Existence of Chitinase Activity in Mature Corn Kernels (*Zea mays* L.)

Joseph N. Neucere,* Thomas E. Cleveland, and Charles Dischinger

Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70124

Reducing infection by *Aspergillus flavus* group species that produce aflatoxins in corn is an area of intense interest. One speculation is that chitinase could be a resistance factor involved in inhibiting or reducing fungal growth. Mature kernels of yellow corn (*A. flavus* resistant) and white corn (*A. flavus* susceptible) were assayed for chitinase activity. Results showed higher activity in germ tissue than in the endosperm with disparity between the two varieties. The highest activity per quantity of protein was observed in the germ of white corn and in salt fractions from whole kernel corn precipitated with 20% ammonium sulfate. From gel permeation chromatography, major chitinase activity occurred in the fractions with molecular size ranging from 14 to 25 kDa. Isoelectric focusing on acrylamide gels showed major chitinase activity in protein zones at pH 3-4. These data provide evidence that the catalytic components exist in several isozyme forms that are acidic and highly associated in the native state.

Several studies have shown that seeds and vegetative tissues of higher plants contain chitinases (Grassman et al., 1934; Powning and Irzykliewicz, 1965; Wargo, 1975; Boller, 1985; Broekaert et al., 1988). Studies with various model systems have shown that chitinases are also induced by pathogen attacks and chemical treatments that impart stress on plants (Boller et al., 1983; Metraux and Boller, 1986). All of these studies provide circumstantial evidence that chitinases exist in different forms in plants and may be somehow involved in host defense against fungal pathogens (Payne et al., 1990). Our interest in chitinases in corn arises from the critical problem of aflatoxin contamination caused by the fungi *Aspergillus flavus* and *A. parasiticus*. The complex relationship between fungal infection and aflatoxin formation is an area of intense research worldwide. Currently, one of the objectives in solving the problem is the identification of resistance genes that could be transferred within the species through classical breeding or genetic engineering. Hence, if intrinsic chitinase is indeed a pertinent resistance factor in corn, then appropriate strategies could be coordinated to improve the health and safety of this major crop.

In this preliminary study, our objective was to develop a similarity profile of chitinase activity in the endosperm and the germ of two varieties of mature corn kernels. We chose kernels from open-pollinated varieties with extremes in aflatoxin contamination due to *A. flavus* infection, namely Yellow Creole (highly resistant) and Huffman (highly susceptible white endosperm variety) in field studies reported earlier (Zuber et al., 1983). Certain protein fractions from these two varieties were shown to have antifungal properties in an earlier study (Neucere and Zeringue, 1987).

MATERIALS AND METHODS

Extraction of Corn Tissues and Protein Fractionation. For preliminary experiments, kernels of the two varieties were sectioned by cutting the lower third of whole kernels, which included most of the germ and the root cap, away from the endosperm. Two hundred milligram samples of each tissue (endosperm and germ) were homogenized with a mortar and pestle by using 2.0 mL of 0.6 M citric acid-1.2 M Na_2HPO_4 buffer, pH 5.1. The homogenates were centrifuged at 12000*g* for 30 min at 10 °C. Isolated supernatants were dialyzed against the same buffer overnight and stored at 5 °C prior to experimentation. For preparative fractionation of protein, 50-g kernel samples were blended with a Tekmar homogenizer using 500 mL of the citric acid-phosphate buffer followed by centrifugation. A 300-mL volume of supernatant was treated with ammonium sulfate in quantities to yield 0-20, 20-40, 40-60, and 60-90% saturation at 25 °C. Each fraction was obtained by centrifugation at 12000*g* for 30 min at 10 °C. The precipitates were dissolved in deionized water, dialyzed exhaustively against deionized water for 3 days at 5 °C, and freeze-dried. A 50-mL volume of the original extract was also dialyzed and freeze-dried for use as a control.

