

Aryl-Glycosidase Activities in Germinating Maize

Peter Biely,¹ Jeffrey A. Ahlgren,^{2,3} Timothy D. Leathers,^{2,4} Richard V. Greene,⁵ and Michael A. Cotta²

ABSTRACT

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Soluble protein extracts of germinating maize seedlings exhibited a limited ability to hydrolyze purified xylans, and specific assays were unable to confirm the presence of endo- β -1,4-xylanase activity. However, extracts contained a variety of aryl-glycosidase activities, including β -glucosidase, β -xylosidase, and α -L-arabinofuranosidase. These activities peaked in three- to four-day seedlings and were particularly concentrated in shoot and root tissues. Maximal levels of β -glucosidase were two

orders of magnitude greater than those of β -xylosidase or α -L-arabinofuranosidase. Isoelectric focusing gels revealed multiple forms of these enzymes. The principal β -glucosidase and α -L-arabinofuranosidase protein species were clustered at pI 4.8–4.9 and pI 5.8–6.0, respectively. β -Xylosidase activity appeared to be associated with both of these enzymes, and no evidence was obtained for a distinct β -xylosidase.

Cereal germination is accompanied by the mobilization of a battery of degradative enzymes, including various amylases, glucanases, lipases, and proteases (Fincher 1989). Enzymes secreted by the seed scutellum and aleurone layers participate in the breakdown of β -glucans and arabinoxylans in the walls of the endosperm, making starch available to support seed germination. Numerous studies have been made of β -glucanases in germinating cereals, including recent studies of β -glucanases from germinating maize (Inouhe and Nevins 1997, 1998; Inouhe et al 1999, 2000). However, cereal xylanases are far less understood. Arabinoxylans are major components of cereal endosperm cell walls (Fincher and Stone 1986; Izydorczyk and Biliaderis 1995), including those of maize (Seckinger et al 1960). Xylanolytic enzymes have been reported in germinating wheat (Fincher and Stone 1974; Schmitz et al 1974) and characterized more extensively in germinating barley (Preece and MacDougall 1958; Taiz and Honigman 1976; Dashek and Crispeels 1977; Slade et al 1989; Banik et al 1997). However, to our knowledge xylanolytic enzymes have not been studied in germinating maize seedlings. An endoxylanase was recently identified as the predominant protein on the surface of maize pollen (Bih et al 1999). This xylanase may play a specific role in the penetration of the stigma surface, as the gene specifying this enzyme was expressed only in the tapetum of developing anthers (Bih et al 1999).

Xylans are complex heteropolysaccharides that require a suite of enzymes for complete hydrolysis (Saha and Bothast 1999). Arabinoxylan from maize pericarp appears to be highly substituted and recalcitrant to degradation by microbial enzymes (Saulnier et al 1995; Hespell et al 1997; Hespell 1998). Although corn fiber xylan was partially saccharified by enzymes from a highly xylanolytic strain of *Aureobasidium* sp., relatively high enzyme levels were required (Leathers and Gupta 1996). The current study was initiated to seek new sources of xylanolytic enzymes with improved specificity for maize arabinoxylan. Maize

xylanases may be uniquely suited to attack this substrate. Such enzymes could ultimately prove useful in applied bioconversions of agricultural residues and other applications. Furthermore, studies of xylanolytic enzymes will contribute to our fundamental understanding of maize germination and development.

MATERIALS AND METHODS

Plant Materials and Germination Conditions

Maize (*Zea mays* L.) hybrids WW64A \times A619 and Golden Harvest hybrid GH2574 were the gifts of M. Muhitch and P. Dowd of the Agricultural Research Service, USDA, Peoria, IL. Kernels were surface-sterilized by gentle shaking for 5 min in 70% ethanol (v/v) followed by gentle shaking for 15 min in 15% (v/v) household bleach solution in sterile water (final concentration 0.8% sodium hypochlorite) containing 0.1% Tween 20. Treated kernels were rinsed five times with sterile water.

