CURRENT MICROBIOLOGY Vol. 48 (2004), pp. 102-107
DOI: 10.1007/s00284-003-4063-z

Current Microbiology
An International Journal

Distribution of Chitinases in the Entomopathogen *Metarhizium anisopliae* and Effect of *N*-Acetylglucosamine in Protein Secretion

Cristine Chaves Barreto, Charley Christian Staats, Augusto Schrank, Marilene Henning Vainstein

Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Campus do Vale, P.O. Box 15005, 91501-970 Porto Alegre, RS, Brazil

Received: 16 January 2003 / Accepted: 19 May 2003

Abstract. For a long time, fungi have been characterized by their ability to secrete enzymes, mostly hydrolytic in function, and thus are defined as extracellular degraders. Chitin and chitinolytic enzymes are gaining importance for their biotechnological applications. Particularly, chitinases are used in agriculture to control plant pathogens. *Metarhizium anisopliae* produces an extracellular chitinase when grown on a medium containing chitin, indicating that synthesis is subject to induction by the substrate. Various sugar combinations were investigated for induction and repression of chitinase. *N*-acetylglucosamine (GlcNAc) shows a special dual regulation on chitinase production. *M. anisopliae* has at least two distinct, cell-bound, chitinolytic enzymes when cultured with GlcNAc as one of the carbon sources, and we suggest that this carbohydrate has an important role in protein secretion.

Entomopathogenic fungi are widely distributed throughout the fungal kingdom. Some insect-pathogenic fungi have restricted host ranges, while others have a wide host range, with individual isolates being more specific [5]. Several species of fungi are very potent biocontrol agents of plant pathogenic fungi and arthropods. *Metarhizium anisopliae* is a broad-host-range entomopathogenic Deuteromycete, first recognized as a potential candidate for biological control of agricultural pests in the 1880s. Efforts to develop biological control methods for ticks by using *M. anisopliae* have been pursued [6]. Like most fungal pathogens, *M. anisopliae* uses a combination of enzymes and mechanical force to penetrate the host cuticle and access the nutrient-rich hemolymph. The mycoparasitic and entomopathogenic fungi produce chitinases for invasion and as one of the host-killing components [4, 13].

Previous studies demonstrate that *M. anisopliae* produces several cuticle-degrading enzymes during penetration of the host cuticle; they appear to be associated with pathogenesis by virtue of their early production in high levels, during infection [4, 7, 8, 15, 17, 25–27]. One of them, the extracellular subtilisin-like protease PR1, was suggested to be a determinant of pathogenicity [16, 23], and it was shown to improve insect infection efficiency when constitutively over-expressed [26]. The relevance of chitinase production and secretion during the penetration of host cuticle by fungal pathogens is far from being totally understood. In fungi, chitinases have a physiological role in hyphal growth and morphogenesis [27]. Among the factors affecting chitinase production in *M. anisopliae*, GlcNAc plays an important role as an inductor of synthesis [1, 5, 17, 22]. However, little information is available concerning chitinase secretion, structure, regulation by other carbon sources, and gene control for this fungus. Two chitinolytic enzymes from *M. anisopliae* were purified and described as chitinase and β-*N*-acetylglucosaminidase; the former was shown to have no activity on *p*-nitrophenol-β-*N*-acetylglucosamide, while the latter enzyme had no activity on either crystalline or swollen chitin [24]. Pinto et al. [17] purified a chitinase (CHIT30) capable of degrading chitin to completion, producing mainly *N*-acetylglucosamine, independently of the substrate used, with an endoacting activity producing also oligomers from swollen chitin. In nature, such a mechanism would favor a rapid and complete degradation of chitin microfibrils, producing monomers for nutrition and induction of further enzyme synthesis. To date, some other chitinases have also been purified from *M. anisopliae* [10]. Genes coding for two chitinases were isolated from *M. anisopliae*, gene *chit1* [1] (codes

Correspondence to: M.H. Vainstein; *email:* mhv@dna.cbiot.ufrgs.br

for a predicted 42-kDa chitinase with pI 5.0) and *chi11* (codes for a 58-kDa chitinase) [11].

