APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1988, p. 2699–2704
0099-2240/88/112699-06$02.00/0

Vol. 54, No. 11

N-Acetyl-D-Glucosamine-Mediated Regulation of Extracellular Protease in the Entomopathogenic Fungus *Beauveria bassiana*

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Received 12 May 1988/Accepted 26 July 1988

The entomopathogenic fungus *Beauveria bassiana* GK2016 grown in a liquid medium incorporating gelatin as the sole carbon and nitrogen source produced an extracellular serine protease (molecular weight, 35,000; pI ca. 10). Without gelatin, *B. bassiana* could utilize *N*-acetyl-D-glucosamine (GlcNAc; 2-acetamido-2-deoxy-D-glucose) as the sole source of carbon and nitrogen, and GlcNAc availability increased the storage carbohydrate content in mycelia. Synthesis of protease was repressed in gelatin medium containing GlcNAc at levels of >1.07 μmol mg of fungal dry weight^{-1}. At levels below this, protease synthesis was initiated; subsequently, free amino nitrogen appeared in the medium and diauxic growth was observed. Slow feeding with GlcNAc (35.34 μg ml^{-1} h^{-1}) did not repress protease synthesis nor did GlcNAc accumulate in the medium above 0.5 mg ml^{-1}. Increasing the rate of release of GlcNAc (83.51 μg ml^{-1} h^{-1}) resulted in the accumulation of GlcNAc in the medium to 2.0 mg ml^{-1}, a 45% increase in growth and a decrease in protease synthesis by about 81%. Free amino acids generated from the hydrolysis of gelatin did not repress protease synthesis. These data are interpreted in terms of known interaction of *B. bassiana* with insect cuticular components. We suggest that the action of extracellular chitinases synthesized by *B. bassiana* on insect cuticle, and pursuant release of GlcNAc, may have important consequences on the regulation of other extracellular catabolic enzymes such as the protease.

Beauveria bassiana is an entomopathogenic fungus which infects its host insect by passage through the cuticle (14). Cuticle penetration is considered to occur by a combination of mechanical means accompanied by enzymatic action, but the relative importance of each is not known. The major cuticular components of insects are protein and chitin (13). Extracellular enzymes such as protease and chitinase produced by the entomopathogenic fungi *B. bassiana*, *Metarhizium anisopliae*, and *Verticillium lecanii* have been shown to hydrolyze locust, *Schistocerca gregaria*, cuticular proteins, and chitin (23). Once these fungi get past the cuticular layers, they can invade the body cavity, grow, and kill the insect.

The exoskeleton and hemolymph of an insect provide numerous microenvironments for exploitation by entomopathogenic fungi. As the entomopathogenic fungus passes from the epicuticle to the hemolymph, various substances such as proteins, chitin, fatty acids, amino acids, amino sugars, carbohydrates, and inorganic substances may be encountered (9). *B. bassiana* can utilize many carbon- and nitrogen-containing components of cuticle and hemolymph such as C_{10} to C_{24} fatty acids (17), trehalose and glucose (6), amino acid combinations (26), chitin, glucosamine, and *N*-acetyl-D-glucosamine (GlcNAc) (20). It is therefore desirable to ascertain whether these components regulate the synthesis of enzymes involved in cuticle penetration.

Until recently, very little was known about the regulation of enzymes involved in cuticular degradation. Synthesis of extracellular chitinase in *M. anisopliae* and *B. bassiana* is regulated by levels of GlcNAc through an inducer-repressor mechanism (21, 24). We isolated and characterized an extracellular serine protease from *B. bassiana* (2). Synthesis of this enzyme is controlled by a multiple regulatory circuit similar to that found in *Neurospora crassa* (1) in which certain carbon sources together with a nitrogen source repress extracellular protease synthesis (3). Here we extend our previous studies by examining the regulatory role of GlcNAc, a major component of insect cuticle, on extracellular protease synthesis by *B. bassiana* in both regular closed batch and fed batch culture conditions. The closed batch condition was used to define the time course of extracellular protease synthesis during fungal development and GlcNAc depletion. The fed batch condition was used to examine synthesis under constant rates of GlcNAc diffusion in shake flasks. An understanding of the effects of GlcNAc on extracellular protease and chitinase could provide a framework for the mechanism of coordinate regulation of the two insect cuticle-hydrolyzing enzymes.

