Colloidal gold-complexed chitosanase: a new probe for ultrastructural localization of chitosan in fungi

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A chitosanase was purified from the intercellular fluid extract of chemically stressed barley (*Hordeum vulgare*) leaves. Purification was achieved by preparative PAGE involving separation under native conditions at pH 4.3 (Reisfeld system) followed by denaturing PAGE in the presence of SDS. One basic chitosanase with a molecular mass of 19 kDa could hydrolyse chitosan and glycol chitosan of crustacean origin in addition to fungal chitosan isolated after treatment of *Mucor mucedo* cells with sodium hydroxide followed by extraction in acetic acid. This enzyme did not exhibit activity towards β -1,4-polymers such as chitin, cellulose or peptidoglycan (from *Micrococcus luteus*) after SDS-PAGE. The chitosanase was conjugated to colloidal gold at pH 9.5 and applied to fungal tissue sections or to isolated glycol chitosan, chitosan, cellulose or chitin. The chitosanase–gold complex reacted intensely with glycol chitosan and chitosan but did not bind to chitin or cellulose. The enzyme–gold complex also labelled chitosan isolated from *M. mucedo*. However, it did not react with *M. mucedo* cells in tissue sections. Labelling with the chitosanase–gold complex was easily detected over cell walls of *Fusarium oxysporum* f. sp. *radicis-lycopersici* spores and hyphae as well as over cell walls of *Ophiostoma ulmi*, *Aspergillus niger* and *Colletotrichum lindemuthianum*. No binding was observed with *Pythium ultimum*.

Introduction

Chitin, chitosan and cellulose are the most ubiquitous (1 $\rightarrow$ 4)- β -linked polysaccharides of fungal cell walls (Wessels & Sietsma, 1981). Chitin [a polymer of *N*-acetylglucosamine; (1 $\rightarrow$ 4)-2-acetamido-2-deoxy- β -D-glucan] can be easily revealed at the ultrastructural level with the use of chitinase- or wheat germ agglutinin-gold complexes (Benhamou & Asselin, 1989). Cellulose (1 $\rightarrow$ 4- β -D-glucan) can also be visualized using an exoglucanase-gold complex (Benhamou, 1989). By contrast, chitosan (partially or fully *N*-deacetylated chitin) has, until now, been difficult to localize by cytochemical approaches because of the absence of specific probes. 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.*, 1977b). It also occurs in the spore and hyphal walls of *Agaricus* (Novaes-Ledieu & Garcia Mendoza, 1981), in spores of rust (Hadwiger & Line, 1981) and *Saccharomyces* (Briza *et al.*, 1988), and in *Fusarium* hyphae and spores (Hadwiger *et al.*, 1981). In some

Zygomycetes, it has been demonstrated that chitosan derives from *N*-deacetylation of nascent chitin by chitin deacetylase (Araki & Ito, 1988; Trudel & Asselin, 1990). Exogenous chitosan and chitosan oligosaccharides from fungal cell walls can elicit various defence reactions in plants, including the synthesis and accumulation of callose (Kendra *et al.*, 1989).

Moreover, chitosan has become a natural biodegradable cationic polymer with commercial applications ranging from pharmaceutical to cosmetic and agricultural uses (Sandford & Hutchings, 1987). Despite the recognized biological significance of chitosan and chitosan derivatives, there is still no probe allowing *in situ* detection of this polysaccharide.

In this paper, we report the conditions required for tagging a barley chitosanase with colloidal gold particles and the use of the gold complex for localizing chitosan in spore and hyphal cell walls of several fungi.

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 R250 were from Bio-Rad. Protein concentration was determined with the Bio-Rad protein assay reagent.

Abbreviations: IF, intercellular fluid; PDA, potato dextrose agar; PR, pathogenesis-related; TEM, transmission electron micrograph.

Glycol chitosan, chitosan and all other chemicals were from Sigma. Glycol chitin was synthesized as previously described (Trudel & Asselin, 1989). The degree of deacetylation of substrates has been reported previously (Grenier & Asselin, 1990).

Plant material, induction of pathogenesis-related (PR) proteins and intercellular fluid extracts. All plants were grown under greenhouse conditions. Barley seedlings (*Hordeum vulgare* cv. Léger) were grown for 25 d before 5 cm leaf sections were made and floated for 3 d on 100 µM-AgNO₃ (Asselin *et al.*, 1985). Intercellular fluid (IF) extracts were prepared by vacuum infiltration followed by low-speed centrifugation (Parent & Asselin, 1984).

