Chapter 2

Expression of Soluble and Catalytically Active Plant (Monocot) ß-Glucosidases in *E.coli*

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ABSTRACT

Complementary DNAs encoding mature and precursor ß-glucosidase proteins Glu1 and Glu2 of maize were amplified by the polymerase chain reaction (PCR) and cloned into the expression vector pET-21a. Both Glu1 and Glu2 isozymes were expressed in high yield (~3.8% of the total soluble protein and 32% of the total expressed protein) in *E. coli*. Recombinant enzymes showed activity towards a variety of artificial and natural substrates at levels similar to those of their native counterparts isolated from maize seedlings. Western blot analysis confirmed that both recombinant isozymes were immunoreactive with maize anti-ß-glucosidase sera and their molecular sizes were identical to those of the native maize Glu1 and Glu2 isozymes. Zymogram assays in native gels revealed that recombinant enzymes had the same electrophoretic mobility and substrate specificity as their native counterparts. In addition, the precursor forms of both maize enzymes as well as the mature forms of oat and sorghum ß-glucosidases were expressed in active and soluble form using the same host-vector system.

Keywords: ß-glucosidase, maize, sorghum, oat, pET21, expression, *E.coli*

INTRODUCTION

In plants, ß-glucosidases (EC 3.2.1.21) have been implicated in a variety of processes such as defense, growth, and development. Specifically, one of the important functions of plant ß-glucosidases is thought to involve defense against pathogens and herbivores by releasing toxic aglycones (e.g., hydroxamic acid, coumarins, thiocyanides, terpenes, saponins and cyanide) from glucosides. Cuevas et al. (1992) showed that maize ß-glucosidase cleaves hydroxamic acid glucosides (HxGlc), particularly 2-O-ß-*D*--glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOAGlc). The aglycone DIMBOA is present as a glucoconjugate (DIMBOAGlc) in intact tissues of maize, rye and wheat. DIMBOA is a potent, toxic aglycone which is shown to inhibit the electron transport system and phosphorylation reactions in bovine mitochondria (Niemeyer et al., 1986) and spinach chloroplasts (Querolo et al., 1983). It has also been shown that HxGlc content of some Gramineae (e.g., maize and wheat) increases when the plant is subjected to pathogen attack (Gutierrez et al., 1988; Niemeyer et al., 1989; Thackray et al., 1988). The purification, characterization and the sequence of the first 20 N-terminal amino acids of maize ß-glucosidase Glu1 have been reported (Esen, 1992). The enzyme is an autodimer made up of 60 kD monomers; it has the highest catalytic activity at pH 5.8 and at 50^{o}C; and its pI is 5.2. The enzyme is an abundant protein in maize seedling parts, as well as growing portions of the mature plant. Substrate specificity studies have shown that the maize ß-glucosidase readily hydrolyzes a variety of aryl ß-glucosides thus showing broad specificity for the aglycone moiety of the substrate but it does not hydrolyze glycosyl glucosides like cellobiose and gentiobiose (Babcock and Esen, 1994). In this respect, it is similar to rubber tree and white clover ß-glucosidases (Pocsi et al., 1989; Selmar et al., 1987). The same study also indicated that maize ß-glucosidase was inhibited by various aglycones and ß-glucosides. However, monosaccharide glycones (e.g., glucose and fructose) were poor inhibitors (Babcock and Esen, 1994). cDNAs for maize ß-glucosidase Glu1 have been cloned and characterized by Brzobohaty et al. (1993) and Esen and Shahid (1995, direct submission). Similarly, a cDNA encoding another maize ß-glucosidase (i.e., Glu2) has been sequenced (Bandaranayake and Esen, 1996) and the corresponding enzyme was purified and characterized in our laboratory (Shahid et al., unpublished data). The full-length *glu1* and *glu2* cDNAs each code for a 566 and 563 amino acid-long precursor protein, respectively. The Glu1 and Glu2 precursor proteins have 54 and 51-amino-acid-long transit peptides, respectively, for plastid targeting. Thus, both mature proteins are 512-amino acid long. Even though there is 90% sequence

identity between the Glu1 and Glu2 isozymes (Fig. 1), they have different spatial and temporal regulation in that Glu1 is expressed in all seedling parts, while Glu2 is expressed in leaves only starting at day 6 after germination. The two isozymes also differ from each other with respect to substrate specificity in that the Glu1 isoform hydrolyzes various artificial aryl ß-glucosides with high catalytic efficiency while the Glu2 isoform hydrolyzes the same substrates poorly or not all. In the present study, complementary DNAs encoding mature ß-glucosidase proteins Glu1 and Glu2 from maize (K55) were amplified by the polymerase chain reaction (PCR) and cloned into the expression plasmid vector pET-21a. The Glu1 and Glu2 isozymes were expressed in high yield and active form in *E. coli*, and characterized with respect to substrate specificity, immunological, and electrophoretic properties. In addition, the precursor forms of both enzymes with their N-terminal presequence as well as the mature form of oat ß-glucosidase (avenacosidase) and sorghum dhurrinase-1 (Dhr1) were expressed in active and soluble form using the same host-vector system as that used for mature maize ß-glucosidase proteins.

