Partial purification and N-terminal amino acid sequencing of a β-1,3-glucanase from sorghum leaves

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Abstract

A protein with an apparent molecular mass of 30 kDa that cross-reacts with barley glucanase antiserum was detected in healthy leaves of sorghum (Sorghum bicolor (L.) Moench). When sorghum leaves were infected with Exserohilum turcicum, the causal agent of leaf blight, the 30-kDa glucanase was substantially induced. The 30-kDa glucanase was partially purified from sorghum leaves using ammonium sulfate fractionation and anion exchange chromatography on DEAE-sephacel. The N-terminal amino acid sequence of the 30-kDa glucanase shows homology to glucanases of maize, barley, bean, soybean, tobacco and pea. The purified 30-kDa glucanase showed antifungal activity against Trichoderma viride.

Additional key words: pathogenesis-related protein, Sorghum bicolor, Trichoderma viride.

Introduction

When plants are infected by pathogens, a number of biochemical changes occur, including the accumulation of phenolics (Velazhahan and Vidhyasekaran 1994), phytoalexins (Kuc 1995), lignins (Ride 1975), pathogenesis-related (PR) proteins (Velazhahan et al. 1998, 2000) and hydroxyproline-rich glycoproteins (Bradley et al. 1992). These biochemical alterations result in strengthening of cell wall barriers against further infection or in direct killing or weakening of the invading pathogens. There is considerable evidence to suggest that the involvement of PR-proteins in plant defense (Datta and Muthukrishnan 1999). These PR-proteins have been classified into 14 families based on amino acid sequences, serological relationship, and/or enzymatic or biological activity (Van Loon and Van Strien 1999). The PR-proteins of family 2 are known as β-1,3-glucanases. These enzymes catalyze the hydrolysis of β-1,3-glucan which is a major component of the cell walls of many fungi (Wessels and Sietsma 1981). Kim and Hwang (1997) demonstrated that β-1,3-glucanase isolated from pepper stems caused lysis of germinating zoospores and inhibited hyphal growth of Phytophthora capsici in vitro. Hence, β-1,3-glucanases are thought to play a direct role in the defense of plants against pathogenic fungi. The significance of β-1,3-glucanases in plant defense is further highlighted by the demonstration that transgenic plants with elevated expression of β-1,3-glucanase are more resistant to pathogens. Lusso and Kuc (1996) demonstrated that constitutive expression of glucanase to enhance resistance of tobacco against Phytophthora parasitica var nicotianae and Peronospora tabacina. Similarly, Nakamura et al. (1999) reported that kiwifruits transformed with a soybean β-1,3-endo-glucanase gene showed elevated resistance to Botrytis cinerea. Although glucanases have been isolated and characterized from a number of dicotyledonous plants the information on glucanases of monocotyledonous plants are limited (Kragh et al. 1991, Akiyama et al. 1996, Kini et al. 2000). Therefore, we have initiated a study on glucanase of sorghum which is an agriculturally important monocot. Krishnaveni et al. (1999) previously reported that healthy plants of both susceptible and resistant
sorghum hybrids had trace amounts of a β-1,3-glucanase with an apparent molecular mass of 32.5 kDa. This glucanase was substantially induced by infection with *Fusarium moniliforme*, infestation by aphids and by mechanical wounding. In this paper, we report for the first time the purification and characterization of a constitutively expressing β-1,3-glucanase from sorghum leaves.

**Materials and methods**

**Plants:** The seeds of sorghum [*Sorghum bicolor* (L.) Moench] cv. C401 were obtained from the Department of Agronomy, Kansas State University, Manhattan, Kansas, USA. The seeds of sorghum cv. CO-26 were obtained from the Millets Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India. Plants were grown in pots under greenhouse conditions.

**Fungal culture:** The fungus *Trichoderma viride* was obtained from the Biological Control Laboratory, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India and maintained on potato dextrose agar (PDA) medium. The fungus, *Exserohilum turcicum* (Pass.) Leonard and Suggs was isolated from leaf blight-infected sorghum leaves and maintained on PDA medium.

**Induction of β-1,3-glucanase in sorghum in response to fungal infection:** Leaves of 4-week-old sorghum seedlings (cv. CO-26) were inoculated with a conidial suspension of *E. turcicum* at 1 × 10⁶ spores per cm² with 0.1 % *Tween* 20 as a wetting agent. The conidial suspension was applied as a fine mist with an atomizer. The plants were incubated in a humidity chamber at 22 °C (Tuleen and Frederiksen 1977). Leaf samples were collected 2 d after inoculation, proteins were extracted in 50 mM sodium phosphate buffer (pH 6.9) and analyzed by Western blotting using barley glucanase antiserum as follows.

**Western blotting:** Proteins (250 µg) were separated by 12 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to a PVDF membrane using a Bio-Rad semi dry transfer cell (Bio-Rad, Hercules, USA). The membrane was then blocked in Tris-buffered saline containing 0.05 % (v/v) *Tween*-20 supplemented with 2.5 % (m/v) gelatin. Antiserum raised against barley glucanase (a gift of Dr. M. Ballance, University of Manitoba, Winnipeg, MB) was used as a primary antibody at 1:1000 dilution. Detection of glucanase on the membrane was performed according to Winston et al. (1987) using a 1:1000 dilution of horseradish peroxidase conjugated goat-anti rabbit IgG (Bio-Rad). Color development was with 4-chloro-1-naphthol (Bio-Rad).

