SURFACE COMPONENTS OF *BEAUVERIA BASSIANA* AND *HELIOTHIS ZEA* INVOLVED IN THE INFECTION PROCESS

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PREFACE

The fungus Beauveria bassiana has been recognized as an entomopathogen since 1835 when Agostino Bassi showed it to be the organism responsible for white muscardine of the silkworm (Pramer, 1965). Since then, it has been found to be a widely distributed, nonspecific entomopathogen (Barson, 1977; and Macleod, 1954).

Conidia of B. bassiana are able to germinate on the surface of the corn earworm (CEW), Heliothis zea (Boddie), and infection is a result of direct penetration of germ tubes of hyphae through the cuticle (Pekrul and Grula, 1979). It is interesting that the cuticle is penetrated by the fungus since it projects such a formidable barrier, consisting mainly of chitin and protein. The fungus is able to penetrate the cuticle with the help of enzymes (Pekrul and Grula, 1979). In fact, a sequence of enzyme activities is necessary for cuticle degradation (Smith et al., 1981).

Once the fungus enters the epithelium and haemocoel, the larva is helpless in overcoming it. Death is thought to be a consequence of starvation, since the larvae fail to continue feeding and pathological changes are observed in gut tissue (Cheung and Grula, 1982). A toxin could be involved, but no evidence for this has been obtained

(Champlin and Grula, 1979).

The primary target organism in this study, the corn earworm is the most important pest of corn and the second most destructive insect in the United States (Swan and Papp, 1972). Also known as the cotton bollworm and the tomato fruitworm, this insect can be found worldwide feeding on a variety of vegetables and flowers.

This study is divided into two parts. The first part deals with compounds found on the CEW surface which can be utilized by B. bassiana for germination and growth. The second part is an attempt to show the presence of a molecular receptor system on the surfaces of B. bassiana and H. zea which facilitate adhesion of conidia to larvae and penetration of the cuticle by the emerging germ tubes. From the data presented in these two areas, the initiation of the infection process may be better understood.

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PART I

UTILIZABLE SURFACE NUTRIENTS ON *HELIOTHIS ZEA* AVAILABLE FOR GROWTH OF *BEAUVERIA BASSIANA*

INTRODUCTION

Utilizing scanning electron microscopy, it was previously shown by Pekrul and Grula (1979) that washed conidia of Beauveria bassiana germinate and grow extensively over the surface of the corn earworm (Heliothis zea). This suggests, but does not prove, that the nutrients required for growth of the fungus are secreted and normally available on the larval surface. The possibility of a misinterpretation exists since all infected larvae in the experiment were grown on a complex organic diet where transfer of nutrients to the corn earworm surface could readily occur.

The growth requirements of B. bassiana are not complex; indeed, carbon-energy source compounds having great chemical variability (from glucose to a complex wax) will allow extensive growth of the fungus (Smith and Grula, 1981).

The purpose of this study is to detail the procedures used to determine which nutrients are present on the corn earworm surface. Qualitative and quantitative data are presented to show that the corn earworm surface does indeed secrete chemicals and these can be used for germination and growth of B. bassiana. In addition, it is shown that the chemicals present on the larval surface can differ depending

on the larval instar and the environment in which a larva is present.

MATERIALS AND METHODS

Surface Extractions

Heliothis zea, corn earworm, larvae were allowed to grow on CSM diet until they were ready to molt into the fourth or fifth instar (Burton, 1970). Before the visible molting process began, each larva was placed in an individual acid-cleaned test tube. At various times after molting, tubes were immersed in an ice bath for at least 20 min before individual extracts were made by suspending each larva in deionized water for 2-4 min at 4°C. By slowing larval reflexes with chilling, spit and fecal contamination of extracts was largely decreased or perhaps eliminated. Also, by using only larvae that had molted in clean glass tubes, carryover of contaminating diet nutrients that might have adhere to their surface was negated.

Instead of immobilization by chilling, some larvae were frozen, etherized, or chloroformed before extraction. Larvae taken from ears of corn were extracted individually after chilling, but were not allowed to molt in tubes. The most consistent results were obtained when extracts of 50 or more larvae were combined and after lyophilization, solubilized in 1 ml of deionized water for analysis. Some

samples were hydrolyzed (6N HCl at 100-105°C for 24 hr for amino acids or 4N HCl at 100-105°C for 4 hr for sugars).

Thin-Layer Chromatography

Thin-layer chromatography (TLC) of amino acids was done on glass plates (20x20 cm) layered with cellulose MN300 (15 g/100 ml H_2O). Two dimensional chromatograms were run in the solvent systems of Heathcote and Jones (1965) and sprayed with 0.4% ninhydrin in acetone to detect amino acids, glucosamine and other ninhydrin-positive compounds. Dansylated extracts of amino acids were prepared by treating 10 mg lyophilyzed sample with 40 ul dansyl chloride solution (5 mg dansyl chloride in 1 ml acetone) for 1 hr at 37°C. Silia G thin-layer plates were run in pyridine:benzene:acetic acid (20:80:2 v/v) and observed under ultraviolet light (Morse and Horecker, 1966). Dansyl derivativies show up as yellow fluorescing spots.

TLC for carbohydrates was performed using silica gel H Plates (50 g/125 ml 0.15 M KH_2PO_4) run in acetone:n-butanol:water (5:4:1 v/v) at 37°C. Before applying samples, the plates were heat-activated in an oven at 110°C for 1 hr. Carbohydrates were detected by spraying with a 4:1 solution of 2% aqueous sodium metaperiodate and 1% $KMnO_4$ in 2% aqueous Na_2CO_3 (Dawson et al., 1979). The presence of glucose was determined by treating the plates with glucose oxidase-peroxidase-o-diansidine (Sigma; PGO Enzyme Kit).

Amino Acid Analysis

Free amino acids and other ninhydrin-positive compounds were identified and quantitated using an amino acid analyser. Analyses were accomplished utilizing a Durum DC6A column eluted with citrate buffer at 60°C (Liao et al., 1973).

Gas Chromatography

Gas chromatography of carbohydrates was accomplished by detecting their derived alditol acetates (Pope, 1977). They were prepared by reacting 25 ul fresh sodium borohydride (20 mg/ml 3 M NH_4OH) with 50 ul reconstituted extract or 100 ug sugar standard in small vials for 1 hr at room temperature. The reaction was stopped by adding glacial acetic acid dropwise until the bubbling stopped. The solution was evaporated to dryness and 100 ul methanol-HCl (0.1 ml concentrated HCl in 100 ml absolute methanol) was added and again evaporated. All evaporation was done under filtered air or nitrogen in a temperature block at 40°C. After adding 100 ul methanol-HCl and evaporating at least 3 more times, the samples were dessicated *in vacuo* over P_2O_5 and KOH overnight. To each sample 50 ul acetic anhydride was added and allowed to react for 1 hr at 121°C in a closed vial. One to 5 ul of cooled sample was used for analysis.

Gas chromatography was accomplished with a Perkin-Elmer Sigma 2 unit equipped with a hydrogen flame detector. A 3 foot x 2 mm glass column packed with 3% SP-2340 on 100/120 Supelcoport was used (Suppelco, Inc.).

B. bassiana Infections

Mutants of *B. bassiana* were obtained by irradiating (UV) conidial suspensions, then selecting for differential proteolytic ability by plating on Sabouraud's dextrose agar (SDA) containing litmus mild (Grula et al., 1978; Cheung and Grula, 1982). Cultures were maintained on SDA.

Conidia of *B. bassiana* mutant E_1, a highly pathogenic mutant, were harvested from 14-21 day cultures grown at 25°C on SDA using 0.03% Triton X-100 in distilled water. After washing 3 times with distilled water, the conidia were resuspended in phosphate buffered saline (PBS; 0.1 M phosphate buffer in 0.9% saline, pH 7.2). Newly-hatched larvae were infected by immersion in the conidial suspension for 5 min or by crawling over wet conidia-coated filter paper for 10-20 min. Some larvae were washed with deionized water or PBS for 30 min and allowed to dry before being infected. Infected larvae were placed in acid-cleaned test tubes in a humidity chamber at 25°C. Larvae were prepared for scanning electron microscopy by fixing 2-4 hr in 4% glutaraldehyde in cacodylate buffer (0.2 M at pH 7.2), critical-point drying, and coating with gold-palladium

(Pekrul and Grula, 1979). Examination was with a JOEL JSM 35 scanning electron microscope.

Growth of *B. bassiana* in Defined Media

Defined growth media contained a basal salt solution consisting of 0.30 g NaCl, 0.3 g $MgSO_4 \cdot 7\ H_2O$, and 0.30 g K_2HPO_4 per liter deionized water. Two amino acid combinations were used in two concentrations (Table I). Conidia were harvested and washed as stated above then resuspended in deionized water to an optical density (O.D.) of 1.0 at 540 nm (approximately $2x10^8$ conidia/ml). Ten ml of medium was inoculated with 0.25 ml of this conidial suspension (approximately $5x10^6$ conidia/ml medium). Cultures were incubated at 25°C on a platform shaker with a rotational speed of 180 rpm. The appearance of germ tubes in stained (crystal violet) samples was used as the indicator for germination.

Growth of *B. bassiana* and *Aspergillus niger* in Extracts

Semi-solid medium (0.7% agar) was made using the combined extract of 30 fifth instar larvae. Total volume was 0.5 ml. This medium, along with SDA (0.5 ml and also containing 0.7% agar) was inoculated with conidial suspensions (0.05 ml) of *B. bassiana* and *A. niger* (O.D. of 1.0 at 540 nm). Tubes were incubated at 25°C in humidity chamber.

TABLE I

COMPOSITION OF DEFINED MEDIA USED FOR GROWTH OF *B. BASSIANA*[a]

Amino Acid	Fifth Instar[b]	Fifth Instar[c] from Corn
asp		16.1
thr	43.5	4.7
ser		19.4
glu		41.8
pro		40.1
gly	3.7	13.5
ala		22.0
val	2.4	13.4
met	3.2	4.8
ile	2.1	5.7
leu		15.3
phe		6.2
his		4.9
lys	14.1	3.0
trp		38.0
arg		2.8
$gluNH_2$[d]	0.6	0.0

[a]Amount and kind of amino acid present is based on nmol/ml amino acid found in the combined extracts of 10 larvae (see Table II). Only those amino acids present in the majority of extracts averaged were used to prepare these media.

[b]Amino acids present in fifth instar larvae which were extracted 24-72 hr after molting in test tubes.

[c]Amino acids in extracts of fifth instar larvae collected from field ears of corn.

[d]Fifth instar medium was made with and without glucosamine to observe its influence.

RESULTS

B. bassiana conidia are able to germinate and grow on the surface of newly-hatched CEW larvae within 18-24 hr when infected larvae are grown on CSM diet (Pekrul and Grula, 1979). Germination takes somewhat longer (24-30 hr) when infected larvae are placed in test tubes devoid of any extra nutrients (Fig. 1a). However, when larvae are washed with deionized water or PBS prior to infection, no germination occurs before 48 hr (Fig. 1b). By 50 hr, limited germination has occurred, but the germ tube lengths indicate growth of only 1-2 hr (Fig. 1c). It should be noted that *B. bassiana* grows saprophytically on dead larva and newly-hatched CEW larvae will survive about 72 hr before dying of starvation; hence, only live larvae were selected for examination.

In order to determine which nutrients are removed with water, epicuticle extracts were taken from live fourth and fifth instar CEW larvae. Contamination was kept to a minimum by individually extracting chilled larvae which had been allowed to molt in test tubes. This avoided the presence of larval diet, hemolymph, regurgitation, and fecal material. Extracts from 50 or more larvae were pooled before being studied.

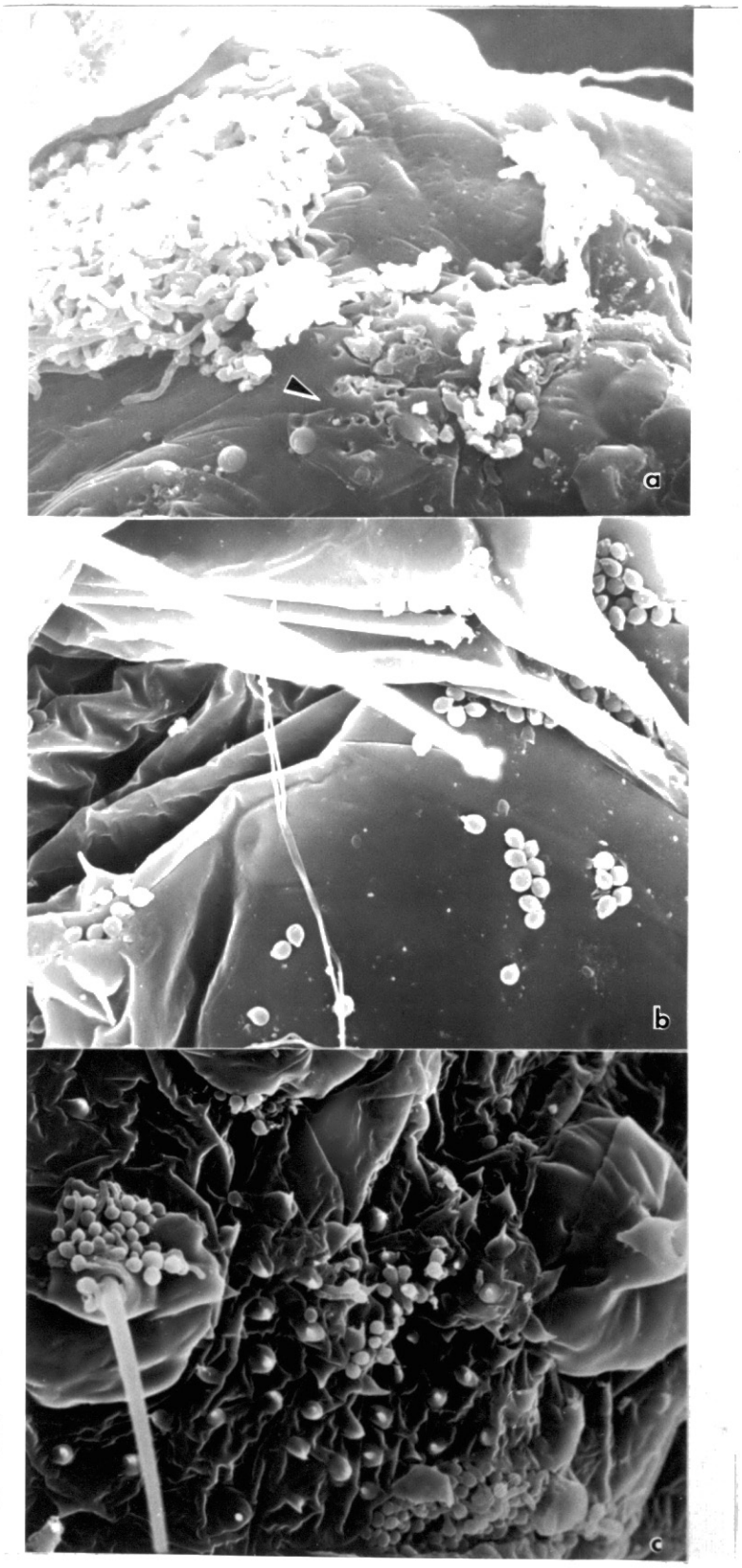
Figure 1. Infections of CEW Larvae by B. bassiana Mutant E_1.

After application of conidia, larvae were placed in test tubes.

a. Control infection at 48 hr. Larvae were not washed prior to infection. Note the enzymatic degradation of the cuticle by germ tubes (arrow). Some depressions were made by germinating conidia which were removed by tissue preparation for SEM (1100x).

b. Larvae washed with water before application of conidia. Sample was fixed 48 hr post-infection.

c. Larvae washed with water before application of conidia. Sample was fixed to hr post-infection (1100x).


a
b
c

Numerous extracts were examined for amino acid content using TLC. Amino acids could be readily detected either by their reaction to ninhydrin or as the dansyl chloride derivitives; however, more spots were detected with dansylation (12-15) than with ninhydrin (8-10). This was probably due to the greater sensitivity of the dansyl chloride procedure. Several of the ninhydrin-positive compounds migrated to areas of the thin-layer plates which made us suspect that some of the compounds could be amino sugars or peptides.

Amino acid analyses revealed some interesting similarities as well as differences among the extracts. Fourth instars appear to have much smaller amounts of amino acids per larva than fifth instars (Table II). Because of this, it was decided to concentrate greater effort toward analysis of fifth instar extracts where more reliable data could be obtained. Even within the fifth instars, the amino acid composition varied according to the time of the extraction following completion of the gross molting process (Table II). Extracts taken within 24 hr after molting had a very high serine, tryptophan, glutamic acid, and lysine content but lacked threonine. This is in sharp contrast to extracts taken 24-72 hr post-molt which had a high content of threonine and lysine; however, serine, tryptophan, and glutamic acid were absent. Amino acid composition appeared to be fairly consistent after 24 hr.

TABLE II

AMINO ACIDS AND GLUCOSAMINE CONTENT OF VARIOUS EXTRACTS AND LARVAL MATERIAL[a]

Amino Acids	Fourth[b] Instar(3) nmol/ larva	Fifth[c] Instar(3) 24 hr nmol/ larva	Fifth[d] Instar(4) 24-72 hr nmol/ larva	Fifth[e] Instar(2) CORN nmol/ larva	Regurg-itate (2) nmol/ larva[f]	Haemo-lmyph[g] %
asp	0.46	0.76		1.67	100.16	0.7
thr	0.27		4.35	0.47	44.41	
ser		4.86		1.94	411.97	28.0
glu	0.20	3.00		4.18	84.91	0.1
pro	0.27			4.01		13.0
gly		1.80	0.37	1.35	136.57	4.3
ala		1.37		2.20	71.82	2.7
cysl/2						0.1
val	0.29	1.14	0.24	1.34	137.52	5.0
met	0.26	1.06	0.32	0.48	81.91	0.7
ile	0.15	0.63	0.21	0.57	96.08	1.6
leu	0.14	0.86		1.53	203.04	4.6
tyr					47.71	5.5
phe				0.62	82.64	1.7
his	0.04			0.49	4.85	13.6
lys	0.03	2.09	1.41	0.30	103.50	12.0
trp	0.19	6.32		3.80	251.39	0.4
arg		0.37		0.28	43.03	5.6
$gluNH_2$			0.06		3.07	

[a]Amino acids present in the majority of extracts averaged.

[b]The number in parentheses indicates the number of analyses averaged.

[c]Samples from larvae extracted less than 24 hr after molting into fifth instar.

[d]Samples from larvae extracted 24-72 hr after molting into fifth instar.

[e]Extractions from larvae removed from ears of corn.

[f]10-fold dilution of regurgitate from fifth instar larvae.

[g]Data of Cheung and Grula, 1981.

Possible extract contaminants (hemolymph and regurgitation material) were analyzed for their amino acid content and compared to data obtained by our procedure for extraction of water-soluble surface compounds (Table II). These materials have a high serine content similar to fifth instar extracts taken within 24 hr of molting, but that was the only relationship between extracts, hemolymph and regurgitated materials. Feces contained no amino acids, but did have a few unidentified amines. Therefore, it appeared the chilled extraction technique was effective in eliminating spit, fecal, and hemolymph contaminants. Since contaminants contained almost all amino acids and a high serine content, it was easy to recognize and discard a contaminated sample.

Free amines were also present in the extracts as revealed by the amino acid analyzer. These probably were the ninhydrin- and dansylation-positive spots observed in TLC which were not amino acids. No attempt was made to identify these amines. Glucosamine was present in small amounts; however, it was detected in less than half of the extracts examined. It averaged 0.06 nmol/larva in fifth instars and was present in regurgitate at a concentration of 3.07 nmol/ml.

Numerous attempts were made to detect other carbohydrates using both thin-layer and gas chromatography. It proved to be very difficult to obtain precise data. Positive reactions usually occurred on TLC plates in the

poorly migrating regions (near the origin). It is possible that carbohydrate polymers or glycopeptides are responsible for such reactions.

Larvae collected from ears of corn were extracted and found to have an amino acid content significantly different from larvae molted in test tubes (Table II). The most striking differences were the high glutamic acid, tryptophan, proline, and alanine contents of larvae taken from corn. In addition, the total amounts of amino acids were generally much higher than amounts from "clean" larvae. Glucosamine was never detected; however, glucose and a glucose-positive nonmigrating spot (possibly starch) were observed after treating TLC plates with the PGO enzymes.

Using data that were available, we attempted to calculate the number of molecules present on a um^2 of larval surface. Measurements of ten extracted fifth instar larvae revealed a surface area of roughly 240 mm^2 (surface area was determined as for a non-involuted cylinder; average length = 20.4 mm, average circumference = 10.9 mm, and average diameter = 3.5 mm). Using these types of data and calculations, the number of molecules per um^2 of larval surface was calculated (Table III).

Throughout this study the presence of peptide(s) on the larval surface appeared to be probable, particularly because the TLC studies continually showed ninhydrin-positive compounds that either did not move or that had Rf values uncharacteristic of amino acids. In order to determine if

TABLE III

10^6 MOLECULES PER um^2 OF LARVAL SURFACE[a]

Amino Acids	Fifth Instar 24-72 hr post-molt	Fifth Instar from ears of corn
asp		4.19
thr	10.92	1.18
ser		4.87
glu		10.49
pro		10.07
gly	0.93	3.39
ala		5.52
val	0.60	3.36
met	0.80	1.20
ile	0.53	1.43
leu		3.84
phe		1.56
his		1.23
lys	3.54	0.75
trp		9.54
arg		0.70
$gluNH_2$	0.15	

[a]Amino acids present in the majority of extracts averaged.

these spots could be peptides, total surface extracts were examined before and after hydrolysis. TLC of hydrolyzed extracts showed a loss of the poorly migrating ninhydrin-positive areas (Fig. 2). Amino acid analyses showed an increase in a number of amino acids (Table IV). It appears that peptides are present and their content of glycine, aspartic acid, alanine, glutamic acid, valine, proline, isoleucine, leucine, and threonine is relatively high. Part, but not all, of the increases in aspartic acid and glutamic acid could have been due to degradation of free or peptide bonded asparagine and glutamine. Partial hydrolysis for carbohydrates gave a weak reaction for galactose which could not be routinely confirmed.

In order to determine if the amino acids found on the larval surface could indeed support germination and growth of B. bassiana in vitro, defined media were made to duplicate the extracts (Table I). All media containing amino acids found in extracts yielded some germination and growth of B. bassiana conidia (Table V). As shown in Table II, larvae grown on field corn contained more amino acids and it was not surprising to observe that a medium composed of these amino acids was superior to the combination from fifth instar (prepared after molting) even after it was supplemented with glucosamine. In spite of substantial germination and growth in all media, extensive growth of hyphae was observed only in media simulating extracts made from larvae grown on corn (Fig. 3). All cultures contained

Figure 2. Two-Dimensional TLC of Amino Acids in Fifth Instar Extract.

