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In vitro evaluation of antifungal activity of soybean (*Glycine max*) seed coat proteins

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ABSTRACT

Proteins in the soybean seed coat have previously been characterized; however, the function of these proteins is unknown. We show that a soybean seed coat protein fraction was able to inhibit the growth of *Fusarium lateritium* and *Fusarium oxysporum* phytopathogenic fungi. The antifungal fraction isolated by DEAE-Sepharose chromatography revealed the presence of peroxidase, vicilin and a 24 kDa protein homologous to acid phosphatases. Germination experiments revealed that both acid phosphatase and peroxidase were exuded during seed imbibition. We suggest that the set of seed coat antifungal proteins may help protect seeds from colonization by phytopathogenic fungi.

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1. Introduction

The seed coat is an important tissue for the regulation of imbibition and maintenance of the integrity of the seed (Bewley and Black, 1994) and is also the first seed barrier encountered by pests and pathogens (Moise et al., 2005). However, this tissue has been exclusively considered as a physical barrier and the involvement of seed coat molecules in this defensive role has not been considered. Seed cotyledons contain an array of proteins that may be involved in the protection of quiescent seeds against fungi. A 2S albumin-homologous protein from passion fruit seeds inhibited growth of the phytopathogenic fungi *Fusarium oxysporum* Schltdl. and *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, and the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Agizzio et al., 2003). Chitinases and β-1, 3-glucanases from seeds of *Vigna unguiculata* (L.) Walp. seeds inhibited growth of *C. lindemuthianum* and *Colletotrichum musae* (Berk. & M.A. Curtis) Arx, (Gomes et al., 1996). Vicilins (7S storage proteins) from seeds of *V. unguiculata* were also able to inhibit fungal development (Gomes et al., 1997). Further reports have shown the presence of vicilin-like 7S storage proteins, normally thought to be expressed only in the embryo, in the seed coat of *Canavalia ensiformis* (L.) DC (Oliveira et al., 1999), *Phaseolus lunatus* L. (Moraes et al., 2000) and *Phaseolus vulgaris* L. (Silva et al., 2004). All these authors suggest that storage proteins play an important role in seed defense mechanisms against pests and pathogens. Soybean seed coat proteins have previously been identified, among these a 41 kDa peroxidase enzyme (Buttery and Buzzel, 1968; Gijzen, 1997), a 32 kDa class I chitinase (Gijzen et al., 2001) and a 21 kDa trypsin inhibitor (Kunitz, 1945; Koide and Ikenaka, 1973) and an 8 kDa hydrophobic protein (Gijzen et al., 1999). However, the functions of these proteins in this tissue are not known. The objective of our study is to investigate the presence of proteins with antifungal activity in the seed coat of *Glycine max* (L.) Merrill, which is a highly valuable edible crop throughout most of North and South America.

2. Materials and methods

2.1. *Glycine max seeds and preparation of fungal inoculum*

Soybean seeds were commercially acquired from local markets (Campos dos Goytacazes, RJ, Brazil) and stored at 4 °C prior to use. Fungal isolates utilized were *Fusarium lateritium* Nees and *F. oxysporum*, which were kindly supplied by CNPAF/EMBRAPA, Goiânia, Goiás, Brazil. The fungi were maintained on Sabouraud agar (1% peptone, 2% glucose and 1.7% agar–agar). For the preparation of *F. oxysporum* and *F. lateritium* conidia, the fungal cultures were transferred to Petri dishes containing Sabouraud agar, and allowed to grow for 12 days. After this period, 0.15 M NaCl solution (10 mL) was added to the dishes and these were

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gently agitated for 1 min for spore liberation with the help of a Drigalski loop. Spores were quantified in a Neubauer chamber for appropriate dilutions.

2.2. Isolation of the antifungal fraction

Seed coats were separated from cotyledons and ground to a fine powder; proteins were extracted (1:10 flour:buffer ratio) with 100 mM phosphate buffer with 500 mM NaCl, pH 7.6 for 3 h at 4 °C and centrifuged at 10,000 × *g* for 30 min. The supernatant obtained was treated with ammonium sulphate at 90% saturation for 24 h at 4 °C and centrifuged at 10,000 × *g* for 30 min at 4 °C. The precipitated fraction was dialysed against water for 24 h at 4 °C using dialysis tubing of molecular weight cut-off of 7 kDa. After dialysis, the precipitated fraction (ammonium sulphate precipitate fraction) and low molecular weight fraction (LMW) containing the molecules excluded during the dialysis were recovered by freeze-drying. The resulting ammonium sulphate precipitate fraction was fractionated using DEAE-Sepharose ion exchange chromatography. The sample was applied to a 14 × 2.5 cm column equilibrated with 50 mM potassium phosphate buffer, pH 7.6. After elution of the non-retained fraction (DI), adsorbed proteins were sequentially desorbed by 0.25 and 0.5 M NaCl (DII and DIII fractions, respectively).

2.3. Effects of seed coat fractions on fungal growth

To assay the effects of different chromatographic fractions (DI, DII and DIII) and LMW fraction on fungal growth, spores ($1 \times 10^4\,mL^{-1}$ in 0.15 M NaCl solution) were incubated at 28 °C in microplates containing 100 μL of Sabouraud broth (6 g/100 mL) in the presence of 100 μL of each chromatographic fraction (400 μg P/mL) or 100 μL of LMW fraction (10 mg/mL) in 0.1 M sodium acetate buffer, pH 5.0. Optical readings at 600 nm were taken at zero time and at 6 h intervals for the following 48 h (Broekaert et al., 1990). Cell growth without addition of fractions was also determined. Experiments were run in triplicate and values obtained were used to calculate averages and standard deviations. The results were analysed through Student's *t*-test and significant differences were determined at $P<0.05$ (Bridge and Sawilowsky, 1999).

