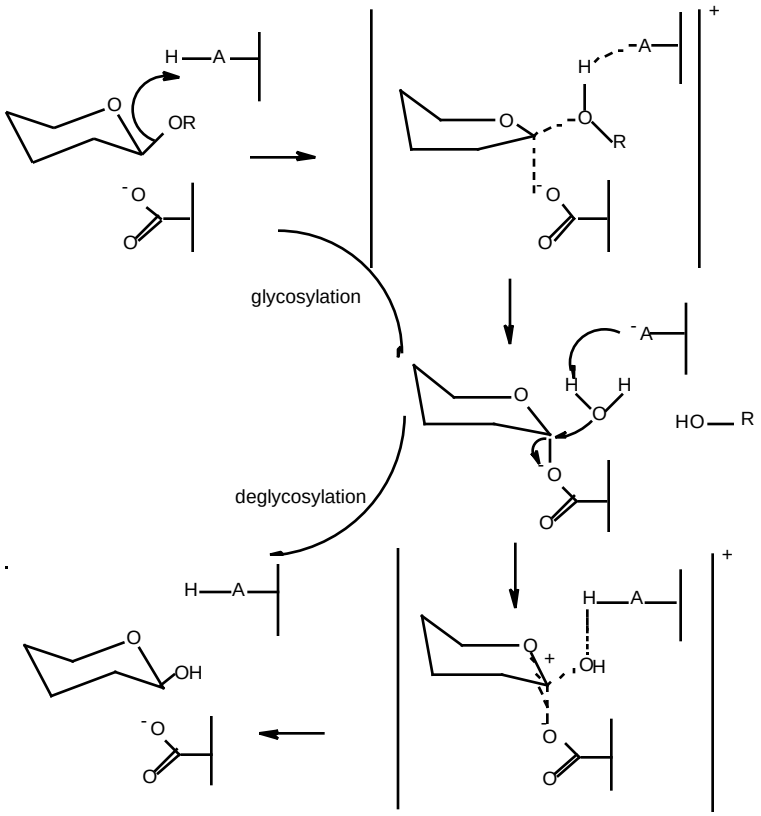

Fig.1. Proposed catalytic mechanism for a retaining ß-glucosidase (34). A covalent α-glycosyl-enzyme intermediate is formed and then hydrolyzed via oxocarbenium-ion-like transition states. R can be either a glucose residue or alkyl or aryl group.

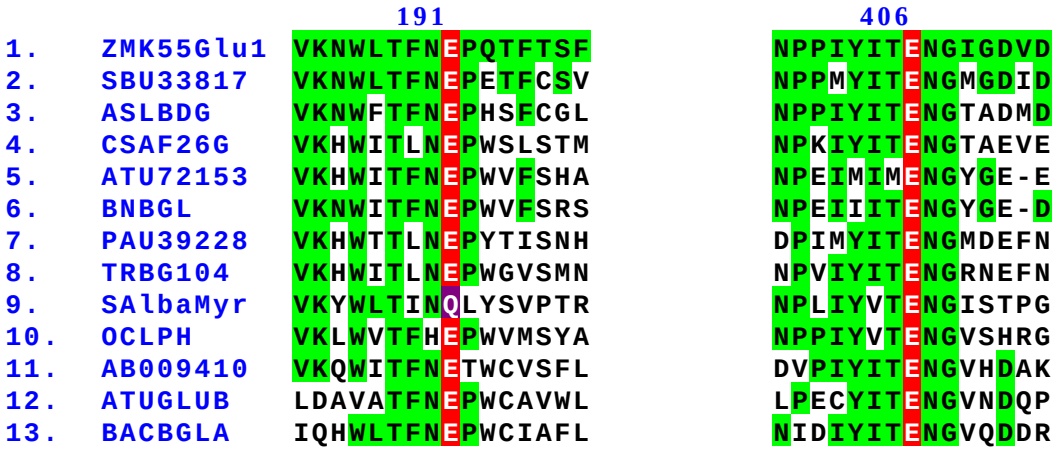

Fig. 2. Sequence alignment in Family 1 ß-glycosidases. Only the acid/base catalyst and the nucleophile containing portions of each polypeptide are shown. Their Genbank codes are indicated in blue. 1, *Zea mays*; 2, *Sorghum bicolor*; 3, *Avena sativa*; 4, *Coctus speciosus*; 5, *Arabidopsis thaliana*; 6, *Brassica napus*; 7, *Prunus avium*; 8, *Trifolium repens*; 9, *Sinapsis alba* (myrosinase); 10, *Rabbit* (lactose pyrolozin hydrolase); 11, *Bacillus spp.*; 12, *Agrobacterium tumafaciens*; 13, *Bacillus polymyxa*.

