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PATHOGENICITY OF SELECTED RESIDENT MICROORGANISMS OF LYMANTRIA DISPAR (L.) AFTER INDUCTION FOR CHITINASE

A Dissertation Presented

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D E D I C A T I O N

To my wife for her love, support and encouragement throughout my graduate studies, and to my parents for their sacrifice made for my educational endeavors. Also to my children in the hope their world will benefit from my small effort.

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A B S T R A C T

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Directed by: Professor Haim B. Gunner

Chitinolytic microorganisms have been isolated from healthy instar III, IV and V L. dispar larvae. The acquisition of the chitinolytic microflora appears to be correlated with an increase of mobility by the maturing larvae. Selected isolates, two identified as Bacillus coagulans and two Streptomyces species were inducible for chitinase by the insect host tissue. Stock chitinases produced by the four isolates readily attacked the chitinous integuments of the larvae, releasing N-acetylglucosamine. In vitro studies showed that the peritrophic membrane was very susceptible to the chitinase. In vivo studies, demonstrated that B. coagulans was lethal to the larvae when these were fed chitinase induced whole cultures of the bacteria; when the chitinase was fed with sublethal doses of B. thuringiensis, significant mortality was observed. When ingested, the chitinase caused localized dissolution and ulceration of the peritrophic membrane. The pH of the mesenteron appears to have a limiting effect on the in vivo chitinase activity on the peritrophic membrane.

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INTRODUCTION

Numerous studies have been reported on the use of entomopathogenic microorganisms to control specific insect pests. Against Lymantria dispar (L.) larvae, a voracious leaf-chewing Lepidoptera infesting hardwood forests (particularly oak stands) of the northeast United States, extensive field and laboratory studies have been reported using the known insect pathogen, Bacillus thuringiensis (27, 60, 61). The Baculovirus, L. dispar Nuclear Polyhedrosis Virus (NPV) has also been shown to be an effective microbial agent against this insect pest (67, 96, 97). Serratia marcescens (86), Streptococcus faecalis (19) and Proteus myxofaciens (20) have also been reported as pathogens to this insect. Their mode of action has not been elucidated.

Recent studies suggest that the addition of chitinase to commercial preparations of B. thuringiensis substantially increases its effectiveness against the spruce budworm, Choristoneura fumiferana (Clem.) (108, 76). The presumed mode of action of the chitinase is its ability to disrupt the protective chitinous peritrophic membrane which lines the mesenteron wall. This membrane acts as a protective barrier preventing particulates such as bacteria from injuring the delicate epithelial gut wall cells and penetrating into the hemolymph where they would readily grow and cause septicemia. The source of chitinase used in these studies was from chicken

gut.

The insect pathogen S. marcescens is readily inducible for chitinase (74). The ability to hydrolyse chitin, such as is found in the peritrophic membrane tissue, had been suggested as a possible mechanism by which the bacteria penetrate into the hemolymph of L. dispar larvae and cause septicemia (86). Numerous microbial species that are part of the forest microflora are also inducible for chitinase (39). L. dispar larvae as well as other forest insects usually have a microflora reflecting their environment. That is, their integuments and alimentary tract are contaminated by those same microbial species which make up the forest microflora (116). Campbell (12, 13) determined that disease played a major role in the decline of dense populations of L. dispar larvae. Microorganisms isolated from dead and moribund larvae (85) were found to be species common to a forest environment. Other than identifying a few of the isolates as B. thuringiensis of unknown variety and certain motile pigmented Streptococcus sp. as pathogens, none of the other isolates were found to be pathogenic when fed to larvae after cultivation on enriched media. No attempts were made to determine whether any of these isolates would become pathogenic after induction for chitinase. More recently Dubois and Gunner (26) reported that several Bacillus spp. isolated from healthy L. dispar larvae were, after induction for chitinase lethal to their host, but were not after cultivation in a non-inducing medium. Furthermore,

the chitinase produced by these isolates hydrolysed the host's chitinous exoskeleton releasing N-acetylglucosamine as a consequence of chitin depolymerization.

