Regulation of chitinase synthesis in *Trichoderma harzianum*

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(Received 12 December 1990; revised 24 May 1991; accepted 3 June 1991)

The production of chitinase by *Trichoderma* species is of interest in relation to their use in biocontrol and as a source of mycolytic enzymes. Fourteen isolates of the genus were screened to identify the most effective producer of chitinase. The best strain for chitinase was *Trichoderma harzianum* 39.1, and this was selected for study of the regulation of enzyme synthesis. Washed mycelium of *T. harzianum* 39.1 was incubated with a range of carbon sources. Chitinase synthesis was induced on chitin-containing medium, but repressed by glucose and *N*-acetylglucosamine. Production of the enzyme was optimal at a chitin concentration of 0.5%, at 28 °C, pH 6.0 and was independent of the age of the mycelium. The synthesis of chitinase was blocked by both 8-hydroxyquinoline and cycloheximide, inhibitors of RNA and protein synthesis, respectively. The mode of chitinase synthesis in this fungus is discussed.

Introduction

Chitin, a β -1,4-linked polymer of *N*-acetylglucosamine, is a major component of the cell wall of many fungi (Austin *et al.*, 1981; Peberdy, 1990). The enzymic degradation of chitin by micro-organisms occurs in two consecutive steps: first the hydrolysis by chitinase [poly(1,4-(*N*-acetyl- β -D-glucosaminide)) glycanohydrolase; EC 3.2.1.14] to oligomers, mainly dimers, followed by their degradation to free *N*-acetylglucosamine by chitinase (*N*-acetyl- β -glucosaminidase; EC 3.2.1.30). The monosaccharide released can then be metabolized by many organisms (Jeuniaux, 1966).

These enzymes are widely distributed in nature and have been detected in bacteria and fungi (Cabib, 1987). Fungal chitinases are involved in gross autolysis associated with the release of spores and stipe elongation in some basidiomycetes (Iten & Matile, 1970) and autolysis of mycelium in cultures (Isaac & Gokhale, 1982). They may have a nutritional role (de Vries & Wessels, 1973) and a morphogenetic function in hyphal growth and differentiation of fungi (Bartnicki-Garcia, 1973; Burnett, 1979; Wessels, 1984).

In recent years several workers have suggested that chitinase-producing fungi, e.g. species of *Trichoderma*, can be effective as biological control agents against fungal pathogens (Tokimoto, 1982; Chet, 1987). The main mechanism involved in the antagonism of *Trichoderma* spp. and pathogenic fungi appears to be the release of lytic enzymes, including chitinases.

Chitinases are produced by *Trichoderma harzianum* when it is grown in the presence of chitin or isolated fungal cell walls (Elad *et al.*, 1983; Ridout *et al.*, 1988; Tokimoto, 1982); however, other nutritional conditions that promote synthesis of this enzyme have not been investigated. Furthermore, it is not known whether the induction of chitinase by chitin in *T. harzianum* is effected by the same inducer-repressor mechanism as has been proposed for other hydrolases produced by *Trichoderma* strains (Merivuori *et al.*, 1985). The objective of this study was to determine the physiological conditions which induce and repress chitinase production in *T. harzianum*.

Methods

Organism and culture conditions. All the *Trichoderma* strains used were from the University of Nottingham collection, except the *Trichoderma hamatum* strains (40.1, 40.2 and 40.3), which were obtained from Dr L. Maczinger, Department of Microbiology, Attila Jozsef University, Szeged, Hungary. Spores obtained from slant cultures on a malt/yeast/glucose agar medium (0.2% yeast extract, 0.2% malt extract, 2% glucose and 2% agar; all w/v) were collected in sterile water, centrifuged at 2000 r.p.m., washed twice and used as an inoculum [1.0×10^7 spores per ml of culture medium (TM)]. TM medium contained (g l⁻¹): bacto-peptone, 1.0; urea, 0.3; KH₂PO₄, 2.0; (NH₄)₂SO₄, 1.4; MgSO₄·7H₂O, 0.3; CaCl₂·6H₂O, 0.3; and 0.1% (v/v) trace element solution containing Fe²⁺, Mn²⁺, Zn²⁺ and Co²⁺. The fungus was grown in conical flasks (250 ml) containing 25 ml TM medium supplemented with the appropriate carbon source (0.3–2.0%, w/v) and incubated with shaking (180 r.p.m.) at 28 °C for an appropriate time.