Amino acids were detected with ninhydrin.

a. Untreated extract.

b. Hydrolyzed extract.

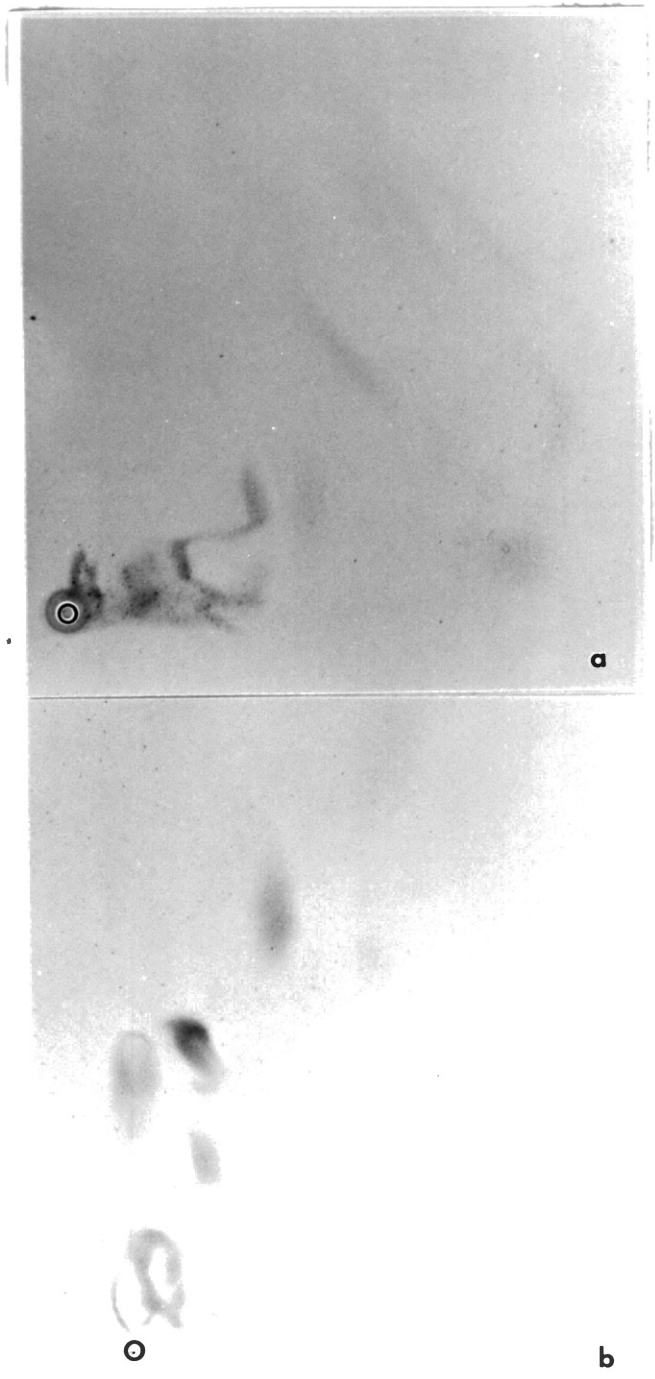

a
b

TABLE IV

AMINO ACIDS IN HYDROLYZED FIFTH INSTAR EXTRACT[a]

Amino Acids	Fifth Instar (not hydrolyzed)	Fifth Instar (hydrolyzed)	Increase in Amino Acids
asp	1.17	8.12	6.95
thr	2.02	3.59	1.57
ser	4.91	3.71	-1.20
glu	4.22	9.58	5.36
pro	0.79	3.55	2.76
gly	2.32	13.34	11.02
ala	1.59	7.31	5.72
val	1.32	4.14	2.82
met	1.28	0.62	-0.66
ile	0.78	3.32	2.54
leu	1.19	3.66	2.47
tyr	0.61	0.18	-0.43
phe	0.50	1.23	0.73
his	0.41	1.04	0.63
lys	2.63	2.44	-0.19
trp	7.73	0.00	-7.73
arg	0.26	0.86	0.60

[a]All amino acids in the three extracts are averaged and given in nmol/larva.

TABLE V

GROWTH OF *B. BASSIANA* IN DEFINED MEDIA

Amino Acid[a] Combination	Amino Acid[b] Concentration (larva/ml)	Percent Germination at 48 hr	Description of Germ Tubes at Five Days
Fifth Instar[c]	10	40	long (15-35 um) with some lysis
	1	20	short (18 um) with some lysis
Fifth Instar with Glucosamine	10	50	5-25 um long with lysis of longer germ tubes
	1	20	short (10 um) with some lysis
Fifth Instar Larvae From Corn	10	50	branched hyphae as long as 100 um with some lysis
	1	35	hyphae of various lengths (7-55 um) with some branching and lysis
Basal Salts	0	0	no germination

[a]Compositions of media are given in Table I.

[b]Concentrations are equal to those found in the extracts. The amount of each amino acid per ml of medium is the same as that found on 1 or 10 extracted larvae.

[c]Amino acids on fifth instar larvae extracted 24-72 hr after molting.

[d]Composition of basal salts is given in text.

Figure 3. Germination and Growth of *B. bassiana* in Defined Media at Five Days (2600x).

a. Basal salt medium.

b. Fifth instar medium.

c. Fifth instar medium supplemented with glucosamine.

d. Fifth instar larvae collected from corn medium.


a
b
c
d

lysing germ tubes of varying lengths; the richer media (corn larvae and fifth instar supplemented with glucosamine) yielded best hyphal development. In all media, however, nutrient exhaustion with eventual lysis of hypae did occur. None of the media caused lysis of ungerminated conidia.

A semi-solid medium made directly from fifth instar extracts was inoculated with washed conidia of B. bassiana and A. niger to aid in determining possible toxic effects of amines and peptides. Both organisms germinated and covered the surface with limited growth (Table VI). A. niger was able to sporulate much faster than B. bassiana in such a medium.

TABLE VI

GROWTH OF *B. BASSIANA* AND *A. NIGER* ON CONCENTRATED FIFTH INSTAR EXTRACT

Medium	Organism	Growth[a] 2 Days	Growth[a] 5 Days	Description of Culture at 5 Days
Extract[b]	*B. bassiana*	1	2	small mycelia over surface; no sporulation
	A. Niger	1	2.5	very little elevated growth; some sporulation
SDA[c]	*B. bassiana*	2	3	good mycelial growth; some sporulation
	A. niger	2	4	excellent growth and sporulation

[a]Growth is based on a scale of 1-4 with 1=poor or limited covering of the medium and 4=excellent or complete coverage.

[b]Combined extracts of 30 fifth instar larvae concentrated into 0.5 ml and containing 0.7% agar as the solidifying agent.

[c]Control (0.5 ml SDA containing 0.7% agar).

DISCUSSION

Water-soluble nutrients, amino acids and glucosamine, are present on the surface of CEW larvae in sufficient quantities to support germination and limited growth of B. bassiana. The amounts found, however, are not sufficient to support prolonged hyphal growth. Prolonged growth is not essential for infection since the most pathogenic mutants of B. bassiana penetrate the CEW cuticle very soon after germination (Pekrul and Grula, 1979). Once penetration has occurred, large and varied amounts of nutrients are available in the epithelium and haemolymph (Cheung and Grula, 1982).

Larvae collected from field ears of corn contain numerous surface amino acids and possible starch. Conidia of B. bassiana germinate readily in media prepared from these amino acids indicating that any lack in field infections is most likely not due to lack of nutrients or presence of water-soluble inhibitory compounds.

Since surface nutrients are easily removed with water, it is interesting to note their reappearance after a few hours (as evidenced by B. bassiana's ability to germinate after 50 hr; Fig. 1c). Deposition of the epicuticle is considered to be fairly complete as soon as the deposition of the procuticle begins; however, secretions to the

epicuticle through the pore canals can possibly continue until the cuticle is fully developed (Zacharuk, 1976). If amino acids are secreted in this way, it is conceivable they would soon be replenished in concentrations sufficient to support germination of invading microorganisms.

It is interesting that surface amino acid concentrations are high and variable within 24 hr after molting. It is likely this "new" surface contains residual molting fluid and cuticular debris which would eventually be removed and replaced by normal secretions.

Nonpathogenic fungi, such as A. niger, do not germinate on the surface of CEW (Pekrul and Grula, 1979). It was assumed that either an inhibitor or the lack of nutrients was responsible. As shown in this study, however, the amino acids present are sufficient for germination of A. niger and the peptides and amines present are not at all inhibitory. It appears that surface germination is inhibited by hydrophobic compounds in the epicuticle (Smith and Grula, 1982).

The presence of peptide(s) on the CEW surface is very interesting; their possible function is not known or predictable. Numerous insect peptides have been found possessing predominately hormonal functions (Mordue and Stone, 1981). Peptides could have a specific purpose on the larval surface; but, until additional data are available, it would appear that their presence is a simple consequence of cuticle production and secretion.

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PART II

POSSIBLE INVOLVEMENT OF SURFACE RECEPTORS IN THE ENTOMOPATHOGENICITY OF *BEAUVERIA BASSIANA*

INTRODUCTION

Following germination of Beauveria bassiana conidia on the surface of the corn earworm (CEW), Heliothis zea, emerging germ tubes are able to enzymatically penetrate the cuticle (Pekrul and Grula, 1979). With highly pathogenic strains, penetration occurs soon after germination; visible portions of penetrating germ tubes are quite short (about 3 um). Intermediate pathogens penetrate with longer germ tubes (>6 um). Penetration is rarely observed with the least pathogenic strains and must eventually occur by very long hyphae. Since all mutants produce enzymes capable of degrading the larval cuticle (even though activities vary among mutants), degrees of pathogenicity are not entirely dependent upon enzymatic capabilities (Pekrul and Grula, 1979). Sequential enzyme activities are required for effective cuticle degradation, yet even differences in these activities cannot account for variation among mutants in their times of effective cuticle penetration (Smith, Pekrul, and Grula, 1981). It was therefore speculated that recognition on a molecular level occurs between germinating conidia and larvae which allows highly pathogenic mutants to penetrate the CEW earlier than other mutants.

Many bacterial pathogens are known to have molecular mechanism allowing them to adhere to specific tissues

(Smith, 1977). Most of these mechanisms involve bacteria with pili or fimbriae such as Escherichia coli (Wevers et al., 1980; and Eden and Hanson, 1978), Neisseria gonorrhoeae (Meyer et al., 1982; and Punsalang and Sawyer, 1973), Vibrio cholerae (Jones and Freter, 1976), Pseudomonas aeruginosa (Woods et al., 1980), and Salmonella typhimurium and Shigella flexneri (Lindley, 1980). In most of these cases, the proteins of the pili or fimbriae responsible for attachment behave like lectins.

Lectins are divalent or multivalent carbohydrate-binding proteins or glycoproteins which bind to cells having surface saccharides (Lis and Sharon, 1973). Many lectins, such as Concanavalin A (Con A), require metal ions. Lectins are also referred to as haemagglutinins since they are primarily detected and assayed by their ability to agglutinate erythrocytes by binding to surface glycoproteins or glycolipids (Leffler and Eden, 1980). This binding is reversible and is inhibited in the presence of specific competitive carbohydrates. Sugar specificity can be for a single or an entire class of carbohydrates (Goldstein and Hayes, 1976). Consequently, the cell types to which a lectin can bind may vary drastically. With some of the bacterial pathogens mentioned above, effective binding occurs with only certain tissues which may account for the selective invasiveness of these pathogens (Lindley, 1980).

Lectins were originally discovered in plant seeds, but have now been isolated from a variety of organisms. In

addition to bacteria, lectins have been found in fungi, invertebrates and even some vertebrates (Barondes, 1981). The functions of these lectins are, for the most part, poorly understood. Most are thought to be on cell surfaces which indicates a role ion recognition of other cell surfaces. This is quite obvious for bacterial pathogens and Rhizobium's attachment to legume roots (Barondes, 1981). However, such functions have not been found in the normal environment for a number of lectins. Suprising applications for some lectins have been found in vitro, though. The most important are their mitogenic effects and their ability to agglutinate malignant cells (Lis and Sharon, 1973). These two findings, along with many other technical applications, are most responsible for the vast lectin research which has been initiated in the last 20 years. As a consequence, many new lectin sources have been found.

Haemagglutinins were first isolated from fungi in 1953 (Bernheimer and Farkas, 1953). Since then a number of fungi have been found to produce lectins. Their functions are not always evident, as in the case of a N-acetyl-D-glucosamine-(NAG-) and chitin-binding lectin from Neurospora crassa (Prick and Diekmann, 1979). Other fungal lectins appear to be produced in certain stages of a life cycle and are responsible for cell aggregation. One of these lectins is discoidin, a protein produced by the slime mold Dictyostelium discoideum upon starvation (Frazier et al., 1975). It has haemagglutination activity (HA) inhibited by

galactose, glucose, frucose, N-acetyl-D-galactosamine, NAG, and a glycoprotein fraction from slime mold homogenates (Rosen et al., 1973). This lectin seems to be necessary for the aggregation of amoebae into a multicellular structure prior to formation of the fruiting body. Agglutination factors with lectin-like qualities have also been isolated from Hansenula wingei, Pichia amethiona, and Saccharomyces kluyeri (Burke et al., 1980). These factors appear to be involved in the aggregation of opposite mating cell types before conjugation.

Some fungi produce lectins which aid in their binding to other organisms. The three-dimensional net formed by Arthrobotrys aligospora is covered with an adhesive material which specifically binds nematodes. N-Acetyl-D-galactosamine completely inhibits the nematode-trapping process indicating the presence of a lectin (Nordbring-Hertz and Mattiasson, 1979). Species of an insect pathogen, Conidiobolus, also produce a soluble haemagglutinin for all blood types (Ishikawa et al., 1979). This HA activity is inhibited by chitin and NAG. Hence, the fungus may use a lectin to bind to chitin in insect cuticles.

A lectin may also be produced by the entomopathogenic fungus B. bassiana. Such a protein, if present on the cell surface, could aid the fungus in binding and penetrating larvae. This study is an effort to detect haemagglutinins, or lectins synthesized by B. bassiana and, if found, to determine their effects on the infection process.

MATERIALS AND METHODS

Test Organisms

Mutants of *Beauveria bassiana* were obtained in this laboratory by irradiating conidial suspensions, then selecting for differential proteolytic activity by plating on Sabouraud's dextrose agar (SDA; Difco Labs.) containing litmus milk (Grula et al., 1978). Batch cultures were grown on SDA or in Sabouraud's dextrose broth (SDB). Conidia were harvested with 0.03% Triton X-100 from 14-28 day cultures grown on SDA at 25°C. Cells were collected from SDB cultures (grown at 25° C) with centrifugation. Blastospore and conidia were removed from cellular masses by filtration through five layers of cheesecloth. After washing three times with distilled water, cells were resuspended in phosphate-buffered saline (PBS; 0.1 M phosphate buffer in 0.9% saline, pH 7.2)

The saprophytic fungus *Aspergillus niger* was isolated from dead corn earworm larvae. It was maintained on SDA incubated at 25°C. Conidia were prepared by the same method used for *B. bassiana*.

Preparation of Crude Haemagglutinins

Batch cultures of B. bassiana were grown in 500 ml SDB on a rotary shaker (180 rpm) for 9-14 days. Cells were separated from spend medium by centrifugation (Sorvall RC2-B) at 5000 rpm for 10 min or by filtration through 0.45 um Millipore filters. Cell material was reserved for sonication procedures which are discussed later. Spent medium was concentrated approximately 30-fold by dialysis against polyethylene glycol (400 g/2 L; Sigma) at 4°C. The concentrated medium was centrifuged if either residual cells or precipitant was present. The resulting crude agglutination sample was used in HA assays and gel filtration chromatography.

Conidia and/or cell material from SDB cultures were resuspended in PBS and sonciated using a Branson sonifier at setting 8 (3-8 times for 20 sec with 1 min cooling periods). Some cells were then frozen, thawed, and resonicated. Supernatant was collected after vortexing 1-3 min. The samples were concentrated by lyophilization or dialysis against polyethylene glycol. After resuspension in distilled water and dialysis against PBS for 24 hr, the crude agglutinin samples were ready for HA assays and affinity chromatography.

Haemagglutination Assays

Chicken (Leghorn) blood was collected by cardiac

puncture and stored in Alsever's solution (2.05 g glucose, 0.8 g sodium citrate, 0.42 g NaCl, 0.02 g citric acid, 100 ml H_2O; pH 6.1). Human blood, which was collected in citrate vacuum tubes, was generously donated from excess supplies by Stillwater Medical Center. Red blood cells (RBC) were washed three times with PBS. The final concentration of RBC suspensions varied among assays.

HA activity was assayed by mixing two-fold serial dilutions of the crude or chromatographically-purified agglutinin with an equal volume of 2% chicken RBC suspension (ChRBC). The activity was expressed as HA titer; i.e., the reciprocal value of the highest dilution at which HA was observed in two hr. In some cases, the activity was rated on a scale of one to four according to titer and microscopic observation. Sugar inhibition and mineral cation enhancement assays were performed on agglutinin diluted in PBS containing carbohydrate (1mg/ml) or 5 mM metal cation.

Fungal cell adherence to erythrocytes was also examined. RBC suspensions were prepared as above in a final concentration having 3% cells or an optical density (O.D.) of 1.0 at 620 nm. Some conidia were germinated in SDB (12 hr on a rotary shaker at 25°C), while others were sonicated (three times for 30 sec with one minute cooling periods). Blastospores, germinating blastospores, and hyphae were collected from three wk SDB cultures. Inhibition or enhancement assays were done using PBS buffers containing: 0.1 M glucose, 50mM $CaCl_2$, 50 mM ethylenediaminetetra-acetic

acid (EDTA), PBS made with distilled water, and PBS made with deionized water. Particulate substitutions included swollen Sephadex G-100 for RBC and A. niger conidia for B. bassiana conidia. Solutions observed contained 25 um fungal cells with 25 um RBC; inhibition and enhancement assays contained an additional 50 um buffer. Photographs were made after two hr using phase contrast or Hoffman modulation contrast microscopy.

HA activity was also determined by measuring the absorbance of unsedimented RBC (Lis and Sharon, 1972; and Lierner, 1954). This assay is a modification of Lierner's technique and is based on the assumption that agglutinated cells sediment much faster than normal free cells. If conidia and RBC form aggregates, an increase in the rate of sedimentation should result. The test solution contained 0.5 ml conidia suspension (0.60 O.D. at 540 nm) and 0.5 ml PBS; control tubes contained only 1 ml PBS. Sugar inhibition was determined with a solution of 0.5 ml conidia and 0.5 ml 0.2 M glucose or NAG in PBS. Sugar control tubes had 0.5 ml PBS instead of conidia. One ml of RBC suspension (1.30 O.D. at 620 nm) was added to each 10 x 75 mm test tube. After vortexing, the tubes were incubated at room temperature in a rack which held them perfectly vertical. O.D.'s were read at 620 nm hourly. Care was taken not to agitate the contents. A standard curve was made to convert the O.D. into percentage of RBC in solution; this is a

linear relationship. RBC percentages in test solutions were subtracted from control tubes and the difference subtracted from 100. This was done to negate the normal sedimentation of free cells. Some solutions were examined with phase microscopy after three hr.

Chromatography

Gel filtration of 0.5 ml crude medium agglutinin was done on a column of Sephadex G-75 (1 x 45 cm) equilibrated with PBS. Six ml fractions were collected at a rate of 15 ml/hr at 4°C. Each fraction was lyophilized, resuspended in 1 ml distilled water, and dialyzed against PBS for three days at 4°C. Protein concentrations were determined by absorbance at 280 nm and by colorimetric determination using the BioRad protein assay (BioRad Labs.; absorbance at 595 nm of 0.1 ml fraction in 1 ml BioRad solution and 4 ml water). HA titers of each fraction were determined using 2% ChRBC. Agglutination was also checked microscopically.

Affinity chromatography was accomplished with Sephadex (G-15, G-25, and G-75) equilibrated with PBS. Columns had one cm diameters and varied in lengths from 10-45 cm. After application of supernatant from sonicated cells, the columns were washed thoroughly with PBS at 25°C. The bound substances were eluted with 0.2 to 0.5 M glucose in PBS. After dialysis against PBS for 24 hr at 4°C, the fractions were examined for total protein content and HA activity. Corrections were made for the concentration factor.

Infections

One ml aliquots of a conidial suspension from a four wk SDA culture of mutant E_1 were resuspended in PBS containing the following: 0.4 M glucose, 0.4 M NAG, 80 um/ml Con A, or 0.01 M each of $CaCl_2$, $MnSO_4 \cdot H_2O$, and $CuSO_4 \cdot 5\ H_2O$. Control and germinated conidia (15 hr SDB culture) were resuspended in PBS. Some conidia were just sonicated using a Branson sonifier at setting 8 (four times for 20 sec with one min cooling period) while others were sonicated, frozen, thawed, and then resonicated (four times for 20 sec with one min cooling period). The one ml conidial suspensions were poured on Whatman #1 filter paper in petri dishes. Newly-hatched CEW and fall armyworm (FAW), Spodoptera fragiterda, larvae were allowed to crawl on the filter paper for ten min, then were placed in paper-capped or cotton-plugged test tubes or on CSM diet (Burton, 1970). Some larvae were treated with Con A prior to infections. After 48 hr, live larvae were prepared for scanning electron microscopy.

Electron Microscopy

SEM samples were fixed in 2-4% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.3 for 2-6 hr. After dehydration in an ethanol series, they were critical point dried, and coated with gold-palladium. A JEOL JSM microscope was used for examination.

Samples prepared for transmission electron microscopy (TEM) were first fixed for one hr at 4°C in a triple aldehyde solution containing 3% paraformaldehyde, 3% glutaraldehyde, and 3% acrolein in 0.2 M cacodylate buffer, pH 7.3. Post fixation was in 1% osmium tetroxide for one hr at 4°C. After dehydration in an ethanol series (70% ethanol contained 2% uranyl acetate) and propylene oxide, the cells were embedded in Polybed 812 or Spurr resin and sectioned with glass knives. Sections were stained with uranyl acetate and lead citrate and examined with an RCA EMU-3G microscope.

