Extracellular Proteins Associated with Induction of Differentiation in Bean Rust Uredospore Germlings

Lynn Epstein, Lucille Laccetti, R. C. Staples, H. C. Hoch, and Wendy A. Hoose

First three and fifth authors: Boyce Thompson Institute, Cornell University, Tower Road, Ithaca, NY 14853. Fourth author: New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456.
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ABSTRACT

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The bean rust fungus (*Uromyces appendiculatus*) has a contact-sensitive response to the stoma, the fungal penetration site on a leaf. Stomatal recognition is followed by mitotic nuclear division and differentiation of the first infection structure, the appressorium. Our evidence suggests that proteins in the extracellular matrix of the germ tube may be involved in the thigmotropic response. Nuclear division of germlings incubated in vitro on a normally inductive scratched surface was significantly reduced by 500 μg of either pronase E or trypsin per milliliter but not by heat-denatured enzymes, trypsin mixed with trypsin inhibitor, or 500 μg of either α- or β-glucosidase, α-mannosidase, or lipase per milliliter. Incubating germlings with pronase E at any time prior to nuclear division inhibited germling response to the scratched surface. Pronase E at 200 μg/ml did not decrease rates of germination or germ tube elongation, but adhesion of the germlings to the substrate was significantly reduced. The data suggest that extracellular proteins may bind the germ tube to an inductive surface, and that binding may be necessary for induction of infection structures.

Additional key words: *Uromyces phaseoli*.

Many fungal plant pathogens form infection structures before penetrating their hosts. With many of the rust fungi, such as the bean rust fungus [*Uromyces appendiculatus* (Pers.) Unger.] [=*Uromyces phaseoli* (Pers.) Wint.], uredospores pass through a series of distinct morphological stages while invading the host plant. Once the germination self-inhibitor is removed, the spores germinate and produce a simple, vacuolated germ tube. On a smooth surface, the dikaryotic germ tube may reach 1 mm in length, but never undergoes nuclear division or forms infection structures. On leaves or other plant surfaces, however, germ tubes that contact stomatal guard cells quickly undergo mitotic nuclear division and form appressoria, which are terminal cells from which the host is colonized (12,17,23). This initial process of differentiation (i.e., formation of the appressorium, infection peg, vesicle, and infection hyphae) can also be induced efficiently by a stomate from a nonhost dicot, a polystyrene replica of a stomate, a groove in glass or a variety of plastics, as well as on a collodion membrane containing oil (2–4,23). Thus, a physical (thigmotropic) stimulus is capable of inducing differentiation.

Since the thigmodifferentiation process appears to require firm attachment of the germlings to the inductive surface (24), we postulate that a component of the fungal extracellular matrix is involved in the pathogen's response to a surface contact stimulus. Data from Mendgen et al (14), Trocha and Daly (21), and Trocha et al (22) suggest the bean rust fungus may have chitin, glucans, mannans, lipids, and proteins on the hyphal surface.

The purpose of the research reported in this paper was to utilize enzymes to identify extracellular polymers that may be involved in thigmodifferentiation of uredospore germlings of the bean rust fungus.

MATERIALS AND METHODS

Bioassay. Uredospores of *U. appendiculatus* were produced, collected, stored, and washed as described previously (18). Alternatively, in lieu of washing, 30 mg of spores were enclosed for 20 min in a 50 cm^3 jar with a vial containing 200 μl of β-ionone

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(5,11). We used a CO_2 gun to disperse 30 mg of spores upward into a closed 0.5 × 0.4 × 1.0-m chamber; polystyrene petri plates were placed on the bottom of the chamber. After 2 min, the spores had settled (approximately 3,000 spores per square centimeter) onto the plates. A Preval atomizer (Precision Valve Corporation, Yonkers, New York) was used to deposit approximately 0.025 μl/mm^2 of the indicated aqueous solution onto the plates. The germination solution was: 10 mM (2-[*N*-morpholino]ethanesulphonic) acid (MES), pH 7.0; 2 mM $MgSO_4$, 3 mM $CaCl_2$, and 100 μM streptomycin sulfate. When specifically indicated, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (HEPES), pH 7.5, was substituted for MES.