Conditions for enzyme induction in washed mycelium. *T. harzianum* 39.1 was grown from a spore inoculum on TM medium using glucose (2%, w/v) as the carbon source. The mycelium was harvested after 18–24 h incubation, washed thoroughly with 250 ml 10 mM-potassium phosphate buffer pH 6.0 and transferred to conical flasks containing 25 ml minimal medium (MM; KH_2PO_4 , 2.0 g l⁻¹; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g l⁻¹; and $\text{CaCl}_2 \cdot 7\text{H}_2\text{O}$, 0.3 g l⁻¹). This was immediately supplemented with the substrates tested for their potential for induction. Mycelial suspensions were incubated with shaking (180 r.p.m.) at 28 °C for up to 24 h, unless stated otherwise.

Enzyme preparations. For chitinase production the mycelium was harvested by filtration onto Whatman no. 1 filter paper, washed with 250 ml 10 mM-potassium phosphate buffer, pH 6.0, frozen at -70 °C and freeze-dried. Lyophilized mycelium was weighed and then ground to a fine powder with liquid nitrogen. The samples were dialysed against 50 mM-sodium acetate buffer pH 5.2 and centrifuged at 12000 g for 15 min at 4 °C. The supernatant was used as a soluble fraction and the pellet as the insoluble cell-bound fraction. The culture filtrate was dialysed overnight at 4 °C against 50 mM-sodium acetate buffer pH 5.2, stored at -20 °C and used as extracellular enzyme preparation.

Chitinase assay. The assay is based on the estimation of reducing sugars released during the hydrolysis of swollen chitin. The reaction mixture, containing 1 ml 0.5% swollen chitin (suspended in 50 mM-sodium acetate buffer pH 5.2, containing 0.02% sodium azide) and 1 ml enzyme solution, was incubated for 1–24 h at 40 °C with shaking, and then centrifuged at 4000 r.p.m. for 5 min. The amount of reducing sugar released in the supernatant was determined by the method described by Miller (1959), using *N*-acetylglucosamine as standard. One unit (U) of activity was defined as the amount of enzyme which catalyses the release of 1 µmol reducing sugar in 1 h at 40 °C. Specific activity is given in units (mg protein)⁻¹.

Chitobiase activity. Chitobiase activity was determined by estimating the amount of *p*-nitrophenol released when enzyme preparations were incubated with *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine. The reaction mixture comprised 0.2 ml 5 mM-substrate, 0.7 ml 10 mM-potassium phosphate buffer pH 6.0, and 0.1 ml enzyme solution. After 10 min at 37 °C, 2.0 ml 0.25 M- Na_2CO_3 was added to stop the reaction. The amount of *p*-nitrophenol liberated was measured spectrophotometrically at 405 nm. One unit (U) of activity was defined as the amount of enzyme that liberates 1 µmol of the reaction product from the substrate min⁻¹.

Enzyme purification. All operations were carried out at 4 °C. Ammonium sulphate was added to the supernatant, from cultures grown on a chitin-containing medium, to give 75% saturation. The precipitate formed was collected by centrifugation and after dialysis (against 50 mM-sodium acetate buffer pH 5.5: buffer A) was applied to a Q-Sepharose column (1.6 × 37 cm) previously equilibrated with buffer A. The chitinase which passed through the column in the void volume, was collected, concentrated by lyophilization and applied to a Sephadex G-100 column (2.6 × 92 cm). The column was equilibrated and eluted with buffer A, at flow rate of 15 ml h⁻¹. Fractions containing chitinase activity were pooled and applied to a Phenyl-Sepharose CL-4B column (1.5 × 6.0 cm) equilibrated with 10 mM-potassium phosphate buffer pH 6.7, containing 0.8 M-ammonium sulphate. The column was washed with a linear gradient of 0.8–0 M-ammonium sulphate and active enzyme was eluted with a buffer solution containing no ammonium sulphate. Fractions showing chitinase activity were pooled and stored at -70 °C.

Analytical methods. Mycelium was lyophilized overnight for dry weight determination. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard. SDS-PAGE was carried out according to Laemmli (1970), using an

11% (w/v) gel. Molecular mass markers were: bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa) and trypsin inhibitor (20 kDa).

Reagents and chemicals. Size markers for electrophoresis, chitobiose, *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine, cycloheximide, chitin and chitosan were commercial products from Sigma. 8-Hydroxyquinoline was purchased from FSA. Swollen chitin used in the chitinase assay was prepared from chitin by the method described Snell (1981). All other chemicals used in the experiments were of the highest reagent grade.

