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SHORT COMMUNICATION

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Characterization of antifungal glycoprotein in red-light-irradiated broadbean leaflets

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Abstract Red-light treatment of broadbean leaflets resulted in the production of antifungal substance(s) against *Botrytis cinerea*. The antifungal substance(s) was positively charged, as the antifungal constituent was removed by the cation exchanger CM cellulose. Treatment of infection droplets with glycosidases (α-mannosidase, β-galactosidase, β-glucosidase), glycol-specific reagent periodate ($NaIO_4$), and proteinase K completely eliminated antifungal activity, suggesting that both protein and carbohydrate are active components. The protein content of infection droplets was 0.148 mg/ml. The HPLC gel column analysis of infection droplets resulted in four fractions; all the fractions showed antifungal activity.

Key words Antifungal substance · Glycoprotein · Red light · *Vicia faba*

Phytoalexins play a vital role in host resistance against plant pathogens and are produced as the result of host–parasite interactions (Cruickshank 1963). Ultraviolet (UV) irradiation (253 nm) has been reported to induce phytoalexin in plants (Bridge and Klarman 1973; Kato et al. 1994). Recently, Islam et al. (1998) reported that irradiation of broadbean leaflets by red-light (600–700 nm) induced resistance against the chocolate spot disease pathogen *Botrytis cinerea* and the accumulation of antifungal substance(s) in broadbean leaflets. It has been demonstrated that inoculation of red-light-irradiated broadbean leaflets with conidia of *B. cinerea* significantly increased the accumulation of the antifungal substance(s) in leaflets. Islam et al. did not find antifungal activity in infection droplets collected from red-light-untreated broadbean leaflets (leaflets kept in darkness). This antifungal substance(s) was reported to be water-soluble, autoclavable, and fungi specific (Islam et al. 1999). The objective of the study was to characterize this red-light-induced antifungal substance(s) produced in broadbean leaflets.

Broadbean (*Vicia faba* L.) plants were grown in a glasshouse, and detached leaflets were inoculated with conidia of *B. cinerea* (Islam et al. 1998). Five to eight drops of 50 µl *B. cinerea* conidial suspension (2×10^5 spores/ml) were placed on either the upper or lower surface of each leaflet washed with water, and placed in plastic boxes lined with moist paper. The leaflets were incubated under continuous red-light irradiation from a fluorescent tube (FL·20S R-F, 600–700 nm, maximum 650 nm, 287 µW/cm^2; National, Osaka, Japan) hung 25 cm above the leaflets. After 48 h irradiation, infection droplets were recovered from the broadbean leaflets with a micropipette and were centrifuged (770 *g* for 10 min) to remove the spores. The infection droplets, recovered from red-light-irradiated broadbean leaflets, were used throughout the study unless otherwise mentioned.

The antifungal activity in the infection droplets or HPLC fractions of infection droplets was monitored either by inhibition of conidial germination of *B. cinerea* added in infection droplets or chromatogram inhibition assays using thin-layer chromatography (TLC) plates spotted with fractions and sprayed with conidial suspension of *Cladosporium paeoniae* as test fungus (Islam et al. 1999).

Acid, enzymatic, and periodate sensitivity of centrifuged infection droplets or HPLC fractions was assayed (Song et al. 1993). These assays employed incubation with either proteinase K (0.05 mg/ml, Sigma; 30 min at 37°C followed by autoclaving), α-mannosidase (38 U/ml, Sigma; 6 h at 25°C), β-galactosidase (1 U/ml, Sigma; 5 h at 37°C), or β-glucosidase (32 U/ml, Sigma; 5 h at 37°C), treatment with a final concentration of 1 N HCl for 3 h at 100°C, and treatment with 35 mM sodium periodate ($NaIO_4$) for 48 h at 25°C. The antifungal activity of treated samples was checked as previously stated. The carbohydrate-specific phenol-ethanol-H_2SO_4 test was also performed for the detection of carbohydrate content in infection droplets or in