Myrosinases (ß-S-glucosidases) deviate from the norm in that they lack the peptide motif TFNEP and thus an acid-base catalyst. Instead they have the homologous motif TINQL in which Q has replaced E. In the 3-D structure of the enzyme-glucone complex, Q occupies a position within the active site equivalent to that of the acid-base catalyst E of O-glycosidases. Wang and Withers (35) and Burmeister et al. (32) proposed that the hydrolysis of ß-glucosinolates (S-glucosides) by myrosinases does not require an acid-base catalyst because glucosinolate aglycones are good leaving groups. Similarly, the deglycosylation step does not require a base catalyst E because Q by itself in the myrosinase active center positions the water, which can hydrolyze the covalent bond between glucose and the enzyme.

Identification of the amino acid residues that line the active site of an enzyme and participate in substrate recognition, binding and catalysis is key to understanding the mechanism of catalysis and the biological function of that enzyme. Such information is also crucial to engineering enzymes to use novel substrates or existing substrates with improved catalytic efficiency. The purpose of the studies reported in this paper was to test the hypothesis that the acid/base catalyst in maize ß-glucosidase isozymes Glu1 and Glu2 is the glutamic acid residue 191 (E191) in the motif TFNEP, and that it cannot be replaced by an isosteric amino acid such as glutamine or another amino acid with carboxyl functionality such as aspartic acid. To this end, we produced E191Q and E191D mutants of both enzymes by site directed mutagenesis. The mutant enzymes and their wild type counterparts were produced in *E. coli* pLyS, purified, and characterized with respect to catalytic activity and substrate specificity using the physiological substrate DIMBOAGlc and various artificial substrates such as *p*NPG, *o*NPG, 4MUGlc, and 6BNGlc.

MATERIAL and METHODS

Mutagenesis and preparation of constructs

The vector-cDNA constructs pET21a-c10 (Glu1) and pET21a-g09 (Glu2) were prepared by ligating the mature protein coding regions of the *glu1* and *glu2* cDNAs, respectively, to the expression plasmid pET21a. The cDNAs were expressed in *E. coli* pLyS to produce the wild type maize ß-glucosidase isozymes Glu1 and Glu2 as described (36). The recombinant plasmids (pET21a-c10 and pET21a-g09) containing the wild type *glu1* and *glu2* cDNAs were used as templates for mutating E191 to Q191 and D191,

respectively, by site-directed mutagenesis. Mutagenesis was performed as described by using mutated oligonucleotides as primers in combination with a 5' or a 3'-end vector specific primer in PCR and then fusing the resulting PCR products by overlap extension as described (37). In order to convert E191 to Q191, the vector-specific 5'-primer T7 (STA ATA CGA CTC ACT ATA GGG, derived from a region in the vector upstream to the 5'end of *glu1* or *glu2* cDNA insert) and gene-specific 3'-primer ß-Glu144 (AAT TGG TTS ACC TTT AAY CAG CCC, antisense, derived from cDNA region encoding the peptide sequence TFNEP) were used to amplify the 5'-fragment. The 3'-fragment was amplified using the gene-specific forward primer ß-Glu145 (GGG CTG RTT AAA GGT SAA CCA RTT, the sense version of ß-Glu144) and reverse primer T7ter (ATG CTA GTT ATT GCT CAG CGG T, derived from a region in the vector downstream to the 3'end of *glu1* or *glu2* cDNA insert). The resulting DNA fragments were electrophoresed in a 1% agarose gel, excised, and then purified using the Gel Extraction Kit QIAGEN II (Hilden, Germany). The purified fragments were fused by overlap extension using the vector-specific T7 and T7ter primers. The amplified PCR product was cloned into *Sma* I site of pBluescript SK(+) and transformed into DH10B cells. Plasmid DNA was isolated and digested with the restriction endonucleases *Nhe* 1 and *Xho* 1. The resulting product from *Nhe* 1 and *Xho* 1 digestion was gel purified and cloned into the expression plasmid pET21a. To back mutate the Glu1 and Glu2 E191Q mutants to the wild type (E191), gene-specific sense ß-Glu171 (GGC TCA TTA AAG GTC AAC CA) and antisense ß-Glu64 (TGG TTG ACC TTT AAT GAG CC), primers were designed and used to amplify the 5'- and 3'- portions of *glu1* and *glu2* cDNAs by PCR. The fragments were fused and cloned into pET21a as described above.

