Influences of Metabolic Inhibitors and Hydrolytic Enzymes on the Adhesion of Appressoria of Pyricularia oryzae to Wax-coated Cover-glasses

Manabu Ohtake, Hirotaka Yamamoto, and Takeo Uchiyama*

Faculty of Agriculture, Niigata University, Ikarashi, Niigata 950-2102, Japan

Received November 13, 1998; Accepted January 26, 1999

For discovering the components that contributed to the bonding strength of the glue substances produced by appressoria of Pyricularia oryzae on wax-coated cover-glasses, the influences of metabolic inhibitors and hydrolytic enzymes were investigated. The bonding strength of appressoria was assessed by the ratio of the remaining appressoria after sonication to the appressoria formed before sonication. Remaining appressoria decreased with increasing concentrations of cerulenin, an inhibitor of lipid synthesis, but isoprothiolane and compactin showed no influence on bonding strength. Tunicamycin, an inhibitor of glycoprotein synthesis, weakened the bonding strength of appressoria, but castanospermin had no effect. Of the hydrolytic enzymes tested, protease particularly weakened the bonding strength of appressoria. On the surfaces of substrata, the appressoria’s bonding strength was higher on the hydrophobic surfaces than on the hydrophilic. These results suggest that lipid components and glycoprotein were closely associated with appressoria bonding strength to the surface of wax-coated cover-glass.

Key words: Magnaporthe grisea; Pyricularia oryzae; glue substances; adhesion of appressorium; appressorium

Under suitable conditions, fungal spores arriving on a host surface begin to adhere and germinate. The germ tubes respond to physical and chemical signals on the host surface and differentiate an appressorium, which is an essential structure for the infection of the host plant. For invading the host cell, a penetration peg is formed under the appressorium and breaks down the host cell wall by physical and chemical means.

For establishing these infection processes on the host surface, adhesion of the spore, germ tube, and appressorium are critical steps before the initiation of infection. The chemical constituents of extracellular substances with adhesive properties are suggested to be glycoproteins and are believed to be important roles for establishing infection.

Pyricularia oryzae Cavara (anamorph of Magnaporthe grisea Barr.) is the causal fungus of rice blast disease. Before penetration through the cell wall of the host plant, the fungus must firmly adhere by its appressorium onto the host surface to prevent the leakage of wall-degrading enzymes and to prevent the lessening of mechanical pressures. This fungus has a ability to form its appressoria not only on leaf surfaces of host plants, but also on many different artificial surfaces, and adheres firmly onto surfaces using glue substances.

In our previous investigation, we reported the chemical composition of the glue substance that adhered the appressoria on artificial wax-coated cover-glasses and found that the substances were rich in lipids (neutral lipids and glycolipids), which may be held by some cross-linked polymers such as glycoproteins.

These experiments were undertaken to discover the components in the glue substances that primarily contributed to the adhesive forces of the appressoria. We report here the influences of metabolic inhibitors and hydrolytic enzymes on the bonding strength of the appressoria.

Materials and Methods

Optimizations and preparation of germination substrata. Pyricularia oryzae P2 was used in this experiment. The culture and conidial suspension were prepared as described previously. Wax-coated cover-glasses, parafilm M, and polyethylene sheets were used as hydrophobic surfaces. Dry agar sheet was used as a hydrophilic surface. Wax-coated cover-glasses were prepared as described previously. Parafilm M and polyethylene sheets (18 × 18 mm) were bonded to the cover-glasses. Dry agar sheets were prepared by dipping the cover-glasses into agar solution (1.5%), followed by raising and drying it in an electric oven at 60°C for 3 h.