MATERIALS AND METHODS

Growth conditions. *B. bassiana* GK2016 conidia were harvested from cultures grown on yeast extract-peptone-glucose agar at 27°C for 7 to 10 days. Liquid cultures were grown in Erlenmeyer flasks containing 1% (wt/vol) gelatin in a basal salts solution composed of $MgSO_4 \cdot 7H_2O$ and NaCl (each 0.3 g/liter) and 0.2 M potassium-phosphate buffer (pH 7). The culture was grown on a rotary shaker (180 rpm) at 27°C. In one set of experiments, an admixture of gelatin and a gelatin hydrolysate was used. The ratio of gelatin to the hydrolysate was varied, but the total concentration of the admixture was maintained at 1% (wt/vol).

Supply of GlcNAc. GlcNAc was supplied to *B. bassiana* in liquid cultures by two methods. In the first method, GlcNAc was added to a closed batch culture at 0.02 to 1.0% (wt/vol). In the second method, the fed batch culture condition, dialysis tubing (6,000-molecular-weight cutoff; 14.6-mm cylinder diameter; Spectrum Medical Industries Inc., Los Angeles, Calif.) was filled with 2 ml of either 50- or 180-mg ml^{-1} solutions of GlcNAc, sealed, and placed in 50 ml of

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gelatin-basal salts medium in a 125-ml Erlenmeyer flask. This provided mean diffusion rates of 35.34 ± 5.92 standard deviation and 83.51 ± 8.75 μg ml^{-1} h^{-1}, respectively, over 18 h. For longer-term experiments, the dialysis bags were replaced twice daily. GlcNAc was supplied at a rate which was less than, greater than, or equal to the rate of utilization by the fungus. This application is similar to that used by Pirt (15) in which nutrients and hence growth to *Escherichia coli* were limited by the use of diffusion capsules. Culture media and dialysis tubing were sterilized by autoclaving at 121°C for 15 min. GlcNAc was filter sterilized (0.45-μm pore size, type HA; Millipore Corp., Bedford, Mass.) before use.

Inocula. Closed batch cultures were inoculated with 1% (vol/vol) of a stock suspension of 10^8 conidia ml^{-1}. In the fed batch cultures, mycelia rather than conidia were used. The use of mycelia as the inocula in the GlcNAc fed batch cultures avoids the consequences of exposing conidia to the various diffusion rates of GlcNAc. Otherwise, different germination responses due to different rates of nutrient supply may complicate interpretation of the results. Standardized mycelial inocula for fed batch cultures were prepared from 3-day shake cultures grown in yeast extract-peptone-glucose broth. Mycelia were removed by membrane filtration (0.45-μm pore size; Millipore Corp.) and then washed twice with sterile distilled water. The mycelial mats were used as the inoculum for each fed batch culture. The mean dry weight of the mycelia which was used as the inoculum was 155 mg ± 6.5 (standard deviation; $n = 6$). This level of reproducibility allowed comparisons within and between experiments.

Assays of cultures. Samples were taken from each culture flask, filtered through preweighed membrane filters, dried at 90°C for 4 h, and weighed. Filtrates were analyzed for GlcNAc (16) and free amino nitrogen (18). Assays for extracellular protease activity and protease units were done by the method of Bidochka and Khachatourians (2). Mycelia were analyzed for storage carbohydrate, using an anthrone reagent (10) with glucose as a standard. To analyze for protein content, 1 to 5 mg of mycelia was placed in a test tube with 1.0 ml of 0.5 M NaOH; the test tube was placed in a boiling-water bath for 15 min and cooled, and 0.5 ml of 1.0 M HCl and 0.5 ml of 1.0 M potassium-phosphate buffer (pH 7) were added sequentially. The concentration of proteins was measured by the Bradford method (5), with bovine serum albumin as a standard.

Electrophoresis. Protein from culture supernatant was precipitated with 95% $(NH_4)_2SO_4$. The precipitate was suspended in distilled water and dialyzed in dialysis tubing (6,000-molecular-weight cutoff) for 24 h at 5°C against two changes of 2 liters of distilled water. The dialysate was lyophilized and stored at −20°C until used. The protein profiles of the culture supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 12% gels and the buffer system described by Laemmli (11). One milligram of lyophilized protein was loaded into each well. Low-molecular-weight (14,400 to 96,000) standards (Bio-Rad Laboratories, Richmond, Calif.) were run in parallel. Gels were stained with silver stain reagent or Coomassie blue R-250 from Bio-Rad. Gels were scanned at 540 nm, using a scanning densitometer (E-C Apparatus Corp., St. Petersburg, Fla.).