These observations suggest that the microflora of healthy L. dispar larvae could, after induction for chitinase, become pathogenic to their host. The pathogenicity of resident micro-organisms found on healthy L. dispar larvae after induction for chitinase is the subject of this study.

HISTORICAL REVIEW

Early History

It would not be appropriate in this review to dwell on the early observations of the interactions between insects and microorganisms. Briefly, however, we can say that the concept of insect pathology, and microbial control of pest insect was articulated about 100 years ago (114). The first recorded observation of disease and abnormalities in insects is found in Aristotle's "Historia Animalium" written around 332 B.C. For almost two thousand years, the only insect disease of interest were those of the silkworm (Bombyx mori) and the honey bee (Apis mellifera) which were historically two insects of great economic importance. From 1834 to 1836 Bassi made three important contributions to the development of insect pathology: 1) he was the first to show experimentally that a microorganism, Beauvaria bassiana was the etiological agent of the muscardine disease of silkworm; 2) he showed that insects other than silkworms were susceptible to infection; 3) he suggested that microorganisms be used to destroy harmful insects. Pasteur, in 1870, published his monumental work "Études sur la Maladie des Vers A Soie" where he identified the pebrine and flasherie disease of silkworm and also established the relationships between susceptibility to microbial disease and health, proper diet, and a contamination-free insect environment. In 1874 Le Conte, an American entomologist, also strongly suggested

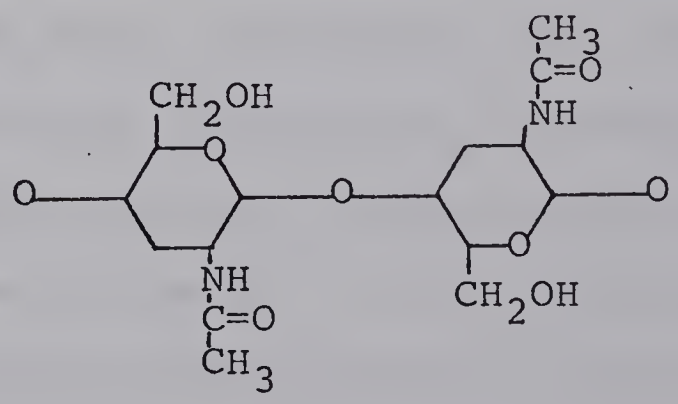
the use of microorganisms to cause diseases in various pests and thus destroy them. In 1879 Metchnikoff reported on the natural infection of the wheat cockchafer (Anisoplia austriaca Hbst.) by Metarrhizium anisoplia (Metch.). He also expressed an appreciation of the natural epizootic in reducing the pest population and tested the feasability of using this pathogen in an active pest control program.

Before 1900, the recognition of the effect of entomopathogenic microorganisms on the depletion of natural insect population was already recognized, and the use of these environmentally native agents to control pest insects had been suggested. It remains for the twentieth century to understand the mechanisms associated with invasion of the insect host by the pathogen and to effectively utilize these natural biological control agents.

Metalnikov and Chorine (68) first demonstrated the susceptibility of L. dispar larvae to B. thuringiensis. This pest insect was first introduced into the northeast United States from France in 1868-1869 (30). It is a member of the Class Insecta and belongs to the Order Lepidoptera. Like other arthopods, a large proportion of its integuments including part of the exoskeleton and internally, the peritrophic membrane, is composed of chitin, an inanimate tissue of considerable structural and protective importance.

Chitin

Chitin was discovered by Braconnot (8) in 1811 and named by him as fungine. Twelve years later, Odier (1823) proposed the name chitin (80) for this structural polysaccharide of considerable strength made up principally of N-acetylglucosamine (N-acetyl-2-amino-2deoxy-D-glucose, hereafter abbreviated as NAG) units. The NAG monomers are linked together in a straight chain polymer by a β 1 $\longrightarrow$ 4 glycosidic bond. Frankel (32) was the first to describe the NAG monomer in 1902. The hydrolysis of 85% of a crab chitin preparation by snail gut enzyme and the identification of NAG as the principal hydrolytic product provided the first clue of the polymeric structure of chitin (124). Meyer and Mark (69) in 1928 proposed the following structure for chitin:



In 1931 Bergmann and his colleagues (5) also Zechmeister and Toth (124) eventually isolated chitobiose, the corresponding dissacharide of NAG.