Kernels were germinated in the dark at 28°C between four layers of sterile wipers (WypAll L40, Kimberly-Clark, Roswell, GA) moistened with sterile water. Of the WW64A \times A619 seedlings, \approx 1–2% showed apparent *Fusarium* contamination and were discarded. None of the GH2574 seedlings showed contamination after surface-sterilization. Germination was interrupted at various times, and whole or dissected seedlings were homogenized for protein extracts.

Homogenization

Whole seedlings or separated tissues were homogenized in 0.05M sodium acetate buffer (pH 5.5) containing 5 mM Na₃, 10 mM EDTA, and 2 mM dithiothreitol, using an homogenizer (Polytron model PT 10/35, Brinkmann Instruments, Westbury, NY). Samples were maintained on wet ice and homogenized at maximal power for repeated cycles of 20–30 sec. Homogenates were clarified by centrifugation at 35,000 \times g for 30 min at 2°C. Supernatants were either assayed directly or desalted and concentrated \approx 10-fold by ultrafiltration using centrifugal filter devices (Centricon YM-10, Millipore Corp., Bedford, MA).

Enzyme and Protein Assays

Total xylanase activity was assayed on birchwood glucuronoxylan or oat spelt arabinoglucuronoxylan (Sigma Chemical Co., St. Louis, MO) by the dinitrosalicylic acid reducing sugar method of Miller (1959) modified as previously described (Leathers 1986; Bailey et al 1992). Individual sugars were identified by thin-layer chromatography (TLC) on silica gel plates developed with 1-butanol and ethanol and water (10:8:7, v/v) and on cellulose-coated plates developed with ethyl acetate and acetic acid and water (3:2:2, v/v). Sugars were detected with *N*-(1-naphthyl)ethylendiamine dihydrochloride (Bounias 1980) or aniline phthalate reagent (Hough et al 1950) on silica gel or cellulose plates, respectively.

¹ Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic.

² Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, USDA, Peoria, IL 61604. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

³ Current address: Wyatt Technology Corp., Santa Barbara, CA 93117.

⁴ Corresponding author. Phone: 309-681-6377. Fax: 309-681-6427. E-mail: leathetd@ncaur.usda.gov.

⁵ Office of International Programs, Agricultural Research Service, USDA, Beltsville, MD 20705.

Endo- β -1,4-xylanase activity was estimated viscosimetrically using a 1.0% solution of carboxymethylxylan in 0.05M acetate buffer, pH 5.5 (Gorbacheva and Rodionova 1977). Carboxymethylxylan (DS 0.5) was prepared by M. S. Dudkin, Odessa Institute of Technology, Odessa, Ukraine, and supplied by I. V. Gorbacheva, Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia. Changes in specific fluidity ($1/\eta = t_0/t_t - t_0$, where t_0 is the flow rate of the buffer without the substrate, and t_t is the flow rate of the substrate buffer solution after time t) were followed over time using a semimicro viscometer, 1 mL capacity (Cannon Instrument Co., State College, PA). Endoxylanase activity was also tested on the chromogenic substrate, Remazol Brilliant Blue dyed xylan (RBB-xylan), as previously described (Biely et al 1985).

β -Glucosidase, β -xylosidase, and α -L-arabinofuranosidase were assayed on 4-nitrophenyl β -D-glucopyranoside, 4-nitrophenyl β -D-xylopyranoside, and 4-nitrophenyl α -L-arabinofuranoside, respectively (Sigma). Extracts were incubated at 30°C with substrates (2 mM) in 0.05M sodium acetate buffer at pH 5.5. Reactions were terminated and developed by the addition of two volumes of saturated sodium tetraborate solution and read at 400 nm. α -Glucuronidase was assayed on 4-nitrophenyl 2-(4-O-methyl- α -D-glucopyranuronosyl)- β -D-xylopyranoside in a β -xylosidase-coupled assay (Biely et al 2000). One unit of activity was defined as the amount of enzyme liberating 1 μ mol of 4-nitrophenol/min.