When a differentiation-inducing surface was needed, the plates were scratched (before the spores were deposited) with either a diamond pencil or Grade 1 steel wool. Plates scratched with these materials, and then misted with either germination solution, routinely induced approximately 65 and 45% of the germlings to undergo at least one round of nuclear division, for the diamond pencil and steel wool scratched plates, respectively. Percentage nuclear division was assessed as follows: germlings were fixed at 4 C in 2.5% gluteraldehyde in 50 mM cacodylate buffer (pH 6.8) for 30 min, carefully rinsed in cacodylate buffer, and then stained with 0.05% mithramycin complex in 0.3% $MgCl_2$, and examined at ×400 on a Zeiss epifluorescence photomicroscope (18). Generally, one hundred germlings of each of three replicates were assessed.

To count germination, germlings were stained in 0.2% (w/v) trypan blue in 20% (v/v) melted phenol, 20% (v/v) lactic acid, and 40% (v/v) glycerol and 20% (v/v) H_2O. One hundred spores for each of four replicates were scored microscopically.

To measure growth, germlings were stained as for germination. Pictures of 186 germlings per treatment were taken on Kodak Plus-X film at ×100. Measurements of germ tubes were made from 76 × 127 mm prints with a digitizer.

Enzymes. The enzymes used were obtained from Sigma (St. Louis, MO); the catalog and E. C. numbers were: α-glucosidase (G-4634) (EC 3.2.1.20), β-glucosidase (G-8625) (EC 3.2.1.21), α-mannosidase (M-7257) (EC 3.2.1.24), protease (P-5147) (often called pronase E), trypsin (T-0134 or T-2395) (EC 3.4.21.4), and lipase (L-1754) (EC 3.1.1.3). α-Mannosidase was desalted with an Amicon (Danvers, MA) Centricon ultrafiltration unit prior to use; trypsin (T-0134) was desalted by gel filtration in a 0.9 × 11-cm column of Sephadex G-25 (Pharmacia, Piscataway, NJ) coarse beads.

Adhesion. Bean rust uredospores, dispersed on unscratched 60× 15-mm polystyrene plates, were misted with germination solution containing 10 mM MES, pH 7, and the appropriate pronase E (200 μg/ml) preparation, as in the enzyme studies. After 6 hr at 20 C, 5 ml of the germination solution was added to the plates, which were then rotated on a shaker at 50 rpm for 5 min. After the solution was removed, the washing procedure was repeated and the germlings were fixed in 3% formaldehyde for 5 min., rinsed with H_2O, stained with 4,6-diamidino-2-phenylindole (DAPI) at 1 μg/ml for 5 min, rinsed again with H_2O and then mounted in 75% (v/v) glycerin and 25% 0.1 M phosphate buffer, pH 8.0. The number of germlings in 10 microscopic fields at ×160 was determined for six replicates with the Zeiss epifluorescent photomicroscope (6).

Experimental design. All experiments were conducted at least two times.

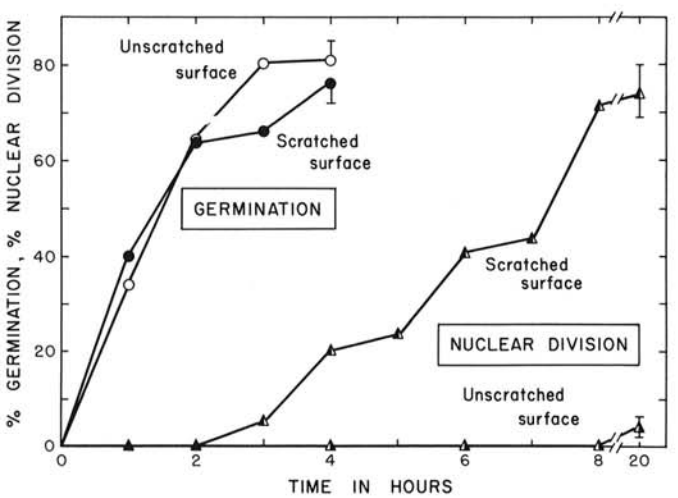

Fig. 1. Time course of germination and nuclear division of bean rust uredospores misted with "germination solution" and incubated at 20 C on scratched or unscratched polystyrene petri plates. The error bars represent ± standard deviation of two replicates.