Isoelectric focusing was performed on lyophilized culture supernatant proteins. Gel tubes containing Ampholine (pH 3 to 10) were used (Pharmacia, Uppsala, Sweden). The extracellular protease was specifically located in the gel tube by application of the gel onto X-ray film (NS2T; Eastman Kodak Co., Rochester, N.Y.) for 0.5 h at 37°C. Once washed, the film showed the presence of protease activity where the gelatin had been dissolved.

TABLE 1. Relationship between GlcNAc and culture growth[a]

GlcNAC concn (mg ml^{-1})	Dry wt (mg 10 ml^{-1})	Mycelial carbohydrate (μg mg^{-1}, dry wt)	Mycelial protein (μg mg^{-1}, dry wt)
0	2.7	181.3	806.3
0.2	5.9	223.6	574.7
1.0	6.6	243.2	468.5
2.0	10.6	243.5	459.1
5.0	11.4	270.2	472.7
10.0	11.7	345.4	452.2

[a] GlcNAc was added to 1% (wt/vol) gelatin in buffered basal salts medium. Cultures were grown for 2 days.

Chemicals. All chemicals used were of analytical grade and were supplied by Sigma Chemical Co., St. Louis, Mo. Gelatin (Difco Laboratories, Detroit, Mich.) was of the highest grade and free of carbohydrate. Gelatin hydrolysate (peptone no. 190) was supplied by GIBCO Diagnostics (Life Technologies, Inc., Madison, Wis.). The purity of GlcNAc was assessed by paper chromatography and found to be free of glucosamine or glucose.

RESULTS

Growth, mycelial protein and carbohydrate content, and extracellular protease activity of *B. bassiana* in closed batch cultures containing GlcNAc. *B. bassiana* can utilize GlcNAc as the sole source of carbon and nitrogen. After 2 days of growth in 0.5% (wt/vol) GlcNAc-basal salts medium, fungal biomass attained 8.4 mg ml^{-1} (data not shown). When *B. bassiana* was grown in unbuffered medium, initially at pH 7, containing GlcNAc as the sole carbon and nitrogen source, the pH fell to 6.6 after 2 days. When 1% (wt/vol) glucose was added to unbuffered gelatin medium, the pH dropped to 4.8 after 2 days. Therefore, a 0.2 M potassium-phosphate buffer (pH 7) was used in the growth medium.

The growth characteristics of *B. bassiana* when GlcNAc was added to the gelatin-basal salts medium were examined. Cultures were harvested and analyzed for dry weight, mycelial carbohydrate content, and protein content. Culture filtrates were analyzed for extracellular protease activity and remaining GlcNAc. Dry weight and mycelial carbohydrate content were positively correlated with GlcNAc for concentrations up to 10 mg ml^{-1} (Table 1). GlcNAc availability increased the storage carbohydrate content in *B. bassiana*. Derepression of extracellular protease synthesis by *B. bassiana* occurred for GlcNAc concentrations below 0.5 mg ml^{-1} (Fig. 1). After 2 days, cultures initially containing 0.2 to 1.5 mg of GlcNAc ml^{-1} had <30% of their GlcNAc remaining (Fig. 1A). These cultures showed the highest extracellular protease activity (Fig. 1B). After 3 days, the culture initially containing 3.0 mg of GlcNAc ml^{-1} had 0.3 mg of GlcNAc ml^{-1} remaining and showed the highest protease activity in the extracellular fluid (Fig. 1B). Little protease activity was observed in cultures containing >0.5 mg of GlcNAc ml^{-1}. By day 4, none of the cultures had >0.5 mg of GlcNAc ml^{-1} (Fig. 1A). Highest extracellular protease activity was observed in cultures initially containing 5 or 10 mg of GlcNAc ml^{-1} (Fig. 1B). Extracellular protease activity which was synthesized in medium with GlcNAc concentrations of <0.5 mg ml^{-1} was related to the amount of growth in the culture. Again, the latter depended on the initial GlcNAc concentration in the medium.