Protein was determined using bovine serum albumin as standard (BCA Protein Reagent, Pierce Chemical Co., Rockford, IL).

Isoelectric Focusing and Detection of Activities in Gels

Isoelectric focusing (IEF) gels (Novex, pH 3–7 and 3–10) and electrophoresis apparatus (Novex XCell II Mini-Cell) were from Invitrogen Corp., Carlsbad, CA. Following electrophoresis, gels were washed twice for 10 min in 0.2M sodium acetate buffer, pH 5.5, and immediately used for enzyme activity determinations. RBB-xylan overlay gels were utilized for the detection of endo- β -1,4-xylanase as previously described (Biely et al 1985). Endo-

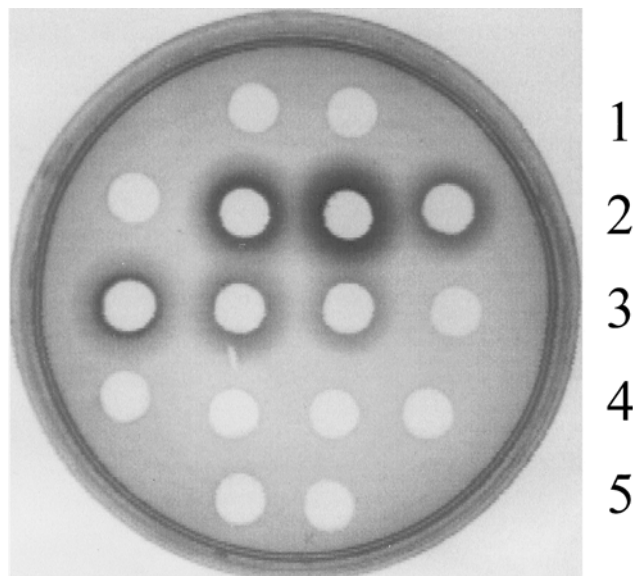


Fig. 1. Reaction of Remazol Brilliant Blue dyed Xylan (RBB-xylan) with agarose catalyzed by maize extracts. Extracts were incubated for 3 hr in wells cut into agarose gels containing 0.1% RBB-xylan. Gels were then washed overnight with water. Row 2 (left to right): extraction buffer; two-day kernel extract; four-day kernel extract; four-day shoot extract. Row 3 (left to right): six-day shoot extract; four-day root extract; six-day root extract; heat-denatured two-day kernel extract. Row 4: heat-denatured shoot and root extracts. Extracts appeared to catalyze the formation of dark halos or rings around the wells, suggesting that the substrate was cross-linked or bound to the agarose. Heat-treated extracts did not produce this effect.

xylanase activity was also tested using the fluorogenic substrates 4-methylumbelliferyl β -D-xylobioside (Umb-Xyl₂) and 4-methylumbelliferyl β -D-xylotrioside (Umb-Xyl₃), synthesized as previously described (Biely et al 1992). Fluorogenic substrates for the detection of β -xylosidase, β -glucosidase, endo- β -glucanase, and α -L-arabinofuranosidase were 4-methylumbelliferyl β -D-xyloside (Umb-Xyl), 4-methylumbelliferyl β -D-glucoside (Umb-Glc), 4-methylumbelliferyl β -D-cellobioside (Umb-Cel), and 4-methylumbelliferyl α -L-arabinofuranoside (Umb-Araf) (Sigma). Gels were treated with 2 mM (or saturated) aqueous solutions of fluorogenic substrates, and the liberation of 4-methylumbelliferone was followed under UV light. Total proteins were stained with Coomassie Brilliant Blue R.

