GROWTH AND SELECTED PARAMETERS RELATING TO ENZYMATIC PENETRATION OF THE INTEGUMENT OF THE CORN EARWORM (*HELIOTHIS ZEA*) BY *BEAUVERIA BASSIANA*

By

REBECCA JUNE SMITH

Bachelor of Science
Oklahoma State University
Stillwater, Oklahoma
1976

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1979

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of the Oklahoma State University
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Thesis Approved:

E. A. Grula
Thesis Adviser

George V. Odell

Mary M. Grula

Norman N. Durham
Dean of the Graduate College

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NOMENCLATURE

SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
SDA	Sabouraud's dextrose agar
TCA	trichloroacetic acid
TLC	thin-layer chromatography
NaOH	sodium hydroxide
KOH	potassium hydroxide
EDTA	ethylenediamine tetracetic acid
NH_4OH	ammonium hydroxide
NAG	N-acetylglucosamine
GLN	D-glucosamine
SDB	Sabouraud's dextrose broth
PI	powdered integument medium
Ch	Chitin salts medium

CHAPTER I

INTRODUCTION

One area of concern to the United States as well as other progressive countries is that of biological control of insects. Such a method would benefit not only the environment, but the health and well-being of humans and animals as well.

A potential biological insecticide, Beauveria bassiana, has been under study by several investigators since the middle 1800's because of its ability to infect a wide variety of insects (Pramer, 1965). Additionally, Beauveria bassiana is a fungus that exhibits no pathogenicity for mammals or plants. This is, of course, a critical characteristic; other than the target insect, a biological insecticide should not have any adverse effects upon other organisms within an environment. Many insects that cause farmers much grief can be infected by B. bassiana, including the corn earworm (Heliothis zea), the fall armyworm (Spongiterda fruda), and the pecan weevil (Curculio caryae) (Champlin et al., 1981).

Because of the wide variety of insects infected by B. bassiana, it is important to elucidate the mechanism of infectivity at the molecular level. Such information would aid in preparation of a "super mutant" of B. bassiana that could effectively infect a wide range of insects and also survive the sometimes harsh natural environment.

Work in our laboratory has centered around the biochemical events associated with entomopathogenicity of Beauveria bassiana and the

development of a super mutant for biological control of insects. The target insect is the corn earworm, Heliothis zea. Early work showed that B. bassiana germinated and penetrated the integument of the corn earworm and indications were that the penetration was effected by extracellular enzymes (Pekrul and Grula, 1979). One of the early areas of research done by the writer was investigation of the nutritional requirements for germination and growth of B. bassiana. It was important to define the basic nutritional needs of this fungus for two reasons: (1) to determine how well the fungus could survive in nature when used as a biological insecticide, and (2) to allow the compounding of chemically defined growth media.

The results of this study are reported in Appendix A. It was found that the nutritional requirements for germination and growth of B. bassiana are relatively simple. It requires a carbon, nitrogen and energy source for germination, and continued hyphal growth. A wide variety of compounds can serve as carbon-energy sources for the fungus, including chitin, lanolin, glucose, starch, and hydrocarbons present in crude oil. Inorganic salts of ammonia, glucosamine, N-acetylglucosamine or selected amino acids serve quite well as nitrogen sources and, if necessary, carbon and energy as well.

It was further observed that conidia of B. bassiana undergo active metabolism fairly early (1-3 hr) after being placed in a suitable growth medium, although germination does not occur until at least 14 hours after inoculation. Because of these findings, it was concluded that B. bassiana could survive very well in nature, using a wide variety of carbon-energy and nitrogen sources for germination and continued growth.

Once the nutritional requirements for germination and growth had been defined, additional studies were done to determine if the larval surface contained any toxic compounds that might prevent germination and thus infectivity of B. bassiana. It had been reported (Koidsumi, 1957; Evalakova, 1962) that some cuticular lipids of other larval forms contained ether-soluble fatty acids and that such compounds are mycotoxic. Because of such findings, the larval surface of the corn earworm was examined for free fatty acids that might prove toxic to B. bassiana. Results from this investigation are given in Appendix B. Caprylic acid was found to be the major free fatty acid on the larval surface of Heliothis zea. It was also found to inhibit germination of Beauveria bassiana. Other fatty acids were found in addition to caprylic acid, and these (nonanoic acid) also possess mycostatic activity toward B. bassiana. Interestingly, it was also observed that inhibition of germination and/or growth of the fungus by these fatty acids is related to the carbon-energy source present in the growth medium.

Another area of research pursued by the writer is that of enzymatic penetration by the fungus. Information was desired pertaining to the kind and number of enzymes required for complete penetration of the corn earworm integument by B. bassiana. A procedure was developed to obtain what appear to be pure protein-chitin complexes from corn earworm larvae. These were termed ghosts, and such ghosts were then utilized for investigation of the enzymes needed to degrade the ghost structure. The findings of this study are given in Appendix C.

It was determined that a combination of proteolytic and chitinolytic activities is needed to completely penetrate and bring about dissolution of the integument of the corn earworm. These activities are required in

sequence, with the proteolytic activity needed first, because chitin in the insect integument is surrounded and protected by protein(s). The survival of the protein-chitin complex in rather harsh chemical and enzymatic treatments suggests that protein is covalently bonded to the chitin.

Two other parameters related to the general problem of penetration of the integument of B. bassiana are addressed in this thesis: (1) the nature of the protein-chitin complex in the corn earworm integument, and (2) the induction of chitinase in B. bassiana and its role in pathogenicity.

It has been shown that growing germ tubes of B. bassiana penetrate the cuticle of corn earworm larvae (Pekrul and Grula, 1979). This penetration is effected by production of exocellular enzymes, particularly a chitinase and a protease. These enzymes must act in a sequential manner; i.e., proteolysis followed by chitinolytic activity in order for complete penetration of the larval cuticle to occur (Smith, Pekrul and Grula, 1981). Characterization of these enzymes, particularly chitinase, is of great interest. Also, it would be beneficial to determine the nature of the protein-chitin complex in the corn earworm since this has not been determined. Additionally, it still is not understood how the proteins are bonded to chitin. Elucidation of the chemical bonding between protein and chitin in the insect integument should aid significantly to our basic knowledge about the structuring and synthesis of non-vertebrate life forms.

Occurrence of Protein-Chitin Complexes in Nature

It appears that chitin is always found complexed with protein in

nature (Hunt, 1970). Chitin is chemically defined as a linear polymer of unbranched chains of beta 1,4 linked, 2-acetamido-2-deoxy-D-glucose residues; however, the natural state of chitin has approximately one out of every six N-acetylglucosamine residues deacetylated to the glucosamine form (Hackman, 1962). Chitin has not been demonstrated in tissues of vertebrates; but is a major constituent of molluscan-arthorpods, lower plants, and it is is found in the cell walls of several classes of fungi. In the case of fungi, protein has been found to be linked to chitin in the hyphal walls of Schizophyllum commune (Seitsma and Wessels, 1977).

The term protein-chitin complex will be used in the context of naturally occurring chitin, since chitin cannot be found in its "pure" form (Hunt, 1970). There are three molecular forms of chitin: alpha, beta, and gamma. The two major components of insect cuticles are protein and alpha chitin; the ratio between the two differs in various species of insects (Hackman, 1976). Alpha chitin consists of two antiparallel chains of N-acetylglucosamine polymers having opposite direction and being directly linked via their carboxyl and amino groups as well as by hydrogen bonding (Rudall, 1963; Muzzarrelli, 1977). Rudall (1967) reported that these chitin sheets were approximately 33 angstroms apart, and Neville (1967) suggested that the secondary bonding in chitin keeps the cuticle in insects from being too brittle because of the extensive covalent bonding.

It was found that a progressive extraction of soft insect cuticles resulted in removal of different groups of proteins (Hackman, 1976). He reported that after sequential treatment with water, salt, urea, and cold dilute sodium hydroxide, 56% of the total protein remained firmly associated with the chitin component of the cuticle. He proposed a covalent

binding between this protein and the chitin chains. Picken (1960), however, argues that their harsh treatment of the cuticle may not indicate covalent binding of protein to chitin, but the stability of tanned proteins in the cuticle. Hackman (1976) further demonstrated that cuticular proteins are covalently bonded to the chitin microfibrils, while others are associated through hydrogen and van der Waals bonds. He proved this by using lithium thiocyanate to dissolve cuticles and found that the residual extracted chitin still had protein bound to, or at least associated with it. He also reported that the protein removed was able to rebind to native chitin during TCA precipitation.

Hackman (1962) has offered considerable evidence for the covalent binding of protein to chitin by demonstrating that the protein-chitin complex in certain insect cuticles and crab shells is resistant to mineral acids and alkali, as well as lithium thiocyanate.

Kimura et al. (1976) also demonstrated the stability of protein-chitin complex to alkali and enzymatic attack when they used the larval and puparial cuticles of Sarcophaga bullata (cockroach) in an attempt to identify peptochitodextrins. Use of chemical and enzymatic methods to cleave glycopeptides proved unsuccessful. Because of their stability to chemical and enzymatic treatments, they suggested that chitin exerts a shielding action on the peptides.

Atwood and Zola (1967) treated cuticles of maggots (Calliphora sp.) with 1 N NaOH rather extensively (3 days; 100°C). They identified 15 amino acids still present with the chitin after this treatment; all were present only in trace amounts when compared to the amount of glucosamine. They also propose that these amino acids were bound covalently to the chitin due to their survival after the alkali treatment.

Hackman (1960, 1962) treated the shell of the marine crayfish, Jasus verreauxi and the puparial chitin of Lucilia cuprina (blowfly) with harsh acids and lithium thiocyanate solutions and hot alkali. He stated that these treatments removed all protein except for the amino acids he assumed to be next to and associated with the chitin. In all situations he was able to detect only small amounts of aspartic acid and histidine in his hydrolyzates utilizing paper chromatography.

Kimura et al. (1976) reported that after exhaustive chemical treatments of protease, chitinase and sodium in liquid ammonia, the major amino acids left were glycine, alanine, glutamic acid, and serine. Lipke et al. (1965) were in close agreement with Kimura et al. (1976) with their findings of leucine, alanine, glycine, and glutamic acid after treatment of the cuticle of the cockroach (Sarcophaga bullata) with dilute alkali and 19 N formic acid.

Most studies of protein-chitin complex have been made by attempting to isolate the proteins intact from the chitin chains by a variety of sequential treatments such as acids (Kipke et al., 1965; Lipke and Geoghegan, 1971), alkali (Hackman and Goldberg, 1971), and urea (Hackman and Goldberg, 1978; Hackman and Goldberg, 1979), and then analyzing the amino acids comprising the proteins. When these studies have been attempted, Hackman and other researchers have found that the amino acids present correlate with the amino acid composition of resilin and elastin, two structural proteins found in animal connective tissue and insect integumental tissue.

Hackman and Goldberg (1971) reported that cuticular protein in a variety of insect groups is heterogeneous, and the amounts of dicarboxylic acids, proline and tyrosine are higher than in fibrous proteins.

Alanine and glycine were the major amino acids reported. In a later study of numerous arthropod cuticular proteins, they found that in Galleria mellonella (greater wax moth) larvae, alanine, proline, glutamic acid, valine, and serine were the major amino acids present (Hackman and Goldberg, 1976). Pant and Sharma (1974) analyzed extracts of the cuticles of Philosamie ricini (giant silkworm) during various stages of larval development. They found that glycine and alanine were the predominant amino acids in all fractions. Srivastava (1971) also found that glycine and alanine were the major amino acids in cuticular extracts of Galleria mellonella. Using a sequence of water, borax buffer, water, ethanol, ether, urea, and sodium hydroxide, he extracted 80% of the protein of the cuticle; 42.5% was left in the sodium hydroxide extract.

In a more recent study Hackman and Goldberg (1979) examined the proteins extracted from the lipid-free late instar larvae of Calliphora vicina (maggot) and found the proteins to be about 14% alpha helix and and 20% in the beta pleated sheet conformation. They suggest that these cuticular proteins have a property typical of insect silks, in that they exist in disordered conformation when in solution. Silk protein has as its predominant amino acids glycine, alanine and serine (Hunt, 1970).

It appears that the amino acid compositions of these proteins extracted from larval cuticles are similar to the amino acid compositions of resilin and elastin. Resilin is an elastic protein that is insoluble in usual protein solvents, but is susceptible to proteolytic cleavage. Resilin contains no methionine, cystine, or hydroxyproline and has as its major amino acids glycine, alanine and aspartic acid (Anderson and Weis-Fogh, 1964). Elastin is a protein that connects the tissues of vertebrates and is also a rubber-like protein. Elastin is also insoluble

in solvents and the major amino acids present in elastin are glycine, alanine and valine. Elastin does have hydroxyproline (Partridge et al., 1963).

Because of the reported differences regarding protein-chitin complexes in invertebrates, it was of interest to determine how proteins are bound to chitin in the corn earworm. Also, such information could aid in characterizing the type of protease needed to initiate hydrolysis of the protein and open the chitin to hydrolytic attack by chitinase when B. bassiana is penetrating the integument.

Properties of Chitinases

Several investigators have demonstrated the production of exocellular enzymes by B. bassiana. Kucera and Samsinakova (1968), Kucera (1971), Leopold (1970), Grula et al. (1978), and Pekrul and Grula (1979) presented evidence relating to the production of exocellular chitinase, proteinases, and lipases by B. bassiana.

The insect integument is a complex structure consisting of two main layers. The main body of the cuticle is the thick inner procuticle and covering this is the epicuticle, which is composed of tanned protein, waxes and phenols (Hunt, 1970). The procuticle is made up of chitin and protein. Since the cuticle of larvae of H. zea is a soft cuticle, it appeared possible that perhaps a proteinase was sufficient to create enough cuticular damage for a germ tube to mechanically enter into the haemocoel. This was shown not to be true by Smith, Pekrul and Grula (1981) who proved that removal of 71% of the protein by sodium dodecyl sulfate (SDS) did not create enough cuticular damage to allow the

entrance of a germ tube; chitinase was also needed to complete the penetration process.

Further investigation of the chitinase produced by B. bassiana was continued to determine the nature of its production, e.g., whether it is a constitutive or inducible enzyme. Additional experimentation was done to completely characterize the chitinase of mutants of B. bassiana for further studies to aid in production of a super mutant that would effectively penetrate the larval cuticle of the corn earworm and be used as a biological insecticide.

Claus (1961) reported that chitinase is located intracellularly in the conidia and hyphae of B. bassiana. However, most other investigators have reported that chitinase of B. bassiana is indeed an exocellular enzyme, as well as chitinases from other species (Grula et al., 1978; Price and Storck, 1975; Lysenko, 1976).

Evidence does not exist which related specifically to the inducibility of chitinase of B. bassiana; variable results have been reported for other organisms. Bennet and Hood (1979) reported that chitinase of Bacillus megaterium was induced by glucosamine; however, N-acetylglucosamine, chitobiose, and chitosan could not induce the enzyme. Monreal and Reese (1969) investigated the inducible system of the chitinase from Serratia marcesens, and found the opposite, e.g., chitinase was induced by N-acetylglucosamine, chitobiose, chitosan, and other N-acetylglucosamine polymers, but not glucosamine. Price and Storck (1975) have purified and characterized an extracellular chitosanase from Streptomyces and found it to be induced by glucosamine.

Clarke and Tracey (1956) reported that chitinase in bacteria is constitutive because growth media containing no chitin, glucosamine, or

N-acetylglucosamine polymers also contained chitinase. In an interesting study of cuticular chitinase, Bade and Stinson (1978) found that the enzyme is present in molting fluid of Manduca and is induced by molting fluid component(s). Jeaniaux (1966) has concluded that, in general, chitinase is both constitutive and inducible.

Breuil and Kushner (1976) studied the inducibility of cellulase, another macromolecular degrading enzyme, and showed that either cellulose or the related but soluble molecule carboxymethyl cellulose, can serve as inducers of cellulase. Additionally, they made the interesting observation that induction of cellulase appears to be dependent upon contact of the cell surface with the inducer.

Rho et al. (1982) reported that addition of cellulose to a culture of Schizophyllum commune greatly enhanced production of cellulose-degrading enzymes. They screened many carbohydrates for cellulase induction and showed that at least four compounds (thiocellobiase, carboxymethylcellulose, cellobiose, and xylan) can induce cellulase. They proposed that a mechanism of induction of cellulase exists where the yeast produces small constitutive amounts of cellulase, which then converts a part of the cellulose into soluble molecules that act as inducers for synthesis of large amounts of cellulase.

Because there appears to be a lack of consensus concerning inducibility and location of fungal chitinase, and since chitinase is important for invasion and penetration of the integument of the corn earworm and other insects by B. bassiana and probably other entomopathogenic fungi, we have attempted to determine if the enzyme is inducible, identify inducers of the enzyme, determine which inducer is the most effective, and, also, which of the mutants produce most of the enzyme. We also wished to identify the enzyme on the basis of migration in PAGE.

CHAPTER II

MATERIALS AND METHODS

Mutants of *Beauveria bassiana*

Mutants of *Beauveria bassiana* used in this study were originally obtained by ultraviolet irradiation in this laboratory (Grula et al., 1978), except for R_1, which was obtained during a visit to the USSR in 1978 (NSF-Sponsored Group I Science Exchange Team of which Dr. E. A. Grula was a member). The CC mutant strains were obtained by larval passage through the corn earworm. The wild type strain was exposed to UV light (GE germicidal lamp) at a distance of 46 cm for 5 min with occasional stirring. Mutants were selected for varying degrees of proteolytic activity on casein-trypticase-soy agar. Cultures of the fungus were routinely grown on Sabouraud's dextrose agar (SDA; Difco Manual) supplemented with 0.3% yeast extract at 25°C until sporulation had occurred. (approximately 9 days).

Target Insect

The corn earworm *Heliothis zea* (Boddie) was used as the major target insect in these studies. Larvae were reared on a corn-soy-milk diet developed by Burton (1970). Individual, newly-hatched first instar larvae were placed in diet cups and maintained at 25°C under constant light and allowed to grow until the desired instar was attained.

Acid Hydrolysis

Samples to be analyzed for amino acids were hydrolyzed using 6 N HCl at 105°C *in vacuo* for 18 hours. The hydrolyzates were air-dried and washed three times with distilled water (to remove HCl) prior to amino acid analysis.

Preparation of Larval Ghosts

Third instar corn earworm larvae were boiled for two hours in 1% sodium dodecyl sulfate (SDS). After this time such larvae consist only of proteins and chitin, and we have referred to them as "ghosts" (Smith et al., 1981). These ghosts were subjected to further chemical and enzymatic treatments as described in Appendix C, and also were treated in a sequential manner utilizing the procedure of Austin et al. (1981). Additionally, ghosts were treated with 5% trichloroacetic acid (TCA) for 72 hours at 0-4°C.

Thin-Layer Chromatography (TLC)

TLC plates coated with powdered cellulose (MN 300; 15 gm/100 ml deionized water) were used for separation of amino acids. The cellulose plates were pretreated by development overnight in isopropanol:water:formic acid (80:20:4). This treatment accumulates impurities present in the cellulose at the leading edge of the plates (solvent front) where they can then be scraped off with a razor blade. Two-dimensional chromatography was performed using the solvent system developed by Heathcote and Jones (1965). Amino acids and amino sugars were detected by spraying

the plates with 0.4% ninhydrin in acetone, and heating in an oven at 100°C for 2-4 min.

Amino Acid Analysis

Free amino acids were identified and quantitated using an amino acid analyzer located in the Department of Biochemistry. Mrs. Rebecca Ting was the person who performed the analysis at 60°C on a Durum DC6A column using a citrate buffer (Liao et al., 1973).

Glucosamine Assay

Glucosamine was determined quantitatively by using the procedure of Rondle and Morgan (1955). A standard curve was utilized for all determinations; limit of detection was 5.0 µg/ml of glucosamine.

Conidial Suspensions

Conidia of B. bassiana were obtained by growth on Sabouraud's dextrose agar. This usually took about 9 days and sporulation was judged by the observation of aerial hyphae collapsing into a powder. They were harvested by adding 0.03% Triton X-100 in water and gently suspending the conidia using a sterile loop prior to centrifugation. Washing was then accomplished by two additional centrifugations (3500 rpm for 15 min) in sterile distilled water. This washing procedure will remove or greatly decrease both nutrient and detergent residues. Aliquots of such washed suspensions were always used to inoculate various media for growth and enzyme production by the fungus.

Basal Salts Medium

A basal salts medium was utilized in several phases of this study. This consists of a salt solution with the following amounts per liter of distilled water: NaCl, 0.30 g; $MgSO_4 \cdot 7\ H_2O$, 0.30 g; and K_2HPO_4, 0.30 g. To this, various carbon-energy and nitrogen sources were added usually at the level of 1 or 0.1%.

Liquid Media for Chitinase Induction

Powdered chitin, gelatin, N-acetylglucosamine, D-glucosamine (final concentration was 1.0%) were added per 100 ml of the basal salts solution, depending on the type of enzyme protein response desired. The medium was sterilized in an autoclave for 15 min at 15 pounds pressure. N-Acetylglucosamine and D-glucosamine were filter-sterilized and added separately. An amino acid medium consisting of alanine, phenylalanine, and valine (all at the concentration of 0.1% each) was also utilized to analyze for induction of chitinase (Smith and Grula, 1981). All media preparations were inoculated with the same amount of a conidial suspension of B. bassiana and incubated at 25°C on a rotary shaker (180 rotations/min) for the desired amount of time (usually 6 days).

Powdered Integument Medium

Integuments were obtained by skinning fifth-instar corn earworm larvae and boiling them in 1% SDS for 2 hours. They were then water-washed, oven-dried (105°C) overnight, and ground into a fine powder using a mortar and pestle. These integuments consist primarily of chitin and protein (see Appendix C).

Protein Determinations

The amount of total protein was determined quantitatively in samples by use of the Bio-Rad assay procedure (BioRad Inc., Richmond, California).

Chitinase Assays

A modified method of Jeaniaux (1966) was utilized for all chitinase assays. Samples with suspected chitinase activity were incubated with 10 mg of powdered purified chitin (Sigma Chemical Co.) in phosphate buffer (see Column Chromatography) at 37°C on a rotary shaker (150 revolutions/min) for 4 hours. Beta-Glucosidase (Sigma) was always present at a concentration of 0.3 mg/ml in the assay mixture. The mixture was boiled for 10 min, spun at 3500 x g for 10 min, and 0.5 ml of the supernatant was added to 0.1 ml of 0.8 M potassium tetraborate. The detection of released N-acetylglucosamine was performed according to the procedure of Reissig et al. (1955). One unit of chitinase activity was defined as 12.6 micrograms of N-acetylglucosamine released in 4 hours by 100 µg of Streptomyces griseus chitinase (Sigma Chemical Co.).

Polyacrylamide Gel Electrophoresis (PAGE)

Tube polyacrylamide gels (900 mm x 5 mm) were prepared having the following composition: 6.5% acrylamide, 2.22 mg/ml bisacrylamide, 0.31% glycine, 0.25 mg/ml Tris, 0.7 mg/ml ammonium persulfate, and 0.35 µl tetraethylenemethylenediamine (TEMED). The gels were allowed to polymerize and individual samples (100 µg protein; 5:1 in 50% glycerol/gel) were layered onto the gels. Electrophoresis was performed using a glycine/Tris buffer (0.25% glycine, 0.06% Tris), pH 8.10, at 2 mA/tube for

approximately one hour. Gels were fixed for one hour in 10% trichloroacetic acid and stained with 0.2% Coomassie Blue for protein visualization.

Commercial Chitin Treatments

Commercial chitin (Sigma Chemical Co.; powdered crab shells) was treated in various ways to assess its ability to support growth of B. bassiana. The chitin was autoclaved, filtered and dialyzed against deionized water (12,000 dalton exclusion limit) and oven-dried (105°C; 24 hr).