Since the ability to secrete chitinases could be a significant factor in determining the degree of virulence, knowledge of how its production is regulated could be highly relevant to understanding the pathogenic process. The aim of this work is to study the regulation of the chitinolytic enzymes in *M. anisopliae*, by combinations of different carbon sources. We show that this microorganism has at least two distinct, cell-bound, chitinolytic enzymes when cultured with GlcNAc as one of the carbon sources, and we suggest that this carbohydrate has an important role in protein secretion.

Materials and Methods

Organism and culture conditions. *Metarhizium anisopliae* strain E6 isolated from *Deois flavopicta* in Espirito Santo State, Brazil, was maintained on agar slants in Cove's complete medium (MCc) as described [17]. To induce chitinase production, 10^6 spores mL^{-1} were inoculated in 100 mL of minimal medium (MM; 0.1% KH_2PO_4/0.05% $MgSO_4$). When glucose was used as carbon source, 0.6% of $NaNO_3$ was added as nitrogen source. The flasks were incubated at 28°C on a rotating shaking platform (180 rpm). Incubation proceeded for 144 h.

Preparation of enzyme fractions. Two enzyme fractions were derived from the cultures, namely, cell-bound and extracellular fractions. After *M. anisopliae* growth, the mycelium was harvested by filtration on Whatman #1 filter paper, ground with liquid nitrogen, resuspended in TE (10 mmol L^{-1} Tris-HCl pH 8.0/1 mmol L^{-1} EDTA) and centrifuged at 13,000 *g* for 30 min at 4°C. The supernatant obtained after centrifugation was used as the cell-bound protein fraction. Culture filtrates were used as the extracellular protein fraction. All fractions were dialyzed overnight against TE at 4°C.

Chitinase assay. The reaction mixture had 0.5 mL of swollen chitin (5 mg mL^{-1} in 50 mM acetate buffer pH 5.5) [20], 0.25 mL enzyme sample and 0.5 mL of 50 mmol L^{-1} acetate buffer pH 5.5. After incubation at 37°C for 5 h, it was centrifuged at 2000 *g* for 5 min, and the amount of *N*-acetylglucosamine (GlcNAc) released in the supernatant was determined by using GlcNAc as standard [18]. One unit (U) of chitinase was defined as the amount of enzyme that catalyzes the release of 1 μmol of GlcNAc · min^{-1} at 37°C. The protein content was determined by the method of Bradford [2], with known concentrations of BSA as standard.

Electrophoresis procedures. SDS-PAGE (13%) was used to analyze the protein fractions under denaturing conditions, as described by Laemmli [12]. For the detection of chitinase activity, electrophoresis was performed in a 7.5% SDS-PAGE containing 0.01% glycol chitin, prepared according to Molano et al. [14], and 0.1% SDS. Gel loading buffer was the same as described by Laemmli [12], but samples were not boiled before running. After electrophoresis, gels were incubated for 2 h at 37°C with shaking, in 50 mmol L^{-1} sodium acetate buffer pH 5.4 containing 1% Triton X-100. Chitinase lytic zones were revealed by incubation of the gel in a freshly prepared 0.01% calcofluor white solution (Fluorescent brightener 28, Sigma) in 500 mmol L^{-1} Tris-HCl pH 8.9. The calcofluor white solution was removed; the gels were incubated for about 1 h at room temperature in distilled water [28] and visualized under UV light.

Table 1. Effect of different carbon sources on chitinase synthesis and secretion by *M. anisopliae*

Carbon source(s) and concentrations	Extracellular chitinase activity (U · g^{-1} micelia dry weight)	Cell-bound chitinase activity (U · g^{-1} micelia dry weight)
Glucose 1.0%	1.29 ± 0.08	0.13 ± 0.009
GlcNAc 0.05%	72.60 ± 1.244	5.75 ± 0.126
GlcNAc 0.075%	15.34 ± 0.418	2.79 ± 0.13
GlcNAc 0.1%	15.70 ± 0.418	2.88 ± 0.063
GlcNAc 0.5%	3.40 ± 0.05	0.88 ± 0.037
Chitin 0.8%	3.18 ± 0.186	1.71 ± 0.092
Chitin 0.8% + Glucose 0.1%	3.20 ± 0.228	0.34 ± 0.021
Chitin 0.8% + Glucose 0.5%	0.67 ± 0.014	0.11 ± 0.007
Chitin 0.8% + Glucose 1.0%	0.52 ± 0.031	0.05 ± 0.003
Chitin 0.8% + Glucose 2.0%	0.46 ± 0.011	0.04 ± 0.002
Chitin 0.8% + GlcNAc 0.01%	3.82 ± 0.213	3.81 ± 0.158
Chitin 0.8% + GlcNAc 0.05%	2.04 ± 0.183	2.66 ± 0.106
Chitin 0.8% + GlcNAc 0.1%	1.48 ± 0.105	1.01 ± 0.034
Chitin 0.8% + GlcNAc 0.5%	0.95 ± 0.057	0.12 ± 0.003
Chitin 0.8% + GlcNAc 1.0%	0.88 ± 0.049	0.08 ± 0.007