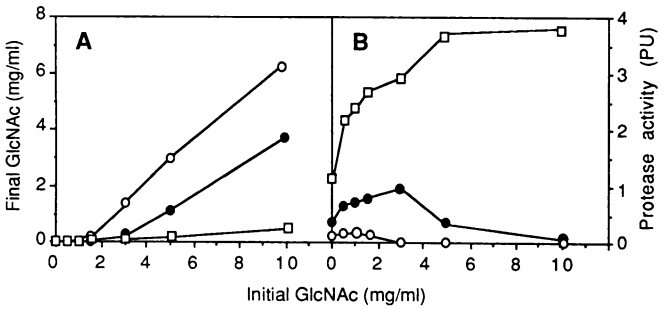

FIG. 1. Relationship between initial GlcNAc and final GlcNAc concentration (A) or protease activity (B) after 2 (○), 3 (●), and 4 (□) days of growth in closed batch cultures. PU, Protease units.

Extracellular protease production in closed batch cultures containing GlcNAc. Closed batch cultures containing 5 mg of GlcNAc ml^{-1} in gelatin-basal salts medium were assayed for dry weight, GlcNAc concentration, extracellular protease, and free amino nitrogen during a time course of growth (Fig. 2A). During the first 2 days of culture growth, GlcNAc depletion occurred slowly, with an average rate of 21.0 μg ml^{-1} h^{-1}. Between 2 and 3 days, GlcNAc was depleted at a rate of 107.9 μg ml^{-1} h^{-1}. This corresponded to the exponential growth phase for *B. bassiana*, with a rate of 21.7 μg (dry weight) ml^{-1} h^{-1}. In this case, during exponential growth, *B. bassiana* utilized 4.97 μg of GlcNAc for every 1-μg increase in dry weight. At day 4, GlcNAc concentration in the medium was 420 μg ml^{-1}. Between 4 and 4.5 days, the growth rate decreased to 6.7 μg (dry weight) ml^{-1} h^{-1}. However, with the depletion of GlcNAc the growth rate increased to 14.2 μg ml^{-1} h^{-1}. At this time, extracellular protease activity and free amino nitrogen appeared in the extracellular fluid. These results indicate that diauxic growth occurred during the time course of this experiment. However, diauxic growth was not pronounced as generally observed with some other microbial systems. Indeed, the diauxie reported here is probably correct but marginal since the second phase of growth is small (1.8 to 2.1 mg ml^{-1}). First, *B. bassiana* utilized the preferred substrate (GlcNAc), after which protease was synthesized, gelatin was hydrolyzed, and the amino acids or peptides were utilized for additional growth. Figure 2B shows the time course of extracellular protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis during GlcNAc depletion. At day 1, high-molecular-weight proteins are present which do not migrate past the stacking gel and corresponded to unhydrolyzed gelatin. At 3 days, an array of proteins ranging from about 10 to >100 kilodaltons appeared, possibly resulting from limited proteolysis by low background levels of extracellular protease. At 5 and 7 days, as judged by the silver-stained gels, 20 distinct bands of proteins appeared in the culture supernatants. The gel front was also stained, indicating the presence of peptides or amino acids, obviously the result of further protease hydrolysis of gelatin. At 5 and 7 days, a major protein band appears at the 35-kilodalton position which coincides with that of an extracellular serine protease (2). Isoelectric focusing zymograms revealed increasing intensities of clearing of the gelatin layer on the X-ray film at 5 and 7 days (Fig. 2C). The position of the zones of clearing corresponded to a pI of about 10.0.

Growth and protease production in closed batch cultures containing various proportions of gelatin and gelatin hydrolysate. Results so far indicated that, once extracellular protease is synthesized, the levels of free amino nitrogen in the