Results

Screening for extracellular chitinase and chitobiase production

The *Trichoderma* strains were grown in TM medium, supplemented with 0.3% (w/v) glucose and 0.5% (w/v) chitin, at 28 °C and shaken at 180 r.p.m. After 48 h the cultures were harvested and protein, chitinase and chitobiase present in the culture filtrates were determined. All 14 *Trichoderma* strains tested produced chitinase and chitobiase when grown on chitin-containing medium supplemented with 0.3% (w/v) glucose (Table 1). The best strains for chitinase production were *T. harzianum* 39.1 and *Trichoderma* sp. 41.4, which produced nearly 3–17 times more enzyme than any other strain. After 48 h the level of chitinase and chitobiase released to the medium was similar in both strains (Table 1). The kinetics of chitinase production are illustrated in

Table 1. Screening for extracellular chitinase and chitobiase production by *Trichoderma* strains

All strains were grown on TM medium supplemented with 0.3% glucose and 0.5% chitin and harvested after 48 h incubation. Each result is the mean of three replicates ± SD.

Strain*	Specific activity [U (mg protein) ⁻¹]	
	Chitinase	Chitobiase
<i>T. viride</i> 37.1	0.49 ± 0.23	1.87 ± 0.22
<i>T. viride</i> 37.2	0.54 ± 0.11	3.34 ± 0.06
<i>T. viride</i> 37.3	0.64 ± 0.02	22.35 ± 1.00
<i>T. viride</i> 37.4	0.29 ± 0.01	14.29 ± 0.46
<i>T. viride</i> 37.5	0.30 ± 0.01	3.04 ± 0.90
<i>T. aureoviride</i> 38.1	0.28 ± 0.01	3.28 ± 0.10
<i>T. harzianum</i> 39.1	2.06 ± 0.70	8.67 ± 0.83
<i>T. hamatum</i> 40.1	0.37 ± 0.05	2.93 ± 0.14
<i>T. hamatum</i> 40.2	0.38 ± 0.01	5.16 ± 0.45
<i>T. hamatum</i> 40.3	0.59 ± 0.06	17.74 ± 0.60
<i>Trichoderma</i> sp. 41.1	0.20 ± 0.02	36.88 ± 0.60
<i>Trichoderma</i> sp. 41.2	0.12 ± 0.04	0.95 ± 0.01
<i>Trichoderma</i> sp. 41.3	1.33 ± 0.09	1.84 ± 0.02
<i>Trichoderma</i> sp. 41.4	2.00 ± 0.10	9.26 ± 0.40

* The strain numbers are those of the University of Nottingham collection.

Fig. 1. Specific activity of chitinase (○, □) and chitobiase (●, ■) produced by *T. harzianum* 39.1 (○, ●) and *Trichoderma* sp. 41.1 (□, ■) during growth in liquid TM medium containing chitin as carbon source.

Table 2. Effect of different carbon sources on growth, extracellular protein and chitinase production by *T. harzianum* 39.1

All cultures were inoculated with 10^7 conidia ml^{-1} and harvested after 48 h incubation. Each result is the mean of three replicates $\pm$ SD.

Carbon source	Growth (mg dry wt) per culture	Protein ($\mu\text{g ml}^{-1}$)	$10 \times$ Chitinase (U)
Glucose (0.3%)	44.0 ± 1.6	13.7 ± 1.1	0.18 ± 0.02
Glucose (0.5%)	65.4 ± 2.0	12.7 ± 0.3	0.24 ± 0.02
GlcNAc (0.3%)	15.3 ± 1.5	7.2 ± 0.6	0.08 ± 0.01
GlcNAc (0.5%)	43.1 ± 2.0	12.7 ± 0.7	0
GlcN (0.3%)	14.5 ± 1.0	15.4 ± 1.1	0.29 ± 0.03
GlcN (0.5%)	34.5 ± 3.5	17.8 ± 1.5	0.37 ± 0.02
Sucrose (0.3%)	49.2 ± 2.0	9.4 ± 0.5	0.06 ± 0.01
Mannose (0.3%)	40.3 ± 1.7	16.9 ± 2.1	0.14 ± 0.02
Lactose (0.3%)	49.3 ± 1.2	7.2 ± 0.8	0
Xylose (0.3%)	41.3 ± 1.5	16.5 ± 2.8	0.20 ± 0.02
Chitin (0.5%)	ND	20.1 ± 0.2	1.11 ± 0.03
Chitin (1.0%)	ND	34.6 ± 1.3	1.87 ± 0.08

ND, Not determined.

* GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine.

Table 3. Effect of different carbon sources on the production of chitinase by washed mycelium of *T. harzianum* 39.1

Washed mycelium was incubated with the substrate for 24 h at 28 °C and both cell-bound and extracellular chitinase were assayed. The results are means of three replicates $\pm$ SD.

Carbon source*	$10^3 \times$ Chitinase [U (mg mycelium) $^{-1}$]		
	Cell-bound	Extracellular	Total
None	0.33 ± 0.03	1.80 ± 0.27	2.13 ± 0.30
Glucose (10^{-2} M)	0.56 ± 0.03	1.67 ± 0.29	2.23 ± 0.32
GlcNAc (10^{-2} M)	0.47 ± 0.08	1.39 ± 0.28	1.86 ± 0.36
Glucosamine (10^{-2} M)	0.56 ± 0.03	2.77 ± 0.28	3.33 ± 0.31
Sucrose (10^{-2} M)	0.47 ± 0.01	2.50 ± 0.28	2.97 ± 0.28
Lactose (10^{-2} M)	0.56 ± 0.00	3.88 ± 0.28	4.44 ± 0.28
Xylose (10^{-2} M)	0.74 ± 0.02	3.05 ± 0.25	3.79 ± 0.27
Mannose (10^{-2} M)	0.56 ± 0.04	3.60 ± 0.28	4.16 ± 0.32
Cellobiose (10^{-2} M)	0.45 ± 0.03	2.00 ± 0.25	2.45 ± 0.28
Chitobiose (10^{-4} M)	0.60 ± 0.01	1.85 ± 0.55	2.45 ± 0.56
Chitin (0.50%)	1.50 ± 0.05	7.20 ± 0.20	8.70 ± 0.25
S-chitin (0.50%)	2.70 ± 0.25	5.30 ± 0.25	8.00 ± 0.50
Cellulose (0.50%)	0.20 ± 0.05	1.85 ± 0.20	2.05 ± 0.25
Chitosan (0.50%)	0.25 ± 0.00	0.50 ± 0.01	0.75 ± 0.01

* GlcNAc, *N*-acetylglucosamine; S-chitin, swollen chitin.

conditions previously described, harvested and analysed for growth, extracellular protein and chitinase activity. Enzyme production was not related to mycelial biomass, as most of the non-inducing sugars supported good growth. However, high chitinase activities were found only in cultures supplied with chitin (Table 2). Biomass produced on chitin-containing medium was not determined, because of inaccuracies due to the mycelium being adsorbed onto chitin. Growth on *N*-acetylglucosamine, at concentrations of 0.3 and 0.5% (w/v), resulted in less enzyme production than was obtained with other non-chitinous substrates. In contrast, significant chitinase production, up to 20% of that of cultures grown on chitin, was observed in glucosamine-containing media (Table 2).

Effect of various carbon substrates on the synthesis and secretion of chitinase in washed mycelium

To verify the importance of chitin for chitinase induction an experimental system was used in which non-induced, glucose-grown mycelium was incubated in the presence of various carbon substrates. Washed mycelium was incubated in minimal medium supplemented with a range of other substrates for 24 h at 28 °C, and activities of cell-bound and extracellular chitinase were determined. Synthesis of the enzyme was influenced by the sugar used as a carbon source, but significant levels of chitinase were found when mycelium was incubated with chitin, with yields fourfold higher than on glucose (Table 3). This suggested that enzyme synthesis was induced by

Fig. 1. Synthesis of chitinase increased rapidly after 24 h, peaking at 48 h; longer periods of culture resulted in an increase in extracellular chitobiase activity and a decrease in chitinase activity. *T. harzianum* 39.1 was selected for further studies on chitinase synthesis.

Chitinase synthesis during growth on different carbon sources

The synthesis of chitinase in the presence of its substrate raised the question of the regulation of synthesis. This was investigated using a range of alternative carbon sources (Table 2). Cultures were grown for 48 h under

Fig. 2. Factors affecting chitinase production in washed mycelium of *T. harzianum* 39.1: (a) temperature (●, 24 °C; ○, 28 °C; △, 34 °C); (b) mycelium age (○, 18 h; ●, 24 h); (c) pH.