MATERIALS AND METHODS

Preparation of expression constructs. The regions of *glu1* (U25157) and *glu2* (U44087) cDNAs coding for the mature maize ß-glucosidase proteins were amplified by the polymerase chain reaction (PCR) using oligonucleotide primers (ß-glu-111, ACTACAGCTAGCGCAAGAGTAGGCAGCCAAAAT, sense for both *glu1* and *glu2*; and ß-glu-113, CTA TCT CGA GTT AAG CTGGCGTAAGAATCTTC, antisense for both *glu1* and *glu2*) containing *Nhe* I (5'-end) and *Xho* I (3'-end) restriction enzyme sites (Fig. 2). The thermostable DNA polymerase *pfu* was used in PCR and the PCR products were blunt end ligated into *Sma* I site of pBlueScript SK (+). The construct was used to transform the *E. coli* strain 'Nova Blue', and the presumptive positive (white) colonies were screened by PCR. Vector-specific primers (T3 and T7) flanking the cloning site were used to determine if they had an insert of expected size (Gussow and Clackson, 1989). Recombinant colonies were isolated and grown for plasmid isolation. After large scale recombinant plasmid isolation, the inserts were cut with *Nhe* I and *Xho* I, gel purified and cloned into the expression vector pET-21a that had been double-digested with *Nhe* I and *Xho* I. The *E.coli* host strain pLys was transformed with the recombinant plasmid constructs (pET-21a-c10 for rGlu1 and pET-21a-g09 for rGlu2, respectively) and used in

Fig. 1. Deduced amino acid sequence identity among mature maize (MzGlu1 and MzGlu2), sorghum (SorGlu) and oat (OatGlu) ß-glucosidases. The deduced amino acid sequences were aligned using the clustal multiple alignment program (MEGALIGN) of the DNASTAR software package. Sequences against gray background indicate regions of amino acid identity among MzGlu1, MzGlu2, SorGlu, and OatGlu. The two peptide motifs that contribute to the glycon recognition and catalysis at the active site are boxed and they are essentially invariant in Family A ß-glucosidases.

expression studies. Recombinant colonies containing the cDNA insert were identified by PCR using vector-specific (T7 promoter and T7 terminator) primers.

Induction and Protein Expression.

Expression of rGlu1 and rGlu2 proteins was induced by IPTG after transformation with the recombinant plasmid constructs pET-21a-c10 and pET-21a-g09. For this, fresh *E.coli* cultures were diluted with 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 0.5-0.6, 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 3 h. Induced cultures were harvested by centrifugation and the cell pellets were lysed, and the lysates were used for protein profile analysis by SDS-PAGE. Specifically, total protein profiles were analyzed by pelleting cells from liquid culture (1 mL) in a microcentrifuge and resuspending the pellet in 100 µl of 125 mM Tris-HCl, pH 6.8/15% glycerol/1% SDS/ 5.6%2-ME (a modified version of 1X sample buffer; Laemmli, 1970). The suspension was boiled for 5 min and 7 µl aliquots were loaded onto 12% SDS-polyacrylamide gels. After electrophoresis, proteins were visualized by staining with the general protein dye Coomassie Brilliant Blue R250. The amount of ß-glucosidase expressed as percent of total protein were determined by densitometric scanning of the soluble and total protein electrophoretic profiles.

Enzyme Extraction, Purification, and Assay.

Protein extractions for enzyme activity assays were performed using 25 mL induced recombinant cultures. The cell cultures were pelleted by centrifugation at 2700g at 4˚C for 10 min, washed with fresh LB, repelleted, and stored frozen at -70˚C until used. The extraction conditions that yielded the highest amount of soluble and active recombinant enzyme were determined by extracting the cell pellets with buffers of different pH, salt and SDS concentration. The pellet was thawed and suspended in an optimized extraction buffer (100 mM Tris HCl/50 mM NaCl, pH 8.0) containing 0.05% SDS/40 µg/mL DNase I/0.2 mM PMSF/2 mM $MnCl_2$/2 mM $MgCl_2$ and incubated at room temperature for 30 min. The extraction buffer volume (mL) to cell pellet weight (g) ratio was 10:1 in all extractions. The soluble protein fraction was recovered by centrifugation at 13,000xrpm in microcentrifuge for 10 min and subjected to electrophoretic analysis by SDS-PAGE and for enzyme activity by spectrophotometric and native PAGE assays. The same extraction buffer was used to extract the native Glu1 isozyme (MzGlu1) from 3 day-old etiolated shoots and native Glu2

isozyme (MzGlu2) from 7 day-old leaves of the maize inbred B73. In this case, the buffer volume (mL) to tissue fresh weight (g) ratio was 3:1. Recombinant ß-glucosidase activity was initially analyzed by measuring the amount of *p*-nitrophenol (*p*NP) released from the hydrolysis of the artificial substrate *p*-nitrophenyl ß-D-glucopyranoside (*p*NPGlc). Each assay was performed in quadruplicate in the wells of a microtiter plate in a total volume 140 µl containing 70 µl 5 mM *p*NPGlc and 70 µl of extract (diluted 50X with the assay buffer 50 mM citrate-100 mM phosphate, pH 5.8) as enzyme source. The reaction mixture was incubated at room temperature for 5 min and stopped by adding 70 µl of 0.4 M Na_2CO_3. The amount of *p*NP released was determined by absorbance measurement in a microplate reader at 410 nm. In addition, DIMBOAGlc, *p*NPGlc, *o*NPGlc, and 4-methyl umbelliferyl ß-D-glucoside (4MUGlc) were used as substrates in specific activity assays by the coupled Peroxidase-Glucose-Oxidase (PGO) assay of Raabo and Terkildsen (1960). The protein content of each extract was determined using a Bradford protein assay kit (Bio-Rad). The specific activity towards each of these substrates was expressed as µmoles substrate hydrolyzed mg protein^{-1} h^{-1}. Fifty µL aliquots containing 250 nmole (1.25 mM final concentration) substrates in the assay buffer were placed in quadruplicate in the wells of a microtiter plate and tested for hydrolysis by adding 50 µL enzyme solution containing 1 µg protein from crude extracts of rGlu1 and rGlu2 clones, 50 µL PGO and 50 µL ABTS (2,2'-azinobis-3-ethylbenzthiazolinesulfonic acid). The reaction mix was incubated at 37 °C for 15 min, and the absorbance was measured in a microplate reader at 410 nm. Kinetic parameters (K_m and V_{max}) were determined by plotting 1/*v* against 1/*s* using *p*NPGlc and *o*NPGlc as substrate at concentrations varying from 0 to 50 mM in 50 mM citrate -100 mM PO_4 buffer, pH 5.8. The amount of *p*NP and *o*NP released after 15 min was determined by measuring absorbance at 410 nm.Enzyme activity was also assayed by zymogram techniques in gels after native PAGE. The soluble protein fractions were electrophoresed into 6% alkaline gels and zymograms were developed using the fluorogenic substrate 4MUGlc (Martin et al., 1992) and chromogenic substrate 6-bromo-2-naphthyl-ß-D-glucoside (6BNGlc) as described before (Esen and Cokmus, 1990). The native counterparts (referred to as MzGlu1 and MzGlu2, respectively) of the recombinant enzymes were isolated from maize seedlings and included in all analysis for comparison and as positive controls.