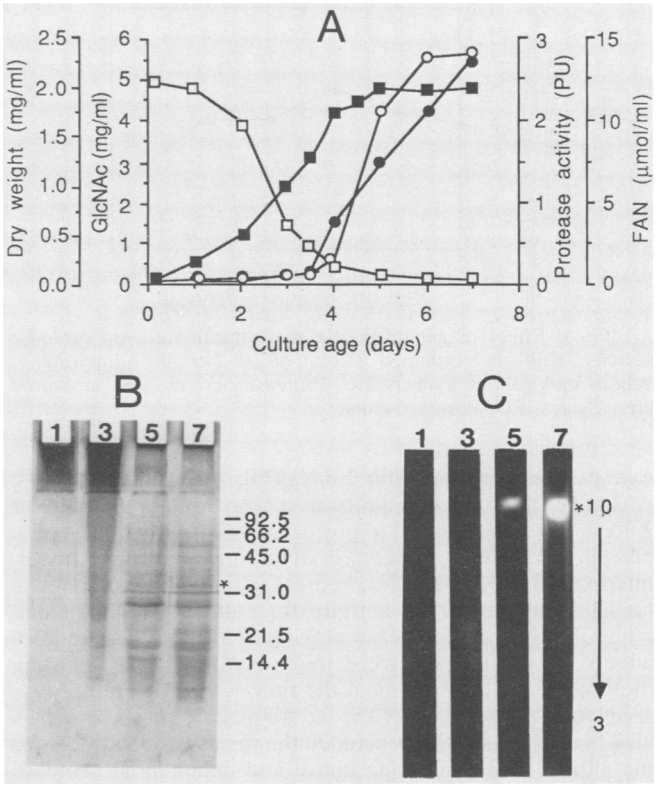

FIG. 2. Time course of development in closed batch cultures containing 1% (wt/vol) gelatin and 0.5% (wt/vol) GlcNAc in basal salts medium. (A) Changes in dry weight (■), GlcNAc concentration (□), protease activity (○), and free amino nitrogen (FAN) (●). PU, Protease units. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracellular proteins. Low-molecular-weight standard sizes (10^3) are indicated at the right. (C) Isoelectric focusing zymograms of extracellular protease activity. pH of the gels is indicated at the right. Numbers above lanes refer to culture age (days). Asterisk shows position of extracellular protease.

extracellular fluid increased as a result of gelatin hydrolysis. This may have some effects on the regulation of extracellular protease synthesis. We tested the effects of a commercial preparation of gelatin hydrolysate on extracellular protease production. The proportion of gelatin to the hydrolysate was varied, but the total concentration was maintained at 1% (wt/vol). Two-day-old batch cultures containing various proportions of gelatin to gelatin hydrolysate were assayed for dry weight and extracellular protease activity (Fig. 3). An increase in the proportion of gelatin hydrolysate resulted in a three-fold increase in fungal biomass. However, only a slight increase in extracellular protease production per milligram of mycelial dry weight was observed with a gelatin/gelatin hydrolysate ratio of 0:1 versus 1:0. Which peptides or amino acids in the gelatin are acting as inducers is not known. Gelatin consists primarily of glycine (25.5%), proline (18.0%), hydroxyproline (14.1%), glutamic acid (11.4%), and arginine (8.1%) (25).

Effect of GlcNAc addition during extracellular protease synthesis. To test for repression of extracellular protease synthesis, aliquots of the culture containing 1% (wt/vol) gelatin-basal salts medium were removed and 2% (wt/vol) GlcNAc was added. These cultures were then reincubated for an additional 1 to 2 days. The residual culture did not contain GlcNAc and showed an exponential increase in

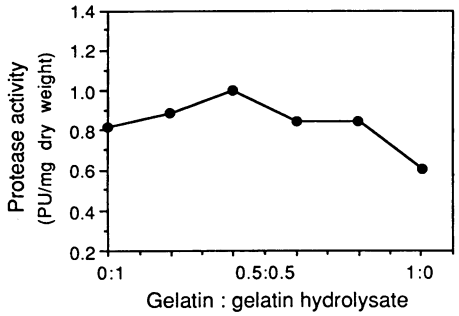

FIG. 3. Effect of gelatin/gelatin hydrolysate ratio on protease activity. Cultures contained a total of 1% (wt/vol) admixture of gelatin and gelatin hydrolysate. Protease activity was measured after 2 days. PU, Protease units.

extracellular protease after 2 days (Fig. 4A). When GlcNAc was added to culture aliquots at various time intervals over 2 to 4 days, extracellular protease activity remained at a plateau for the next 1 to 2 days. This suggests that extracellular protease synthesis was repressed in these cultures. The final levels of protease activity for cultures supplemented with GlcNAc, and protease activity at the time the culture aliquot was removed, were log transformed. By plotting these protease levels against the time when the culture was supplemented with GlcNAc, we obtained two straight parallel lines (Fig. 4B). We excluded the results from day 2 since the culture was in late lag phase and exponential protease synthesis occurred after that time. The upper line was displaced by 15% higher protease activity. This represents the amount of protease synthesized before complete repression takes effect. There could be several explanations, the most obvious of which is residual protease synthesis until the mRNA is degraded. If so, the mRNA half-life could be estimated from the time displacement between the two lines, which is 3.6 h.