In order to mutate E191 to D191, the vector-specific 5'-primer T7 (see above) and gene-specific mutated 3'-primer ß-Glu173 (GG ATC ATT AAA GGT CAA CCA antisense, derived from cDNA region encoding the peptide sequence TFNEP) were used to amplify the 5'-fragment. The 3'-fragment was amplified using the gene-specific mutated sense primer ß-Glu 174 (TGG TTG AAC TTT AAT GAT CC) and antisense primer T7ter (see above). The resulting PCR products were fused, digested, and cloned as described above. In all cases, positive clones were identified by PCR screening of colony lysates. The mutant colonies were screened by PCR using T7/T7ter primer pairs and three positive colonies representing each mutant were selected and expressed in 25 mL cultures and extracted as described (36).

Expression and protein purification

For protein production, fresh *E. coli* cultures harboring the recombinant plasmid were diluted 100 fold with 3-4 L LB broth containing 50 µg ampicilin/mL and 38 µg/mL chloramphenicol and grown in a shaker-incubator at 37 °C. After the OD of the cultures reached about 0.6, IPTG was added to a final concentration of 0.6 mM and the cultures were incubated at room temperature (25-26 °C) for an additional 4 h. Induced cultures were harvested by centrifugation at 2700g at 4 ˚C and stored frozen at - 70 ˚C prior to use. The pellet was then thawed and suspended in an 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 and incubated at room temperature for 30 min. The soluble protein fraction was recovered by centrifugation at 12,000 rpm for 20 min and analyzed for enzyme activity by spectrophotometric and native PAGE zymogram assays. ß-glucosidase activity was initially analyzed by measuring the amount of pNP released from p-nitrophenyl ß-D-glucopyranoside (*p*NPG). Each assay was performed in quadruplicate in the wells of a microtiter plate in a total volume of 140 µl containing 70 µl 5 mM *p*NPG and 70 µl enzyme solution. The reaction was incubated at room temperature for 5 min and stopped by adding 70 µl 0.4 M Na_2CO_3. The absorbance was read in a microplate reader at 410 nm.

The wild type and mutant proteins were purified using the crude cell extracts as starting material. The protein fraction containing ß-glucosidase was precipitated from crude extracts by a 45 to 65% ammonium sulfate cut and dissolved in 50 mM Na-acetate buffer, pH 5. The enzyme solutions were adjusted to 0.5 M ammonium sulfate prepared in Na-acetate buffer, pH 5, before loading onto a hydrophobic interaction chromatography (Toyo pearl-butyl-650) column (1.5x24 cm). The protein solution was centrifuged to remove precipitated bacterial proteins, loaded first onto Toyo pearl butyl-650 column, washed with 0.5 M ammonium sulfate in acetate buffer, pH 5 to baseline absorption, and eluted with a reverse gradient of 0.5 M to 0.1 M ammonium sulfate in 2 hrs. The fractions were assayed for *p*N*P*Gase and *o*NPGase activities and analyzed for general protein spectrum by SDS-PAGE. The resulting fractions containing activity were pooled. The combined fractions were adjusted to 0.6 M ammonium sulfate and loaded onto Toyo pearl-phenyl-650 hydrophobic interaction chromatography column and eluted by reverse salt gradient from 0.5 M to 0.1 M ammonium sulfate. The resulting fractions were further analyzed by *p*NPGase assay and SDS-PAGE (Fig. 3).