Several reviews on the structure and composition of chitin from arthropods have been published (92) and only a brief description will be given here. Meyer and Pankow (70) described

a rhombic cell with the dimensions along the axes as a = 9.40Å, b = 10.46Å and c = 19.25Å. Lotmar and Picken (64) in x-ray diffraction pattern studies have reported slightly different values for the three axes. X-ray diffraction patterns of chitin from different sources including the hard chitin of crabs and the so-called soft cuticle chitin of insects resulted in the reporting of several patterns of infolding of the polymer chain (99). This lead to the proposal of three structural forms of chitin, α, β, and γ chitin.

The proposed arrangement of the α-chitin structure is that the long straight polymer chain is repeatedly folded upon itself. Carlstrom (15) in reexamining the α-chitin considered the indexing of some of the weaker reflections and arrived at an orthorhombic cell of dimensions at half the value previously reported by Meyer and Pankow (70). The proposal of this antiparallel structure of α-chitin is further supported by infrared absorption showing a high perpendicular dichroism and strong absorption of the NH .....C = 0 bonding (99).

The less stable configuration of chitin is described as β-chitin where the chains of the polymer are parallel to each other. Dissolution of β-chitin in acid and reprecipitation usually results in the shrinking of the β form to the more stable α form.

Jeuniaux (53) indicated that regardless of the native structure of chitin (α, β, or γ), acid hydrolysis and reprecipitation results in the formation of the more stable α form.

Furthermore, β-chitin is more easily penetrated by chemical reagents and is more amenable to enzyme attack than α-chitin suggesting a lower degree of packing and a more open type of crystalline structure (45). In 1955 Rudall (98) showed that water played a significant part in the crystal structure of β-chitin. Dweltz (28) in a more recent study placed one water molecule per NAG residue in his model of β-chitin. He also found a simple cell with only one chain passing through it and concluded that all the chains in a crystallite must have the same direction and that the cell is essentially rectangular. X-ray measurement of dried and rehydrated cuticular chitin further supports the proposed structure of β-chitin where one water molecule per residue may approximate the monohydrate.

The γchitin was proposed when it was observed that chitin from the squid (Loligo) pen (98) (when examined by x-ray crystallography) showed periodicity and hydration of the β-form and also the dehyrated αform. Little direct evidence is presented in the literature for this form of chitin and much of the proposed structure is deduced from data obtained on studies of both the αand β forms of chitin. Below is presented a schematic diagram of the three forms of chitin:

Physically, crustacean chitin has been reported to have a density of 1.398 to 1.420, a tensile strength (dry) from 9.5 - 58 kg/mm^2 and an isoelectric point at pH 2.6 by several investigators (cf. 92).

Investigations (36) have shown that chitin is not exclusively a polymer of NAG but is composed of 82.5% NAG, 12.5% glucosamine and 5% water. This would give a chain structure of about one glucosamine residue for every 6 or 7 NAG residue in addition to firmly bound water. Rudall (99) on the basis of x-ray diffraction diagrams, infrared-absorption spectra, and density measurements, concluded that chitin may depart from an idealized poly-NAG structure and have one deacetylated residue for every 6 or 7 NAG residue. This structure, with bound water replacing the missing acetyl groups to maintain density and crystallographic properties, is consistent with that of poly-NAG. Hackman and Goldberg (45) in their own investigation of the cuttlefish shell and squid "pen", which is a β chitin, concurred with these interpretations.

The NAG monomer is also present as a component of a) hyaluronic acid where the polymer is made of alternating units of glucuronic acid and NAG linked by a β 1 → 3 glycosidic bond, b) blood group A substance, c) chondroitin sulfate A and C,

and d) heparin where the acetate group may sometimes be substituted by sulfate to form a sulfamic acid group (-N-SO_2OH) (33). Furthermore, in bacterial cell walls, NAG appears as a monomer unit alternating with N-acetylmuramic acid and linked to it by a β 1 $\rightarrow$ 4 glycosidic bond. The dimer with the tetrapeptide attached on the number three carbon on N-acetylmuramic acid and the pentaglycine cross linking bridge is the basic structural component of bacterial cell walls.

