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# Some Pathogenesis-Related Proteins Are Chitosanases with Lytic Activity Against Fungal Spores

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**Proteins from intercellular fluids (IFs) of nonstressed and stressed barley leaves, cucumber cotyledons, and tomato leaves were analyzed for chitinase and chitosanase activity in sodium dodecyl sulfate (SDS)-polyacrylamide gels. Glycol chitin incorporated in the gel matrix was used as the substrate for detection of chitinases, while glycol chitosan was the substrate of chitosanases. Chitinases with molecular masses between 25 and 35 kDa were stimulated in stressed barley and cucumber. Chitosanases with molecular masses between 10 and 24 kDa were stimulated in stressed barley, cucumber, and tomato. Chitosanase activities in IF extracts of stressed tissue were also analyzed after two-dimensional gel electrophoresis involving separation in native gels at pH 8.9 (Davis system) and 4.3 (Reisfeld system) in the first dimension and SDS gels in the second dimension. In stressed barley leaves, four acidic (three at 22 kDa, one at 19 kDa) and two basic (one at 22 kDa, one at 19 kDa) chitosanases were detected. Four acidic chitosanases (one at 10 kDa, one at 12 kDa, and two at 14 kDa) were also observed in stressed cucumber cotyledons, while one basic chitosanase (24 kDa) was found in stressed tomato. Chitosanases were also evaluated for their capacity to hydrolyze fungal spores of pathogens. Spore suspensions were embedded in polyacrylamide gels, and lysis was observed visually by transparency through the opaque spore suspension. In one-dimensional SDS gels, lysis of *Fusarium oxysporum* f. sp. *radicis-lycopersici* was observed with stressed barley and tomato IF extracts. In tomato, the activity lysing spores corresponded to the major basic chitosanase at 24 kDa. In barley, the spore lytic activity migrated exactly as did basic chitosanase 6 (19 kDa) after analysis in a two-dimensional gel system. *Verticillium albo-atrum* and *Ophiostoma ulmi* spores were also lysed by the same chitosanases. By using these new gel assays, up to six chitosanase activities of low molecular mass (below 24 kDa), distinct from chitinase activities, can be detected in IF extracts of stressed tissue.**

*Additional keyword*: stress.

Pathogenesis-related (PR) proteins are host-encoded proteins induced by some pathogens or certain types of stress (for review, see Carr and Klessig 1989). Up until now, PR proteins and PR genes have been most studied in tobacco (Lotan *et al.* 1990; Ohshima *et al.* 1990). There are five families or groups of tobacco PR proteins (Carr and Klessig 1989) that are used as references for nomenclature. Family 1 is represented by PR-1 tobacco proteins (~16 kDa) of unknown function. Family 2 refers to $\beta$-1,3-glucanases (Kauffmann *et al.* 1987; Côté *et al.* 1989), and family 3 refers to chitinases (Legrand *et al.* 1987; Trudel *et al.* 1989). Family 4 is made up of two low molecular mass (~13 kDa, ~15 kDa) proteins of unknown function, while the family 5 protein (~22 kDa) has sequence homology with thaumatin (Pierpoint *et al.* 1987; Richardson *et al.* 1987).

In addition to tobacco (*Nicotiana tabacum* L.), PR proteins have been found in a variety of plant species including tomato (*Lycopersicon esculentum* Mill.), potato (*Solanum tuberosum* L.), bean (*Phaseolus vulgaris* L.), cucumber (*Cucumis sativus* L.), cowpea (*Vigna sinensis* (Torner) Savi), and several other dicotyledonous species (cited in Carr and Klessig 1989). Barley (*Hordeum vulgare* L.) (Fischer *et al.* 1989a; Bryngelsson and Green 1989) and maize (Nasser *et al.* 1988) are the most studied plants among monocotyledonous species for PR proteins.

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Recently, much attention has been given to PR chitinases and $\beta$-1,3-glucanases because they represent potential antifungal proteins (Benhamou *et al.* 1989; Mauch *et al.* 1988a, 1988b; Roberts and Selitrennikoff 1988; Schlumbaum *et al.* 1986; Trudel *et al.* 1989). In the context of identifying and evaluating potential antimicrobial hydrolases associated with PR proteins, we report that some proteins in intercellular fluid (IF) extracts from barley, cucumber, and tomato leaves under chemical stress (and viral stress in the case of cucumber) show chitosanase activity as detected by lysis of glycol chitosan by proteins separated in various polyacrylamide gel systems. Chitosanases (EC 3.2.1.99) (Monaghan *et al.* 1973) (acting on chitosan without activity on chitin) were distinguished from chitinases (acting on chitin without activity on chitosan) by their molecular weight and substrate specificity. Chitosan (poly (1→4)-$\beta$-D-glucosamine) can be defined as deacetylated chitin. Chitosanases thus represent another type of hydrolase in IF extracts of stressed plant tissue with activity toward a specific fungal cell wall polysaccharide. To our knowledge, this is the first report of extracellular stress-related plant chitosanases distinct from chitinases that can be lytic to fungal spores of some plant pathogens.

