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Barley Pathogenesis-Related Proteins with Fungal Cell Wall Lytic Activity Inhibit the Growth of Yeasts[1]

Jean Grenier, Claude Potvin, and Alain Asselin*

Département de Phytologie, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Québec, Canada G1K 7P4

Proteins from intercellular fluid extracts of chemically stressed barley (*Hordeum vulgare L.*) leaves were separated by native polyacrylamide gel electrophoresis at alkaline or acid pH. Polyacrylamide gels contained *Saccharomyces cerevisiae* (bakers' yeast) or *Schizosaccharomyces pombe* (fission yeast) crude cell walls for assaying yeast wall lysis. In parallel, gels were overlaid with a suspension of yeasts for assaying growth inhibition by pathogenesis-related proteins. The same assays were also performed with proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. In alkaline native polyacrylamide gels, only one band corresponding to yeast cell wall lytic activity was found to be inhibitory to bakers' yeast growth, whereas in acidic native polyacrylamide gels one band inhibited the growth of both yeasts. Under denaturing nonreducing conditions, one band of 19 kD inhibited the growth of both fungi. The 19-kD band corresponded to a basic protein after two-dimensional gel analysis. The 19-kD protein with yeast cell wall lytic activity and inhibitory to both yeasts was found to be different from previously reported barley chitosanases that were lytic to fungal spores. It could be different from other previously reported lytic antifungal activities related to pathogenesis-related proteins.

PR proteins are host proteins induced by various pathogens and stress-related factors (for recent reviews, see Carr and Klessig, 1989; Bol et al., 1990; Lotan and Fluhr, 1990; Linthorst, 1991). They have been classified into five families or groups (Carr and Klessig, 1989; Bol et al., 1990). Group 1 corresponds to PR-1 tobacco proteins of unknown function (approximately 16 kD). There are recent reports of the antifungal activity of PR-1-type proteins (Lawton et al., 1993; Niderman et al., 1993). Group 2 has been recently subdivided into 2a (β-1,3-glucanases of about 33–41 kD) and 2b (β-1,3-glucanase of about 25 kD) (Bol et al., 1990). Group 3 is composed of chitinases (approximately 28–34 kD), and group 4 consists of low molecular mass (13 and 14.5 kD) proteins of unknown function (Linthorst et al., 1991). Tobacco PR4 protein is homologous to potato Win stress proteins (Friedrich et al., 1991). Group 5 is also subdivided into 5a and 5b and includes osmotins (about 24 kD) (thaumatin-like proteins) and another protein of unknown function (approximately 45 kD) (Bol et al., 1990). Some group 5 PR proteins have been reported to have membrane-permeabilizing activities (Bol et al., 1990; Linthorst et al., 1991; Vigers et al., 1991). There is also one report on the antifungal activity of PR-5-type proteins (Woloshuk et al., 1991).

Some of the PR proteins are thus enzymes with antimicrobial potential. In addition to β-1,3-glucanases and chitinases, chitosanases have also been recently described as stress-related PR proteins in barley (*Hordeum vulgare*) leaves, cucumber cotyledons, and tomato leaves (Grenier and Asselin, 1990). Although the precise role of PR proteins is still debated, some PR proteins have been shown to be antifungal in vitro (Mauch et al., 1988; Woloshuk et al., 1991; Vigers et al., 1992). Seed proteins related to PR proteins have also been reported to be antifungal in vitro (for reviews, see Borgmeyer et al., 1992; Hejgaard et al., 1992). In many cases, PR proteins or seed PR-like proteins seem to act synergistically (chitinase plus β-1,3-glucanase or protein plus antibiotic-like nikkomycin) for inhibiting the active growth of fungi. In this manuscript, some barley PR proteins are shown to inhibit the growth of bakers' and/or fission yeast without the addition of complementary inhibitory factors.

MATERIALS AND METHODS

Induction of PR Proteins

Barley (*Hordeum vulgare* cv Léger) leaves were stressed with $AgNO_3$ as previously described (Grenier and Asselin, 1990). IF extracts (Parent and Asselin, 1984) were used as sources of PR proteins. Protein concentration was determined by using the Bio-Rad protein assay reagent. Control barley leaves were floated on water instead of $AgNO_3$ (Grenier and Asselin, 1990).