PAGE. Proteins were separated in one-dimensional native or denaturing 15% (w/v) polyacrylamide gel systems as previously described (Trudel *et al.*, 1989). Proteins were stained with Coomassie Brilliant Blue R250 followed by aqueous silver nitrate.

Detection of hydrolase 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% glycol chitosan or 0.05% *M. mucedo* chitosan as substrate in the gel matrix. Bands were detected with Calcofluor White M2R staining, as for chitinase activity, or by staining the intact glycol chitosan with Coomassie Brilliant Blue R250 (Grenier & Asselin, 1990). After electrophoresis, SDS-gels were first incubated at room temperature in 200 ml sodium acetate buffer (0.01 M, pH 5.0) with 1% (v/v) purified Triton X-100 (Grenier & Asselin, 1990). After 20 min, the buffered Triton X-100 solution was replaced and gels were incubated for 18 h at 37 °C with gentle shaking. Chitosanase activity was also detected after native PAGE. Chitosanase activity in gels run at pH 4.3 was detected by an overlay 7.5% (w/v) polyacrylamide gel (0.75 mm thick) containing 0.01% 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 for SDS-gels. Hydrolase activity against carboxymethyl (CM)-cellulose was evaluated after SDS-PAGE by Calcofluor staining (Côté *et al.*, 1991). Detection of lysozyme activity after SDS-PAGE was performed as previously described (Audy *et al.*, 1989).

Isolation and analysis of chitosan from *Mucor mucedo*. Chitosan was isolated from a 2-d-old *M. mucedo* culture after autoclaving in NaOH and solubilizing in acetic acid (White *et al.*, 1979). Chitosan was hydrolysed in 6 M-HCl for 18 h at 105 °C. TLC was performed with cellulose-coated plates for 7 h in *n*-butanol/pyridine/0.5 M-HCl (5:3:2, by vol.). Spots were detected with ninhydrin.

Purification of a single barley chitosanase. IF extracts (80 ml) from chemically stressed barley leaves were concentrated tenfold by freeze-drying. IF extracts were subjected to native PAGE in 15% (w/v) polyacrylamide gels (10 gels, 800 µl per gel) at pH 4.3 (Reisfeld system). The lower band corresponding to chitosanase activity was cut out and the protein was eluted in distilled water (5 ml per gel slice) overnight at 4 °C. The eluate was filtered through a Millipore membrane (0.22 µm) and freeze-dried. The eluate was resuspended in 6 ml distilled water and subjected to SDS-PAGE in 15% (w/v) polyacrylamide gels containing 0.01% glycol chitosan (seven gels, 800 µl eluate per gel). Bands corresponding to chitosanase activity at 19 kDa were cut out and incubated twice for 15 min in 0.01 M-sodium acetate (pH 5.0) in 25% (v/v) propan-2-ol. After rinsing gel pieces with water, they were incubated overnight at 37 °C with shaking in 0.01 M-sodium acetate (pH 5.0). The eluate was filtered through a Millipore membrane (0.22 µm) and concentrated by freeze-drying. One milligram of purified chitosanase was recovered.

Fungal isolates and culture conditions. The isolate of *Colletotrichum lindemuthianum* (Sacc. and Magn.) Briosi and Cav. used in this study

was kindly provided by Dr D. Mazau, Centre de Physiologie Végétale, Université Paul Sabatier, Toulouse, France. It was cultured at 23 °C in a defined, solid medium containing (g per 1000 ml H₂O): glucose (2.8), MgSO₄·7H₂O (1.3), KH₂PO₄ (2.7), mycological peptone (2.0), yeast extract (10.0) and agarose (20.0).

The isolate of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, Jarvis and Shoemaker was obtained from Mr P. O. Thibodeau, Complexe Scientifique, Ste-Foy, Québec, Canada. It was cultured at 22 °C on potato dextrose agar (PDA, Difco) and periodically re-inoculated and re-isolated from ripe tomato fruits.

The isolate of *Ophiostoma ulmi* (Buism.) Nannf. was provided by Dr G. Ouellette, Centre forestier des Laurentides, Ste-Foy, Québec, and was isolated from an infected elm tree in Québec, and periodically re-inoculated and re-isolated from elms. It was grown at 22 °C on PDA medium.

The isolate of *Aspergillus niger* Van Tiegh was obtained from the culture collection maintained at the Département de Phytologie, Université Laval. The fungus was cultured on PDA medium at 22 °C.

