Enzymic activity of the mycoparasite *Verticillium fungicola* on *Agaricus bisporus* fruit body cell walls

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The *in vitro* production of different hydrolytic enzyme activities by *Verticillium fungicola*, a mycoparasite of *Agaricus bisporus* fruit bodies, was examined in cultures grown with various carbon sources (glucose, fructose, sucrose and *A. bisporus* cell walls). Several of the identified enzymes were influenced by the carbon source. The results of *A. bisporus* cell wall digestion by these enzymes *in vitro* have been compared with *V. fungicola* infection of *A. bisporus* fruit bodies *in vivo*. Evidence supporting mycoparasite enzymic action on the host cell walls is presented.

Keywords: *Agaricus bisporus* cell walls, lytic enzymes, mycoparasitism, *Verticillium fungicola*

INTRODUCTION

Verticillium fungicola has been found to cause dry bubble disease in crops of the white button mushroom *Agaricus bisporus*. The infection process is not understood because of its complexity, involving the presence of *V. fungicola* spores and subsequent interactions between the mycelium of the pathogen and *A. bisporus* mycelium. In a recent study North & Wuest (1993) described the presence of *V. fungicola* hyphae growing intercellularly inside the *A. bisporus* fruit body, as previously reported by Ware (1933) and Matthews (1983). More recently Dragt *et al.* (1996) also found *V. fungicola* growing intracellularly in the *A. bisporus* fruit body hyphae.

The cell walls of the parasitized *A. bisporus* fruit bodies are thin in comparison to the thicker and more electron-dense walls of healthy cells, supporting the view that cell-wall-degrading enzymes may play an important role in the infection process, as previously postulated by Michaels (1973), Trigliano & Fergus (1979) and Kalberer (1984).

The cell wall of fungi is a complex structure composed of glucans (α and β), glycans, chitin and lesser quantities of proteins and lipids (Bartnicki-García, 1968). Successful cell wall degradation may therefore depend on the activity of more than one enzyme. The present work was undertaken to investigate the enzymes secreted by *V. fungicola* grown on a minimal synthetic medium supplemented with glucose, fructose, sucrose or cell walls of *A. bisporus* as the sole carbon source and to

investigate the effect of these extracellular enzymes on cell walls in an attempt to correlate the results with the *in vivo* infection process.

METHODS

Organisms and culture conditions. *A. bisporus* mushrooms (strain H25) were grown at 21 °C in the Centro de Investigación, Experimentación y Servicios del Champiñón (CIES, Quintanar del Rey, Cuenca, Spain). *V. fungicola* var. *fungicola*, isolated from naturally infected mushrooms, was grown at 25 °C and maintained on Raper agar (Raper *et al.*, 1972).

Preparation of hyphal cell walls. *A. bisporus* fruit bodies, free of lamellae, were sliced and disrupted by the action of a Polytron homogenizer and the walls purified as described by Avellán *et al.* (1986). The cell walls were stored in a lyophilized state.

Conditions for enzyme production. *V. fungicola* was grown on minimal synthetic medium (MSM) containing (g l⁻¹): MgSO₄·7H₂O, 0·2; K₂HPO₄, 0·9; KCl, 0·2; FeSO₄·7H₂O, 0·002; MnSO₄, 0·002; and ZnSO₄, 0·002. The medium was supplemented with 0·1% (w/v) of the appropriate carbon (glucose, fructose, sucrose and *A. bisporus* cell walls) and nitrogen (NH₄NO₃) sources. The pH of the medium was adjusted to 6·3. Flasks (250 ml) containing 50 ml culture medium were heat sterilized, inoculated with *V. fungicola* conidia and incubated statically at 25 °C for various time periods (24 to 168 h). The cultures were filtered through Whatman 3MM paper in a Millipore filtration apparatus, the filtrate centrifuged at 6000 g for 10 min and the supernatant extensively dialysed against distilled water at 4 °C. These dialysed solutions were routinely used for enzyme assays. The enzyme solutions were then lyophilized for further analysis.