DIMBOAGlc Purification.

Thirty grams of frozen seedlings were ground in liquid N_2, extracted with 70 mL 100% MeOH and centrifuged at 16,000*g* for 20 min at 4°C. The resulting supernatant was

freeze-dried and the powder was dissolved in 70% MeOH and fractionated on a Sephadex LH-20 gel-filtration column (2.6X56 cm) at room temperature (~23-25°C). The fractions which formed a blue complex (positive test for the presence of hydroxamic acids and their glucosides) with the ferric chloride reagent (0.6% $FeCl_3$, 7.5 mM HCl in ethanol) were combined and refractionated on a longer LH-20 gel filtration column (2.6X88 cm). The fractions that reacted with the $FeCL_3$ reagent were combined and freeze-dried. The resulting dried DIMBOAGlc was dissolved in 70% MeOH. After determining the molarity of DIMBOAGlc present in this solution, aliquots with known DIMBOAGlc content were dried in microfuge tubes, dissolved in the enzyme assay buffer, and used for activity assays.

Thin Layer Chromatography.

An artificial substrate *p*NPGlc and the natural substrate DIMBOAGlc were used in activity assays by thin-layer-chromatography (TLC). Thin-layer-chromatography was performed using 0.25 mm silica coated Whatman® PE SIL G/UV plates. The rGlu1 and rGlu2 extracts were diluted 10X in 10 mM citrate-20 mM phosphate buffer, pH 5.8, and incubated with 5 mM final concentrations of DIMBOAGlc and pNPGlc in 10 mM citrate-20 mM phosphate buffer, pH 5.8 at room temperature for 6 hr. Ten µl of the reaction mix was spotted on the TLC plate and chromatographed vertically using the acetonitrile/H2O (85/15) mixture as the mobile phase for 45 min (Robyt and White, 1990). The plate was sprayed with MeOH/H2SO4 (4:1; v/v), and then baked at 110oC for 10 min to visualize the reaction products. For each substrate, a "no enzyme control" was included in the assay.

SDS-PAGE and Western Blotting.

The soluble extracts were mixed with one-fourth volume of the 4X SDS-gel sample buffer (Laemmli, 1970) and heated at 100oC for 5 min. Volumes containing 7-µg protein from recombinant bacterial lysates and laß-grown maize seedling extracts were loaded onto 12% SDS gels and electrophoresed. After electrophoresis, the gels were soaked in 1X blotting buffer (25 mM Tris-125 mM glycine/0.25% SDS/ 20% MeOH) and the proteins were transferred onto a PVDF membrane (Millipore Corporation, Bedford, MA) (Pluskal et al., 1986). Immunodetection was carried out using an anti-maize ß-glucosidase serum (R681) as described by Mohammed and Esen (1989).

RESULTS

The primer pair (ß-glu-111-113 for both glu1 and glu2 cDNA amplifications) containing Nhe I (5'-end) and Xho I (3'-end) restriction sites, respectively, yielded amplicons of the expected size (1559 bp) by the PCR. The amplicons contained the coding sequence for the mature maize ß-glucosidase proteins MzGlu1 and MzGlu2. The restriction enzyme Nhe I site was chosen for the 5' cloning site in order to minimize the incorporation of additional amino acids encoded by the plasmid vector to the N-terminus of rGlu1 and rGlu2 polypeptides. Consequently, the recombinant ß-glucosidase mature proteins synthesized in E. coli each contained only one additional amino acid residue (alanine) at their N-termini after the initiator methionine. Thus, the first two codons (AUG and GCU) at the 5'-translated region of the ß-glucosidase mRNAs were transcribed into them from the plasmid vector template. After the initiator is cleaved, the recombinant proteins were 513 amino acid-long as opposed to their 512 amino-acid-long native counterparts that occur in the maize plant (Fig. 1). At the 3'-end, the first stop codon UAA was from the maize ß-glucosidase cDNA, and it was five nucleotides upstream of the Xho I site that was designed into the antisense primer p113 (Fig. 2). Thus, our expression constructs were designed to produce ß-glucosidase polypeptides that are essentially identical to those that occur in the maize plant. This was to avoid any potential negative effects of a fusion protein or affinity tags on enzyme activity. The induction experiments using 0 to 1 mM final concentrations of IPTG in culture medium showed that there was no constitutive expression in the absence of the inducer (i.e. IPTG); see Fig. 3A, T0 lane, and the amount of ß-glucosidase polypeptides in cell lysates increased as IPTG concentration increased from 0.1 to 0.6 mM, and remained constant thereafter. Thus, 0.6 mM IPTG concentration was chosen as optimum for use in all subsequent experiments. The induction and extraction data indicated that the recombinant maize ß-glucosidases rGlu1 and rGlu2 were expressed at high levels (approx. 11.3% of the total protein for Glu1 and 8.8% of the total protein for Glu2). Approximately 32% of the total expressed ß-glucosidase was in soluble form (Fig. 3B). In fact, specific ß-glucosidase activity in E. coli lysates attributed to rGlu1 and rGlu2 was about 4.0-4.4 and 5.2-7.3 times (depending on the substrate), respectively, higher than those measured in extracts of the maize inbred line B73 from which both ß-glucosidase cDNAs had been isolated (Table I). As for the influence of temperature on expression level, there were no significant differences between 25-26˚ and 37˚C with respect to the amount of the total ß-glucosidase monomer on SDS gel profiles. However, when cultures were grown at temperatures around 25-26˚C, approximately 32% of the total expressed ß-glucosidase was soluble and catalytically active as opposed to little or no soluble protein being produced at 37˚C. In other