RESULTS AND DISCUSSION

Enzyme Activities

Soluble protein extracts were prepared as described from whole germinating seeds and isolated organs of two maize hybrids. Extracts were assayed for total xylanolytic activity using natural and dyed xylans as substrates. Xylanase assays based on the production of reducing sugars gave slight positive reactions. Shoot and root extracts liberated free xylose and arabinose from xylans. However, TLC analyses were unable to detect xylooligosaccharides in the hydrolysates. The production of oligosaccharides is often characteristic of the action of endo- β -1,4-xylanases. Viscosimetric assays, chosen as one of the most sensitive endoglycanase assays, also failed to confirm the presence of endo- β -1,4-xylanase activity. Shoot and root extracts of Golden Harvest hybrid GH2574 promoted no apparent change in the viscosity of carboxymethylxylan solutions during extended incubations at 30°C. This finding was rather unexpected because endoxylanases previously were described in germinating wheat and barley (Preece and MacDougall 1958; Fincher and Stone 1974; Schmitz et al 1974; Taiz and Honigman 1976; Dashek and Crispeels 1977; Slade et al 1989; Banik et al 1997).

RBB-xylan is a sensitive and specific substrate for the detection of endoxylanases (Biely et al 1985). However, a remarkable and unexpected activity in maize extracts rendered this substrate useless. When extracts were incubated in wells cut into an agarose gel containing RBB-xylan, no zones of clearing were observed. On the contrary, the extracts appeared to catalyze the cross-linking of this substrate or its attachment to the agarose gel, producing darkened halos (Fig. 1). Shoot and root extracts were active in promoting this phenomenon, and heat-treated extracts had no effect. Halos were enhanced by destaining of the gels and could not be removed by incubation with microbial xylanases, which indicates that maize extracts contain enzymes that catalyze coupling of the xylan-dye conjugate to agarose through the dye molecule.

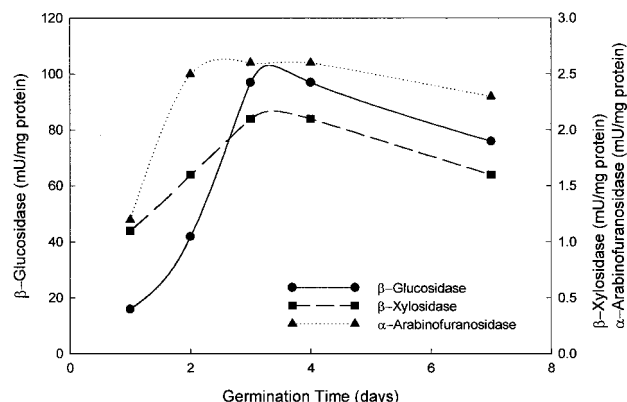


Fig. 2. Time course of β -glucosidase, β -xylosidase, and α -L-arabinofuranosidase specific activities in whole maize seedlings of hybrid WW64A x A619 during germination.

TABLE I
Specific Activities of Aryl-glycosidases in Extracts of Germinating Maize

Hybrid and Sample	Days	β -Glucosidase (mU/mg)	β -Xylosidase (mU/mg)	α -L-Arabinofuranosidase (mU/mg)
WW64A \times A619				
Kernels	3	120	1.3	1.5
Shoots	3	210	2.4	8.4
Roots (primary)	3	420	5.0	4.6
Roots (secondary)	3	320	3.9	4.0
Shoots	4	270	1.5	12
Roots	4	620	5.2	13
GH2574				
Kernels	2	48	1.1	1.4
Shoots	2	280	3.3	4.4
Kernels	4	43	0.6	3.2
Shoots	4	410	3.5	4.8
Roots	4	420	3.5	2.9
Kernels	6	35	0.7	1.4
Shoots	6	50	1.5	4.1
Roots	6	210	0.7	1.4