Protein concentration was determined according to Bradford (1976) using the Bio-Rad protein assay dye reagent and BSA as a protein standard. Fungal growth was followed by determining mycelial dry weight.

Enzyme assays and substrates. Activities of 1,3- β -glucanase, 1,4- β -glucanase, 1,6- β -glucanase, 1,3- α -glucanase, 1,4- α -glucanase and 1,4- β -xylanase were assayed on, respectively, laminarin (Koch-Light), carboxymethylcellulose (Sigma), pustulan (Calbiochem), 1,3- α -glucan of *Aspergillus nidulans*, amylose (Sigma) and xylan (Koch-Light) by measuring the release of reducing groups (Nelson, 1957). Reaction mixtures contained 33 μ l substrate solution (5 mg ml⁻¹) at pH 5.2 (50 mM sodium acetate buffer) and 33 μ l enzyme solution; enzyme activity was stopped after 16 h at 37 °C by adding the Nelson-Somogyi alkaline copper reagent (Somogyi, 1952). Activities (U) are expressed as μ mol glucose or xylose released h⁻¹ as assessed from standard curves prepared for each sugar.

Chitinase activity was assayed with 60 μ l of 5 mg chitin ml⁻¹ (Sigma) in 50 mM sodium acetate buffer pH 5.2, mixed with 140 μ l enzyme solution. The mixture was incubated for 16 h at 37 °C and the reaction was stopped by boiling for 5 min. Samples were centrifuged at 5000 *g* for 10 min and 100 μ l supernatant was incubated with 20 μ l of 3% (w/v) helicase at 37 °C (De la Cruz *et al.*, 1992). After 1 h incubation, 100 μ l samples were taken and the *N*-acetylglucosamine liberated was measured according to Riessig *et al.* (1955).

Activities of β -D-glucosidase, α -D-glucosidase, β -D-mannosidase, α -D-mannosidase, β -D-xylosidase, *N*-acetyl- β -D-glucosaminidase and *N*,*N'*-diacetyl- β -D-chitobiosidase (chitobiosidase) were determined by measuring the rate of hydrolysis of the corresponding *p*-nitrophenylglycosides (Sigma). Reaction mixtures contained 50 μ l glycoside solution (0.5 mg ml⁻¹ in 50 mM sodium acetate buffer, pH 5.2) and 50 μ l enzyme solution. After 16 h at 37 °C reactions were stopped by adding 0.9 ml 0.37 M Na₂CO₃. Absorbance was measured at 410 nm. Activities (U) are expressed as μ mol *p*-nitrophenol released by the enzyme solution h⁻¹ at 37 °C.

Protease activity was assayed with 100 μ l casein solution (1% in 50 mM potassium phosphate buffer pH 8.0) mixed with 0.8 ml of 0.2 M Tris/HCl buffer pH 8.0, 0.3 ml enzyme solution and distilled water to a total volume of 1 ml. The mixture was incubated for 16 h at 30 °C. Samples were then mixed with 1.5 ml 5% trichloroacetic acid, kept for 1 h at room temperature and then centrifuged to remove undigested casein. The absorbance of the clear supernatant was measured at 280 nm. Activity (U) is expressed as an increase in *A*₂₈₀ of 0.001 under the described assay conditions.

Appropriate controls of substrates and enzyme solutions incubated separately were included in each group of activity assays.

Detection of 1,3- β -glucanase and chitinase activities on PAGE. The 1,3- β -glucanase and chitinase activities were separated by PAGE under native conditions at pH 8.9 according to the method of Davis (1964), using 5% (w/v) polyacrylamide stacking gel and 6–15% (w/v) polyacrylamide gradient resolving gel, containing 0.01% (w/v) glycol chitin synthesized from glycol chitosan (Trudel & Asselin, 1989) in the case of chitinase activity. Chitinase activity after PAGE was identified by incubating the gel for 2 h at 37 °C in 50 mM sodium acetate buffer pH 5.2 and staining with calcofluor white M2R according to the method of Trudel & Asselin (1989). Detection of 1,3- β -glucanase activity after PAGE was performed by incubating the gel 2 h at 37 °C with 0.6% (w/v)

laminarin in 10 mM sodium acetate buffer pH 5.2, the reducing sugars formed being detected with 0.15% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma) in 1 M NaOH according to the method of Pan *et al.* (1989). All chemicals for electrophoresis were obtained from Bio-Rad.