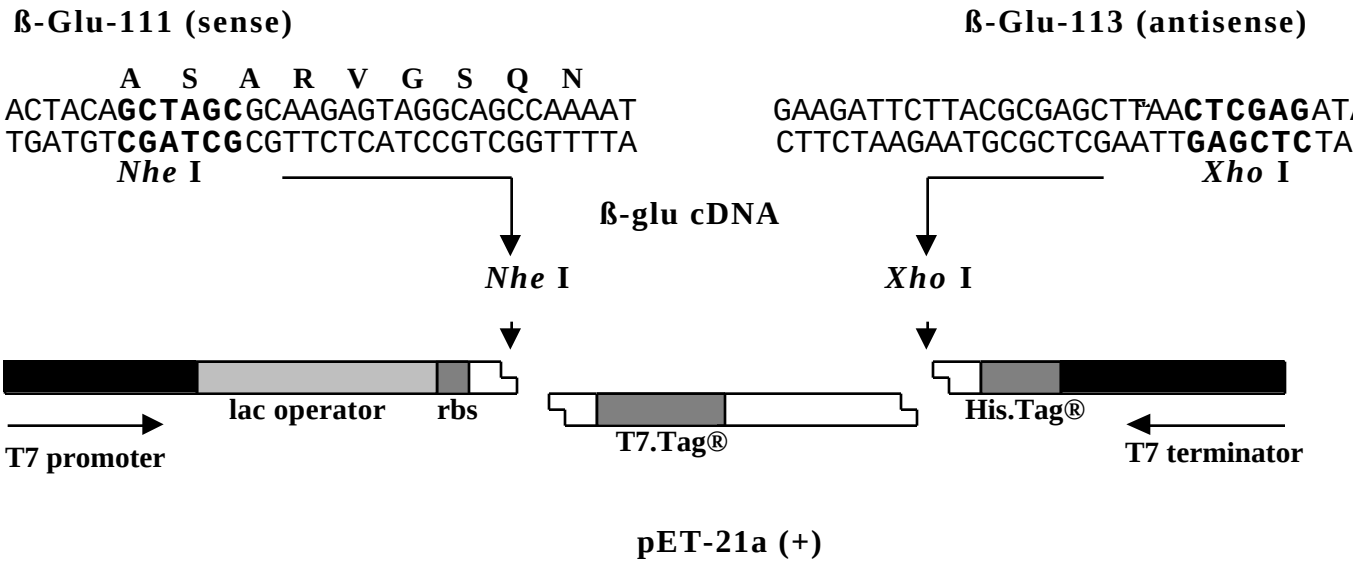

Fig. 2. Maize *glu1* and *glu2* cDNA expression constructs showing the locations and sequences of the sense (ß-glu111, 5'-end) and antisense (ß-glu113, 3'-end) primers and the engineered restriction sites (in bold). rGlu1 (or rGlu2) cDNA coding for mature maize ß-glucosidase protein is inserted into the multiple cloning site of the plasmid expression vector pET-21a between the *Nhe* I and *Xho* I sites, replacing the fragment containing the T7 Tag.

words, almost all of the expressed protein was directed to inclusion bodies at 37˚. In view of these results, room temperature (25-26˚C) was selected for routine use.

Comparisons of the protein profiles of lysates from induced and uninduced cultures by SDS-PAGE showed that induced cultures containing pET-21a-c10 and pET-21a-g09 constructs had an intense band in the 58-59 kD region of the gel; this band was absent from the profiles of uninduced cultures as well as from those of induced cultures containing pET-21a plasmid without a ß-glucosidase cDNA insert (negative control). The mobility of rGlu1 and rGlu2 polypeptides on SDS gels was equal to those of their native counterparts isolated from maize (Fig. 3C). However, both native Glu2 and recombinant Glu2 polypeptides had a slightly faster mobility than the native Glu1 and rGlu1 polypeptides even though both Glu1 and Glu2 polypeptides were of the same length. Western blotting after SDS-PAGE and probing the blots with a monospecific anti-ß-glucosidase serum revealed a single immunoreactive band of identical electrophoretic mobility (58 to 59 kD) in lysates of the E. coli cultures harboring pET-21a-c10 (expressing rGlu1) and in extracts of maize seedlings expressing the native MzGlu1 (Fig. 3C). The same was the case in lysates of cultures harboring the recombinant plasmid pET-21a-g09 (expressing rGlu2) and maize extracts containing native MzGlu2 (Fig. 3C) except that the maize leaf extract showed two immunoreactive bands due to the transient occurrence of the Glu1 and Glu2 isozymes in the same organ -- leaves of the 6-8 day old post germination seedlings. The control lysate from cells with nonrecombinant pET-21a showed no immunoreactive polypeptides (Fig. 3C, E. coli lane).