## MATERIALS AND METHODS

**Chemicals.** All chemicals for electrophoresis, analytical grade mixed bed resin AG 501-X8 (20–50 mesh), protein molecular mass markers, and Coomassie Brilliant Blue R

250 were from Bio-Rad (Mississauga, Ontario, Canada). Protein concentration was determined with the Bio-Rad protein assay reagent. Glycol chitosan, chitosan, and all other chemicals were from Sigma Chemical Co. (St. Louis, MO). Glycol chitin was synthesized as previously described (Trudel and Asselin 1989) from the same lot of glycol chitosan used for the assay of chitosanase activity. The degree of deacetylation of substrates was determined by titration according to Sannan *et al.* (1976).

**Fungal spores.** *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker and *Verticillium albo-atrum* Reinke & Berthier were supplied by N. Benhamou (Université Laval). Both fungi were maintained on potato dextrose agar at 22° C. For production of spores (conidia), both fungi were grown with shaking at 22° C in potato dextrose broth. Spores were separated from hyphae by filtering through one layer of Miracloth and concentrated by centrifugation (10,000 × *g*, 10 min, 10° C). *Ophiostoma ulmi* (Buisman) Nannf. was provided by G. B. Ouellette (Agriculture Canada, Ste-Foy, Québec, Canada) and grown on potato dextrose agar. Spores were obtained by flooding the petri dishes with sterile distilled water. The concentration of spores was determined with a hemacytometer.

**Plant material.** All plants were grown under greenhouse conditions. Barley (*H. vulgare* cv. Léger) leaves were grown for 25 days. Cucumber (*C. sativus* SMR-18) was grown to the cotyledon stage, and tomato (*L. esculentum* cv. Rutgers) leaves were used 40 days after germination.

**Induction of PR proteins and IF extracts.** Barley leaves (5-cm sections) were floated for 3 days on 100 $\mu$M $AgNO_3$ and cucumber cotyledons on 50 $\mu$M $AgNO_3$. Tomato leaves (5-cm sections) were stressed on 1 mM serine as previously described (Asselin *et al.* 1985). Controls were run as previously described (Asselin *et al.* 1985). IF extracts were prepared by vacuum infiltration followed by low-speed centrifugation (Parent and Asselin 1984). Cucumber cotyledons were also infected with $U_2$-tobacco mosaic virus (Trudel *et al.* 1989) for induction of PR proteins.

**Polyacrylamide gel electrophoresis (PAGE).** Proteins were separated in one-dimensional native or denaturing 15% (w/v) polyacrylamide gel systems as previously described (Trudel *et al.* 1989). They were also separated in two-dimensional gels involving first-dimension native PAGE at pH 4.3 or pH 8.9 exactly as described by Trudel *et al.* (1989) except for the use of 15% (w/v) polyacrylamide instead of gradient gels for the second dimension. Proteins were stained with Coomassie blue (Trudel *et al.* 1989).

**Detection of chitinase and chitosanase activity after PAGE.** Chitinase activity was detected by calcofluor white M2R staining after lysis of glycol chitin as substrate as described for tobacco PR proteins (Trudel *et al.* 1989). Chitosanase activity was detected after hydrolysis of 0.01% (w/v) glycol chitosan as substrate in the gel matrix. Detection was with calcofluor white staining as was done for chitinase activity or by staining the intact glycol chitosan with Coomassie blue. After electrophoresis, sodium dodecyl sulfate (SDS) gels were first incubated at room temperature in 200 ml of sodium acetate buffer (0.01 M at pH 5.0) with 1% (v/v) purified Triton X-100 (Trudel and Asselin 1989). After 20 min, the buffered Triton X-100 solution was replaced and incubated for 18 hr at 37° C with gentle shaking. Staining with calcofluor white or Coomassie blue was previously described (Trudel *et al.* 1989). Chitosanase activity was also detected after native PAGE. Gels run at pH 8.9 contained 0.01% (w/v) glycol chitosan. After electrophoresis, gels were first incubated as was done for SDS gels, except for incubation during 10 instead of 20 min. Gels were then placed on a glass plate and incubated in sodium acetate buffer in a plastic container under moist conditions for 4 hr. Chitosanase activity in gels run at pH 4.3 was detected by an overlay 7.5% (w/v) polyacrylamide gel (0.75 mm in thickness) containing 0.01% (w/v) glycol chitosan. Transfer of proteins to the overlay gel was made by blotting according to Audy *et al.* (1988). Staining of native gels was exactly as was done for SDS gels.