Table 4. Influence of chitin concentration on the production of chitinase by washed mycelium of *T. harzianum*

Washed mycelium was incubated with chitin for 18 h at 28 °C. Cell-bound and extracellular chitinase were assayed. The results are means of three replicates $\pm$ SD.

Chitin (%)	$10^2 \times$ Chitinase activity (U ml ⁻¹)		
	Cell-bound	Extracellular	Total
0	2.4 $\pm$ 0.2	2.0 $\pm$ 0.1	4.4 $\pm$ 0.3
0.1	2.3 $\pm$ 0.0	18.0 $\pm$ 0.9	20.3 $\pm$ 0.9
0.25	3.1 $\pm$ 0.4	24.0 $\pm$ 1.2	27.1 $\pm$ 1.6
0.5	3.9 $\pm$ 0.3	35.0 $\pm$ 0.9	38.9 $\pm$ 1.2
1.0	3.7 $\pm$ 0.3	33.0 $\pm$ 0.5	36.7 $\pm$ 0.8
2.0	2.9 $\pm$ 0.1	29.0 $\pm$ 0.7	31.9 $\pm$ 0.8

the substrate and the levels produced on glucose are constitutive. Clearly such a mechanism of control is not absolute because significant levels of production were obtained on lactose, xylose and mannose. Chitinase synthesis was repressed when other polymers such cellulose and chitosan were used as a substrate. Significant chitinase activity was present when the fungus was incubated in chitin-containing minimal medium, in the absence of any other compounds.

Culture conditions for maximum chitinase synthesis in washed mycelium

The production of chitinase in washed mycelium in chitin-containing medium was temperature dependent, levels detected at 28 °C being higher than at 34 °C and 24 °C (Fig. 2a). The physiological age of the mycelium was not a factor; both young and old mycelium, harvested at 18 h (linear phase) and 24 h (early stationary phase), respectively, showed similar production of extracellular chitinase (Fig. 2b). Production of chitinase was markedly affected by pH, with the optimum at 6.0 (Fig. 2c). Chitinase production was substrate concentration dependent (Table 4); above 0.5% (w/v) chitin there was no further promotion of synthesis.

Fig. 3. Effect of glucose and *N*-acetylglucosamine on the induction of chitinase in *T. harzianum* 39.1. (a) Washed mycelium was incubated with 0.5% chitin and after 6 h glucose (▲) or *N*-acetylglucosamine (●) was added to give a final concentration of 1% (w/v). The control (■) contained no glucose or *N*-acetylglucosamine. (b) Concentrations of glucose (▲) and *N*-acetylglucosamine (●) in the culture medium.

There was no appreciable accumulation of cell-bound chitinase before the appearance of the enzyme in the medium (Tables 3 and 4), indicating that synthesis and secretion of chitinase are closely associated events occurring only under conditions of favourable temperature and pH.

Effect of glucose and *N*-acetylglucosamine on chitinase induction

Experiments were carried out with washed mycelium in which chitin medium was supplemented with 1.0% (w/v) glucose or 1.0% (w/v) *N*-acetylglucosamine at 0 h and 6 h of incubation. A decrease in chitinase production was observed in the presence of both carbon sources (Table 5, Fig 3). Repression was greatest when glucose or *N*-

Fig. 4. SDS-PAGE analysis of proteins secreted by *T. harzianum* 39.1 after transfer of the mycelium from non-inducing conditions to media with different carbon sources: 1, glucose (1%); 2, *N*-acetylglucosamine (1%); 3, chitin (1%); 4, chitin (1%) + glucose (1%); 5, chitin (1%) + *N*-acetylglucosamine (1%); 6, GlcNAc (1%) + glucose (1%). Ch, chitinase purified from culture filtrate of *T. harzianum*. The sizes of marker proteins are shown on the left.

Table 5. Effect of single and mixed carbon sources on chitinase production by washed mycelium of *T. harzianum* 39.1

Each substrate or mixed substrate was added at onset of the experiment and cultures were harvested after 24 h incubation. All values are means of three replicates; standard deviations were less than 10% of the mean.

Carbon source*	Chitinase [U (mg protein) ⁻¹]	
	Cell-bound	Extracellular
Glucose (1%)	0.005	0.60
GlcNAc (1%)	0.018	0.59
Chitin (0.5%)	0.050	6.55
Chitin (0.5%) + Glucose (1%)	0.020	0.42
Chitin (0.5%) + GlcNAc (1%)	0.010	0.65
GlcNAc (1%) + Glucose (1%)	0.012	0.67

* GlcNAc, *N*-acetylglucosamine.