Specific activity and kinetic parameters of the native and recombinant ß-glucosidases were studied using various ß-glucoside substrates as shown in Table I. Both recombinant and native enzymes exhibited similar substrate specificity toward the physiological substrate DIMBOAGlc and nonphysiological substrates pNPGlc and oNPGlc in solution assays. However, the activity toward DIMBOAGlc could not be quantified by spectrophotometric measurement because either DIMBOA or its breakdown products interfered with the PGO assay, yielding reddish color rather than green with ABTS. The data also show that specific activities of rGlu1 and rGlu2 in bacterial lysates, respectively, were approximately 4.0-4.4- and 5.2-7.3-fold greater than those of their native counterparts in crude extracts of maize tissues (Table I). The data in Table II indicate that both Glu1 and Glu2 isozymes hydrolyzed oNPGlc with the highest efficiency followed by pNPGlc and MUGlc as evident from their Vmax values. However, the Km values for pNPG and oNPG were 5 and 1.4 fold higher for the Glu2 isozyme than those for the Glu1 isozyme. In addition, the kinetic data indicate

that native and recombinant ß-glucosidases do not differ from each other with respect to substrate specificity and the rate at which a given substrate is hydrolyzed as evident from their similar Km and Vmax values (Table II).

Comparative substrate specificity data obtained with rGlu1 and rGlu2 isozymes and their native counterparts were confirmed also by zymograms assays in 6% native gels (Fig. 4), and thin layer chromatography (Fig. 5). For example, the zymogram data showed that both recombinant enzymes (rGlu1 and rGlu2) had the same electrophoretic mobility and substrate specificity as their native counterparts (Fig. 4A-B). The Glu1 isozymes hydrolyzed both 4MUGlc and 6BNGlc, while the Glu2 isozymes hydrolyzed 4MUGlc but not 6BNGlc (Fig. 4A-B). The TLC data also showed clearly that rGlu1 and rGlu2 hydrolyzed the physiological substrate DIMBOAGlc and pNPGlc at levels similar to those of their native counterparts isolated from maize seedlings (Fig. 5). The E. coli lysate from cultures transformed with nonrecombinant plasmid (pET-21a, negative control) showed no detectable activity towards DIMBOAGlc and pNPGlc (Fig. 5).

DISCUSSION

This paper reports the expression of two recombinant maize ß-glucosidase (rGlu1 and rGlu2) isozymes in soluble and catalytically active forms and at high levels in E. coli, as well as their comparison with the native Glu1 and Glu2 isozymes with respect to substrate specificity, kinetic, electrophoretic and immunological properties. The results showed that the catalytic, electrophoretic, and immunological properties of the recombinant enzymes were identical to those of their native counterparts isolated from maize (Figs. 3-5; Tables I and II). The extractability data indicated that a higher percentage of the total expressed ß-glucosidase was soluble when cultures were grown and induced at room temperature than that at 37oC. This suggested that a high percentage of the foreign protein was targeted to the inclusion bodies at 37oC than at 25-26oC (Schein, 1989). Moreover, the addition of SDS to the extraction buffer at a final concentration of 0.05% resulted in (~10-30%) increase in extractable ß-glucosidase activity. This was likely due to the breakup of inclusion bodies by SDS. However, the most of the inclusion body-bound protein was misfolded and inactive because SDS concentrations above 0.05% resulted in 2-3 fold increase in extractability of the ß-glucosidase monomers based on densitometric analysis after SDS-PAGE but no increase in

Table I. Comparison of activities of the recombinant (rGlu1 and rGlu2) maize ß-glucosidase isozymes with their native (MzGlu1 and MzGlu2) counterparts.

	Specific activity (µmol hydrolyzed substrate. mg^{-1} h^{-1})		
Enzyme	***p*-NPG**	***o*-NPG**	**4-MUG**
rGlu1	1050±50	2960±10	1900±07
MzGlu1	260±07	720±06	430±02
rGlu2	210±04	860±10	870±04
MzGlu2	40±06	130±01	120±07

Table II. Comparison of kinetic parameters of the recombinant (rGlu1 and rGlu2) maize ß-glucosidase isozymes with their native (MzGlu1 and MzGlu2) counterparts

	K_m (mM)		V_{max} (µmol h^{-1})		V_{max}/K_m	
Enzyme	***p*NPG**	***o*NPG**	***p*NPG**	***o*NPG**	***p*NPG**	***o*NPG**
rGlu1	0.41±0.01	1.42±0.02	225.4±3.0	282.7±1.0	549.7	199.0
MzGlu1	0.40±0.02	1.34±0.07	261.0±8.0	288.5±1.3	652.5	215.2
rGlu2	0.84±0.05	1.25±0.05	52.8±0.7	168.3±1.8	62.8	134.6
MzGlu2[a]	0.88±0.04	1.32±0.05	ND	ND	-	-

[a] Partially purified (40% to 70% ammonium sulfate cut) used in the assay.