Native One-Dimensional PAGE

Proteins were separated by native 15% (w/v) PAGE at pH 4.3 (Reisfeld system) (Reisfeld et al., 1962) or at pH 8.9 (Davis system) (Davis, 1964) as previously described (Grenier and Asselin, 1990). Polyacrylamide gels contained 2 mg mL^{-1} of *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* crude cell walls prepared by extraction of washed yeast cells in 3% (w/v) NaOH at 75°C for 6 h (Bacon et al., 1969). Detection of yeast cell wall lysis in gels was as follows. After electro-

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* Corresponding author; fax 1–418–656–7806.

Abbreviations: ATCC, American Type Culture Collection; CM, carboxymethyl; 2-D, two-dimensional; IF, intercellular fluid; PR, pathogenesis-related protein; YPD, yeast potato dextrose.

phoresis, gels were incubated in 10 mM sodium acetate (pH 5.0) for 20 min at 37°C. Gels were transferred to a fresh solution of sodium acetate buffer and incubated at 37°C for 100 min. Gels were then stained in 0.005% (w/v) aniline blue in 150 mM K_2HPO_4 (pH 8.6) for 15 min at room temperature (Côté et al., 1989). Gels were destained for 2 min in distilled water at room temperature. Lytic zones were revealed by fluorescence using a long-wave UV transilluminator (C-62; UV Products, San Gabriel, CA). Gels were photographed (Polaroid film No. 667) as previously described (Côté et al., 1989).

Denaturing One-Dimensional PAGE

Proteins were subjected to denaturing SDS-polyacrylamide gels (15%, w/v) under nonreducing conditions (Grenier and Asselin, 1990). As for native gels, denaturing gels contained crude yeast walls for detection of cell wall lysis. Gels containing fungal walls were incubated after electrophoresis in 10 mM sodium acetate (pH 5.0) and 1% (v/v) purified Triton X-100 (Grenier and Asselin, 1990) for 5 h following the initial 20-min incubation. Lysis zones were visualized as for native gels.

2-D PAGE

2-D gels involved first-dimension native gels run at pH 4.3 or 8.9, followed by SDS-gel electrophoresis under nonreducing conditions in the second dimension (Grenier and Asselin, 1990). A set of gels was stained for protein with Coomassie blue, followed by aqueous silver nitrate (Grenier and Asselin, 1990). With another set of gels run in parallel, the 2-D gels contained 2 mg mL^{-1} of *Saccharomyces* or *Schizosaccharomyces* crude cell walls. In addition to fungal walls, 2-D gels also contained 0.01% (w/v) glycol chitosan for detection of chitosanase activity (Grenier and Asselin, 1990). Chitosanase activity was detected by calcofluor white M2R staining as previously described (Grenier and Asselin, 1990). Fungal wall lysis was detected as clear bands when gels were observed against a dark background. Bands of lysis were photographed against a black background (Grenier and Asselin, 1990).

Growth-Inhibition Assays after PAGE

After native PAGE, gels were incubated twice for 5 min at room temperature in Bacto YPD broth. The gel was transferred to a sterile Petri plate and air dried for 2 min under laminar air flow. A sterile No. 3MM paper impregnated with a yeast cell suspension (10^8 cells mL^{-1} in YPD broth) was gently laid on the surface of the gel. After 1 min, the No. 3MM paper was removed, and the gel was incubated in a sealed Petri plate at 30°C for 48 h. Gels were then photographed against a black background to help visualize the growth-inhibition zones. After denaturing PAGE, gels were incubated twice in 25% (v/v) isopropanol in 10 mM sodium acetate (pH 5.0) at 37°C for 25 min. After incubation, denaturing gels were processed as for native gels.

Substrate Specificity Assays

Cell suspensions of *Candida utilis* (*Torula* yeast; Sigma), *Candida pseudotropicalis* (Sigma; ATCC-2512), *S. pombe* (fission yeast), and *S. cerevisiae* (Fleischmann, commercial; isolate YPH-274; isolate ATCC-4109) were used in 15% (w/v) SDS-polyacrylamide gels at a final concentration of 5 mg mL^{-1} to yield an opaque background. *S. cerevisiae* cells were also chemically treated to prepare crude cell wall preparations enriched in glucan components (Bacon et al., 1969) as embedded substrates into SDS-gels. Commercial bakers' yeast (15 g *S. cerevisiae*; Fleischmann) were suspended in 500 mL of 3% (w/v) NaOH and incubated with occasional stirring at 75°C for 6 h. After cooling at room temperature, cells were centrifuged at 10,000*g* for 10 min and washed twice in 200 mL of 25 mM sodium phosphate buffer at pH 6.5. After an additional wash in distilled water (200 mL), cells were resuspended in 200 mL of 3% (v/v) acetic acid and incubated with occasional stirring at 90°C for 4 h. After cooling at room temperature, crude walls were centrifuged at 10,000*g* for 10 min. The acetic acid treatment was repeated, and crude cell walls were finally washed twice in 25 mM sodium phosphate (pH 6.5) and once in distilled water as described above. Crude cell walls were freeze dried.