The isolate of *Pythium ultimum* Trow was kindly provided by Dr R. Bélanger, Université Laval. It was cultured on PDA medium at 22 °C.

Mucor mucedo Linn. ex Fr. was described previously (Trudel & Asselin, 1990).

Tissue processing for electron microscopy. Samples from actively growing colonies of each fungus, or polysaccharides in agarose plugs, were fixed with 3% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.2) for 2 h at room temperature, dehydrated in a graded ethanol series and embedded in Epon 812. Some samples were post-fixed with 1% (w/v) osmium tetroxide in sodium cacodylate buffer for 1 h at 4 °C, before dehydration. Ultrathin sections were collected on Formvar-coated nickel grids and processed for gold cytochemistry.

Preparation of chitosanase-gold complex. Colloidal gold with an average particle diameter of 15 nm was prepared according to Frens (1973) by reducing aqueous tetrachloroauric acid solution (0.01%, w/v) with 1% (w/v) sodium citrate. The optimal pH for binding the colloidal gold sol to chitosanase was estimated to be 9.5. The minimal amount of chitosanase necessary for full stabilization of the gold sol was determined by adding 100 µl colloidal gold (pH 9.5) to 10 µl of serial dilutions of the enzyme. A few minutes later, 100 µl 10% (w/v) NaCl was added and flocculation estimated visually. The minimum concentration of chitosanase necessary to stabilize the gold sol at pH 9.5 was estimated to be 3 µg ml⁻¹. A fivefold excess (150 µg) was used to fully stabilize 10 ml colloidal gold.

For preparation of the chitosanase-gold complex, about 150 µg of the enzyme was dissolved in 100 µl distilled water and mixed with 10 ml colloidal gold at pH 9.5. To further stabilize the gold sol, five drops of PEG 20000 (1%, w/v) were added to the mixture. After centrifugation at 13000 r.p.m. for 60 min, the supernatant was discarded and the dark-red pellet was resuspended in 0.5 ml 0.1 M-PBS (pH 6.0) containing 0.02% PEG 20000. The gold-complexed chitosanase was stored at 4 °C until required.

Preparation of exoglucanase- and ovomucoid-gold complexes. The exoglucanase, a β-1,4-cellobiohydrolase (β-glucosidase; EC 3.2.1.21), was purified in a five-step procedure from a cellulase produced by the fungus *Trichoderma harzianum* Rifai (Benhamou, 1989). It was complexed to colloidal gold at pH 9.0 as previously described (Benhamou *et al.* 1987). Briefly, 500 µg of the enzyme was mixed with 10 ml colloidal gold at pH 9.0, and the pellet recovered after centrifugation was resuspended in 0.5 ml 0.1 M-PBS/PEG (pH 6.0).

Wheat germ agglutinin (WGA), a lectin with *N*-acetylglucosamine binding specificity, was used in a two-step procedure for the localization of chitin. Due to its low molecular mass, this lectin could not be directly complexed to colloidal gold (Benhamou, 1988). Ovomucoid, a high molecular mass protein isolated from egg white,

Fig. 1. Electrophoretogram of barley intercellular fluid extract, purified barley chitosanase and chitosanase activity against *M. mucedo* chitosan. Prestained molecular mass markers (lane A, 10 μ l, 100 μ g) (110 kDa, phosphorylase *b*; 84 kDa, BSA; 47 kDa, ovalbumin; 33 kDa, carbonic anhydrase; 24 kDa, soybean trypsin inhibitor; 16 kDa, lysozyme), barley intercellular fluid extract (lane B, 10 μ l, 60 μ g) and electrophoretically purified barley 19 kDa chitosanase (lane C, 10 μ l, 6 μ g) were separated by SDS-PAGE (15%, w/v). Proteins were stained with Coomassie Brilliant Blue R-250 followed by silver nitrate. Purified barley chitosanase (10 μ l, 6 μ g) was also subjected to SDS-PAGE in a gel containing *M. mucedo* chitosan (see Methods) at a final concentration of 0.05% (lane D). *M. mucedo* chitosan was initially dissolved in 10% (v/v) acetic acid. After renaturation and lysis by chitosanase for 18 h at 37 °C in buffered Triton X-100, chitosanase activity was detected by Coomassie Brilliant Blue R-250 staining (Grenier & Asselin, 1990). Lysis appears as a white band against a blue background.

was used as a second-step reagent because of its high affinity for WGA. The protein was complexed to gold at pH 5.3, and the pellet recovered after centrifugation was resuspended in 0.5 ml PBS/PEG (pH 6.0).