Electron microscopy. Cell walls of *A. bisporus* fruit bodies were suspended in 0.05 M sodium acetate buffer pH 5.0 (5 mg ml⁻¹) and incubated with the enzyme solutions (3 mg ml⁻¹ in the same buffer) obtained from cell walls or fructose-grown cultures. Digestions were incubated at 37 °C for 16, 24 and 72 h. Tissue from infected and healthy *A. bisporus* fruit bodies together with the digested cell walls were fixed in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.0 for 4 h at room temperature. The samples were washed four times with phosphate buffer and postfixed in 2% (w/v) osmium tetroxide in the same buffer for 1 h at room temperature. After several washes, the samples were dehydrated in acetone and embedded in Vestopal W resin. Ultrathin sections (50 nm) were collected on 300-mesh copper grids coated with Formvar and stained with uranyl acetate and lead citrate. Samples were examined in a Philips EM300 transmission electron microscope.

Digestion of *A. bisporus* cell walls by the enzyme extracts obtained from fructose or *A. bisporus* cell wall culture medium was also quantified by gravimetric analysis.

RESULTS

Enzyme production of *V. fungicola* grown on different carbon sources

Growth of *V. fungicola* was determined on different carbon sources (Fig. 1). Maximum growth was obtained on sucrose and glucose media compared with that obtained on fructose. Growth on medium containing *A. bisporus* cell walls was not determined because of the inability to separate the insoluble cell wall material from *V. fungicola* mycelium.

The production of *V. fungicola* lytic activities was

Fig. 1. Growth of *V. fungicola* on MSM supplemented with 0.1% (w/v) fructose (■), glucose (○) or sucrose (●) as sole carbon source. Mycelia grown on different carbon sources (50 ml culture medium) were collected and dried at 60 °C. Growth was estimated as mycelial mass (mg). Each value represents the mean of three separate determinations.

Fig. 2. Time-course curves of enzyme activities (U ml^{-1}) produced by *V. fungicola* growing in MSM supplemented with 0.1% (w/v) *A. bisporus* cell walls (□), fructose (■), glucose (○) or sucrose (●) as sole carbon source. (a) 1,3- β -glucanase; (b) 1,6- β -glucanase; (c) 1,4- α -glucanase; (d) 1,3- α -glucanase; (e) 1,4- β -glucanase; (f) 1,4- β -xylanase; (g) *N*-acetylglucosaminidase; (h) protease.

significantly influenced by the carbon source. Fig. 2 shows the time course of (a) 1,3- β -glucanase, (b) 1,6- β -glucanase, (c) 1,4- α -glucanase, (d) 1,3- α -glucanase, (e) 1,4- β -glucanase, (f) 1,4- β -xylanase, (g) *N*-acetylglucosaminidase and (h) protease production by *V. fungicola* mycelia grown in synthetic medium supplemented with 0.1% (w/v) *A. bisporus* cell walls, fructose, glucose or sucrose. Whereas 1,3- β -glucanase and 1,4- α -glucanase were, remarkably, detected with all the carbon sources used, the other activities were only significantly produced with *A. bisporus* cell walls (1,4- β -glucanase was induced only in this medium). Proteolytic and 1,3- β -glucanase activities were induced earlier in the medium containing *A. bisporus* cell walls than in the media with other carbon sources. The maximum extracellular protein production and the highest enzyme activities were obtained after 72 h incubation in all cases. Non-inoculated cell wall medium contained some basal protein ($0.9 \mu\text{g ml}^{-1}$). This protein may have come from

fragments that were released from the cell walls by heat sterilization. However, after 72 h of *V. fungicola* growth the protein reached $3.1 \mu\text{g ml}^{-1}$ in the same medium but a part of the basal protein could have been digested by the action of the parasite. The fructose, glucose and sucrose media after 72 h of *V. fungicola* growth contained 1.1, 2.5 and $2.0 \mu\text{g protein ml}^{-1}$, respectively.