**Detection of lysis in polyacrylamide gels containing fungal spores.** After one-dimensional SDS-PAGE or two-dimensional PAGE in 15% polyacrylamide gels containing $2\text{–}4 \times 10^7$ spores per milliliter (concentration sufficient to yield an opaque background), gels were incubated for 18 hr in sodium acetate as described above. Lysis zones appeared as clear bands against the opaque spore-containing matrix when visualized with light coming from the back of the gels. Bands of lysis were photographed against a black background provided by a black cloth as previously described (Audy *et al.* 1989).

## RESULTS AND DISCUSSION

**Protein patterns in IF extracts of nonstressed and stressed barley, cucumber, and tomato.** IF extracts of nonstressed and chemically stressed barley leaves, cucumber cotyledons, and tomato leaves were first analyzed by SDS-PAGE for the presence of PR proteins (Fig. 1A). Several low molecular mass proteins were detected in IF extracts after chemical stress of green tissue in barley (Fig. 1A, lane 1), cucumber (Fig. 1A, lane 3), and tomato (Fig. 1A, lane 5). Similar IF extracts from nonstressed barley (Fig. 1A, lane 2), cucumber (Fig. 1A, lane 4), and tomato (Fig. 1A, lane 6) yielded small amounts of fewer proteins with molecular masses different from proteins in stressed tissues. Aqueous silver nitrate (barley and cucumber) and serine (tomato) were thus very effective for inducing the accumulation of extracellular stress proteins. With cucumber cotyledons, infection by tobacco mosaic virus (strain $U_2$) yielded the same type of protein pattern after electrophoretic analysis of IF extract (not shown). Chemical stress was used because it yielded highly reproducible protein patterns as previously reported in tobacco (Asselin *et al.* 1985).

Induction of barley PR proteins (Fig. 1A, lane 1) was highly efficient with $AgNO_3$ especially when compared to $CdCl_2$, $ZnCl_2$, $MnCl_2$, $MgCl_2$, and salicylate. All these chemicals had been shown not to induce PR proteins in barley (Fischer *et al.* 1989a). Induction of cucumber PR proteins by $AgNO_3$ (Fig. 1A, lane 3) was less efficient and did not produce significant amounts of cucumber PR protein E (or $\gamma$) of 22 kDa. This PR protein was reported after hypersensitive reaction to tobacco necrosis virus (Wagih and Coutts 1982). There are several cases (cited in Carr and Klessig 1989) where abiotic inducers (including salicylate) are not equally effective in inducing the

accumulation of all PR proteins or PR protein families. Induction of tomato PR proteins (Fig. 1A, lane 5) by serine yielded numerous proteins in addition to tomato PRp14 protein (main band migrating at the level of 14 kDa). Tomato PRp14 protein is the best studied tomato PR protein and is homologous to tobacco PR-1 proteins (in Carr and Klessig 1989).