Metabolic inhibitors. Metabolic inhibitors of lipids, cerulenin (Sigma chemical company), compactin (Sigma Chemical Company), and isoprothiolane (Wako Co. Ltd.), were tested, and as the metabolic inhibitors of glycoproteins, castanospermine (Wako), and tunicamycin (Sigma) were used. Inhibition tests were done in the concentration ranges of inhibitors in which conidial germination and appressoria formation were scarcely influenced. For evaluating the influences of metabolic inhibitors to the bonding strength of appressoria formed on the surfaces of the wax-coated cover-glasses, droplets (50 μl) of conidial suspensions (2 × 10⁶ conidia/ml) in deionized water or containing metabolic inhibitors were placed on the surfaces of wax-coated cover-glasses and kept in petri dishes at 25°C with high humidity for 24 h in darkness. After using a light microscope to count the numbers of the germinated conidia and formed appressoria, appressoria on the substrata were submerged in

* To whom correspondence should be addressed. Fax: +81-25-262-6854; E-mail address: uchiyama@agr.niigata-u.ac.jp
wax-coated cover-glasses. Cerulenin\(^{19}\) prevented the chain elongation of fatty acid by inhibiting the condensation reaction between acyl and malonyl thioesters. On the other hand, isoprothiolane\(^{20}\) inhibits fatty acid synthesis by preventing the incorporation of acetic acid into fatty acids, and also prevents fatty acid incorporation into triglycerides in the mycelia of \(P.\ oryzae\). If the lipid components are important to the adhesive properties of appressoria, their bonding strength must be weakened by the inhibition of fatty acid synthesis. Consequently, any lowering of bonding strength was estimated from the ratio of the remaining appressoria unremoved from the substrate by sonication (30 sec).

As shown in Fig. 1, the ratio of the remaining appressoria gradually decreased with increasing concentrations of cerulenin, and dropped to 18\(\pm\)6.4\% at 20 ppm of cerulenin for 49\(\pm\)3.6\% of remaining appressoria at 0 ppm. The germination of conidia and appressoria formation were scarcely influenced at the tested range of cerulenin concentration.

On the other hand, isoprothiolane did not show an effect on the adhesive properties of appressoria. The ratio of the remaining appressoria was within the range from 44.4\(\pm\)5.6\% at 0 ppm to 48\(\pm\)4.5\% at 100 ppm. Neither germination of conidia (91.7\(\pm\)3.4\% at 0 ppm to 91.0\(\pm\)3.6\% at 100 ppm) nor appressoria formation (84.3\(\pm\)4.0\% at 0 ppm to 80.4\(\pm\)9.0 at 100 ppm) were influenced in the range of isoprothiolane concentration tested.

These results may indicate that the inhibition of fatty acids synthesis in the appressoria weakened the bonding strength of appressoria, and also may indicate that the fatty acids were important in intensifying the bonding strength of the glue substance in the lipid components.

### Table 1. Induction of Appressorium Formation on the Cover-glass by cAMP

<table>
<thead>
<tr>
<th>Substrata</th>
<th>Incubation time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Cover-glass</td>
<td>Germination</td>
</tr>
<tr>
<td>No treatment</td>
<td>92.8(\pm)4.1</td>
</tr>
<tr>
<td>Wax-coated (%)</td>
<td>93.3(\pm)7.2</td>
</tr>
<tr>
<td>cover-glasses (%)</td>
<td>91.0(\pm)2.1</td>
</tr>
<tr>
<td>Apsorpsirum</td>
<td>46.3(\pm)4.6</td>
</tr>
</tbody>
</table>

The stock solution of cAMP was mixed in conidial suspension to a final concentration of 0.1, 1 and 10 mM. The conidial suspensions were placed on the cover-glasses, and incubated in moistened glass petri dishes at 24°C in darkness.