RESULTS

Before any attempt was made to isolate haemagglutinins from B. bassiana, an indirect test was done to determine if a molecular receptor system could be present. Infections were examined in which the fungus or larvae were first treated with the lectin Con A. Con A should bind to any glucose or mannose polymer and could serve as a physical blockage for a receptor. If a glucose or mannose lectin is involved in the infection, interference by Con A should have drastic effects. There was a general loss in adhesion, but it was not as great as expected except for conidia germinated in SDB prior to infection (Table I). Penetration also seemed to be affected, but to small degree. The lack of germination was probably due to inhibitory effects of Con A on fungal metabolism (Barkai-Golan et al., 1978). With these indications, attempts were made to isolate B. bassiana haemagglutinins.

Medium from a ten day SDB culture of B. bassiana was concentrated 30-fold and found to contain HA activity with ChRBC (Table II). It should be noted the titers did vary with each preparation, but the activity was always present. Sugar inhibition was best when NAG was present, yet most carbohydrates tested brought about some inhibition (Table

TABLE I

INFLUENCE OF CONCANAVALIN A ON INFECTIONS BY *B. BASSIANA*[a]

Treatment of Conidia[b]	Treatment of Larvae[b]	Adhesion[c]	Germination[c]	Penetration[c]
none	none	++++	+++	+++
resuspended in Con A[d]	none	+	-	-
rinsed with Con A	none	++	+	-
resuspended in Con A[e] and glucose	none	+++	+	-
none	rinsed with Con A	++	-	-
none	rinsed with PBS	+++	-	-
germinated conidia[f] resuspended in PBS	none	++++	+++	+
germinated conidia[f] resuspended in Con A	none	-	-	-

[a]Observations of larvae fixed 48 hr after infection were made with scanning electron microscopy.

[b]Conidia and larvae were treated with the solutions prior to infection.

[c]Rated on a scale of 1-4 with 4=excellent.

[d]Con A suspension was 80 um/ml in PBS.

[e]1 mg/ml glucose was added to the Con A suspensions.

[f]Germinated conidia were collected from a 14 hr culture grown in SDB.

TABLE II

HAEMAGGLUTINATING ACTIVITY OF CONCENTRATED MEDIUM FROM 10-DAY SDB CULTURE OF *B. BASSIANA* MUTANT E_1[a]

Buffers Used for Serial Dilutions of Agglutinin[b]	Titer with 2% Chicken RBC[c]
PBS	128
glucose	32
glucosamine	8
NAG	4
galactose	128
galactosamine	16
mannose	64
EDTA	256
$MgCl_2$	1024
$CaCl_2$	512
$MnCl_2$	256

[a]Crude preparation of medium was concentrated 30-fold by dialysis against polyethylene glycol.

[b]Carbohydrate concentrations were 1 mg/ml in PBS; concentrations of EDTA and metal ions were 5mM in PBS.

[c]Titers given are the reciprocal of the highest dilution which agglutinated RBC in microtiter plates.

II). HA activity was notably enhanced in the presence of Mg^{++} and Ca^{++}. Gel filtration of spent medium on Sephadex G-75 at 4°C revealed the activity eluted within the first and second protein peaks (Fig. 1). It appears B. bassiana releases into the medium a large haemagglutinin which is somewhat specific for NAG; it could be composed of multiple subunits requiring metal cations.

Sonication will also release HA activity from cells of B. bassiana. The relatively mild sonciation used in these studies does not cause lysis of conidia or blastospores. Cells (mycelia and blastospores) from a ten day SDB culture of mutant E_1 released a haemagglutinin which, when concentrated 30-fold, gave a titer of 32 with ChRBC (Table III). It did not exhibit specificity with regard to sugars since glucose, NAG, glucosamine, and galactosamine all showed identical inhibition. Calcium was the only metal cation which substantially enhanced the activity. Affinity chromatography with Sephadex G-75 at 25°C gave a small peak eluted with 0.25 M glucose which contained most of the recovered HA activity (Fig. 2). Such data suggest that a nonspecific haemagglutinin is present on the surface of B. bassiana cells which can be removed with sonication.

Since proteins released from B. bassiana agglutinate RBC, it seemed probable the fungus itself should adhere to erythrocytes. Various cell types of mutant E_1 were added to ChRBC suspensions. Adherence occurred with dormant conidia but not hyphae or conidiophores (Fig. 3). Erythrocytes also

Figure 1. Gel Filtration Chromatography of Spent Medium From Mutant E_1. Five ml of 30-fold concentrated medium from a ten day SDB culture was applied to a Sephadex G-75 column (1 x 45 cm). Absorbance was continuously monitored at 280 nm (———). Six ml fractions were concentrated (lypohilization) and analyzed for protein content (BioRad;-○-) and HA activity (□ ; 2% ChRBC in PBS made with tap water). (HA activity was never observed when assayed using PBS made with deionized water; EDTA inhibited all activity in concentrated fractions).

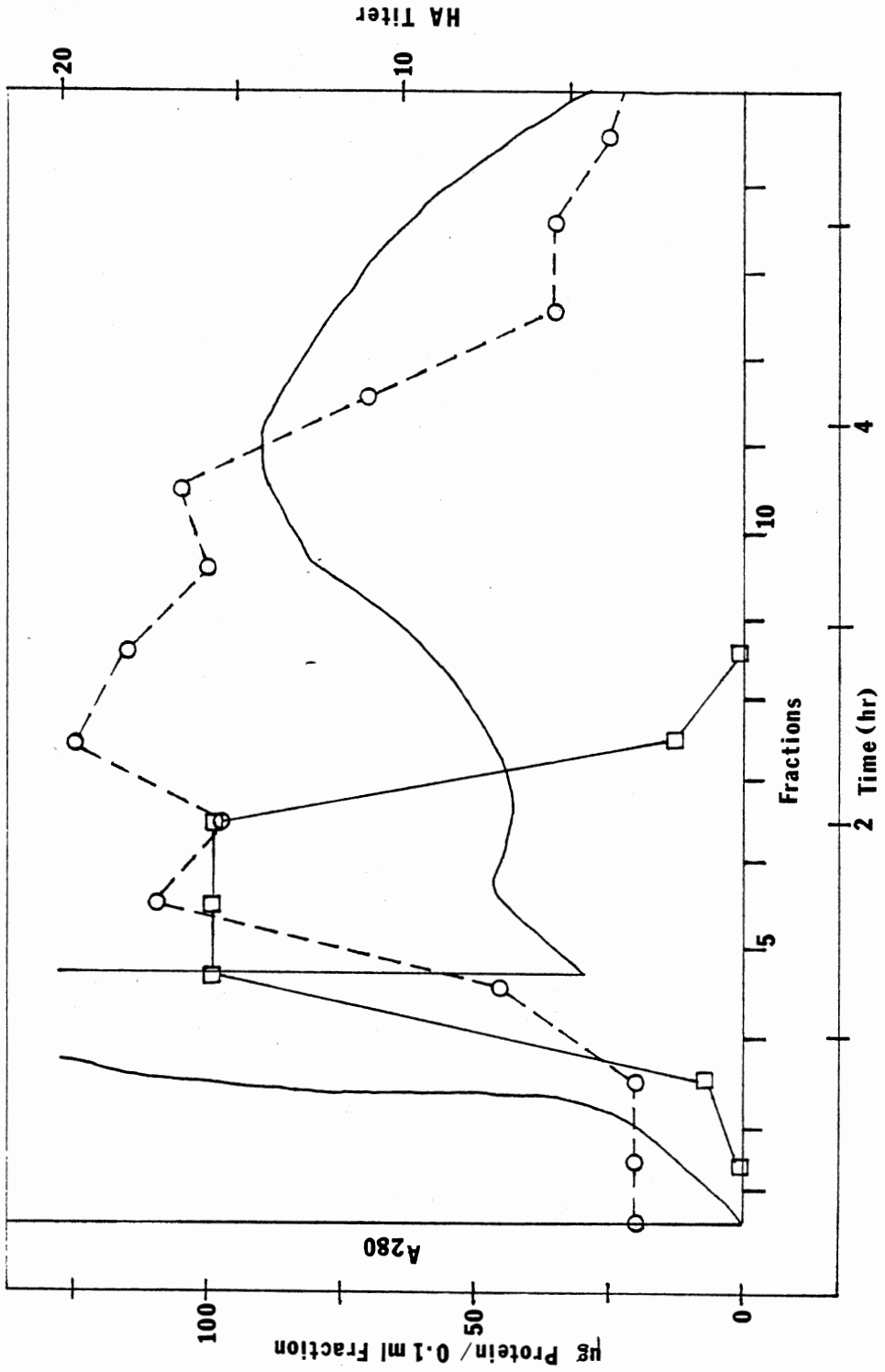

HA Titer
20
10
μg Protein/ 0.1 ml Fraction
100
50
0
A280
Fractions
5
10
2 Time (hr)
4

TABLE III

HAEMAGGLUTINATING ACTIVITY OF SUPERNATANT FROM SONICATED CELLS OF TEN-DAY CULTURE OF *B. BASSIANA* MUTANT E_1[a]

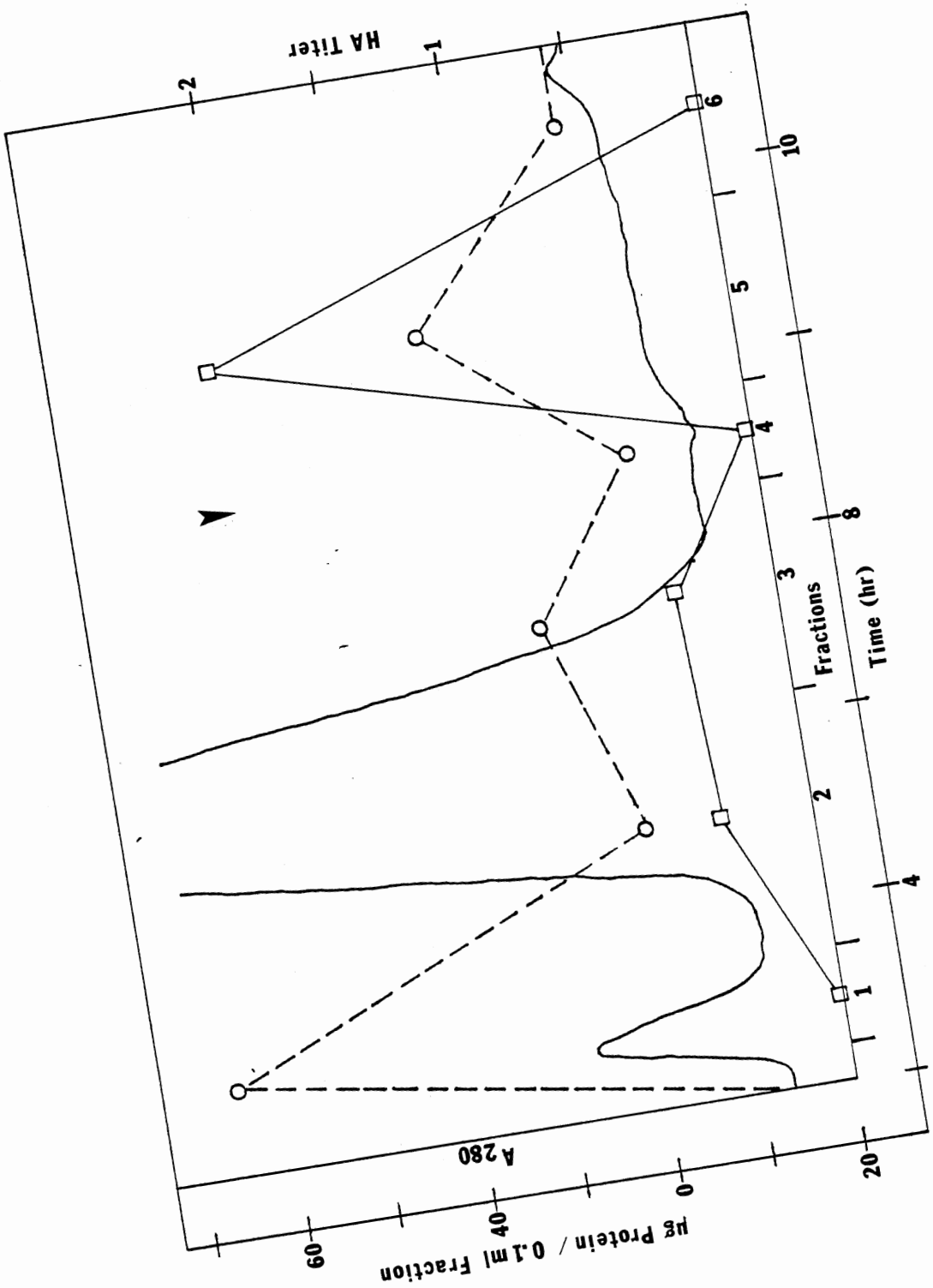
Buffers Used for Serial Dilutions of Agglutinin[b]	Titer with 2% Chicken RBC[c]
PBS	32
glucose	8
glucosamine	8
NAG	8
galactose	32
galactosamine	8
mannose	16
EDTA	64
$MgCl_2$	64
$CaCl_2$	128
$MnCl_2$	64
50 mM $CaCl_2$	256

[a]Crude preparation of supernatant from sonicated cells was concentrated 20-fold by lyophilization.

[b]Carbohydrate concentrations were 1 mg/ml in PBS; concentrations of EDTA and metal ions were 5 mM in PBS except for the last $CaCl_2$ buffer.

[c]Titers given are the reciprocal of the highest dilution which agglutinated RBC in microtiter plates.

Figure 2. Affinity Chromatography of Supernatant From Sonicated Mutant E_1 Cells. Seven ml of 30-fold concentrated supernatant from sonicated (3 times for 20 sec with one min cooling period) cells of a ten day SDB culture was applied to a Sephadex G-75 column (1 x 45 cm). The bulk of the protein was eluted with PBS at 25° C. Bound proteins were eluted with 0.25 M glucose in PBS (glucose buffer was started at the point indicated with arrow). Absorbance was continuously monitored at 280 nm (——). Large fractions were concentrated (dialysis) and analyzed for protein content (-○-; BioRad) and HA activity (□ ; 2% ChRBC in PBS made with tap water). (HA activity was never observed when assayed using PBS made with deionized water; EDTA inhibited all HA activity in concentrated fractions.)


HA Titer
2
1
μg Protein / 0.1 ml Fraction
60
40
0
20
A 280
Fractions
1
2
3
4
5
6
8
10
Time (hr)
4

bound germinated conidia and blastospores, but not the emerging germ tubes (Fig. 3). Glucose had a small inhibitory effect with dormant conidia and sonicated also had less affinity for RBC (Fig. 4). It should be noted that some dormant conidia treated with glucose or sonication still did bind to ChRBC; however, large aggregates were observe only with untreated conidia.

Conidia also had a high affinity for swollen beads of Sephadex gel, a glucose polymer. This adherence was drastically inhibited with glucose (Fig. 5).

These data indicate a surface haemagglutinin is present on conidia and blastospores and is released, to some extent, by sonication. Conidia from mutant E_1 were harvested from SDA plates and sonicated. Stained (crystal violet) sonicated conidia showed no lysis. However, stain did collect around clumps of conidia suggesting the accumulation of a negatively-charged substance such as protein. Supernatants from sonicated conidia and from well-washed sonicated conidia were concentrated by dialysis against polyethylene glycol and run on a Sephadex G-25 affinity column. HA activity was eluted from both supernatants with glucose (Fig. 6). The amount of protein and HA activity was much higher in the peak from the supernatant of well-washed conidia. In fact, most of the protein in the second supernatant was released with glucose. Based on these data, it seems evident that HA activity of conidia can be dislodged by sonication and repeated washes in buffer.

Figure 3. Adherence of ChRBC to Cells of Mutant E_1.

a. Dormant conidia (154x).

b. Conidia on conidiophores (154x).

c. Germinating conidia. Emerging germ tubes have no affinity for ChRBC. Note the adherence of a germinated conidium to a lysing RBC (arrow) (154x).

d. Blastospores (750x).

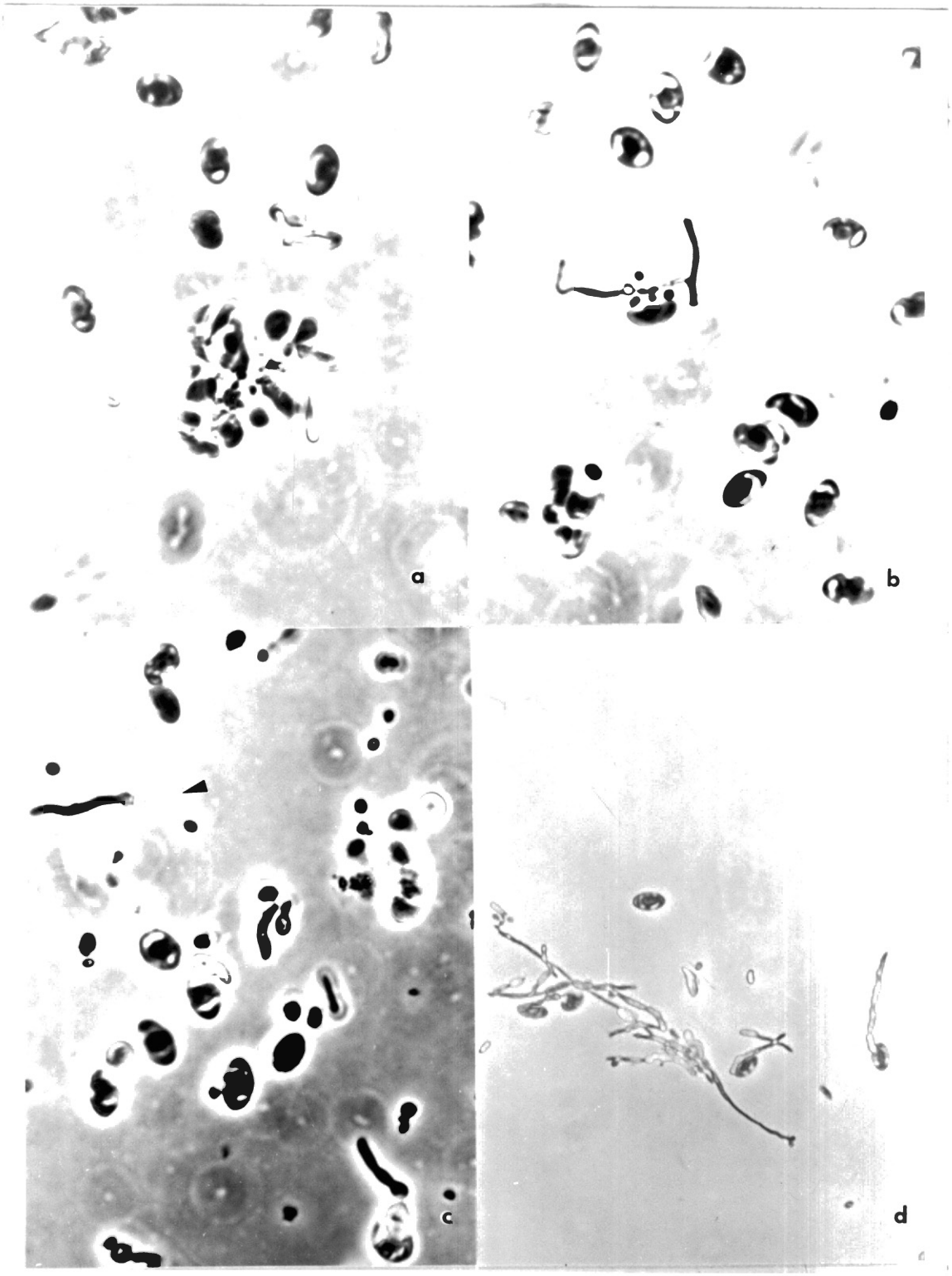

a
b
c
d

Figure 4. Adherence of ChRBC to Treated Conidia of Mutant E_1.

a. In presence of glucose (750x).

b. Sonicated conidia (790x).

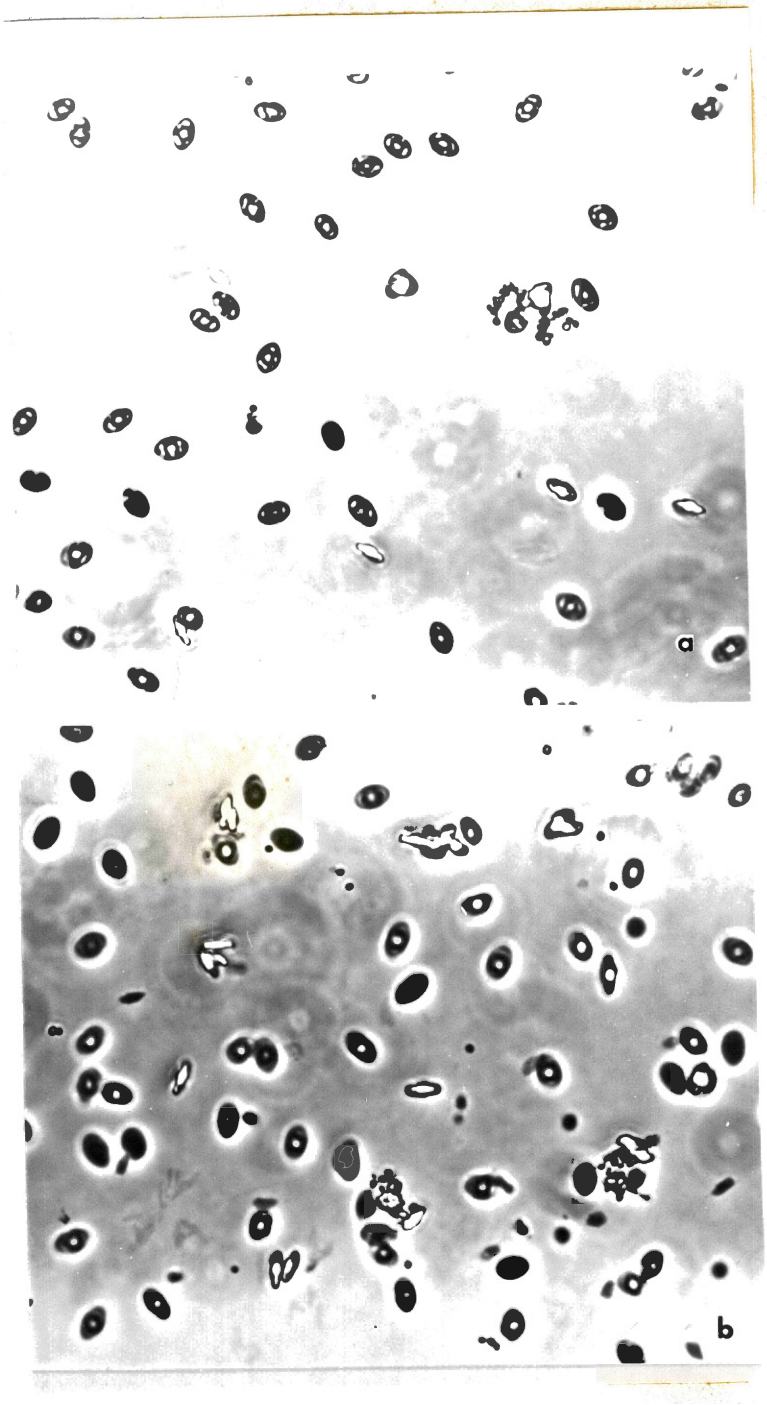

a
b

Figure 5. Affinity of Conidia From Mutant E_1 for Sephadex G-100 Beads (1600x).

a. In PBS.

b. In the presence of glucose.