ND: not determined

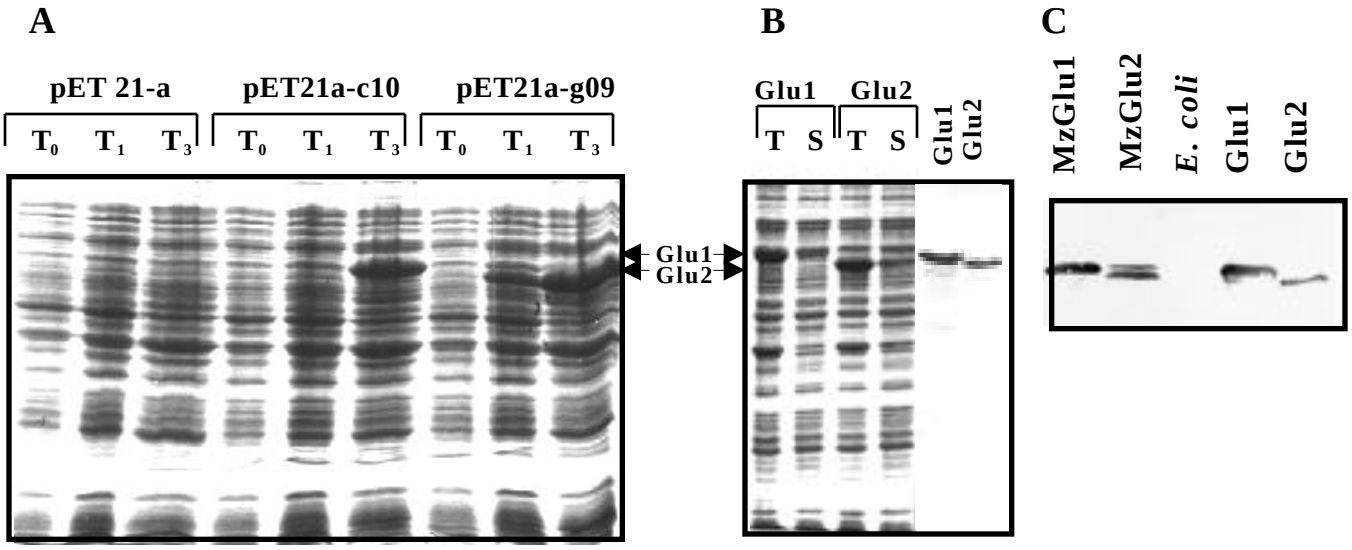

Fig. 3. A, SDS-PAGE profiles of cell lysates from *E. coli* transformed with nonrecombinant plasmid (pET-21a, negative control) and the recombinant plasmids pET-21a-c10 (rGlu1) and pET-21a-g09 (rGlu2) after incubation in the presence of 0.6 mM IPTG for 0 (T_0), 1 (T_1), and 3 (T_3) hours. The arrows point to positions of the ß-glucosidase monomers (60 kD).). **B**, SDS-PAGE profiles of the total (T) and soluble (S) protein fractions isolated from *E. coli* transformed with the recombinant plasmids pET-21a-c10 (rGlu1) and pET-21a-g09 (rGlu2), showing the amount of rGlu1 and rGlu2 monomers present in each fraction. Note that only about one-third of the total expressed ß-glucosidase is in the soluble fraction. **C**, Immunoblots of native maize ß-glucosidase isozymes (MzGlu1 and MzGlu2) and their recombinant counterparts (rGlu1 and rGlu2) expressed in *E. coli*. Native MzGlu1 isozyme was isolated from 3 day-old etiolated shoots and MzGlu2 from 7 day-old leaves of the maize inbred B73. The *E. coli* lane denotes the lysate of *E. coli* transformed with pET-21a plasmid lacking a ß-glucosidase cDNA insert (negative control). Note that the MzGlu2 preparation is contaminated with some Glu1 isozyme because spatial transition (which typically occurs at day 10-11) from Glu1 and Glu2 was not completed in 7-day-old leaves used for protein isolation. Note also that both native and recombinant Glu2 monomers move slightly faster in SDS-PAGE than the Glu1 monomer although they both have the same relative molecular masses (~58.5 kD).

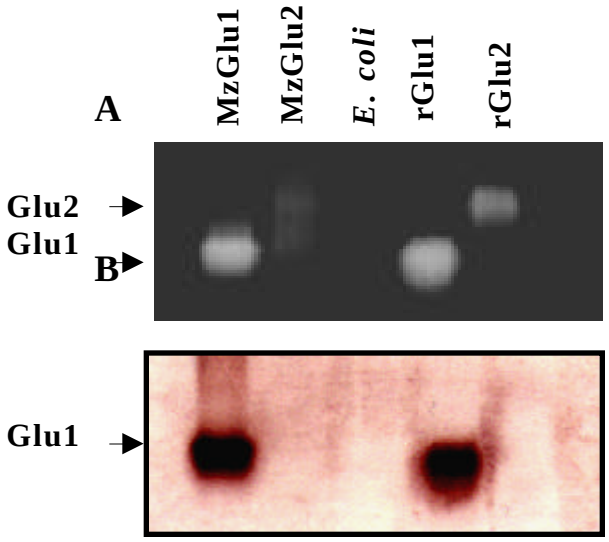

Fig. 4. Native PAGE (6%) gel zymograms of native maize ß-glucosidase isozymes (MzGlu1 and MzGlu2) and of their recombinant counterparts (rGlu1 and rGlu2) expressed in *E. coli.* **A,** a zymogram developed with the fluorogenic substrate 4MUGlc (4-methylumbelliferyl-ß-D-glucoside) which is hydrolyzed by both isozymes. **B,** a zymogram developed with the chromogenic substrate 6BNGlc (6-bromo-2-naphthyl-ß-D-glucoside) which is hydrolyzed by the Glu1 isozyme but not by the Glu2 isozyme. Note that the recombinant isozymes exhibit the same electrophoretic mobility and substrate specificity as the native counterparts and the lysate of *E. coli* transformed with pET-21a plasmid lacking a ß-glucosidase cDNA insert has no enzyme activity detectable with either substrate.