Enzyme assays and kinetic characterization

Standard ß-glucosidase assay against *para*-and *ortho*-NPG was performed as described by Cicek and Esen (1999). Kinetic parameters for wild type and mutant ß-glucosidases were measured by varying substrate concentration from 0.098 to 16.66 mM. The enzyme protein concentration was adjusted (normalized) by dilution before all activity measurements. K_m and k_{cat} values of wild type and mutant ß-glucosidases were determined from the amount of *p*NP and *o*NP released from *p*NPG, *p*NPGal, *p*NPFc, *o*NPG, *o*NPGal, and *o*NPFc. Each assay was performed in quadruplicate in the wells of a microtiter plate in a total volume of 140 µl containing 70 µl substrate and 70 µl diluted enzyme solution. The reaction was incubated at room temperature (~25 ˚C) for 10 min and stopped by adding 70 µl 0.4 M Na_2CO_3. The absorbance was read in a microplate reader at 410 nm. All kinetic data were plotted by using the Enzyme Kinetics ® software (Trinity Software, FL). The protein concentrations were determined by the method of Bradford (BioRad Kit) using bovine serum albumin as standard.

Since our E191Q mutants somewhat mimic myrosinases, which have the motif TINQL instead of TFNEP of ß-O-glycosidases and thus lack the acid/base catalyst E, we tested the E191Q mutants for activity towards the natural myrosinase substrate sinigrin (isolated from white mustard). The sinigrin hydrolysis assay was performed as described (28) both in the presence and absence of 1 mM ascorbic acid in the reaction mix. Both wild type enzyme and the D191 mutant were also assayed for activity towards sinigrin.

For activity assays in native PAGE gels, purified enzymes were electrophoresed into 6% alkaline gels, and zymograms were developed using the artificial fluorogenic substrate 4MUGlc. Electrophoresis and gel equilibration were performed as previously described (36). The gel was incubated in 4MUGlc solution (1mM 4MUGlc in 50 mM citrate-100 mM phosphate buffer, pH 5.8) under agitation on a shaker platform, and enzyme bands were detectable under UV light within 5 to 10 min. The gel was washed twice in citrate-phosphate buffer for 1 min each wash, and immediately photographed under UV light (Fig. 4).

Thin-layer-chromatography was performed using 0.25 mm silica coated Whatman® PE SIL G/UV plates. The Glu1 and E191Q enzymes were adjusted to 0.1µg protein in 50 µl reaction mix containing 1 mM final concentration of the natural substrate DIMBOAGlc and incubated at 37˚C for 48 hrs. Ten µl of the reaction mixture was spotted on the TLC plate and chromatographed vertically using as mobile phase an acetonitrile/dH_2O (85/15) mixture for 45 min (38). The plate was sprayed with MeOH/H_2SO_4 (4:1; v/v), and then baked at 110 °C for

10 min to visualize any unhydrolyzed glucoside and the reaction products (glucose and DIMBOAGlc) (Fig. 5). For DIMBOAGlc, no enzyme was included in the assay. DIMBOAGlc was purified from the 4 day-old seedlings as described (36).

The effect of pH on activity was studied using 50 mM sodium citrate (pH 4.0-4.5), 50 mM sodium acetate (pH 5.0-5.5), 50 mM MES (pH 6.0-6.5), 50 mM PIPES (pH 7.0), 50 mM MOPS (pH 7.5), 50 mM Tris (pH 8.0-8.5), 50 mM CHES (pH 9.0-9.5) and 50 mM CAPS (pH 10.0-11.0). Following the standard ß-glucosidase assay described above, time-dependent activity enhancement was measured in Glu1 and E191Q mutant after adding Na-carbonate to the reactions. The absorbance was recorded in different pH solutions.

RESULTS

The recombinant Glu1 and Glu2 isozymes produced in *E. coli* were essentially identical to the native enzymes produced in maize except the N-terminal amino acid alanine as an add-on in recombinant forms.