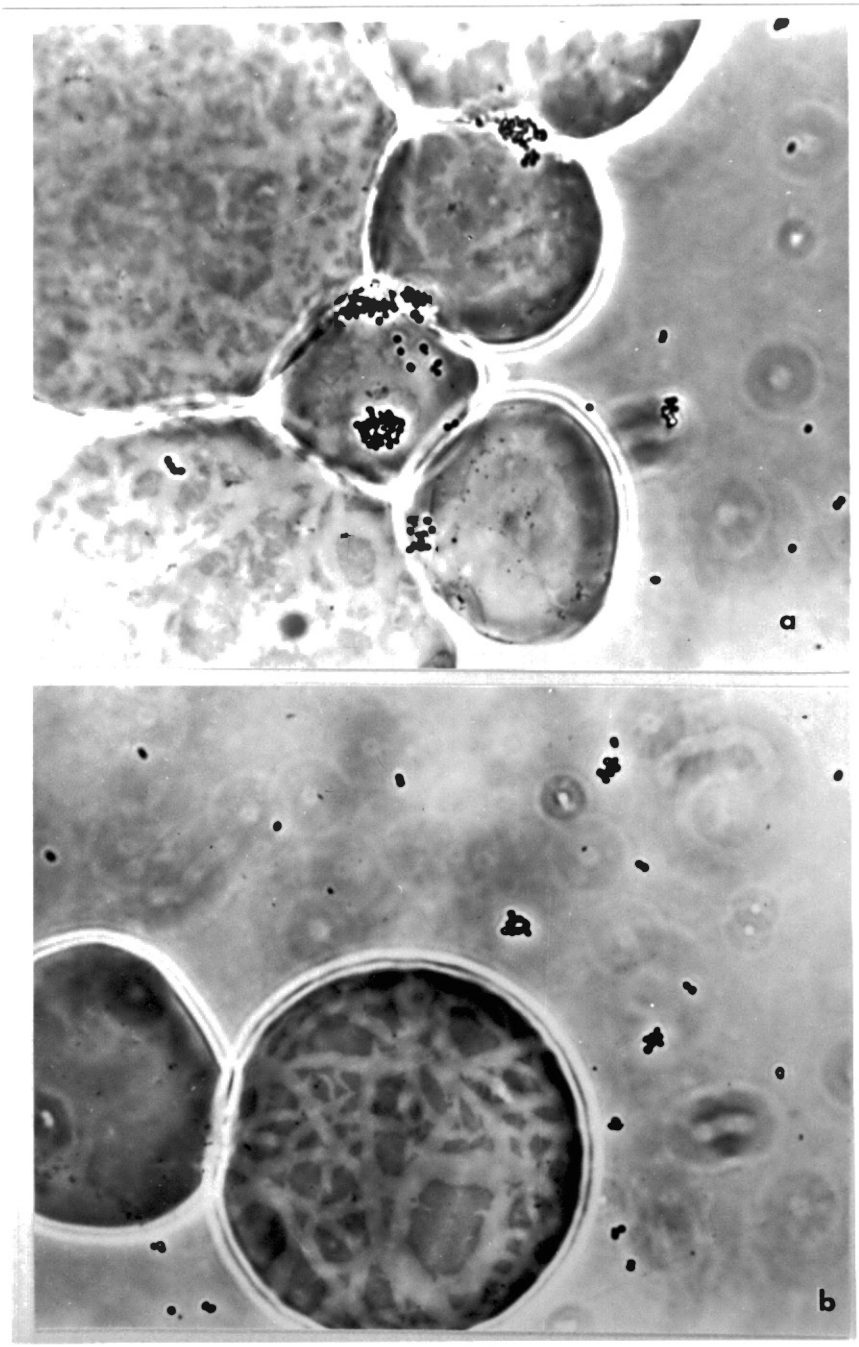

a
b

Figure 6. Affinity Chromatography of Supernatants From Sonicated Mutant E_1 Conidia. Eleven ml of supernatant from sonicated (five times for 20 sec with one min cooling period) conidia from three wk SDA culture was applied to a Sephadex G-25 column (1x45 cm) at Point A. Two ml of ten-fold concentrated supernatant from well-washed sonicated conidia (rinsed three times in PBS with vigorous vortexing) was applied to the same column at Point B. Both samples were eluted with PBS followed by 0.25 M glucose in PBS (arrows). The second sample was also eluted with 0.5 M glucose in PBS (small arrow). Between runs the column was rinsed with 0.2 M glycine-HCl buffer, pH 2.2, and equilibrated with PBS. Absorbance was continuously monitored at 280 nm (———). Large fractions were concentrated (dialysis) and analyzed for protein content (-○-; BioRad) and HA activity (;□ 2% ChRBC in PBS made with tap water). (HA activity was never observed when assayed using PBS made with deionized water; EDTA inhibited all activity in concentrated fractions.)

HA Titer
2
1
μg Protein / ml Fraction
A280
40
20
0
Fractions
Time (hr)
1
2
3
4
5
6
7
8
2
4
8
12
A
B

In order to determine what part of the conidial wall might be removed by sonication, dormant and germinating conidia were sectioned and observed using TEM. Dormant conidia have a very thick (approximately 0.2 um) wall composed of five or six layers (Fig. 7). This structure makes sectioning very difficult. Upon germination, the outermost layers are eventually removed (Fig. 8). This same process can be observed with SEM (Fig. 9). Thin sections of sonicated conidia, however, did not show a consistent loss of these outer layers although the walls were thinner (approximately 0.15 um) (Fig. 10). What exactly is removed from the wall by sonication is unknown.

Infected larvae were observed to determine if the agglutinin served a specific role in the initiation of the infection process. Conidia treated with glucose or NAG prior to infection showed a drastic loss in ability to adhere to CEW larvae (Fig. 11). Conidia treated with Ca^{++} and Mn^{++} did not have an enhanced binding or penetrating ability (Fig. 11). In fact, cation-treated conidia behaved like conidia from poorly pathogenic mutants; i.e., they germinate and grow extensively over the surface of larvae without any obvious penetration. Sonicated conidia did not exhibit a great loss in adherence, but did undergo germination with some penetration (Fig. 12). Their ability to penetrate was lost if conidia were sonicated, frozen, and resonicated (Fig. 12). Numerous infections with conidia

Figure 7. Thin Sections of Dormant Conidia. Outer layers of the wall are partially removed.

a. Dormant conidium with thick wall (19,900x).

b. Dormant, swollen, and germinating conidia (10,200x).

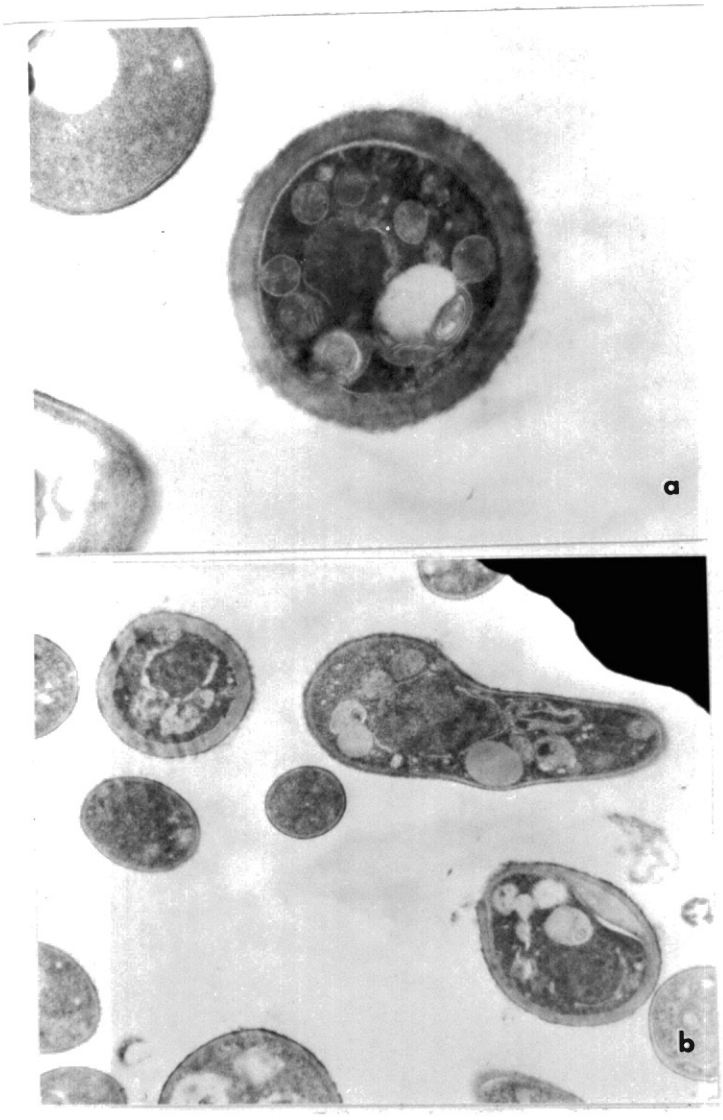

a
b

Figure 8. Thin Sections of Germinating Conidia.

a. Outer layers of wall are partially removed (46,700x).

b. Outer layers of the wall are completely removed (35,000x).

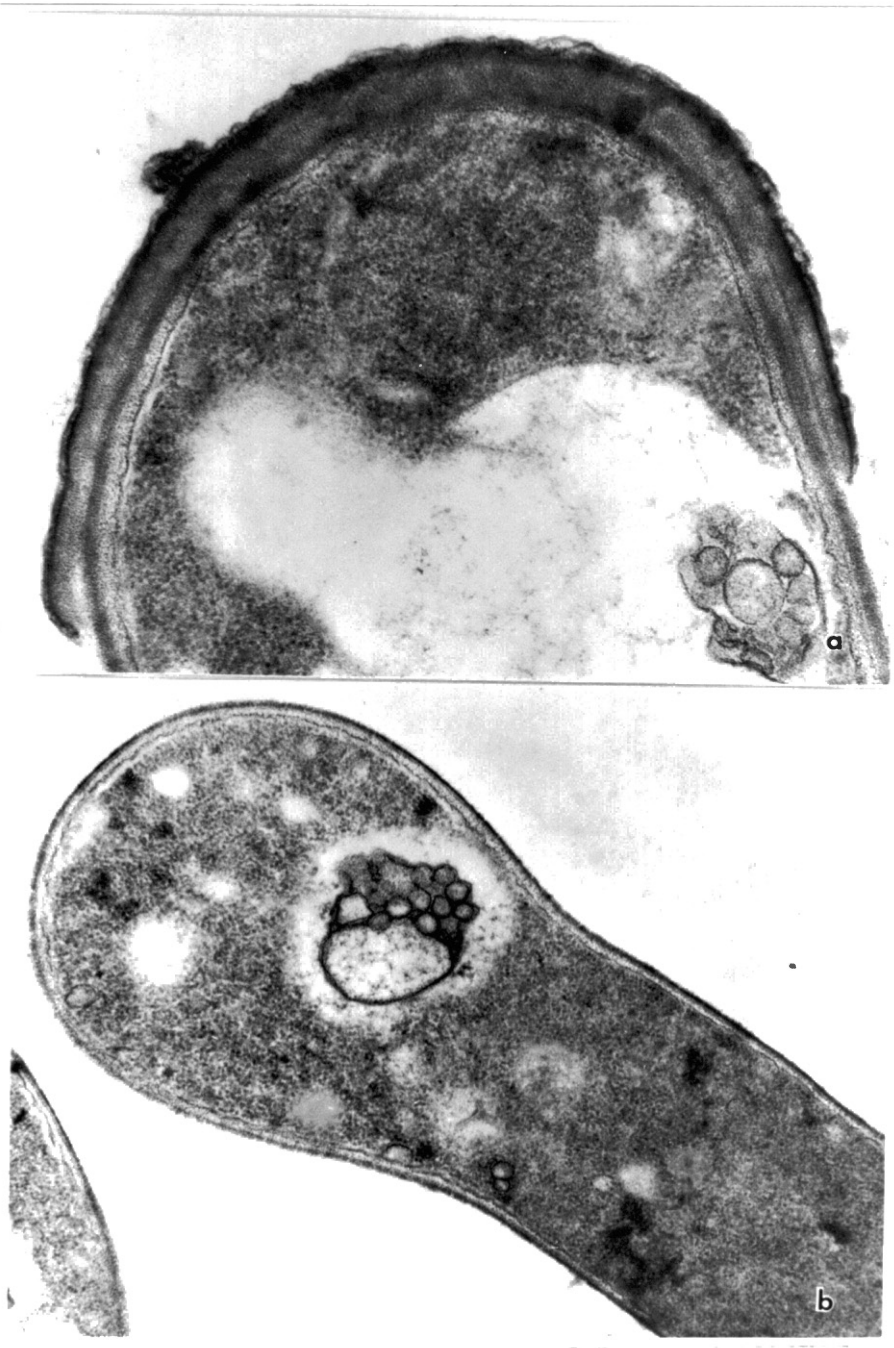

a
b

Figure 9. Germinating Conidium as Viewed with SEM. Note the fragment of wall (arrow) and the rough surface of the conidium (12,200x).

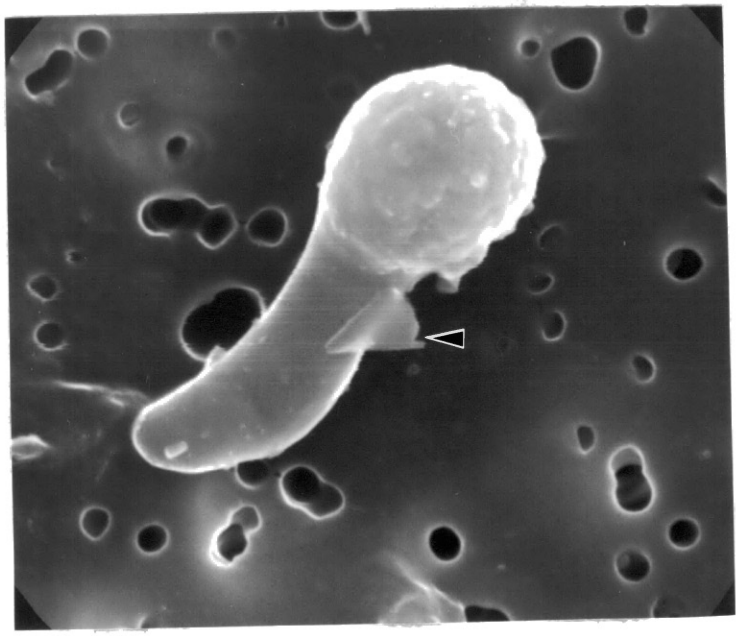


Figure 10. Thin Section of Sonicated Conidia.
Conidia were collected from three wk cultures and sonicated three times for 20 sec with one min cooling period. The walls appear to be intact (68,700x).

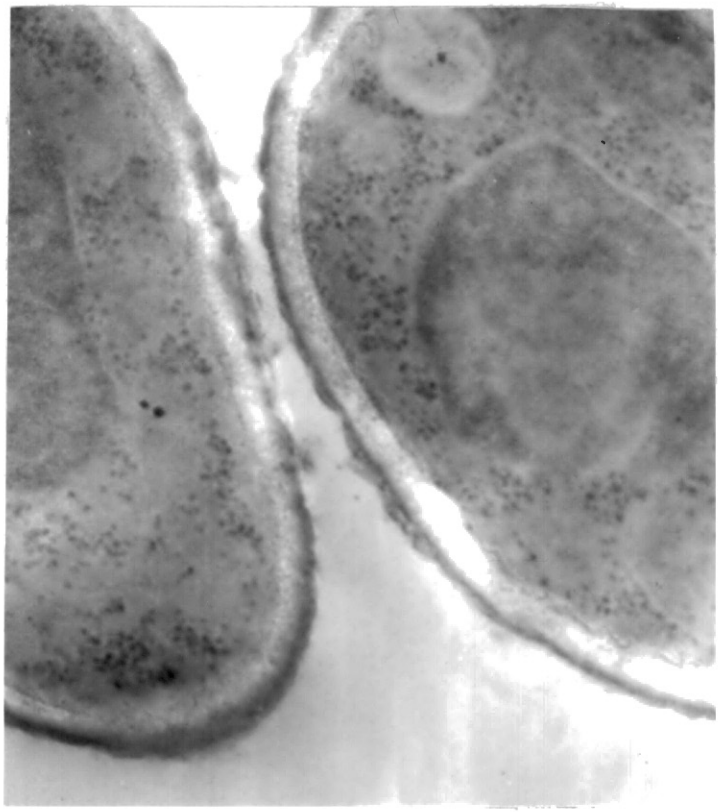


Figure 11. Infections of CEW with Treated Conidia of Mutant E_1.

a. Conidia treated with glucose prior to infection (1300x).

b. Conidia treated with NAG prior to infection (2200x).

c. Conidia treated with mineral cation solution prior to infection (540x).

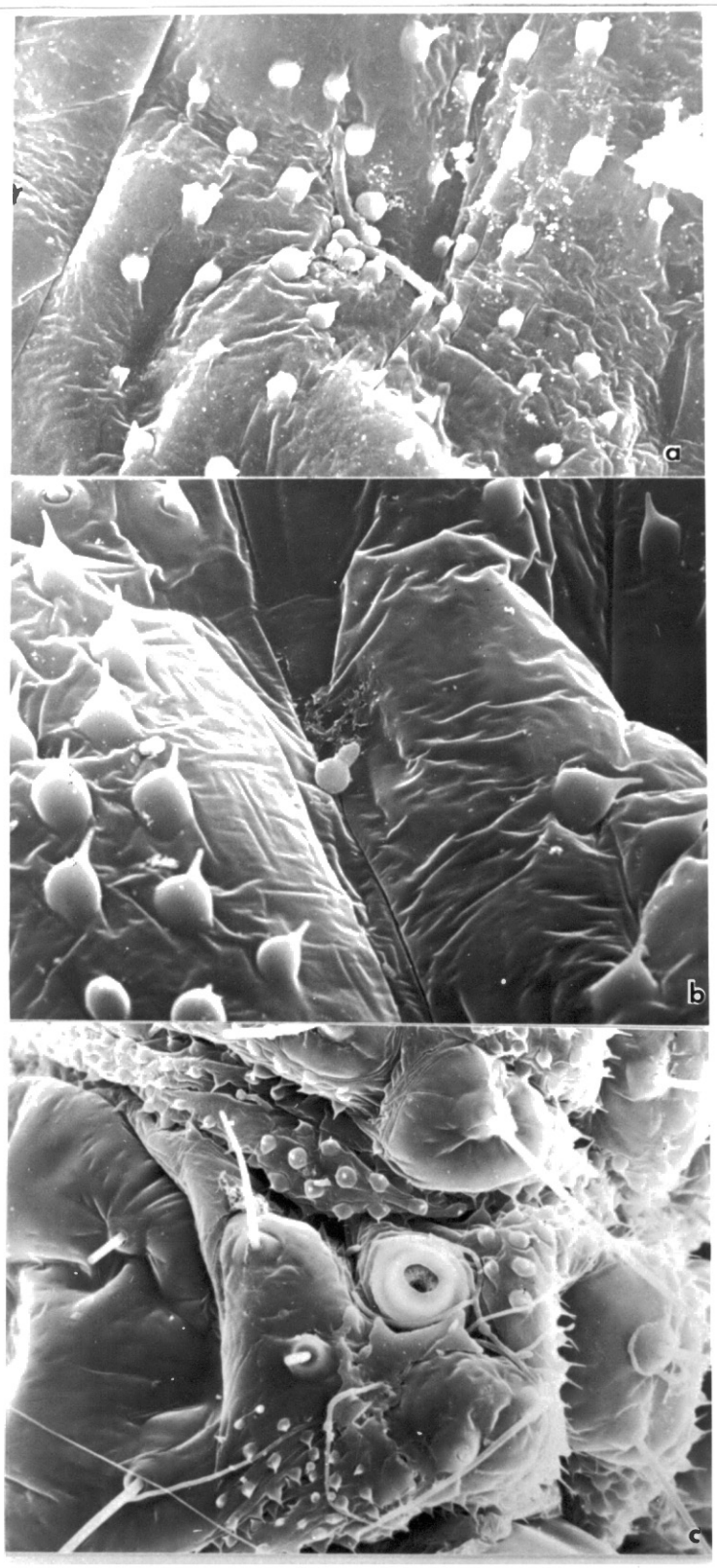
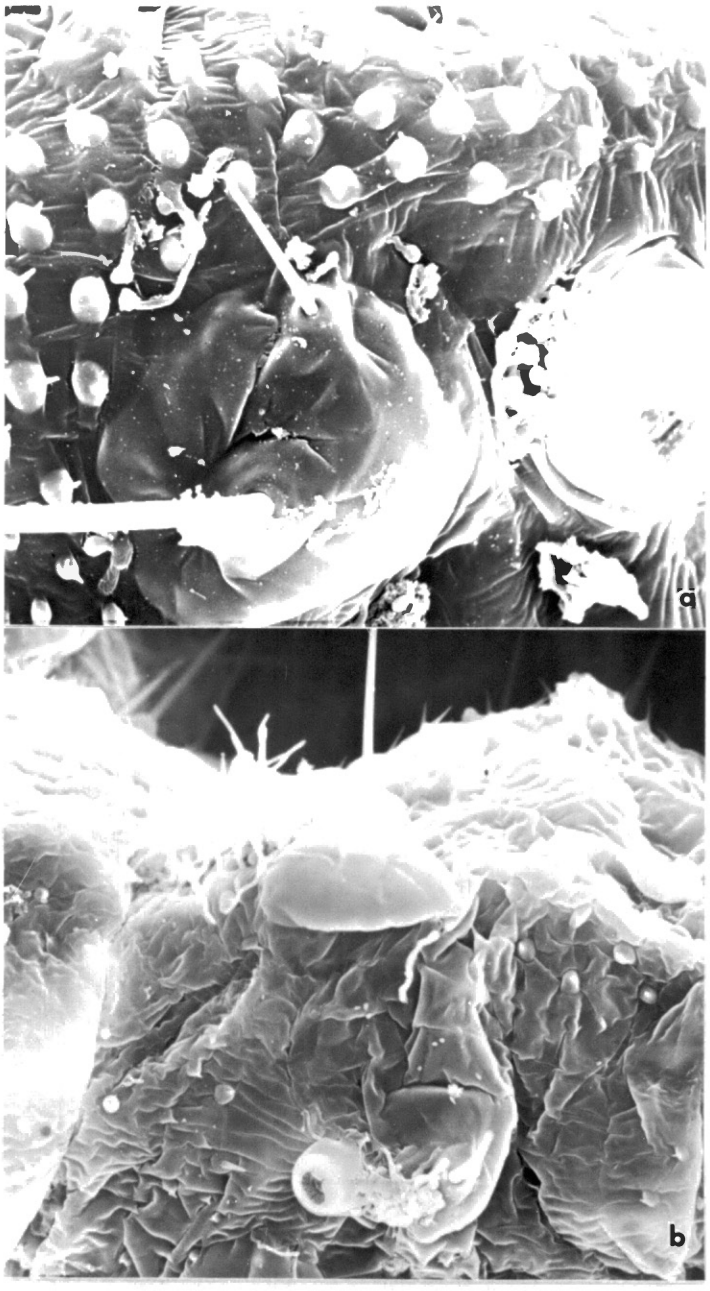

a
b
c

Figure 12. Infections of CEW with Sonicated Conidia of Mutant E_1.