In one experiment, crude bakers' yeast cell walls were also treated with sodium *m*-periodate and borohydride to chemically destroy (Smith degradation) glucans without β-1,3 linkages. Crude walls (8 mg treated with NaOH and acetic acid) were suspended in 27 mM sodium *m*-periodate for 3 d at 4°C in the dark with occasional stirring. The suspension was then centrifuged at 8,500*g* for 10 min and resuspended in distilled water. This washing procedure was repeated twice before resuspension in 27 mM sodium *m*-periodate for 3 d at 4°C in the dark with occasional stirring. The suspension was then centrifuged at 8,500*g* for 10 min and resuspended in distilled water. This washing procedure was repeated twice before centrifugation (8,500*g*, 10 min), and cell walls were washed three times in distilled water. Bakers' yeast crude cell walls, treated with periodate or untreated, were used as embedded substrates into polyacrylamide gels at a final concentration equivalent to 5 mg mL^{-1} of nontreated cells. Gel (SDS-PAGE) assays of lysis of laminarin, pustulan, and CM-cellulose were performed as previously described (Côté et al., 1991). This was the same for glycol chitin hydrolysis (Trudel and Asselin, 1989).

Yeasts

S. cerevisiae isolate W303a was provided by Dr. M. Fortin (McGill University, Montreal, Canada) and *S. pombe* (isolate CSCT-20) was provided by Dr. R. Lévesque (University of Laval, Quebec, Canada). Both yeasts were grown in YPD broth at 30°C. Other yeasts were from Sigma, and Fleischmann bakers' yeast was bought in local stores.

RESULTS

Growth Inhibition of Bakers' Yeast by Stressed Barley IF Extracts

In preliminary experiments, *S. cerevisiae* W 303a cells were grown in the presence of various amounts of stressed barley IF extracts containing PR proteins. After yeast cells were incubated with stressed barley IF extracts for 6 h, up to 74% of yeast cells could not be recovered as live cells (not shown).

This inhibition was not detected with autoclaved IF extracts or IF extracts from nonstressed barley leaves. Thus, the inhibition effect seemed to be related to the presence of stress-related components sensitive to autoclaving. Proteins in IF extracts (PR proteins) from barley leaves were studied accordingly for their possible involvement in growth inhibition of yeasts.

Growth Inhibition and Lysis of Yeast Cell Walls after Native PAGE at Alkaline pH

Stressed barley IF extracts containing PR proteins were subjected to native PAGE at pH 8.9 in polyacrylamide gels containing crude yeast walls for assaying fungal wall lysis because some PR proteins have been previously shown to be lytic to certain fungal cell wall components. At pH 8.9, this electrophoretic system is mostly efficient for separating acidic or neutral proteins (Davis, 1964). Three sets of gels embedded with two types of yeast walls were treated as follows. The first set (Fig. 1, lanes 1) of gels was stained with Coomassie blue for protein detection. Silver staining of proteins was not feasible in gels embedded with fungal walls because of interfering background staining of fungal walls. The second set of gels (Fig. 1, lanes 2) was incubated after electrophoresis in sodium acetate buffer at pH 5.0 for detection of fungal wall lysis. Detection of fungal wall lysis was made after aniline blue staining of walls. Dark bands (Fig. 1, lanes 2) corresponded to lysis zones. The third set of gels (Fig. 1, lanes 3) corresponded to gels overlayed with a suspension of live *Saccharomyces* (Fig. 1A) or *Schizosaccharomyces* (Fig. 1B) cells for subsequent assay of growth inhibition. When barley IF leaf extracts containing PR proteins induced by chemical stress (Grenier and Asselin, 1990) were separated by native PAGE at pH 8.9, growth inhibition of *S. cerevisiae* was observed (Fig. 1A, lane 3) at a level corresponding in electrophoretic mobility to a *Saccharomyces* wall lysis band (Fig. 1A, lane 2, upper dark band) and to a band stained lightly after Coomassie blue (Fig. 1A, lane 1, arrow). No growth inhibition of *Schizosaccharomyces* was observed (Fig. 1B, lane 3) even if wall lysis activities acting on *Schizosaccharomyces* walls were present (Fig. 1B, lane 2). The lytic band inhibitory to *Saccharomyces* (Fig. 1A, arrow) was not inhibitory to *Schizosaccharomyces* (Fig. 1B, arrow). Diagonal dark bands in Figure 1A reflect insufficient contact areas of the No. 3MM paper with the gel surface. Growth-inhibition assays with gels were repeated at least three times for each electrophoretic system.