Site-directed mutagenesis was used to alter the active site acid/base catalyst E191 to Q and D. When crude extracts were analyzed for ß-glucosidase activity by standard enzyme assay using *p*NPG, the results showed that all three mutant crude extracts had very little or no detectable activity as compared to the wild type. To confirm that the E191Q mutant was indeed the intended mutant, the appropriate region was sequenced. In addition, the Q residue was back-mutated to E and D using the isolated plasmid with the E191Q mutant construct as template. The activity assays on lysates from wild type showed that complete activity was restored after back-mutation of Q191 to E191, but not in mutation of Q191 to D191 (Fig. 4). The Q191D mutant was totally inactive towards all substrates listed in Table 1 under the same assay conditions. The mutant inserts were sequenced to confirm the presence of the desired single mutation in the TFNEP region. The sequencing the appropriate coding region of the mutant E191Q and EQ191D cDNA clones showed that there were no mutant sites other than the single mutation that was induced. The E191Q mutant showed reduced but detectable activity towards the natural substrate DIMBOAGlc (Fig. 5), as well as the artificial substrates listed in Table 1. The TLC results clearly showed that the E191Q mutant hydrolyzed DIMBOAGlc, at a 14.5-fold reduced rate, when compared to the wild type enzyme, which completely hydrolyzed DIMBOAGlc, as expected (Fig. 5).

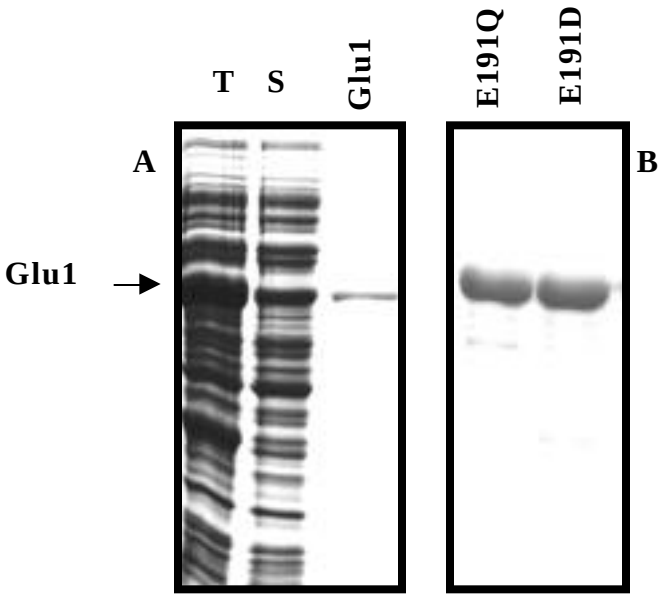

Fig. 3. **A**, SDS-PAGE profiles of the total (T) and soluble (S) fractions, and purified wild type ß-glucosidase isolated from *E. coli* transformed with the recombinant plasmid pET-21a-c10 (Glu1); **B**, SDS-PAGE profiles of purified mutant ß-glucosidases.