Concentration of Supernatants

Supernatants of growth media were dialyzed against 20% Carbowax 6000 (12,000 dalton exclusion limit) to dryness. Contents inside the membrane tubing were resuspended in 1 ml of deionized water for further analysis.

Column Chromatography

Sephadex G-75 filtration was performed utilizing concentrated spent growth medium. The column had a 200 ml bed volume and an 80 ml void volume. Phosphate buffer (0.05 M K_2HPO_4; 0.04 M $KHPO_4$), pH 6.8, was utilized as the eluant. Flow rate was 1 ml/min. Fractions (1.5 ml/fraction) were collected and monitored using an ultraviolet detector. When peaks were observed, pooled fractions were lyophilized and the various enzymatic activities were determined.

Dansylation

Ghosts of third instar corn earworm larvae were dansylated and hydrolyzed for N-terminal amino acid analyses. Dansylation was accomplished utilizing the modified procedure of Gray and Hartley (1963). Dansylated ghosts were hydrolyzed for amino acids (6 N HCl; 24 hr) and such hydrolyzates were chromatographed in one dimension on Silca Gel G plates utilizing benzene, pyridine and acetic acid as the solvent (80:20:2). Plates were observed under ultraviolet light for dansylated amino acid spots. Dansylated standard amino acids were chromatographed each time with the samples.

Enzymes

All enzymes utilized in this study were purchased from Sigma Chemical Co., St. Louis, Missouri.

CHAPTER III

RESULTS

The Chitin-Protein Complex in the Corn Earworm Integument

A major objective of this work involving penetration of the corn earworm integument by B. bassiana was to determine the type(s) of enzyme activity required for such penetration. We had previously shown that at least a proteinase and a chitinase are required (Smith et al., 1981). During this investigation it was discovered that intact protein-chitin complexes of corn earworm integuments can readily be prepared by boiling larvae in 1% sodium dodecyl sulfate (SDS) for 2 to 4 hours. These complexes were termed "ghosts" and were further utilized in this study to elucidate the molecular structure of protein-chitin, specifically which amino acids are most closely associated with chitin. Because the protein covers and protects the chitin from enzymatic degradation by chitinase enzymes, it became important to determine what type of enzyme will most expeditiously remove this protein.

Amino acid analyses were performed on water-washed corn earworm integuments and compared to analyses of SDS ghosts in order to ascertain if the amino acid composition differed after the SDS treatment (Tables I and II). The major amino acids in hydrolyzates of these water-washed integuments are glycine, alanine, aspartic and glutamic acids, and proline. These five amino acids make up 58% of the total. Even after the

TABLE I

AMINO ACIDS IN WATER-WASHED INTEGUMENTS
OF THIRD INSTAR CORN EARWORM LARVAE

Amino Acid	Concentration (nmoles/0.1 ml)[a]	Molar Percent of Total
ASP	328.9 ± 12.2	11.2
THR	202.0 ± 1.0	6.9
SER	253.8 ± 11.5	8.7
GLU	305.6 ± 11.2	10.4
PRO	305.6 ± 2.1	10.4
GLY	432.5 ± 7.8	14.8
ALA	328.9 ± 9.2	11.2
VAL	178.7 ± 2.2	6.1
MET	25.9 ± 0.2	0.9
ILE	67.3 ± 3.9	2.3
LEU	152.8 ± 6.3	5.2
PHE	88.1 ± 0.7	3.0
HIS	101.0 ± 12.4	3.5
LYS	75.1 ± 5.4	2.6
ARG	80.3 ± 3.0	2.7
NH_3	1546.2 ± 72.2	
TOTAL	4472.7	

[a]Three ghosts (of the same approximate size) were acid-hydrolyzed (6N HCl; 18 hr; 100°C, *in vacuo*) and the hydrolyzate was resuspended in 0.5 ml of deionized H_2O. An aliquot (0.1 ml) was then analyzed. The concentration given represents the average of two determinations.

TABLE II

AMINO ACIDS IN SDS-PRODUCED "GHOSTS" OF THIRD INSTAR CORN EARWORM LARVAE

Amino Acid	Concentration (nmoles/0.1 ml)[a]	Molar Percent of Total
ASP	40.3 ± 2.1	4.5
THR	25.3 ± 0.8	2.8
SER	48.7 ± 4.8	5.4
GLU	104.8 ± 5.6	11.7
PRO	62.7 ± 5.4	7.0
GLY	126.4 ± 3.6	14.1
ALA	148.8 ± 6.3	16.6
VAL	86.1 ± 0.7	9.6
MET	20.6 ± 1.2	2.3
ILE	35.6 ± 2.9	4.0
LEU	79.6 ± 4.1	8.9
PHE	---	---
HIS	42.1 ± 0.7	4.7
LYS	42.2 ± 2.2	4.7
ARG	31.8 ± 5.3	3.6
NH_3	378.4 ± 24.2	
TOTAL	1273.4	

[a]Three ghosts (of the same approximate size) were acid-hydrolyzed (6N HCl; 18 hr; 100°C *in vacuo*) and the hydrolyzate was resuspended in 0.5 ml of deionized H_2O. An aliquot (0.1 ml) was then analyzed. The concentration given represents the average of two determinations.

rather harsh SDS treatment, all commonly occurring amino acids are still present in hydrolyzates of ghosts. The amino acids present in greatest amounts are alanine, glycine, glutamic acid, and leucine, making up a total of 49%. Amounts of aspartic acid and proline are greatly decreased in ghosts; phenylalanine was not detectable.

Because most major amino acids were still present in SDS ghosts of corn earworm larvae (third instar utilized for all experiments), these ghosts were subjected to further chemical treatments chosen because of their ability to remove protein from chitin. SDS ghosts were treated using a sequence of EDTA, urea, and three NaOH washings (Austin et al., 1981; Figure 1). Also, SDS ghosts were treated with 1 M KOH at 56°C for 72 hours. Results from amino acid analyses of these chitin-protein complexes are given in Table III.

It is evident that more protein is removed (90%), and some amino acids are completely missing after these treatments. Threonine, proline and arginine are absent in hydrolyzates of ghosts treated by the Austin procedure, while phenylalanine and arginine were completely removed by the KOH treatment. The major amino acids present in Austin-treated ghosts are glycine, alanine, leucine, valine and glutamic acid (74% of the total); while the major amino acids in KOH-treated ghosts are leucine, alanine, glycine, valine and lysine (58% of the total). It is important to note, however, that glycine and alanine are two of the major amino acids present, no matter which type of treatment is utilized.

Three amino acids are present in significantly higher amounts in KOH-treated ghosts as compared to ghosts obtained by the sequential treatment. These are aspartic acid, lysine and proline.

Figure 1. The modified Austin (1981) treatment of corn earworm larvae used to remove protein from chitin.

MODIFIED AUSTIN TREATMENT OF CORN EARWORM LARVAE

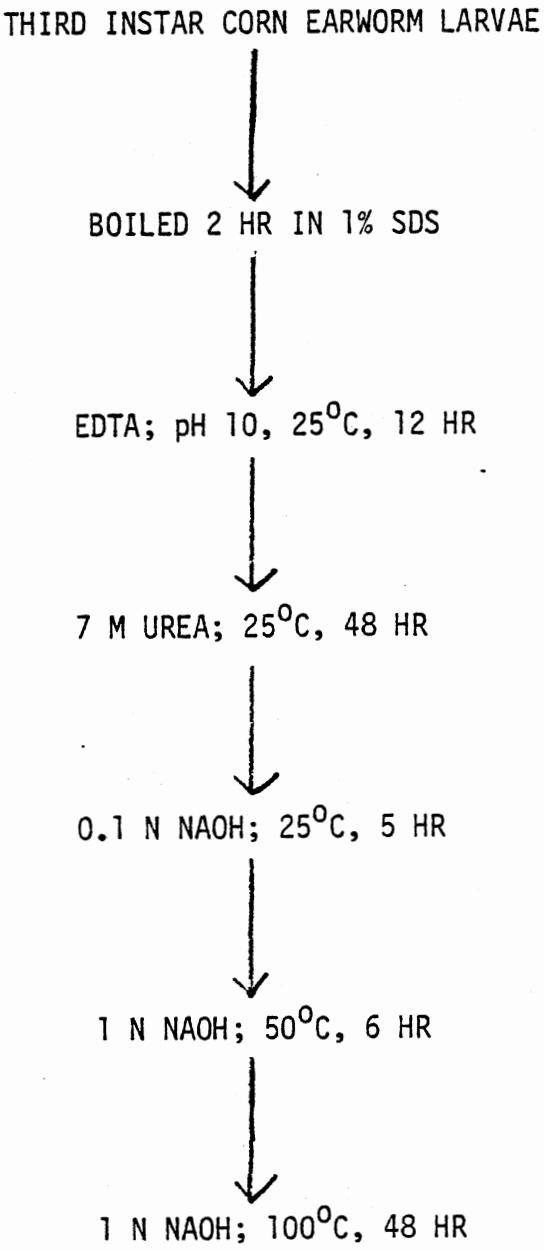


TABLE III

AMINO ACIDS IN GHOSTS OF THIRD INSTAR CORN EARWORM LARVAE EXTRACTED USING THE AUSTIN PROCEDURE OR ALKALI

Amino Acid	Treatment of Austin et al.[b] Nmoles/ 0.1 ml[a]	Treatment of Austin et al.[b] Molar Percent of Total	1 M KOH[c] Nmoles/ 0.1 ml[a]	1 M KOH[c] Molar Percent of Total
ASP	1.4 ± 1.3	0.7	5.5 ± 1.3	4.4
THR	---	---	1.9 ± 1.4	1.5
SER	11.2 ± 1.8	5.6	4.3 ± 1.7	3.5
GLU	22.7 ± 2.4	11.3	10.6 ± 1.5	8.7
PRO	---	---	10.2 ± 0.6	8.2
GLY	64.5 ± 2.1	32.1	13.3 ± 1.9	10.7
ALA	25.4 ± 3.7	12.6	16.5 ± 1.9	13.2
VAL	17.6 ± 3.8	8.7	12.8 ± 0.6	10.3
MET	4.9 ± 0.4	2.4	2.7 ± 0.5	2.2
ILE	14.9 ± 0.4	7.4	4.8 ± 0.2	3.9
LEU	27.1 ± 4.4	13.4	17.1 ± 2.4	13.7
PHE	7.0 ± 1.2	3.5	---	---
HIS	12.5 ± 3.3	6.2	11.4 ± 0.4	9.1
LYS	6.9 ± 0.2	3.4	13.5 ± 1.2	10.8
ARG	---	---	---	---
NH_3	165.1 ± 25.4		159.4 ± 12.6	
TOTAL	366.3		284.0	

[a]Three ghosts (of the same approximate size) were acid-hydrolyzed (6N HCl; 18 hr; 100°C, *in vacuo*) and the hydrolyzate was resuspended in 0.5 ml of deionized H_2O. An aliquot (0.1 ml) was then analyzed. The concentration given represents the average of two determinations.

[b]See Figure 1 and Austin et al., 1981.

[c]56°C for 72 hr.

Since most amino acids were present in hydrolyzates of these ghosts, even after the detergent and harsh alkali treatments, ghosts were treated even more extensively in order to remove as much protein as possible from the chitin. Trichloroacetic acid (TCA; 5%, 72 hr) was added to ghosts that had already been subjected to the Austin treatment and they were incubated at 0-4°C. The results from hydrolyzates made from these ghosts were intriguing (Table IV).

It was observed that the additional treatment with TCA removed several amino acids. Threonine, serine, proline, valine, methionine, phenylalanine, and arginine were not detected using either thin-layer chromatogarphy or the amino acid analyzer. Four amino acids were present in greatest amount. These were glycine, leucine, alanine, and aspartic acid, which make up 80.5% of the total. A rather surprising and unexplained finding was the increased amount of aspartic acid in the TCA-treated ghost when compared with the Austin-treated ghost (Table III).

Ghosts subjected to combined treatment with SDS, alkali, and then TCA (Table IV, column 1) have very few amino acids still present; these must be the ones closest to the chitin since the ghosts remain intact throughout the harsh chemical treatments.

In order to determine how these amino acids are bonded to the chitin, 4.0 N ammonium hydroxide was added to three of the TCA ghosts, and the suspension incubated for 1 hour at 45°C. Heptinstall et al. (1970) reported that such treatment removes O-ester bonded alanine from teichoic acid in cell walls of Bacillus subtilis. This supernatant was concentrated to dryness (air-drying) and resuspended in 0.5 ml of deionized water. This suspension, as well as the ghosts themselves, was analyzed using two-dimensional thin-layer chromatography. When the supernatant

TABLE IV

AMINO ACIDS IN GHOSTS OF THIRD INSTAR CORN EARWORM LARVAE EXTRACTED USING THE AUSTIN PROCEDURE FOLLOWED BY TCA AND/OR NH_4OH

Amino Acid	TCA		TCA + NH_4OH[a]
	Nmoles/ 0.1 ml[b]	Molar Percent of Total	
ASP	18.19	12.4	-
THR	---	---	-
SER	---	---	-
GLU	8.85	6.0	+[c]
PRO	---	---	-
GLY	58.13	39.5	+
ALA	19.87	13.5	+
VAL	---	---	+
MET	---	---	-
ILE	9.11	6.2	+
LEU	22.23	15.1	+
PHE	---	---	-
HIS	5.02	3.4	-
LYS	5.59	3.8	-
ARG	---	---	-
NH_3	64.65		
TOTAL	211.60		

[a]5% TCA, 72 hr at 0-4°C; followed by 4 N NH_4OH, 1 hr at 45°C.

[b]Three ghosts (of the same approximate size) were acid-hydrolyzed (6 N HCl; 18 hr; 100°C, in vacuo) and the hydrolyzate was resuspended in 0.5 ml of deionized water. An aliquot (0.1 ml) was then analyzed.

[c]Amino acids have been identified on a thin-layer chromatography plate only.

was subjected to TLC without prior acid hydrolysis, one large spot appeared (Figure 2). After this supernatant was hydrolyzed (6 N HCl, 24 hr, 105°C) and chromatographed, the spots shown in Figure 3 were observed. Glucosamine appears to be the major component in this large spot, as it is also the major spot appearing after hydrolysis. Additionally, some small peptides must be present with glucosamine, because several amino acids (alanine, leucine, glycine, and glutamic acid) appear on the TLC plate after hydrolysis. The ammonium hydroxide ghosts (termed "limit" ghosts) also contained these same amino acids after acid hydrolysis (Table IV).

Based on data obtained using the amino acid analyzer, the amount of protein removed by each treatment was determined. Water-washed integuments were used for such calculations as 100% protein (Table VI). The SDS treatment removed 71.6% of the protein from water-washed integuments; however, when alkali (KOH) or the Austin treatment was performed on SDS ghosts, 91-94% of the protein was removed. Approximately 95% of the protein was removed by TCA extraction following Austin treatment. Further treatment with ammonium hydroxide results in removal of all proteinaceous material except for a few amino acids (Table V).

In order to determine the N-terminal amino acids of the peptide still bonded to chitin after the ammonium hydroxide treatment, dansylation of these ghosts was performed. TCA ghosts and ammonium hydroxide ghosts were dansylated, hydrolyzed for amino acids, and chromatographed. The results of this study are given in Table VI. Glycine appeared to be the major N-terminal amino acid in both TCA and ammonium hydroxide ghosts. There is a small amount of N-terminal alanine as well in the ammonium hydroxide ghosts.

Figure 2. Diagrammatic representation of the thin-layer chromatographic plate from NH_4OH-TCA-Austin ghosts

Solvent system: Heathcote and Jones (1965)

Detection system: 0.4% ninhydrin in acetone

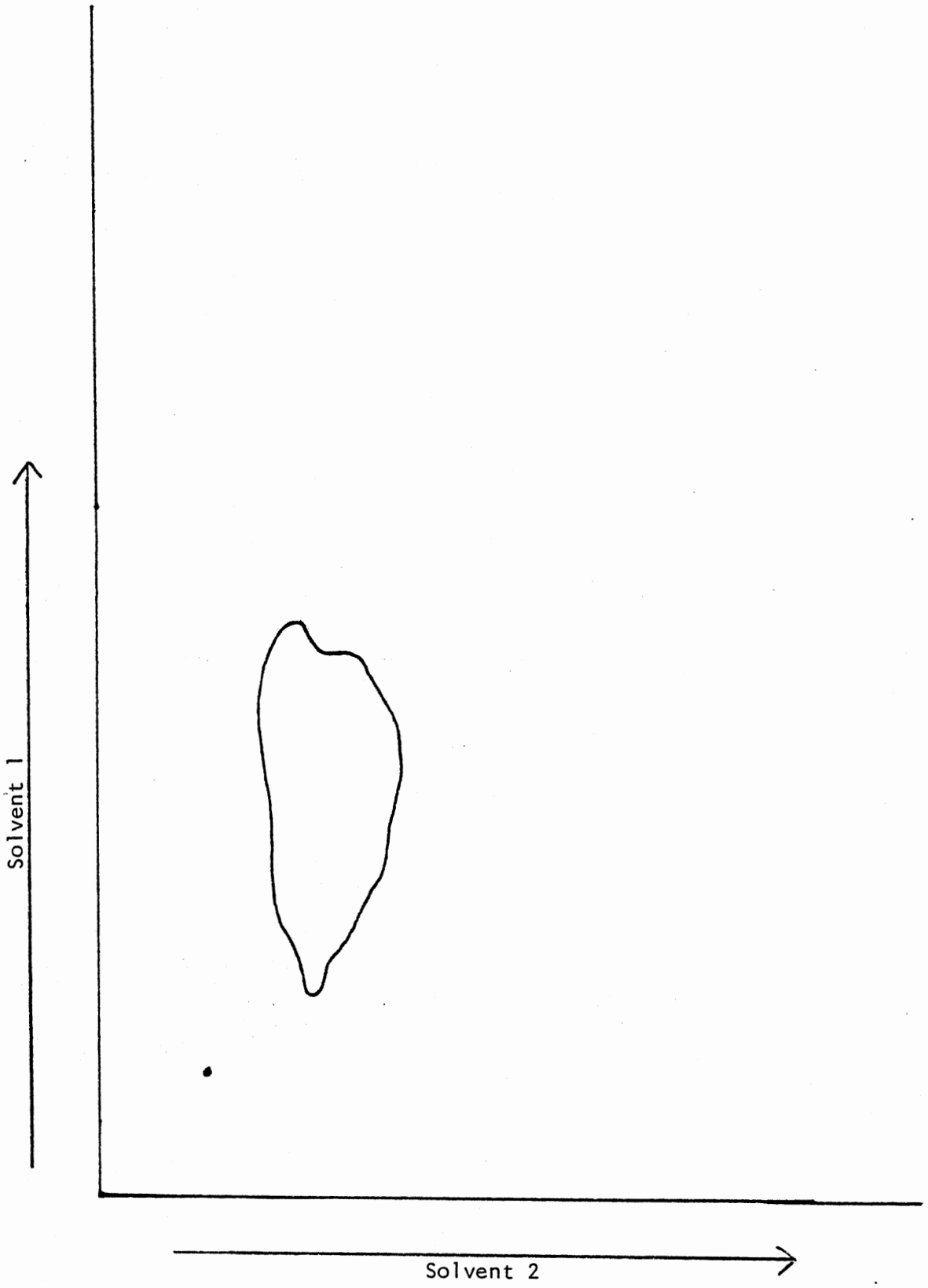

Solvent 1
Solvent 2

Figure 3. Diagrammatic representation of the thin-layer chromatographic plate from NH_4OH-TCA-Austin ghosts that were subjected to acid hydrolysis (6 N HCl; 18 hr; 100°C; *in vacuo*)

Solvent System: Heathcote and Jones (1965)

Detection System: 0.4% ninhydrin in acetone

ILE = Isoleucine
LEU = Leucine
ALA = Alanine
GLU = Glutamic acid
GLY = Glycine
GLN = Glucosamine

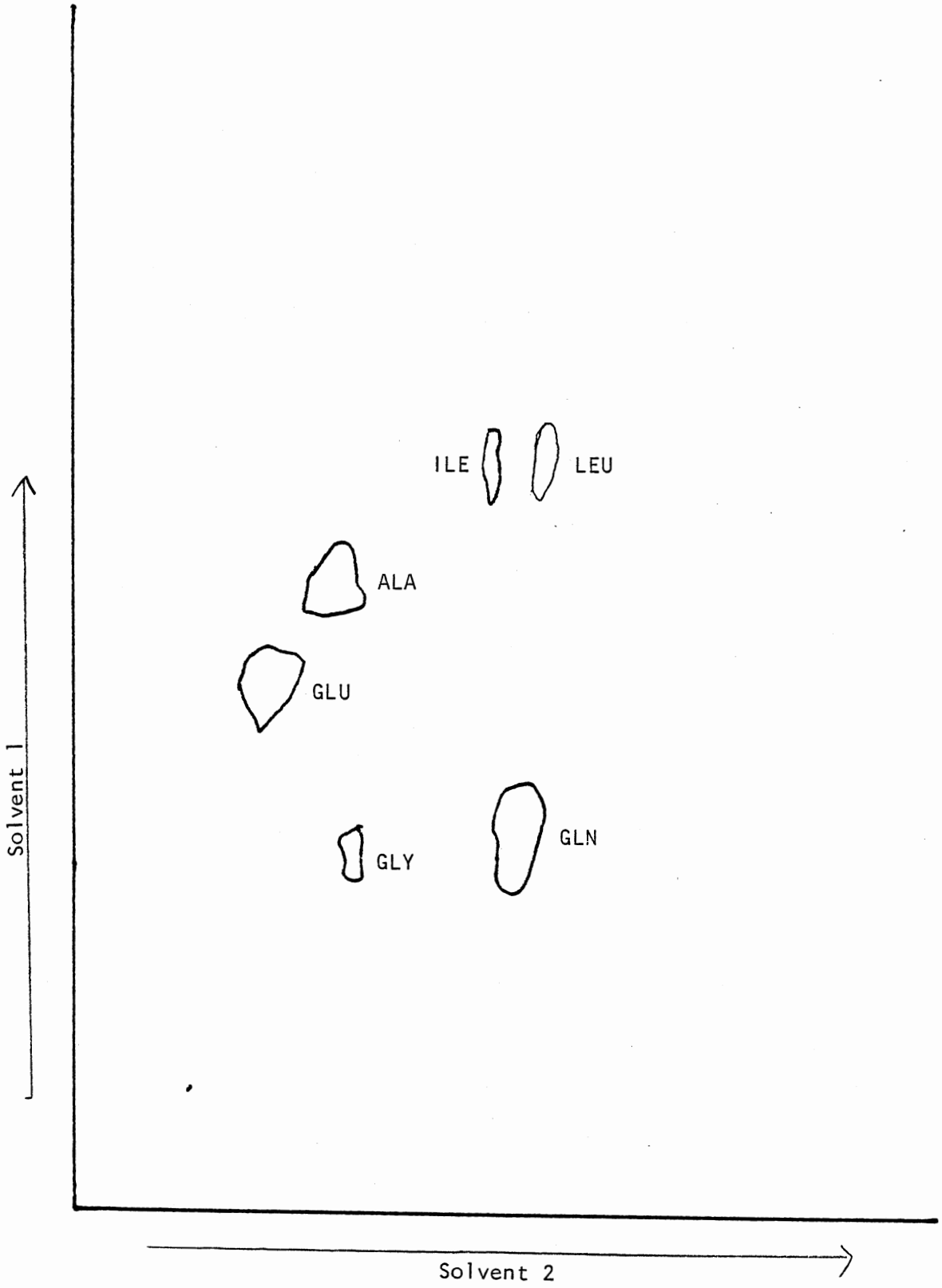

ILE
LEU
ALA
GLU
GLY
GLN
Solvent 1
Solvent 2

TABLE V

RELATIVE AMOUNT OF PROTEIN REMOVED BY VARIOUS TREATMENTS ON CORN EARWORM LARVAL INTEGUMENTS

Treatment	Percent Protein Removed[a] (%)
Water-washed	0
SDS (2 hr boiling-3rd instar larval)	71.6
Modified Austin Treatment[b]	91.8
KOH[c]	93.7
TCA[d]	95.3
NH_4OH[e]	99.0

[a]3rd instar larvae.