The production of β -D-glucosidase, α -D-glucosidase, β -D-xylosidase, β -D-mannosidase, chitobiosidase and *N*-acetylglucosaminidase together with endolytic activities were also determined (Table 1). β -D-Glucosidase and α -D-glucosidase activities were detected in all the culture media whereas β -D-xylosidase and chitobiosidase were found only in media containing cell walls and fructose; chitobiosidase activity was considerably greater in the medium containing cell walls. Maximum specific activities [$\text{U (mg extracellular protein)}^{-1}$] of the enzymes assayed (Table 1) were obtained when the carbon source was *A. bisporus* cell walls, except in the case of 1,3- β -glucanase, 1,4- α -glucanase and 1,4- β -xylanase, which reached their maximum values in medium containing fructose as the carbon source.

Native PAGE

PAGE analysis of the *V. fungicola* protein extracts obtained in different culture media (Fig. 3a) revealed that the mycoparasite presents different protein patterns depending on whether it is growing on *A. bisporus* cell walls or on glucose, sucrose or fructose. Extract from non-inoculated cell wall medium used as control did not show any distinct band when analysed by electrophoresis (Fig. 3a, lane 0). Three (A–C) and seven (A–G) bands corresponding to, respectively, 1,3- β -glucanase and chitinase activities were developed on PAGE (Fig. 3b, c). Depending on the carbon source used, different isoforms of both enzymes were detected. 1,3- β -Glucanase isoform C was only found in culture filtrates of medium containing cell walls. Isoforms A and B were detected in all culture media but were significantly increased in cell wall culture filtrate. In the case of chitinase, identical electrophoretic profiles (isoforms A and B) were detected in culture filtrates of media containing glucose, sucrose or fructose. A completely different profile (isoforms A–G) was detected in culture filtrates of medium containing *A. bisporus* cell walls.

Electron microscopy

Pieces of healthy and infected *A. bisporus* fruit bodies were examined by transmission electron microscopy. Fig. 4(a), as control, is an ultrathin section of healthy *A. bisporus* fruit body hyphae, while Fig. 4(b) shows the invasion of the host hyphae by *V. fungicola*. The remaining micrographs (Fig. 4c–f) show the interaction zone between *V. fungicola* and *A. bisporus* and part of the host wall where clear signs of cell wall degradation can be seen.

Fig. 5(c–h) shows the sequential degradation of isolated *A. bisporus* fruit body cell walls by the *V. fungicola* enzyme extracts (only from media with cell walls or

Table 1. Extracellular enzyme activities produced by *V. fungicola* grown in MSM with different carbon sources

Enzyme activities were measured after 72 h incubation. Results are means of at least three independent experiments. U, total activity ($\mu\text{mol h}^{-1}$); U mg^{-1} , specific activity.