Extracellular protease synthesis in GlcNAc fed batch cultures. To test for the relationship between GlcNAc levels and rate of availability on protease synthesis, GlcNAc was supplied through a dialysis membrane at diffusion rates equal to or greater than the consumption rate of the fungus. In the control flask, GlcNAc was omitted from the membrane tubing. Cultures of *B. bassiana* were grown for 2 days with GlcNAc supplied from membrane tubing at a mean rate of 35.34 or 83.51 μg ml^{-1} h^{-1}. Increasing the rate of supply caused a 45% increase in growth (Fig. 5A) and a decrease in extracellular protease synthesis by about 81% (Fig. 5C). When GlcNAc was supplied at 35.34 μg ml^{-1} h^{-1}, the concentration of GlcNAc never exceeded 400 μg ml^{-1} in the culture fluid (Fig. 5B). At the higher supply rate, however, GlcNAc accumulated in cultures to as much as 2.07 mg ml^{-1}. These values suggest that GlcNAc concentration in excess of the immediate growth requirements also causes repression of the extracellular protease.

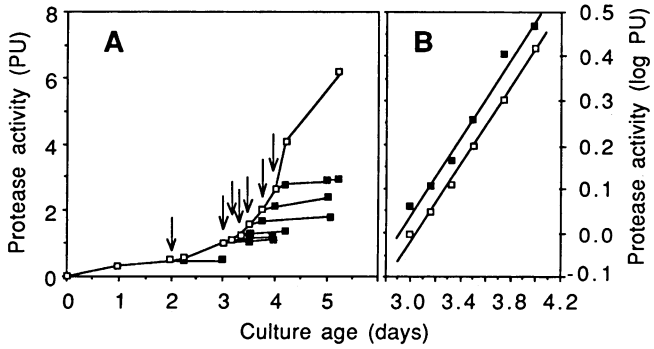

FIG. 4. Time course of extracellular protease production and response to GlcNAc. (A) Closed batch cultures were grown in a medium containing 1% (wt/vol) gelatin in basal salts solution. Arrows indicate the time at which culture aliquots were removed and 2% (wt/vol) GlcNAc was added. (B) Final protease activity (■) for GlcNAc-supplemented cultures are superimposed onto the time the culture aliquot was removed and protease activity (□) was measured. Protease activity in panel B was log transformed to show the exponential nature of synthesis. PU, Protease units.

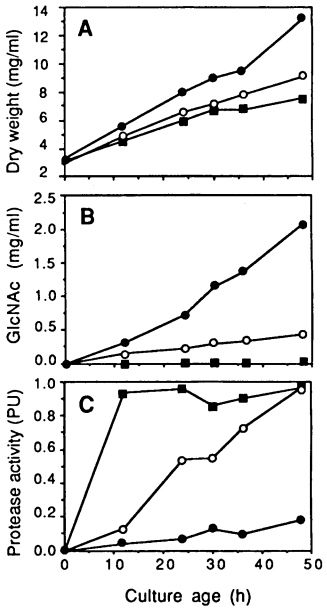

FIG. 5. Effect of diffusion-limited supply of GlcNAc on (A) culture dry weight, (B) GlcNAc accumulation in the medium, and (C) protease activity. GlcNAc was supplied at 0 (■), 35.34 (○), or 83.51 (●) μg ml^{-1} h^{-1}. PU, Protease units.

DISCUSSION

The extracellular protease of *B. bassiana* is a pivotal enzyme in the degradation of cuticle layers of susceptible insects (23). We are interested in the early events of grasshopper infection by *B. bassiana*. This paper presents our data regarding the synthesis of extracellular protease in vitro and its regulation by GlcNAc. The reason we explored the effects of GlcNAc on extracellular protease synthesis was that, along with extracellular protease, chitinase is involved in cuticle digestion (23) and the product of the latter reaction is GlcNAc. High levels of chitinase activity on chitin in the growth microenvironment could generate enough GlcNAc to satisfy the carbon and nitrogen requirement of the fungus. GlcNAc could affect the synthesis of extracellular protease. Therefore, not only the presence of GlcNAc but also the rate of availability and amount are important.

The importance of the functional interplay between fungal extracellular protease and chitinase is recognized in light of the structure of insect cuticle. In the insect cuticle, proteins surround chitin fibrils (4). Sequential treatment of insect cuticle with protease and chitinase from commercial preparations (19) or from *M. anisopliae* (23) showed that chitin

hydrolysis by chitinase was enhanced when the cuticle was pretreated with a protease. When *B. bassiana* is grown in culture containing insect cuticle as the sole carbon and nitrogen source, extracellular protease appears first followed by chitinase (22). The most obvious interpretation is that this could be the result of induction of chitinase by GlcNAc subsequent to degradation of the surrounding protein by extracellular protease. Initially, low levels of GlcNAc could be provided to the fungus by the action of chitinase present at a basal constitutive level. Another possible interpretation would be that there are extrinsic factors, such as peptides released by the action of protease, that serve to enhance chitinase synthesis.