Cytochemical labelling. For cytochemical labelling with chitosanase and exoglucanase-gold complexes, grids were first incubated for 5 min on a drop of PBS/PEG (pH 6.0) and then transferred to a drop of the enzyme-gold complex for 30 min at room temperature in a moist chamber. Grids were thoroughly washed with PBS (pH 7.4), rinsed with distilled water and contrasted with uranyl acetate and lead citrate before examination with a JEOL 1200 EX electron microscope at 80 kV.

For cytochemical labelling with the WGA/ovomucoid-gold complex, grids were first incubated on a drop of PBS (pH 7.4) for 5 min, then transferred to a drop of WGA (25 μ g ml⁻¹ in PBS, pH 7.4) for 1 h at room temperature, and finally incubated on a drop of ovomucoid-gold complex diluted 1:30 in PBS/PEG (pH 6.0). After washing, grids were counterstained as described above.

Cytochemical controls. Specificity of the labellings with the different probes was assessed by several control tests: (1) incubation with the chitosanase or the exoglucanase-gold complex to which an excess of the corresponding substrate had been previously added; (2) incubation with the uncomplexed enzyme followed by incubation with the gold-complexed enzyme; (3) incubation with WGA to which was previously added *N,N,N'*-triacylchitotriose followed by incubation with the ovomucoid-gold complex; (4) incubation with the ovomucoid-gold

Fig. 2. TLC of crustacean and isolated *M. mucedo* chitosan after HCl hydrolysis. Commercial crustacean chitosan and isolated *Mucor* chitosan (see Methods) were subjected to 6 M-HCl hydrolysis at 105 °C for 18 h (10 μ g μ l⁻¹, final concentration). D-Galactosamine (lane A), D-glucosamine (lane B), *Mucor* hydrolysed chitosan (lane C) and commercial hydrolysed chitosan (lane D) (all 10 μ g) were separated by TLC on a cellulose-coated TLC plate (20 $\times$ 20 cm) (see Methods). Spots were detected with ninhydrin.

complex, the WGA step being omitted; and (5) incubation with a protein-gold complex such as BSA-gold complex.

Results

Purification of a basic chitosanase from intercellular fluid extracts of stressed barley leaves

We recently reported that several plant chitosanases accumulated in the IF extracts of chemically stressed leaf tissue (Grenier & Asselin, 1990). Certain basic chitosanases even exhibited some lytic activity against fungal spores. This prompted the idea that some plant chitosanases from IF extracts could be valuable probes for localizing chitosan in fungal cells. One basic chitosanase of 19 kDa was purified by two cycles of preparative PAGE (native PAGE followed by SDS-PAGE). The source of the enzyme was from barley IF extracts (Fig. 1,

Fig. 3. Transmission electron micrographs (TEMs) of embedded commercial chitosan (a, d), glycol chitosan (b, e), chitin (c) and embedded chitosan isolated from *M. mucedo* cell walls (f, g, h). Samples were incubated with the chitosanase-gold complex (a, b, c, f), the gold-complexed exoglucanase (d, e, g) or the WGA/ovomucoid-gold complex (h). Bars, 0.25 μm.

lane B). The purified chitosanase exhibited a single band at 19 kDa after SDS-PAGE and staining with Coomassie Brilliant Blue followed by silver nitrate (Fig. 1, lane C) when compared to molecular mass markers (Fig. 1, lane A). The purified chitosanase also exhibited a single band of activity (Fig. 1, lane D) when assayed in a gel system containing fungal chitosan as substrate (Grenier & Asselin, 1990).

Substrate specificity of purified chitosanase

By using new gel assays (Trudel & Asselin, 1989; Grenier & Asselin, 1990, Côté *et al.*, 1991), the purified chitosanase was tested for its activity against several polysaccharides containing a β -1,4-glucan backbone. The chitosanase did not exhibit activity towards chitin (this work and Grenier & Asselin, 1990), CM-cellulose or

Fig. 4. TEMs of *F. oxysporum* f. sp. *radialis-lycopersici* in culture labelled with the chitosanase-gold complex. (a) Spore, (b) young hypha and (c, d) older hyphae. Abbreviations: Cy, cytoplasm; FW, fungal wall; L, lipid body; M, mitochondrion; N, nucleus; R, ribosomes; S, septum; SW, spore wall. Bars, 0.5 μ m.