HPLC Conditions. The HPLC system from ISCO (Lincoln, NE) consisted of the V^4 absorbance detector and Model 2350 pump. Data acquisition and analysis, as well as system control, were accomplished by using ISCO's Chemresearch software with an IBM System 2, Model 30, computer (Engel et al., 1989). For gel permeation, a GPC-100 Synchropak (Lafayette, IN) column (250 × 4.6 mm) was used. The column was loaded with 1500 μg of protein from the 0-20 fraction. Isocratic elution was done with 10 mM sodium phosphate, pH 7.0, containing 100 mM sodium chloride at an absorbance of 280 nM and a 1 mL min^{-1} flow rate. Fractions were collected every 15 s for subsequent chitinase assay.

Nonequilibrium Isoelectric Focusing (IEF). The solution for casting vertical polyacrylamide gels for IEF consisted of 5% acrylamide (w/v), 0.5% *N,N'*-methylenebis(acrylamide) (w/v), 80 μL of 10% (w/v) ammonium persulfate, 50 μL (v/v) of *N,N'*-tetramethylenediamine, and 1.5 mL of ampholytes (1.25 mL, pH 3-10; 0.25 mL, pH 2.5-5) (all from Sigma) per 30 mL of total volume (Cleveland and McCormick, 1987). Two slab gels (14 × 14 × 0.08 cm) were cast by pouring the solution between two vertical glass plates clamped on a vertical electrophoresis apparatus and allowing polymerization to occur for 1 h. For each slab, 500 μg of protein from the 0-20 fraction was layered at the anode (top) side of the apparatus along the entire width of the gel. Upper and lower reservoir solutions contained 10 mM H_3PO_4 and 20 mM NaOH, respectively. IEF was performed at 4 °C for 3 min at 200 V and for 3 h at 400 V. The gels were sliced at 2-cm invervals, extracted with 3.0 mL of citrate-phosphate buffer, pH 5.1, and tested for chitinase activity.

Chitinase Assay. Chitinase activity was determined according to the method of Hackman and Goldberg (1964), using Chitin Azure (Sigma) as substrate.

Protein Determinations. Protein content was performed according to the Lowry et al. (1951) procedure using BSA (Sigma) as standard.

RESULTS AND DISCUSSION

The results in Table I show the differences in chitinase activity detected in the germ and the endosperm of the

Table I. Measurement of Chitinase Activity in the Germ (G) and Endosperm (E) of Two Varieties of Corn

variety	protein content, mg/mL of crude extract	chitinase act.,[a] units/mL of crude extract	chitinase sp act.
Yellow Creole (G)	4.2	2.50	0.60
Yellow Creole (E)	2.8	0.10	0.04
Huffman (G)	4.4	5.30	1.22
Huffman (E)	1.6	0.50	0.31

[a] One unit of chitinase activity liberates 1 mg of *N*-acetyl-D-glucosamine from chitin in 48 h, pH 5.1, 37 °C. Colorimetric procedure using Chitin Azure as substrate.

Table II. Chitinase Activity in Ammonium Sulfate Fractions Obtained from Extracts of Whole Kernels of Yellow Creole

fraction[a]	protein content, % of dry wt	chitinase act.,[b] units/mg of dry wt	chitinase sp act.
control (35)	42	2.76	6.57
0–20 (43)	49	14.29	30.41
20–40 (98)	62	4.39	7.08
40–60 (35)	53	2.76	5.21
60–90 (23)	14	0.0	0.0

[a] Numbers in parentheses refer to yields in milligrams of gross dry weight in each isolated fraction following dialysis and freeze drying. [b] Chitin Azure procedure.

two varieties of corn and the protein content in each extract. The highest activity was found in the Huffman germ, which was twice as high as that found in the Yellow Creole germ. For both varieties, the content of protein per unit weight is higher in the germ than in the endosperm.

For partial fractionation of chitinase, whole kernels of Yellow Creole were extracted with a citrate–phosphate buffer from which fractions were obtained by ammonium sulfate precipitation (Table II). The major activity was in the 0–20 (i.e., 0–20% ammonium sulfate saturation) fraction and represented a 5-fold purification over the original extract (total). The other fractions, at increasing percentages of saturation, contained decreasing amounts of chitinase activity with no activity in the highest (60–90%) saturation.

Results of fractionation of the 0–20 fraction by gel permeation chromatography to estimate molecular weights of active components is shown in Figure 1. Major chitinase activity (dashed curve) occurred in the 14–25 kilodalton (kDa) fractions. A minor peak of activity was also observed around 43 kDa. Presumably, the enzyme system exists as a series of isozymes of lower molecular weight.