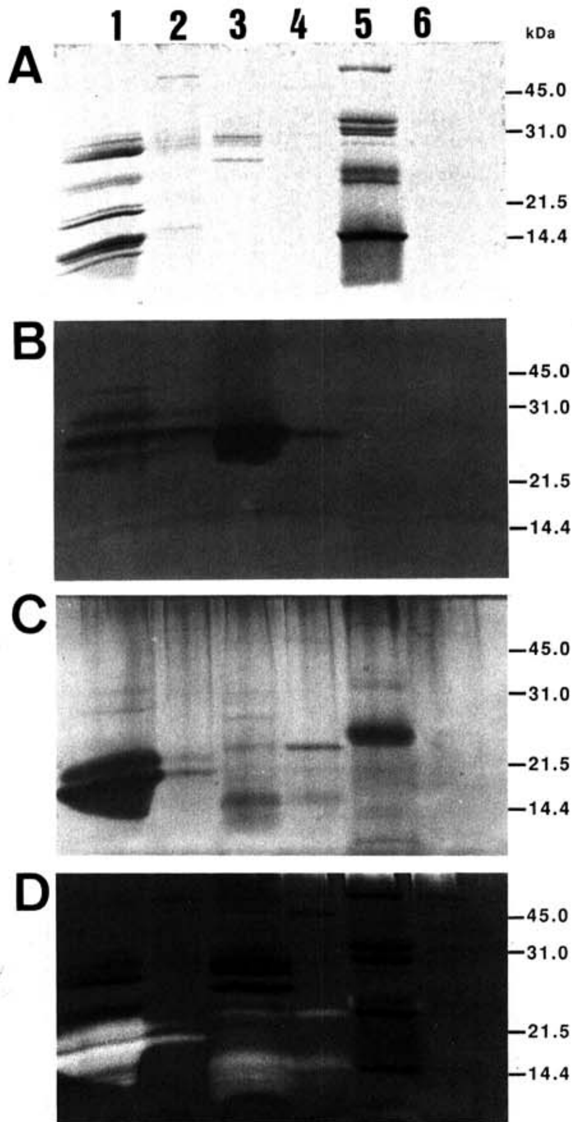


**Fig. 1.** Proteins, chitinase, and chitosanase activities after electrophoresis in 15% (w/v) polyacrylamide-sodium dodecyl sulfate (SDS) gels. Intercellular fluid (IF) extracts (20 μl) of chemically stressed barley ($AgNO_3$) leaves (20 μg of protein) (lane 1), cucumber ($AgNO_3$) cotyledons (2 μg) (lane 3), and tomato (serine) leaves (25 μg) (lane 5) were compared to IF extracts from nonstressed barley (2 μg) (lane 2), cucumber (0.2 μg) (lane 4), and tomato (0.1 μg) (lane 6) tissue. In panel **A**, proteins were stained with Coomassie Brilliant Blue R 250. In panel **B**, the gel contained 0.01% (w/v) glycol chitin as substrate for chitinase activity. In panels **C** and **D**, the gels contained 0.01% (w/v) glycol chitosan as substrate for chitosanase activity. After electrophoresis, gels with substrate (**B**, **C**, and **D**) were incubated in 0.01 M sodium acetate (pH 5.0) with 1% (v/v) purified Triton X-100 for 18 hr. Gels with glycol chitin (**B**) and glycol chitosan (**C**) were stained with calcofluor white M2R as previously described (Trudel and Asselin 1989). In panel **D**, the gel was stained with Coomassie blue instead of calcofluor white. Molecular mass markers (kDa) (5 μg) denatured with SDS and mercaptoethanol are indicated on the right. Proteins in IF extracts were denatured by SDS alone (Trudel *et al.* 1989).

**Chitinase versus chitosanase activities in IF extracts of nonstressed and stressed barley, cucumber, and tomato.** IF extracts were analyzed after SDS-PAGE for chitinase activity with glycol chitin as substrate in the gel matrix. Chitinase activity was visualized as dark (nonfluorescent) bands against a UV fluorescent background as previously described for tobacco chitinases (Trudel *et al.* 1989). Stressed barley leaf IF extract showed four bands of chitinase activity at 25, 28, 30, and 35 kDa (Fig. 1B, lane 1). The activities at 28 and 30 kDa were present in smaller amounts (Fig. 1B, lane 2) in nonstressed IF extracts. One large band of chitinase activity was detected at 28 kDa in stressed cucumber cotyledon (Fig. 1B, lane 3), while the same activity was present in much smaller amounts in nonstressed IF extract (Fig. 1B, lane 4). No chitinase activity could be detected after SDS-PAGE of stressed (Fig. 1B, lane 5) or nonstressed (Fig. 1B, lane 6) tomato leaf IF extracts. This is surprising since tomato PR chitinases have been reported to accumulate in parallel with tomato PRp14 (Fischer *et al.* 1989b). It could well be related to the nature of the inducer (serine). However, serine was utilized as the inducer of tomato PR proteins because it yielded very good signals of chitosanase activity. A faint chitinase signal across the gel at the 14.4-kDa level was exactly the same signal as the one already described after SDS-PAGE for lysozyme activity (Audy *et al.* 1989). This signal probably corresponds to contaminating lysozyme-chitinase activity even though we previously identified it as an electrophoretic artifact (Audy *et al.* 1989).