Table 6. The effect of inhibitors of RNA and protein synthesis on extracellular protein and chitinase activity in washed mycelium of *T. harzianum* 39.1.

Inhibitor ($\mu\text{g ml}^{-1}$)	Protein ($\mu\text{g ml}^{-1}$)	$10^3 \times$ Chitinase activity* [U (mg mycelium) ⁻¹]
None	21.4	8.02
8-Hydroxyquinoline		
20	22.2	3.02
50	21.5	2.50
100	10.8	2.37
Cycloheximide		
5	13.9	1.05
10	16.6	0.32
20	17.7	0.39

* The results are means of two replicates.

acetylglucosamine was added to the chitin-containing medium at the onset of the experiment. No induction could be detected and the level of chitinase activity was the same as that produced in the presence of 1.0% (w/v) glucose or 1.0% (w/v) *N*-acetylglucosamine alone (Table 5). The proteins secreted into the culture filtrate were concentrated by precipitation with 75% saturation ammonium sulphate, separated by 11% SDS-PAGE gel and visualized by staining with Coomassie blue (Fig. 4). One protein band, with an apparent molecular mass of 40 kDa, was found only when chitin was used as carbon source. This protein has the same apparent molecular mass as purified chitinase from *T. harzianum* (39.1 kDa; Fig. 4). In the presence of glucose and *N*-acetylglucosamine this protein band was not observed.

Addition of the sugars 6 h after the onset of induction also repressed chitinase production (Fig. 3a). The rate of utilization of the two sugars was similar (Fig. 3b); exhaustion of glucose prompted the production of enzyme, but this was not observed with *N*-acetylglucosamine.

Inhibition of RNA and protein synthesis and its effect on enzyme induction

To determine whether the production and secretion of chitinase was dependent on *de novo* protein synthesis, the effects of 8-hydroxyquinoline and cycloheximide, which inhibit RNA and protein synthesis respectively (McKeehan & Hardesty, 1969; Fraser & Creanor, 1974), on chitinase production during induction were investigated. The results are shown in Table 6. Both inhibitors

exhibited a strong effect on chitinase production in washed mycelium. The induction of chitinase and protein synthesis was suppressed far more with cycloheximide than with 8-hydroxyquinoline.

Discussion

A wide range of prokaryotic and eukaryotic micro-organisms have the potential to produce chitinolytic enzymes when chitin is present in the growth medium (Jeuniaux, 1966; Monreal & Reese, 1969). Formation of chitinase in micro-organisms is thought to be controlled by a repressor-inducer system in which chitin or products of degradation (oligomers) serve as an inducer (Monreal & Reese, 1969). Since chitin is insoluble, the micro-organisms are unable to utilize it unless it has been hydrolysed to soluble oligomers or *N*-acetylglucosamine. Several proposals have been made to explain how induction may occur in this situation.

Monreal & Reese (1969) suggested that the most probable inducers of chitinase in *Serratia marcescens* are soluble oligomers derived from chitin, but not the monomer (*N*-acetylglucosamine). In contrast, St Leger *et al.* (1986), using slow-feeding with sugars or alanine in a carbon-deficient medium, demonstrated that the most effective inducer of chitinase in *Metarhizium anisopliae* was *N*-acetylglucosamine, when supplied at a rate of about 20 µg ml⁻¹ h⁻¹. Smith & Grula (1983), working with *Beauveria bassiana*, reported that the sterilization of chitin by autoclaving or boiling causes release of glucosamine and *N*-acetylglucosamine from the macromolecule and the solubilized components function as the inducers for chitinase synthesis. Roby *et al.* (1987) showed that some chitin oligomers (hexamer to nonamer), at high concentrations, enhanced chitinase activity in melon plants by a factor of 3 to 5.

The work described in this paper shows that chitinase synthesis in *T. harzianum* 39.1 is also regulated by products of chitin degradation. High chitinase activity was found only in cultures supplied with chitin but not with other polymers such as cellulose and chitosan, which is further indicative of induction. Interestingly, removal of the acetyl group produces a non-susceptible substrate (chitosan) and substitution of the *N*-acetyl group with OH (=cellulose) similarly yields a non-substrate. Neither chitobiose nor *N*-acetylglucosamine promoted enzyme production in *T. harzianum* 39.1 under the conditions of these experiments. The levels obtained were comparable with those produced with glucose. However, it seems unlikely that chitobiose could function as an inducer of chitinase activity since extracellular constitutive chitobiase activity would probably

degrade chitobiose to *N*-acetylglucosamine before it entered the cell.