The results are means of at least three determinations, each corresponding to two independent experiments. Unit (U) was defined as μM GlcNAc · mL^{-1} · min^{-1} · mg^{-1} protein. Specific activity: U per mycelia dry weight (g).

Results and Discussion

The existence of external hydrolytic enzymes in microorganisms might be attributed to the impermeability of the cell membrane to their corresponding substrate. Some of these enzymes are formed only in the presence of their specific substrates. This situation may pertain to chitinase formation in *M. anisopliae*, where the enzyme appeared at high levels in response to the presence of chitin, its usual substrate. One of the major roles of chitinases found in fungi is to modify the organism's structural constituent chitin, present in their cell walls [9, 20], but the extracellular chitinolytic enzymes produced by *M. anisopliae* have also been suggested to be pathogenicity determinants involved in host invasion [7, 17]. The effect of different carbon sources on chitinase synthesis and secretion is shown in Table 1. When compared with cultures with chitin as sole nitrogen and carbon source, extracellular chitinase activity was progressively reduced (from 4.7- to 6.9-fold) when glucose (ranging from 0.5% to 2%) was added in the culture medium with 0.8% chitin. At 0.1% glucose, the extracellular activity was not reduced. In addition, the cell-bound chitinase activity was drastically reduced from 5 (0.1% glucose) to 42-fold (2% glucose) in the same culture system. In these conditions, at concentrations higher than 1.0%, glucose