Conidia were collected from a three wk culture on SDA.

a. Conidia sonicated (three times for 20 sec with one min cooling period) prior to infection (1200x).

b. Conidia treated as above then frozen, thawed, and resonicated (same treatment) prior to infection (600x).


a
b

pregerminated in SDB (12-15 hr cultures) failed to show any penetration (Fig. 13).

An interesting phenomenon was observed when fall armyworms (FAW) were infected with mutant E_1 conidia. Conidia adhered to and germinated on all parts of the body, but penetration only occurred on the head (Fig. 14). In fact, early penetration (soon after germination) was accomplished by almost every conidium which germinated there. As pointed out previously (Pekrul and Grula, 1979), penetration by B. bassiana on the head of CEW larvae was never observed.

Most of the data presented above strongly suggest the presence of a lectin on the conidial surface which facilitates adherence and possibly early penetration of larvae. The absence of such a lectin could account for the extensive growth without penetration which is observed with poorly pathogenic mutants (Pekrul and Grula, 1979). A variety of tests were conducted to determine differences in amounts and behavior of lectins among mutants of B. bassiana. Three mutants were used: E_1, a good pathogen (LD_{50}= 15); R_1, an intermediate pathogen (LD_{50}= 100); and 14, a very poor pathogen (LD_{50}= 320) (Pekrul and Grula, 1979).

Visual adherence of conidia to erythrocytes was used as the simplest test for the presence of a surface haemagglutinin. Unfortunately, conidia from all mutants formed aggregates with ChRBC (Fig. 15). The only notable

Figure 13. Infection of CEW with Mutant E_1 Conidia

Pregerminated in SDB.

Conidia were collected from a 15 hr SDB culture. Note the lack of penetration (720x).

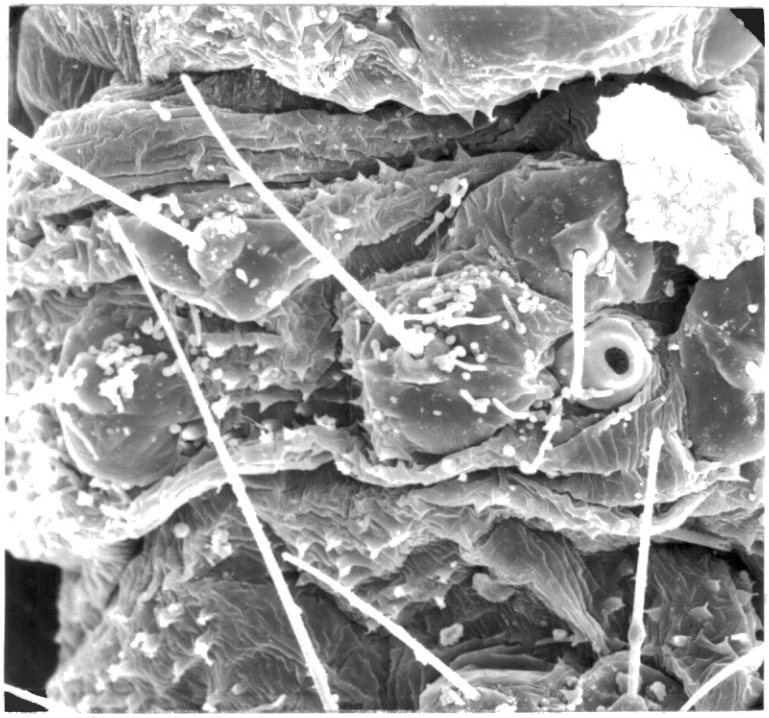


Figure 14. Infection of the FAW with Conidia of Mutant E_1.

a. Penetrating conidia on FAW head (320x).

b. Higher magnification of penetrating conidia (2200x).

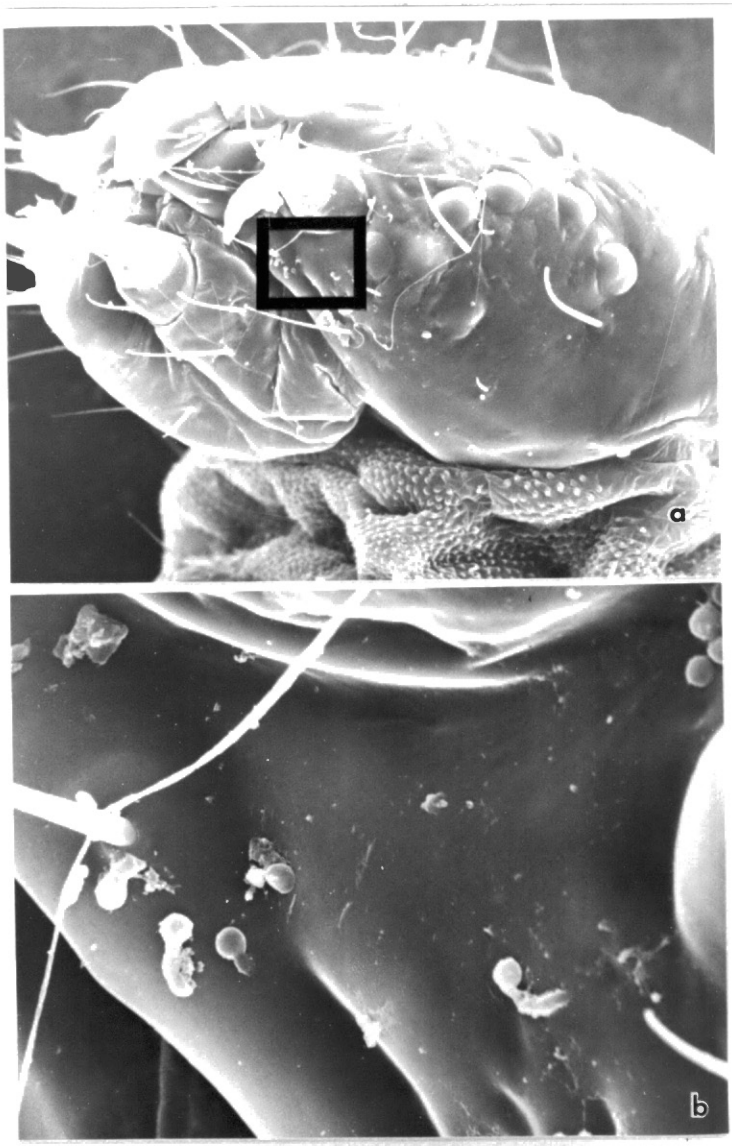

a
b

Figure 15. Adherence of ChRBC to Conidia of *B. bassiana* and *A. niger*.

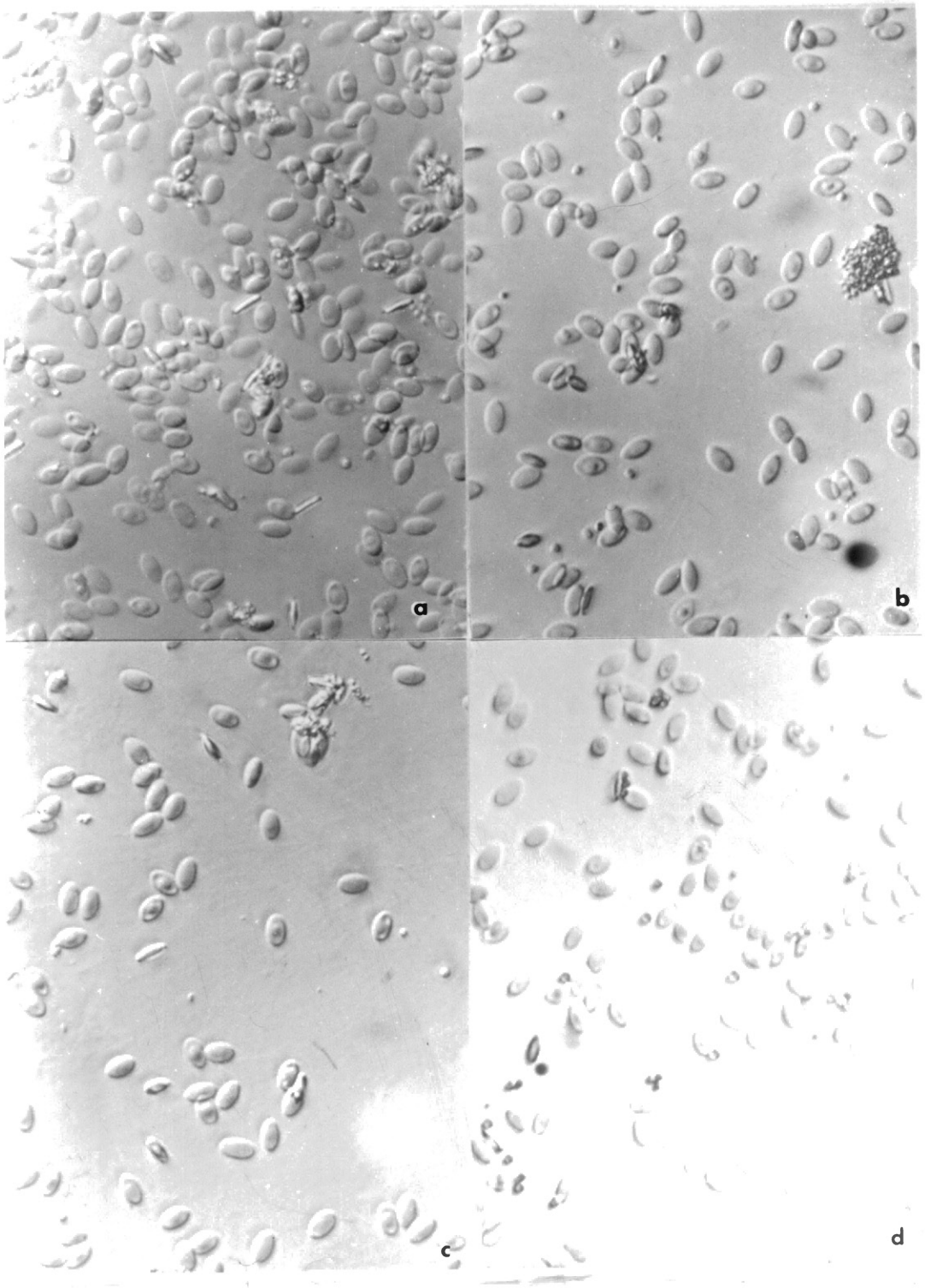
Photographs were made using Hoffman modulation contrast microscopy (770x).

a. Mutant E_1.

b. Mutant R_1.

c. Mutant 14.

d. *A. niger*.


a
b
c
d

differences were in the size of aggregates and in the number of free, unbound conidia. The best pathogen, E_1, had the largest aggregates and the fewest free conidia. Conidia of A. niger, a nonentomopathogenic fungus, had no affinity for ChRBC (Fig. 15). Because of the subtle differences among B. bassiana mutants, it was decided impractical to quantitate surface HA activity using this method.

Lectins, by causing agglutination, increase the rate of RBC sedimentation as determined by a decrease in O.D. at 620 nm. Since conidia form large aggregates with erythrocytes, they should increase their sedimentation rates. The O.D. of suspensions of RBC and conidia were monitored for three hr (Figs 16 and 17). Four blood types in three buffers were used with three mutants. The largest decrease in O.D. resulted with all three mutants in ChRBC at one hr; NAG inhibited this increased sedimentation rate while glucose did not (Fig. 16). Sedimentation rates of human RBC did not increase greatly in the presence of conidia, especially when compared to supposedly sugar-inhibited suspensions; these data are exemplified by human type B cells (Fig. 17). Because of its inconsistencies, this method was also rejected as a reliable and reproducible method to compare mutants.

Supernatants of sonicated conidia were compared using affinity chromatography. Equal amounts of similar concentrations were run on an 11 ml Sephadex G-15 column (Fig. 18). Peaks from each mutant were eluted with glucose.

Figure 16. Sedimentation of ChRBC in the Presence of *B. bassiana* Conidia.

Percentages given have been corrected against sedimentation of control ChRBC. Conidia of Mutants E_1 (○), R_1 (▽), and 14 (□) were used.

a. Cells suspended in PBS.

b. Cells suspended in 0.05 M NAG in PBS.

c. Cells suspended in 0.05 M glucose in PBS.

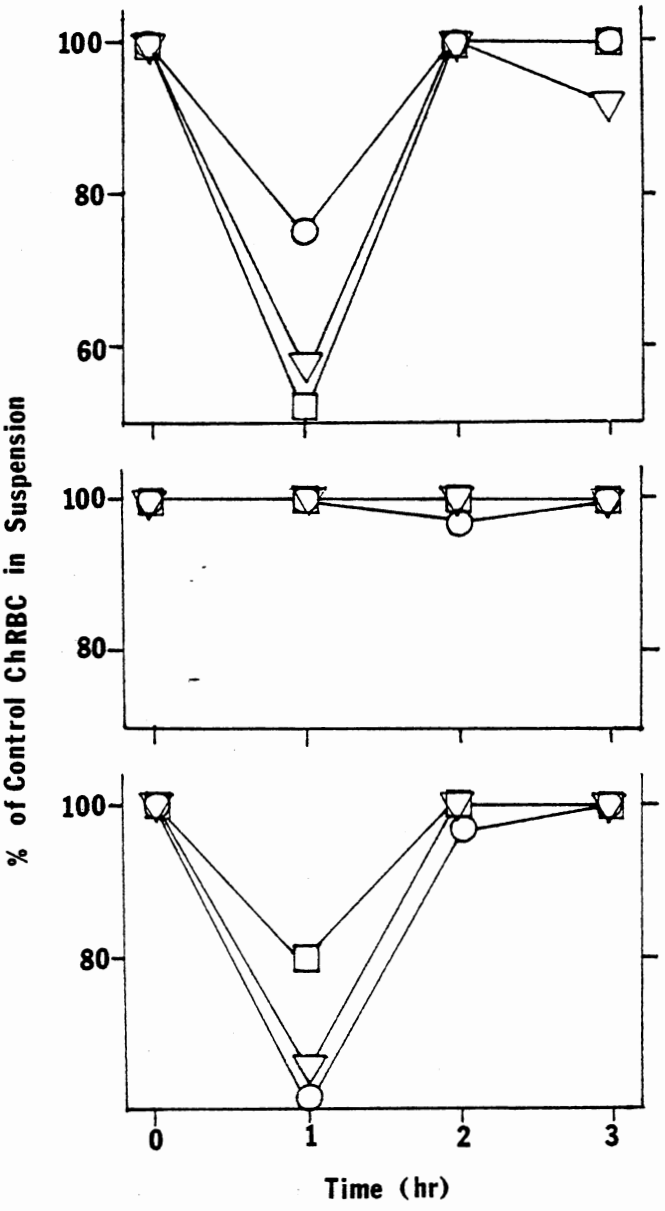

100
80
60
100
80
100
80
0
1
2
3
% of Control ChRBC in Suspension
Time (hr)

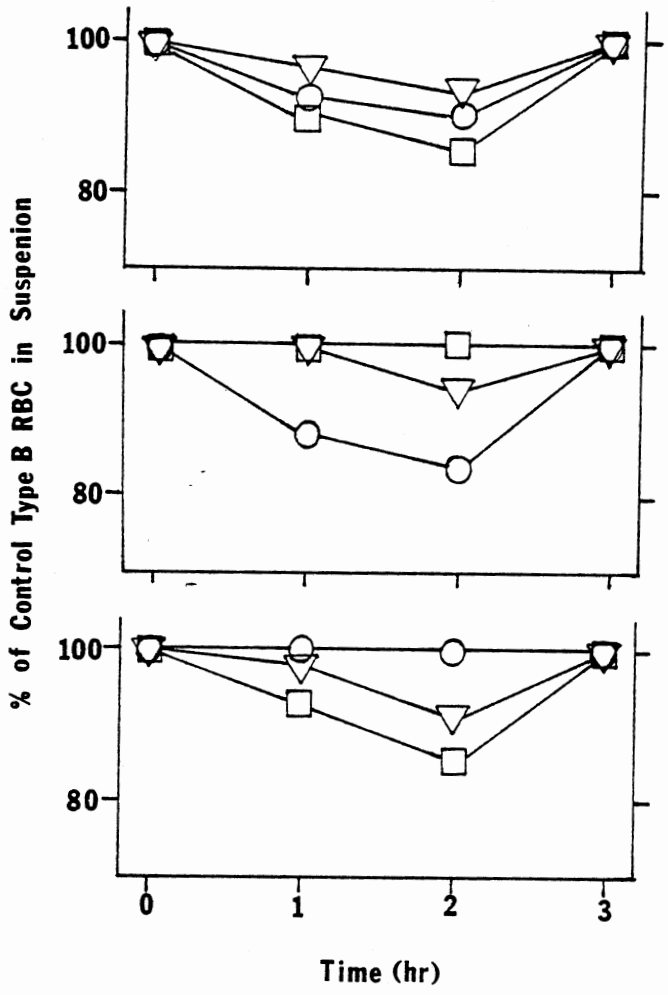
Figure 17. Sedimentation of Human Type B Erythrocytes in the Presence of B. bassiana Conidia.

The results with type B cells are representative for human type A and O cells. Percentages given have been corrected against sedimentation of control RBC. Conidia of Mutants E_1 (○), R_1 (▽), and 14 (□) were used.

a. Cells suspended in PBS.

b. Cells suspended in 0.05 M NAG in PBS.

c. Cells suspended in 0.05 M glucose in PBS.


100
80
100
80
100
80
0
1
2
3
% of Control Type B RBC in Suspenion
Time (hr)

Unfortunately, the peaks did not vary much in size and, therefore, the amount of protein released with glucose was almost the same for each mutant.

Finally, comparisons were made with agglutinin preparations from large SDB cultures of the three mutants. Spent medium and two supernatants were prepared by sonication from each mutant. All concentrations were kept constant among the mutants. Relatively low titers were observed with ChRBC (Table IV). R_1 and 14 had the highest titers among spent medium samples. The highest HA activity in sonicated samples was in the first preparation obtained from E_1. The only mutant to show activity in supernatants from sonicated frozen conidia was R_1. No large differences among the mutants were ever observed.

Figure 18. Affinity Chromatography of Supernatants of Sonicated Conidia of Mutants E_1, R_1, and 14.

Conidia of each mutant were collected from four wk SDA cultures and sonicated in 20 ml PBS (three times for 15 sec with one min cooling period). Four ml of supernatant was applied to a Sephadex G-15 column (1 x 11 cm) at Point A. The bulk of the protein was eluted with PBS at 25°C. Bound proteins were eluted with 0.2 M glucose in PBS at Point B. Absorbance was continuously monitored at 280nm.

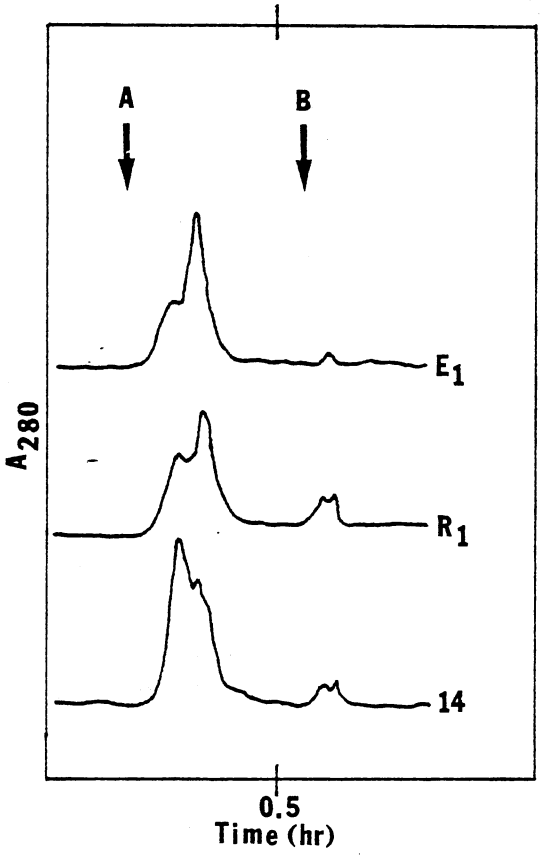

A
B
E_1
R_1
14
A_{280}
0.5
Time (hr)

TABLE IV

HAEMAGGLUTINATING ACTIVITIES OF PREPARATIONS FROM TEN-DAY SDB CULTURES OF B. BASSIANA MUTANTS E_1, R_1, AND 14

Agglutination Preparation	Mutant	Titer With 2% ChRBC[a]	Microscopic Observations[b]
Spent Medium[c]	E_1	4	+
	R_1	8	+++
	14	8	+++
Supernatant from Sonicated Cells[d]	E_1	16	+++
	R_1	4	++
	14	4	++
Supernatant from Sonicated Frozen Cells	E_1	-	+
	R_1	8	+
	14	-	-

[a]Titers given are reciprocals of highest dilution which agglutinated RBC in microtiter plates.

[b]Observations with phase microscope at two hr. Ratings are on a scale of 1-4 with 4=excellent.

[c]Spent medium was concentrated 30-fold by dialysis against polyethylene glycol.

[d]Supernatant from sonicated cells (eight times for 20 sec with one min cooling period) was concentrated 20-fold by dialysis against polyethylene glycol.