Another explanation for chitinase induction is that chitinolytic micro-organisms have trace quantities of constitutive enzymes which are continuously released even under starvation conditions. The constitutive level of chitinase activity may be sufficient to initiate chitin degradation and release soluble oligomers. Vasseur *et al.* (1990) reported the isolation of a mutant of *Aphanocladium album* with a high level of constitutive chitinase activity, which allowed an early degradation of chitin and early induction of chitinase when compared to the wild-type. Evidence that *T. harzianum* 39.1 produces chitinase constitutively was obtained in our experiments. Constitutive enzyme levels were produced when the mycelium was incubated with glucose, *N*-acetylglucosamine, glucosamine, sucrose, mannose or xylose, or even in the absence of a carbon source. These results lead us to the view that the probable inducers of chitinase in *T. harzianum* 39.1 are soluble oligomers derived from the chitin preparation or by the action of constitutive chitinase.

Another possible suggestion, so far unsubstantiated, is that the signal for induction of synthesis of chitinase could result from the physical contact between the cell surface and the insoluble substrate, as has been proposed for cellulase biosynthesis (Berg & Petterson, 1977; Binder & Ghose, 1978).

The inductive formation of chitinase in washed mycelium of *T. harzianum* 39.1 was markedly affected by addition of glucose or *N*-acetylglucosamine, suggesting that catabolic repression may be involved in the regulation of chitinase synthesis. The synthesis of various catabolic enzymes in micro-organisms is repressed when glucose or other readily metabolizable compounds are added to the culture. In *Trichoderma viride* (Nisizawa *et al.*, 1972) and *Trichoderma reesei* (Merivouri *et al.*, 1985) the formation of some hydrolases, including α -amylase, β -glucosidase, xylanase and cellulase, was affected by glucose. This phenomenon has been called the glucose effect (Epps & Gale, 1942), or catabolic repression (Neidhardt, 1960; Holzer, 1976). It appears that the formation of certain types of enzymes in fungi is affected by catabolic repression to varying degrees, irrespective of whether they are inducible or constitutive (Nisizawa *et al.*, 1972).

Induction of chitinase was inhibited by addition of 8-hydroxyquinoline and cycloheximide, suggesting that *de novo* transcription and translation are necessary for induction to occur.

C.J.U. was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil. We wish to thank D. R. West and J. B. Green for technical support, and B. V. Case for photographic work.