Chitosanase activity (Fig. 1C) was analyzed by incorporating glycol chitosan in the gel matrix, and lysis of glycol chitosan was detected after calcofluor white staining exactly as was done for chitinase activity. Barley showed two large main bands at 19 and 22 kDa in stressed tissue (Fig. 1C, lane 1), while the same bands were faint in nonstressed tissue (Fig. 1C, lane 2). Stressed tissue of cucumber showed a stimulated activity near 14 kDa (Fig. 1C, lane 3), while nonstressed cucumber extract had one band (23 kDa) more prominant than that shown from stressed tissue. One major band of stimulated chitosanase activity was observed at 24 kDa in stressed tomato (Fig. 1C, lane 5), while the same band was not observed in nonstressed tissue (Fig. 1C, lane 6). Except for one band in cucumber, chitosanase activity was significantly stimulated in all tissues and did not correspond in electrophoretic mobility to chitinase activity in the case of the stimulated signals showing the highest lytic activity. Some chitosan preparations yielded the same bands as were seen with glycol chitosan (not shown), indicating the glycolation of chitosan is not the target of enzymatic activity. Glycol chitosan was used routinely because of its solubility in water; chitosan is insoluble in water but is soluble in dilute acids.

Chitosanase activity was also assayed by staining with Coomassie blue (Fig. 1D) because there are some limitations when using calcofluor with chitosan. Calcofluor white binds very strongly to glycol chitosan (or chitosan) and requires rather prolonged destaining (Trudel and Asselin, in press). After prolonged destaining in SDS gels,

some false positive signals were observed as was also reported for chitin deacetylases (Trudel and Asselin, in press). These false positive signals were always associated with large amounts of protein in bands. These false signals were never observed in native gels or after using Coomassie blue for staining instead of calcofluor white. Intact glycol chitosan was intensely stained with Coomassie blue while lysis zones remained unstained (white bands) (Fig. 1D). Intense staining of glycol chitosan with Coomassie blue is not unusual since several anionic dyes have been shown to interact strongly with chitosan. Monosulfonated, disulfonated, and trisulfonated dyes were easily bound to chitosan in a stoichiometric manner (Maghami and Roberts 1988). Coomassie blue is a sulfonic acid dye interacting with amino groups of proteins (De Moreno *et al.* 1986). Relatively high affinities of Coomassie blue with cationic polyamino acids have been reported (De Moreno *et al.* 1986). We extrapolated that it is similar with the cationic polyglucosamine polymer (chitosan).

Staining with Coomassie blue was thus used as a second most reliable and necessary criterion to verify chitosanase activity after SDS-PAGE. By using Coomassie blue, the same pattern of activity of the stimulated major bands was detected after SDS-PAGE of all extracts (Fig. 1D, lanes 1 to 6 versus Fig. 1C, lanes 1 to 6). The major band at 24 kDa in stressed tomato (Fig. 1D, lane 5) appeared as a white signal between two close protein bands also stained with Coomassie blue. One faint band near 10 kDa was detected with the two staining procedures in stressed tomato leaf IF extract (Fig. 1D, lane 5 versus Fig. 1C, lane 5). We interpreted as chitosanases only the bands detected with both staining procedures. Some faint bands after calcofluor white staining were not revealed by Coomassie blue and were thus not identified as chitosanases. It is noteworthy that protein bands in gels containing glycol chitosan appear darker (Fig. 1D) than in gels without chitosan (Fig. 1A). It could well be related to the fact that glycol chitosan binds Coomassie blue very tightly. Chitosan and glycol chitosan were previously shown to bind calcofluor (fluorochrome stain) very tightly (Trudel and Asselin, in press). This is exactly the same for glycol chitosan stained with Coomassie blue. Finally, gels containing glycol chitosan were also subjected before staining to a nitrous acid treatment (Trudel and Asselin, in press), which is known to specifically depolymerize chitosan. Such treatment induced the disappearance of the substrate indicating that contaminants are not responsible for the staining with Coomassie blue or calcofluor white.

Chitosanase activity has been reported in plant extracts containing chitinase or lysozyme activity (Abeles *et al.* 1971; Bernasconi *et al.* 1987; Molano *et al.* 1979; Nichols *et al.* 1980; Pegg 1988). In our study, we used highly (95%) acetylated glycol chitin versus highly deacetylated glycol chitin as glycol chitosan (94% deacetylation as determined by titration) as substrates to easily differentiate chitosanase from chitinase activity. Higher levels of acetylation in chitosan can bring results that would not necessarily allow differentiating hydrolysis of glycol chitin versus glycol chitosan. However, the distinction between chitinase and chitosanase activity might not be absolute. There could be enzymes able to hydrolyze both substrates because there are several plant chitinases able to act as lysozymes.

Chitosan has been reported to occur in the cell walls of *Phycomyces, Mucor, Zygorhynchus*, and *Choanephora* (Bartnicki-Garcia 1968; Letourneau *et al.* 1976; Datema *et al.* 1977). It also occurs in the spore and hyphal walls of *Agaricus* (Novaes-Ledieu and Garcia Mendoza 1981), with terminated growth of rust spores on wheat (Hadwiger and Line 1981), and in pea-*Fusarium* (Hadwiger *et al.* 1981) and gall mite-*Solanum* interactions (Bronner *et al.* 1989).