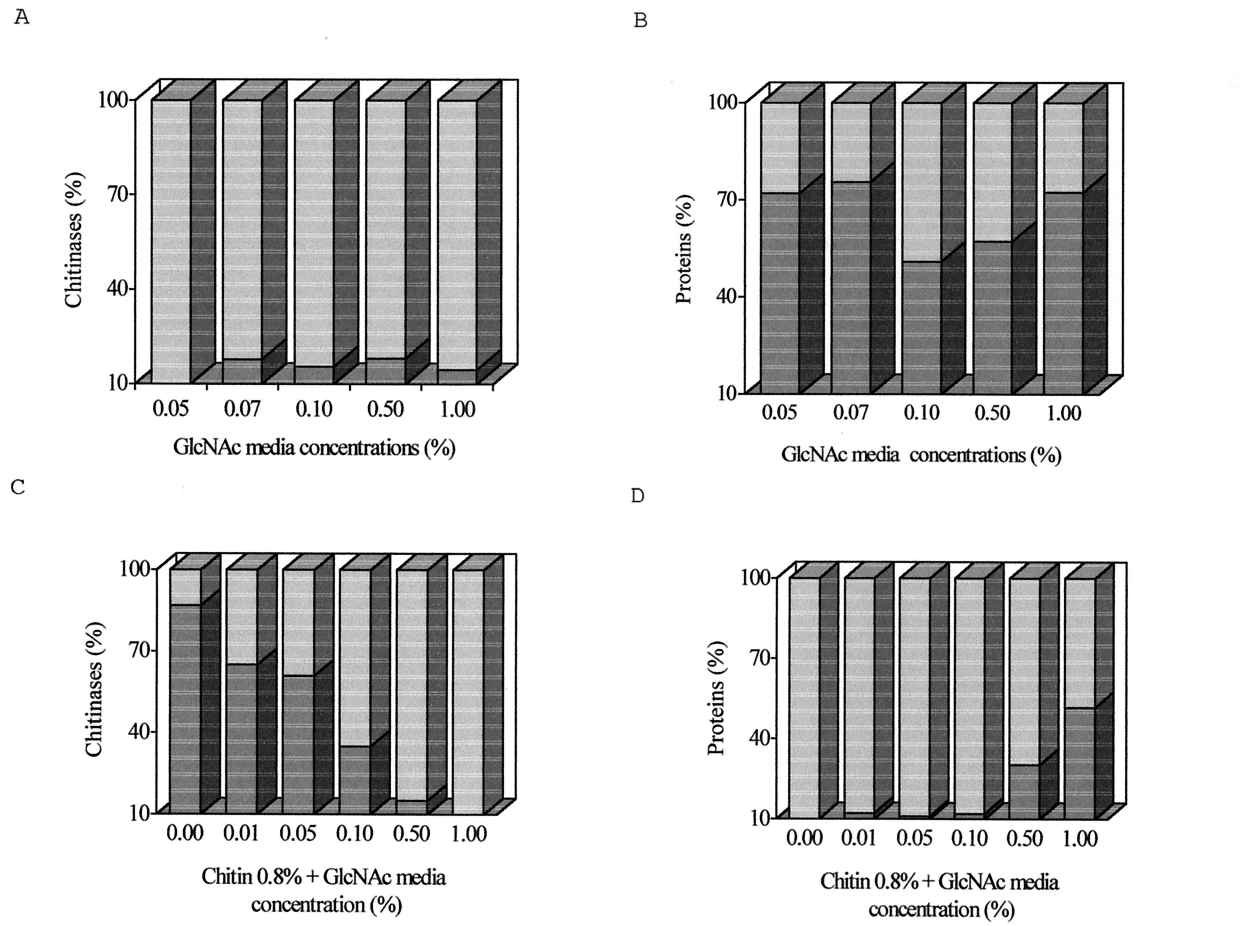

Fig. 1. Percentage of chitinase (A and C) and total protein (B and D) present in different fractions of *M. anisopliae* during growth on media containing crescent concentrations of GlcNAc (A and B) or GlcNAc in combination with 0.8% chitin (C and D). Extracellular fraction (light shading); cell-bound fraction (dark shading).

completely abolished chitinase activity, overcoming the chitin induction effect. The effect of glucose repression was previously described for proteins utilized in the carbohydrate degradation pathways [19]. GlcNAc shows a special dual regulation on chitinase production. At low concentrations (0.05%), it induced the production and secretion of the enzyme, but repressed chitinase secretion at higher concentrations (over 0.5%). This effect was observed for the extracellular as well as for the cell-bound fractions. When GlcNAc was added to media containing chitin, similar results were observed in a more moderate scale (Table 1). In this respect, the same dual regulation by the hydrolysis product occurs with *Saccharomyces cerevisiae* invertase, where production of the secreted enzyme is highly regulated by the concentration of glucose present in the medium [3].

Secretion of chitinases by mycelium grown on media containing GlcNAc alone or in combination with chitin was expressed as a percentage of the total enzyme activity. When GlcNAc alone was present in the culture medium (no added chitin), no effect on chitinase secretion was observed. In this condition, over 90% of the total chitinase activity (extracellular and cell bound), was present in the extracellular protein fraction (Fig. 1A). It is important to note that a variation in GlcNAc concentration altered total protein secretion. GlcNAc at a 0.05% concentration inhibited protein secretion, where 70% of the total protein in the system was cell bound (Fig. 1B). With 0.1% GlcNAc, protein secretion was raised up to 50%, and higher concentrations of GlcNAc gradually decreased secretion until it again reached 30% (at 1.0% GlcNAc, Fig. 1B).