peptidoglycan (*Micrococcus luteus*). However, it exhibited lytic activity not only against glycol chitosan (94% deacetylation) and chitosan (74% deacetylation) of

crustacean origin (data not shown), but also against chitosan isolated from *Mucor mucedo* (Fig. 1, lane D). *M. mucedo* represents one of the best sources of fungal

Fig. 5. TEMs of *O. ulmi* (a), *C. lindemuthianum* (b), *A. niger* (c) and *P. ultimum* (d) in culture labelled with the chitosanase-gold complex. Abbreviations are defined in the legend to Fig. 4 except: EM, extracellular material; Va, vacuole. Bars, 0.5 μ m.

chitosan (White *et al.*, 1979) and chitosan from Mucorales is highly deacetylated (70–95%) (Fenton & Eveleigh, 1981; McGahren *et al.*, 1984). In order to assess the

identity of the isolated *Mucor* chitosan, the extract was subjected to HCl hydrolysis and analysed by TLC. The hydrolysed *Mucor* chitosan yielded D-glucosamine (Fig.

2, lane C) in a way similar to that of commercial crustacean glycol chitosan (Fig. 2, lane D). In this TLC system, reference D-galactosamine could be separated from D-glucosamine (Fig. 2, lanes A and B, respectively).

Cytochemical localization of chitosan

Application of the gold-complexed chitosanase to sections of embedded commercial chitosan (Fig. 3a) and glycol chitosan of crustacean origin (Fig. 3b) resulted in the deposition of numerous gold particles. By contrast, chitin failed to react positively with the gold-complexed enzyme as judged by the very few scattered gold particles observed over sections of embedded material (Fig. 3c). Both chitosan and glycol chitosan were unlabelled following incubation with the exoglucanase–gold complex (Fig. 3d, e), indicating that cellulosic subunits were absent.

Chitosan extracted and purified from *M. mucedo* cell walls was substantially labelled with the chitosanase–gold complex (Fig. 3f) but did not react with either the exoglucanase–gold complex (Fig. 3g) or the WGA/ovomucoid–gold complex (Fig. 3h). *M. mucedo* chitosan has been shown in this work to be sensitive to purified barley chitosanase (Fig. 1, lane D).

Incubation of sections from spores of *F. oxysporum* f. sp. *radicis-lycopersici* with the gold-complexed chitosanase, resulted in a heavy labelling of cell walls (Fig. 4a). Labelling appeared to be uniformly distributed although in some cases a preferential accumulation of gold particles was observed over the innermost wall layers (Fig. 4a). Cytoplasm, nucleus, lipid bodies and other organelles were always free of labelling. The thick wall of young emerging hyphae of *F. oxysporum* f. sp. *radicis-lycopersici* were also intensely and specifically labelled (Fig. 4b). In apparently older hyphal cells, the thinner wall was labelled by scattered gold particles (Fig. 4c, d). Although weak compared to that of spores, this labelling was specifically associated with cell walls and septa, and did not occur over cytoplasm and organelles (Fig. 4d).

Application of the gold-complexed chitosanase to sections of *O. ulmi* resulted in the deposition of gold particles over the outermost wall layers (Fig. 5a). Almost no labelling occurred over the cytoplasm or organelles. A significant cell wall labelling was also observed over *C. lindemuthianum* cell walls (Fig. 5b). Gold labelling appeared to be of higher intensity at the triangular junction between the wall and septum (Fig. 5b). Labelling was found to be mainly associated with the outermost wall layers of *A. niger* hyphal cells, whereas the extracellular, fibrillar matrix was free of gold particles (Fig. 5c). Of all the fungi tested, *P. ultimum* was the only one that did not react with the chitosanase–gold complex

as judged by the absence of significant labelling (Fig. 5d).

Adsorption of the gold-complexed chitosanase with glycol chitosan prior to section labelling yielded negative results with *O. ulmi* (data not shown). By contrast, labelling was neither eliminated nor reduced when the gold-complexed enzyme was previously incubated with glycol chitin or β -1,4-glucans from barley (data not shown). All other control tests resulted in an absence of labelling over both material sections and embedding resin.

Discussion

Chitosanases have been identified as a class of enzymes different from chitinases (e.g. EC 3.2.1.14) and lysozymes (e.g. EC 3.2.1.17) (Monaghan *et al.*, 1973). Up to now, chitosanases have been characterized mostly from bacterial (Pelletier & Syguch, 1990, and references therein) and fungal sources (Reyes *et al.*, 1985). More recently, some plant PR proteins have been shown to be extracellular chitosanases (Grenier & Asselin, 1990).