References

- AUSTIN, R. R., BRINE, C. J., CASTLE, J. E. & ZIKAKIS, J. P. (1981). Chitin: New facets of research. *Science* **212**, 749–753.
- BARTNICKI-GARCIA, S. (1973). Fundamental aspects of hyphal morphogenesis. In *Microbial Differentiation*, pp. 245–268. Edited by J. M. Ashworth & J. E. Smith. Cambridge: Cambridge University Press.
- BERG, B. & PETTERSON, G. (1977). Location and formation of cellulases in *Trichoderma viride*. *Journal of Applied Bacteriology* **42**, 65–75.
- BINDER, A. & GHOSE, T. K. (1978). Adsorption of cellulose by *Trichoderma viride*. *Biotechnology and Bioengineering* **19**, 1187–1199.
- BRADFORD, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- BURNETT, J. H. (1979). Aspects of structure and growth of hyphal wall. In *Fungal Walls and Hyphal Growth*, pp. 1–25. Edited by J. H. Burnett & A. P. J. Trinci. Cambridge: Cambridge University Press.
- CABIB, E. (1987). The synthesis and degradation of chitin. *Advances in Enzymology* **59**, 59–101.
- CHET, I. (1987). *Trichoderma* – application, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control*, pp. 137–160. Edited by I. Chet. New York: Wiley.
- ELAD, Y., CHET, I., BOYLE, P. & HENIS, Y. (1983). Parasitism of *Trichoderma* sp. on *Rhizoctonia solani* and *Sclerotium rolfsii* – scanning electron microscopy and fluorescence microscopy. *Phytopathology* **73**, 85–88.
- EPPS, H. M. R. & GALE, E. F. (1942). The influence of the presence of glucose during growth on the enzymic activities of *Escherichia coli*. Comparison of the effect with that produced by fermentation acids. *Biochemical Journal* **36**, 619–623.
- FRASER, R. S. S. & CREANOR, J. (1974). Rapid and selective inhibition of RNA synthesis in yeast by 8-hydroxyquinoline. *European Journal of Biochemistry* **46**, 67–73.
- HOLZER, H. (1976). Catabolic inactivation in yeast. *Trends in Biochemical Sciences* **1**, 178–181.
- ISAAC, G. & GOKHALE, A. V. (1982). Autolysis: a tool for protoplast production from *Aspergillus nidulans*. *Transactions of the British Mycological Society* **78**, 389–394.
- ITEN, W. & MATILE, P. (1970). Role of chitinase and other lysosomal enzymes of *Coprinus lagopus* in the autolysis of fruiting bodies. *Journal of General Microbiology* **61**, 301–309.
- JEUNIAUX, C. (1966). Chitinases. *Methods in Enzymology* **8**, 644–650.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- McKEEHAM, W. & HARDESTY, B. (1969). The mechanism of cycloheximide inhibition of protein synthesis in rabbit reticulocytes. *Biochemical and Biophysical Research Communications* **36**, 625–630.
- MERIVOURI, H., SIEGLER, K. M., SANDS, J. A. & MONTENECOURT, B. S. (1985). Regulation of cellulase biosynthesis and secretion in fungi. In *Biochemical Society Transactions*, pp. 411–414. Edited by D. C. Watts. London: The Biochemical Society.
- MILLER, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry* **31**, 426–428.
- MONREAL, J. & REESE, E. T. (1969). The chitinase of *Serratia marcescens*. *Canadian Journal of Microbiology* **15**, 689–696.
- NEIDHARDT, F. C. (1960). Mutant of *Aerobacter aerogenes* lacking glucose repression. *Journal of Bacteriology* **80**, 536–543.
- NISIZAWA, T., SUZUKI, H. & NISIZAWA, K. (1972). Catabolite repression of cellulase formation in *Trichoderma viride*. *Journal of Biochemistry* **71**, 999–1007.
- PEBERDY, J. F. (1990). Fungal cell walls. A review. In *Biochemistry of Cell Walls and Membranes in Fungi*, pp. 5–30. Edited by P. J. Kuhn, A. P. J. Trinci, M. J. Jung, M. W. Goosey & L. G. Copping. London: Springer-Verlag.
- RIDOUT, C. J., COLEY-SMITH, J. R. & LYNCH, J. M. (1988). Fractionation of extracellular enzymes from mycoparasitic strain of *Trichoderma harzianum*. *Enzyme and Microbial Technology* **10**, 180–187.
- ROBY, D., GADELLE, A. & TOPPAN, A. (1987). Chitin oligosaccharides as elicitor of chitinase activity in melon plants. *Biochemical and Biophysical Research Communications* **143**, 885–892.
- SMITH, R. J. & GRULA, E. A. (1983). Chitinase is an inducible enzyme in *Beauveria bassiana*. *Journal of Invertebrate Pathology* **42**, 319–326.
- SNEH, B. (1981). Use of rhizosphere chitinolytic bacteria for biological control of *Fusarium oxysporum* f. sp. *dianthi* in carnation. *Phytopathologische Zeitschrift* **100**, 251–256.
- ST LEGER, R. J., COOPER, R. M. & CHARNLEY, A. K. (1986). Cuticle-degrading enzymes of entomopathogenic fungi. Regulation of production of chitinolytic enzymes. *Journal of General Microbiology* **132**, 1509–1517.
- TOKIMOTO, K. (1982). Lysis of the mycelium of *Lentinus edodes* caused by mycolytic enzymes of *Trichoderma harzianum* when the two fungi were in an antagonistic state. *Transactions of the Mycological Society of Japan* **23**, 13–20.
- VASSEUR, V., ARIGONI, F., ANDERSEN, H., DEFAGO, G., BOMPEIX, G. & SENG, J.-M. (1990). Isolation and characterization of *Aphanocladium album* chitinase-overproducing mutants. *Journal of General Microbiology* **136**, 2561–2567.
- DE VRIES, O. M. H. & WESSELS, J. G. H. (1973). Release of protoplasts of *Schizophyllum commune* by combined action of purified α -1,3-glucanase and chitinase derived from *Trichoderma viride*. *Journal of General Microbiology* **76**, 319–330.
- WESSELS, J. G. H. (1984). Apical hyphal wall extension. Do lytic enzymes play a role? In *Microbial Cell Wall Synthesis and Autolysis* (FEMS Symposium 27), pp. 31. Edited by C. Nombela. Amsterdam: Elsevier.