The presence of chitin also induced protein secretion and, as GlcNAc, it had no effect on chitinase secretion (Fig. 1C, 1D). However, when GlcNAc was present in the culture medium in combination with chitin, the effect

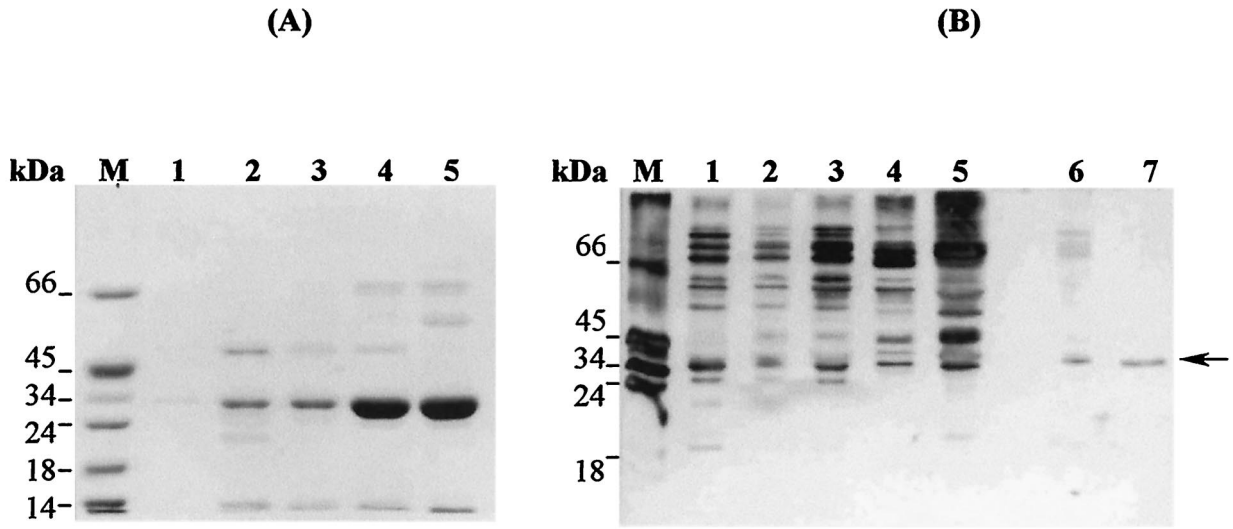

Fig. 2. SDS-PAGE on a 12% gel of the proteins secreted by *M. anisopliae* grown on media containing 0.8% chitin added at different concentrations of GlcNAc. (A) Secreted proteins. M: molecular mass marker; 1: 0.8% chitin; 2: 0.8% chitin + 0.02% GlcNAc; 3: 0.8% chitin + 0.05% GlcNAc; 4: 0.8% chitin + 0.1% GlcNAc; 5: 0.8% chitin + 1% GlcNAc. (B) Intracellular proteins. M: molecular mass marker; 1: 0.8% chitin + 0.01% GlcNAc; 2: 0.8% chitin + 0.05% GlcNAc; 3: 0.8% chitin + 0.1% GlcNAc; 4: 0.8% chitin + 0.5% GlcNAc; 5: 0.8% chitin + 1% GlcNAc; 6: 0.8% chitin; 7: purified 30-kDa chitinase from *M. anisopliae.*

on protein secretion was stronger, depending on the GlcNAc concentration. At concentrations higher than 0.5%, a negative effect on protein secretion occurred. On the other hand, when GlcNAc concentration was raised, an opposite effect was observed on chitinase secretion: the release of chitinase to the culture supernatants was more prominent. When these results were compared with others concerning the production of chitinases in the cell-bound and extracellular fractions (Table 1), it seems that, although 1.0% of GlcNAc repressed chitinase production, it also stimulated secretion, so that almost all chitinases produced were secreted. The intracellular transport of secreted proteins in the fungal hypha is not yet well understood, so the repression caused by GlcNAc has no obvious explanation.

In a previous work, we purified a 30-kDa chitinase from *M. anisopliae* [17] with relative mobility, in SDS-PAGE, identical to the most abundant protein of the extracellular fractions from *M. anisopliae* chitin plus GlcNAc growing cultures (Fig. 2). SDS-PAGE incorporated with glycol chitin was used to detect chitinase activity in cell-bound protein fractions (Fig. 3A, 3B). In such experiments, we found that when the GlcNAc concentration was raised in the culture medium, with or without chitin, it was possible to identify two clear zones (bands) of chitinolytic activity with distinct mobility. The analysis of samples from cultures with combined carbohydrates (chitin plus GlcNAc) showed a decrease of intensity of the chitinase with the lowest electrophoretic mobility, and totally abolished when 1% GlcNAc was present in the culture medium (Fig. 3A). This indicates a reduction of chitinase production associated with the stimulation of its secretion (Table 1 and Fig. 2C). The opposite effect was observed in intracellular fractions obtained from GlcNAc cultures (Fig. 3B). Electrophoretic analysis for chitinase activity showed that whenever GlcNAc was used as carbon source, two distinct chitinase activities were detected, but just the one with the lowest electrophoretic mobility seemed to be regulated, since the other was always present independent of the GlcNAc concentration.