[b]3rd instar larvae as in Figure 1.

[c]3rd instar larvae 1 M KOH, 72 hr, 56°C.

[d]3rd instar larvae Figure 1 plus 5% TCA, 72 hr, 0-4°C.

[e]3rd instar larvae Figure 1 plus TCA plus 4 N NH_4OH, 1 hr, 45°C.

TABLE VI

R_f VALUES OF AMINO ACIDS FROM DANSYLATED GHOSTS OF THIRD INSTAR CORN EARWORM LARVAE TREATED WITH TCA OR TCA + NH_4OH[a]

Ghost	Amino Acid[b]	R_f Value (cm)
TCA	Glycine	0.27
TCA + NH_4OH	Glycine	0.27
TCA + NH_4OH	Alanine	0.42

[a]5% TCA, 72 hr; 0-4°C, and +4 N NH_4OH, 45°C, 1 hr.

[b]Amino acids were identified on the basis of comparison of standard dansylated amino acids, as well as their retention times.

Chitinase Induction in *Beauveria bassiana*

Mutants of *B. bassiana* differing in pathogenicity for the corn earworm were first analyzed for chitinolytic activity using powdered chitin (Sigma Chemical Co.) as the growth substrate. It was found that a number of different proteins were produced extracellularly when supernatants of growing mutants were analyzed by polyacrylamide gel electrophoresis (PAGE; Figure 4). When the band patterns of the mutants are compared, no unique proteins are present in the supernatants of the more effective entomopathogens. Indeed, one of the least effective entomopathogens, mutant 14, produces a large amount of exocellular protein (4.0 mg/ml as compared to 2.6 mg/ml for the best entomopathogen, E_1). Thus there appears to be no "specific" protein(s) secreted by the better pathogenic mutants that could be identified as *the* penetrating enzyme(s).

The exocellular protein pattern of E_1, the most entomopathogenic mutant of *B. bassiana* (LD_{50} = 15 conidia/larva), grown in five different media, is shown in Figure 5. When E_1 is grown in chitin salts, the fungus produces the greatest number of proteins, whereas in the N-acetylglucosamine medium, only four faint bands appear on the gel. The large number of bands found in media from cells growing in chitin was difficult to understand until analysis showed that large amounts of protein exist in association with chitin. Because of this finding, it appears logical to assume that, in addition to chitinases, several of the proteins appearing in the concentrated chitin growth medium are proteinases.

When the exocellular protein pattern of fungi that have grown in either SDB, gelatin, N-acetylglucosamine, or powdered integument medium are compared to growth in chitin salts (Figure 5), it can be seen that

Figure 4. Polyacrylamide gels showing exocellular proteins produced by various mutants of Beauveria bassiana

6.5% polyacrylamide gels were run at 2 mA/tube in a tris-glycine buffer (pH 8.0).

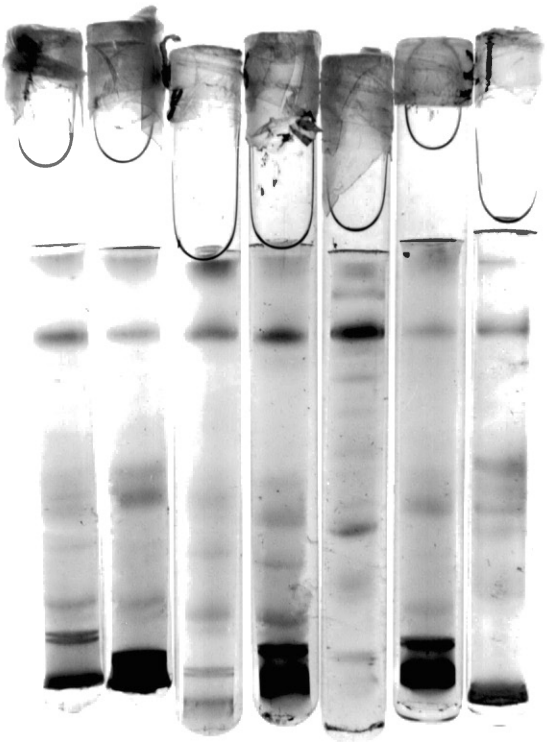
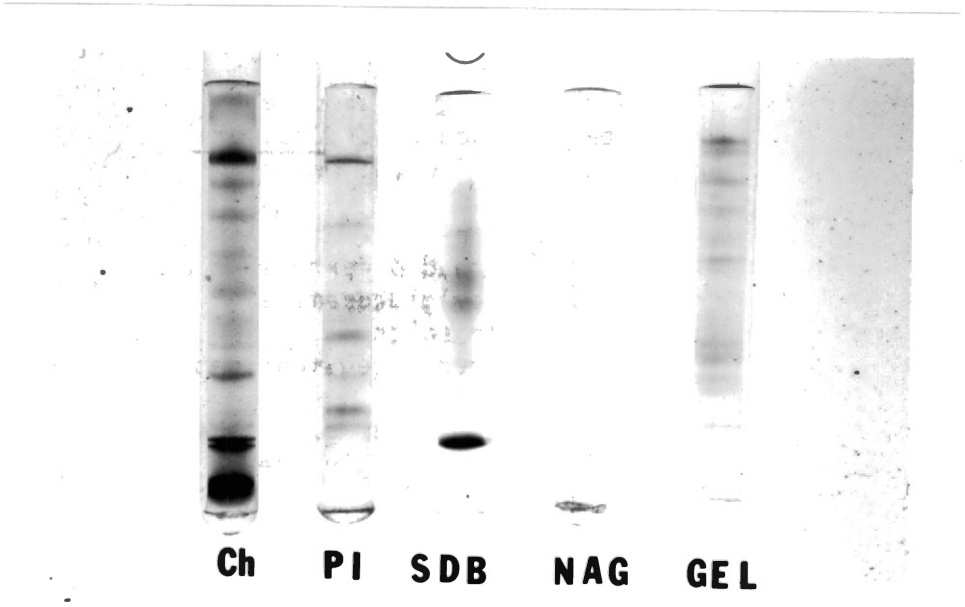


Figure 5. Polyacrylamide gels showing exocellular proteins produced by Beauveria bassiana E_1 in different media

Gel = Gelatin
SDB = Sabouraud's dextrose broth
NAG = N-acetylglucosamine
Ch = Chitin salts
PI = Powdered integument medium

6.5% polyacrylamide gels were run at 2mA/tube in a tris-glycine buffer (pH 8.0).


Ch
PI
SDB
NAG
GEL

the production of exocellular proteins is dependent on composition of the growth medium. The fungus synthesizes different proteins in different media, indicating that most of the proteins are probably inducible.

To further establish the inducibility of the B. bassiana chitinase, powdered chitin was added to cultures of mutant E_1 growing in different media (Figure 6). Presumably the fungus would exhaust the original nutrients present and begin metabolizing the chitin. Additionally, a proteinase would also have to be produced in order to degrade the protective protein surrounding the chitin. In all three situations examined, the addition of chitin to the medium induced the production of a greater number of exocellular proteins.

Because of the presence of protein in all types of chitin, it appeared necessary to examine the growth response of B. bassiana when commercial chitin was treated in several different ways. Surprisingly, when commercial chitin was autoclaved, it was found that approximately 140 µg N-acetylglucosamine and 80 µg glucosamine are released from 1 gram of chitin. Additionally, amino acids are released (Cheung and Grula, unpublished) (Table VII).

When commercial chitin was subjected to dialysis, oven-drying, and chloroform extraction and added to the medium without autoclaving, the growth responses of B. bassiana mutant E_1 were very poor (Table VIII). Such evidence indicates that an inducer of chitinase synthesis is released from chitin by autoclaving and this material is of small molecular dimensions since it can be dialyzed out. Reautoclaving will allow some growth of the fungus; however, growth is never as good as after the first autoclaving. It was also shown that concentrated soluble supernatant

Figure 6. Polyacrylamide gels showing exocellular proteins produced by Beauveria bassiana E_1 when powdered chitin is added to various media

6.5% polyacrylamide gels were run at 2 mA/tube in a tris-glycine buffer (pH 8.0).

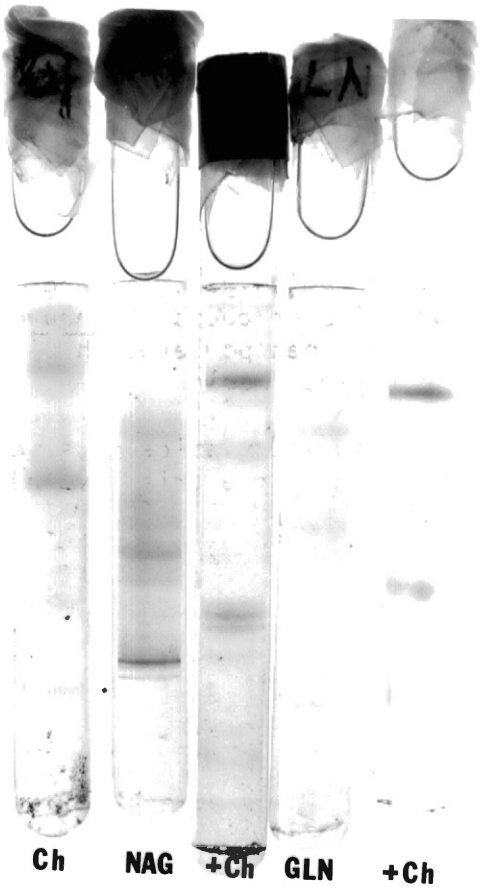

Ch
NAG
+Ch
GLN
+Ch

TABLE VII

COMPARISON OF AMINO ACIDS FOUND IN COMMERCIAL CHITIN, WATER-WASHED, AND SDS-TREATED INTEGUMENTS OF THE CORN EARWORM

Amino Acid	Molar Percent of Total		
	Commercial Chitin	Water	SDS
ASP	12.0	11.2	4.3
THR	5.0	6.9	2.7
SER	6.0	8.7	5.2
GLU	13.0	10.4	11.2
PRO	8.0	10.4	6.7
GLY	12.0	14.8	13.5
ALA	12.0	11.2	15.9
VAL	8.0	6.1	9.2
MET	1.0	0.9	2.2
ILE	3.0	2.3	3.8
LEU	4.0	5.2	8.5
PHE	5.0	3.0	---
HIS	5.0	3.5	4.5
LYS	2.0	2.6	4.5
ARG	6.0	2.7	3.4

Concentration in nmoles was not available for commercial chitin.

TABLE VIII

GROWTH OF *BEAUVERIA BASSIANA* MUTANT E_1 ON TREATED CHITINS

Medium Used	Germination[a]	Growth
Chitin Salts*	+	+
Chitin; 4 N HCl; 100°C; 4 hr	-	-
Colloidal Chitin[b]	-	-
Filtrate from Autoclaved Chitin[c]	+	+
Dialyzed Chitin[d]	+	-
Chloroformed Chitin	+	-
Dialyzed, Oven-Dried Chitin	-	-
Chitosan*	-	-
Chitobiose	+	+

[a]Germination was scored as the visible protuberance of a germ tube from a conidium.

[b]Colloidal chitin is prepared by numerous concentrated HCl treatments followed by extensive washing with distilled water.

[c]Chitin is autoclaved for 15 min, then filtered through two layers of Whatman No. 1 chromatography paper. Filtrate was lyophilized and resuspended in 10 ml of deionized water for growth observations.

[d]Dialyzed chitin is prepared by dialyzing 3 days against 3 changes of deionized water (12,000 molecular weight exclusion membrane). Ratio of 20 to 1 was used during each dialysis.

*Denotes autoclaving; 15 min at 15 lbs/in.^{2}.

material from autoclaved chitin allows ready growth and induction of chitinase synthesis by B. bassiana.

Utilizing chitinase assay procedures, it was found that N-acetylglucosamine, D-glucosamine, chitobiose, and the supernatant from autoclaved chitin can induce synthesis of chitinase (Table IX). Interestingly, a three amino acid mixture (alanine, phenylalanine, valine) which supports excellent growth of B. bassiana (Smith and Grula, 1981), cannot induce chitinase synthesis, nor can chitosan, the deacetylated product of chitin.

Another type of protein-chitin complex, corn earworm integument ghosts produced using SDS and autoclaved (Smith et al., 1981) were readily utilized as a growth substrate for B. bassiana. Mutants of B. Bassiana are unable to initiate germination and subsequent growth when KOH-treated ghosts are present as the sole source of carbon-nitrogen and energy even after autoclaving of such ghosts. This indicates that alkali removes the same type of material as autoclaving. This finding also shows that one of our purest chitin preparations (see Table V) cannot induce synthesis of chitinase (even after autoclaving).

"Sparking" experiments were done to investigate what compounds and concentrations thereof were necessary to initiate germination and growth of B. bassiana utulizing the alkali-treated ghosts (Table X). These experiments corroborated the chitinase assay determinations and established the inducers of chitinase synthesis to be N-acetylglucosamine, D-glucosamine, chitobiose, and autoclaved chitin supernatant. A concentration of 10 µg/ml of the inducer is sufficient to allow excellent germination and growth of B. bassiana E_1 on alkali-treated ghosts. Also, as shown in Table X, no combination of amino acids would induce chitinase or germination and growth of B. bassiana when present in sparking amounts.

TABLE IX

INDUCERS OF CHITINASE SYNTHESIS IN BEAUVERIA BASSIANA

Compound[a]	µg NAG Released/ 4 hr at 37°C	Total Units[c]
Amino Acid Mixture[b]	0	0
N-Acetylglucosamine	36	2.8
D-Glucosamine	54	4.3
Chitobiose	42	3.3
Chitosan (Autoclaved)	0	0
Chitin (Autoclaved)	62	4.8

[a]All cultures were grown in the basal salts medium (see Materials and Methods) and contained the compound indicated at a level of 1.0%. Growth of the cultures was allowed to continue for 4 days at 25°C with shaking prior to assay using the procedure of Jeaniaux (1966).

[b]Alanine, phenylalanine, and valine (0.1% each in basal salts).

[c]Total units of chitinase are based on commercial chitinase from Sigma. One unit of chitinase activity = 12.6 µg NAG released in 4 hr from 100 µg of commercial chitinase at 37°C.

TABLE X

"SPARKING" EXPERIMENTS FOR INDUCTION OF CHITINASE IN *BEAUVERIA BASSIANA* USING KOH-TREATED GHOSTS

Compound Added to Ghosts	Concentration of "Sparking" Compound	Growth of E_1[a]
None	---	---
D-Glucosamine	5 µg/ml	-
D-Glucosamine	10 µg/ml	4+
N-Acetylglucosamine	10 µg/ml	4+
Chitobiose	10 µg/ml	3+
Autoclaved Chitin Supernatant	10 µg/ml	3+
Chitosan	10 µg/ml	-
Alanine	10 µg/ml	-
Phenylalanine	10 µg/ml	-
Valine	10 µg/ml	-
Ala + Phe	10 µg/ml each	-
Ala + Val	10 µg/ml each	-
Phe + Val	10 µg/ml each	-
Ala + Phe + Val	10 µg/ml each	-

[a] 4+ designates excellent growth; (-) designates no growth. Cultures were grown for 96 hr at 25°C on the rotary shaker.

A number of different mutants of B. bassiana were grown in autoclaved chitin salts medium in order to determine the relative amounts of extracellular chitinase produced (Table XI). When the total units of chitinase activity were compared to commercial chitinase (obtained from Sigma Chemical Co.), it appears that all mutants are better chitinase producers. There is no positive correlation between entomopathogenicity (LD_{50}) and total units of chitinase. When the specific activity of the mutants were calculated, all of the better entomopathogens exhibit a specific activity greater than 2.0; the two least effective pathogens, 14 and 9, have specific activities of less than 1.0.

To determine total as well as specific activity of chitinase production by various mutants of B. bassiana, five mutants having LD_{50} values from 10 to 500 conidia/larva were grown for three days in four media known to induce chitinase activity. Results of this study are given in Table XII. Mutant CC_3, a potent pathogen, produces the greatest amount of chitinase in all four media. N-Acetylglucosamine appears to be the most effective inducer of chitinase based on specific activity measurements when mutants were grown for three days. When total units are compared, ghosts and glucosamine are the best inducers.

Surprisingly, corn earworm SDS ghosts were able to induce good chitinolytic activity in mutants CC_3 and 14. In both total units and specific activity, N-acetylglucosamine, D-glucosamine, and SDS ghosts are better inducers of chitinase than chitin after three days growth. Again, no correlation appears to exist between entomopathogenicity and chitinase activity. For example, in N-acetylglucosamine medium, CC_3 and 14 are roughly equal in specific activity. In the SDS ghost medium, 14 is better than E_1, the most potent pathogen for the corn earworm.

TABLE XI

AMOUNTS OF CHITINASE ACTIVITY PRODUCED BY VARIOUS MUTANTS OF *BEAUVERIA BASSIANA* IN AUTOCLAVED CHITIN-SALTS MEDIUM[a]

Mutant	Protein[b] (µg/0.1 ml)	µg NAG/4 hr	Total Units[c]	Specific Activity[d]	LD_{50}[e]
E_1	160	59	4.68	2.93	15
CC_3	100	81	6.43	6.43	25
HP_1	160	42	3.33	2.08	10
CC_2	220	82	6.51	2.96	18
R_1	420	26	2.06	0.49	100
8	140	29	2.30	1.64	200
14	450	49	3.89	0.86	320
9	720	60	4.76	0.66	500

[a]Mutants were grown in chitin salts (pH 7.2) for 7 days. Mutants are arranged in order of entomopathogenicity for the corn earworm (Cheung, unpubl.).

[b]Protein is determined by the Bio-Rad assay method.

[c]Total units of chitinase are based on commercial chitinase from Sigma. One unit of chitinase activity = 12.6 µg NAG released in 4 hr from 100 µg of commercial chitinase.

[d]Specific activity = total units of chitinase activity/100 µg protein.

[e]LD_{50} = Avg. number of conidia/larva at the LC_{50}.

TABLE XII

CHITINASE ACTIVITY IN BEAUVERIA BASSIANA AFTER THREE DAYS GROWTH IN DIFFERENT MEDIA

Mutant	Protein[a] (µg/ml)	µg NAG/ 4 hr	Total Units[b]	Specific Activity[c]	LD_{50}[h]	Medium
CC_3	140	39	3.10	2.20	25	NAG[d]
E_1	230	23	1.80	0.78	15	NAG
R_1	160	15	1.20	0.75	100	NAG
14	170	36	2.90	2.04	320	NAG
9	240	31	2.50	1.03	500	NAG
CC_3	230	57	4.50	1.96	25	GLN[e]
E_1	320	33	2.60	0.81	15	GLN
R_1	280	27	2.10	0.76	100	GLN
14	550	44	3.50	0.64	320	GLN
9	480	51	4.10	0.85	500	GLN
CC_3	190	74	5.90	3.10	25	Ghost[f]
E_1	490	41	3.30	0.67	15	Ghost
R_1	530	43	3.40	0.64	100	Ghost
14	310	55	4.40	1.42	320	Ghost
9	470	38	3.80	0.81	500	Ghost
CC_3	450	38	3.00	0.67	25	Chitin[g]
E_1	390	12	0.95	0.24	15	Chitin
R_1	420	16	1.30	0.31	100	Chitin
14	770	22	1.80	0.23	320	Chitin
9	690	13	1.00	0.14	500	Chitin

[a]Determined by the Bio-Rad assay method.

[b]Total units of chitinase are based on commercial chitinase from Sigma. One unit of chitinase activity = 12.6 µg NAG released in 4 hr from 100 µg commercial chitinase.

[c]Specific activity = total units/100 µg protein.

[d]N-Acetylglucosamine-salts medium.

[e]D-Glucosamine-salts medium.

[f]Corn earworm SDS ghosts-salts medium.

[g]Chitin-salts medium.

[h]LD_{50} = See Table XI (e).

Concentrated supernatants of B. bassiana mutants after growth in chitin salts, N-acetylglucosamine, D-glucosamine and amino acid media were examined using gel filtration. Supernatants were separated utilizing a Sephadex G-75 column, and fractions were assayed for chitinolytic activity and subjected to PAGE. This was done in order to locate the chitinase enzyme on a polyacrylamide gel, and to obtain a "pure" chitinase for further studies. Preliminary results from these experiments have been encouraging.

When the concentrated supernatant of mutant E_1 grown in N-acetylglucosamine salts or D-glucosamine salts medium was subjected to gel filtration, the same type of peak pattern was observed (Figure 7). Enzyme analyses revealed that the chitinolytic activity is concentrated at peak 2, while no detectable chitinolytic activity was found in peaks 1 or 3. PAGE analysis of these fractions showed that two bands appeared in the peak 2 fraction (Figure 8).

When mutant 14 was grown in the N-acetyl glucosamine salts medium, concentrated and subjected to gel filtration, alone and simultaneously with commercial chitinase, it was found that the second peak (Peak No. 2) increased in intensity (Figure 9).

The concentrated supernatant of mutant E_1 grown in an amino acid mixture (Smith and Grula, 1981) gave, after gel filtration, a different peak pattern from the amino sugar runs (Figure 10). No chitinolytic activity was detected in any of the pooled fraction groups, or in the concentrated total supernatant.

Figure 7. Gel filtration of the concentrated supernatant from B. bassiana mutant E_1 grown in NAG medium. The column packing utilized was Sephadex G-75, bed volume was 200 ml, and the flow rate 1 ml/min. The void volume (80 ml) was always collected before each scan. 2.5 mg of protein was layered onto the column.

