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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 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 agronomically important monocot. Krishnaveni *et al.* (1999) previously reported that healthy plants of both susceptible and resistant

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Abbreviation: PDA - potato dextrose agar.
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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 moniliformae*, 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 1×10^4 spores per cm^3 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 dodecylsulphate 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 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 cm^3 of 50 mM sodium phosphate buffer (pH 6.9). The homogenate was centrifuged at 10 000 *g* for 20 min at 4 °C. To the supernatant solid ammonium sulphate was added to a final concentration of 60 % saturation at 4 °C. After incubation for overnight with gentle stirring, this solution was centrifuged at 10 000 *g* for 20 min at 4 °C. The pellet was dissolved in 25 cm^3 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 cm^3 volumes were collected. Fractions containing protein were detected by monitoring at A_{280} 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 × 8 cm × 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* (5×10^3 conidia cm^{-3}) was prepared in sterile distilled water and mixed with 20 cm^3 of molten

potato dextrose agar (PDA) medium and poured onto the Petri dishes (90 mm in diameter). Sterile filter paper discs (6 mm in diameter) were laid on the agar surface at 2 cm away from periphery of the Petri dish and glucanase solutions equivalent to 2, 5 and 10 µg protein were applied to each disc. After the plates were incubated at room temperature (28 ± 2 °C) for 48 h, inhibition of *T. viride* growth was observed.

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

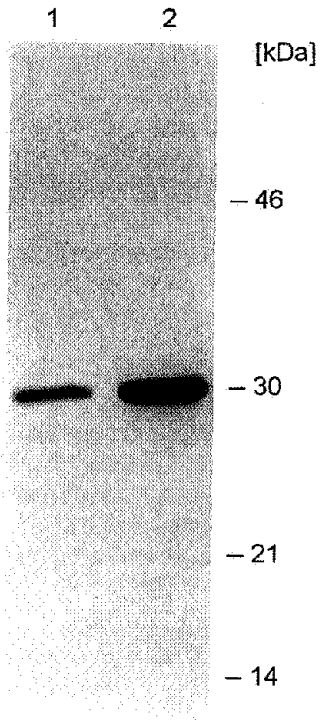

Fig. 1. Induction of β-1,3-glucanase in sorghum leaves in response to inoculation with *Exserohilum turcicum*. Aliquots of total proteins (250 µg) extracted from control (*lane 1*) and *E. turcicum*-inoculated sorghum leaves were analyzed by western blotting after SDS-PAGE using barley glucanase antiserum. Molecular mass markers are shown on the right.

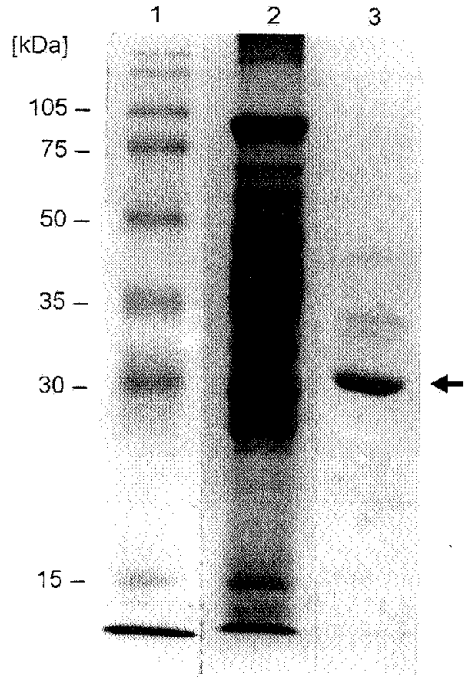

Fig. 2. SDS-PAGE analysis of β-1,3-glucanase purified from sorghum leaves. *Lane 1* - marker, *lane 2* - total proteins from sorghum leaves (200 µg), *lane* 3 - peak fraction of DEAE-Sephacel column (2 µg). The molecular mass of marker proteins is indicated on the left.

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. P23535), soybean (GenBank accession No. Q03773, tobacco (GenBank accession No. P23547) and pea (GenBank accession No. Q03467).

Species	N-terminal amino acid sequence																				
Sorghum	G	V	C	Y	G	V	N	G	D	N	L	P	S	A	S	D	V	V	K	L	Y	Q
Maize	G	V	C	Y	G	V	N	G	D	N	L	P	P	A	S	D	V	V	Q	L	Y	Q
Barley	G	V	C	Y	G	M	V	G	D	N	L	P	S	R	S	D	V	V	Q	L	Y	K
Bean	G	V	C	Y	G	M	M	G	N	N	L	P	S	A	N	E	V	I	N	L	Y	R
Soybean	G	V	C	Y	G	R	L	G	N	N	L	P	T	P	Q	E	V	V	A	L	Y	
Tobacco	G	V	C	Y	G	K	H	A	N	N	L	P	S	D	Q	D	V	I	N	L	Y	
Pea	G	I	C	Y	G	M	M	G	N	N	L	P	P	A	N	E	V	I	A	L	Y	K

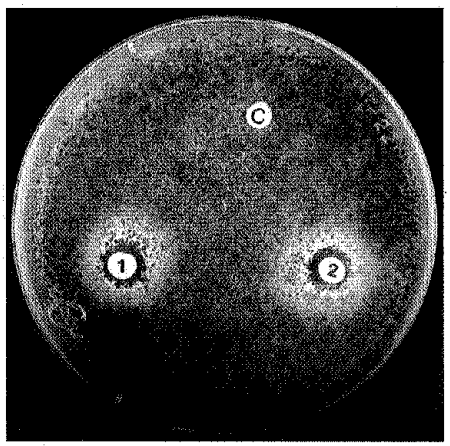

Fig. 3. *In vitro* antifungal activity of the 30-kDa glucanase purified from sorghum leaves against *Trichoderma viride*. Buffer (*C*) or 10 μg of glucanase purified from sorghum leaves (*1* and *2*) were applied to the sterile filter paper discs.

(Vogeli-Lange *et al.* 1994). It has been demonstrated that only class I vacuolar isoforms of tobacco glucanase were effective in inhibiting the growth of *Fusarium solani*. In contrast, the class II glucanases PR-2a, PR-2b and PR-2c did not exhibit antifungal activity (Sela-Buurlage *et al.* 1993). The purified 30-kDa glucanase from sorghum leaves showed low antifungal activity against *T. viride* even at a dose of 10 μg of protein per disk. This constitutively expressing glucanase may be involved in inducing resistance in plants by releasing elicitors from the cell walls of invading fungi as suggested by Mauch and Staehelin (1989). A β-1,3-glucanase from soybean has been found to release oligosaccharide fragments from fungal cell walls that elicit phytoalexin synthesis (Ham *et al.* 1991). It has been demonstrated that leaves of transgenic tobacco transformed with a soybean β-1,3-glucanase gene induced the transcription of a plant defense gene, phenylalanine ammonia-lyase, in response to fungal attack to a greater extent than untransformed leaves (Yoshikawa *et al.* 1993).

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