The chitinase activity detected for the extracellular protein fractions prepared from cultures containing glucose might be a result of fungus autolysis. This probably occurs, since *M. anisopliae* reached the stationary phase in this medium on the fourth day of culture (data not shown). Autolysis was also expected for cultures with high GlcNAc concentrations. Analysis of total protein secretion from extracellular fractions obtained when GlcNAc was used at a concentration of 0.1% showed that secretion was increased to 50%. According to this hypothesis, it is difficult to explain why higher GlcNAc concentrations gradually decreased secretion until it reached 30%, at a concentration of 1.0% of GlcNAc, the same value obtained for 0.05% of this monosaccharide.

Regarding the analysis by SDS chitinase activity gels, some disagreement with the results obtained for chitinase activity assays in vitro was expected, since glycol chitin was used as substrate for the gels and swollen chitin for the in vitro assays. Besides, the pattern

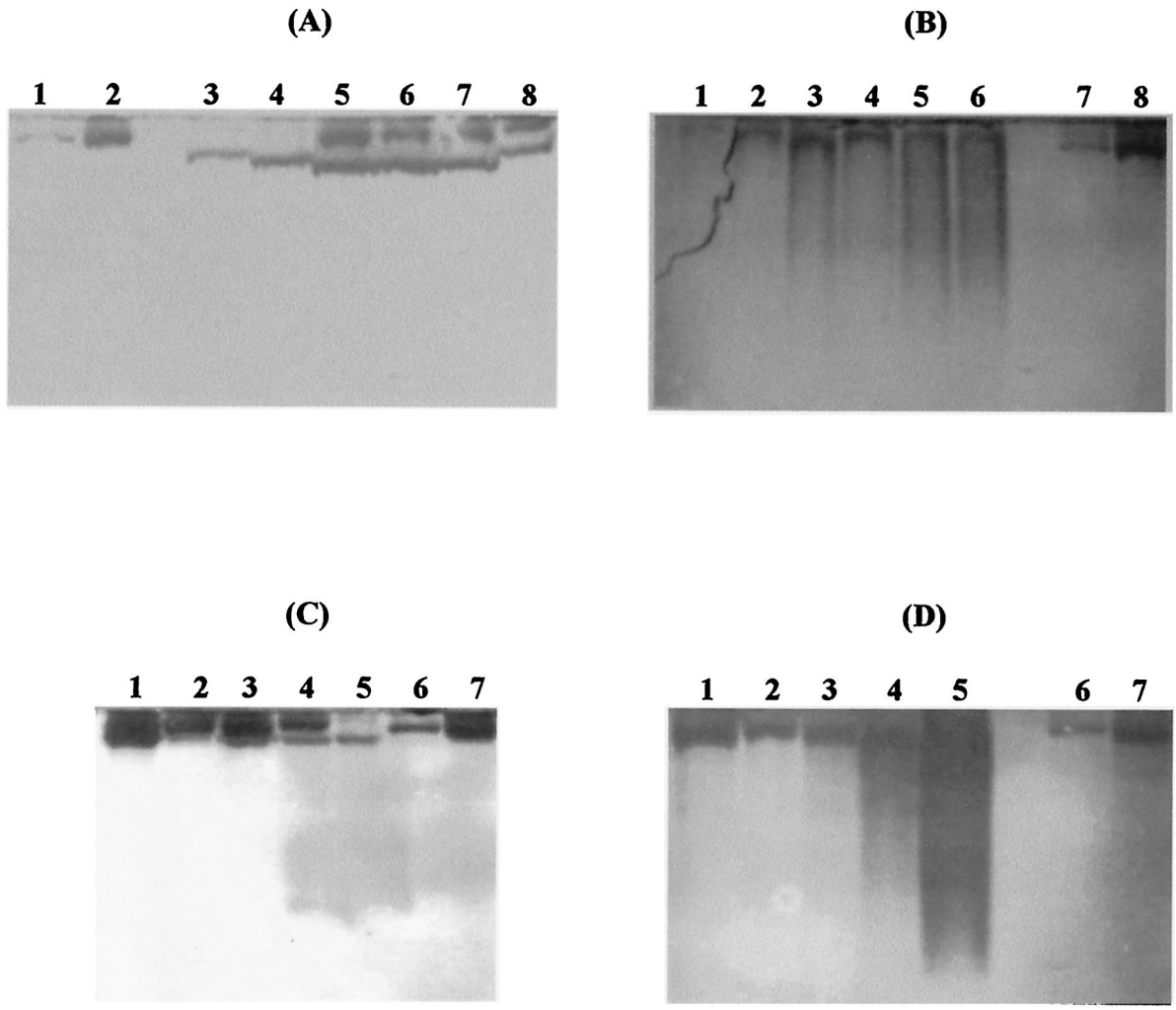

Fig. 3. Electrophoretic analysis in a 7.5% SDS non-denaturing gel of the chitinolytic activity of *M. anisopliae* intracellular (A and C) and secreted (B and D) proteins. (A) Proteins extracted from mycelia grown on different GlcNAc concentrations. 1: purified 30-kDa chitinase from *M. anisopliae*; 2: 0.8% chitin; 3: 0.025% GlcNAc; 4: 0.05% GlcNAc; 5: 0.075% GlcNAc; 6: 0.1% GlcNAc; 7: 0.5% GlcNAc; 8: 1.0% GlcNAc. (B) Secreted proteins from *M. anisopliae* grown on different GlcNAc concentrations. 