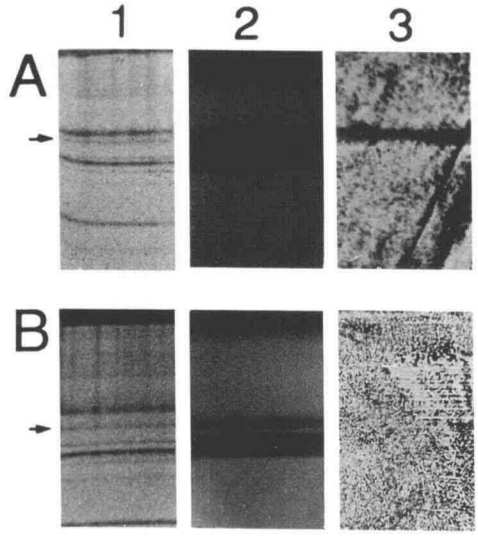

Figure 1. Detection of barley PR proteins and yeast wall lytic and antifungal activities after native PAGE at alkaline pH. Stressed barley IF proteins (50 μg) were subjected to native PAGE at pH 8.9 in 15% (w/v) polyacrylamide gels containing 2 mg (wet weight) mL^{-1} of NaOH-treated yeast crude walls (A, *S. cerevisiae* walls; B, *S. pombe* walls). After electrophoresis, barley PR proteins were stained with Coomassie blue G-250 (lanes 1). Yeast wall lytic activities (lanes 2) were revealed by UV fluorescence after aniline blue staining following incubation of gels in sodium acetate buffer at pH 5.0. Antifungal activities (lanes 3) were detected by using an overlay suspension of yeast cells (A, *S. cerevisiae;* B, *S. pombe*) growing for 48 h. The arrow in A corresponds to the lytic band inhibitory to *S. cerevisiae*. The arrow in B corresponds to the same lytic band not inhibitory to *S. pombe*.

Growth Inhibition and Lysis of Yeast Walls after Native PAGE at Acidic pH

Stressed barley IF extracts were subjected to native PAGE at pH 4.3. This electrophoretic system was designed to separate mostly basic proteins (Reisfeld et al., 1962). After native PAGE at acidic pH, one zone of *Saccharomyces* growth inhibition (Fig. 2A, lane 3) was observed at the level of a *Saccharomyces* wall lytic activity (Fig. 2A, lane 2, dark band at the level of the growth-inhibition zone). The zones of wall lysis and growth inhibition corresponded in electrophoretic mobility to a major Coomassie blue-stained band (Fig. 2A, lane 1, arrow). It is noteworthy that the protein bands appear like diffuse lysis zones and are not as well resolved as in the native PAGE system at alkaline pH. This can be explained by interactions between some proteins and the embedded fungal walls during electrophoresis at pH 4.3. This phenomenon does not seem to be as important at alkaline pH. Fungal wall lysis and growth inhibition of yeasts were best detected at pH 5.0 (not shown), which is close to the pH used for electrophoresis. Contrary to what was observed after native PAGE at pH 8.9, *Schizosaccharomyces* growth was inhibited (Fig. 2B, lane 3) in a zone corresponding to *Schizosaccharomyces* wall lysis (Fig. 2B, lane 2, dark zone at the level of the *Schizosaccharomyces* growth-inhibition zone) and to a protein band stained with Coomassie blue (Fig. 2B, lane 1, arrow at the level of the lysis and growth-inhibition zones).