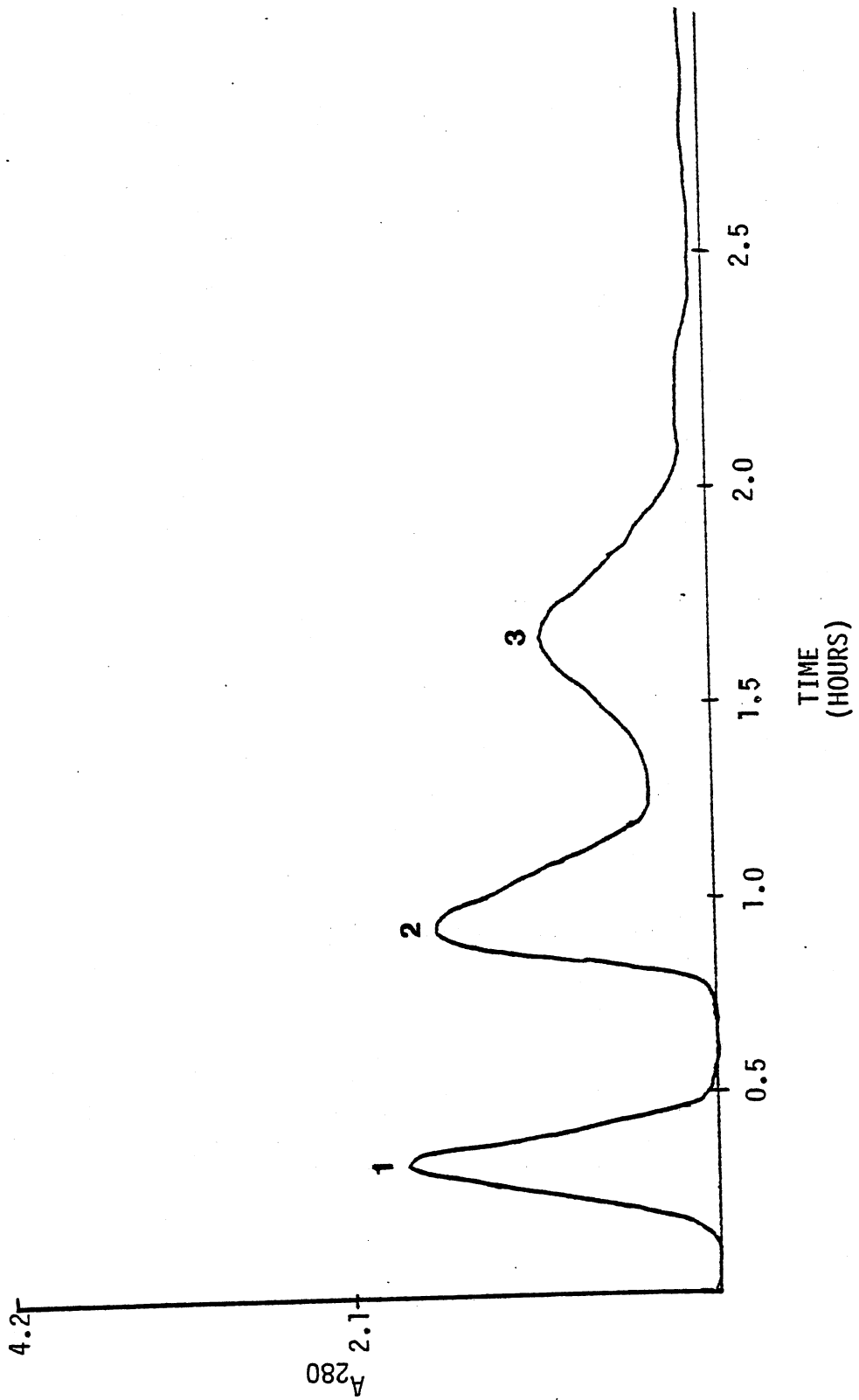

4.2
2.1
A_{280}
1
2
3
0.5
1.0
1.5
2.0
2.5
TIME
(HOURS)

Figure 8. A band representation of proteins obtained from gel filtration of concentrated supernatant from peak 2 in Figure 7

Orig = Original concentrated supernatant
Pk 2 = Peak 2 from the gel filtration
C'ase = Commercial chitinase from Sigma

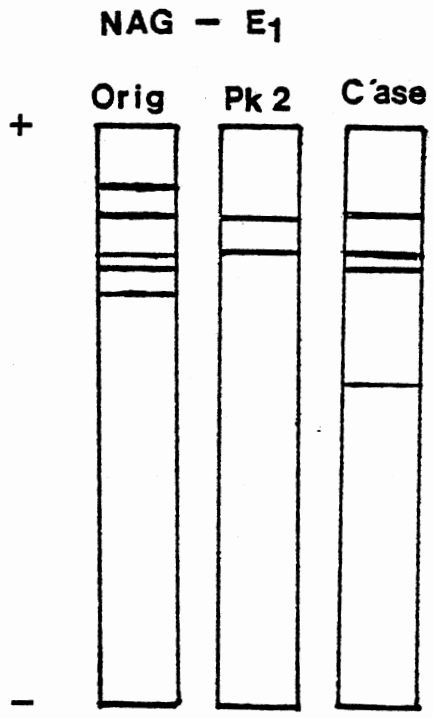

NAG – E1
Orig
Pk 2
C´ase
+
–

Figure 9. Gel filtration of concentrated supernatant from B. bassiana mutant 14 grown in NAG medium and simultaneously subjected to gel filtration with commercial chitinase. Column packing was Sephadex G-75, bed volume was 200 ml, and flow rate was 1 ml/min. The void volume (80 ml) was always collected before each scan. 2.5 mg of protein from B. bassiana supernatant and 1.0 mg of commercial chitinase was layered onto the column.

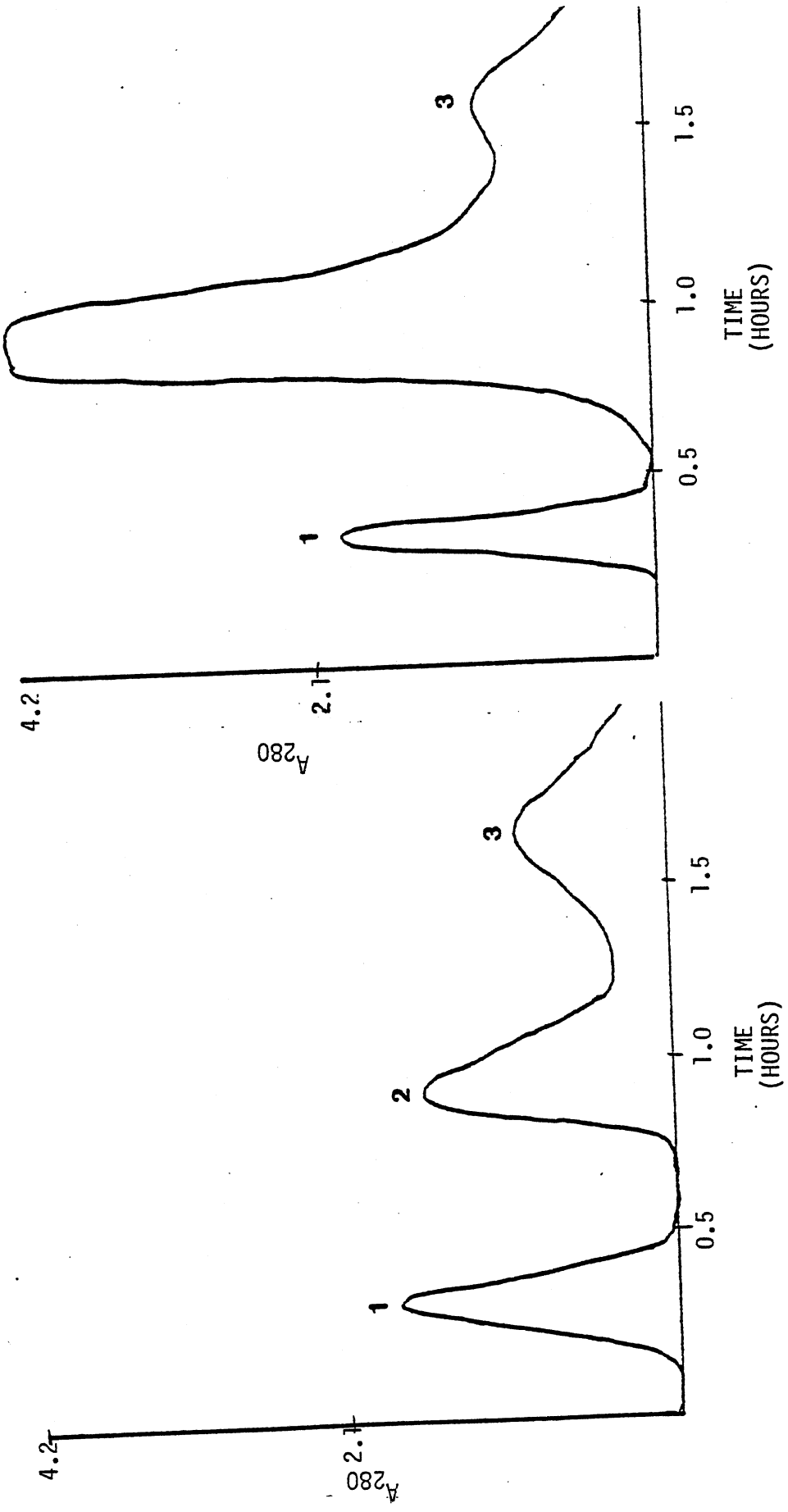
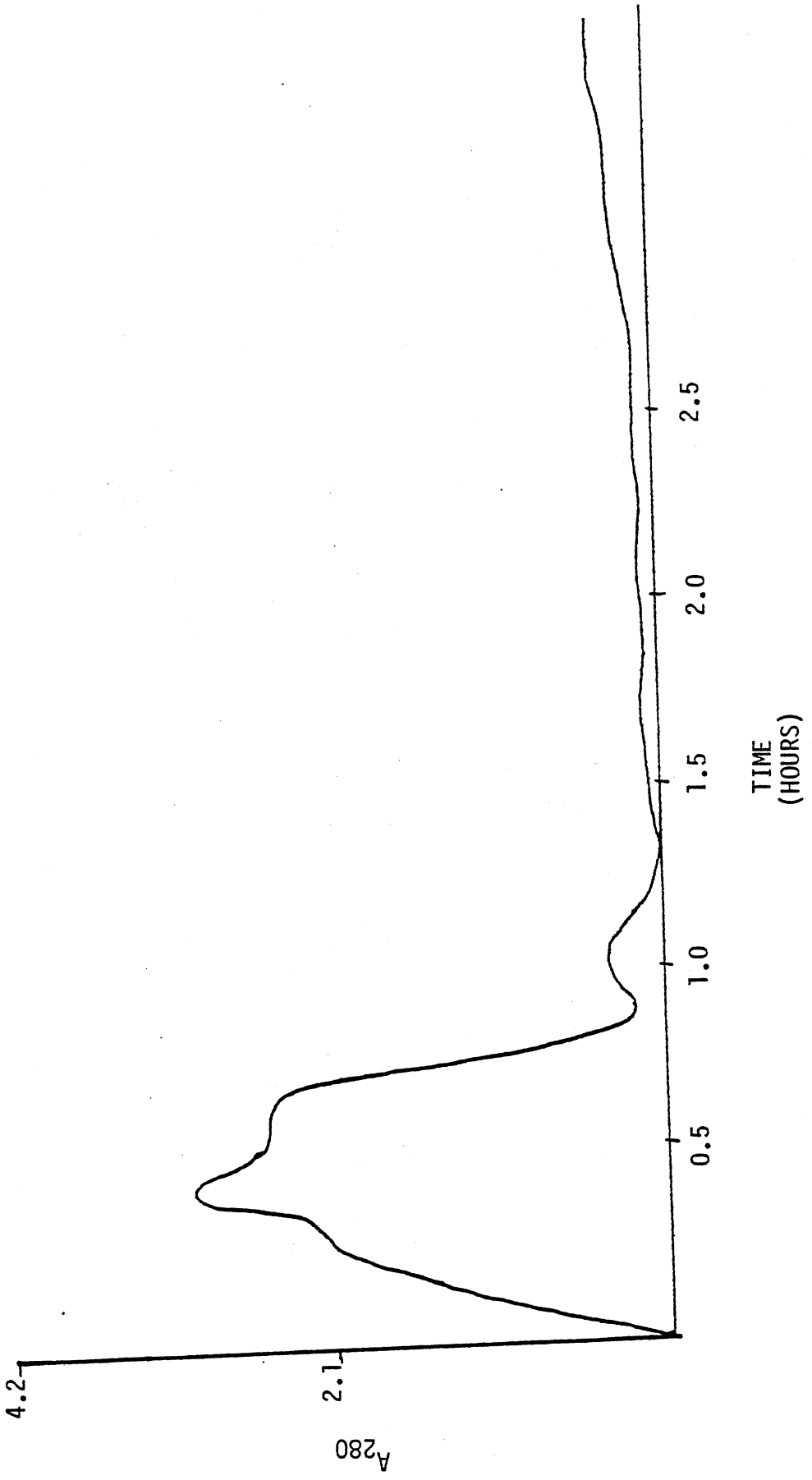

4.2
2.1
A_{280}
1
2
3
0.5
1.0
1.5
TIME
(HOURS)
4.2
2.1
A_{280}
1
2
3
0.5
1.0
1.5
TIME
(HOURS)

Figure 10. Gel filtration of concentrated supernatant from B. bassiana E_1 grown in amino acid medium. Column packing was Sephadex G-75, bed volume was 200 ml, and flow rate was 1 ml/min. The void volume (80 ml) was always collected before each scan. 2.5 mg of protein was layered onto the column.


4.2
2.1
A_{280}
0.5
1.0
1.5
2.0
2.5
TIME
(HOURS)

CHAPTER IV

DISCUSSION

Chitin-Protein Bonding in the Corn Earworm Integument

When the amino acid composition of the variously treated larval ghosts of third instar corn earworm larvae are compared, it is evident that the different chemical treatments remove more protein when used in sequence (see data in Table V). Two of the major amino acids always found in water-washed, SDS, Austin-treated, KOH, or TCA-Austin-treated corn earworm integument proteins are glycine and alanine (Tables I-IV). Although in some instances, leucine, aspartic acid, and lysine could also be considered major amino acid residues surviving the treatments, alanine and glycine were always found to be major amino acids within the total remaining. Such a finding indicates that these two amino acids, plus possibly glutamic acid and leucine may be more closely linked to the chitin. When alkali compounds were utilized to remove protein from the SDS ghosts (KOH, NaOH, NH_4OH), it appears that the molar percent of serine and threonine are decreased (Tables III and IV); indeed after TCA has been added to the Austin-treated ghosts, serine and threonine are completely removed. Since alkali and TCA preferentially remove O-ester bonded protein (Hackman, 1960; Heptinstall, 1970), one possible protein linkage to chitin in the corn earworm integument could be via an ester

bond of serine or threonine; these bonds are thus cleaved by these treatments, thereby releasing protein.

Although not addressed specifically in this study, the writer takes the position that covalent bonding exists between some proteins and chitin in the corn earworm integument. The most favorable argument for this position is that some of the same amino acids continue to be associated with chitin despite treatments which are known to break ester, and, to some extent, peptide bonds (Hackman, 1976; Atwood and Zola, 1967).

Kimura et al. (1976) reported that the amino acids left in integuments of Sarcophaga bullata (cockroach) after exhaustive chemical and enzymatic treatments involving proteolytic enzymes, chitinase, and sodium in liquid ammonia, were glycine, alanine, glutamic acid, and serine. Pant and Sharma (1974) report that alanine and glycine predominate all protein fraction amino acids of Philosamia ricini (round-head wood borer) after sequential treatments with water, sodium sulfate, urea, and sodium hydroxide. Srivastava (1971) also reported the predominance of glycine and alanine in larval cuticles of Galleria mellonella (greater wax moth) treated with borax, ethanol, urea, and sodium hydroxide in sequence.

Hackman (1960) reports, however, that after treatment of various shells of crayfish, the blowfly Lucilia cuprina, and larval skins of Agrianome spinicollis (giant silkworm) with sodium hydroxide (1 N; 100°C; 60 hr), only aspartic acid and histidine could be found along with glucosamine after acid hydrolysis. This has not been corroborated by other investigators (Hunt, 1970; Atwood and Zola, 1967).

Alanine and glycine are the two major amino acids present in the proteins elastin and resilin, two structural type proteins. Resilin has been found in several larval integuments, and is reportedly responsible

for the elasticity of these structures (Hunt, 1970). Thus it appears that the predominance of glycine and alanine in cuticular proteins of the corn earworm (Heliothis zea) could be related to the amount of resilin present in this integument (Table XIII). However, the persistence of glycine and alanine after exhaustive chemical treatments would indicate that several residues of these amino acids are directly bonded to or in close proximity to the chitin chains (see Figure 12). Findings in our laboratory indicate that B. bassiana produces significant amounts of elastase; thus data for elastin are included in Table XIII.

Findings from this study permit various protein-chitin bonding possibilities to be hypothesized (Figure 11). Hunt (1970) has postulated that protein could be bound only to the anomeric carbon of each N-acetylglucosamine molecule (carbon #1) because many glycoproteins have their protein and sugar moieities linked in this manner. This implies that protein could only be bound at the ends of the chitin chains, or that amino acids could be situated between molecules of N-acetylglucosamine (Figure 11A). Another possibility could be the presence of intervening protein between chitin chains; bonding would be of the O-ester type or of the glycosylamine type (Figure 11B). Aspartic acid or glutamic acid in the protein-chitin complex of the corn earworm could be O-ester linked because alkali treatments (KOH and NaOH) result in significant removal of these amino acids (see Tables II and III). If, as Hackman (1962) has reported, there is one glucosamine molecule for every six N-acetylglucosamine molecules in chitin, then another type of bonding could exist; an amide type linkage between the free amino group of glucosamine and the carboxyl end of the protein (Figure 11C). beta-1,6 Branching of the poly-N-acetylglucosamine chains is not a possibility because Hunt (1970) and

TABLE XIII

AMINO ACID COMPOSITIONS OF RESILIN AND ELASTIN*

Amino Acid	Resilin[a]	Elastin[a]
4-Hydroxyproline	---	11.4
Aspartic Acid	102.0	7.7
Threonine	29.6	8.6
Serine	78.6	8.0
Glutamic Acid	50.4	15.2
Proline	79.4	109
Glycine	376.0	331
Alanine	111.0	223
Valine	25.6	141
Cystine/2	---	---
Methionine	0	Trace
Isoleucine	20.4	27.0
Leucine	25.6	63.9
Tyrosine	29.2	7.7
Phenylalanine	27.4	35.0
Lysine	4.9	3.2
Histidine	6.5	0.6
Arginine	33.6	7.0
Tryptophan	---	0.5
NH_3	69.2	---

*Taken from Hunt (1970).

[a]Residues per 1000 total residues.

Figure 11. Structures of Possible Amino Acid Bonding to Chitin in the Corn Earworm Integument

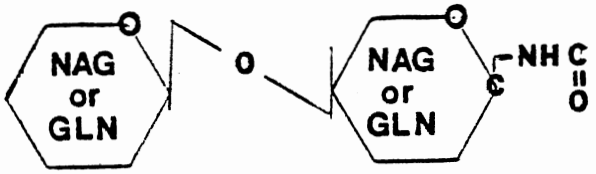
A. Amino acid bonding through Asn or Gltn to the anomeric carbon of the amino sugar, or between the amino sugar chains.

B. Intervening protein between amino sugar chains. Bonding to C#4 would be of the ester type, whereas bonding to the #6 carbon of the second amino sugar chain could be an ester linkage or a glycosyl-amine type bond.

C. Amide linkage to glucosamine instead of N-acetylglucosamine. Any amino acid could be bonded here.

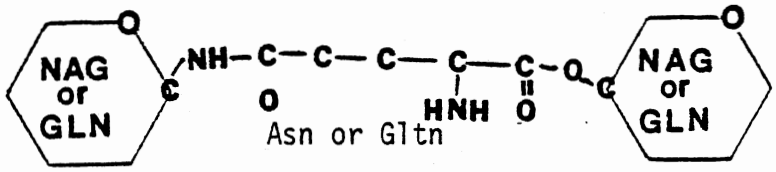
ASN = Asparagine
GLTN = Glutamine
NAG = N-acetylglucosamine
GLN = Glucosamine

A.

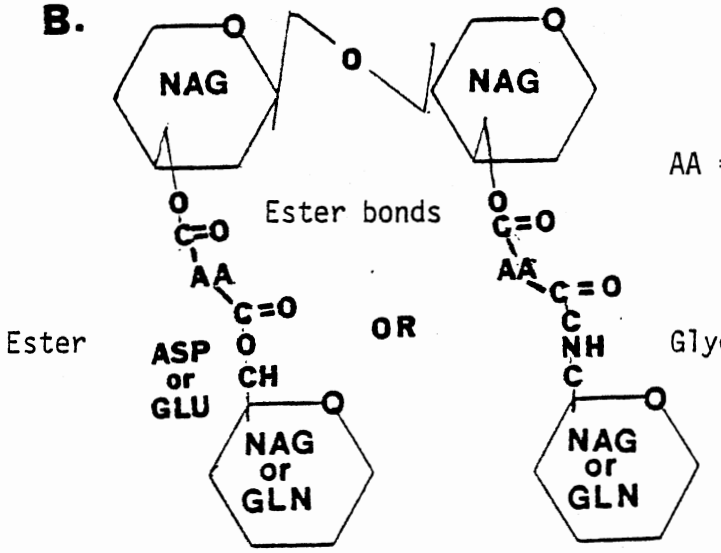


Amido group of Asn or Gltn (Glycosylamine bond)

OR



B.

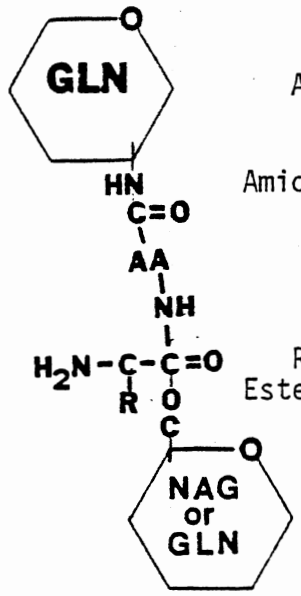


AA = Glycine, Alanine, Leucine

Ester

Glycosylamine

C.



AA = Glycine, Alanine, Leucine

Amide Bond

R = Side chain of Ala, Leu, or Gly

Ester Bond

Rudall (1967) could not find evidence for such structuring utilizing X-ray diffraction technology.

The use of ammonium hydroxide on the TCA-Austin-treated ghosts (produces limit ghosts) revealed several interesting things about this amino acid-chitin complex. Even after the ammonium hydroxide treatment, the ghosts were still intact structures. When these limit ghosts were hydrolyzed, some amino acids were still found associated with the chitin (Table IV). These same amino acids were also removed by the ammonium hydroxide and were found in the supernatant. Thus, some of the same amino acids that remain attached to the limit ghosts are also removed by the alkali treatment.

Dansylation of the "limit" ghost as well as the TCA ghosts (Table VI) revealed that the major N-terminal amino acid was glycine. Alanine was also found to be an N-terminal amino acid in smaller quantities (1/3-1/2 as much based on color intensity on the thin-layer plate) in the limit ghosts. Therefore, all amino acids in the limit peptide(s), including other residues of alanine and glycine, have their amino groups somehow bonded either to each other or to the amino sugar residues of chitin.

There are two types of carbohydrate-protein covalent linkages found in vertebrate glycoprotein: (a) a glycosylamine bond involving the amide groups of asparagine or glutamine with the anomeric carbon of the amino sugar (Figure 11A), or (b) an O-glycosidic bond between the amino sugar and one of the hydroxy aliphatic amino acids (serine or threonine). No evidence for this particular type of bonding was obtained in this study because serine and threonine were never found in the limit ghosts. Hunt (1970) makes the interesting observation that it would be presumptuous

to assume that carbohydrate-protein bonding in invertebrate chitin would have to be the same type linking as found in vertebrate forms.