Enzyme activity	Substrate	Carbon source							
		Cell wall		Fructose		Glucose		Sucrose	
		U	U mg^{-1}	U	U mg^{-1}	U	U mg^{-1}	U	U mg^{-1}
1,3- β -Glucanase	Laminarin	63.80	405.98	31.15	551.81	53.65	418.48	36.35	353.77
1,6- β -Glucanase	Pustulan	31.35	199.49	4.85	85.91	4.10	31.98	3.75	36.49
1,4- β -Glucanase	CM-cellulose	10.00	63.63	0	0	0	0	0	0
1,3- α -Glucanase	1,3- α -Glucan	15.75	100.22	5.35	94.77	7.35	57.33	5.50	53.52
1,4- α -Glucanase	Amylose	26.80	170.50	11.95	211.69	18.45	143.91	20.7	201.45
1,4- β -Xylanase	Xylan	11.1	70.63	4.30	76.17	4.75	37.05	4.60	44.76
β -D-Glucosidase	pNP- β -D-Glc	7.60	48.36	1.00	17.71	0.75	5.85	0.95	9.24
α -D-Glucosidase	pNP- α -D-Glc	0.50	3.18	0.10	1.77	0.10	0.78	0.1	0.97
β -D-Xylosidase	pNP- β -D-Xyl	0.40	2.54	0.10	1.77	0	0	0	0
β -D-Mannosidase	pNP- β -D-Man	0.45	2.86	0	0	0	0	0	0
Chitinase	Chitin	6.35	40.40	0.85	15.05	0.85	6.63	0.85	8.27
N-Acetylglucosaminidase	pNP-GlcNAc	6.55	41.67	1.80	31.88	1.35	10.53	0.35	3.40
Chitobiosidase	pNP-(GlcNAc) ₂	4.00	25.45	0.1	1.77	0	0	0	0
Protease	Casein	841.0	5351.5	135.25	2395.92	32.35	252.34	32.35	314.84

Fig. 3. PAGE (6–15% gradient) of (a) extracellular proteins (85 μg of each sample), (b) 1,3- β -glucanase activities (20 μg of each sample) and (c) chitinase activities (20 μg of each sample) secreted by *V. fungicola* grown on media containing either *A. bisporus* cell walls (lane 1), glucose (lane 2), sucrose (lane 3) or fructose (lane 4); non-inoculated medium containing *A. bisporus* cell walls was used as control (lane 0, 70 μg).

fructose because glucose and sucrose cultures produced poorer cell wall digestions), together with the corresponding controls (Fig. 5a, b). The lytic effect of the enzyme extract obtained with *A. bisporus* cell walls

appeared significantly higher than that obtained with fructose as the carbon source and this was confirmed by gravimetric analysis of the cell walls after digestion. After 16 h the percentage weight loss of the cell walls

Fig. 4. Transmission electron micrographs of *V. fungicola* (V) infection of *A. bisporus* (A). (a) Healthy *A. bisporus* fruit body hyphae (bar, 2 μ m); (b) invasion of host hyphae by *V. fungicola* (bar, 2 μ m); (c), interaction zone between *V. fungicola* and *A. bisporus* (bar, 1 μ m); (d, e) details of the interaction site before penetration, showing signs of *A. bisporus* cell wall degradation (bars, 1 μ m); (f) penetration of *A. bisporus* by *V. fungicola*. Note disappearance of wall structure at penetration site (bar, 1 μ m).

Fig. 5. Transmission electron micrographs of *A. bisporus* cell walls incubated with enzyme extracts of *V. fungicola*. (a, b) *A. bisporus* cell wall controls, 0 h and 72 h respectively; (c, e, g) cell walls after 16, 24 and 72 h, respectively, of incubation with the enzyme extract of *V. fungicola* grown in medium containing *A. bisporus* cell walls as carbon source; (d, f, h) cell walls after 16, 24 and 72 h, respectively, of incubation with the enzyme extract of *V. fungicola* grown in medium containing fructose as carbon source. Bars, 1 μ m.

incubated with the enzyme extracts from medium containing fructose and that containing cell walls was 7.6% and 18.9%, respectively; after 24 h it was 9.6% and 26.7%; and after 72 h it was 16.7% and 40.0% (each value represents the mean of three separate determinations).