Growth Inhibition and Lysis of Yeast Walls after Denaturing PAGE

Stressed barley IF extracts were subjected to denaturing SDS-PAGE under nonreducing conditions (without DTT or β-mercaptoethanol) to estimate the apparent molecular mass of active proteins. The addition of reductants precluded the detection of active lytic or inhibitory proteins (not shown). Under nonreducing conditions, SDS-PAGE allowed the detection of two inhibition zones when *Saccharomyces* growth was studied (Fig. 3A, lane 3). The two inhibition zones

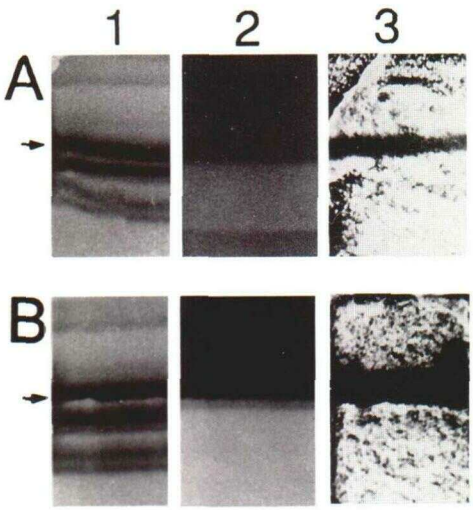

Figure 2. Detection of barley PR proteins and yeast wall lytic and antifungal activities after native PAGE at acidic pH. Stressed barley IF proteins (50 μg) were subjected to native PAGE at pH 4.3 in 15% (w/v) polyacrylamide gels containing 2 mg (wet weight) mL^{-1} of NaOH-treated yeast crude walls (A, *S. cerevisiae*; B, *S. pombe*). After electrophoresis, barley PR proteins were stained with Coomassie blue G-250 (lanes 1). Yeast wall lytic activities (lanes 2) were revealed by UV fluorescence after aniline blue staining following incubation of gels in sodium acetate buffer at pH 5.0. Antifungal activities (lanes 3) were detected by using an overlay suspension of yeast cells (A, *S. cerevisiae*; B, *S. pombe*) growing for 48 h. The arrow in A corresponds to the lytic band inhibitory to *S. cerevisiae*. The arrow in B corresponds to the same lytic band also inhibitory to *S. pombe*.

corresponded in migration to lysis activities (Fig. 3A, lane 2, one small band above the 18.5-kD marker and one large band below the same marker). The upper band corresponded to a Coomassie blue-stained band (Fig. 3A, lane 1, arrow). This was not the case for the lower diffuse band (Fig. 3A, lane 1, area corresponding to the lower diffuse band in lane 2). When proteins were assayed for *Schizosaccharomyces* growth inhibition, only one growth-inhibition band was observed near 19 kD (Fig. 3B, lane 3) as in the case of the upper band observed with *Saccharomyces* (Fig. 3, B versus A, lanes 3). As for *Saccharomyces*, the *Schizosaccharomyces* wall lysis zone (Fig. 3B, dark band in lane 2 versus lane 3) corresponded to a protein stained with Coomassie blue (Fig. 3B, arrow).

Growth Inhibition and Lysis of Yeast Walls after 2-D PAGE and Comparison with Chitosanase Activities

A 2-D PAGE system was used to detect barley PR proteins, fungal wall lysis, and growth-inhibition activities (Fig. 4). The 2-D gel system was a combination of native PAGE at pH 4.3 or 8.9 in the first dimension followed by SDS-PAGE (under nonreducing conditions) in the second dimension. The 2-D SDS-gel contained yeast walls and glycol chitosan in some assays because some barley PR proteins have been previously identified as chitosanases lytic to fungal spores (Grenier and Asselin, 1990). The presence of yeast walls and glycol chitosan in such gels did not significantly influence the migration of proteins in the presence of SDS. The previously observed band near 19 kD (Fig. 3) exhibiting growth inhibition against both yeasts was also detected after 2-D gel analysis (Fig. 4). Growth inhibition of *Saccharomyces* after 2-D gel separation (Fig. 4D, L, right panel) was observed at the same spot as *Saccharomyces* wall lytic activity (Fig. 4B, L, right panel). This activity corresponded to a protein band stained with Coomassie blue (Fig. 4A, L, right panel). The yeast growth-inhibition activity and the yeast wall lytic activity did not co-migrate with previously reported lytic chitosanases (Fig. 4C, barley chitosanases 3, 4, 5, and 6 versus L spot). In addition to the basic 19-kD lytic and inhibitory barley protein corresponding to a major PR protein (as detected by Coomassie blue staining) and distinct from chitosanase lytic activities, one *Saccharomyces* growth-inhibition spot was also observed in the 2-D gel system when proteins were first separated at alkaline pH. This activity (Fig. 4D, L, left panel) did not, however, correspond after 2-D separation to yeast wall lysis activities (Fig. 4B, left panel) or to chitosanase (Fig. 4C, left panel) or the Coomassie blue-stained band (Fig. 4A, left panel).