The regulation of extracellular protease and chitinase in entomopathogenic fungi may have important ramifications in insect pathogenesis. Chitin exists as a polymer of GlcNAc and forms 25 to 40% of the insect cuticle (13). Synthesis of chitinase has been induced in *B. bassiana* cultures containing GlcNAc (21). In cell-free concentrated (50-fold) media from 4-day closed batch cultures of *B. bassiana* containing 1% (wt/vol) GlcNAc, 36 μg of GlcNAc was released after a 4-h chitinase assay period (21). Chitinase was also induced in fed batch cultures (GlcNAc supplied at 20 μg ml^{-1} h^{-1}) of *M. anisopliae* (24). Increasing the rate of supply of GlcNAc to 80 μg ml^{-1} h^{-1} decreased chitinase synthesis by 87%. Chitinase synthesis is regulated by products of chitin degradation through an inducer-repressor mechanism. Our data show that extracellular protease was synthesized in fed batch cultures (GlcNAc supplied at 35 μg ml^{-1} h^{-1}) of *B. bassiana* (Fig. 5C). A 2.37-fold increase in the rate of supply resulted in a decrease of extracellular protease synthesis by 81%. Extracellular protease synthesis is repressed in closed batch cultures if the GlcNAc concentration is >1.07 μmol mg of fungal dry $weight^{-1}$ or approximately 2.26 μmol mg of $mycelial\ protein^{-1}$ (Fig. 2A). Extracellular protease was not repressed in the presence of gelatin hydrolysate (Fig. 3). Cooper (7) has suggested that in phytopathogenic fungi specific regulation of extracellular fungal protease by amino acids is unlikely in view of the heterogeneity of monomer composition and the nonspecific action of some proteases.

Concerted regulation of extracellular enzymes involved in the sequential hydrolysis of complex polysaccharides has been reported in some fungi. For example, a single gene was demonstrated in the regulation of cellulase, mannanase, and xylanase of the wood-rotting fungus *Polyporus adustus* (8). Similarly, *N. crassa* cellulase and cellobiase were under a single regulatory mechanism (12). Insect cuticle, however, is composed of more heterogeneous components than the polysaccharides hydrolyzed by the above cases. Yet an analogous situation of sequential hydrolysis of insect cuticle by entomopathogenic fungi exists, and concerted regulation by functionally dissimilar extracellular enzymes may be the case here. Out data show that extracellular protease synthesis is regulated by products of chitin degradation. One possibility is for *B. bassiana* extracellular protease and chitinase to be under the control of the multiple regulatory system described earlier (3).

Controlling the induction-repression of extracellular chitinase and protease by means of a single regulatory system would be the most effective way of adjusting the rate of insect cuticle degradation. During cuticle penetration, extracellular proteases would hydrolyze proteins. Chitin fibrils would be exposed, with the subsequent induction of chitinase and release of GlcNAc. If GlcNAc levels were high, the synthesis of extracellular protease would be repressed. An analysis of the stoichiometric relationships between regulation of protease synthesis and GlcNAc levels for *B. bassiana* in various physiological states and in situ should give insight into its functional role.

ACKNOWLEDGMENTS

Research performed in this study was supported by the Saskatchewan Department of Agriculture through the Agriculture Development Fund. The award of a Canadian Wheat Board Fellowship to M.J.B. is gratefully acknowledged.

We thank G. A. Jones for constructive review of the manuscript.