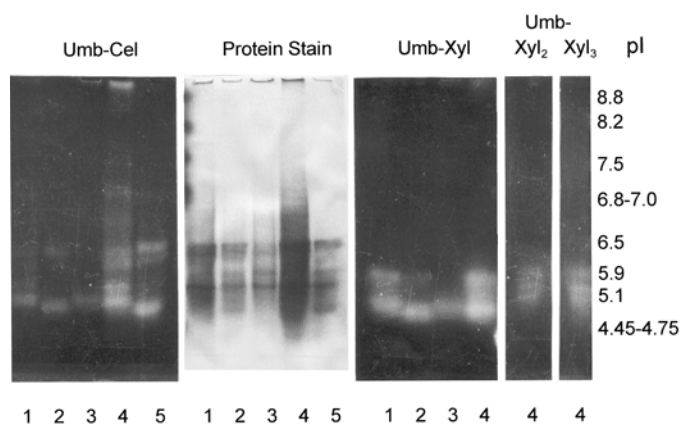


Fig. 3. Broad range (pH 3–10) isoelectric focusing gels of proteins from four-day-old maize seedlings. Lanes 1 and 3, shoot extracts of hybrid WW64A \times A619; lane 2, root extract of WW64A \times A619; lane 3, shoot extract of hybrid GH2574; lane 4, shoot extract of hybrid GH2574; lane 5, root extract of GH2574. Replica gels were stained with Brilliant Blue R to reveal total proteins, or incubated with synthetic fluorogenic substrates to reveal enzymatic activities. 4-Methylumbelliferyl β -D-cellobioside (Umb-Cel); 4-methylumbelliferyl β -D-xyloside (Umb-Xyl); 4-methylumbelliferyl β -D-xylobioside (Umb-Xyl₂); 4-methylumbelliferyl β -D-xylotrioside (Umb-Xyl₃).

Separate experiments with the free dye (Remazol Brilliant Blue R) also gave positive results, although the binding was less pronounced. This interesting phenomenon may have practical applications and deserves further study.

Extracts were subsequently assayed for specific levels of aryl-glycosidases using synthetic substrates as described above. As shown in Fig. 2, the specific activities of β -glucosidase, β -xylosidase, and α -L-arabinofuranosidase from whole seedlings were maximal at about three to four days after germination. Table I shows the specific activities of these enzymes in dissected kernels, shoots, and roots of two maize hybrids. Generally, the highest specific activities were found in homogenates of shoots and roots after three to four days of germination. Enzyme levels were similar in both maize hybrids tested. Notably, maximal β -glucosidase levels were approximately two orders of magnitude higher than those of β -xylosidase and α -L-arabinofuranosidase. No evidence of α -glucuronidase activity was found in selected shoot and root samples using the β -xylosidase-coupled assay.

Isoelectric Focusing of Enzymes

Protein extracts from shoots and roots of the two maize hybrids tested were separated on broad-range (pH 3–10) IEF gels. As revealed by Brilliant Blue R staining, the majority of proteins in

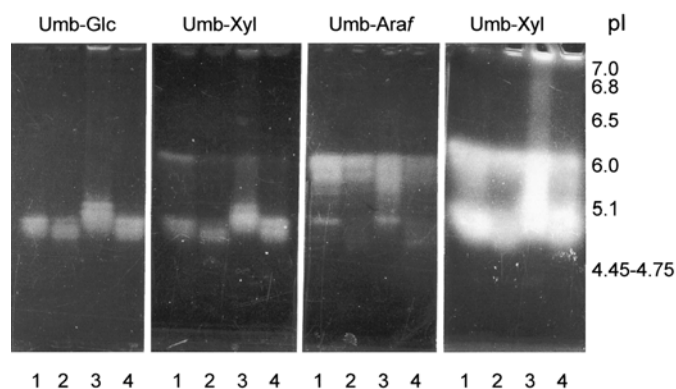


Fig. 4. Acidic range (pH 4–7) isoelectric focusing gels of proteins from four-day-old maize seedlings. Lane 1, shoot extract of hybrid WW64A \times A619; lane 2, root extract of WW64A \times A619; lane 3, shoot extract of hybrid GH2574; lane 4, root extract of GH2574. Replica gels were incubated with synthetic fluorogenic substrates to reveal enzymatic activities. 4-Methylumbelliferyl β -D-glucoside (Umb-Glc); 4-methylumbelliferyl β -D-xyloside (Umb-Xyl); 4-methylumbelliferyl α -L-arabinofuranoside (Umb-Araf). Panel far right illustrates extended incubation of the Umb-Xyl replica.