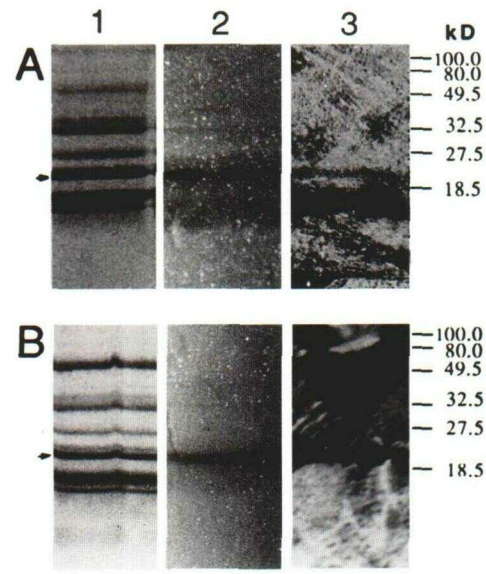

Figure 3. Detection of barley PR proteins and yeast wall lytic and antifungal activities after SDS-PAGE. Stressed barley IF proteins (50 μg) were subjected to SDS-PAGE under nonreducing conditions in 15% (w/v) polyacrylamide gels containing 2 mg (wet weight) mL^{-1} of NaOH-treated yeast crude walls (A, *S. cerevisiae*; B, *S. pombe*). After electrophoresis, barley PR proteins were stained with Coomassie blue R-250 (lanes 1). Yeast wall lytic activities (lanes 2) were revealed by UV fluorescence after aniline blue staining following incubation of gels in sodium acetate buffer at pH 5.0 preceded by isopropanol treatment. Antifungal activities (lanes 3) were detected by using an overlay suspension of live yeast cells (A, *A. cerevisiae*; B, *S. pombe*) growing for 48 h. Molecular mass markers are indicated on the right. The arrow in A corresponds to the 19-kD lytic band inhibitory to *S. cerevisiae*. The arrow in B corresponds to the same 19-kD lytic band also inhibitory to *S. pombe*.

