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Endo-β-1,4-xylanase inhibitors in leaves and roots of germinated maize ☆

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Abstract

Extracts of both leaves and roots of germinated maize were found to contain endo–β–1,4–xylanase inhibitors, previously reported only from whole maize meal. The inhibitors seem to be of the xylanase inhibitor protein (XIP) type, since they inhibit endoxylanases of families 10 and 11 and also show some other characteristics similar to XIP inhibitors described in other cereals. Inhibitors from leaves and roots appeared to be similar. A novel property of the inhibitors described in this work is their unusual thermostability. The half-life of inhibitors at pH 4.5 and 100 °C is greater than 10 h. However, the inhibitors are less thermostable at higher pH levels. Because they did not inhibit a plant endoxylanase, the inhibitors may play a role in maize defense against phytopathogens.

Keywords: Germination; Maize; Xylan; Xylanase Inhibitor; *Zea mays*

1. Introduction

Our study of hemicellulolytic enzymes in germinated maize detected several aryl-glycosidase activities that may be involved in hydrolysis of highly branched maize arabinoglucuronoxylans (Biely et al., 2003). However, no evidence was reported for a similar mobilization of endo-β-1,4-xylanase (EC 3.2.1.8). This is in contrast to the situation in germinated barley, in which endoxylanases are expressed in the aleurone layer to hydrolyze the endosperm walls (Fincher, 1989). We were unable to detect endoxylanase in germinated maize leaf and root extracts using a very sensitive viscosimetric assay with carboxymethylxylan as substrate (Biely et al., 2003). The first and the only endoxylanase thus far described in maize is the predominant protein on the surface of pollen (Bih et al., 1999). This organ-specific endoxylanase may play a specific role in the penetration of the stigma surface. However, when our xylanase-negative maize extracts were supplemented with active microbial endoxylanases as positive controls, they still exhibited no activity in viscosimetric assays. This clearly suggests that the maize extracts contain endoxylanase inhibitors. Three general types of proteinaceous endoxylanase inhibitors have been described to date from various cereals, XIP-type, TAXI-type, and TL-XI (Beaugrand et al., 2006; Goesaert et al., 2004; Juge and Delcour, 2006; Juge and Svensson, 2006). Thus far maize is reported to contain only the xylanase inhibitor protein I type of endoxylanase inhibitor (XIP-I), and only at very low concentrations compared with other cereals such as wheat and rye (Goesaert et al., 2004). In these previously

Abbreviations: CM, carboxymethyl; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IEF, isoelectric focusing; RBB-xylan, 4-O-methyl-D-glucurono-D-xylan-remazol brilliant blue; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TAXI, *Triticum aestivum* xylanase inhibitor; TL-XI, thaumatin-like xylanase inhibitor; XIP, xylanase inhibitor protein.

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published studies, the XIP-I was isolated from whole maize meal by affinity chromatography on a column with an immobilized microbial endoxylanase (Elliott et al., 2003; Goesaert et al., 2003). No information is available on the presence of endoxylanase inhibitors in other parts of the maize plant. Here we report on the occurrence of endoxylanase inhibiting proteins in leaves and roots of germinated maize.

2. Experimental

2.1. Plant materials, germination, and homogenization

Maize (*Zea mays* L.), hybrid Golden Harvest GH2574, was obtained courtesy of P. Dowd of the Agricultural Research Service, USDA, Peoria, IL. Before germination the kernels were surface sterilized in 70% ethanol (v/v) and then 5% (v/v) household bleach solution as previously described (Biely et al., 2003). Kernels were germinated in the dark between layers of sterile moist sheets at 28 °C for 6 days, also as previously described (Biely et al., 2003). Separated leaves and roots were homogenized in 0.05 M sodium acetate buffer (pH 5.5) containing 5 mM NaN_3 and 10 mM EDTA in the presence or absence of 2 mM dithiothreitol. The homogenates were clarified by centrifugation and desalted and concentrated ≈10-fold by ultrafiltration (Centricon YM-10, 10 kDa cutoff, Millipore Corp., Bedford, MA) as previously described (Biely et al., 2003).