[e]Cells were sonicated (eight times for 20 sec with one min cooling period), frozen overnight, thawed, and resonicated (three times for 20 sec, with one min cooling period). The supernatant was not concentrated.

DISCUSSION

HA activity from B. bassiana can be demonstrated using spent medium and supernatant from sonicated cells. This activity is most likely located in the walls of conidia and blastospores (unsoluble form), but appears to be released into the medium (soluble form). It is possible these activities are due to the same protein, even though differences in sugar specificities were observed. The soluble agglutinin could be released with the outer wall during germination. If this is the case, sugar specificity may vary due to conformational changes of the protein. While embedded in the wall, the agglutinin may be nonspecific; yet, once released, its conformation may alter to a NAG-specific form (Lis and Sharon, 1973). If the proteins are the same, the agglutinin could be a large protein judging from its elution time on gel filtration. It might be low in tyrosine and tryptophan since it gives lower values when quantitated by absorbance at 280 nm as opposed to the BioRad protein assay (based upon increased absorbance of a dye which binds to protein; Bradford, 1976).

The surface (insoluble) agglutinin is most likely in one of the outer conidial wall layers. The most likely layer is the outermost, wavy, electron-dense one (Fig. 8).

This layer does not appear to be firmly attached to the rest of the wall and might easily be removed with sonication. However, this was not clearly evident when sections of sonicated conidia were examined using TEM (Fig. 10). It is possible that the agglutinin is present in large quantities elsewhere in the conidium and sonication is not the most effective method of removal.

It is questionable whether the agglutinin is substantially enhanced in the presence of metal cations. These did not increase HA activities greatly unless the agglutinin samples had first been dialyzed or lyophilized. Extensive dialysis against PBS could remove a high concentration of metal ions. Crude preparations also showed less inhibition in the presence of EDTA than dialyzed fractions. The type of water used in making PBS affected titers; tap water gave a higher activity than deionized water. Our tap water contains 39.93 mg Ca^{++}, 14.96 mg Mg^{++}, and 29.75 mg Na^{+} per liter (data supplied by Dr. B. Burks). Considering such data, it is possible the agglutinin is enhanced by Ca^{++} and Mg^{++}.

One of the most disturbing recurrences was the loss of activity and protein after chromatography, dialysis, and/or lyophilization. Fractions had to be so highly concentrated that only small volumes were available for analysis. Fractions containing large quantities of HA activity should be analyzed with polyacrylamide gel electrophoresis to determine purity, size, and nature (glycoprotein or

lipoprotein) of the agglutinin.

The presence of an agglutinin on the surface of B. bassiana conidia and blastospores establishes a new perspective on the mechanism of infection initiation. The purpose of the agglutinin, if it is related to pathogenicity, could be to satisfy a critical requirement for conidial adhesion followed by germination and penetration.

Adhesion to the larval surface seems to be enhanced by the agglutinin. Conidia treated with glucose, NAG, or Con A prior to infections did not accumulate on the larvae as well as control conidia. In fact, these conidia were very difficult to find using SEM. Mineral cations enhanced the binding activity only a small degree. From these observations, it appears that the agglutinin is important in binding a conidium to a larva.

Germination could also be affected by the agglutinin, although no substantial evidence for this has been obtained. Many lectins influence both inter- and intracellular processes after their binding to cell surfaces (Barondes, 1981; and Lis and Sharon, 1973). It is possible but not very likely, that attachment of the agglutinin to a receptor helps initiate germination. This could be partially responsible for inhibition of germination by nonpathogenic fungi, such as A. niger (Part I; and Smith and Grula, 1982).

Without question, the most interesting function of the

agglutinin could be enhancement of early penetration. As mentioned before, mutants of B. bassiana penetrate larvae at different times following germination. The best pathogens penetrate soon after germination (early penetration) while the poorest pathogens penetrate only after extensive growth of their hyphae (Pekrul and Grula, 1979). The agglutinin could anchor a highly pathogenic conidium such that if the emerging germ tube is oriented toward the cuticle, it is forced to continue growing in that direction with the aid of cuticle degrading enzymes (Smith, et al., 1981). Conidia lacking the agglutinin would be mobile and allow emerging germ tubes to grow upon the surface; penetration by these unanchored hyphae would occur only if hyphal growth was blocked by cuticle. A model of this mechanism is presented in Fig. 19.

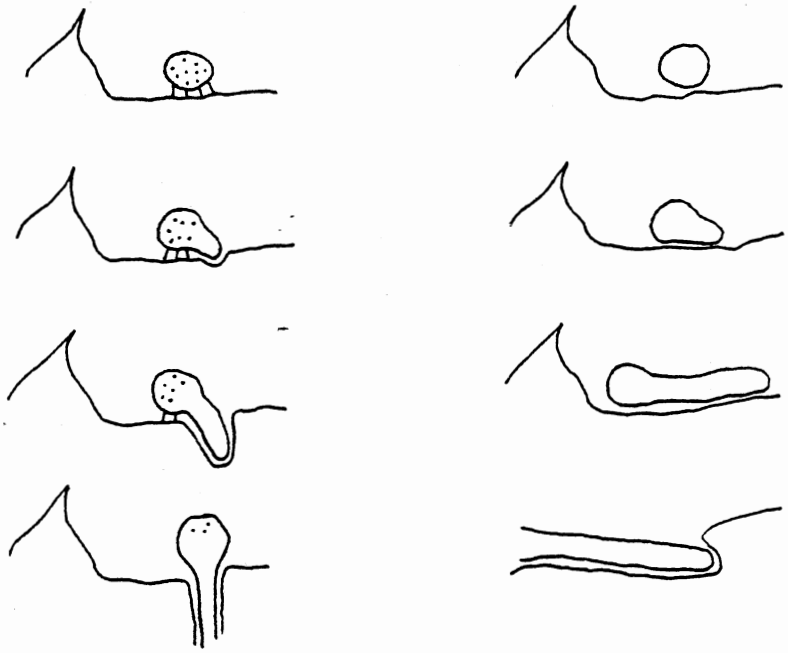
This mechanism could explain why conidia germinated in SDB prior to infection were never observed penetrating the cuticle. Soon after germination (12-15 hr cultures), the outer walls of conidia are shed. If the agglutinin is also shed at this time, it would not be able to secure conidia to the surface. This would force penetration to be fortuitous.

If an agglutinin enhances attachment and penetration, there must be receptors on the larval surface. These would most likely be glycoproteins or glycolipids judging from the composition of the cuticle (Hackman, 1974). These receptors could be localized on certain parts of larvae. Conidia adhered to all regions of the CEW, but few were usually

Figure 19. Possible Mechanism for Enhancement of Early Penetration of the Cuticle by the Agglutinin.

HIGHLY PATHOGENIC
MUTANT WITH
LECTIN ON CONIDIUM

LESS PATHOGENIC
MUTANT WITHOUT
LECTIN ON CONIDIUM



PENETRATION IS
SOON AFTER
GERMINATION

PENETRATION IS
FORTUITOUS
DUE TO ENZYMES

observed on the head and penetration was never seen there. Attachment by conidia also occurred everywhere on the FAW, yet penetration (which occurred immediately after germination) was only observed on the head. Thus it appears that receptors are located only on the head of the FAW whereas they are located only on the body of the CEW. With infections of the pecan weevil, penetration was observed only with long hyphae (Champlin et al., 1981). Receptors may be lacking or hidden (by soil materials) on the pecan weevil surface.

In order to determine if penetration was enhanced by the agglutinin, mutants with different penetrating abilities were compared. No easy and reliable method was found which enable one to show a difference in amounts of agglutinin on conidia. All mutants appear to produce agglutinin in seemingly equal amounts. Conidia of each mutants tested were able to adhere to ChRBC. However, conidia from E_1, a good pathogenic mutant, did seem to form larger aggregates when mixed with RBC and individual conidia of this mutant usually bound to erythrocytes. Far more unattached conidia were seen with the worst pathogen, 14. This leads one to believe that more agglutinin is present on the walls of highly pathogenic conidia. However, it was impossible to accurately quantitate that difference using such a visual assay.

The sedimentation rate of erythrocytes was not increased greatly by any conidia that were tested. In fact,

a substantially increased rate of sedimentation was only observed with ChRBC. Sedimentation rates of the human RBC types were not greatly affected by conidia. The largest differences between test and control suspensions occurred at the first hour reading; later readings were all nearly identical. This could result from the hydrophobic tendencies of conidia; their buoyancy could deter the sedimentation of aggregates.

Sonicated preparations from all three mutants gave similar results with affinity chromatography. Protein peaks eluted with glucose all appear to be of equal size. The only significant difference in the runs was that R_1 had a slightly larger agglutinin peak. From this, it appears that all mutants produce, or at least release with sonication, comparable amounts of agglutinin.

HA titers of preparations from the mutants were also quite similar. It may be of interest that E_1 gave the highest titer with sonication supernatant, whereas R_1 and 14 gave the highest titers when using concentrated spent medium. Activity from sonicated frozen conidia was only recovered from R_1. These differences could be an indication that the agglutinin has various degrees of attachment to the conidial wall. Agglutinin from R_1 and 14 may be easily released into the medium, whereas E_1 agglutinin can only be released by trauma or, perhaps, some type of enzymatic cleavage. Another possible explanation is that R_1 and 14

simply produce more germinating blastospores which release the agglutinin with the outer layers of the wall. In either case, it is evident that the amount of agglutinin obtained from each mutant will vary with the method of preparation.

These preliminary data strongly suggest the presence of haemagglutinin on the surface of B. bassiana conidia and blastospores which enhances adherence to larvae. More tests must be conducted to unequivically confirm this. The most obvious need is the purification of large quantities of protein for characterization and specific assays. Once the protein is purified, antibody can be produced and used to localize the lectin within the fungus. More specific assays should be used to determine the amount of agglutinin produced by each mutant. Functions of the agglutinin, if any, must be clarified. If the agglutinin is indeed proven to enhance pathogenicity, either by increasing adherence or early penetration, its presence could be used as a determining factor in the selection of highly effective mutants for use as biological insecticides.

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APPENDIXES

APPENDIX A

MODE OF INFECTION OF THE CORN EARWORM (*HELIOTHIS ZEA*) BY *BEAUVERIA BASSIANA* AS REVEALED BY SCANNING ELECTRON MICROSCOPY

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Mode of Infection of the Corn Earworm (*Heliothis zea*) by *Beauveria bassiana* as Revealed by Scanning Electron Microscopy

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Conidia from highly pathogenic mutants of *Beauveria bassiana* germinate quickly (within 18 hr) on the surface of corn earworm larvae (*Heliothis zea*) and immediately begin penetration of the cuticle. Enzymes produced by the penetrating hyphae degrade the cuticle since holes are formed at the point of entry. Clustering of conidia around nodules of larvae is often seen, but penetration is not restricted to such areas. Direct evidence is presented to show that conidia can also germinate inside of spiracle openings and could invade larvae by this route. Once inside the hemocoel, the fungus multiplies extensively; however, larval death occurs with only minimal breakdown of internal tissues. During mummification, outgrowths of fungal hyphae occur first and most extensively in the intersegmental regions of larvae. Conidia from mutants exhibiting low levels of pathogenicity are either significantly delayed or do not germinate on larval surfaces. When germination does occur, hyphae from such mutants do not penetrate immediately; instead, extensive surface hyphal growth, with or without eventual penetration of the integument, is evident.

KEY WORDS: *Beauveria bassiana*, germination and growth of, penetration and infection by; *Heliothis zea;* corn earworm; larval surface, fungal growth on; scanning electron microscopy.

INTRODUCTION

Beauveria bassiana has been shown to be an effective entomopathogen and is being developed in our laboratory for use as a mycoinsecticidal agent. Infection by this fungus has generally been regarded to occur as a result of direct penetration of the integument by growing hyphae (Lefebvre, 1934; Vey and Fargues, 1977; Ferron, 1978) and appears to be facilitated by both mechanical and enzymatic activity. Penetration through the alimentary tract (Broome et al., 1976) and the respiratory system (Clark et al., 1968; Hedlund and Pass, 1968) has also been reported.

An interesting ultrastructure study of infections by *Metarrhizium anisopliae* reveals that penetration occurs through the integument from appressoria (Zacharuk, 1970a,b). It was observed that appressorial cells contain numerous mitochondria, dictyosomes, ribosomes, and endoplasmic reticulum, suggesting a high level of metabolic activity. Zacharuk concluded that penetration of the epicuticle was primarily enzymatic in nature, but was initiated by mechanical activity. A later scanning electron microscopic (SEM) study of infection by *M. anisopliae* shows that hyphae grow entensively over the cuticle before forming appressoria and penetrating the host larva (Schabel, 1978). It has also been reported that *B. bassiana* produces appressorial-like structures during infection of *Leptinotarsa decemlineta* (Vey and Fargues, 1977).

In the present study, infection of *Heliothis zea* by mutants of *B. bassiana* having high and low levels of pathogenicity were observed using SEM to precisely determine the location and nature of the initial penetration. Light microscopy was also utilized to aid in determining the extent of destruction of intralarval cells and tissues.

MATERIALS AND METHODS

Eleven mutants of *B. bassiana* were obtained in this laboratory by irradiating (UV) conidial suspensions, then selecting for varying levels of proteolytic activity by plating on Sabouraud's dextrose agar

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(SDA) containing litmus milk (casein protein). Pathogenicity (as defined by LD_{50} and LC_{50} values) toward the major target pest, *Heliothis zea*, was determined by probit analysis using 150–200 larvae for each of three to four concentrations (threefold dilutions beginning with 10^6 conidia/ml) of fungal conidia (Cheung and Grula, unpubl.).

Conidia of *B. bassiana* were collected from 7-day cultures grown at 25°C on SDA. To obtain infections, first-instar larvae were allowed to crawl on filter paper containing conidia for 10 min. No attempt was made to apply a constant amount of conidia to each larva and both wet (water-washed) and dry (nonwashed) conidia were placed on filter paper. Following such treatment, the larvae were placed on Burton's CSM diet (Burton, 1970) in which D-chloramphenicol (0.5 g/3.8 liter) was used to replace the antifungicides (formaldehyde, sorbic acid, and *p*-hydroxymethylbenzoic acid) normally employed. Untreated larvae were used as controls and grown on the same medium at 25°C.

Larvae for the SEM study were killed and fixed in 4% glutaraldehyde for 2–4 hr followed by 1% osmium tetroxide for 2 hr using 0.2 M cacodylate buffer at pH 7.3 (Hayat, 1970). After dehydration with an ethanol series (30–100%), the larvae were critical-point-dried and coated with gold–palladium (Hayat and Zirkin, 1973). The samples were then examined using a JEOL JSM 35 microscope.

Samples to be examined by light microscopy were killed and fixed in alcoholic Bouin's and dehydrated in an ethanol and tertiary butyl alcohol series. After embedding in paraffin, the larvae were sectioned at 4–10 μm and stained with lactophenol cotton blue and Mayer hemalum–eosin (Grimstone and Skae, 1972).

RESULTS

Infections Using Highly Pathogenic Strains

With the infection process used, conidia were usually distributed on the ventral side of the insect and commonly found in the vicinity of the setae and the noncellular, horn-like nodules (Figs. 1–7). When the inoculum is large, conidia adhere to all regions of the cuticle and exhibit a tendency to clump (Fig. 2).

Conidia are able to germinate on all regions of the cuticle. Hyphae from the germinating conidia exhibit a strong orientation (positive chemotaxis) toward the cuticle and penetrate very soon after germination (Figs. 1, 4, 6, 7, 12). Penetrating hyphae have an average visible length of 1.4 μm. Penetration is observed as early as 16–18 hr after infection. Due to the location of the conidia, penetration usually occurs at the base of the nodules (Figs. 1, 3, 7), but can be observed elsewhere (Figs. 4, 5, 6, 8). It should be noted that multiple infections (penetration of a single larva by more than one germinating conidium) occur.

Penetration through the cuticle occurs without the formation of any visible appressorial-like structures. In some cases, there are slight thickenings of the penetrating germ tubes (Fig. 4), but appressoria have not been observed. A clean circular hole can easily be seen around the germ tube at the point of penetration of the cuticle (arrows in Figs. 1, 4, 6, 7, 8).

Germinating conidia have been seen on the heads of heavily infected larvae, but penetration of the structure has never been observed. Instead, the hyphae grow extensively in an errant manner over the surface (Fig. 9). Such growth is in striking contrast to that observed on other regions of larvae.

In addition to direct penetration through the integument, the fungus can enter through the respiratory system. *B. bassiana* conidia are small enough (average diameter is 2.5 μm) to be trapped inside a spiracle (diameter of 5.3 μm or larger) and are able to germinate there (Fig. 10). Germ tubes of conidia on the outside surface are also able to enter the spiracle both through the spiracle opening or by penetration through the side of the spiracle (Fig. 11).

Extensive fungal growth is evident in the hemocoel within 48 hr after the exposure to

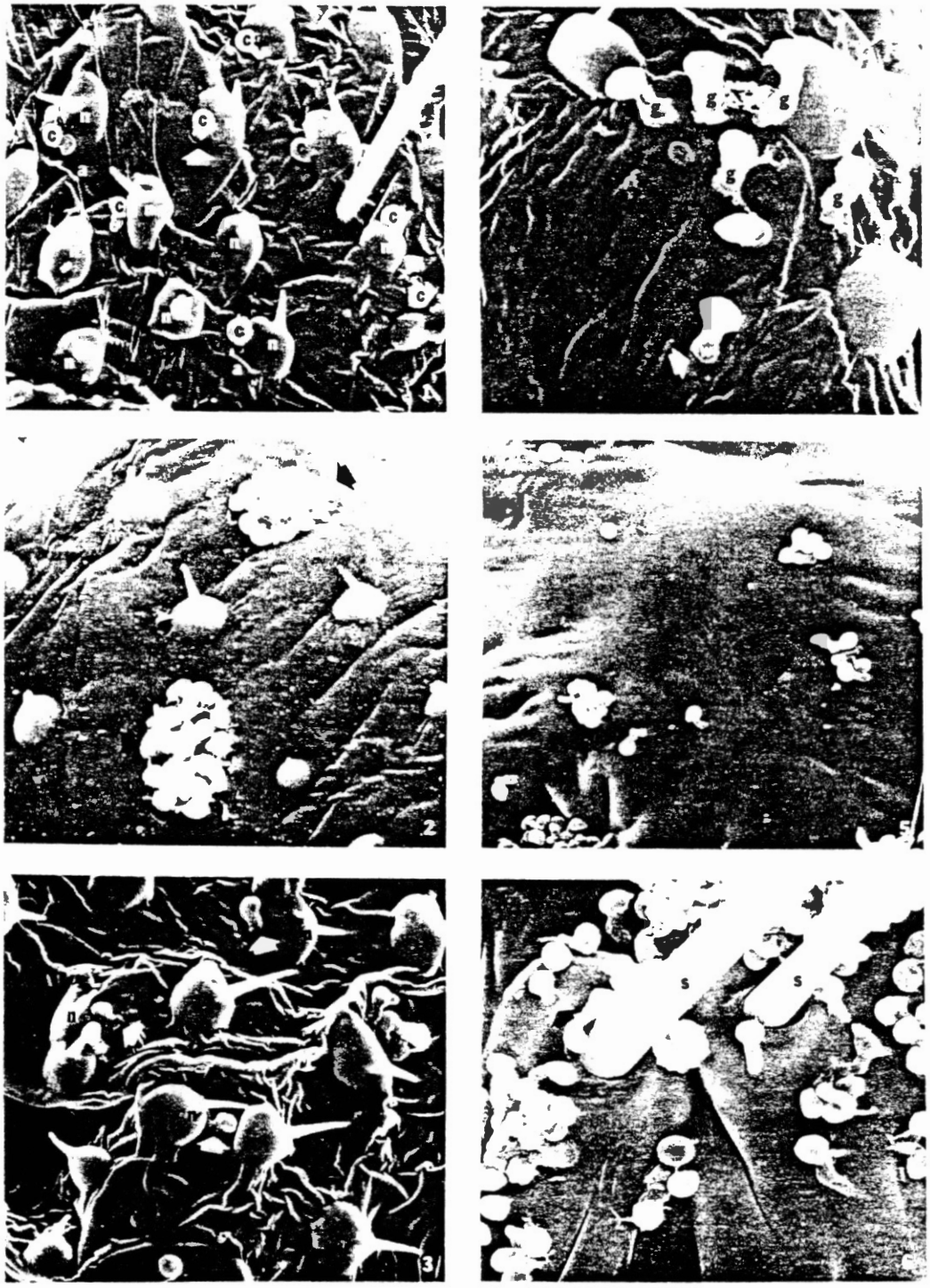

FIG. 1. Distribution of conidia (c) near the horn-like nodules (n). Enzymatic degradation of the integument is shown by the hole at the point of penetration by the germ tube (arrow). Artifactual wrinkling of the integument is angular (a). Mutant E_1. (Fixed 18 hr postinfection.) ×1660.

FIG. 2. Accumulation of conidia near the nodules (arrows). Mutant 10 (24 hr postinfection). ×1494.

FIG. 3. Penetration (arrows) of the integument at the base of the nodules (n). Mutant E_1 (24 hr). ×1660.

fungal conidia (Fig. 13). This growth takes place with only minimal tissue damage. When tissue infiltration occurs, the fat body is the first tissue to be infected and show signs of deterioration. This does not occur, however, until about 60–72 hr after infection. Some degradation of gut tissue and Malpighian tubules can also be seen; however, such activity is not evident until the fourth to sixth day, at which time death has occurred and mummification is usually evident. Tissues which still appear unaffected, even in extensively mummified larvae, are the silk glands, muscle, and tracheae.

Hemocytes could be observed in all stages of the infection; however, due to the extensive hyphal growth in the hemocoel, relative numbers of these cells and their activities could not be determined.

Although, as pointed out, some tissues are affected late in the infection cycle, it was interesting to note that death and mummification can actually occur with no apparent tissue damage (Fig. 14). The hyphae can be protruding through the integument while the gut, muscle, tracheae, silk glands, and some fat bodies are still intact. During this stage the hemocoel is completely filled with fungal growth.

Study of mummified larvae reveals that hyphae exit first at the intersegmental regions (Fig. 15), but eventually appear over the entire insect. No structures have been seen either at the hyphal tips or at the point of exit (Fig. 16).