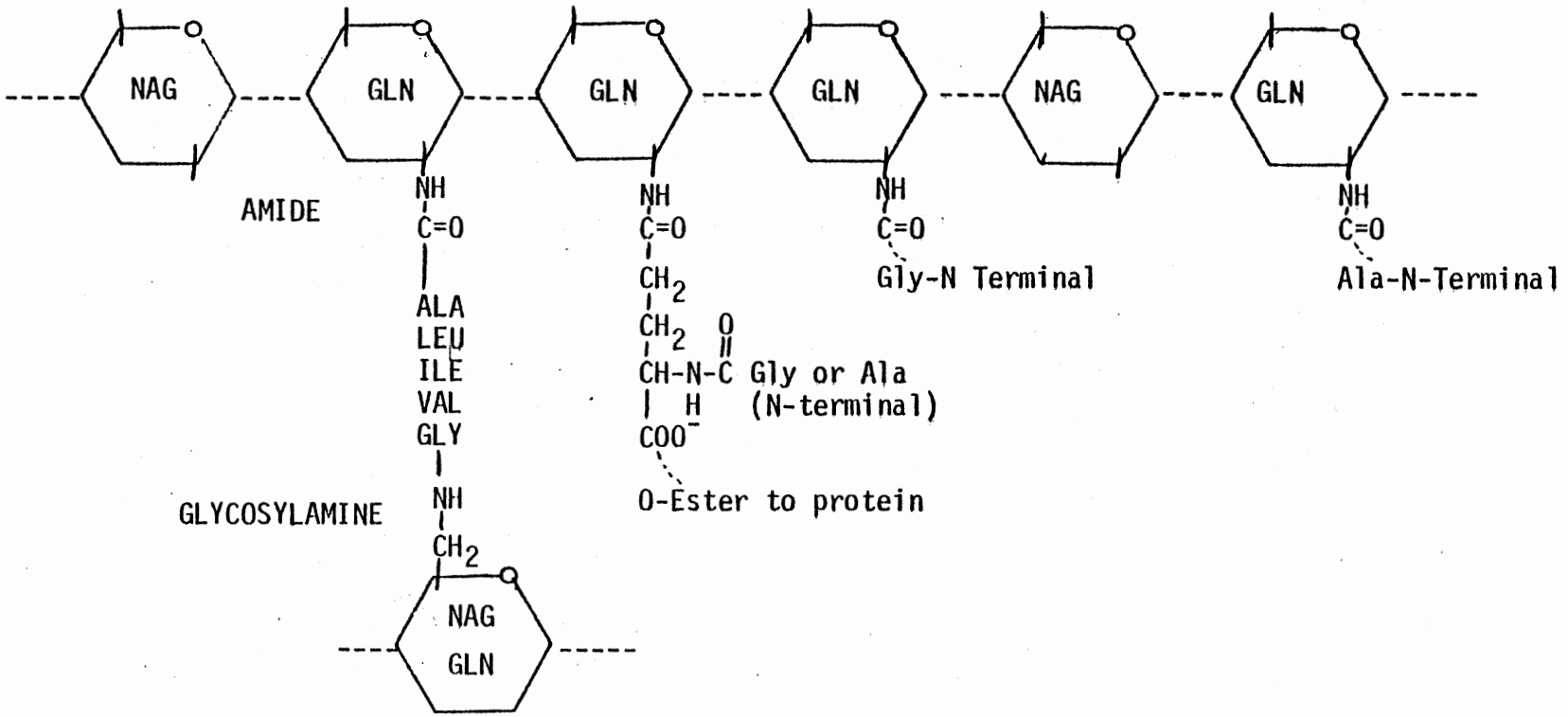
Several disadvantages have been overcome with the use of corn earworm ghosts for the elucidation of protein-chitin linkages. Utilizing the ammonium hydroxide ghosts as the "limit substrate" consisting of the minimal amino acids and chitin, it should now be possible to determine whether the bonding is of the O-ester type, glycosylamine linking, or whether the glucosamine amino group is involved in an amide type linkage, by adding specific peptidases to this structure.

Carboxypeptidase A (Sigma) was incubated with the limit ghosts and these ghosts were hydrolyzed and analyzed for amino acids. It was observed (thin-layer chromatography) that glutamic acid was completely removed by the carboxypeptidase. This finding indicates that there is a free carboxyl group present on the glutamic acid that allows for carboxypeptidase attack. However, since glutamic acid was the only amino completely removed by this enzymatic treatment, this implies that glutamic acid is not bonded to any other amino acid, since these amino acids would also be cleaved by the carboxypeptidase treatment. When leucine aminopeptidase was incubated with the limit ghosts, and the ghosts were hydrolyzed and analyzed for amino acids, all amino acids present in the control limit ghost were still found after the enzymatic treatment.

One possible model of the limit amino acid-chitin bonding in the corn earworm integument is given in Figure 12. This model accounts for the resistance of the amino acids found in the limit peptide; i.e., if ester bonded, the alkali treatment should have removed them, but amide bonding is more resistant to these chemical treatments. The finding that carboxypeptidase cleaves all of the glutamic acid residues from the limit

Figure 12. Model of Possible Amino Acid Bonding to Chitin in the Corn Earworm Integument

NAG = N-acetylglucosamine
GLN = D-glucosamine
ALA = Alanine
LEU = Leucine
ILE = Isoleucine
VAL = Valine
GLY = Glycine


NAG
GLN
GLN
GLN
NAG
GLN
AMIDE
NH
C=O
ALA
LEU
ILE
VAL
GLY
NH
CH2
NAG
GLN
GLYCOSYLAMINE
NH
C=O
CH2
CH2
CH-N-C
H
COO-
O-Ester to protein
Gly or Ala
(N-terminal)
NH
C=O
Gly-N Terminal
NH
C=O
Ala-N-Terminal

ghost can be explained by its bonding to the amino group of glucosamine via either its alpha or gamma carboxyl group. The presence of the free carboxyl could indicate that protein was ester-bonded to this group through a hydroxyl group from a serine or threonine residue in the protein, and this type of bonding was cleaved by the alkali treatment. Additionally, the model accounts for the presence of N-terminal glycine and alanine in the limit ghosts as was shown by dansylation of this structure (Table VI). Some evidence for the bonding of these amino acids to glucosamine instead of N-acetylglucosamine has been obtained in this study. Because of the stability of these limit amino acid linkages when subjected to alkali and TCA, it appears more likely that an amide bond is present instead of the more labile ester bond that would be responsible for the linkage if N-acetylglucosamine was linked to these amino acids. Secondly, dansylation of the limit ghost revealed no glucosamine N-terminal residues; therefore, this amino group could be bonded to the amino acids, and thus not be available for dansylation.

Once the actual linkages are elucidated, it should then be possible to determine the type of proteinase required by B. bassiana to remove all amino acids from chitin, and thus allow for chitinolytic attack and preparation of pure chitin.

Induction of Chitinase in B. bassiana

From the data presented here, it appears that there is no correlation between the amount of extracellular protein produced by mutants of B. bassiana and their relative entomopathogenicities for the corn earworm. Mutants that exhibit very little pathogenicity (14 and 9) produced

approximately the same numbers and types of extracellular proteins as did the excellent entomopathogens (E_1 and CC_3) when grown in chitin salts.

Chitin salts medium is an excellent growth medium for production of exocellular proteins by B. bassiana; however, it cannot be assumed that these proteins are only chitinases; since, even in commercial chitin, protein surrounds and protects the chitin chains from chitinolytic enzymes. Dry weight determinations of this commercial chitin before and after treatment with 1.0% sodium dodecyl sulfate (4 hr; 100°C) revealed that at least 32% of this preparation is actually protein.

Mutants of B. bassiana appear to synthesize different proteins depending on the type of growth medium utilized, and also synthesized more of them when commercial chitin was added to these growth media. Such data demonstrate that at least some extracellular proteins produced by B. bassiana are inducible.

It was found that monomeric units of chitin, i.e., N-acetylglucosamine or D-glucosamine, as well as chitobiose or autoclaved commercial chitin induce chitinase synthesis in mutants of B. bassiana (Table IX). Neither amino acids nor chitosan induce synthesis of chitinase. It was also observed that when SDS ghosts were utilized as the sole source of carbon, nitrogen, and energy, chitinase synthesis was also induced (Table XII).

When the various inducers of chitinase synthesis were compared, it was found that commercial chitin was a relatively poor inducer of chitinase synthesis after only three days growth (Table XII). N-Acetylglucosamine, D-glucosamine, and SDS-ghosts can induce synthesis of more total units of chitinase activity than commercial chitinase. When the specific activities of the mutants are compared, again there appears to be no

correlation between entomopathogenicity and amount of chitinase activity (Table XII).

When commercial chitin is autoclaved, and the supernatant from this medium is used as a growth substrate for B. bassiana, it was observed that such supernatants readily induce chitinase synthesis. This was not surprising when it was shown that the supernatant contained free N-acetylglucosamine and glucosamine. When commercial chitin was subjected to four repeated autoclavings, washed by centrifugation, and utilized in the salts medium as the sole source of carbon, nitrogen, and energy, the growth response of B. bassiana mutant E_1 decreased after such successive autoclavings, indicating that chitin cannot act as an inducer for chitinase synthesis. More evidence for this hypothesis was obtained when dialyzed commercial chitin permitted little or no growth when used as the sole source of carbon, nitrogen, and energy.

Additional information relating to this problem was obtained utilizing autoclaved KOH-treated ghosts. Mutants of B. bassiana are unable to grow when such ghosts are used as the sole source of carbon, nitrogen, and energy in a salts medium; yet, when small amounts of compounds known to induce chitinase synthesis (N-acetylglucosamine and D-glucosamine) were added to the medium, the mutants were able to grow very well (Table X). Such data reinforce the premise that chitinase induction, and hence synthesis, occurs only when a compound such as N-acetylglucosamine or D-glucosamine is present; and that the insoluble macromolecule of chitin cannot alone induce synthesis of chitinase in B. bassiana.

The above findings raise the interesting question of what actual inducers for synthesis of chitinase are present on the surface of the corn earworm since it is obvious that chitinase synthesis must occur for

fungal hyphae to gain entrance to the larval haemolymph. Additionally, it is also of interest to determine how such components become available on the surface of larvae.

Recent findings in our laboratory show that amino sugars such as N-acetylglucosamine, and some small peptides consisting mainly of amino acids such as serine and tryptophan, are present on the larval surface. These data are not surprising when one considers the components of the integument of the corn earworm (Smith et al., 1981). The presence of N-acetylglucosamine is possibly due to its oozing out during synthesis of chitin. The peptides might also have a common origin; i.e., during the synthesis of various integumental proteins, amino acids or small peptides could have migrated to the larval surface.

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APPENDIX A

NUTRITIONAL REQUIREMENTS FOR CONIDIAL GERMINATION AND HYPHAL GROWTH OF *BEAUVERIA BASSIANA*

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Nutritional Requirements for Conidial Germination and Hyphal Growth of *Beauveria bassiana*

REBECCA J. SMITH AND E. A. GRULA

Department of Cellular, Molecular and Developmental Biology, Oklahoma State University, Stillwater, Oklahoma 74078

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Nutritional requirements for germination and growth of the entomopathogenic fungus *Beauveria bassiana* are not complex. For germination to occur, a utilizable source of carbon must be present; however, a nitrogen source is needed for continued hyphal growth, otherwise lysis ensues. Compounds that can serve as utilizable carbon–energy sources for germination include glucose, *N*-acetylglucosamine, glucosamine, chitin, starch, lanolin, hydrocarbons in crude oil, and some longer-chain fatty acids. Both organic and inorganic sources of nitrogen are readily utilized for growth. Conidia undergo active metabolism soon after being placed in a suitable growth medium, indicating that conidia are released from their state of dormancy several hours before emergence of the germ tube can be observed. Because of the nutritional versatility of *B. bassiana*, this fungus should be able to survive and be infective in several types of natural environments.

KEY WORDS: *Beauveria bassiana*, germination and growth of, nutritional requirements; corn earworm; larval surface, relationships to entomopathogenicity.

INTRODUCTION

Work in this laboratory has centered on biochemical events that can be positively correlated to entomopathogenicity of *Beauveria bassiana*. Our target insect is the corn earworm, *Heliothis zea*, one of the most important agronomic pests in the United States (Pramer, 1965).

It has been shown that conidia of *B. bassiana* germinate on the surface of corn earworm larvae and the growing germ tube enzymatically penetrates the integument (Grula et al., 1978; Pekrul and Grula, 1979). The integument is of primary importance in pathogenicity of fungi because this structure functions as a physical barrier to hyphal penetration; in addition, chemicals are present which probably can either permit or inhibit conidial germination.

The larval integument contains, as its major macromolecular components, proteins and chitin; these are, at least in part, in a complexed state (Muzzarelli, 1973). Other components include phenolic precursors of quinones that link amino acid chains to form sclerotin, and various waxes and lipids, depending on the species of insect. These waxes and lipids are usually long-chain hydrocarbons, with smaller amounts of fatty acids, alkyl esters, and other fatty alcohols (Richards, 1978). Thus, although more detailed studies are needed, it appears that a number of different types of compounds are present on or near the larval surface that could be utilized either to permit or inhibit the germination and subsequent growth of fungal entomopathogens such as *B. bassiana*.

The primary objective of the work being reported was to establish the nutritional requirements for germination of conidia and the subsequent growth of hyphae of *B. bassiana* and, also, to formulate a defined germination medium for use in further studies. After the basic nutritional needs had been defined, attempts were made to correlate growth under some nutritional conditions with pathogenicity toward corn earworm larvae.

MATERIALS AND METHODS

Mutants of *B. bassiana* were obtained by ultraviolet irradiation of conidia (Grula et

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al., 1978). Stock cultures were kept on Sabouraud's dextrose agar (SDA) slants at 25°C, and transferred approximately every 3 weeks to maintain maximum viability. All liquid cultures (Erlenmeyer flasks or test tubes, 20 × 150 mm) were incubated at 25°C on a platform shaker having a rotational speed of 180 rpm. Larvae of the corn earworm were allowed to grow in 1-oz clear plastic containers on the corn–soy–milk diet developed by Burton (1970). Containers were kept under constant light at 25°C, and allowed to grow until the desired stage of development was reached.

A basal salt solution consisting of the following amounts per liter of distilled water was used in all defined medium formulations: NaCl, 0.30 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; and K_2HPO_4, 0.30 g. To this, various carbon-energy and nitrogen sources were added, including powdered corn earworm integuments. These integuments were obtained by skinning fifth-instar larvae and boiling them in 1% sodium dodecyl sulfate for 2 hr. They were then water-washed, oven-dried (105°C) overnight, and ground into a fine powder using a mortar and pestle. These integuments consist primarily of chitin and protein.

Conidia of *B. bassiana* were obtained by growth on Sabouraud's dextrose agar. This generally took about 9 days and sporulation can readily be judged since the aerial hyphae collapse into a powdery mass. They were harvested by adding 0.03% Triton X-100 in water and gently suspending the conidia using a sterile loop prior to centrifugation. Washing was then accomplished by two additional centrifugations (3500 rpm for 15 min) in sterile distilled water. This washing procedure will remove or greatly decrease both nutrient and detergent residues. Aliquots of such washed suspensions were always used to inoculate various media for observation of germination and growth of the fungus.

Germination was scored as the visible protuberance of a germ tube from the spherical conidium (length equal to about one-half the diameter of a conidium). Staining was accomplished using crystal violet (30 sec). Kodak Panatomic-X film was utilized for all light photography.

Enzymes used in these studies were obtained from Sigma Chemical Company and are as follows: lipase (Type VII from *Candida cylindracea*), proteinase (Type IV from *Streptomyces caespitosus*), chitinase, lysing enzymes (Type III, from *Cytophaga dissolvens*), and β-glucuronidase (Type H-2 from *Helix pomatia*).

D-[U-^{14}C]glucose (sp act: 3.4 mCi/mmol; New England Nuclear Co.) was added to give a final activity of 0.1 μCi/ml in growth medium.

RESULTS

Lefebvre (1931) reported that he obtained 10–30% germination of *B. bassiana* conidia in the center of distilled water droplets, and as high as 90% at the edge of such droplets. After germination, extensive hyphal growth was observed in the droplets within 24–48 hr. If his observations are correct, it could be concluded that germination of conidia and significant hyphal outgrowth can occur utilizing only endogenous carbon–energy–nitrogen reserves. Because he further reported that 2% peptone greatly enhanced the percentage of germinating spores, one has to question the extensive germination and growth he obtained in distilled water. Apparently Lefebvre grew the fungus on potato–dextrose agar and directly transferred conidia to distilled water without washing. Lack of washing would permit the carryover of nutrients that could be utilized for germination and growth. This interpretation receives support from the work of Gabriel (1959) who tried to germinate *B. bassiana* in rainwater, saline, and peptone. He reported none to very low germination in rainwater or saline, and abundant germination in 2% peptone. During the early phases of our work, direct transfers from litmus milk, lanolin, and Sabouraud's dextrose agar to distilled water always resulted in some germination of conidia.

Contrary to the report of Lefebvre (1931), however, we have continually observed that washed conidia of *B. bassiana* cannot germinate and grow in distilled water; indeed, conidial lysis occurs (Figs. 1A, B). Actually washed conidia cannot even germinate and grow in the basal salts medium. Addition of a carbon-energy source such as glucose to the basal salts medium will allow germination of conidia; however, lysis of the outgrowing germ tubes soon ensues (Figs. 1C, D). When conidia are grown in basal salts plus glucose and a nitrogen source such as ammonium chloride, extensive germination and growth are obtained. These findings illustrate that a utilizable exogenous carbon–energy source is necessary for germination; however, a nitrogen source must also be present to sustain growth of hyphae and prevent their autolysis. The nitrogen present in amino sugars such as *N*-acetylglucosamine or glucosamine can readily be utilized since luxuriant growth occurs when either of these compounds is present as the sole source of carbon, nitrogen, and energy.

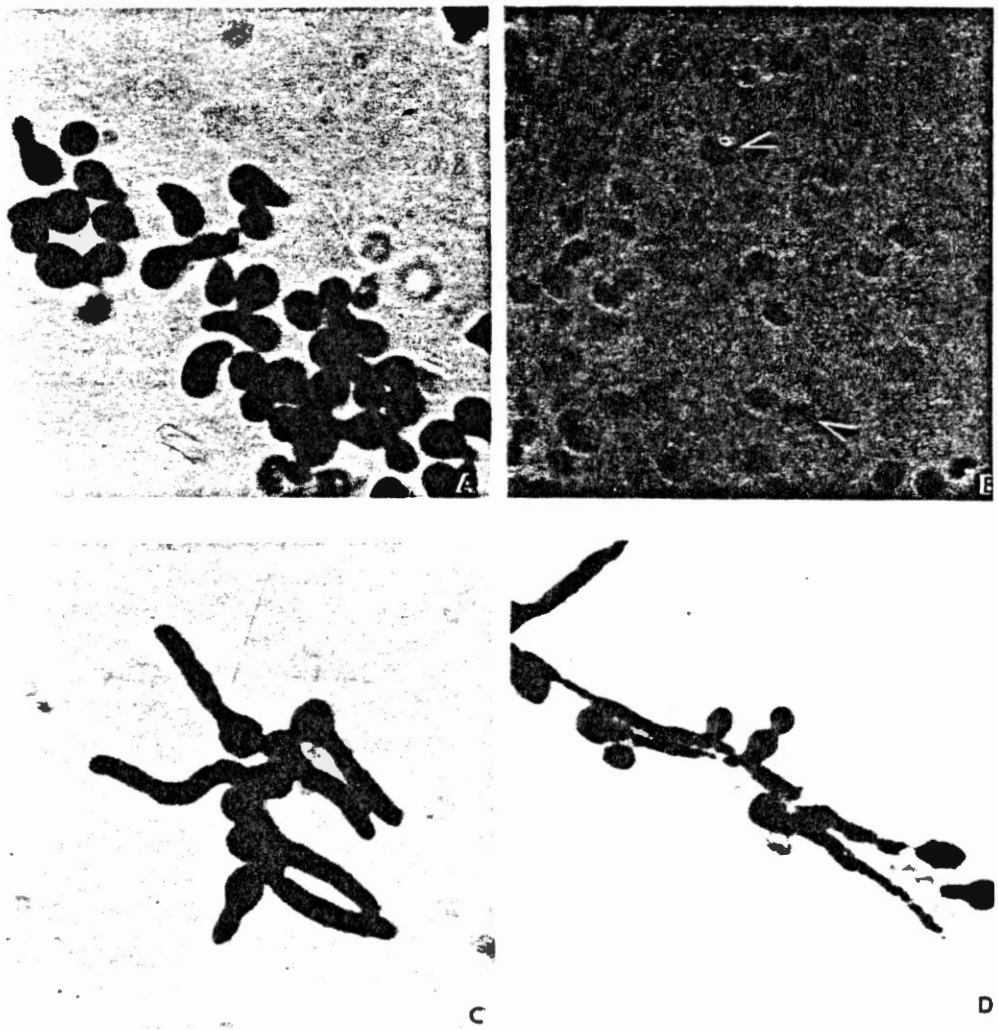

FIG. 1. Conidia of *Beauveria bassiana* E_1 germinating in *N*-acetylglucosamine medium (A), and lysing (arrows) in distilled water (B), at 16 hr (1600×). Conidia of *Beauveria bassiana* E_1 growing in glucose and ammonium chloride (C), and hyphal and germ tube lysis in glucose alone (D) at 22 hr. (1600×).

A nonutilizable analog of glucose, *O*-methylglucose, will not support either conidial germination or hyphal growth, further illustrating that a utilizable carbon–energy source is absolutely required for germination of conidia. *O*-Methylglucose is not toxic since the presence of this compound does not inhibit normal germination and growth when adequate amounts of glucose, for example, are present.

Amino acid studies were conducted to determine if these compounds can also be utilized as sole sources of carbon, nitrogen, and energy. Eventually, it will probably be necessary to determine what water-soluble compounds are present on larval surfaces to support germination of *B. bassiana* conidia and basic information relating to amino acid nutrition will be valuable and aid in interpretation of data from such studies. The amino acids were grouped according to their metabolic families (Table 1) and tested at the level of 0.01% each (final concentration) in the basal salts medium.

Amino acids in families 1 and 4 allowed the best germination and hyphal growth. Data obtained using microscopical observation are given in Table 2. A minimum of at least three amino acids is necessary for good germination and subsequent growth. The best combination is alanine, phenylalanine, and either leucine or valine. A combination of alanine, aspartic acid, and phenylalanine is also satisfactory. Tyrosine can be substituted for phenylalanine; however, germination is not as rapid or extensive. No single amino acid or combination of two amino acids was found to be satisfactory although some germination and growth occurred when alanine, histidine, and phenylalanine were individually tested (data not shown). As shown in Table 2, the absence of alanine always resulted in poor germination and hyphal outgrowth. In no case were any amino acids found to be toxic to the fungus at the concentrations tested.

In summary, although the fungus appears to have no specific requirements for individual amino acids, certain of them seem to be preferentially utilized, and alanine is one that appears to enter into the carbon–nitrogen flow of the organism's metabolism better than all others.

The ability of various fatty acids to serve

TABLE 1
AMINO ACID GROUPINGS FOR STUDY OF GERMINATION OF *Beauveria bassiana* CONIDIA

Family	Amino acids[a]
1	Alanine, valine, leucine
2	Aspartic acid, lysine, methionine, isoleucine, threonine
3	Serine, glycine, cysteine
4	Phenylalanine, tyrosine, tryptophan
5	Glutamic acid, proline, arginine
6	Histidine

[a] Each amino acid (L-isomer except for glycine) was tested at the level of 100 μg/ml in the basal salts medium: *p*H was always adjusted to 6.0. All amino acid families were tested separately and in all possible combinations for ability to allow germination and continued growth of *B. bassiana*.

TABLE 2
AMINO ACID REQUIREMENTS FOR GERMINATION AND GROWTH OF *Beauveria bassiana*

Amino acid[a]						
Ala	Val	Leu	Phe	Tyr	Germination[b]	Growth[b]
+	+	+	+	+	4	4
+	+	+	+	–	2	3
+	+	+	–	+	2	2
+	+	–	+	+	3	3
+	–	+	+	+	3	3
–	+	+	+	+	1	1
+	+	+	–	–	2	2
+	–	–	+	+	2	3
+	+	–	+	–	4	4
+	+	–	–	+	3	4
+	–	+	+	–	4	4
+	–	+	–	+	4	3
–	+	+	+	–	1	2
–	+	+	–	+	1	—[c]
–	–	+	+	+	2	1

[a] Level of each amino acid was 100 μg/ml.
[b] 4 = maximum germination or growth response. Cultures were observed at 16 hr for germination and at 72 hr for growth.
[c] No growth.

as carbon–energy sources for germination and growth of *B. bassiana* was also studied. An additional compelling reason exists for testing such compounds since Koidsumi (1957) and Evalakova and Chekhourina (1962) have reported that fatty acids (not identified) are present on the surface of larvae and are toxic to germination and growth of *Aspergillus flavus* and *B. bassiana*.