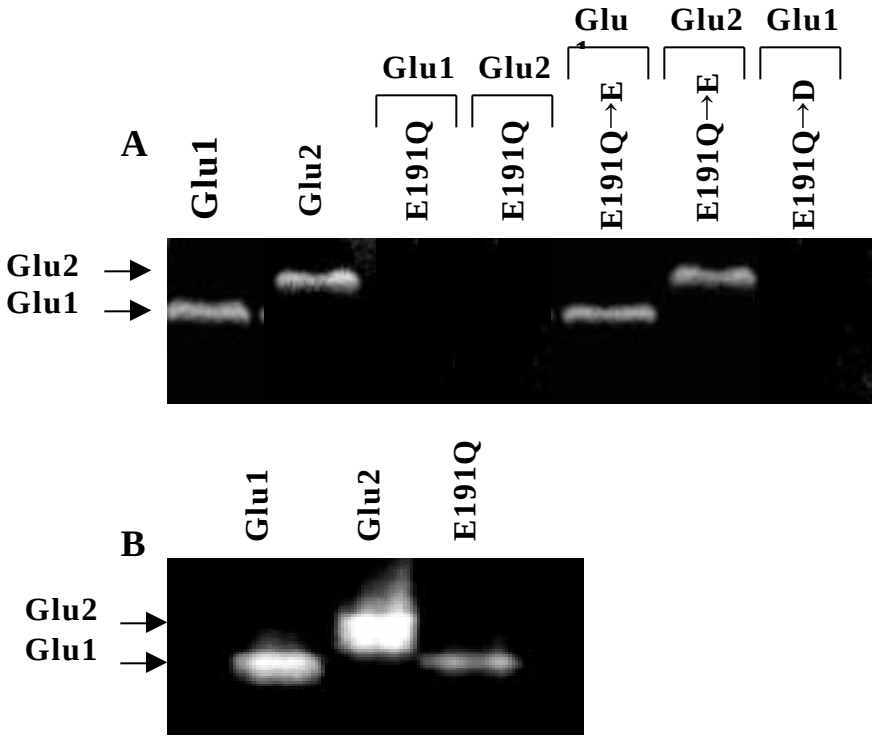

Fig. 4. **A,** a zymogram developed with the fluorogenic substrate 4MUGlc (4-methylumbelliferyl-ß-D-glucoside), which is hydrolyzed by both Glu1 and Glu2. The Q mutants of Glu1 and Glu2 do not hydrolyze 4MUG. Note that full activity was restored in back mutants (E191Q→E) of E191Q of Glu1 and Glu2, but not in E191Q →D mutant; **B**, a zymogram developed with 4MUGlc after normalizing the *p*NPGase activity of purified recombinant Glu1, Glu2 and mutant E191Q.

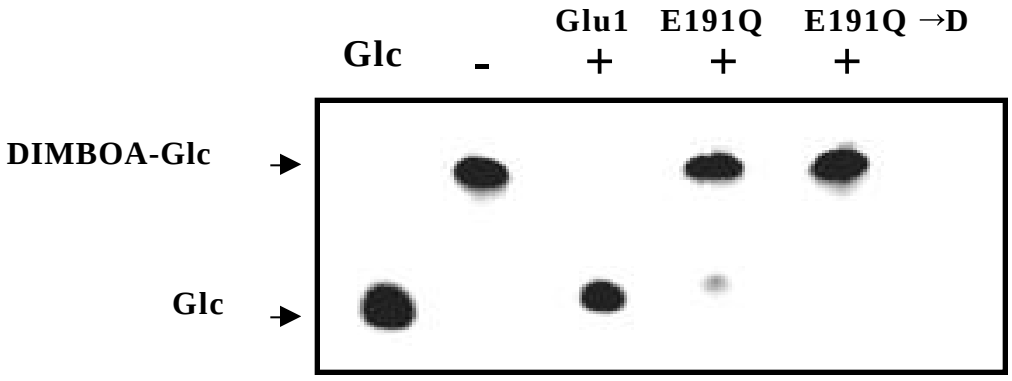

Fig. 5. TLC chromatogram showing the activity of wild type Glu1, E191Q and E191Q→D mutants towards physiological substrate DIMBOAGlc. The plus sign (+) denotes incubation of substrate with enzyme, whereas the minus sign (-) denotes incubation of substrate without enzyme.

The substitution of E by Q in Glu1 and Glu2 was intended to mimic the catalytic site of a ß-S-glucosidases (i.e., myrosinases). As mentioned before, myrosinase contain Q in place of E, the acid/base catalyst in the motif TFNE/QP region of the active site, which provides protonation assistance to aglycone departure in all O-ß-glucosidases. The Q and D mutants did not show any detectable activity towards the myrosinase natural substrate sinigrin. The inclusion of ascorbic acid at 1 mM final concentration in the reaction mix had no effect on the result.

SDS-PAGE and immuno blot analysis of cell lysates from small-scale cultures showed that the mutant ß-glucosidases were expressed at levels (~10% of total *E. coli* protein) equivalent to their wild type counterparts (data not shown). Moreover, there was no detectable difference between the mutant and the wild type enzymes with respect to ammonium sulfate concentration required for precipitation and binding and elution behavior during purification by hydrophobic interaction chromatography. The purification protocol employed yielded near 95% homogeneity as judged by SDS-PAGE analysis. The mutant enzymes also displayed the same electrophoretic mobility in SDS-PAGE gels (58 kDa) and immunoreactivity as their wild type counterparts (Fig. 3).