LITERATURE CITED

1. **Abbot, R. J., and G. A. Marzluf.** 1984. Major extracellular protease of *Neurospora crassa*. J. Bacteriol. **159**:505–510.
2. **Bidochka, M. J., and G. G. Khachatourians.** 1987. Purification and properties of an extracellular protease produced by the entomopathogenic fungus *Beauveria bassiana*. Appl. Environ. Microbiol. **53**:1679–1684.
3. **Bidochka, M. J., and G. G. Khachatourians.** 1988. Regulation of extracellular protease in the entomopathogenic fungus *Beauveria bassiana*. Exp. Mycol. **13**:161–168.
4. **Blackwell, J., and M. A. Weih.** 1980. Structure of chitin-protein complexes: ovipositor of the Ichneumon fly *Megarhyssa*. J. Mol. Biol. **137**:49–60.
5. **Bradford, M. M.** 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.
6. **Campbell, R. K., G. L. Barnes, B. O. Cartwright, and R. D. Eikenbary.** 1983. Growth and sporulation of *Beauveria bassiana* and *Metarhizium anisopliae* in basal medium containing various carbohydrate sources. J. Invertebr. Pathol. **41**:117–121.
7. **Cooper, R. M.** 1977. Regulation of synthesis of cell wall-degrading enzymes of plant pathogens, p. 163–211. *In* B. Solheim and J. Raa (ed.), Cell wall biochemistry related to specificity in host-plant pathogen interactions. Universitetsforlaget, Oslo.
8. **Eriksson, K.-E., and E. W. Goodell.** 1974. Pleiotropic mutants of the wood-rotting fungus *Polyporus adustus* lacking cellulase, mannanase, and xylanase. Can. J. Microbiol. **20**:371–378.
9. **Hepburn, H. R.** 1985. Structure of the integument, p. 1–58. *In* G. A. Kerkut and L. I. Gilbert (ed.), Comprehensive insect physiology, biochemistry and pharmacology, vol. 4. Pergamon Press, Inc., Elmsford, N.Y.
10. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 226–272. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press, Inc. (London), Ltd., London.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
12. **Myers, M. G., and B. Eberhart.** 1966. Regulation of cellulase and cellobiase in *Neurospora crassa*. Biochem. Biophys. Res. Commun. **24**:782–785.
13. **Neville, A. C.** 1975. Biology of the arthropod cuticle, p. 71–123. Springer-Verlag KG, Berlin.
14. **Pekrul, S., and E. A. Grula.** 1979. Mode of infection of the corn earworm (*Heliothis zea*) by *Beauveria bassiana* as revealed by scanning electron microscopy. J. Invertebr. Pathol. **34**:238–247.
15. **Pirt, S. J.** 1971. The diffusion capsule, a novel device for the addition of a solute at a constant rate to a liquid medium. Biochem. J. **121**:293–297.
16. **Reissig, J. L., J. L. Strominger, and L. F. Leloir.** 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. **217**:959–966.
17. **Saito, T., and J. Aoki.** 1983. Toxicity of free fatty acids on the larval surfaces of two lepidopterous insects towards *Beauveria bassiana* (Bals.) Vuill. and *Paecilomyces fumoso roseus* (Wize) Brown et Smith (Deuteromycetes:Moniliales). Appl. Entomol. Zool. **18**:225–233.
18. **Samples, D. R., R. L. Richter, and C. W. Dill.** 1984. Measuring proteolysis in cheddar cheese slurries: comparison of Hull and trinitrobenzene sulfonic acid procedure. J. Dairy Sci. **67**:60–

63.
19. **Samsinakova, A., S. Misikova, and J. Leopold.** 1971. Action of enzymatic systems of *Beauveria bassiana* on the cuticle of the greater wax moth larvae (*Galleria mellonella*) J. Invertebr. Pathol. **18**:322–330.
20. **Smith, R. J., and E. A. Grula.** 1981. Nutritional requirements for conidial germination and hyphal growth of *Beauveria bassiana*. J. Invertebr. Pathol. **37**:222–230.
21. **Smith, R. J., and E. A. Grula.** 1983. Chitinase is an inducible enzyme in *Beauveria bassiana*. J. Invertebr. Pathol. **42**:319–326.
22. **St. Leger, R. J., A. K. Charnley, and R. M. Cooper.** 1986. Cuticle degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. J. Invertebr. Pathol. **48**:85–95.
23. **St. Leger, R. J., R. M. Cooper, and A. K. Charnley.** 1986. Cuticle degrading enzymes of entomopathogenic fungi: cuticle degradation in vitro by enzymes from entomopathogens. J. Invertebr. Pathol. **47**:167–177.
24. **St. Leger, R. J., R. M. Cooper, and A. K. Charnley.** 1987. Cuticle-degrading enzymes of entomopathogenic fungi: regulation of production of chitinolytic enzymes. J. Gen. Microbiol. **132**:1509–1517.
25. **Windholz, M.** 1976. The Merck index, 9th ed., p. 564. Merck & Co., Inc., Rahway, N.J.
26. **Woods, S. P., and E. A. Grula.** 1984. Utilizable surface nutrients on *Heliothis zea* available for growth of *Beauveria bassiana*. J. Invertebr. Pathol. **43**:259–269.