Infection Using Low Pathogenic Mutants

The growth of mutants exhibiting low pathogenicity is in striking contrast to that described above. Nongerminating clusters of conidia (Fig. 17) can be observed as late as 28 hr postinfection. In addition, the majority of conidia that do germinate yield hyphae that grow errantly over the surface of larvae (Figs. 18–20). Penetration by a few hyphae is sometimes observed. When it occurs, some hyphae may penetrate soon after germination; however, penetration is more often effected by hyphae that have undergone significant elongation (as much as 20–30 μm (Fig. 19)). Extensive elongation of hyphae prior to penetration is rarely or never seen using highly pathogenic mutants. As with highly pathogenic strains, when penetration occurs, it is due to enzymatic activity since clean circular holes are produced at the point of entry (arrow in Fig. 19) and appressoria are never observed. Nonpathogenic fungi, such as *Penicillium chrysogenum* and *Aspergillus niger*, do not germinate on the surface of larvae (Figs. 21, 22) even after 6 days contact.

Enzymatic Profiles and LD_{50} Values of Mutants

Data presented in Table 1 compare the LD_{50} values and SEM ranking of pathogenicity with the amount of exocellular enzymes (chitinases, proteases, and lipases) elaborated by *B. bassiana*. Regarding interpretation of these data, we wish to caution that, depending on composition of the growth medium, elaboration of exocellular enzymes by *B. bassiana* can vary widely (Grula et al., 1978). Nevertheless, the data presented in Table 1 strongly suggest that low entomopathogenicity (errant type of surface hyphal growth) is not simply a consequence of the lack of what is commonly accepted as the enzyme "cocktail" (Samsinakova et al., 1971) supposedly required for penetration of insect integuments. All mutants possess varying levels of the three major enzyme activities regardless of their pathogenicity and some

FIG. 4. Orientation of germ tubes (g) toward the integument. Penetration does not always occur at the base of the nodules and some penetrating hyphae exhibit a slight increase in width (w). Formation of a hole is occurring at the base of one penetrating hypha (arrow). Mutant E_1 (24 hr). ×2988.

FIG. 5. Several germ tubes penetrating soon after germination. R_1 strain (26 hr). ×1079.

FIG. 6. Conidia adhering and penetrating near setae (s). Arrow indicates hole formation. Mutant E_1 (18 hr). ×2158.

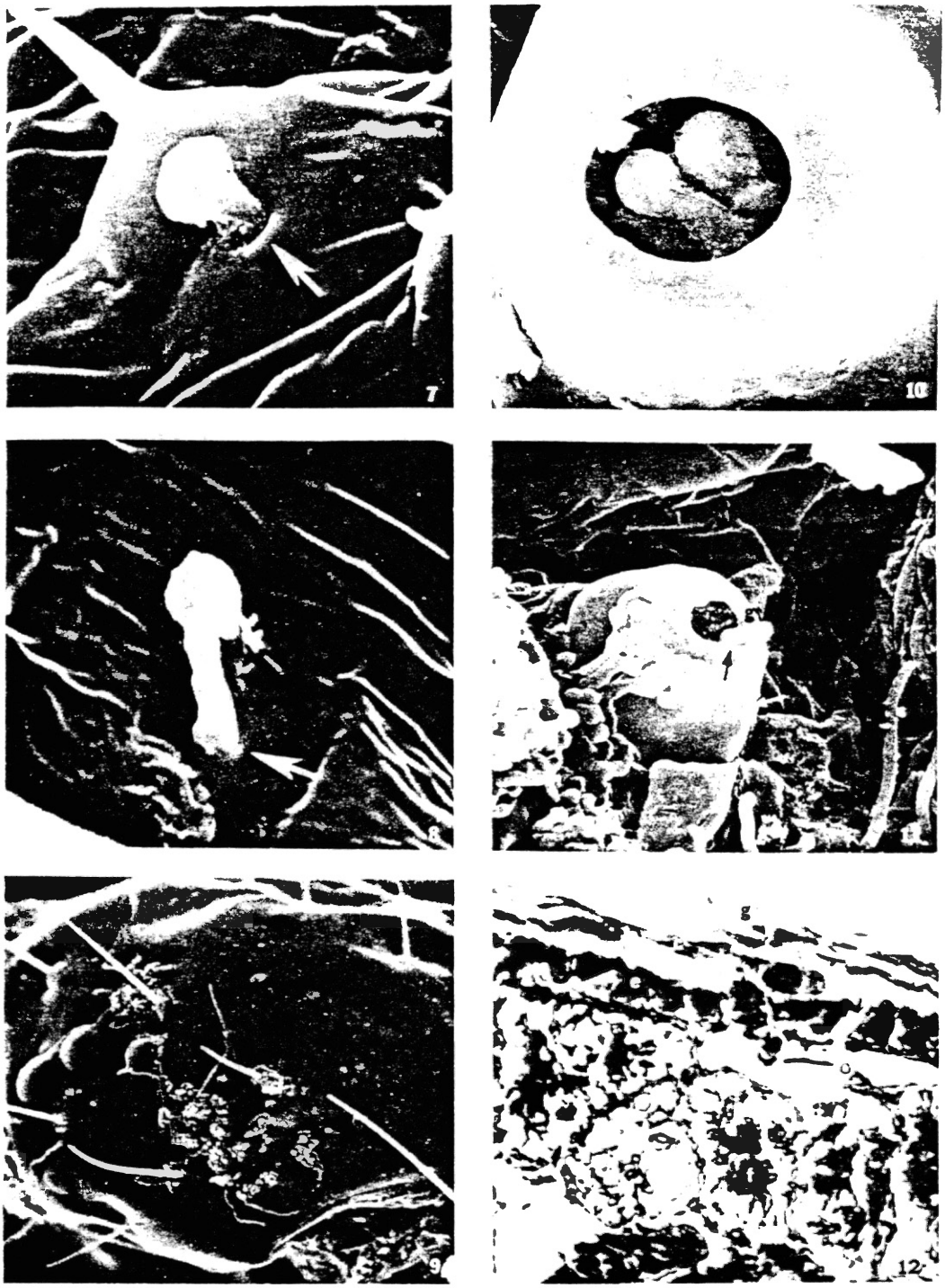

FIG. 7. Enzymatic degradation and production of a hole in the integument by an invading germ tube (arrow). Mutant E_1 (18 hr). ×6474.

FIG. 8. Hole formation by a penetrating hypha (arrow) of the R_1 strain. Some hyphal growth is evident prior to penetration (26 hr). ×4814.

FIG. 9. Hyphae from germinated conidia growing errantly and unable to penetrate the head. Mutant E_1 (20 hr). ×299.

mutants (20 and 14, for example) contain very high levels of certain enzyme activities yet are poor pathogens.

DISCUSSION

Using larval death as an endpoint, reliable data (LD_{50} or LC_{50} values) can be obtained to show that a certain number of conidia must be placed on a larval surface to successfully infect and kill an insect and thus determine the degree of pathogenicity of a culture. However, such analyses can only determine whether a viable conidium has germinated and penetrated thus establishing an active infection or has failed to penetrate. Observations reported in this paper obtained using the SEM have clarified and extended our data since growth behavior of germinated conidia on the larval surface can be readily followed. At least three different situations have been observed after germination of conidia from mutants *B. bassiana:* (1) immediate penetration, (2) errant growth of hyphae without penetration, and (3) a limited amount of errant growth followed by eventual penetration.

The data are interesting and provocative and could shed new light, at a hitherto unsuspected level, on a basic mechanism involved in penetration of the larval integument by fungi. Additional information relating to the physical–chemical aspects of conidial germination on the surface of larvae should be obtained; however, a molecular understanding of the errant-type growth and penetration phenomenon also requires concerted effort. The existence of such behavior suggests that some chemical recognition between the emerging hyphal tip of germinating conidia and the larval surface is essential before the hyphal tip can become oriented toward penetration. It is obvious from data given in Table 1 that all mutants produce varying amounts of those enzymes considered necessary for penetration of the integument (Samsinakova et al., 1971). It appears that lack of a particular enzyme cannot be the reason a priori for the errant type of hyphal growth observed.

It seems logical that the "focusing" of exocellular fungal enzymes for dissolution of the integument and penetration is probably maximized when the emerging germ tube attaches to the larval surface and exocellular enzymes are elaborated in the immediate vicinity of the hyphal tip. Such a mechanism can be envisioned as an excellent strategy for concentration of those enzymes required for penetration of the integument. Certainly future studies need to be directed toward definition of the recognition factors which appear to be involved in the attachment of hyphal tips to larval surfaces.

In addition to nonpenetrating and errant-type hyphal growth, inhibition of conidial germination appears to be partially responsible for the low pathogenicity exhibited by some mutants. Relatively short-chain fatty acids (C-10 and below) are present on the larval surface and inhibit germination (Smith and Grula, unpubl.); however, the extent of their involvement is yet to be determined.

Since the larval diet contained no antifungal agents, conidia dislodged from the surface of treated larvae remain viable for some time. These conidia could be ingested and invade the larvae via the alimentary tract; however, no evidence for this type of infection process was obtained in this study. *B. bassiana* conidia were never detected within the gut and the gut tissue remained intact even after death and mummification had occurred (Fig. 14). In addi-

FIG. 10. Germinating *B. bassiana* conidia within a spiracle. Mutant E_1 (20 hr). ×4980.

FIG. 11. Germ tubes entering the spiracle opening (g). Note the enzymatic degradation at the edge of the opening near the germ tube (black arrow). Germ tubes are also penetrating through the side of the spiracle (white arrow). Mutant E_1 (30 hr). ×1328.

FIG. 12. Thick section of a germ tube (g) penetrating through the cuticle (c). Note the appearance of the epithelial cells (e) and fat body (f). Mutant E_1 (22 hr). ×1062.

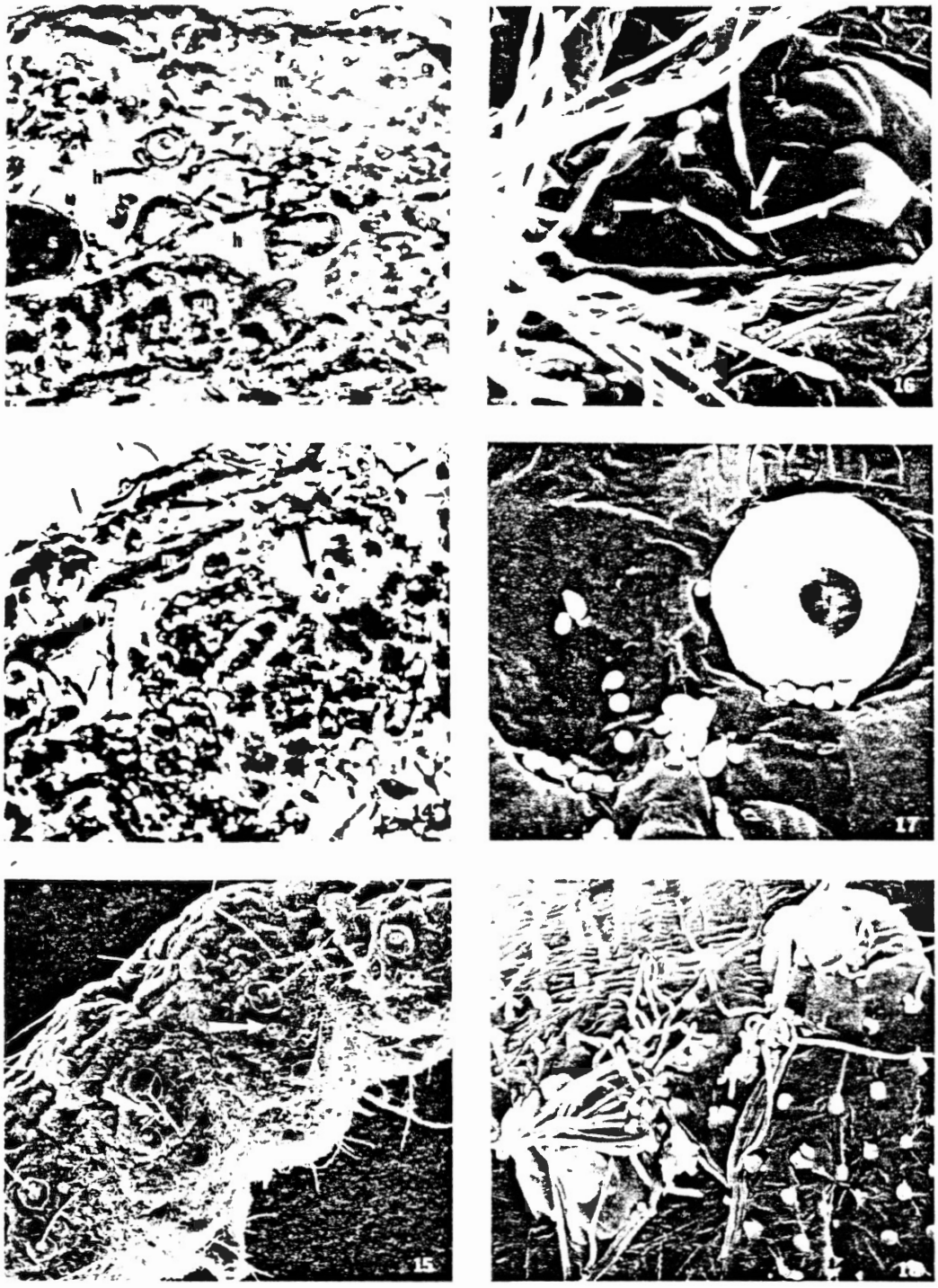

FIG. 13. Extensive fungal growth in the hemocoel (h) with no visible damage to the gut tissue (gu), silk glands (s), and muscle (m). Note the presence of a hypha within a trachea (arrow). Cuticle is labeled (c). Mutant E_1 (48 hr). ×1062.

FIG. 14. Mummified larva with intact fat body (f) and muscle (m). Note the presence of some hemocytes (arrows). Mutant E_1 (12 days). ×1062.

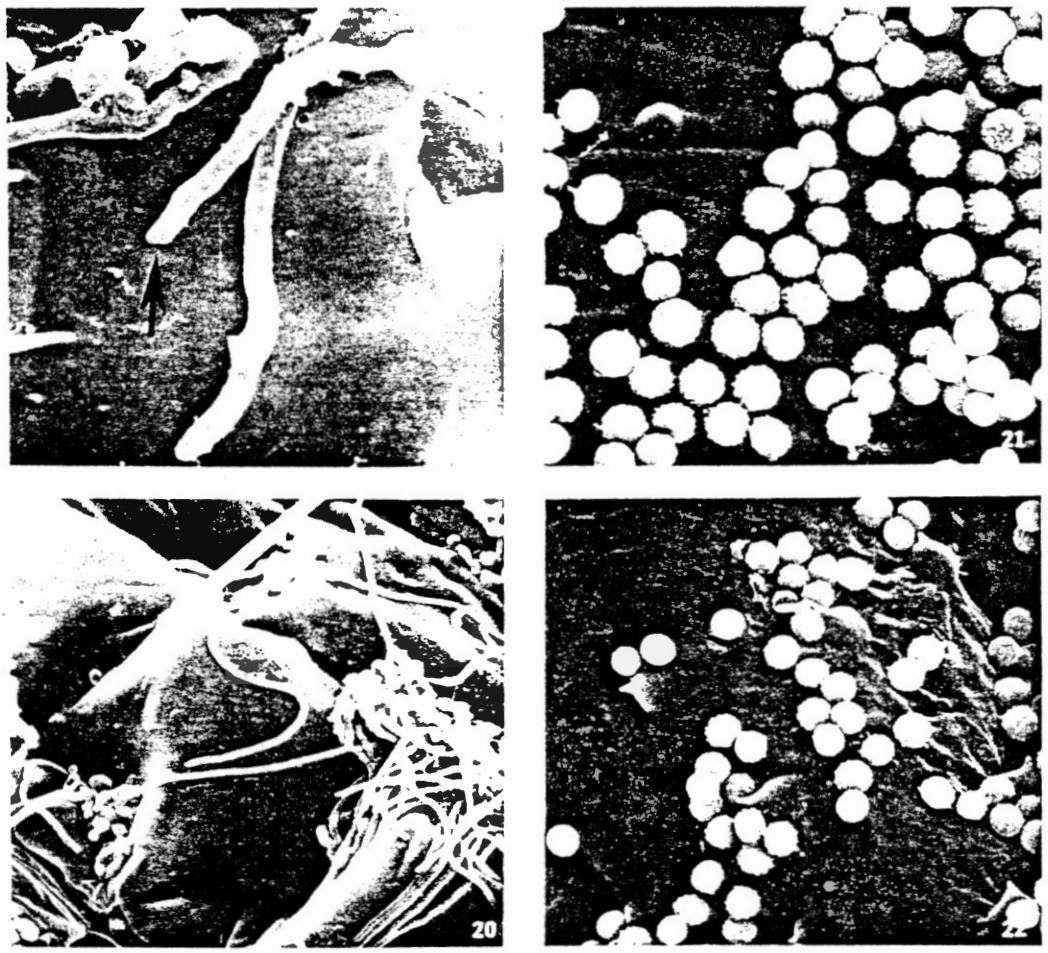

FIG. 19. Extensive growth of hyphae from a low pathogenic mutant with eventual penetration (arrow) by a hypha about 15 μm long. Mutant 14 (26 hr). ×3320.

FIG. 20. Lack of observable penetration by hyphae of mutant with low pathogenicity. Mutant 14 (26 hr). ×830.

FIG. 21. Nongerminating conidia of *Aspergillus niger* (6 days). ×1494.

FIG. 22. Nongerminating conidia of *Penicillium chrysogenum* (6 days). ×1245.

tion, we have observed that germination and growth of the fungus are extremely poor at the high *p*H (near 9.0) existing in the midgut region.

The exact cause of death is difficult to determine. Apparently, soluble components of the hemolymph supply sufficient nutrients for growth and extensive multiplication of the fungus. Degradative enzymes of the fungus (lipases and proteases) are

FIG. 15. Exit of hyphae at the intersegmental regions. Note the lack of fungal growth from the spiracles (arrows). Mutant E_1 (6 days). ×548.

FIG. 16. Exit of fungal hyphae on a mummified larva (arrows). Note the lack of a hole at the point of exit. Mutant E_1 (11 days). ×913.

FIG. 17. Conidia of a low pathogenic mutant unable to germinate on the larval surface. Mutant 23 (24 hr). ×1079.

FIG. 18. Hyphae of low pathogenic mutant growing errantly and unable to penetrate the integument. Mutant 9 (26 hr). ×647.

TABLE 1

COMPARISON OF KILLING, PENETRATION, AND SELECTIVE ENZYME ACTIVITIES OF *Beauveria bassiana* MUTANTS

Mutant	LD_{50}[a] (conidia/larva)	SEM[b] ratings	Proteolytic activity[c] 4-day	Proteolytic activity[c] 7-day	Lipolytic activity[c] 4-day	Lipolytic activity[c] 7-day	Chitinase activity[d] 5-day
E_1	<15	90	1.78	1.80	1.59	1.50	1.10
3	30	50	1.09	1.27	1.33	1.50	0.57
7	35–40	25	1.17	1.82	1.36	1.54	0.82
16	35–40	25	1.55	1.85	1.53	1.33	0.25
23	70–90	50	1.17	1.83	1.54	1.63	0.37
R_1[e]	100	80	1.45	2.54	1.45	1.63	0.33
10	150–180	60	1.36	1.58	1.28	1.37	0.07
1	250	50	1.87	2.18	1.48	1.45	0.18
20	250–300	70	1.57	2.27	1.52	1.38	0.05
14	320	35	1.05	1.60	2.08	2.50	0.21
9	>500	20	1.20	1.30	1.47	1.79	0.07

[a] Obtained by probit analysis (150–200 larvae/test) of the number of dead larvae 4 days after infection at which time at least 95% of those larvae which would die of the initial infection have been killed.

[b] Based on visible penetration, using SEM. The rating of 100 signifies that all germinating conidia penetrated the integument immediately; a zero (0) indicates all germinating conidia yielded hyphae that grew errantly over the surface and never penetrated or that the conidia never germinated on the larval surface. Germination usually occurs on the larval surface within 16–18 hr and these data were obtained 26 hr after infection.

[c] Numbers given are the ratio of clearing (enzyme activity) to colony size. Proteolytic activity was determined using litmus milk–SDA; lipolytic activity was obtained using tributyrin-containing agar. Data are given for two different time periods because of variability in enzyme synthesis and/or activity.

[d] Conidia were germinated and grown in chitin salts minimal medium at *p*H 7.0 (Grula et al., 1978). Enzyme activity was monitored by measuring optical density of the culture at 540 nm. Direct testing of concentrated supernatants for chitinase activity from 3- to 6-day fungal cultures has shown that fungal growth with chitin as the sole source of carbon, nitrogen, and energy is directly proportional to levels of exocellular chitinase (Grula and Grula, unpubl.).

[e] Isolated in these laboratories from Boverin, a mycoinsecticidal preparation of *B. bassiana* used in the USSR. The Boverin was recently obtained from Dr. O. Alyoshina and Dr. N. V. Lappa of the USSR through a cooperative program sponsored by the American Society for Microbiology and administered through a grant from the National Science Foundation. Field and laboratory studies using Boverin against cabbage pests in the USA have been done and will soon be available (Ignoffo et al., 1979).

probably helpful and could aid growth by solubilizing tissues such as the fat body. Due to the extensive growth of the fungus, death of larvae could easily result from exhaustion of nutrients in the hemolymph; however, very little degradation of hemolymph proteins occurs during the infection (Cheung and Grula, unpubl.). Ancillary physiological changes, such as alterations in *p*H and dehydration, are most likely involved; however, their role is yet to be determined. Toxins (nonenzymatic) could also aid the infection by immobilizing the larvae or certain of their life systems; but, methanol- or water-soluble toxins of the beauvericin type are not responsible for larval mortality (Champlin and Grula, 1979).