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HPLC fractions. On silica gel TLC plates, 0.05 ml of infection droplets or fractions was spotted and sprayed with phenol-ethanol-H_2SO_4 solution (phenol 3 g, sulfuric acid 5 ml, ethanol 95 ml) followed by incubation at 110°C for 10 min; change of color of the spot to a chocolatey-black was checked. The treatment of infection droplets or HPLC fractions with glycosidases (α-mannosidase, β-galactosidase, β-glucosidase), glycol-specific periodate ($NaIO_4$), and proteinase K completely eliminated the antifungal activity (Table 1). The antifungal activity of infection droplets was relatively stable even after acid hydrolysis with 1 N HCl for 3 h at 100°C. The carbohydrate-specific phenol-ethanol-H_2SO_4 test also gave a positive reaction.

These results suggest that both protein and carbohydrate are active components of the antifungal substance(s) in the droplets and that the carbohydrate component of antifungal substance is a polysaccharide. The results also suggest that binding of protein and carbohydrate is necessary to express the antifungal activity. A heat-stable, high molecular weight glycoprotein toxin was isolated from culture filtrates of the tomato leaf mold pathogen *C. fulvum* (Lazarovits et al. 1979). Mannose, glucose, galactose, and several other unidentified sugars were also found in this toxin.

In charge analysis, cation exchanger dry carboxymethyl (CM) cellulose (1.5 g) was pretreated and preequilibrated with 0.03 M Na acetate, pH 4, and was added to 100 ml infection droplets and stirred for 2 h at room temperature (Jin et al. 1996). The liquid was then passed through a filter paper (Whatman no. 1) to remove CM cellulose. Antifungal activity of the filtrate was then checked. In the same way, anion exchanger diethylaminoethyl (DEAE) cellulose (1.5 g) was pretreated and preequilibrated with 0.5 M Tris-Cl, pH 7.8, and was added to 100 ml infection droplets and stirred and filtered as was CM cellulose. The antifungal constituent was removed by the cation exchanger CM cellulose but not by anion exchanger DEAE cellulose, which indicated that the antifungal substance(s) was positively charged.

For HPLC analysis, infection droplets (260 ml) were concentrated to 0.5 ml by evaporation. The concentrates were passed through a filter (0.20 μm; Dismic-25cs, Advantec, Tokyo, Japan) and centrifuged at 2310 *g* for 10 min. Then, 0.3 ml of a concentrated infection droplet was applied to an HPLC gel column (Superdex 200 HR 10/30; average particle size, 13 μm; separation range, 10000–600000; length × inside diameter, 300 × 10 mm; Amersham Pharmacia Biotech, Piscataway, USA). The column was pre-equilibrated with ultrafiltered purified water (Milli-Q, >18.0 MΩ·cm). The sample was eluted with the same water at a flow rate of 0.5 ml/min, and four fractions (Fig. 1) were collected. Antifungal activity was observed in all four fractions in inhibition zone analysis with the highest antifungal activity, i.e., clear zone in fraction IV.

Protein content of infection droplets was estimated using the Bio-Rad protein assay system with bovine serum albumin (BSA) as the protein standard (Bradford 1976), and it was estimated as 0.148 mg/ml of infection droplet. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of infection droplets and HPLC fraction IV was performed in 12.5% polyacrylamide gel plates using the procedure of Laemmli (1970). Molecular mass standards included phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). Protein bands in the gel were stained with Coomassie brilliant blue (CBB) R-250. SDS-PAGE analysis of infection droplets showed five separate bands with molecular mass ranging from 14.2 to 38.5 kDa, and fraction IV showed a single band with a molecular mass of 38.5 kDa (Fig. 2). The carbohydrate-