**Purification of β-1,3-glucanase:** Leaves collected from 45-d-old sorghum cv. C401 plants were used for purification of β-1,3-glucanase. Leaves (250 g) were homogenized with a prechilled pestle and mortar at 4 °C with 500 cm³ of 50 mM sodium phosphate buffer (pH 6.9). The homogenate was centrifuged at 10000 g for 20 min at 4 °C. To the supernatant solid ammonium sulfate was added to a final concentration of 60 % saturation at 4 °C. After incubation for overnight with gentle stirring, this solution was centrifuged at 10000 g for 20 min at 4 °C. The pellet was dissolved in 25 cm³ of 50 mM sodium phosphate buffer (pH 6.9). This solution was dialyzed against two changes of the same buffer and subjected to anion exchange chromatography. The sample was applied to DEAE-Sephacel column (Pharmacia, Uppsala, Sweden) equilibrated with 1 mM Tris, pH 7.5. The adsorbed proteins were eluted with a linear 0 - 0.5 M NaCl gradient in the same buffer. The fractions in 2 cm³ volumes were collected. Fractions containing protein were detected by monitoring at A₂₈₀ nm. Anion exchange chromatography resolved a peak at 150 mM NaCl. The peak fractions were analyzed by 12 % SDS-PAGE using a Mighty Small II unit (Hoefer, San Francisco, USA) according to the method of Laemmli (1970). A constant current of 20 mA per gel (0.75 mm x 8 cm x 7 cm) was applied. Gels were stained for protein with 0.25 % (m/v) Coomassie Brilliant blue R-250 in 40 % (v/v) methanol and 10 % (v/v) acetic acid and destained in 40 % methanol and 10 % acetic acid. Apparent molecular mass of proteins was determined by comparison with molecular mass standards (Rainbow markers, Amersham Pharmacia, California, USA). The Coomassie Protein Assay Reagent (Pierce, Rockford, USA) was used for protein quantitation with BSA as the standard.

**N-terminal sequencing:** Purified protein was subjected to 12 % SDS-PAGE and then the protein was electroblotted onto a PVDF (Bio-Rad) membrane using a Bio-Rad semi-dry transblot unit. The protein was subjected to automated Edman degradation using an Applied Biosystems sequencer at the Biotechnology Microchemical Core Facility, Kansas State University, Manhattan, Kansas, USA. Database search was performed with the BLAST 2.0 service from the National Centre for Biotechnology Information World Wide Web server.

**Fungal growth inhibition assay:** A spore suspension of *Trichoderma viride* (5 × 10⁶ conidia cm⁻³) was prepared in sterile distilled water and mixed with 20 cm³ of molten
Results and discussion

β-1,3-glucanases have been purified and characterized from a number of dicotyledonous plants (Simmons 1994). In the case of monocotyledons, β-1,3-glucanases have been reported in rice (Akiyama et al. 1996), barley (Ballance and Svendsen 1988, Kragh et al. 1991, Hrmova and Fincher 1993), wheat (Lai et al. 1993), rye (Ballance and Manners 1978) and pearl millet (Kini et al. 2000). In the present study, we observed induction of a glucanase with a molecular mass of 30 kDa in sorghum leaves in response to inoculation with *E. turcicum*, the leaf blight fungus. Protein extracts from leaves of uninoculated control plants also expressed low levels of 30-kDa glucanase (Fig. 1).

The constitutively expressing 30-kDa glucanase was purified from sorghum leaves using ammonium sulphate fractionation and anion exchange chromatography on DEAE-sephacel. When the peak fractions of anion exchange chromatography were subjected to SDS-PAGE and stained with Coomassie blue, a 30-kDa major polypeptide was found (Fig. 2). The N-terminal amino acid sequence of the 30-kDa sorghum glucanase showed 90 % sequence identity with glucanase of maize, 77 % identity with glucanase of barley, 63 % identity with a bean glucanase, 61 % identity with glucanases of tobacco and soybean and 54 % identity with a glucanase of pea (Table 1). However, the purified 30-kDa glucanase showed antifungal activity against *T. viride* only at 10 µg level (Fig. 3). β-1,3-glucanases are also implicated in diverse physiological and developmental processes in plants including cell division (Fulcher et al. 1976), microsporogenesis (Bucciaglia and Smith 1994), pollen germination and tube growth (Meikle et al. 1991), fertilization (Lotan et al. 1989, Ori et al. 1990), fruit ripening (Hinton and Pressey 1980) and seed germination.
Table 1. The N-terminal amino acid sequence of the 30-kDa glucanase purified from sorghum leaves and its alignment with other glucanases. The N-terminal amino acid sequence of sorghum glucanase was compared with the glucanase sequences of maize (GenBank accession No. P49237), barley (GenBank accession No. Q02438), bean (GenBank accession No. P23533), soybean (GenBank accession No. Q03773), tobacco (GenBank accession No. P23547) and pea (GenBank accession No. Q03467).

<table>
<thead>
<tr>
<th>Species</th>
<th>N-terminal amino acid sequence</th>
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<tbody>
<tr>
<td>Sorghum</td>
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</tr>
<tr>
<td>Maize</td>
<td>GVCGVNGDNLPSASDVVVQLYQ</td>
</tr>
<tr>
<td>Barley</td>
<td>GVCGVNGDNLPSRSDVVQLYK</td>
</tr>
<tr>
<td>Bean</td>
<td>GVCGVNGDNLPSANEVINDLYR</td>
</tr>
<tr>
<td>Soybean</td>
<td>GVCGVNGDNLPSANEDVIVALY</td>
</tr>
<tr>
<td>Tobacco</td>
<td>GCYGMNGNLPSDQDVILNY</td>
</tr>
<tr>
<td>Pea</td>
<td>GCYGMNGNLPSDQDVILNY</td>
</tr>
</tbody>
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Fig. 3. In vitro antifungal activity of the 30-kDa glucanase purified from sorghum leaves against Trichoderma viridae. Buffer (C) or 10 μg of glucanase purified from sorghum leaves (1 and 2) were applied to the sterile filter paper discs.

References


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