1: 0.025% GlcNAc; 2: 0.05% GlcNAc; 3: 0.075% GlcNAc; 4: 0.1% GlcNAc; 5: 0.5% GlcNAc; 6: 1.0% GlcNAc; 7: purified 30-kDa chitinase from *M. anisopliae*; 8: 0.8% chitin. (C and D) Proteins from *M. anisopliae* grown on 0.8% chitin supplemented with different GlcNAc concentrations; (C) intracellular proteins and (D) secreted proteins. 1: 0.8% chitin + 0.01% GlcNAc; 2: 0.8% chitin + 0.05% GlcNAc; 3: 0.8% chitin + 0.1% GlcNAc; 4: 0.8% chitin + 0.5% GlcNAc; 5: 0.8% chitin + 1.0% GlcNAc; 6: purified 30-kDa chitinase from *M. anisopliae*; 7: 0.8% chitin.

of chitinolytic activity found for samples prepared from cultures grown on 0.8% chitin in combination with GlcNAc seemed to match that detected for activity using swollen chitin as substrate. The results obtained from mycelia-growing cultures with GlcNAc as a sole carbon and nitrogen source do not correspond to those obtained for chitinase intracellular fraction activity, but in this case it is important to notice that there were different amounts of protein in each lane of the gel. Samples from cultures on 1% of GlcNAc had at least fivefold more mycelial protein than that present in samples prepared from 0.05%. These different values represent different fungal growth.

The multiplicity of *M. anisopliae* enzymes provides a major challenge to determine the role played by a particular enzyme in adaptation to a new environment or in pathogenicity. The high capacity of the secretion machinery of *M. anisopliae* is still to be exploited for biotechnological purposes. However, our knowledge of the fungal secretion pathway is still at an early stage.

ACKNOWLEDGMENTS

This work was supported by grants and fellowships from PADCT, CNPq, and FAPERGS.