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APPENDIX B

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

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

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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

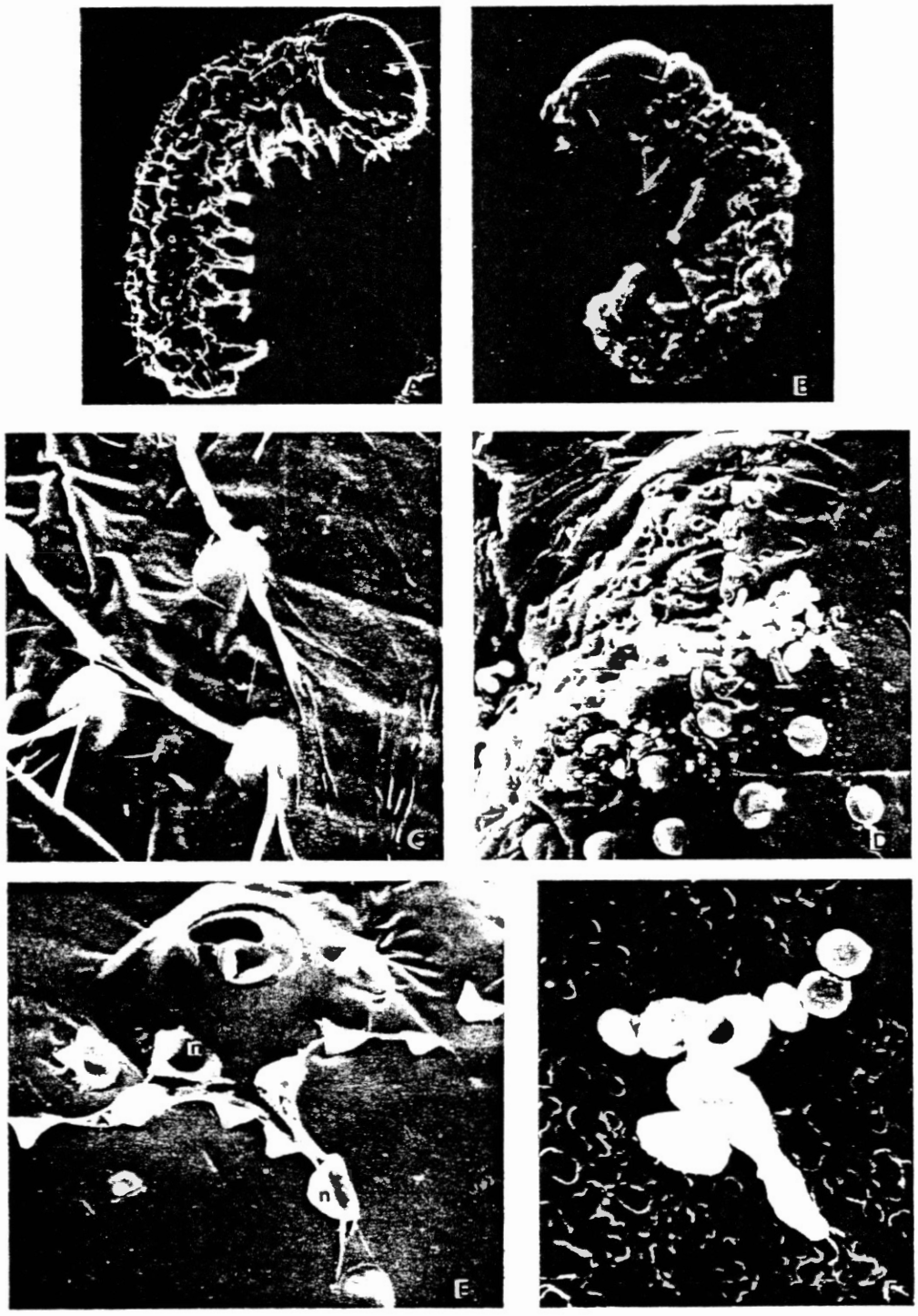
A
B
C
D
E
F

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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APPENDIX C

VIRULENCE OF *BEAUVERIA BASSIANA* MUTANTS FOR THE PECAN WEEVIL

Virulence of *Beauveria bassiana* Mutants for the Pecan Weevil[1,2,3]

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ABSTRACT

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The virulence of five mutant strains of an entomopathogenic fungus, *Beauveria bassiana* (Bals.) Vuill., which exhibit different capacities for pathogenicity in the corn earworm, *Heliothis zea* (Boddie), were established for the pecan weevil, *Curculio caryae* (Horn). An artificial method of infection on soil surfaces was used that approximates the naturally occurring infective process. The mutants exhibited different degrees of virulence for pecan weevil larvae; however, no positive and direct correlation to synthesis of specific extracellular enzymes (chitinases, proteinases, lipases) exists. Scanning electron microscopy revealed that penetration of the integument probably does involve extracellular enzymes of *B. bassiana*. Since the mutants used in this study were previously assayed for virulence against corn earworm larvae, it has been possible to compare some aspects of infection in both larval forms. The greatest difference relates to number of conidiospores required to establish an active and terminal infection. Numbers required are greater for the pecan weevil than the corn earworm, and possible reasons for this are discussed. Caprylic acid, a fatty acid found on the surface of corn earworm larvae which inhibits the germination of *B. bassiana*, could not be demonstrated on the surface of pecan weevil larvae.

An entomopathogenic fungus, *Beauveria bassiana* (Bals.) Vuill., has long been known to be the causative agent of the white muscardine disease of over 30 different insect species (Pramer 1965) and has been reported to be the most common fungal isolate from dead and moribund insects in nature (Macleod 1954). Previous reports from this laboratory have dealt with an investigation into the molecular basis of the entomopathogenicity exhibited by *B. bassiana* when using a lepidopteran, the corn earworm (CEW), *Heliothis zea* (Boddie), as the primary target insect (Grula et al. 1978, Champlin and Grula 1979, Pekrul and Grula 1979). An understanding of the mechanism of infectivity is essential to achieve the ultimate goal of this work, that being the successful development of the fungus as a biological agent for pest control.

To make comparisons of data obtained from both sides of an ordinal barrier, an additional study was undertaken using the pecan weevil (PW), *Curculio caryae* (Horn). In contrast to the CEW, which spends the duration of its life cycle before pupation above ground, the 4th-instar PW emerge from the pecan nut and burrow into the ground where they form earthen cells in which pupation and teneral maturation into the adult form occurs in 1 to 2 years (Harp and Van Cleave 1976). The ability of *B. bassiana* to infect PW larvae in soil has been well established. Swingle and Seal (1931) isolated the fungus from larvae which had a high mortality rate after entering the soil in a laboratory rearing situation, and Tedders et al. (1973) reported a *B. bassiana* strain to be the most virulent of several fungal pathogens studied.

Pekrul and Grula (1979) provided scanning electron microscopic evidence for the enzymatic penetration of the CEW larval integument by growing hyphae of highly pathogenic *B. bassiana* mutant strains when conidiospores were applied to the larval surface. For comparative purposes, we report here on infectivity of PW larvae by using mutants of *B. bassiana* exhibiting high and low pathogenicities for the CEW (Pekrul and Grula 1979) and which produce different levels of extracellular enzymes.

Materials and Methods

Fungal Growth Conditions and Conidiospore Preparation

The five *B. bassiana* mutant strains were obtained by ultraviolet (UV) irradiation of wild-type conidiospore suspensions followed by the selection of colonies exhibiting different degrees of extracellular proteolytic enzyme activity when plated on Sabouraud dextrose agar (SDA, Difco Laboratories) containing casein (Grula et al. 1978). Stock cultures of each mutant were grown and maintained on SDA slants supplemented with 0.3% yeast extract (Difco). Transfers were made every 2 weeks and incubated at 25°C until complete sporulation had occurred (ca. 14 days), after which they were stored at 2 to 4°C. Conidiospores from 14- to 21-day-old cultures were used to inoculate SDA-yeast extract plates which were then incubated under the same conditions to obtain large quantities of spores for testing. Standard suspensions were prepared by suspending freshly sporulated cultures in sterile 0.03% Triton X-100® (nonionic detergent), then washing twice in sterile, distilled water. The detergent allows for a homogenous suspension to be obtained by decreasing the

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amount of clumping, and the following water washes effectively remove the detergent but permit the conidiospores to remain in suspension separated from each other. The conidiospores were resuspended in sterile distilled water to give a concentration appropriate for the given experiment as judged spectrophotometrically at 540 nm. Plate counts were performed on appropriate dilutions by using SDA containing 0.03% Triton X-100 to determine the concentration of viable cells in the undiluted suspensions. Addition of detergent in this amount retards the spreading of fungal colonies so that they can be plated out and counted accurately.

Virulence Test with CEW

The surface application of each fungal mutant was accomplished by allowing 1st-instar CEW to crawl on a piece of Whatman 3MM® filter paper (8.25 cm diameter in a sterile petri plate) saturated with 2 ml of an appropriate dilution of the specified mutant conidiospore suspension. After 5 min, individual larvae were placed in diet cups containing a corn meal, soy flour, and milk solids diet as formulated by Burton (1970). The artificial diet was modified for this study by substituting D-chloramphenicol (0.5 g/2.5 liters of diet) for the antifungal agents *p*-hydroxymethylbenzoate and sorbic acid which inhibit the growth of *B. bassiana*. The infected larvae were reared for 4 days at 25°C, after which time mortalities were scored. Four different conidiospore dilutions were used for each mutant with 150 to 200 larvae per dilution. Probit analysis (Finney 1971) was performed on the mortality data to determine the median lethal concentration (LC_{50}) for each mutant.

Virulence Test with PW

The surface application of each fungal mutant was accomplished by allowing 4th-instar PW (obtained from pecan packaging plants in northeastern Oklahoma) to burrow down into soil which had an appropriate dilution of the specified mutant conidiospore suspension applied to the surface. One large plastic cup (14.5 cm deep, 11.5 cm diameter) containing 12.7 cm of an autoclaved soil-sand mixture (10:1) was used for each dilution. A 10-ml amount of the suspension was applied evenly over the entire soil surface (95 cm^2) with a 10-ml pipette in a dropwise manner. After allowing the suspension to diffuse into the soil (ca. 1.3 cm), larvae were put on the surface and allowed to burrow down through the conidiospore layer. Each cup was then covered with a piece of Parafilm® into which 10 holes had been punched and was incubated at 25°C for 21 days. After 7 days, 5 ml of sterile water was added to each cup to maintain moisture. Four dilutions of each mutant conidiospore suspension were tested in four separate cups containing 25 larvae each. Two to three replicate experiments were performed for each mutant strain, and the percentage of insects which had become mummified and having a white, chalky appearance (indicative of the *B. bassiana* muscardine infection) after the incubation period was scored for each dilution. These data were then used to determine LC_{50} values for each mutant.

Scanning Electron Microscopy (SEM) of Infected PW Larvae

Fourth-instar PW were rinsed in distilled water to remove surface soil; however, large amounts remained as shown in Fig. 1. A conidiospore suspension (obtained from a 14 day *B. bassiana* E_1 culture) was either brushed or poured onto the larvae which were then placed in test tubes inside a moisture chamber at 25°C. After various time intervals, larvae were fixed for 6 h in 4% glutaraldehyde buffered with 0.2 M cacodylate buffer at pH 7.3. Specimens were dehydrated in an ethanol series, and critical-point drying was done under CO_2. The samples were mounted on aluminum stubs with silver conducting paint; some larvae were sliced in half before mounting. A Hummer® evaporator was used to coat the samples with gold-palladium (ca. 400 nm). Observations and photographs were made, using a JOEL 35 JSM® scanning electron microscope and Polaroid® film.

Hexane Extraction of Larval Surfaces

Groups of 50 4th-instar CEW or 4th-instar PW were placed in a beaker containing distilled water and agitated continuously for 30 min with a magnetic stirring bar to remove surface-adhering soil. Larvae were then placed in a beaker containing 20 ml of hexane and extracted for 30 min with intermittent shaking. The extract was cleared by filtration through Whatman no. 1® filter paper and concentrated with a rotary evaporator or under nitrogen gas. The residue was then methylated by the addition of sodium-dried benzene (4 ml), 2,2-dimethoxypropane (0.4 ml), and methanolic HCl (0.5 ml of 2.5% methanol in concentrated HCl, vol/vol). The solution was incubated at 25°C for 18 h, after which it was concentrated by evaporation and stored under nitrogen gas at −20°C. Fatty acid composition was determined by using a Perkin-Elmer Sigma II® gas-liquid chromatography unit equipped with a hydrogen flame detector and a stainless-steel column (1.22 m by 0.635 cm) packed with 3% Sylar® on a Chromosorb® support. Methylated fatty acid standards (Sigma Chemical Company) were utilized as controls.

Results and Discussion

We were able to infect 4th-instar PW with various *B. bassiana* mutants in a controlled laboratory situation by utilizing methodology which approximates the manner in which the disease is acquired in nature. Weevils became infected as they burrowed into the soil through surface layers containing different concentrations of infective conidiospores. A dose-response relationship between the percentage of larvae which would acquire the white muscardine disease and die within 21 days and the concentration of conidiospores applied to the surface was seen to exist. This being the case, it was then possible to rate the relative entomopathogenic potentials of each mutant studied by using probit analysis to ob-

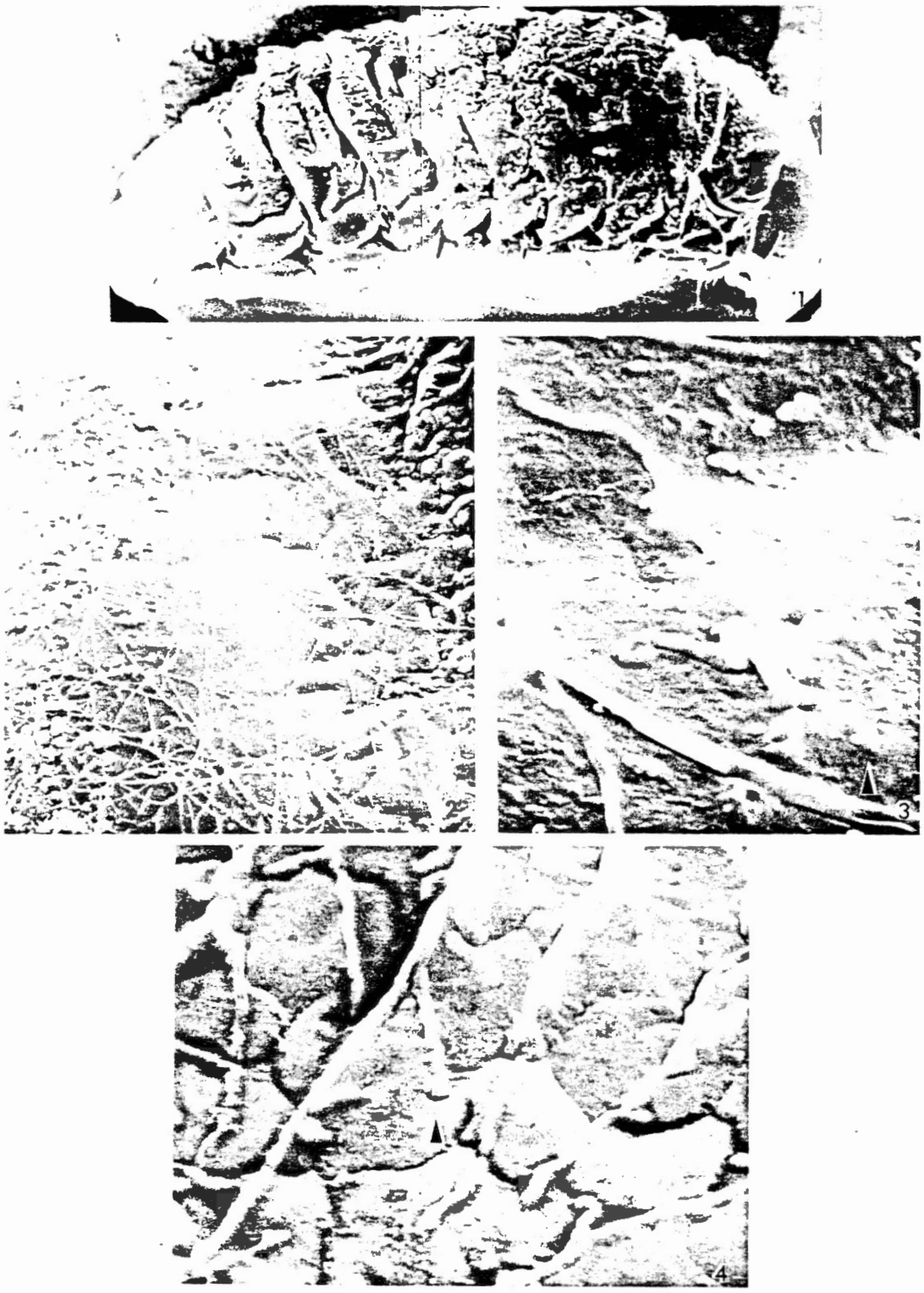

FIG. 1.—4th-instar pecan weevil 53 h postinfection with *B. bassiana* strain E_1 (18.3×).
FIG. 2.—Abundant fungal and bacterial growth on pecan weevil cuticle 75 h postinfection (200×).
FIG. 3.—Penetration of pecan weevil cuticle by a germ tube (arrow) 75 h postinfection (2,000×).
FIG. 4.—Production of a hole in the pecan weevil cuticle by a penetrating hypha (arrow) (1,000×).

Table 1.—Virulence of *B. bassiana* mutants against the pecan weevil and corn earworm

Mutant	4th-instar pecan weevil LC_{50}[a]	1st-instar corn earworm LC_{50}[a]
R_1	$9.72 \pm 3.86 \times 10^6$ (0.41)	$3.43 \pm 0.36 \times 10^5$ (1.12)
E_1	$1.83 \pm 0.44 \times 10^7$ (0.90)	$4.11 \pm 1.10 \times 10^4$ (1.01)
14	$2.17 \pm 0.48 \times 10^7$ (1.06)	$9.84 \pm 0.83 \times 10^6$ (1.31)
HP_1	$2.83 \pm 1.00 \times 10^7$ (0.97)	$1.48 \pm 0.14 \times 10^5$ (1.55)
8	1.00×10^9 (0.06)	$1.39 \pm 0.20 \times 10^6$ (1.47)

[a]Values are reported in conidiospores/ml ± SD, with slope values in parentheses.

tain LC_{50} values. The same dose-response relationship had been observed between *B. bassiana* mutants and infected 1st-instar CEW (Grula et al. 1978).

Although the order of infectivity observed by using various mutants of *B. bassiana* for the CEW does not completely hold when testing the same mutants against the PW (Table 1), there is an overall correlation, in that mutants exhibiting pathogenicity for the CEW are also capable of infecting the PW. The major variation involves dosage levels required to establish a terminal infection.

Differences involved in testing of the two species of insects include the following: infected CEW larvae were incubated in the atmosphere, whereas infected PW larvae were incubated in the soil, and thus the availability of oxygen for growth of the fungus differed; time of exposure to conidiospore infections of the CEW was controlled (5 min), whereas PW larvae laid on and then burrowed through the conidiospore-containing soil for different periods of time; some of the conidiospores picked up by the PW larvae at the soil surface were probably rubbed off as they burrowed down through the soil, and this very likely contributed to a decrease in the infection efficiency; CEW larvae were infected as 1st instars, at which time they are most susceptible to infection by *B. bassiana*, whereas PW larvae were infected as 4th instars, the only time at which their infection can be effected in a field application situation; and complete maturation of the PW requires a relatively long period of time (2 to 3 years) while they are sequestered underground, whereas the CEW proceeds through four larval moltings at a relatively rapid rate (14 to 17 days). These differences must be kept in mind when considering our finding that the *B. bassiana* mutants appear to be 1 to 2 logs less effective as pathogens for the PW as opposed to the CEW (Table 1).

As can be seen in Table 2, all but one of the mutants studied elaborated significant amounts of the extracellular enzyme activities which have been postulated by Samsinakova et al. (1971) to be essential for penetration of the integument of greater wax moth larvae by growing hyphal tips. Extracellular chitinolytic activity could not be demonstrated for *B. bassiana* 8, the mutant which exhibits the least virulence for PW larvae. Again, as with the CEW (Grula et al. 1978, Pekrul and Grula 1979), no positive correlation exists between killing of the insects and amounts of specific enzyme activities elaborated by the fungal mutants.

SEM observations reveal that the fungus germinates and undergoes hyphal elongation on the cuticular surface (Fig. 2–4). In contrast with the situation observed on the CEW larval surface (Pekrul and Grula 1979), a thin film of unknown chemical composition covers much of the PW surface, obscuring some of the fine detail of fungal growth and possible penetration. In addition, numerous soil bacteria (and possibly fungi) are present on the cuticle as contaminants, even though the specimens were washed before fixation (Fig. 2). It was possible, however, to determine that the initiation of germination on the PW surface does occur ca. 36 h after application and that penetration can occur either immediately after germination or after a certain degree of hyphal elongation. Holes produced enzymatically by penetrating hyphal tips are shown in Fig. 3 and 4. As reported by Pekrul and Grula (1979), no appressoria are present at the sites of penetration.

Several free fatty acids have been extracted from CEW larvae by using hexane, and one of these, identified as caprylic acid, is an effective inhibitor of the germination of *B. bassiana* conidiospores (Smith and Grula, unpublished data). In the present study, free fatty acids were not detected on the PW

Table 2.—Proteolytic, lipolytic, and chitinolytic activities of *B. bassiana* mutants

Mutant	Proteolytic activity[a]		Lipolytic activity[b]		Chitinolytic activity[c]
	Litmus milk	Gelatin	Tributyrin	Tween 80	
R_1	2.44	3.00	1.63	1.52	51
E_1	2.00	1.87	1.50	1.47	55
14	2.72	3.14	2.50	1.68	23
HP_1	1.17	1.20	1.00	1.38	49
8	1.01	1.08	2.52	1.72	<1d

[a]Ratio of clearing to colony size at day 7 postinoculation, using litmus milk-SDA and gelatin-SDA plates.
[b]Ratio of clearing to colony size at day 7 postinoculation, using tributyrin-SDA and Tween 80-SDA plates.
[c]Expressed as μg of *N*-acetylglucosamine liberated after 6 h incubation by 1 ml of 200-fold-concentrated spent chitin-salts growth medium (Smith and Grula 1981), using the chitinase assay method of Jeuniaux (1966). Age of the culture at time of harvest was 5 days.
[d]Lower detection limit for chitinase, using the Jeuniaux method.

larval surface, even when the number of larvae extracted was increased twofold (from 50 to 100). This indicates that a possible barrier to infection (inhibiting or slowing of conidiospore germination) existing on the CEW surface is absent from the PW. This finding plus the observation that germination of conidiospores does occur on the surface of PW larvae suggest that the lower virulence (i.e., increased LC_{50} values) of *B. bassiana* for the PW is probably a reflection of other variables in either the test parameters involving the two insects or the infective process itself.

It appears entirely feasible to infect PW larvae in nature by applying suitable suspensions of *B. bassiana* conidiospores to the soil surface, thereby initiating a surface infection as the larvae burrow through to take up residence in their earthen cells. It should prove advantageous to have relatively loose soil to maintain a higher level of oxygen concentration, since fungi such as *B. bassiana* are aerobic-type organisms; however, we have observed many infected PW larvae as deep as 12.7 cm underground. It is our understanding that Chinese and Japanese workers plow into the ground conidiospore inocula previously grown on mixtures of manure and various wood chips (personal communication from J. Aoki, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan). Such growth and inocula could serve to allow a continuous source of nutrients to be present for growth, to provide areas of cover and protection for the fungus, and to permit better aeration of the soil for growth of *B. bassiana*. Tedders et al. (1973) conducted field experiments in which either Vermiculite® or chopped straw was incorporated into the conidiospore mixture to aid oxygen diffusion.

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