**Analysis of chitosanase activities in IF extracts of stressed tissues by two-dimensional gels.** Two-dimensional gels were required to separate all induced PR proteins and chitinases in tobacco leaf tissue (Trudel *et al.* 1989). The same approach was applied to analysis of chitosanases found in IF extracts of stressed tissue. Proteins were first separated in native gels at pH 8.9 (Davis system for acidic proteins) (Fig. 2, arrow a) or at pH 4.3 (Reisfeld system for basic proteins) (Fig. 2, arrow b) and then subjected to SDS-PAGE in the second dimension. Six chitosanases were detected after Coomassie blue detection in two-dimensional gels with barley IF extract (Fig. 2A). Four bands (arrowheads 1 to 4) were found by using the native first-dimension gel for separating acidic proteins, and two bands (arrowheads 5 and 6) were seen after separation in the native gel designed for separating basic proteins. Four acidic chitosanases were also observed with stressed cucumber IF extract (Fig. 2B), while only one tomato chitosanase could be detected after separation for basic proteins in the first dimension (Fig. 2C). At least three bands observed after one-dimensional SDS-PAGE (Fig. 1, C and D) are thus the result of two or more separate proteins. For example, the upper band of chitosanase activity in barley (Fig. 1C, lane 1 and Fig 1D, lane 1) is made up of acidic proteins 1, 2, 4, and basic protein 5 (Fig. 2A). The lower band of chitosanase activity in barley (Fig. 1C, lane 1 and Fig. 1D, lane 1) is composed of one acidic (protein 3) and one basic (protein 6) chitosanase (Fig. 2A). In stressed cucumber tissue, the activity at 14 kDa (Fig. 1C, lane 3 and Fig. 1D, lane 3) is the combination of acidic proteins 3 and 4 (Fig. 2B). Acidic and basic isoforms can thus be found, and the relative abundance of acidic versus basic chitosanases varies between plant species.

**Detection of lysis of *F. o.* f. sp. *radicis-lycopersici* spores by chitosanases separated in gels.** Because chitosan can be found in various fungal cell walls including *Fusarium* (Christian and Hadwiger 1989), chitosanases were evaluated for their capacity to hydrolyze fungal cells. Spores were chosen because they could be embedded uniformly in the gel matrix to serve as potential substrate. Spore suspensions were then incorporated into gels, and lysis was observed by transparency through the opaque spore suspension. Lysis zones appeared darker because photographs were taken against a black background. This is exactly the same approach as the one used with *Micrococcus* cell-containing polyacrylamide gels used for detection of lysozyme activity after electrophoretic separation of proteins (Audy *et al.* 1988, 1989; Potvin *et al.* 1988; Trudel *et al.* 1989). Analysis of lysis of spores was first studied after one-dimensional SDS-PAGE (Fig. 3A). IF extracts of nonstressed and stressed tissues

were subjected to SDS-PAGE and then examined for lysis of spores. Lysis of *F. o.* f. sp. *radicis-lycopersici* spores was observed in stressed barley and tomato IF extracts (Fig. 3A, lanes 1 and 5). In tomato (Fig. 3A, lane 5), the activity lysing spores corresponded to the major chitosanase activity at 24 kDa, which was previously shown to be a basic protein. In barley, the diffuse band in the one-dimensional gel was further analyzed in a two-dimensional gel system similar to the one used with glycol chitosan (Fig. 2). Analysis in a two-dimensional gel system showed that lysis of *F. o.* f. sp. *radicis-lycopersici* spores was only detected at the same level as basic chitosanase 6 (Fig. 3B). So, lysis of *F. o.* f. sp. *radicis-lycopersici* spores has only been observed with basic chitosanases stimulated in stressed tissue.

**Lysis of spores of other fungi by barley chitosanase 6 and tomato chitosanase.** In addition to *F. o.* f. sp. *radicis-lycopersici* spores, *V. albo-atrum* and *O. ulmi* spores were also lysed by those chitosanases (not shown). Spores from some Basidiomycetes (*Ustilago maydis* (DeCandolle) Corda and *Scleroderma aurantium* Pers.) were not lysed, and neither were spores of *Trichoderma* sp. (Deuteromycetes).