RESULTS

Mitotic nuclear division and appressorium formation occurs when a bean rust germling encounters a topographical signal, i.e., a scratch on a membrane or a stomatal pore. On polystyrene plates, germination occurred similarly on scratched and unscratched surfaces, but nuclear division occurred only on scratched surfaces (Fig. 1). As shown previously (17) for all of the experiments reported here, qualitative observations indicated that appressorial formation was correlated with nuclear division.

Bean rust uredospores were dispersed onto scratched polystyrene plates. These were then misted with either α-glucosidase, β-glucosidase, α-mannosidase, pronase E (a nonspecific protease), trypsin, or lipase at 500 μg/ml (Table 1). Pronase E and trypsin significantly ($P = 0.05$) reduced nuclear division. Nuclear division was not affected by heat-denatured pronase E or trypsin, trypsin inhibitor alone, trypsin mixed with trypsin inhibitor (500 μg/ml), or by any of the other enzymes. Thus, it appears that fungal proteins were involved in the induction of nuclear division. We noted that α- or β-glucosidase, α-mannosidase, pronase E, or trypsin at 500 μg/ml of did not induce nuclear division in germlings incubated on smooth polystyrene plates (data *unpublished* except those for pronase E in Fig. 2).

When germlings were incubated on a scratched surface, the suppression of nuclear division was linear with the log of the concentration of pronase E (ED_{50} = 40 μg/ml, **r** = −0.99)(Fig. 2); however, pronase E at 200 μg/ml did not affect either uredospore germination or germ tube elongation (Table 2).

Nuclear division was suppressed in germlings incubated on scratched surfaces provided the enzyme (final pronase E concentration approximately 200 μg/ml) was added prior to the start of mitosis (Table 3). We found, for example, that nuclear

TABLE 1. Effect of selected enzymes (500 μg/ml) on nuclear division of bean rust germlings incubated on scratched surfaces[v]

Assay conditions[w]			% Nuclear division, % of control[d]	
Enzyme	Buffer	pH	Active enzyme	Denatured enzyme
α-Glucosidase	MES	7.0	117 a	ND[y]
β-Glucosidase	MES	5.0	94 a	101 a
α-Mannosidase	MES	5.0	105 a	ND
Pronase E	MES	7.0	11 b	96 a
Pronase E	HEPES	7.5	31 b	88 a
Trypsin	HEPES	7.5	35 b	106 a
Trypsin + trypsin (500 μg/ml)[z]	HEPES	7.5	106 a	ND
Lipase	HEPES	7.5	118 a	120 a

[v] Bean rust uredospores were incubated at 20 C for 10 hr on polystyrene petri dishes that were scratched with a diamond pencil; this surface normally induces nuclear division and formation of appressoria.

[w] The uredospores were misted with germination solution containing 10 mM of the indicated buffer at the pH indicated with or without the indicated active or heat-denatured enzyme (20 min at 90 C).

[x] Trypsin inhibitor (500 μg/ml) alone did not affect nuclear division of the germlings (112% of the control).

[y] ND, not determined.

[z] Percent nuclear division of mithramycin-stained material was determined via epifluorescent microscopy by assessing 100 germlings on three replicate plates. Values represent percentages of a buffer control. Means followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P = 0.05$). S.D. = 11.5.

division of germlings misted with pronase E at either 0 or 2 hr was equally suppressed. In contrast, treatment after 4 or 6 hr of germination (when nuclear division was underway) had little or no effect on nuclear division. We note that application of denatured pronase E at 0, 2, 4 or 6 hr did not significantly ($P = 0.05$) affect nuclear division. Thus, pronase E did not affect nuclear division after the contact response had commenced.