**Enzymes.** Hydrolytic enzymes; lipase (Sigma), pro tease (Sigma) and \(\alpha\)-glycosidase (Sigma); were tested to estimate their influences on the appressoria’s bonding strength. Droplets (50 \(\mu\)l) of conidial suspensions (2 \(\times\) 10\(^6\) conidia/ml) in deionized water were placed on the wax-coated cover-glass and were kept in petri dishes with high humidity at 25°C for 10 h. After confirming appressoria formation (>50\%), water in the spore suspension was replaced by enzyme solution, followed by incubation for 14 hr at 25°C. After counting the germinated conidia and the formed appressorium using a light microscope, appressoria on the substratum were submerged in water and sonicated for 30 sec by a sono cleaner (Kaijo Denki, 45 W, 40 KHz). The appressoria’s bonding strength was assessed as already described, and compared with those without enzymes.

**Induction of appressoria by cAMP.** Three concentration (0.1, 1, and 1 mm) of cAMP (Merck) were tested to find the optimum concentration for appressoria formation.\(^{18}\) Conidial suspensions (2 \(\times\) 10\(^6\) conidia/ml) containing cAMP were placed on the cover-glass (not wax-coated), followed by incubation at 25°C with high humidity in darkness. The effects of cAMP were evaluated by comparison with the formed appressoria on the cover-glasses and wax-coated cover-glasses without cAMP. As shown in Table 1, the optimum concentration of cAMP for appressorium formation on the cover-glasses was estimated at 1 mM.

**Results and Discussion**

**Effects of cerulenin and isoprothiolane upon the bonding strength of appressoria**

Lipids are the major components of the glue substances produced by appressoria of \(P.\ oryzae\) on the

![Fig. 1. Effects of Cerulenin upon Appressoria Bonding Strength to the Wax-coated Cover-glasses.](image-url)
Effects of compactin on the bonding strength of appressoria

No sterols were detected in the lipid fractions of the glue substances, but traces of sterols may be present and may be important in the appressoria’s adhesive properties. Thus the influences of compactin, as an inhibitor of sterol biosynthesis, upon appressoria bonding strength was tested. The ratio of unremoved appressoria varied very little with changes in compactin concentration. The ratio of the remaining appressoria was within the range from 47.5 ± 6.6% at 0 ppm to 48.7 ± 8.2% at 40 ppm. Neither the germination of conidia (93.0 ± 2.6% at 0 ppm to 88.7 ± 2.3% at 40 ppm) nor appressoria formation (84.5 ± 4.3% at 0 ppm to 76.0 ± 6.1 at 40 ppm) were influenced at the tested concentration of compactin.

Because compactin is a potent inhibitor of HMG (3-hydroxy 3-methyl glutaryl)-Co A reductase, inhibition of the enzyme’s activity is closely related to changes in the overall rates of cholesterol synthesis. Yet the appressoria’s bonding strength on the wax-coated substrate was not affected by compactin. These results might be evidence that no sterols were present in the lipid fractions of the appressoria’s glue substances.

Effects of castanospermine and tunicamycin on the bonding strength of appressoria

The glue substances that take part in the adhesion of conidia and germ tubes have been attributed to the extracellular glycoprotein they secrete. Harmer et al.\textsuperscript{22} reported that the mucilage secreted from the tips of the conidia allowed the conidia’s adhesion to the substrate. Xiao et al.\textsuperscript{11} also reported that extracellular glycoprotein fixed germ tubes and appressoria to the substrate surface. Bircher et al.\textsuperscript{23} reported that tunicamycin strongly reduced the adherence of the germing of Phytophthora palmivora to a polystyrene surface and inhibited the appressorium formation of the adhering germings.

The glue substances of appressoria of P. oryzae ought to have contained glycoproteins, which may be important in the adhesive properties of the appressoria as a three-dimensional framework.

Changes in castanospermine concentration did not bring about changes in the ratio of remaining appressoria in all ranges of tested concentrations. The ratio of the remaining appressoria was within the range from 52 ± 5.2% at 0 ppm to 54.± 7.0% at 100 ppm, but the ratio of remaining appressoria decreased with tunicamycin treatment from 47.7 ± 3.7% at 0 ppm to 32.5 ± 4.9% at 4 ppm (Fig. 2).