Data given in Table 3 reveal that a few of the longer-chain fatty acids (capric, undecanoic, stearic, and oleic acids) can function as carbon–energy sources and support germination of conidia; however, none can support continued hyphal growth, as autolysis of the nascent germ tubes occurs. Indeed, several of the shorter-chain fatty acids will inhibit germination of conidia when added to medium formulations that support good germination and growth of *B. bassiana* (Table 4).

Evalakova and Chekhourina (1962) reported that ether extracts from larvae were toxic to growth as well as germination of *B. bassiana*. Koidsumi (1957) reported that free medium-chain (C:8–C:10) saturated fatty acids were the most active fungal inhibitors in ether extracts from larvae of *Bombyx mori*. To determine if those fatty acids inhibitory to germination of conidia are also inhibitory to growth of hyphae, the fatty acids were added (0.02% final concentration) to cultures of *B. bassiana* after germination had occurred (16–24 hr). The medium utilized was the liquid basal salts mixture containing *N*-acetylglucosamine as the carbon–energy–nitrogen source. In no case were the fatty acids toxic to hyphal growth, and we therefore conclude that the toxicity of the fatty acid is expressed primarily during germination of conidia.

TABLE 3
GERMINATION AND GROWTH OF *Beauveria bassiana* IN THE PRESENCE OF VARIOUS FATTY ACIDS[a]

Fatty acid	Germination[b] (16 hr)	Growth[b] (72 hr)
Butyric (C:4)	–	–
Valeric (C:5)	–	–
Caproic (C:6)	–	–
Heptanoic (C:7)	–	–
Caprylic (C:8)	–	–
Nonanoic (C:9)	–	–
Capric (C:10)	+	–
Undecanoic (C:11)	+	–
Stearic (C:18)	+	–
Oleic (C:18-1)	+	–

[a] Final concentration of fatty acid was 1.0%. NH_4Cl (0.1%) was present in all tubes in addition to the basal salts.

[b] (+) At least 50% of conidia were germinating; (–) no visible germination or growth.

TABLE 4
INHIBITION OF GERMINATION OF *Beauveria bassiana* BY VARIOUS FATTY ACIDS[a]

Fatty acid	Germination[b] (16 hr)
Butyric (C:4)	–
Valeric (C:5)	–
Caproic (C:6)	–
Heptanoic (C:7)	–
Caprylic (C:8)	–
Nonanoic (C:9)[c]	–
Capric (C:10)	+
Undecanoic (C:11)	+
Stearic (C:18)	+
Oleic (C:18-1)	+

[a] Present at a final concentration of 0.02% in the basal salts, medium which contained *N*-acetylglucosamine (1.0%) as the carbon–nitrogen–energy source.

[b] (+) Germination of at least 50% of the conidia; (–) no germination.

[c] Causes extensive lysis of conidia.

The above data relate to *B. bassiana* mutant E_1, a potent entomopathogen (LD_{50}: 10–15 conidia/first-instar larva) of the corn earworm (Pekrul and Grula, 1979; Cheung and Grula, unpubl.). When all of our pathogens are tested for ability to germinate and grow on caprylic acid (C:8), which is highly toxic to most of the mutant strains of *B. bassiana*, several of the mutants can germinate and grow in the presence of this compound (Table 5). There is, however, no correlation between ability of the mutant strains to grow on caprylic acid (either 0.05 or 0.1% in solid medium containing *N*-acetylglucosamine as the carbon–nitrogen–energy source) and entomopathogenicity.

Ability of nucleic acid bases or their

TABLE 5
GROWTH OF VARIOUS MUTANTS OF *Beauveria bassiana* IN THE PRESENCE OF CAPRYLIC ACID

Mutant[a]	Time (hr)[b]	
	0.05%	0.1%
E_1	72	148
E_{1-2}	72	148
HP_1	48	148
E_{1-1}	72	316
3	96	—
7	72	—
16	48	316
23	72	316
R_1	48	316
10	72	—
8	72	—
1	96	—
20	48	316
14	96	—
9	120	—

[a] Mutants are ranked in decreasing order of pathogenicity (Cheung and Grula, unpubl.).

[b] The time indicates the approximate number of hours the fatty acid exerted its fungistatic effect at each concentration. (−) No growth for up to 14 days. The basal salts medium contained *N*-acetylglucosamine (1.0%) as the carbon–nitrogen–energy source and agar (1.5%).

nucleoside forms (uracil, guanosine, cytosine, and adenosine at 1% final concentration) to serve as sole sources of carbon, nitrogen, and energy was also tested. None permitted germination of *B. bassiana*. Also urea (1% final concentration) will not serve as a sole source of carbon, nitrogen, and energy.

To aid in understanding availability and possible use of larval surface components in the nutrition of entomopathogenic *B. bassiana*, knowledge of the ability of waxes to support germination and growth would also be desirable. Waxes are heterogenous high-molecular-weight compounds, the principal components of which are usually esters of fatty acids and alcohols. Enzymes able to initiate attack on such compounds would have maximal specificity directed toward *O*-ester bonds rather than specific fatty acids or alcohol residues. For these reasons, lanolin was chosen as the test compound because it is readily available commercially and fairly well characterized (contains 33 high-molecular-weight alcohols and 36 fatty acids; *Merck Index*, 9th ed., p. 5207).

To formulate a solid medium (1.5% agar) whereon the extent of growth and sporulation could be determined, lanolin was aseptically added (after autoclaving) to the basal salts plus ammonium chloride medium to a final concentration of 1%. To aid in emulsifying the wax and to keep it distributed throughout the agar, Duponol C (cationic detergent from Dow Chemical Co.) was also aseptically added (to 1%) after autoclaving (testing showed that this level is not toxic to growth of *B. bassiana*). Data given in Table 6 reveal that conidia from about one-half of our mutant strains can initiate and sustain growth using lanolin as a sole source of carbon and energy under our conditions of testing. Such growth is not due to carryover of nutrients with the inoculum since all of the cultures that grew on the first subculturing have continued to grow through (to date) six transfers. Data given in Table 6 show our mutant strains ranked in decreasing order of pathogenicity for the corn earworm. As can be seen, no direct correlation between pathogenicity and ability to utilize lanolin as a sole source of carbon and energy exists.

To further determine if paraffinic (alkanes)-type compounds which can also exist in waxes will support growth of *B. bassiana*, a light crude oil (designated as Sacatosa, sp grav of 30.0) was added to the basal salts–ammonium chloride medium to a final concentration of 1% (before autoclaving), and the liquid medium inoculated using washed conidia from the various mutant strains of *B. bassiana*. Data given in Table 6 show that some of the mutants are able to germinate and grow very well using the alkanes in crude oil as a sole source of carbon and energy. Complete breakdown, particularly of the heavier-weight components, does not occur (data not shown). As was observed when using either lanolin or

TABLE 6
GROWTH OF *Beauveria bassiana* IN LANOLIN OR CRUDE OIL[a] AS SOLE SOURCE OF CARBON AND ENERGY

Mutant[b]	Growth (144 hr)[c]	
	Lanolin	Crude oil
E_1	4	4
E_{1-2}	4	4
HP_1	4	4
E_{1-1}	4	4
3	—	2
7	4	2
16	3	1
23	—	2
R_1	4	3
10	—	2
8	—	2
1	4	4
20	—	2
14	4	4
9	—	4

[a] Lanolin and crude oil were added to the basal salts medium to a final concentration of 1.0%. NH_4Cl (0.1%) was present in both media as the nitrogen source.

[b] Mutants are ranked in decreasing order of pathogenicity (Cheung and Grula, unpubl.).

[c] 4 = excellent growth; 1 = poor growth.

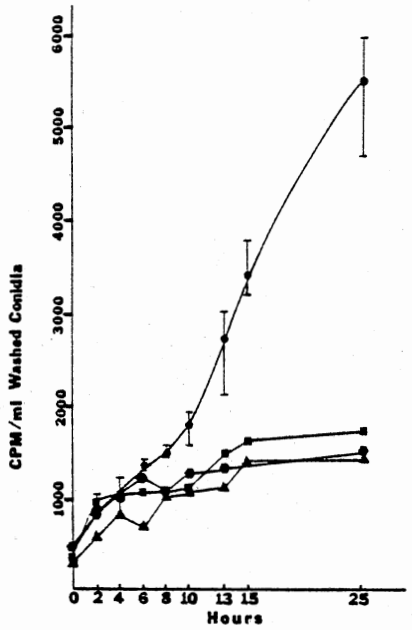

FIG. 2. Uptake and assimilation of [^{14}C]glucose in the presence of various inhibitors. ●, Glucose alone; ●, sodium azide (5 mM); ■, cycloheximide (1 μg/ml); ▲, actinomycin D (1 μg/ml). The amount of inoculum was 1 × 10^5 conidia/ml. Percentage of germinated conidia was: 30% at 12 hr, 60% at 15 hr, and 80–85% at 24 hr.

crude oil as sole source of carbon and energy, no positive correlation with pathogenicity exists.

The β-glucan, pustulan (1→6 linkages) was also tested as a sole source of carbon–energy in the liquid basal salts medium. Although pustulan supports germination and some outgrowth of hyphae, the hyphae soon lyse even if a utilizable source of nitrogen (NH_4Cl) is also present.

Germination and growth of *B. bassiana* are extensive when powdered integuments, starch, or chitin are present as the carbon–energy source in the basal salts medium. Germination does not occur when cellulose is present as the sole source of carbon and energy (Whatman No. 1 chromatography paper or mixed sawdust).

Conidia begin to actually metabolize glucose soon after its addition to the medium (Fig. 2). Visible germination does not occur for at least 12–14 hr, yet there is a significant amount of glucose that is assimilated from the medium before this time. When inhibitors of protein, ATP, and RNA synthesis are added, such metabolic activity does not occur (Fig. 2). Such experiments further establish generalized requirements for synthesis of protein, ATP, and nucleic acids for germination and growth. Uptake experiments utilizing [^{14}C]glucose in the presence of cycloheximide, which inhibits protein synthesis, also illustrate the need for protein synthesis in order for hyphal growth to occur (Fig. 2).

Although data for all mutants are not presented in this paper, it can be pointed out that all of our mutant strains (over 40) grow very well when either glucosamine, *N*-acetylglucosamine, or a three-amino-acid mixture (alanine, phenylalanine, valine) is present as the source of carbon, ni-

trogen, and energy. Further, all grow very well with glucose as the sole source of carbon–energy when nitrogen is added in the form of NH_4Cl.

An additional aim of these studies was to attempt synchronization of conidial germination via nutritional means. Such specific control over the germination process would permit study of specific metabolic events occurring during the initiation of germination. Regardless of the medium utilized, this was not possible. Therefore, some attempts to "shock" the conidia into synchronous germination were made. These studies included heat treatments, and use of certain enzymes capable of degrading components possibly existing in the conidial cell wall. Hopefully, one or more of the enzymes would break down any permeability barriers that might exist and be responsible for the initiation of germination.

In no case could any of the enzymes, with the exception of chitinase, exert a stimulatory effect on the germination event of *B. bassiana* (Table 7). Chitinase appeared to slightly stimulate the percentage of germinating conidia (from 50 to 75%). This effect was observed numerous times, but no actual synchronization was ever observed. Additionally these enzymes did not inhibit germination. When either glucuronidase or the lysing enzyme was added after germination had occurred (16 hr), extensive lysis of the nascent germ tubes was observed within 6–8 hr.

TABLE 7
VARIOUS TREATMENTS AND THEIR EFFECT ON THE GERMINATION OF *Beauveria bassiana*[a]

Treatment	Microscopic observation (16 hr)
Chitinase[b]	Some increase (50 to 75%)[c]
Proteinase	No effect
Lipase	No effect
β-Glucuronidase	No effect
Lysing enzyme	No effect
Alanine (to 1.0%)	No effect
Heat (65°C for 15, 30, 45, and 60 min)	No effect

[a] Conidia were permitted to germinate in the glucose–NH_4Cl–salts medium.

[b] All enzymes were present at a final concentration of 400 μg/ml and buffered in 0.1 M K_2HPO_4, pH 6.5.

[c] The effect observed was one of an increase in the numbers of germinating conidia, not the length of time required for germination.

DISCUSSION

Data presented show that a carbon and energy source is required for germination of conidia of *B. bassiana*. For good germination, a carbon–energy source is needed regardless of the medium from which conidia are obtained. The carbon source must be one that can be metabolized by the fungus, since compounds such as *O*-methylglucose, nucleic acid bases, and certain fatty acids cannot be utilized for the process. For continued growth and extension of the hyphae and to prevent autolysis, an exogenous nitrogen source is also required. Because germination and some growth of the emerging hypha can occur in the absence of an exogenous nitrogen source, it appears that conidia possess ample endogenous nitrogen reserves for synthesis of proteins required for the germination process.

Our data show that *B. bassiana* will germinate and grow luxuriantly using a wide variety of compounds that are relatively simple and plentiful in nature. These include glucose, *N*-acetylglucosamine, glucosamine, several amino acids, chitin, starch, and low levels of some fatty acids. In addition, several of our mutants will grow and sporulate using lanolin or even a crude hydrocarbon mixture as carbon–energy sources. No requirements for B vitamins or compounds that might be difficult to obtain in nature exist. This ability to use relatively large numbers and diverse types of compounds for germination and growth most likely enhances both survival of *B. bassiana* and its infectivity of insect larvae in nature.

Unless utilizable nutrients are incorporated into application formulas, field infections by conidia will involve germination

utilizing those nutrients present on the larval surface. Such compounds could be present as the result of natural secretions or could be picked up from the environment (field crop or diet formulations) in which the corn earworm is growing. To date, no information is available regarding kinds of nutrients present under any conditions. Studies are underway in this laboratory which have as their ultimate goal, the definition of such compounds.

Now that the variability of carbon, nitrogen, and energy sources which satisfy the germination and growth requirements of *B. bassiana* is known, it will permit us to better relate and understand which larval surface components can and will be utilized. Such information will be important in defining the molecular events relating to growth on the surface of larvae prior to hyphal penetration into the hemolymph. Such data may also help explain why some fungal mutants are better pathogens than others.

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APPENDIX B

TOXIC COMPONENTS ON THE LARVAL SURFACE OF THE CORN EARWORM (*HELIOTHIS ZEA*) AND THEIR EFFECTS ON GERMINATION AND GROWTH OF *BEAUVERIA BASSIANA*

JOURNAL OF INVERTEBRATE PATHOLOGY 39, 15–22 (1982)

Toxic Components on the Larval Surface of the Corn Earworm (*Heliothis zea*) and Their Effects on Germination and Growth of *Beauveria bassiana*

REBECCA J. SMITH AND E. A. GRULA

Department of Microbiology, Oklahoma State University, Stillwater, Oklahoma 74078

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Caprylic acid is present on the surface of corn earworm, *Heliothis zea*, and fall armyworm, *Spodoptera fragiterda*, larvae. Because caprylic acid inhibits germination of *Beauveria bassiana*, presence of this compound will be determined to the establishment of an infection of larvae by this fungus. Other free fatty acids present on the surface of the *H. zea* and *S. frugiterda* are tentatively identified as valeric and nonanoic acids; these also possess mycostatic activity toward *B. bassiana*. Depending on concentration, caprylic acid inhibits germination of conidia for different amounts of time (R. J. Smith and E. A. Grula, 1981, *J. Invertebr. Pathol.*, 37, 222–230). We now further report that inhibition and/or growth is also related to the source of carbon, nitrogen, and energy present in the growth medium. This observation of selective toxicity in the presence of different nutrients was also observed using nonanoic acid. Our data therefore make it necessary to interpret the effects of certain fatty acids on germination and growth of *B. bassiana* (and probably other fungi as well) in terms of nutrients for the germination process.

KEY WORDS: Fatty acids on corn earworm surface; mycostatic activity, *Beauveria bassiana;* caprylic acid; nonanoic acid; nutrition and fatty acid toxicity; inhibition of germination.

INTRODUCTION

Invasion of the corn earworm (*Heliothis zea*) by hyphae of the entomopathogenic fungus *Beauveria bassiana* occurs via enzymatic penetration of the larval integument (Grula et al., 1978; Pekrul and Grula, 1979; Smith et al., 1981). Prior to such penetration it is necessary for conidia of the fungus to germinate on the surface of larvae. Successful germination requires that utilizable nutrients be present and either that toxic components not be present in amounts sufficient to inhibit germination, or that the organism be able to ignore or overcome them. The nutritional requirements for germination and growth of *B. bassiana* have been defined (Smith and Grula, 1981).

Koidsumi (1957) reported that cuticular lipids of the silkworm (*Bombyx mori*) and the rice stem-borer (*Chilo simplex*) could be extracted with ether and such extracts were inhibitory to both the germination and growth of *Aspergillus flavus*. The most toxic components of these extracts were straight-chain saturated fatty acids, presumably caprylic or capric acid. Evalakova and Chekhourina (1962) extracted larvae of the sunn pest (*Eurygaster integriceps*) using ether and reported that such extracts inhibited the growth of *B. bassiana*. These findings strongly suggest that the cuticular surface of insect larvae will contain fatty acids which could be inhibitory to germination and growth of conidia and, therefore, eventual penetration of the integument by certain entomopathogenic fungi.

The objectives of the present study were to ascertain which fatty acids are present on the surface of corn earworm larvae, and to determine the effects of such compounds on germination and growth of *B. bassiana* conidia.

MATERIALS AND METHODS

Mutants of *B. bassiana* were obtained by ultraviolet irradiation of washed conidial suspensions, and selecting for varying levels of proteolytic (caseolytic) activity by plating on Sabouraud's dextrose agar

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(SDA) containing litmus milk (Grula et al., 1978). The target pest was *Heliothis zea* (corn earworm). First-instar larvae were placed in 1–oz containers of corn–soy–milk diet (Burton, 1970) and permitted to grow under constant light at 25°C until the desired instar was attained.

Lipid extractions were obtained from third and fourth-instar corn earworm larvae in the following manner: Each group of 50 larvae was placed in a beaker of distilled water and continuously agitated for 30 min (magnetic stirrer) to remove any adhering diet. Control groups of larvae were extracted without the water washing. Larvae were then placed in a beaker containing 20 ml), and methanolic–HCl (0.5 ml of 2.5% v/v, tent shaking for 30 min. The extract was filtered through Whatman No. 1 paper and concentrated to dryness using a rotary evaporator or under nitrogen gas. The residue was then methylated for gas chromatography by the addition of sodium-dried benzene (4 ml), 2,2-dimethoxypropane (0.4 ml), and methanolic–HCl (0.5 ml of 2.5% v/v, methanol/HCl). The solution was kept at room temperature for 18 hr after which time the resulting methyl esters were evaporated and stored under nitrogen at −20°C until time of analysis.

Gas chromatography was accomplished using a Perkin-Elmer Sigma II unit equipped with a hydrogen flame detector. The column was stainless steel (4 ft × ¼ in.) and packed with 3% Sylar on a Chromosorb support. Mass spectrometry was performed on a coupled GC–MS Hewlitt-Packard 5992-B unit. The same column was utilized for all analyses.

Medium for growth of *B. bassiana* consisted of a basal salts solution (NaCl, 0.3 g; $MgSO_4 \cdot 7\ H_2O$, 0.3 g; and K_2HPO_4, 0.3 g/liter distilled water) to which either N-acetylglucosamine (0.1%), glucose (0.1%), and ammonium chloride (0.1%), or an amino acid mixture (alanine, phenylalanine, and valine at 0.1% concentration of each) was added as the carbon-energy and nitrogen source (Smith and Grula, 1981). When a solid medium was desired, agar (1.5%) was added. Germination was scored as the visible protuberance of a germ tube from the spherical conidium. Crystal violet (30 sec) was used to stain the preparations.

Hydrophobic fatty acids were neutralized and made water soluble by addition of approximately 0.2 M Na_2HPO_4 prior to filtration (for sterilization) and addition to the growth media.

All chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri.

RESULTS

When hexane extracts of third and fourth-instar corn earworm larval surfaces were added to conidia of *B. bassiana* in the N-acetylglucosamine salts medium (NAG) inhibition of germination was observed. Normally, conidia will germinate approximately 16 hr after inoculation into this medium. The effect of these extracts was generally fungistatic, i.e., little or no conidial lysis was observed and, at times, growth of some conidia (about 15%) would occur after about 4 weeks incubation. Addition of these extracts to germinated conidia (16 hr) in NAG salts medium did not affect further growth. Thus the effect of components in the hexane extract appear to be directed to inhibition of germination.

Gas chromatographic analysis of these inhibitory extracts showed that several fatty acids were present in differing amounts depending on the batch of larvae extracted; however, one major peak was always present (Fig. 1). This peak exhibited a retention time corresponding to that of caprylic acid, an eight-carbon saturated fatty acid. Cochromatography of caprylic acid and an inhibitory larval surface extract showed that only one peak appeared at the retention time of caprylic acid (Fig. 2).

Mass spectral analysis of the extract major peak component gave a spectrum almost completely identical to the mass spectrum of the methyl ester of caprylic acid (Figs. 3, 4). Based on these data, we

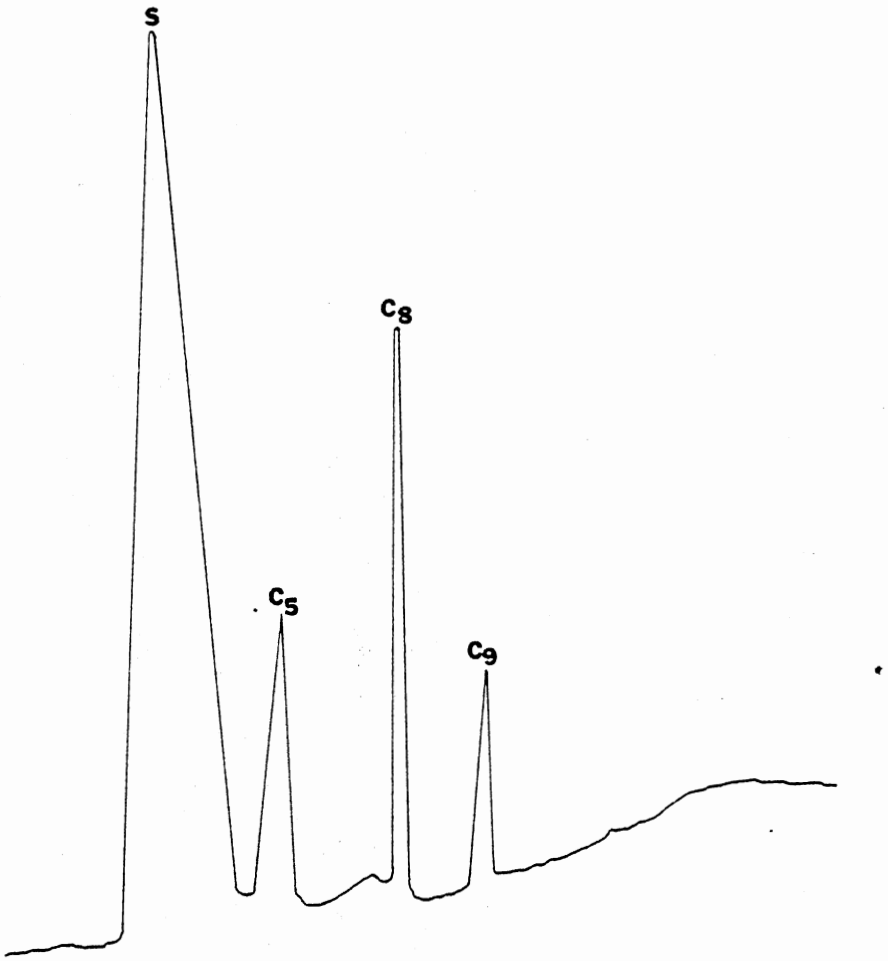

FIG. 1. GC tracing of a mycostatic hexane larval surface extraction of *H. zea*. S, solvent peak; C_5, valeric acid peak; C_8, caprylic acid peak; C_9, nonanoic acid peak.

are identifying the major component in the hexane larval surface extracts as caprylic acid.