The barley chitosanase was chosen as a probe for four reasons. First, it is much easier to purify a chitosanase from an IF extract that contains less than 30 proteins than from whole tissue extracts. Second, the barley extracellular enzyme is resistant to proteolysis as is the case for the well-known PR proteins. Third, barley represents an inexpensive and readily available source of tissue for enzyme purification. In fact, other cereals (such as wheat) can also be used as good sources of plant chitosanases. Fourth, several microbial chitosanases also exhibit cellulase activity (Pelletier & Syguch, 1990) which would preclude their use as specific probes for chitosan localization.

The barley chitosanase–gold complex interacted with fungi known to contain chitosan in their cell walls, including *Colletotrichum* (Siegrist & Kaus, 1990) and *Fusarium* (Hadwiger *et al.*, 1981). However, to our knowledge this is the first evidence for the occurrence of chitosan in the cell walls of *Ophiostoma* and *Aspergillus*. *A. niger* cell walls are known to contain α -(1 $\rightarrow$ 4)-galactosamine polymers. Because such galactosamine polymers display similar conformation and functional properties to chitosan (Yokoyama *et al.*, 1989), the barley chitosanase was tested for its activity against the *Physarum polycephalum* galactosamine polymer in sclerotia (McCormick *et al.*, 1970). No activity could be found against this polygalactosamine polymer (data not shown), thus indicating that chitosan was recognized.

It is important to note that chitosan is operationally defined as partially or fully deacetylated chitin. The specificity of labelling should eventually take into

account the fact that there is evidence for complete (Datema *et al.*, 1977b) or partial deacetylation (Mol & Wessels, 1987) with fungal chitin. It is noteworthy that the basic 19 kDa barley chitosanase (chitosanase 6, Grenier & Asselin, 1990) was found to exhibit lytic activity not only against chitosan but also against fungal spores such as those from *F. oxysporum* f. sp. *radicis-lycopersici*, *Verticillium albo-atrum* and *O. ulmi* (Grenier & Asselin, 1990). Results of the present work confirmed that this chitosanase was able to specifically bind to chitosan present in the cell walls of these fungi (results not shown for *V. albo-atrum*). However, the enzyme was not able to reveal chitosan in *M. mucedo* cell sections while it could easily hydrolyse chitosan isolated from the same fungal culture. Even though chitosan is a major wall component of Zygomycetes, it may not be readily accessible to chitosanase (at least to this barley chitosanase). The substrate may be masked by tightly bound components, such as anionic glycuronans and inorganic polyphosphates (Datema *et al.*, 1977a). This has been well illustrated for chitosan in Mucorales cell walls (McGahren *et al.*, 1984).

Numerous chitosanase-binding molecules were detected in the spore walls of *F. oxysporum* f. sp. *radicis-lycopersici*. Although this observation correlates well with the recent finding that spores of this pathogenic fungus were somewhat lysed by the enzyme in polyacrylamide gels (Grenier & Asselin, 1990), the biological significance of this accumulation is unclear. One might suggest that the appreciable amount of chitosan in the walls of *F. oxysporum* f. sp. *radicis-lycopersici* spores contributes to strengthen the cell surface. This is in line with previous observations by Qureshi & Tague (1970) that the ability of *Fusarium* to withstand adverse conditions was closely related to the presence of particular compounds in its thick-walled spores. Owing to its peripheral localization and its cationic properties, chitosan may also be involved in cell-cell recognition and/or attachment to host cells during pathogenesis.

A significant difference in the amount of chitosan was observed between spores and mycelium of *F. oxysporum* f. sp. *radicis-lycopersici*. A similar heterogeneity was recently reported in the case of cellulosic β -1,4-glucans which were found to be more abundant in spore than in mycelial walls (Benhamou *et al.*, 1990). This raises the question of the relative value that should be given to the chemical composition of spore wall in the taxonomic classification of fungi. In the classification of Bartnicki-Garcia (1968), the Zygomycetes constitute a unique group containing chitosan along with chitin. The present cytochemical observations demonstrate that chitosan is more widely distributed in fungi than was previously thought.

The present results also suggest that the barley

chitosanase may be of potential value not only for investigating the distribution of chitosan in fungi, but also to gain new insights into the role of chitosan during pathogenesis. Noteworthy in this context are the preliminary results showing that the level of wall-bound chitosan in *C. lindemuthianum* grown in culture markedly increases when the fungus develops in its host plant (unpublished results). This is in agreement with the reported involvement of chitosan fragments in plant defence (Kendra *et al.*, 1989).

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