Castanospermine\textsuperscript{21,24} and tunicamycin\textsuperscript{24} are biosynthetic inhibitors of glycoproteins. Tunicamycin inhibits the first step in the lipid-linked saccharide pathway, i.e., the transfer of GlcNac-1-P to dolichol-P. Castanospermine blocks glycoprotein processing by virtue of its inhibition of glucosidase I and glucosidase II.

Inhibition tests indicated that appressoria bonding strength was influenced by tunicamycin, which blocked the first step in the lipid-linked saccharide pathway of glycoproteins synthesis, more than by castanospermin, which blocked glycoprotein processing. These results also indicate the importance of glycoprotein as a frame work of glue substances.

Another metabolic inhibitor, tricyclazole, a blocker of melanin biosynthesis,\textsuperscript{25} was also tested, but showed no effect on the appressoria’s bonding strength and appressoria formation in the range of the tested concentrations (0.1, 1.0, 10 μg/ml).

Influences of cycloheximide, an inhibitor of peptidyl transferase in protein synthesis, was also tested. The ratio of remaining appressoria rapidly dropped from 50% at 0 ppb to 30% at 50 ppb of cycloheximide concentration. Neither the germination of conidia (92.4 ± 3.1% at 0 ppb to 87.1 ± 3.6 at 100 ppb) nor appressoria formation (87.3 ± 2.0% at 0 ppb to 80.9 ± 5.1% at 100 ppb) were influenced at the tested concentration of cycloheximide. This result indicates that bonding strength was weakened by blocking enzyme synthesis associated with the biosynthesis of the components in the glue substances.

Effects of hydrolytic enzymes on the bonding strength of appressoria

Xiao et al.\textsuperscript{11} reported that α-glucosidase, α-mannosidase, and protease inhibited the appressorium formation and conidial adhesion in M. grisea. Bircher et al.\textsuperscript{12} reported that Pronase E reduced appressorium formation in Phytophthora palmivora and its adhesion to polystyrene surfaces. To estimate the relationships between the chemical bond and the constituents of glue substances, the effects of hydrolytic enzymes on the bonding strength of appressoria were tested.

As shown in Fig. 3, three hydrolytic enzymes (lipase, protease, and α-glucosidase) weakened the appressoria’s bonding strength. Protease was somewhat effective in
weakening the adhesion of appressoria. These results suggest that glycoproteins are important for adhesiveness and the construction of frameworks in the glue substances. 11)

Bonding strength of appressoria on the hydrophilic and hydrophobic surfaces
The properties of the substrate’s contact surface and its relationship to the bonding strength of appressoria were investigated. Since appressoria differentiation was poor on hydrophilic substrates such as agar and slide glass, cAMP was used for inducing appressoria formation.

As shown in Table 2, the appressoria’s bonding strength was stronger on the hydrophobic surfaces than on the hydrophilic. Hydrophobic surfaces have been congenial to appressoria formation. 16,18 The results indicate that the glue substances, because of their hydrophobic properties, were a strong affinity to hydrophobic surfaces.

From these experiments, it was clear that lipid components and glycoproteins were important in intensifying the bonding strength of the glue substance of appressoria. The wax on the substrate surfaces also contributed to its adhesion with the glue substance of appressoria. This conclusion was supported by the observation that when the mycelia of Erysiphe graminis f.sp. hordei was removed from leaf surfaces by a glass needle and examined by scanning microscopy, wax granules on the leaf adhered around the mycelia. 26 Hydrophobic surfaces favor the development of appressoria by P. oryzae. 16 Adhesion tests of appressoria on various substrates indicated that the appressoria’s bonding strength was stronger on hydrophobic surfaces than on the hydrophilic. It can be inferred from these evidences that appressoria recognize the properties of substrate surfaces and makes glue substances particularly well-suited for adhesion to them.

Acknowledgment
We thank Dr. Y. Kubo (Kyoto University) for his gift of tricyclazole.

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