To determine if caprylic acid is present on the surface of other larvae, the fall armyworm (*Spodoptera frugiterda*) and pecan weevil (*Curculio caryae*) were also extracted using the hexane procedure. Based on retention times, caprylic acid appears to be a major fatty acid that is also present on the surface of the fall armyworm (Fig. 5). Other fatty acids comparable to the ones found in corn earworm larval surface extracts are also present but in differing amounts. Interestingly, fatty acids could not be demonstrated in pecan weevil larval extracts, even when the number of larvae was increased to 100.

To investigate which short and long-chained fatty acids were toxic to conidial germination and hyphal growth of *B. bassiana*, several fatty acids differing by one or two carbons were added (0.02 and 0.04% final concentrations) to the NAG salts medium. Data given in Table 1 reveal that saturated fatty acids below 10 carbons are highly inhibitory to germination whereas the higher-chain fatty acids (capric, undecanoic, oleic, stearic) are not. When these fatty acids were added to previously germinated conidia (16 hr), no effect on hyphal growth was observed, thus

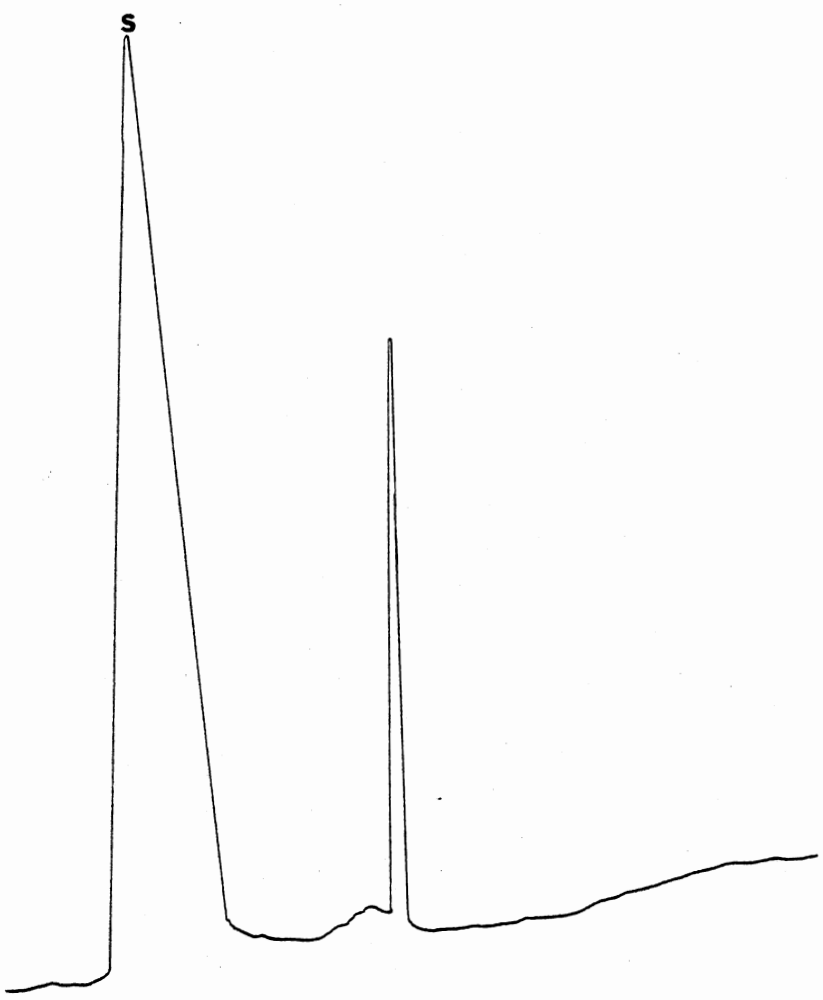

FIG. 2. GC tracing of a mycostatic hexane larval surface extract from *H. zea* cochromatographed with caprylic acid. Solvent peak is indicated by (S).

emphasizing that the effect of these fatty acids is basically on germination. Caprylic acid was found to inhibit condial germination at a minimum concentration of 0.02% in the liquid NAG salts medium for a 1-week incubation period.

The ability of caprylic acid to act as a fungistatic agent was investigated utilizing various nutritional conditions (Table 2) since no one has yet defined which nutrients are actually present on the surface of any insect larval form. This becomes an important point because such nutrients would be the only ones available for germination and growth of any fungal conidia present.

Surprisingly, the presence of different nutrients has a definite influence on the inhibitory effect of this fatty acid. When a complex medium such as Sabouraud's dextrose broth (SDB) was used, conidia were able to overcome (by 48 hr) the fungistatic effect of caprylic acid (0.04%) and grow well. In the amino acid medium, caprylic acid (0.04%) completely inhibited germination and growth of all *B. bassiana* mutants for the total observation period (3 weeks). Results were variable in the glucose–ammonium chloride minimal medium, i.e., the fungistatic effect of caprylic acid on germination and growth of some mutants occurred, but the growth that resulted was not

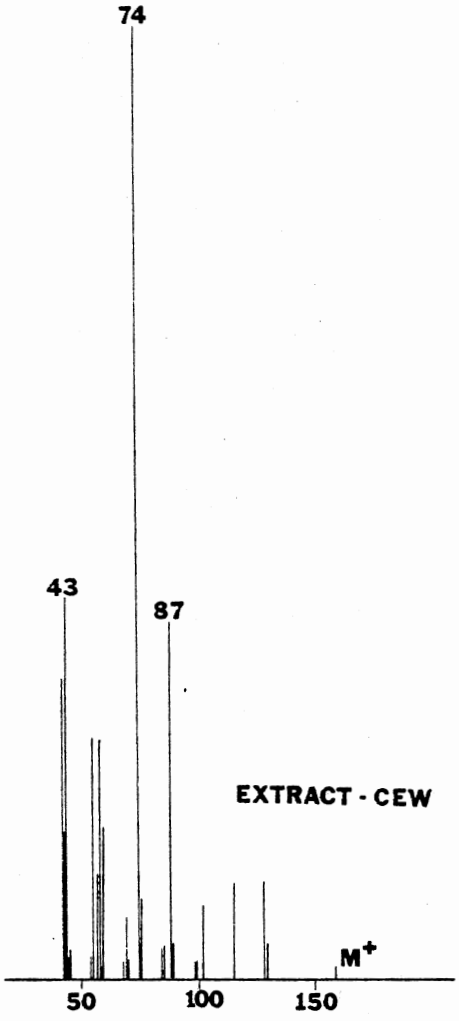

FIG. 3. Mass spectrum of a mycostatic hexane larval surface extraction of *H. zea*. M^+ = 158.1.

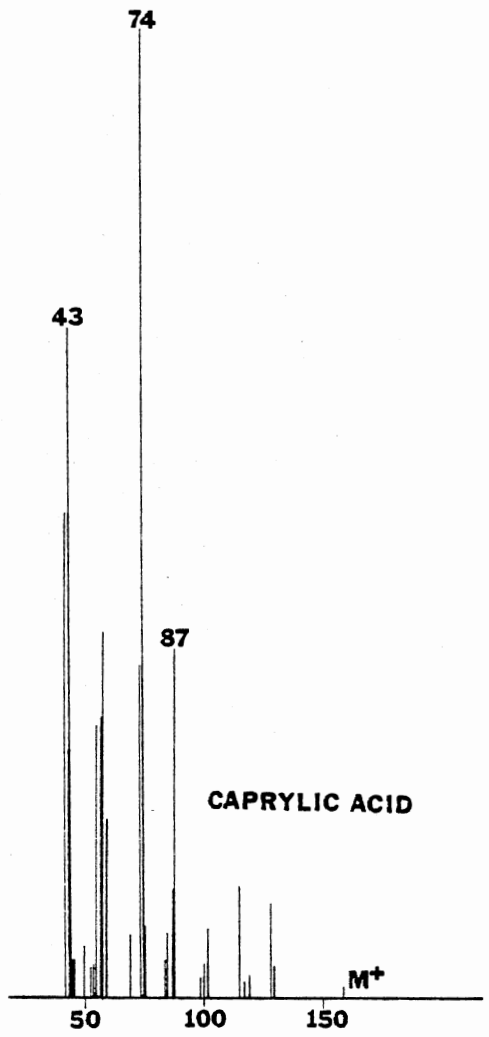

FIG. 4. Mass spectrum of caprylic acid methyl ester. M^+ = 158.1.

as extensive as when germination was permitted in SDB (Table 2).

Due to this variation in inhibitory response exhibited by conidia of some mutants of *B. bassiana* to caprylic acid in different media, one must question whether this variation is due to the presence of different nutrients or to a concentration effect. When the level of the three amino acids was increased four times (0.4% of each), caprylic acid (at 0.02 and 0.04%) still completely inhibited conidial germination for up to 3 weeks.

Nonanoic acid, which may also be present on the surface of corn earworm larvae (Fig. 1) was tested and also found to inhibit conidial germination of *B. bassiana*. This fatty acid was found to be considerably less toxic (Table 3) in the different media. Interestingly, several mutants of *B. bassiana* appeared to overcome the inhibitory effect of nonanoic acid more efficiently in the least complex media (amino acid and glucose–ammonium chloride media).

DISCUSSION

Free fatty acids having mycostatic activity are present on the surface of some species of insect larvae (*H. zea*, *S. frugiterda*). The major fatty acid found on the *H. zea* larval surface is caprylic acid.

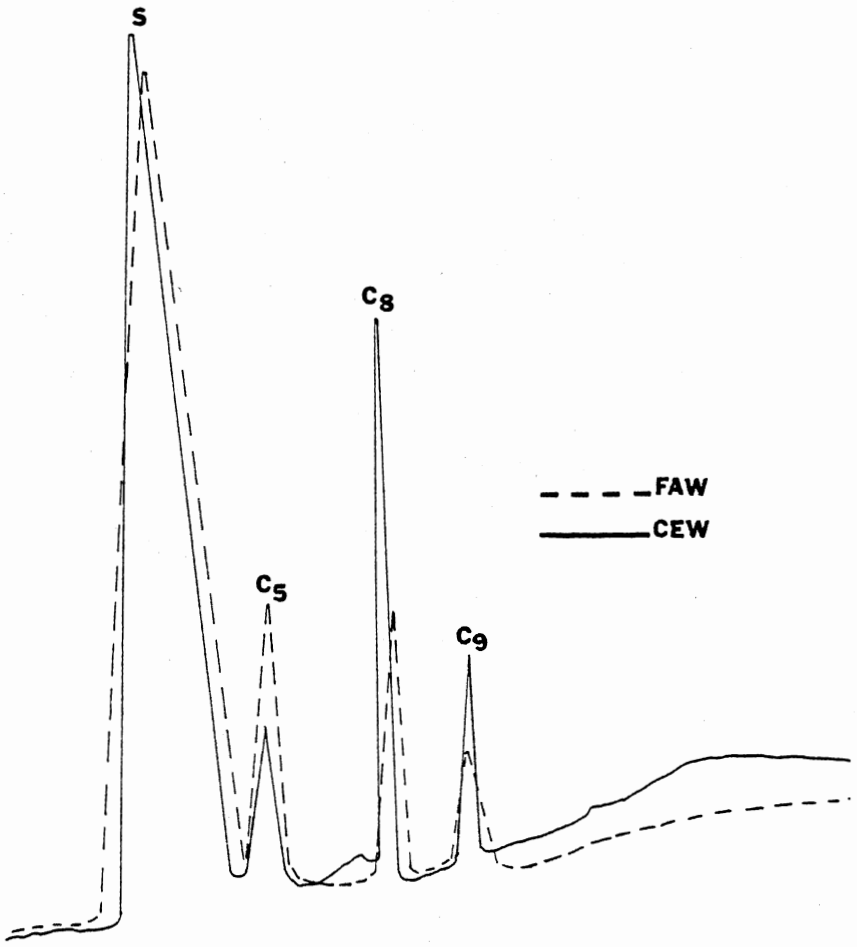

FIG. 5. GC tracing of a hexane extract of the larval surface of *H. zea* compared to the scan from *S. frugiterda*.

TABLE I
INHIBITON OF GERMINATION OF *Beauveria bassiana* BY VARIOUS FATTY ACIDS

Fatty acid[a]	Germination[b]
Butyric (C:4)	−
Valeric (C:5)	−
Caproic (C:6)	−
Heptanoic (C:7)	−
Caprylic (C:8)	−
Nonanoic (C:9)	−
Capric (C:10)	+
Undecanoic (C:11)	+
Stearic (C:18)	+
Oleic (C:18-1)	+

[a] Final concentration of fatty acid was 0.02% in basal salts medium with *N*-acetylglucosamine added at a concentration of 1.0%.

[b] (−) No germinating conidia were ever observed; (+) at least 50% of conidia had germinated (observed at 16 hr).

Identification is based on comigration in gas chromatography, near identical mass spectra fragmentation patterns, and similar mycostatic activity toward germination of conidia of *B. bassiana*. Other fatty acids present on the surface of the *H. zea* have been tentatively identified as valeric acid and nonanoic acid on the basis of retention times during gas chromatography.

Although different batches of *H. zea* larvae contain varying amounts of fatty acids, caprylic acid was always found in extracts, even when only one fatty acid could be demonstrated. Our data reinforce and extend the work of Koidsumi (1957) who reported the presence of free medium-chain saturated fatty acids on the cuticle of the

TABLE 2
GROWTH RESPONSE OF *Beauveria bassiana* TO CAPRYLIC ACID IN DIFFERENT MEDIA

	Medium utilized[a]					
	SDB		AA salts		Glu–NH_3 salts	
Mutant	Control	+C:8[b]	Control	+C:8[b]	Control	+C:8[b]
E_1	0.98	0.89	0.54	0	0.69	0.59
HP_1	0.93	0.95	0.66	0	0.74	0.03
8	0.88	0.81	0.56	0	0.72	0.60
R_1	1.10	0.99	0.68	0	0.78	0.71
14	0.98	0.94	0.47	0	0.62	0.01

[a] SDB, Sabouraud's dextrose broth; AA salts, amino acid medium; Glu–NH_3 salts, glucose–ammonium chloride medium. All compositions are given under Materials and Methods.

[b] Final concentration of caprylic acid was 0.04%. Optical densities were read at 540 nm after 72 hr growth at 25°C on a rotary shaker (180 rpm).

silkworm and which he presumed to be either caprylic or capric acid.

The surface of the *S. frugiterda* possesses similar fatty acids (Fig. 5); however, amounts of the individual fatty acids can differ. It was somewhat surprising to observe that no fatty acids could be extracted from the surface of pecan weevil larvae.

Based on the data presented, it appears logical to conclude that the surface of each species of insect larvae will differ both qualitatively and/or quantitatively as regards free fatty acids. In addition, although precise data are lacking at this time, it is entirely possible that the growth environment of a larva will influence both the number and kind of fatty acids present.

Caprylic acid inhibits germination of conidia of *B. bassiana* for various lengths of time, depending on concentration of the fatty acid and the type of carbon, nitrogen, and energy source present in the growth medium. This finding is somewhat disconcerting since it makes it difficult to predict the effect of caprylic acid on the germination of *B. bassiana* during invasion of *H. zea*. It now appears necessary to determine what nutrients are available on a larval surface that conidia can utilize for germination. Studies are being done in this laboratory to determine the identity of such nutrients on the surface of *H. zea* larvae. Preliminary results show that both amino acids and amino sugars are present in washings of larvae (Pekrul and Grula, unpubl.).

As stated, germination and growth of co-

TABLE 3
GROWTH RESPONSE OF *Beauveria bassiana* TO NONANOIC ACID IN DIFFERENT MEDIA

	Medium utilized[a]					
	SDB		AA salts		Glu–NH_3 salts	
Mutant	Control	+C:9[b]	Control	+C:9[b]	Control	+C:9[b]
E_1	0.98	0.05	0.54	0.43	0.69	0.66
HP_1	0.93	0	0.66	0.57	0.74	0.69
8	0.88	0.08	0.56	0.52	0.72	0.70
R_1	1.10	0.04	0.68	0.65	0.78	0.72
14	0.98	0	0.47	0.39	0.62	0.57

[a] SDB, Sabouraud's dextrose broth; AA salts, amino acid medium; Glu–NH_3 salts, glucose–ammonium chloride medium. All compositions are given under Materials and Methods.

[b] Final concentration of caprylic acid was 0.04%. Optical densities were read at 540 nm after 72 hr growth at 25°C on a rotary shaker (180 rpm).

nidia are absolutely required before an active fungal infection of a larval integument can occur. Once germination is underway, metabolic patterns and accompanying structural syntheses are shifted from a dormant state characteristic of conidia to those of growing hyphae. Because a significant amount of metabolic activity is occurring during germination (Smith and Grula, 1981), it is not surprising that a compound such as caprylic acid would have its activity directed toward this event in the cell cycle.

An interesting study by Teh (1974) showed that short-chain fatty acids (C:5–C:11) inhibit selective transport in *Cladosporium resinae*. He also reported the loss of K^+ and proteins by these cells in the presence of toxic fatty acids, indicating that some type of direct action on the cell membrane was occurring. Cell membrane perturbation by caprylic and nonanoic acids may be occurring; however, such a mechanism cannot completely account for the differences in response exhibited by *B. bassiana* particularly when the organism is growing in the presence of different carbon, nitrogen, and energy sources. Some additional selective type of inhibition involving an intracellular metabolic event is indicated.

Gershon et al. (1973) report that toxicity of fatty acids to fungi is affected by several factors: (1) chain length of the fatty acids, (2) *p*H of the medium, and (3) the presence in test media of adsorbents such as serum albumen. No explanation is given as to why such parameters exist or their possible effects. Since selective toxicity by fatty acids occurs in media containing different sources of carbon, nitrogen, and energy, it should now be possible to design experiments to determine more precisely the basis of fatty acid toxicity in *B. bassiana*.

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APPENDIX C

REQUIREMENT FOR SEQUENTIAL ENZYMATIC ACTIVITIES FOR PENETRATION OF THE INTEGUMENT OF THE CORN EARWORM (*HELIOTHIS ZEA*)

JOURNAL OF INVERTEBRATE PATHOLOGY 38, 335–344 (1981)

Requirement for Sequential Enzymatic Activities for Penetration of the Integument of the Corn Earworm *(Heliothis zea)*

REBECCA J. SMITH, SUE PEKRUL, AND E. A. GRULA

Department of Microbiology, Oklahoma State University, Stillwater, Oklahoma 74078

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When larvae of the corn earworm, *Heliothis zea*, are boiled in 1% sodium dodecyl sulfate for 2 to 4 hr, larval contents are solubilized leaving a thin and transparent but intact skeletal structure that we are naming ghosts. The thinnest and most transparent ghosts are produced by boiling first-instar larvae. As larvae mature, later instar forms possess substantial quantities of melanized areas that remain largely undissolved in the detergent. Chemical analyses reveal that ghosts consist largely, and perhaps exclusively, of chitin and protein. Scanning electron microscopy shows that the chitin–protein complex is a continuous layer that does not possess holes sufficiently large for fungal hyphae to pass through without some type of accompanying enzymatic activity. Digestion of ghosts is possible using the combination of a proteolytic enzyme followed by chitinase. Because this sequence is necessary for dissolution of the structure, the protein material is most likely structural and wrapped about the chitin. Such an arrangement would allow protection of the structure from direct chitinase attack. The number of proteins involved is not known; however, because they survive the detergent–heat treatment this strongly suggests that the protein(s) is covalently bonded to chitin. Several different proteolytic enzymes allow attack of the underlying chitin by chitinase. This indicates that the accompanying protein(s) is exposed and therefore accessible to several types of proteolytic enzymes. Such a conclusion may not be warranted, however, if the accessibility and conformational state of the protein(s), and therefore digestibility, is affected by the relatively vigorous preparative procedure.

KEY WORDS: Sequential enzymatic activity; corn earworm integument; *Beauveria bassiana*; chitin–protein complexes; detergent-produced ghosts; protected chitin.

INTRODUCTION

Work in our laboratory is centered around the elucidation of the molecular events that occur when *Beauveria bassiana* infects the corn earworm, *Heliothis zea*. Grula et al. (1978) and Pekrul and Grula (1979) have shown that conidia of *B. bassiana* germinate on the larval surface of the corn earworm and the nascent germ tubes subsequently penetrate the integument via enzymatic activity.

Analyses of insect cuticles have progressed rapidly in the past 10 years. Most investigators now conclude that the two major constituents of insect cuticles are proteins and chitin (Rudall, 1976). Generally the procuticle is a glycoprotein and varies with the species of insect (Hackman, 1976).

Several investigators have established that *B. bassiana* produces several types of exocellular enzymes, including chitinases, proteases, and lipases (Huber, 1958; Leopold and Samsinakova, 1970; Grula et al., 1978; Pekrul and Grula, 1979). In study by Samsinakova et al. (1971), it is suggested that exocellular enzymes of *B. bassiana* are required and always function as a unit for penetration through the insect integument; however, they state that mechanical action must not be disregarded as a mechanism of infection.

A major objective of our continuing

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study is to determine the type(s) of enzymatic activity that permits *B. bassiana* to infect corn earworm larvae and, also, if a sequence of several different enzymes is involved. Our research has been facilitated by the finding that "ghosts"[1] (chitin–protein complexes) of larval integuments are readily produced by treatment with an anionic detergent.

MATERIALS AND METHODS

Mutants of *B. bassiana* were obtained by ultraviolet irradiation of conidia (Grula et al., 1978). Stock cultures were kept on Sabouraud's dextrose agar (SDA) slants at 25°C and transferred approximately every 3 weeks to maintain maximum viability. Conidial suspensions were obtained by suspending conidia in 0.03% Triton X-100, followed by two washes in sterile distilled water. Larvae of the corn earworm were allowed to grow in 1-oz clear plastic containers on the corn–soy–milk diet developed by Burton (1970). Containers were kept under constant light at 25°C and incubated until the desired stage of development was reached.

Ghost integuments were prepared by boiling (2–4 hr) corn earworm (CEW) larvae (first through fifth instar) in 1% sodium dodecyl sulfate (SDS). These ghosts were then suspended in five changes of sterile distilled water (1 hr per exposure) to remove residual detergent. They were hydrolyzed (6 N HCl for 24 hr or 4 N HCl for 4 hr) for analysis of specific compounds, or prepared for scanning electron microscopy after various enzymatic and chemical treatments.

Two-dimensional paper chromatography was performed on Whatman No. 1 paper using the solvent systems of Heathcote and Jones (1965). Chromatograms were sprayed with ninhydrin reagent to observe amino acids and other ninhydrin-positive compounds such as glucosamine.

Free amino acids in hydrolyzed ghosts were also identified and quantitated using an amino acid analyzer. The analyses were performed at 60°C on a Durum DC6A column using citrate buffer (Liao et al., 1973).

Thin-layer chromatography was performed using glass plates, 20 × 20 cm^2, layered with Silica Gel H. Plates were activated at 100°C for 1 hr immediately before use. These plates were developed in a solvent system consisting of acetone, *n*-butanol, and distilled water (50–40–10, v/v/v), and sprayed either for reducing sugars (Bailey and Bourne, 1959), or amino sugars (Partridge and Westall, 1948).

All samples for scanning electron microscopy were fixed for 3 hr in 4% glutaraldehyde in 0.2M cacodylate buffer at *p*H 7.3. The samples were dehydrated in an ethanol series and critical point dried in CO_2. After mounting on aluminum stubs with double adhesive tape or silver conducting paint, a Hummer evaporator was used to coat the samples with approximately 400 nm gold–palladium. Observations and photographs were made using a JOEL 35 JSM scanning electron microscope and Polaroid film.