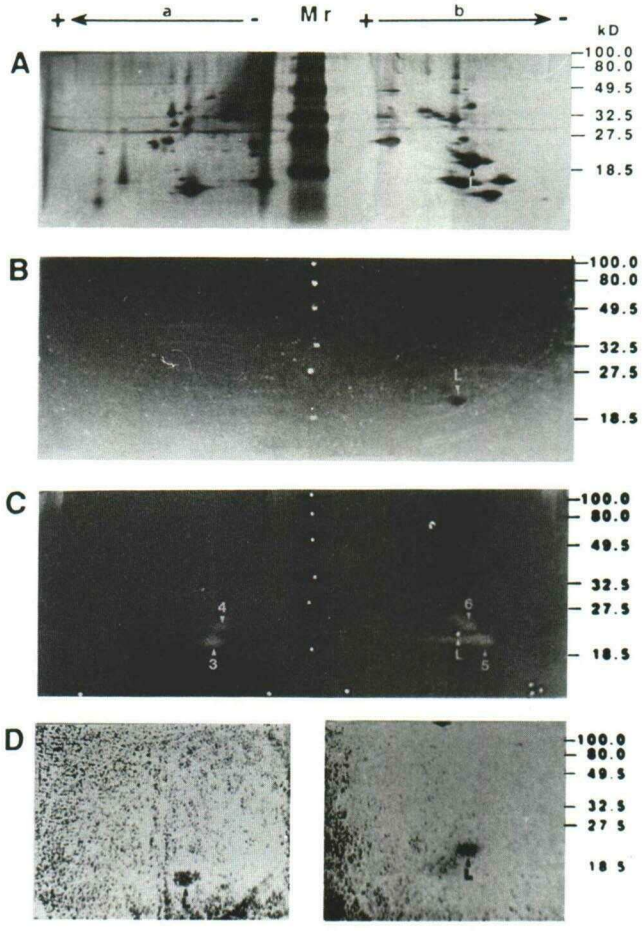

Figure 4. Detection of barley PR proteins and yeast wall lytic, chitosanase, and antifungal activities after 2-D PAGE. Stressed barley IF proteins (50 μg) were subjected in the first dimension to native PAGE at pH 8.9 (upper arrowhead in a) as in Figure 1 or to native PAGE at pH 4.3 (upper arrowhead in b) as in Figure 2. The 2-D gel was run under nonreducing conditions in SDS gels as in Figure 3 in 15% (w/v) polyacrylamide gels without (A and D) or with (B and C) 2 mg (wet weight) mL^{-1} of NaOH-treated crude *Saccharomyces* walls in addition to 0.01% (w/v) glycol chitosan. After electrophoresis, barley PR proteins were stained with Coomassie blue R-250 (A). *Saccharomyces* lytic activity (B, L) was revealed as in the Figures 1 to 3. Chitosanase activities (C; 3, 4, 5, and 6 versus L) were revealed after Coomassie blue staining followed by aqueous silver nitrate staining (Grenier and Asselin, 1990). Antifungal activities (D) were detected by using an overlay suspension of live *Saccharomyces* cells growing for 48 h. Molecular mass markers are indicated on the right. L in panel D corresponds to growth-inhibition zones of *S. cerevisiae*.

Substrate Specificity of the 19-kD Protein

The 19-kD barley protein was subjected to SDS-PAGE under nonreducing conditions and assayed for lysis of various crude yeast cell suspensions in addition to some yeast wall preparations (chemically treated to selectively remove components) and polysaccharides reported to be present in fungal walls. Results are summarized in Table I. The 19-kD barley protein was lytic to *Candida* yeasts in addition to bakers' yeast and fission yeast. Lysis of *Candida* was detected by the presence of a clear band at 19 kD against the opaque yeast cell suspension embedded in the gel matrix. Chemically treated yeast crude walls were also lysed by the barley 19-kD protein (Table I, treated yeasts). In this case, bakers' yeast walls treated extensively with sodium hydroxide, acetic acid, or periodate were still sensitive to lysis. Such walls are mostly enriched in β-1,3-glucans. Finally, laminarin, pustulan, glycol chitin, glycol chitosan, and CM-cellulose were not hydrolyzed by the 19-kD barley protein. An alkali-soluble *Saccharomyces* yeast β-1,3-glucan (Cabib and Bowers, 1971) was also not hydrolyzed by the 19-kD barley protein. Overall, these results suggest that some insoluble β-1,3-glucan (but not laminarin, a soluble oligomeric β-1,3-glucan, or an alkali-soluble yeast glucan) might be the substrate hydrolyzed by the 19-kD barley antifungal protein.

DISCUSSION AND CONCLUSION

The present results demonstrate that some barley PR proteins can inhibit the in vitro growth of two ascomycetous yeasts as detected by an overlay assay after PAGE separation of proteins. A similar overlay technique has been used recently for detecting antifungal activity of α-purothionin, *Urtica dioica* agglutinin, and tobacco chitinase (De Bolle et al., 1991). With the present technique, the yeast growth-inhibition assay could be used not only with native PAGE but also after SDS-PAGE. Moreover, the technique did not require an agar layer spread on top of the polyacrylamide gel. In addition, the detection of antifungal activity could be assessed for cell wall lytic activity by using gels run in parallel and embedded with crude fungal walls. This approach can allow a comparison of antifungal activity with fungal cell wall lysis. Finally, the technique can also be refined by using mixed substrates (for example, crude cell walls plus chitosan) that can allow detection of two lytic activities within the same gel provided that distinct detection methods are available.

To our knowledge, this is the first report dealing with the assay of antifungal activity of plant proteins separated by SDS-PAGE and with the comparative electrophoretic study of antifungal activities for detecting complex or mixed substrate lysis. By using these electrophoretic assays, we could observe two types of barley PR antifungal activities as far as apparent molecular masses are concerned. A 19-kD barley protein was inhibitory to both yeasts (*Saccharomyces* and *Schizosaccharomyces*), and a 15-kD protein was only inhibitory toward *Saccharomyces*. The use of 2-D gels involving native PAGE in the first dimension and SDS-PAGE under nonreducing conditions in the second dimension showed that the 19-kD protein is probably basic, because it is well separated in the Reisfeld electrophoretic system designed for separating basic proteins at acidic pH (Reisfeld et al., 1962). On the other hand, the 15-kD protein behaved like an acidic protein well separated at alkaline pH in the Davis electrophoretic system (Davis, 1964).