2.4. Protein characterization

Protein concentrations in the seed coat fractions were determined by the Bradford method (Bradford, 1976). The protein profiles were analysed by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli (1970) and a 15% non-denaturing gel as described by Davis (1964). Electrophoresis was run at 15 mA and proteins (10 μg) were stained by 0.05% Coomassie Blue and destained in acetic acid (10%). DIII fraction proteins separated by SDS–PAGE were blotted on a PVDF membrane and the N-terminal amino acid sequence of a 24 kDa protein band was determined on a Shimadzu PPSQ-10 Automated Protein Sequencer performing the Edman degradation (Edman, 1950). PTH-amino acids were detected at 269 nm after separation on a reverse-phase C18 column under isocratic conditions.

2.5. Vicilin detection

DIII fraction proteins separated by SDS–PAGE were blotted on a nitrocellulose membrane and submitted to Western blotting detection (Towbin et al., 1979) using a primary antibody (anti-*V. unguiculata* cv. EPACE 10 vicilin antibody produced in rabbit) diluted at 1:2000 and a secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, Sigma) diluted at 1:2000. Immune reactions were developed using a BCIP/NBT tablet (Sigma) dissolved in 10 mL of distilled water for 10 min.

The levels of vicilin-like proteins were measured by the enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlman, 1971) using an anti-*V. unguiculata* cv. EPACE 10 vicilin primary antibody produced in rabbit (diluted 1:2000) and an alkaline phosphatase-conjugated anti-rabbit IgG second antibody (diluted 1:2000). *Vigna unguiculata* cv. EPACE 10 cotyledon vicilins, in concentrations of 5–0.0024 μg/100 μL, were used as standard. Alkaline phosphatase activity was revealed by adding 50 μL of a development solution (5 mg 4-nitrophenyl phosphate-*p*-NPP) in 5 mL of 100 mM glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.2) and the reaction was stopped by the addition of 50 μL 3 M NaOH. Absorbance was read at 410 nm. ELISA experiments were run in triplicate.

2.6. Enzyme detection

The presence of chitinase in the DIII fraction was determined by Western blotting (Towbin et al., 1979), using an anti-*Adenanthera pavonina* L. cotyledon chitinase antibody produced in rabbit, diluted at 1:1000, and a secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, Sigma) diluted at 1:2000. *Adenanthera pavonina* cotyledon chitinase was used as a positive control.

Acid phosphatase activity was measured according to the method of Ferreira et al. (1998), using *p*-nitrophenylphosphate (*p*-NPP) as substrate and 5 μg of the proteins from the DIII fraction. For determination of the optimal enzyme temperature, the reaction mixture was incubated from 20 to 60 °C for 25 min. Optimal time was determined by incubation of the reaction mixture for periods from 0 to 35 min at 37 °C. The optimal pH of the enzyme was analysed by performing the reactions at pH 2.0–9.0 using 100 mM glycine (pH 2.0, 3.0 and 9.0), 100 mM sodium acetate (4.0 and 5.0) and 150 mM Tris (6.0, 7.0 and 8.0). To determine the thermal stability of the enzyme, the fraction was preincubated at 30, 40, 50 and 60 °C for 10 min with further addition of *p*-NPP solution. The reaction was stopped by adding 1 mL of 1 M NaOH and the absorbance was read at 405 nm. One unit of activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol of *p*-NPP per minute.

Acid phosphatase activity was also detected on a 15% non-denaturing gel (Davis, 1964). The gel was washed with 150 mM Tris–HCl, pH 7.6 and incubated with 150 mM Tris–HCl, pH 7.6, 40 mM $CaCl_2$ and 10 mM *p*-NPP for 16 h. After this time, the gel was incubated with 1 M NaOH and calcium phosphate precipitation on the protein band was observed.

Total peroxidase activity was assayed as described by Hammerschmidt et al. (1982). The reaction mixture (1 mL) consisted of 0.25% (v/v) guaiacol in 10 mM sodium phosphate buffer, pH 6.0, containing 10 mM hydrogen peroxide. The tested sample (8.4 μL) was added to initiate the reaction, which was measured spectrophotometrically at 470 nm after 10 min. One unit of peroxidase activity was defined as the amount of enzyme that catalyses an absorbance increase of 0.01 per minute at 470 nm. Peroxidase activity was also detected using diaminobenzidine (DAB) as substrate on a 15% non-denaturing gel (Davis, 1964) and separated proteins were transferred to a nitrocellulose membrane. The membrane was rinsed in PBS (20 mM Na_2PO_4, 140 mM NaCl, pH 7.2), and incubated with the development solution (5 mg DAB, 100 μL of 2 M Tris, pH 7.5, 300 μL 0.1 M imidazole, 4.9 mL water, 5 μL H_2O_2) for 15 min.

Trypsin inhibitor activity was determined following the methodology described by Xavier-Filho and Coelho (1980) using BAPNA as substrate at a final concentration of 1 mM at 1% (v/v) of

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