Proteins may be involved in binding the germ tube to the surface. Pronase E significantly ($P = 0.01$) decreased the adhesion of bean rust germlings to polystyrene plates, while heat-denatured pronase E did not (Table 4).

DISCUSSION

Our evidence suggests that fungal proteins are specifically involved in the thigmotropic response. Neither germination nor growth rate was affected by pronase E, whereas nuclear division was suppressed on a normally inductive surface. It is unlikely that the effect of pronase E is attributable to any properties other than its proteolytic capabilities. Comparable quantities of protein were applied in all of the treatments with the various enzymes and the trypsin inhibitor (Table 1), but only active pronase E and trypsin suppressed nuclear division. Since trypsin inhibitor, known to specifically inhibit trypsin, alone did not affect fungal nuclear division, it is significant that nuclear division occurred when trypsin was mixed with its inhibitor. This strongly suggests that a protease, not a nonproteolytic contaminant, is the effective enzyme, and that a fungal protein is the affected component.

The ability of a fungus to react to a thigmotropic stimulus requires contact between the fungus and the stimulating surface. Dickinson (2) demonstrated that rust uredospore germ tubes adhere tightly to any hydrophobic surface. Wynn (24) showed that when rust uredospores were incubated on waxless leaves of their hosts, the germlings neither adhered to the surface nor reacted to the stomata. Similarly, differentiation does not occur on agar where the germ tubes grow away from the surface (H. C. Hoch, *unpublished*).

Our evidence suggests that extracellular proteins are involved in the thigmotropic response, possibly by attaching the germling to a hydrophobic surface, as on a leaf. The enzymes we used appeared to affect the fungal surface. If pronase E or trypsin could traverse the hyphal wall and reach the plasmalemma, we would expect cellular damage; we have no evidence for this. Moreover, pronase E affected adhesion, an extracellular phenomenon. Extracellular proteins (or glycoproteins) are apparently involved in fungi-substratum adhesion in *Buergenera spartinae* (15) and *Dictyostelium discoideum* (19) as well as in fungi-plant adhesion (10,13).

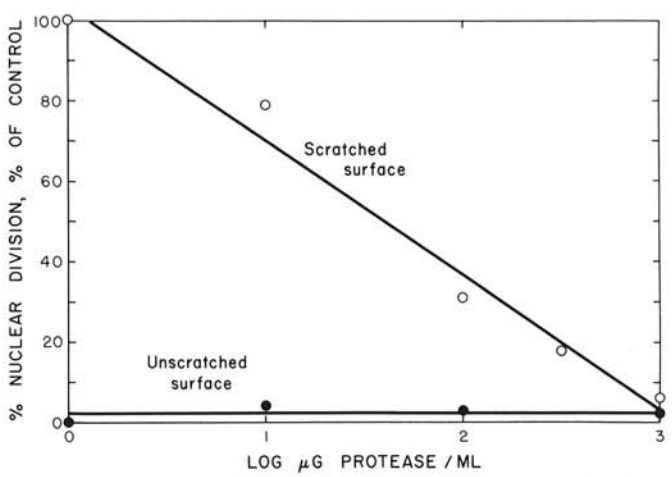

Fig. 2. Effect of the concentration of pronase E on nuclear division of bean rust germlings incubated overnight at 20 C on scratched or unscratched polystyrene petri plates. Pronase E was mixed in "germination solution" and misted onto the plates. The lines are linear regressions with $r = -0.99$ on the scratched surface and $r = 0.38$ on the unscratched surface.

The bean rust extracellular proteins involved in the thigmotropic response have not been well characterized. Mendgen et al (14) used lectins to determine that germ tubes had mannose and glucose residues on the surface; it was not determined whether the residues were a part of the glycoprotein or a nonproteinaceous compound. We note that neither an α-mannosidase nor an α- or β-glucosidase affected the surface response whereas two proteolytic enzymes did (Table 1). Therefore, if the cellular proteins are indeed glycoproteins, it seems likely that the carbohydrate portions of the molecule are not involved in the induction of differentiation. Day and co-workers (1,16,20) have isolated a 74-kdalton protein from the surface of a number of fungi, including the bean rust fungus.