Literature Cited

1. Bogo MR, Rota CA, Pinto Jr H, Ocampos M, Correa CT, Vainstein MH, Schrank A (1998) A chitinase encoding gene (*chit 1 Gene*) from the entomopathogen *Metarhizium anisopliae:* Isolation and characterization of genomic and full-length cDNA. Curr Microbiol 37:221–225
2. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
3. Carlson M, Botstein D (1983) Organization of the *SUC* gene family in *Saccharomyces.* Mol Cell Biol 3:351–359
4. Charnley AK, St Leger RJ (1991) The role of cuticle-degrading enzymes in fungal pathogenesis in insects. In: Cole GT, Hoch HC (eds) The fungal spore and disease initiation in plants and animals. New York: Plenum, pp 267–286
5. Clarkson JM, Charnley AK (1996) New insights into mechanisms of fungal pathogenesis in insects. Trends Microbiol 4:197–204
6. Correia ACB, Fiorin AC, Monteiro AC, Verissimo CJ (1998) Effects of *Metarhizium anisopliae* on the tick *Boophilus microplus* (Acari: *loxididae*) in stabled cattle. J Invertebr Pathol 71: 189–191
7. Frazzon APG, Da Silva Vaz Jr I, Masuda A, Schrank A, Vainstein MH (2000) *In vitro* assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus.* Vet Parasitol 94: 117–125
8. Giménez-Pecci MP, Bogo MR, Santi L, Moraes CK, Corrêa CT, Vainstein MH, Schrank A (2002) Characterization of mycoviruses and analyses of chitinase secretion in the biocontrol fungus *Metarhizium anisopliae.* Curr Microbiol 45:334–339
9. Gooday G (1990) The ecology of chitin degradation. Microb Ecol 10:387–431
10. Kang SC, Park S, Lee DG (1998a) Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae.* J Invertebr Pathol 73:266–281
11. Kang SC, Park S, Lee DG (1998b) Isolation and characterization of a chitinase cDNA from the entomopathogenic fungus, *Metarhizium anisopliae.* FEMS Microbiol Lett 165:267–271
12. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–683
13. Mathivanan N, Kabilan V, Murugesan K (1998) Purification, characterization, and antifungal activity of chitinase from *Fusarium chlamydosporum*, a mycoparasite to groundnut rust *Puccinia arachidis.* Can J Microbiol 44:646–651
14. Molano J, Polacheck J, Duram A, Cabib C (1979) An endochitinase from wheat germ. J Biol Chem 254:4901–4907
15. Moraes CK, Schrank A, Vainstein MH (2003) Regulation of extracellular chitinases and proteases in the entomopathogen and acaricide *Metarhizium anisopliae.* Curr Microbiol 46:205–210
16. Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994) Specific induction of a cuticle-degrading protease of the insect pathogenic fungus *Metarhizium anisopliae.* Microbiology 140:185–189
17. Pinto AS, Barreto CC, Schrank A, Ulhoa CJ, Vainstein MH (1997) Purification and characterization of extracellular chitinase from entomopathogen *Metarhizium anisopliae.* Can J Microbiol 43: 322–327
18. Reissig JL, Strominger JL, Leloir LF (1955) A modified colorimetric method for the estimation of *N*-acetylamino sugars. J Biol Chem 217:959–967
19. Ronne H (1995) Glucose repression in fungi. Trends Genet 11: 12–17
20. Sahai AS, Manocha MS (1993) Chitinases of fungi and plants: their involvement in morphogenesis in host-parasite interactions. FEMS Microbiol Rev 11:317–338
21. Shimahara K, Takigushi Y (1988) Preparation of crustacean chitin. Methods Enzymol 161:417–423
22. St Leger RJ, Cooper RM, Charnley AK (1986) Cuticle-degrading enzymes of entomopathogenic fungi: Regulation of production of chitinolytic enzymes. J Gen Microbiol 132:1509–1517
23. St. Leger RJ, Durrads PK, Charnley K, Cooper RM (1988) Role of extracellular chymoelastase in the virulence of *Metarhizium anisopliae* for *Manduca sexta.* J Invertebr Pathol 52:285–293
24. St Leger RJ, Cooper RM, Charnley AK (1991) Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae.* J Invertebr Pathol 58:415–426
25. St. Leger RJ, Bidochka M, Roberts DW (1994) Isoforms of the cuticle-degrading Pr1 proteinase and production of a metalloproteinase by *Metarhizium anisopliae.* Arch Biochem Biophys 313: 1–7
26. St. Leger RJ, Joshi L, Bidochka M, Roberts DW (1996a) Construction of a mycoinsecticide overexpressing a toxic protease. Proc Natl Sci USA 93:6349–6354
27. St. Leger RJ, Joshi L, Bidochka M, Rizzo MJ, Roberts DW (1996b) Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Appl Environ Microbiol 62:1257–1264
28. Trudel J, Asselin A (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal Biochem 178:362–366