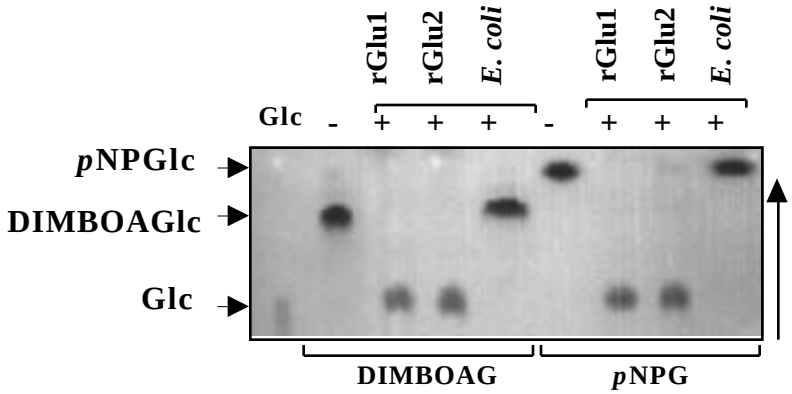

Fig. 5. TLC chromatogram showing the activity and substrate specificity of two maize ß-glucosidase isozymes expressed in *E. coli.* An artificial substrate (*p*NPGlc) and the physiological substrate DIMBOAGlc were tested for hydrolysis by the recombinant rGlu1 and rGlu2 isozymes. The plus (+) denotes incubation of the substrate with either rGlu1, rGlu2 or lysate from an *E. coli* culture transformed with nonrecombinant plasmid (negative control) while the minus (-) denotes incubation of the substrate without any enzyme source (no enzyme control). Note that both recombinant isozymes hydrolyzed the natural substrate DIMBOAGlc and artificial substrate pNPGlc like the native counterparts isolated from maize

enzyme activity. We have not attempted to solubilize the inclusion body-bound protein with urea and guanidine-HCL and renature it by removing the denaturant gradually by dialysis. This is because our previous attempts to renature the urea or GND-HCl denatured native maize ß-glucosidases were not successful. Fortunately, the increase in extractability with SDS was not achieved at the expense of enzyme activity and stability because 0.05% SDS had little or no effect on activity and stability of either the rGlu1 or rGlu2 isozyme. Maize and other ß-glucosidases (e.g., sorghum, rice, almond, and Trichoderma) are resistant to denaturation by SDS and can be extracted and assayed in the presence of SDS (Esen and Gungor, 1993).

To our knowledge this is the first documented report on the expression of an intact plant ß-glucosidase in a prokaryotic host. Brzobohaty et al. (1993) stated that they expressed the maize Glu1 isozyme in E. coli using the pET vector system. However, no data were presented as to what the level of expression and the characteristics of the recombinant enzyme were other than the fact that it hydrolyzed the cytokinin O-glucoside and the kinetin N-3-glucoside zeatin in vitro assays. Recently, Inoue et al. (1996) have reported the expression of a plant ß-glucosidase from another monocot (furostanol glycoside 26-O-ß-glucosidase of Costus specious, the ginger plant) using pT7 blue and pET3d plasmid expression vectors in E. coli . Although bacterial lysates showed detectable activity after IPTG induction towards the natural substrate furostanol 26-O-ß-glucoside, albeit one-hundredth the activity level found in ginger rhizome extracts, the recombinant enzyme could not be detected on gels by either Coomassie staining or zymogram techniques. Similarly, Keresztessy et al. (1996) expressed the mature cassava cyanogenic ß-glucosidase (linamarase) in fusion with glutathione-S-transferase in the plasmid vector pGEX-2T in E. coli. In this case, the induction by IPTG resulted in the production of a 87 kD polypeptide (60 kD linamarase+27 kD GST). Most of the fusion protein was not soluble, having been misfolded and directed to inclusion bodies when cultures were grown at 370C. In addition to misfolding, the recombinant linamarase was extremely sensitive to proteolysis. The soluble fusion protein was catalytically active against the natural substrate linamarin as well as artificial substrates tested. However, the fusion protein had about a 100-200 fold lower Vmax values for linamarin and pNPGlc than did the native linamarase although they both had the same Km values. The fusion protein was found to be tightly associated and thus copurified with the bacterial chaperonin GroEL. Keresztessy et al. (1996) also observed that the dissociation of the chaperonin GroEL and the cleavage of the GST from the fusion protein led to considerable elevation of ß-glucosidase activity. The authors explained the

activity increase with correct folding of the recombinant ß-glucosidase after dissociation from the chaperonin GroEL and removal of GST.

The design of the *glu1* and *glu2* cDNA constructs so as to code for intact rGlu1 and rGlu2 mature ß-glucosidase polypeptides without N-or C-terminal affinity tags and fusion partners was probably the most crucial factor in our successful expression of these isozymes. The presence of an extra Ala residue at the N-termini of rGlu1 and rGlu2 had no apparent effect either on enzyme activity or substrate specificity. Our previous attempts to express rGlu1 and rGlu2 in fusion with 27-kD GST in another host-vector (DH5α-pGEX) system resulted in very little protein production and the expressed fusion protein was catalytically inactive and apparently degraded as evident from a number of immunoreactive, low molecular weight fragments detected on western blots. The fact that the catalytically active form of both isozymes is an autodimer could have presented problems in expressing biologically active protein in surrogate host systems such as *E.coli*. This is because active enzyme production requires that the monomer first fold correctly in the reducing environment of the bacterial cytosol and then interact with another monomer to form the catalytically active dimer.