In conclusion, various molecular forms of chitosanase activity were stimulated in stressed tissue of various plant species. This is analogous to the induction of chitinases and $\beta$-glucanases and of other PR proteins (cited in Trudel *et al.* 1989). By using a simple polyacrylamide gel assay with appropriate substrates, chitosanases were easily distinguished from chitinases. Some basic chitosanases even showed lytic activity toward fungal spores of pathogens by using a new polyacrylamide gel assay to visualize spore lysis. The potential antifungal activity of the various forms of chitosanases (with special emphasis on the basic proteins) has now to be evaluated with purified enzymes against a spectrum of fungal pathogens.

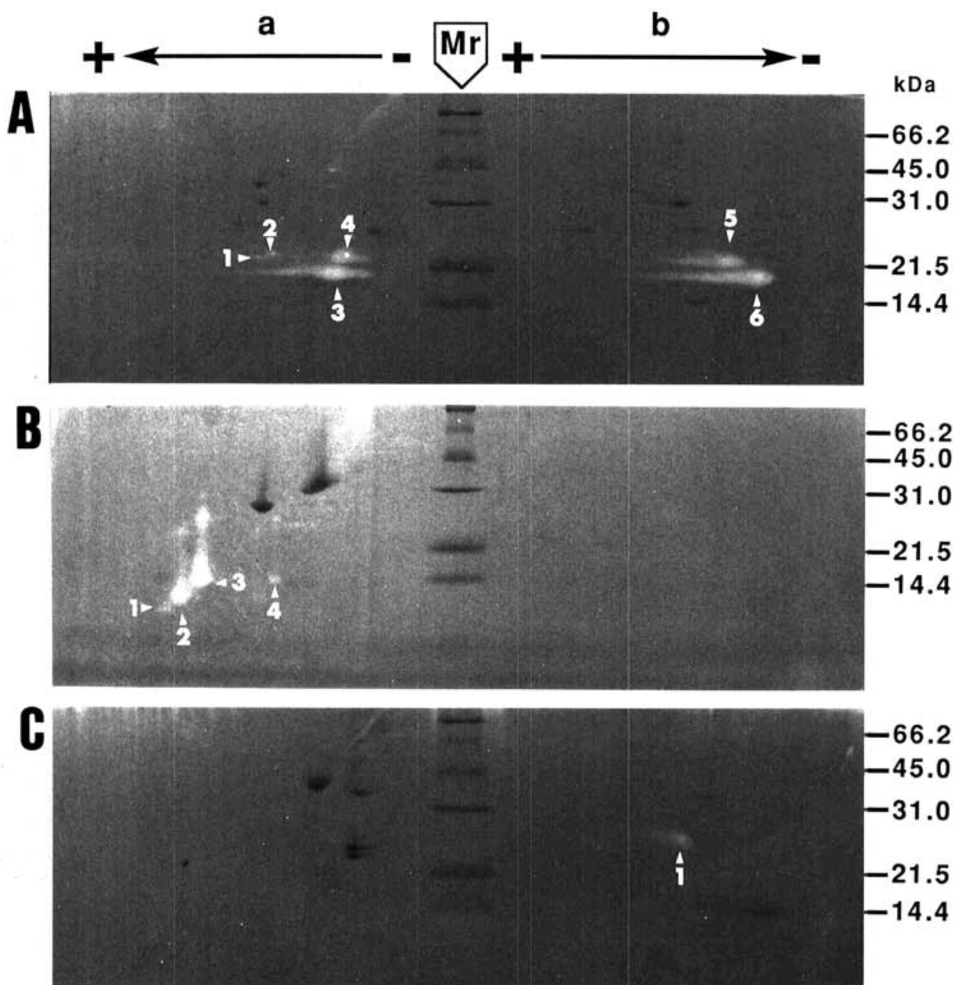


**Fig. 2.** Chitosanase activities after separation of proteins in two-dimensional gels. Intercellular fluid extracts (20 $\mu$l of the same protein amounts as given in Fig. 1) of chemically stressed barley ($AgNO_3$) leaves (panel **A**), cucumber ($AgNO_3$) cotyledons (panel **B**), and tomato (serine) leaves (panel **C**) were first subjected to native gel electrophoresis in 15% (w/v) polyacrylamide gels for acidic proteins (migration in the Davis system, arrow a) or basic proteins (migration in the Reisfeld system, arrow b). After native PAGE, gel slices were boiled for 5 min in sodium dodecyl sulfate buffer (without reducing agents) and subjected to sodium dodecyl sulfate-PAGE as given in Figure 1. The second dimension gels contained 0.01% (w/v) glycol chitosan as substrate for chitosanase activity. After electrophoresis in the second dimension, gels were incubated in sodium acetate buffer and Triton X-100 as given in Figure 1. Gels were then stained with Coomassie Brilliant Blue R 250. Arrowheads indicate the position of chitosanases. Molecular mass markers ($M_r$) were prepared as given in Figure 1.