Estimation of isoelectric points of the enzyme system was made by nonequilibrium IEF on polyacrylamide gels (data not shown). A number of diffused bands were observed within a pH gradient ranging from 3 to 10. Two preparative gels were run, cut into six horizontal slices 2 cm wide, extracted with citrate–phosphate buffer, pH 5.1, and tested for chitinase activity. Only the fraction at 2 cm from the top of the gels (anode) showed measurable activity. Therefore, the isoelectric points of the enzymes are in the pH range 3–4. The activity corresponded to 1 enzyme unit/mL of extract recovered from the gels.

An earlier in vitro study on antifungal properties of water/salt-soluble proteins in these two varieties of corn showed the complexity of the system (Neucere and Zeringue, 1987). Direct correlation between inhibition of fungal growth and lectin activity in one of the salt-eluted fractions was observed only in Yellow Creole, the yellow corn (resistant) variety. The acidic extracts (germ, endosperm, and salt-precipitated fractions) from neither variety in the current study showed inhibition of fungal

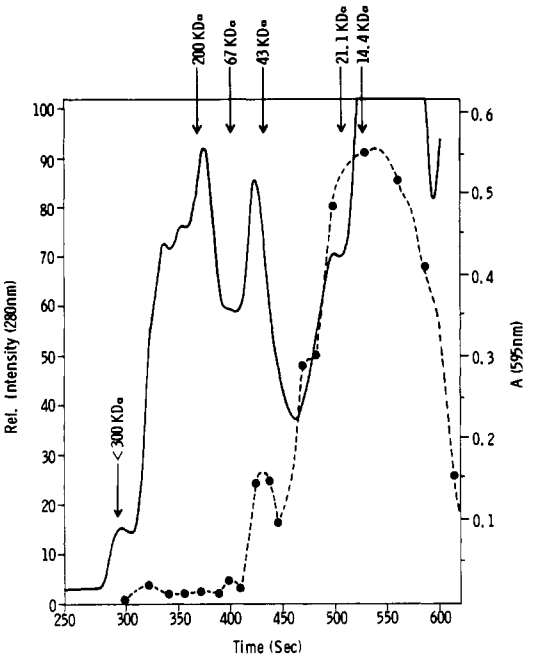

Figure 1. Gel permeation profile and chitinase assay of the 0–20 ammonium sulfate fraction of corn kernels. Solid curve, protein measurement; dashed curve, chitinase activity. Molecular weight markers: 14 400, lysozyme; 21 100, soybean trypsin inhibitor; 43 000, ovalbumin; 67 000, BSA; 200 000, β-amylase; 300 000, DNA. Fifty microliters at 30 μg/μL of protein was applied to the column and monitored for 10 min.

growth (preliminary experiments on solid agar plates) when aliquots containing 0.1–1.0 mg of protein were used. It could be that extreme concentrations of the enzymes are required to adversely affect the germinating spores. Another possibility is that chitinases have no role in the resistance observed in Yellow Creole since no correlation was found between high chitinase levels and resistance in this variety. However, the reason for the preponderance of fungal infection and aflatoxin production in one maize variety over the other examined in this study is difficult to assess on the basis of intrinsic factors such as chitinase; susceptible varieties, for example, may have growth-enhancing factors (sugars and other nutrients) that overcome any inhibitors that are present in the kernels during the course of maturation.

Chitinases related to defense against fungal and bacterial pathogens also include types induced by elicitors following physical damage or pathogenic attack. A recent preliminary study (Mitchell and Gayner, 1989) showed that basic endochitinases exist in diverse germinated tissues of corn. The isozyme profiles showed molecular weight in the range of 35 000. Immunoblotting showed that the relative amounts of chitinase were low. To our knowledge, the current results are the first to show that a relatively high content of acidic chitinases exists in dormant corn kernels. Their possible role, however, in protecting corn from *A. flavus* infection and aflatoxin contamination and their specific interactions with cell walls of fungal spores remain to be determined in future studies. Results of our in vitro studies indicate that chitinase is not important in the potential mechanism imparting resistance under laboratory conditions.

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