S. cerevisiae (bakers' yeast) and *S. pombe* (fission yeast) were chosen because their cell wall compositions are rather well known (Cabib et al., 1982). Moreover, they exhibit an interesting cell wall component difference in that fission yeast does not contain chitin. In addition, the sequential removal

Table I. *Walls of fungi and polysaccharides susceptible or not to hydrolysis by the barley basic 19-kD protein*

Substrate	Treatment or Origin	Susceptibility to Lysis	Ref. for Assay in Gels (SDS-PAGE)
S. cerevisiae	Commercial (Fleischmann)	+	This work
	YPH-274	+	This work
	ATCC-4109	+	This work
C. utilis	Commercial (Sigma)	+	This work
C. pseudotropicalis	ATCC-2512	+	This work
S. pombe	CSCT-20	+	This work
Laminarin (β-1,3-glucan)	Commercial (Sigma)	−	Côté et al., 1989
Pustulan (β-1,6-glucan)	Commercial (Sigma)	−	Côté et al., 1991
Chitosan (β-1,4-glucan) (glycol)	Commercial (Sigma)	−	This work
Chitin (β-1,4-glucan) (glycol)	Trudel and Asselin, 1989	−	Trudel and Asselin, 1989
CM-Cellulose (β-1,4-glucan)	Commercial (Sigma)	−	Côté et al., 1991
Alkali-soluble bakers' yeast (β-1,3-glucan)	Cabib and Bowers, 1971	−	Grenier and Asselin, 1993
C. utilis	NaOH treated	+	This work
C. pseudotropicalis	NaOH treated	+	This work
S. cerevisiae	NaOH (2×) treated	+	This work
	NaOH (27×) treated	+	This work
	m-Periodate treated	+	This work

of yeast cell wall components has been studied in detail. This allows the use of various cell wall preparations enriched in specific cell wall components. For example, bakers' yeast walls are predominantly made of β-1,3-glucans with smaller amounts of β-1,6-glucans in addition to mannan (Cabib et al., 1982; Yamaoka et al., 1989). Various bakers' yeast wall preparations can thus be chosen as substrate because the structure and the sequential chemical removal of the polysaccharide components of yeast cell walls have been the target of numerous studies (Cabib et al., 1982). The biochemistry of yeast cell walls is better known than most other fungi and is thus a very useful substrate for studying fungal wall lytic activity.

The 19-kD barley protein was further studied for its cell wall lytic activity because it acted on both yeast cell walls. By using various gel assays for detection of fungal wall lysis or lysis of known wall polysaccharide components or fractions, we have shown that the 19-kD barley antifungal protein did not hydrolyze laminarin (β-1,3-glucan) or an alkali-soluble bakers' yeast β-1,3-glucan, pustulan (β-1,6-glucan), glycol chitin, glycol chitosan, or CM-cellulose (β-1,4-glucans). However, the 19-kD barley PR protein could easily hydrolyze yeast walls (of both genera) even if yeast walls were treated with periodate to remove non-β-1,3-glucans. Tentatively, the barley 19-kD antifungal protein can thus be identified as a β-1,3-glucanase distinct from laminarinases. There are several examples in which laminarinase activity is not necessarily and uniquely related to the capacity to lyse fungal walls. For example, none of six endogenous β-1,3-glucanases from bakers' yeasts showed any lytic activity against yeast wall, whereas all exhibited laminarinase activity (Hien and Fleet, 1983). Also, it has been shown that laminarinase activity does not necessarily correlate with fungal wall lytic activity in various yeast species including the fission yeast (Phaff, 1977; Jeffries and MacMillan, 1981; Reichelt and Fleet, 1981). Several other studies have shown that β-1,3-glucanases with activity on laminarin are not necessarily endowed with lytic activity on cell walls or alkali-insoluble β-1,3-glucan (Phaff, 1977). There are no clear indications as to why some glucanases are lytic to cell walls and others are not. An important factor could be the high affinity of a lytic enzyme for the insoluble wall glucan (Phaff, 1977). It is noteworthy that the barley 19-kD enzyme is efficiently retarded during native PAGE at acidic pH in the presence of yeast wall (Fig. 2). Moreover, several investigators have selectively adsorbed lytic activities in crude preparations with insoluble walls (Phaff, 1977). Finally, it is interesting to note that a debranching *Rhizopus* glucanase with an estimated molecular mass of 19 kD has been reported (Yamamoto et al., 1972). This enzyme acted as a glucanase on yeast glucan with very little activity on laminarin and was reported to be a β-glucanase (endo) of the debranching type. This fungal enzyme could be similar to the barley 19-kD PR protein reported here.

In summary, two barley PR proteins exhibited antifungal activities toward yeasts. Until now, the 19-kD protein could have corresponded to new PR proteins, and the same is true for the 15-kD barley protein (not shown). The significance and precise characterization of such antifungal lytic activities associated with the PR response of plants to stress remain to be determined, as does their relationship to the various types of PR proteins.

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