Enzymes used in this study were obtained from Sigma, St. Louis, Missouri, and are as follows: lipases (Type VII from *Candida cylindracea*, and Type II from hog pancreas), proteinase (Type IV from *Streptomyces caespitosus*), lysing enzymes (Type III from *Cytophaga dissolvens*), trypsin, elastase (Type I

[1] Portions of this work were reported at the XIII Annual Meeting of the Society for Invertebrate Pathology held in Seattle, Washington, July 27–August 1, 1980 by E. A. Grula. At that time, Dr. Thomas A. Angus, Department of Forestry, Sault Ste. Marie, Ontario, Canada, suggested the name "ghosts" for what we were calling "girdles." Out of respect for Dr. Angus and in appreciation of his many efforts in the area of entomopathology, we are naming the detergent-produced chitin–protein complexes ghosts.

from hog pancreas), chymotrypsin (Type II from bovine pancreas), cathepsin C (Type X from bovine spleen), and pepsin (hog stomach mucosa). All enzymes with the exceptions of trypsin and elastase were buffered in 0.1 M potassium phosphate, *p*H 6.8. Trypsin and elastase were buffered in 0.1 M sodium carbonate, *p*H 8.3. All enzymes were present at the level of 400 μg/ml and incubations were at 25°C for 2 hr except for chitinase which was permitted to react for 48 hr.

RESULTS

In an attempt to repeat the work of Samsinakova et al. (1971), water-washed integuments were prepared by skinning fifth-instar corn earworm larvae and spreading these integuments on glass slides, dorsal side up. The integuments were streaked with a loopful of a conidial suspension of our various *B. bassiana* mutants and incubated (25°C) in a moisture chamber. It was not possible to maintain complete sterility under such conditions, and to no surprise, we continually observed *Aspergillus* and *Penicillium* spp. growing on these integuments. These fungi would even overgrow inoculated *B. bassiana*, but growth of *B. bassiana* could be observed before the *Aspergillus* and *Penicillium* spp. covered the integument. These contaminating fungi have been tested for ability to infect the corn earworm in numerous ways (Grula et al., 1978) and even when conidia were injected directly into the hemocoel, no deaths occurred. Pekrul and Grula (1979) reported that conidia of these *Aspergillus* and *Penicillium* isolates will not germinate on the surface of a live CEW larva. Thus we observed that water-washed integuments prepared as recommended by Samsinakova et al. (1971) are readily attacked by noninfectious fungal contaminants which cannot, however, infect the live larval form.

After the obvious difficulties using water-washed integuments, various detergent treatments were utilized to determine their effects on larval integuments. The detergent method was chosen since Grula et al. (1965) used 1% SDS to isolate a pure cell wall mucopeptide layer from the Gram-negative bacterium *Erwinia carotovora*. Also, Braun (1969) boiled cell walls of *E. coli* in 4% SDS to obtain a mucopeptide containing only covalently linked lipoprotein. We observed that boiling of whole larvae for 2 hr in 1% SDS produces an almost transparent ghost form of the larva. More transparent ghosts are produced from first- as opposed to fifth-instar larvae; also, we have boiled for up to 4 hr and still obtain intact ghost forms.

It is apparent (Figs. A and B) that the SDS treatment of larvae does not degrade the integument completely since the larval ghost is still an intact unit. Chemical analyses of ghosts reveal that it is composed of proteins and chitin. No reducing sugars or glucans can be demonstrated by thin-layer chromatography even when the entire contents of three larval ghosts were chromatographed on one plate; only glucosamine can be observed after hydrolysis. Amino acid analysis of ghost hydrolysates reveal that most protein amino acids are present. Amino acids present are similar to those found in crab shell chitin (Table 1). Because much protein material (numbers of proteins present not known) is not removed during the extensive boiling in detergent, covalent bonding of protein to chitin is strongly suggested.

Ghosts were first utilized to discern whether holes were present in the larval cuticle that were large enough for a germinating hyphal tip of *B. bassiana* to directly pass through. Such holes would eliminate the need for enzymes to degrade the protein–chitin procuticle. No holes were ever observed in any of the SDS-treated larvae that a growing germ tube could penetrate (Fig. C). Such holes

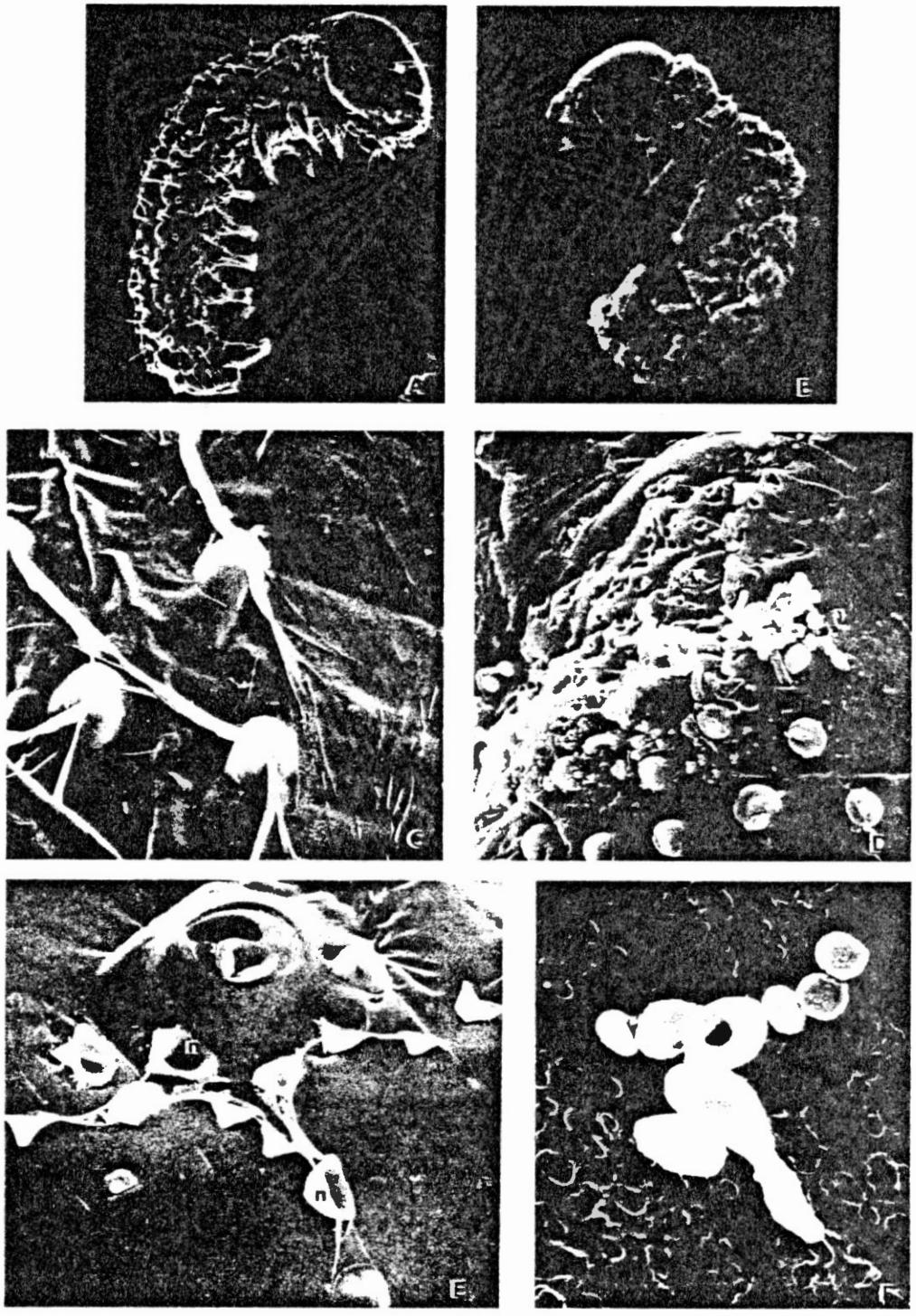
A
B
C
D
E
F

TABLE 1
COMPARISON ON AMINO ACID COMPOSITION OF CORN EARWORM GHOST HYDROLYSATES[a] AND COMMERCIAL CHITIN[b]

Amino acid	Percentage of total	
	Ghosts	Commercial chitin
Glycine	13	12
Alanine	11	12
Glutamic acid	10	13
Aspartic acid	9	12
Serine	9	6
Proline	9	8
Leucine	6	4
Valine	6	8
Lysine	5	2
Threonine	5	5
Tyrosine	4	2
Arginine	4	6
Isoleucine	3	3
Histidine	3	5
Phenylalanine	3	5
Methionine	0.3	1

[a] Second-instar corn earworm larvae were hydrolyzed after SDS treatment.

[b] Commercial chitin was obtained from Sigma Chemical Company.

can only be observed after an infection with *B. bassiana* has occurred (Fig. D). These observations support the idea that enzymatic degradation of the cuticle by *B. bassiana* is necessary for an active infection to occur.

Samsinakova et al. (1971) reported that with the addition of certain enzyme combinations, they were able to obtain complete disintegration of water-washed integuments of the greater wax moth, *Galleria mellonella*. We were unable to obtain any degradation using water-washed corn earworm integuments (Tables 2, 3, 4). Various sequences of lipase, proteinase, and chitinase were added to such integuments under toluene (48 hr) to prevent bacterial contamination which, otherwise, readily occurs and usually results in disintegration of the integuments. No visible degradation was ever observed in the presence of toluene.

Enzymes were added to the larval ghosts in various sequences and observations were recorded both macroscopically and by scanning electron microscopy (Tables 3, 4). To obtain complete disintegration of ghosts, a sequence of only two enzymes is needed. The first must be a proteinase and this can then be followed by chitinase (Tables 3, 4). Although lysing enzyme is generally used to degrade β-1,3-glucans, testing shows that significant proteolytic activity is also present in our preparations. SEM observations of the fragments left by these sequences show that there is considerable damage to the outer layers of the integument, revealing smooth protein–chitin layers underneath. It is very interesting that treatment with lysing enzyme followed by chitinase removes the nodules of the cuticle (Fig. E).

Ghosts were treated with various

FIG. A. First-instar corn earworm larva. 59×.

FIG. B. First-instar corn earworm larva treated with SDS for 2 hr. 61×.

FIG. C. Cuticular surface of a second-instar corn earworm larva treated with SDS for 2 hr. Note the lack of any holes large enough to allow direct penetration by fungal hyphae, and the detergent caused degradation at the base of nodules. 1700×.

FIG. D. Cuticle of a corn earworm larva infected with *B. bassiana*. Holes on the surface are due to cuticle-degrading enzymes produced by fungal hyphae that were knocked off by agitation during preparation of this specimen. Note the hole being produced by a penetrating germ tube (arrow). 1105×.

FIG. E. Surface of a fragment from a second-instar corn earworm larva treated with SDS for 2 hr followed by lysing enzyme and chitinase. The nodules (n) have been removed along with a layer of cuticle (arrow) that holds them to the surface. One of the removed nodules is inverted and is trapped in a spiracle opening. Note smoothness of the surface. 935×.

FIG. F. *B. bassiana* conidia and germinating conidia treated with SDS for 2 hr. The walls of both conidia and hyphae appear intact even at the growing tip although collapsing has occurred. 4080×.

TABLE 2
EFFECTS OF ENZYMATIC TREATMENTS ON WATER-WASHED AND SDS-TREATED LARVAE

Enzymes	Water washed		SDS treated	
	Macroscopic	SEM	Macroscopic	SEM
None	Intact	No degradation	Intact	Outer layer pitted; cracks with inner layers exposed
Trypsin	Intact	Extensive wrinkling; collapsed nodules[b]	Intact	Small cracks at base of nodules
Proteinase	Intact	Extensive wrinkling; collapsed nodules[b]	Intact	Holes in depressions[c]
Lysing enzyme	Intact	Extensive wrinkling; collapsed nodules[b]	Intact	Holes in depressions[c]
Chitinase	Intact	Extensive wrinkling; base of nodules is shrunken	Intact	Small holes over entire surface
Lipase 1[a]	Intact	Large holes in cuticle; top layers removed	Intact	No layering effect;[d] nodules shrunken at base
Lipase 2	Intact	Variety of nodular effects; severe wrinkling	Intact	Variety of nodular effects; pitted areas

[a] Lipase 1 is Type VII from *Candida cylindracea;* Lipase 2 is Type II from hog pancreas.
[b] Collapsed nodules are defined as nodules with shrunken bases.
[c] Holes in depressions are defined as cracks in large wrinkles and intersegmental regions.
[d] Layering effect is defined as degradation and peeling of outer layer with other layers exposed.

chemicals to aid in determining the nature of the layers that were being removed by the SDS preparative treatment. They were dipped in lanolin and subjected to the sequential enzymatic treatments of proteinase–chitinase and lysing enzyme–chitinase. No disintegration such as that seen without the lanolin pretreatment was ever observed (Table 5). It appears that coating the ghosts with a wax such as lanolin can protect them from enzymatic attack. When a crude oil preparation was used to coat the ghosts, followed by treatment with the same enzymes, complete disintegration occurred. Such experiments suggest, but do not prove, that waxes are most likely the components on the larval cuticle that protect it from attack by proteinases and chitinases. If such experiments have validity, they also suggest that surface components of the ghosts are sufficiently hydrophobic to associate with waxy materials. This statement is based on the assumption that significant or complete removal of detergent has occurred during the washing procedure.

Because of frequent reports utilizing alkali to deproteinize complex substrates including chitin (Datema, 1977; Muzzarelli, 1976), ghosts were treated with KOH, NaOH, and ethylenediaminetetracetic acid (EDTA) (Table 6). No macroscopic disintegration was observed; however, KOH treatment results in removal of some of the residual pigmentation in later instar ghosts making them even more transparent. In addition, such mild treatment with KOH results in removal of much of the protein (about 90%) associated with ghosts.

To establish whether *B. bassiana* could utilize the detergent-produced ghosts for germination and growth, a powdered integument medium consisting of a basal salts solution, and powdered, dried (105°C; 24 hr) ghosts (1%) was made (Smith and Grula, 1981). *B. bassiana* grows very well utilizing such ghosts as

TABLE 3
MACROSCOPIC OBSERVATIONS OF SEQUENTIAL ENZYMATIC TREATMENTS ON WATER-WASHED AND SDS-TREATED CORN EARWORM LARVAE

Enzymes[a]	Observation	
	Water washed	SDS treated
Chi–Try	Intact	Intact
Chi–Pro	Intact	Intact
Chi–Lys	Intact	Intact
Try–Chi	Intact	Fragmented
Pro–Chi	Intact	Fragmented
Lys–Chi	Intact	Fragmented
Cat–Chi	Intact	Fragmented
Chy–Chi	Intact	Fragmented
Pep–Chi	Intact	Fragmented
Ela–Chi	Intact	Fragmented
Lip–Pro–Chi	Intact	Fragmented
Lip–Lys–Chi	Intact	Fragmented

[a] Chi, chitinase; Pro, proteinase; Try, trypsin; Lys, lysing enzyme; Cat, cathepsin; Chy, chymotrypsin; Pep, pepsin; Ela, elastase; Lip, lipase (Type VII from *Candida cylindracea*).

the sole source of carbon, nitrogen, and energy.

To investigate the effect of SDS treatment on conidia and hyphae of *B. bassiana*, conidia (dormant and germinated for 16 hr) were also boiled in SDS (1%) for 2 hr and observed using scanning electron microscopy (Fig. F). Two important findings from this portion of the study are that a detergent-resistant basement structure (chitin–protein complex?) is present in both conidial and hyphal walls and the tip of growing hyphae is not visibly "open-ended." Thus if exocellular enzymes are produced only at the tip during growth of hyphae, their secretion must involve passage through this structure.

DISCUSSION

Data presented emphasize the complexity of the corn earworm integument and reinforce the postulated theory that more than one enzyme is required to penetrate the cuticle and that they act in a sequential manner.

Samsinakova et al. (1971) were able to completely disintegrate water-washed integuments of the greater wax moth by the addition of certain enzymatic com-

TABLE 4
SEM OBSERVATIONS OF SEQUENTIAL ENZYMATIC TREATMENTS ON WATER-WASHED AND SDS-TREATED CORN EARWORM LARVAE

Enzymes[a]	Observations	
	Water washed	SDS treated
Chi–Try	Extensive wrinkling; collapsed nodules	Small holes, bases of nodules cracked
Chi–Pro	Extensive wrinkling; collapsed nodules	Small holes, layers peeled back
Chi–Lys	Extensive wrinkling; collapsed nodules	Small holes, large cracks and outer layer stretched
Try–Chi	Extensive wrinkling; collapsed nodules	Large areas of outer layer and a few nodules removed
Pro–Chi	Extensive wrinkling; collapsed nodules	Outer layers degraded, nodules removed; smooth
Lys–Chi	Extensive wrinkling; collapsed nodules	Outer layer with nodules peeled back; smooth
Ela–Chi	Extensive wrinkling; collapsed nodules	Outer layer and a few nodules peeled back
Lip–Pro–Chi	Extensive wrinkling; collapsed nodules	Outer layer and nodules peeled back
Lip–Lys–Chi	Extensive wrinkling; collapsed nodules	Some layered effect

[a] Chi, chitinase; Pro, proteinase; Try, trypsin; Lys, lysing enzyme; Ela, elastase; Lip, (Type VII from *Candida cylindracea*).

TABLE 5
EFFECTS OF SEQUENTIAL ENZYMES ON LANOLIN- AND CRUDE OIL-COATED CORN EARWORM GHOSTS

Enzymes[a]	Macroscopic Observations	
	Lanolin coated	Crude oil coated
Lys–Chi	Intact	Fragmented
Pro–Chi	Intact	Fragmented
Lip–Pro–Chi	Fragmented	Fragmented
Lip–Lys–Chi	Fragmented	Fragmented

[a] Lys, lysing enzyme: Chi, chitinase; Pro, proteinase; Lip, lipase 1.

TABLE 6
EFFECT OF CHEMICAL TREATMENTS ON CORN EARWORM GHOSTS

Treatment[a]	Macroscopic	SEM observations
EDTA	Intact	Areas of layers peeled; variety of nodular degradation
NaOH	Intact	Shrinkage at base of nodules
KOH	Intact; more transparent	Layered effect; much flaking

[a] EDTA treatment was 0.01 M, *p*H 10, at 25°C for 18 hr; NaOH treatment was 0.1 N, at 25°C for 18 hr; and KOH treatment was 0.1 M, at 37°C, for 18 hr.

binations, as well as culture filtrates of *B. bassiana*. We were unable to see visible degradation of water-washed integuments of the corn earworm with any type of sequential enzymatic treatments used in this study. Although Samsinakova et al. (1971) gave the concentration of enzymes used and the sequences of addition, buffers used and *p*H of their system were not reported, nor were any data given to indicate that precautions were taken to prevent bacterial contamination. We found abundant contamination with bacteria when no toluene overlay was used during the extended incubations. Since their samples were left in the open air for 36 hr, it is possible that the degradation observed occurred as a result of bacterial and/or fungal activity. One must also question whether the ability of fungi to degrade larval integuments prepared by water washing should be correlated to infectivity since we have observed that nonentomopathogenic fungi (*Aspergillus* and *Penicillium* spp.) grow luxuriantly and digest such isolated integuments.

Chemical analyses of larval ghosts prepared by boiling in SDS reveal the presence only of protein and chitin. The SDS treatment apparently removes all lipids, glucans, and waxes, and probably some protein not associated through covalent bonds. Scanning electron microscope observations demonstrate there are continuous protein–chitin layers, indicating that this complex acts like a "girdle" even in the intersegmental regions.

As shown, a combination of two enzymatic activities will completely degrade ghosts. One of the enzymes that functions very well is "lysing" enzyme. This enzyme is normally utilized for its high glucanase activity. Because glucan could not be detected in ghosts and since lysing enzyme serves as an excellent first treatment, we assayed for glucanolytic (Miller, 1959), proteolytic (Rindernecht et al., 1968), and chitinolytic activity (Jeuniaux, 1966). Our preparations were found to have a high glucanolytic and proteolytic activity (10 μg of lysing enzyme is equivalent to 1

μg of trypsin activity, Russell and Grula, unpubl.), but very little chitinolytic activity (commercial chitinase has about 17 times greater activity). It thus appears that the proteolytic activity present in this enzyme preparation is involved in the degradation of ghosts. All known proteolytic enzymes tested caused complete disintegration of ghosts when used as a pretreatment with chitinase, providing further evidence that the proteolytic activity in the lysing enzyme is causing the damage.

Based on data presented in Table 5, it appears logical to assume that a "waxase" and/or lipase may also be needed for penetration of the larval integument by *B. bassiana*. We have reported (Smith and Grula, 1981) that several mutants of *B. bassiana* grow adequately when lanolin is the sole source of carbon and energy, thus indicating that a type of waxase can be produced by several mutant strains of this fungus. However, because the actual composition of the outer layers of the corn earworm integument have not been precisely identified, definite conclusions should not yet be made.

An unexpected finding in this study relates to the surface nodules of CEW larvae. Boiling in SDS followed by pretreatment with certain proteolytic enzymes and chitinase, results in their removal and partial degradation. The extent to which they are removed varies with these treatments. Lysing enzyme–chitinase and proteinase–chitinase remove more nodules and leave the surface entirely smooth (Fig. E), whereas the lysing enzyme alone or trypsin–chitinase removes only a few nodules. Since SDS boiling degrades the base of the nodules somewhat (Fig. C), it appears logical to assume that the base structure responsible for holding these nodules to the ghosts consists of a protein, probably bonded covalently to chitin. The nodular base apparently has been weakened and uncovered by the SDS treatment to the extent that the proteolytic enzymes can cleave the remaining bonds and remove the nodule. An additional reason for suspecting that protein is the nodule-cementing material is the finding that ghosts are composed only of protein and chitin. Also alkali treatment will not remove the nodules, eliminating glucan as the cementing material since Sietsma and Wessels (1977) utilized alkali treatment to remove glucans present in the cell wall of *Schizophyllum commune*.

In summary, this study establishes that an intact basement structure consisting of a protein–chitin complex (ghosts) surrounds insect larvae and that an enzymatic sequence consisting of a proteinase followed by chitinase is needed to dissolve the structure. Hence hyphae of *B. bassiana* must produce at least these two types of enzyme activities in order to penetrate and infect the corn earworm. It therefore becomes readily apparent that mutant forms of *B. bassiana* that cannot produce any exocellular proteolytic or chitinolytic enzyme activities will be unable to penetrate the insect cuticle. Thus such enzyme activities become important components in the series of events necessary to the infective process of insect larvae by fungi.

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VITA

Rebecca June Smith

Candidate for the Degree of

Doctor of Philosophy

Thesis: GROWTH AND SELECTED PARAMETERS RELATING TO ENZYMATIC PENETRATION OF THE INTEGUMENT OF THE CORN EARWORM (HELIOTHIS ZEA) BY BEAUVERIA BASSIANA

Major Field: Microbiology

Biographical:

Personal Data: Born in Muskogee, Oklahoma, May 23, 1955, the daughter of Mr. and Mrs. William C. Smith.

Education: Graduated from Checotah High School, Checotah, Oklahoma, in 1973; received the Bachelor of Science degree from Oklahoma State University, Stillwater, Oklahoma, in 1976; received the Master of Science degree at Oklahoma State University in 1979; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in July, 1982.

Professional Experience: Graduate teaching assistant, Microbiology Department, Oklahoma State University, 1977; research assistant, Microbiology Department, Oklahoma State University, 1977-1980; instructor, Microbiology Department, Oklahoma State University, 1981; Visiting Assistant Professor, Microbiology Department, Oklahoma State University, 1982.

Professional Affiliations: Member of the American Society for Microbiology; member of Sigma Xi.