DISCUSSION

In the present work the production of enzymes secreted by *V. fungicola* growing on minimal synthetic medium supplemented with glucose, fructose, sucrose or *A. bisporus* fruit body cell walls as the carbon source has been studied, in an attempt to compare their hydrolytic effect on such walls with the *in vivo* effect of *V. fungicola* on *A. bisporus* fruit bodies. Some of the enzyme activities identified were endopolysaccharidases (1,3- β -glucanase, 1,6- β -glucanase, 1,4- β -glucanase, 1,3- α -glucanase, 1,4- α -glucanase, 1,4- β -xylanase and chitinase), disaccharases (chitobiosidase), exopolysaccharidases (β -D-glucosidase, α -D-glucosidase, β -D-xylosidase, β -D-mannosidase, and N-acetyl- β -D-glucosaminidase) and proteases. Some of these lytic enzymes, which are key enzymes in the lysis of cell walls of higher fungi, are produced by other organisms that are known to attack and parasitize fungi (Elad *et al.*, 1982, 1985).

The results have shown that most of the enzyme activities produced by *V. fungicola* growing on *A. bisporus* walls are also present in the three other media tested, although in lesser amounts, suggesting their partial constitutive nature – 1,4- β -glucanase was induced by cell walls but not by the other substrates. 1,3- β -Glucanase seemed to be less affected by the carbon source than the other activities, showing high values in all the culture media. Cell walls promoted 1,6- β -glucanase production and constitutive enzyme levels were produced when the mycelium was incubated with fructose, glucose or sucrose. Data obtained suggest that enzyme activities might be subject to both induction and catabolite repression since they reach a maximum activity after 72 h and then drastically decrease, except for 1,3- β -glucanase and protease activities, which did not show significant differences after 3 d growth.

The fact that the production of most of the carbohydrases and proteases was higher in the medium containing cell walls is not surprising since *A. bisporus* contains large amounts of different carbohydrates and proteins in its walls (García Mendoza *et al.*, 1987a; Mol & Wessels, 1990). As this cell wall possesses a complex layered structure (García Mendoza *et al.*, 1987b; Mol *et al.*, 1990) the gradual action of the endopolysaccharidases could facilitate the subsequent action of the exopolysaccharidases.

On the other hand, PAGE results showed that *V. fungicola* presented different extracellular protein patterns when growing in medium containing cell walls or with the other carbon sources. Moreover, cultures grown in the cell wall medium gave a higher number of protein bands and some of them may correspond to other specific cell wall lytic activities not identified at

present. 1,3- β -Glucanase and chitinase activities separated by native gel electrophoresis and identified by their specific tests were found to be composed of various isoforms. Obviously, *V. fungicola* produces different isoforms of both hydrolytic enzymes according to the composition of the growth medium, indicating that at least some of those isoforms are induced only in the presence of *A. bisporus* cell walls.

From all these results it can be concluded that the secretion of the enzymes *in vitro* is a good indicator of the kind of enzymes that the pathogen could produce and the role that they play in relation to disease.

Electron microscopy of the sequential enzymic digestion of the isolated *A. bisporus* cell walls showed significant differences between the enzyme extract obtained from cell walls medium and from fructose medium, suggesting that the presence of *A. bisporus* cell walls induces in *V. fungicola* the appropriate enzyme engineering to attack *A. bisporus* cell walls. The micrographs presented here show evidence that this degradation does not take place simultaneously and with the same intensity on both the outer and inner wall surfaces, although at the end of the process, most, and in some zones all, the wall polysaccharidic structure partially disappeared. This *in vitro* multistage wall degradation may be different than that in living cells in which other factors can limit the accessibility of the substrates to the enzymes, but in any case an important enzymic degradation seems to occur which produces softening of *A. bisporus* cell walls by the intercellular *V. fungicola* infection (Dragt *et al.*, 1995) or the breakage of more sensitive points of this wall which permits intracellular penetration (Dragt *et al.*, 1996).

The different behaviour of the *A. bisporus* living cells towards the *V. fungicola* enzymes could be due to particular architectural features of the cell wall related to the morphogenetic process, together with other factors such as the production of brown pigments (melanins), also suggested by Dragt *et al.* (1995), which can inhibit the action of the polysaccharide enzymes by producing resistant chitin-melanin complexes (Bull, 1970).

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