2.2. Purified endoxylanases

Seven purified endoxylanases belonging to two glycoside hydrolase families (http://afmb.cnrs-mrs.fr/CAZY) were used. Endoxylanases of family 10 were products of *Aspergillus oryzae* (provided by M. Tenkanen, University of Helsinki, Finland), *Streptomyces lividans* (XlnA, supplied by D. Kluepfel, Institute Armand Frappier, University of Quebec, Laval, Canada), and *Thermoascus aurantiacus* (supplied by P. Christakopoulos, Technical University of Athens, Greece). Barley endoxylanase (Van Campenhout et al., 2007), also belonging to glycoside hydrolase family10, was a generous gift of K. Gebruers and J.A. Delcour (Katholic University Leuven, Belgium). Endoxylanases of family 11 were products of *Trichoderma reesei* (alkaline endoxylanase of pI 9.0, supplied by M. Tenkanen), *Schizophyllum commune* (XlnA, supplied by M. Yaguchi, NRCC, Ottawa, Canada), and *Thermomyces lanuginosus* (supplied by M. Bhat, Food Research Institute, Norwich, UK). All enzymes were electrophoretically homogeneous. Endoxylanase activity was assayed on beech wood xylan by determination of reducing sugars using the Somogyi-Nelson method (Biely, 2003). One unit of endoxylanase activity is defined as the amount of enzyme liberating reducing sugars equivalent to 1 μmol of xylose in 1 min at 30 °C.

2.3. Assays of endoxylanase inhibitors

Endoxylanase inhibition was measured by three methods. The effect of inhibitors could be visualized in a sensitive viscosimetric endoxylanase assay as previously described (Biely et al., 2003). In this assay the increase in specific fluidity ($1/\eta = t_0/t_0–t_t$, where t_0 is the flow time of the substrate solution at zero reaction time, and t_t the flow time of the solution after time t) of a 1% solution of carboxymethylxylan caused by an appropriate amount of endoxylanase was measured in the absence and presence of maize extracts or proteins in an Oswald-type capillary semimicro viscometer, 1 ml capacity, at 30 °C (Cannon Instrument Co., State College, PA). The slope of $1/\eta$ versus time is directly proportional to the enzyme activity.

A convenient qualitative assay for the presence of inhibitors in various protein samples and chromatographic fractions used a cup-plate method with 2% agar containing 0.05 M sodium acetate buffer (pH 5.5) and 0.5 mM 4-methylumbelliferyl β-D-xylobioside, the fluorogenic substrate of endoxylanases (Biely et al., 1992). Each well was supplied with 10 μl of a test sample. The room temperature reaction was initiated by the addition of 10 μl of solution containing 0.5 mU of purified *A. oryzae* endoxylanase. Substrate and enzyme controls were run in parallel. Endoxylanase inhibitors partially or completely abolished the formation of the fluorescence aglycon as visualized by illumination in ultraviolet light at 366 nm.

A quantitative assay for endoxylanase inhibitors utilized soluble RBB-xylan as a substrate (Biely et al., 1985). A 0.2% solution of the substrate containing 12% dye in 0.05 M sodium acetate buffer, pH 5.5 (0.5 ml) was mixed with test samples (40 μl) and the reaction started with the addition of 10 mU of *A. oryzae* endoxylanase in 10 μl. After 30 min at 30 °C the reaction was terminated by the addition of two volumes of ethanol. After 10 min at room temperature the mixture was centrifuged and the absorbance of the supernatant was measured at 595 nm. One unit of endoxylanase inhibitor is defined as the amount of protein blocking 1 U of endoxylanase activity as defined above.

Inhibition of endoxylanases was also evaluated using 4-nitrophenyl β-1,4-D-xylobioside. Family 10 endoxylanases, including the barley enzyme, were assayed in a final volume of 0.11 ml containing 2 mM substrate in 0.05 M sodium phosphate buffer, pH 5.0, terminated by the addition of 0.9 ml of saturated sodium tetraborate solution. The effect of inhibitors on family 11 endoxylanases was tested under the same conditions using 10 mM 4-nitrophenyl β-1,4-D-xylobioside as substrate. Released 4-nitrophenol was determined at 405 nm (Puchart and Biely, 2007).

2.4. Partial purification of endoxylanase inhibitors from maize leaves and roots

Desalted and concentrated leaf or root extracts were fractionated by cation exchange chromatography on