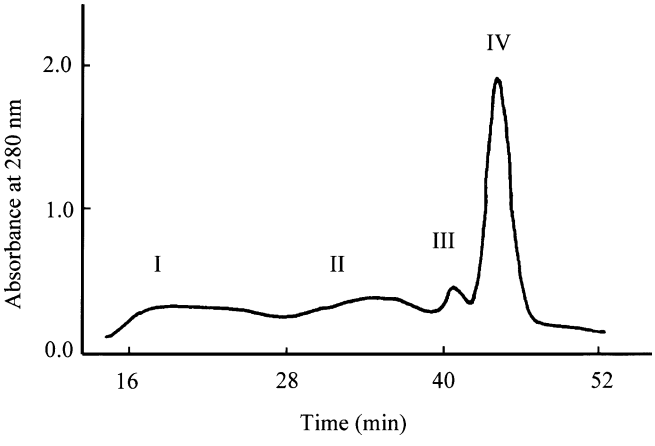

Fig. 1. HPLC gel filtration of concentrated infection droplets from red-light-irradiated broadbean leaflets. Fractions (*I–IV*) were eluted by ultrafiltered purified water (Milli-Q)

Table 1. Carbohydrate- or protein-specific test of red-light-induced antifungal substance(s) produced in broadbean leaflets

Treatment	Conidial germination of *Botrytis cinerea* (%)[a]			Reaction
	Treated sample	Untreated sample	Water control	
Sodium periodate ($NaIO_4$, 35 mM)	82.0 ± 4.3	4.0 ± 1.5	82.0 ± 4.1	Carbohydrate positive
β-Galactosidase (1 U/ml)	89.0 ± 3.0	7.0 ± 1.5	88.0 ± 3.0	Carbohydrate positive
α-Mannosidase (38 U/ml)	86.0 ± 3.6	5.0 ± 1.3	81.0 ± 2.2	Carbohydrate positive
β-Glucosidase (32 U/ml)	79.0 ± 3.0	6.0 ± 1.0	82.0 ± 1.8	Carbohydrate positive
Proteinase K (0.05 mg/ml)	92.0 ± 1.1	7.0 ± 2.1	89.0 ± 1.9	Protein positive
Acid hydrolysis (1 N HCl)	15.0 ± 1.3	13.0 ± 2.8	78.0 ± 3.2	Stable
Phenol-ethanol-H_2SO_4 test[b]	–	–	–	Carbohydrate positive chocolatey spot

All data are means ± SD from three replicates
[a] Spore germination was examined on glass slides after 24 h of incubation
[b] Test was performed on silica gel TLC plate

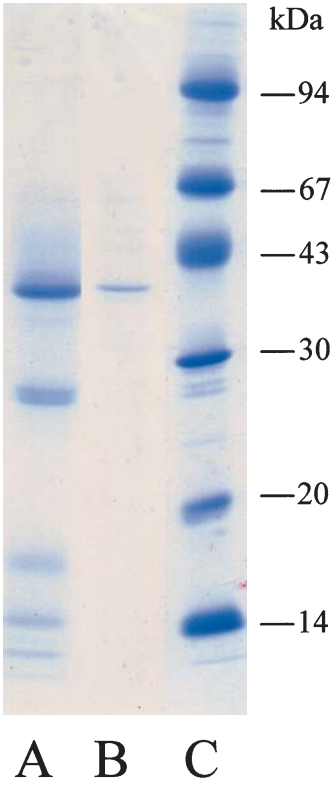

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of infection droplets. *Lane A*, original infection droplets; *lane B*, HPLC–gel column filtrated fraction IV; *lane C*, molecular weight marker

specific phenol-ethanol-H_2SO_4 test showed the presence of carbohydrate in all HPLC fractions. Treatment of fraction IV with glycosidases (α-mannosidase, β-galactosidase, β-glucosidase) and proteinase K completely eliminated its antifungal activity, which indicated that both carbohydrate and protein are essential components of its antifungal property.

These findings suggest the possibility of the presence of more than one red-light-induced antifungal compound in broadbean leaflets. It should be noted that SDS-PAGE provides an estimate of the molecular weight of protein subunits. As it has been found that both the protein and carbohydrate are the components of the antifungal compound in fraction IV, the molecular mass estimated by SDS-PAGE seems to be the total molecular weight of the compound where protein is bonded as one of the components. However, further work on the purification and chemical nature of the red-light-induced antifungal substance(s) is needed to understand the mechanism of red-light-induced resistance in broadbean.

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