maize extracts appeared at pI 4.8–6.5 (Fig. 3). Replica gels were incubated with 4-methylumbelliferyl β -D-xylobioside (Umb-Xyl₂) or 4-methylumbelliferyl β -D-xylotrioside (Umb-Xyl₃) for detection of endo- β -1,4-xylanase activities. These substrates produced only faint bands of apparent activity at pI 5.0–6.0, not clearly different from the more pronounced β -xylosidase activities detected using 4-methylumbelliferyl β -D-xyloside (Umb-Xyl). Because 4-methylumbelliferone can be liberated from Umb-Xyl₂ and Umb-Xyl₃ by the double and triple action of β -xylosidase, this result was inconclusive for the presence of endo- β -1,4-xylanase. Presumptive endo- β -1,4-glucanases were also detected in this pI range using 4-methylumbelliferyl β -D-cellobioside (Umb-Cel), and it is possible that Umb-Xyl₂ and Umb-Xyl₃ were attacked by these enzymes. These endoglucanases are apparently different from those previously described from maize coleoptile cell walls with values of pI 7.3 and 7.8 (Inouhe et al 1999). No presumptive endoxyylanase activity was observed in the alkaline pH region. An alkaline form of the enzyme might be expected based on the predicted value (pI 9.45) of maize pollen endoxyylanase (Bih et al 1999).

Glycosidase activities were characterized further using acidic range (pI 3–7) IEF gels. Replica gels were incubated with 4-methylumbelliferyl β -D-glucoside (Umb-Glc) and 4-methylumbelliferyl α -L-arabinofuranoside (Umb-Araf) to detect β -glucosidase and α -L-arabinofuranosidase activities, respectively. Because β -

glucosidase activities were far greater than those of other glycosidases (Table I), only one-tenth as much protein was loaded onto gels stained with Umb-Glc. As shown in Fig. 4, β -glucosidase activity appeared in multiple protein species clustered around pI 4.8–4.9. Interestingly, samples from shoots of hybrid GH2574 showed a slightly different pattern. Although exoglucanases may exhibit β -glucosidase activity, these enzymes are apparently distinct from the exoglucanase previously purified from maize coleoptile cell walls with a value of pI 7.2 (Inouhe et al 1999).

Gels incubated with Umb-Xyl to reveal α -xylosidase activities showed protein patterns very similar to those stained with Umb-Glc (Fig. 4). This result suggests that most of the β -xylosidase activity in maize extracts is associated with β -glucosidases. On extended incubation with Umb-Xyl, a secondary cluster of protein species became evident at pI 5.8–6.0. These proteins corresponded to a similar group in gels stained with Umb-Araf. Thus, secondary β -xylosidase activity may be associated with a cluster of α -L-arabinofuranosidase species. Additional forms of arabinosidase appeared near pI 4.9 (Fig. 4).

CONCLUSIONS

Maize seedlings were examined for the production of xylanolytic enzymes during germination. Endo- β -1,4-xylanase activity was not confirmed in seedlings under the conditions tested. However, β -xylosidase and α -L-arabinofuranosidase activities were detected that may participate in the degradation of maize arabinoxylan. β -Xylosidase activity did not appear to reside in a distinct enzyme form but instead was associated with a highly active β -glucosidase and, to a lesser extent, with a predominant form of α -L-arabinofuranosidase.

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