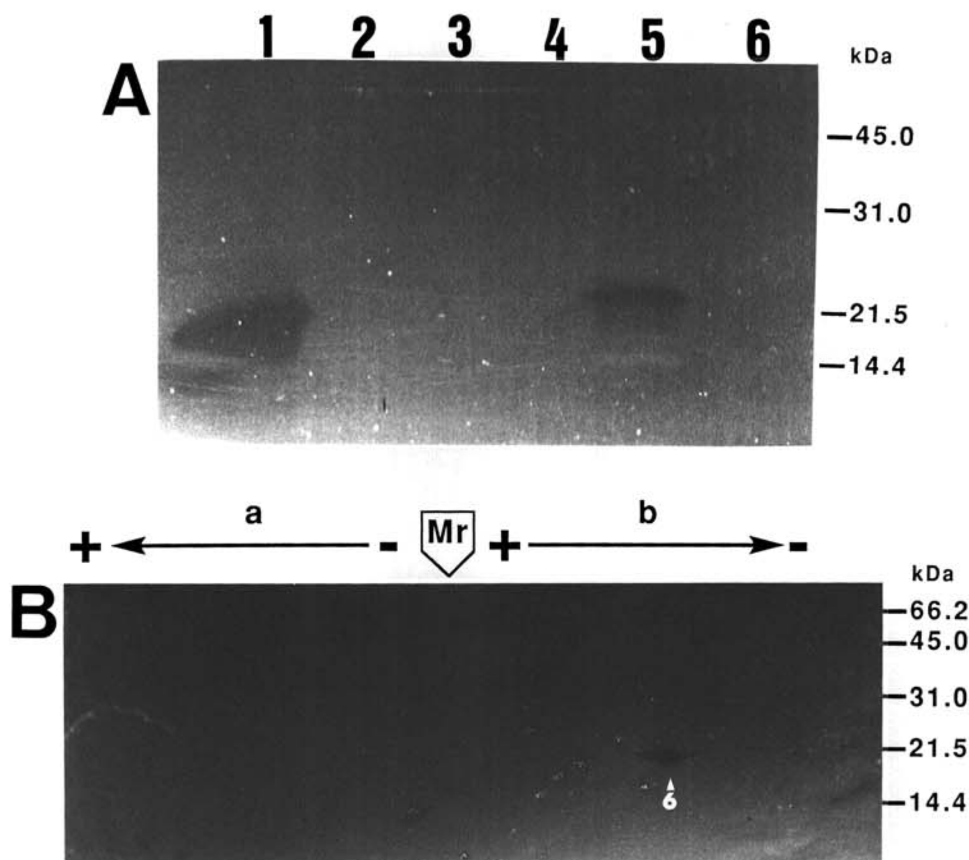


**Fig. 3.** Lysis of *Fusarium oxysporum* f. sp. *radicis-lycopersici* spores by proteins in intercellular fluid (IF) extracts of stressed tissue. In panel **A**, IF extracts (20 μl of the same protein amounts as given in Fig. 1) of chemically stressed barley ($AgNO_3$) leaves (lane 1), cucumber ($AgNO_3$) cotyledons (lane 3), and tomato (serine) leaves (lane 5) were subjected to sodium dodecyl sulfate-PAGE as given in Figure 1 and compared with IF extracts from nonstressed barley (lane 2), cucumber (lane 4), and tomato (lane 6) tissue. In panel **A**, the gel contained $2 \times 10^7$ *Fusarium* spores per milliliter. After electrophoresis, the gel was incubated for 18 hr as given in Figure 1. Spore lysis was visually detected by transparency through the opaque spore suspension. Photographs were taken against a black background. Lysis at the migration level of chitosanases was only observed with stressed barley (lane 1) and stressed tomato (lane 5). In panel **B**, the stressed barley IF extract was analyzed for lysis of *Fusarium* spores after two-dimensional gel electrophoresis as given in Figure 2. Arrow a indicates the direction of migration of the first-dimension gel for acidic proteins, while arrow b indicates the direction for basic proteins in the first dimension. The second-dimension gel was run as given in Figure 2. Renaturation and detection of spore lysis were the same as in panel **A**. Only one basic lytic activity was observed migrating as basic chitosanase 6 of stressed barley tissue. Molecular mass markers are indicated on the right.


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