TABLE 2. Effect of pronase E (200 μg/ml) on germination and growth of bean rust uredospores

Treatment[a]	Germination[b] (%)	Hyphal length, μm[c]
Buffer	80 ± 3	108 ± 4
Pronase E	76 ± 4	116 ± 4
Heat-denatured pronase E	81 ± 2	ND[d]

[a] Uredospores on polystyrene plates were misted with germination buffer with or without 200 μg/ml pronase E; heat-denatured pronase E was pretreated at 90 C for 20 min. Spores were incubated at 20 C for 3 or 4 hr for germination and growth data, respectively.
[b] Percent germination of spores, stained with trypan blue in lactophenol, was determined by assessing 100 spores for four replicates. The *F*-test did not indicate any significant ($P = 0.05$) differences among treatment means. Error expressed as standard error of the mean.
[c] Hyphal length of 186 germlings per treatment was measured from photomicrographs using a digitizer. Statistics were as above.
[d] ND, not determined.

TABLE 3. Effect of pronase E on nuclear division of bean rust germlings incubated on scratched surfaces

Treatment time[a] (hr)	Nuclear division of control at time of treatment (%)	Nuclear division after 10 hr (%)	
		Pronase E[a]	Denatured pronase E[b]
0	0	3	46
2	2	2	44
4	19	24	47
6	41	40	38
10	43	–	–

[a] Uredospores were misted with "germination solution" and incubated at 20 C for 10 hr. Time of treatment is the time after the start of the incubation process when the uredospores were sprayed with the germination solution amended with 400 μg of pronase E per milliliter. S.D. = 3.7.
[b] Denatured pronase E was heat-denatured by treatment at 90 C for 20 min.

TABLE 4. Effect of pronase E on adhesion of bean rust germlings to polystyrene

Treatment[y]	Conc. (μg/ml)	Germlings per microscope field[z]
Buffer	0	10.9 x
Pronase E	200	2.9 y
Heat-denatured pronase E	200	13.6 x
S.D.		3.0

[y] Bean rust uredospores deposited on polystyrene petri plates were misted with "germination solution" amended with the indicated treatment; heat-denatured pronase E was pretreated at 90 C for 20 min. After 6 hr at 20 C, the germlings were covered with the germination solution, and then rotated on a shaker at 50 rpm for 5 min. The liquid was removed. The washing procedure was repeated, and the germlings were fixed and stained for epifluorescent microscopy.
[z] The number of germlings at ×160 in 10 microscopic fields was determined for six replicates. Means in each column followed by the same letter were indistinguishable by the Student-Newman-Keuls multiple range test ($P = 0.01$).

The protein forms structures called fimbriae which are 7 nm wide and vary in length from 0.5 to over 20 μm. Kaminskyj and Day (9) suggested that fimbriae are involved in chemically-induced, but not surface-induced, differentiation since antifimbrial antisera inhibited sugar-induced appressorial formation but not differentiation induced on collodion-oil membranes. While this work is being reevaluated (W. A. Day, *personal communication*), we suggest that surface proteins other than fimbriae may be involved in stomatal recognition.

The bean rust extracellular proteins involved in substrate adhesion are apparently involved in the transmission of surface signals which start nuclear division. Elemants of the bean rust cytoskeleton (microtubules and microfilaments) do seem to be involved in the induction of nuclear division (7,8,18), and it is possible that a connection between the hyphal surface and the cytoskeleton could be part of the sensing mechanism. Therefore, we suggest that the extracellular proteins function by either binding the germling to the surface so that the germ tube can sense the inductive topography or binding and transmitting to the cytoskeleton information about the inductive topography. Too little is known yet to evaluate these possibilities.

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