Our success in expressing the two maize ß-glucosidase isozymes is not a "lucky hit" or exception because we also expressed two other monocot ß-glucosidases (sorghum dhurrinase1 and oat avenacosidase which share 70 and 55% sequence identity, respectively, with maize ß-glucosidases, Fig. 1) in active and soluble form using the same host-vector system as that used for maize rGlu1 and rGlu2 (data not shown). Likewise, we expressed the intact precursor polypeptides of rGlu1 and rGlu2, and both precursors were catalytically active and behaved as their mature proteins with respect to substrate specificity in solution and gel assays. However, a detailed kinetic characterization of the recombinant maize ß-glucosidase precursors as well as the sorghum and oat mature proteins have not been carried out. Nonetheless, the data suggest that the presence of the 51 to 54 amino acid-long N-terminal transit sequences in maize ß-glucosidase precursors does not affect their catalytic activity. As shown in Fig. 1, mature Glu1 and Glu2 polypeptides each are 512 amino-acid-long after cleavage between Arg^{54} and Ser^{55} in Glu1, and between Arg^{51} and Ser^{52} in Glu2 after their precursors reach the target subcellular compartment (i.e. the plastid).

Even though there is very high amino acid sequence identity (90%) between the Glu1 and Glu2 isozymes (Fig. 1), they differ in substrate specificity from each other. Investigations on the substrate specificity of various plant ß-glucosidases show that maize, *Hevea,* and almond ß-glucosidases (Babcock and Esen, 1994; Grover and Cushley, 1977;

Selmar et. al., 1987) exhibit broad substrate (i.e. aglycone) specificity. Both rGlu1 and rGlu2 hydrolyze the physiological substrate DIMBOAGlc with similar catalytic efficiency but show subtle differences in specificity for such artificial substrates as 4MUGlc, *p*NPGlc and 6BNGlc. For example, rGlu1 hydrolyzes 4MUGlc and *p*NPGlc 5 to 6 times more efficiently than rGlu2 when their activities are normalized (equalized) for DIMBOAGlc. Similarly and more dramatically, rGlu1 readily hydrolyzes the chromogenic substrate 6BNGlc and have been used in zymogram assays for years while rGlu2 shows no detectable activity towards this substrate (Fig. 4B). These results are in full agreement with those obtained with native Glu1 and Glu2 isozymes isolated from maize (Fig. 4B). As for the slightly different mobilities of the Glu1 and Glu2 isozymes through SDS-PAGE gels in spite of their identical lengths (Fig. 3A-C), the difference is thought to arise from their amino acid compositions in that the Glu2 isozyme has four more hydrophobic amino acids than the Glu1 isozyme. The additional hydrophobic residues in the Glu2 primary structure would increase the amount of SDS bound to it and thus the net negative charge of the Glu2-SDS complex, resulting in faster migration towards the anode during SDS-PAGE. Examples of anomalous mobility of basic proteins (e.g., cytochromes and histones moving slower than expected for their size) and that of those with high hydrophobic amino acid content (e.g., cereal prolamins moving faster than expected for their size) have long been known (Noelken et al. 1981; Esen, unpublished observations).

It should also be emphasized that the expression of two maize ß-glucosidase cDNAs in *E. coli* allowed the biosynthesis and accumulation of their protein products rGlu1 and rGlu2, respectively, at levels about 4 to 5 times higher than their native counterparts in maize tissues. In fact, this comparison is based on the soluble and catalytically active portion (32%) of the total expressed recombinant protein. This is significant in that some plant ß-glucosidases such as maize Glu2, sorghum dhurrinase2 and rice ß-glucosidases are not abundant proteins, making purification in sufficient quantities from their natural source extremely difficult if not impossible for a thorough characterization. In these cases, cloning the cDNA and expressing it in *E. coli* would be both a feasible and an expedient strategy. We realize that the ß-glucosidase cDNAs we expressed in *E. coli* are from monocotyledonous plants, specifically from grasses, and their protein products are not glycoproteins; they are synthesized on free ribosomes and targeted to the plastid. In contrast, all known dicot ß-glucosidases and thiglucosidases (i.e., myrosinases) are glycoproteins (Falk and Rask, 1995a,b; Li et al., 1992;), co-translationally targeted to the ER via a signal peptide. The only report of a catalytically active dicot ß-glucosidase expression in *E. coli* is

by Keresztessy et al. (1996). In this case, the cassava ß-glucosidase (linamarase) was produced in fusion with GST. The demonstration of catalytic activity in the nonglycosylated linamarase answers part of the important question of whether or not glycosylation is required for activity or stability and targeting of dicot ß-glucosidases. The linamarase data indicate that glycosylation is not essential for activity.

The expression system we constructed and tested sets the stage for a variety of investigations we wished to do but could not do before. Firstly, it allows the production of large amounts of enzyme for purification and further characterization including tertiary structure determination. This is important because in our experience the most challenging aspect of ß-glucosidase purification is to protect the enzyme from modification by endogenous proteases and a slew of phenolic compounds. The absence of an affinity tag in recombinant enzymes is not an insurmountable obstacle to purification because judicious use of differential solubility and preparative chromatographic and electrophoretic procedures may still yield sufficient quantities of homogenous enzyme. Secondly, the questions related to the improvement of catalytic efficiency, mechanism of substrate specificity, site and mechanism of aglycone binding, and the contribution of individual amino acids at the active site to binding and catalysis can now be addressed by making appropriate chimeric and mutant constructs using the procedures of domain swapping and site-directed mutagenesis and expressing and testing them in *E. coli*. These studies would have been virtually impossible in a transgenic plant system considering the number of constructs that one needs to test and thus has not been even attempted before.

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