The results of activity assays with the purified Glu1 E191Q mutant and its wild type counterpart Glu1 using six different nitrophenyl glucosides are presented in Table 1. No data from activity assays with the D mutant are presented because there was no activity measurable with this mutant above background levels. As is evident from the data in Table 1, the K_m values obtained with the mutant were essentially similar to those obtained with the wild type enzyme in the case of each substrate. The k_{cat} data clearly showed that the E191Q mutant activity level was reduced about 100 to 250-fold, depending on the substrate, when compared to that of the wild type. The E191Q mutant yielded the highest k_{cat} value with *p*NPFc as did the wild type enzyme, although its activity was 245-fold lower than that of the wild type. The magnitude of differences between the E191Q mutant and the wild type enzyme were also evident when other measures of catalytic efficiency (e.g., k_{cat}/K_m) were use for comparison (Table 1). The pH optimum of the Q mutant is slightly shifted to acidic side when compared to wild type (Fig. 6). Surprisingly, the E191Q mutant continued to hydrolyze *o*NPG substrates at pH 9 to 10.5 after addition of Na_2CO_3. In other words, the addition of Na_2CO_3 did not stop the activity of the Q mutant when *o*NPG and *o*NPFc substrate were used, and this observation was confirmed by assaying activity in different buffer systems that yielded a final pH of 9 to 10.5 after addition of Na_2CO_3 (Fig. 7). However, no such continued activity was observed with the

wild-type enzyme after the addition of Na_2CO_3. The activity enhancement was monitored in time-course with Q mutant and wild type Glu1.

The inhibition of activity of the E191Q mutant and the wild type enzyme was studied using four different artificial substrates (*p*NPGlc, *o*NPGlc, *p*NPFc, and *o*NPFc) and *p*NP-S-Glc as inhibitor (Table 2). The data showed that the mode of inhibition was competitive in all cases, and the mutant enzyme dissociation constants (K_i) were about 2 (*p*NPFc) to 20-fold (*p*NPGlc) lower than those of the wild type (Table 2).

DISCUSSION

In nature, ß-glucosidases display broad substrate specificity toward natural and artificial substrates. ß-glucosidases share a common catalytic mechanism that requires two carboxylic residues that act as nucleophile and acid/base catalyst in active site. The nucleophilic residue has been first identified in *Agrobacterium* ß-glucosidase (Abg) by employing the mechanism-based inhibitor 2-deoxy-2-fluoro-D-glycopyranoside. The proposed mechanism of glucosidases is based on retention of the configuration of sugar moiety. This is a double displacement mechanism involving an initial binding of the substrate to the enzyme followed by nucleophilic attack of the enzyme upon anomeric center to form a glycosyl-enzyme intermediate. This intermediate is then hydrolyzed by general acid/base-catalyzed attack of water upon the anomeric center, releasing glucose as product and returning the enzyme to its original protonation state.

Studies on glycosyl-enzyme intermediate using 2,4-dinitrophenyl-2-fluoro-ß-D-glucose showed that a nucleophile forms a covalent bond, with the carbenium ion that is formed as an intermediate of the substrate (26). Based on the X-ray analysis and chemical evidence, in all Family 1 ß-glucosidases, the nucleophile was identified as E in the invariant region V/ITENG of the catalytic site. Therefore, in maize ß-glucosidase, the nucleophilic residue was identified as E406 in the corresponding homologous peptide region (Fig. 8A).

Maize ß-glucosidases contain the conserved the motif ^{188}TFNEP^{192}, which contains the proposed general acid/base catalyst (E191), donating its proton to oxygen in the glycosidic bond between glucose and aglycone moiety of the substrate. Site-directed mutagenesis results revealed that E170 of the *Agrobacterium* ß-glucosidase is identified as the acid/base catalyst (29). An equivalent residue in another enzyme of Family 1, E160 of