Distribution of Chitin in Nature

It is estimated that several billion tons of chitin are deposited in the environment annually. In the plant kingdom chitin is present in the cell walls of all fungi except in certain Phycomycetes (Oomycetes and Monoblepharidales) and in the Lambouldeniales of the Ascomycetes (31). There is however some controversy about the presence of chitin in the cell walls of Ascomycetes. Roelofsen and Hoette (95) reported the presence of chitin in the cell walls of asocogonous as well as non-ascogonous Asomycetes but Northcote and Horne (78) in their study of the chemical composition and structure of the cell wall of baker's yeast do not mention the presence of chitin. To date, chitin has not been found in bacteria, actinomycetes or most myxomycetes.

Blumenthal and Roseman (7) estimated the quantity of chitin in fungi to range from 2.6% of the mycelial dry weight

in Neurospora crassa, to 26.2% in Asperigillus parasiticus. Smithies (110) reported the concentration of chitin in Penicillium griseofulvum to be 5.5% of the mycelial dry weight. The results of these investigators also concur with earlier reports (1, 103) that the content of chitin slowly increases with age.

In the animal kingdom, chitin is found in the perisarcs of Coelenterata (Hydromedusa, fossils of Graptozoa), in the jaws, bristles and gut lining of Chaetopoda, Polychaete and Olygochaeta, in the egg shells of Nematoda, in the dorsal shield and jaws of Molusca (Cephalopoda and Gasteropoda) and is in most representative classes of Arthropoda (92) including the Class Insecta (52, 53). In insects, chitin is present throughout the body wall, i.e. the exoskeleton, and is present in the foregut, hindgut and peritrophic membrane (122). It is also present in the tracheal tubes of immature insects and in the scales of adult insect wings. Both protozooa and flagellated algae seem to lack chitin entirely.

In most insects, chitin is the main consitutent of the endocuticle (60% of the dry weight) and in Periplanata Americana (cockroach) it may also be as high as 22% of the exocuticle dry weight. Generally the cuticular chitin is approximately 35% of the cuticle dry weight (92, 122).

In studies on Pieris brassicae (87) with 1-(2, 6 - dichlorobenzoyl)-3-(3, 4-dichlorophenyl)-urea, an inhibitor of chitin synthesis, the following pathway for chitin synthesis

was deduced: Glucose ⟶ glucose-6-P ⟶ glucosamine-6-P ⟶ N-acetylglucosamine-6-P ⟶ N-acetylglucosamine-1-P ⟶ UDP-N-acetylglucosamine ⟶ Chitin (57). Chitin is formed in the exoskeleton (endocuticle) of Lepidoptera by the deposition of microfibrils in a series of layers or lamellae (44, 63). In studies with H^3 glucose (18) a precursor for chitin, the tritiated sugar is deposited at the edge of the epidermis in layers as microfibrils parallel to each other corresponding to the new cuticle synthesized during the period of incorporation. Tritiated amino acids on the other hand are incorporated not only in layers but also diffusely throughout the cuticle. Chitin present in arthropods is not present as a simple polymeric NAG structure but is in fact bound with a protein called arthropodin. Experimental evidence of covalent bonds between chitin and arthropodin show that NAG as well as chitin can react with L-amino acids, particularly tyrosine and with cuticular protein to give complexes, dissociable however by changing pH values (42, 43).

Much of the evidence on the chitin-arthropodin interrelationship indicates that arthropodin is interlaced between and bound to the NAG polymer at the NH_2 residue. As such the arthropodin lies both parallel and perpendicular to the chitin chains in both a cross-grid arrangement as well as in a parallel arrangement of chitin microfibrils and protein chains (93).

Peritrophic Membrane

Of particular interest in this study is the peritrophic membrane. It has the same component as the inner layers of the cuticle, that is, a basis of chitin with protein incorporated (53, 122). This structure can be defined as a cylindrical membraneous envelope, a few microns thick which surrounds the food in the ventriculus and sometimes extends into the proctodaeum (111). It is present in most but not all insects. In Lepidoptera such as L. dispar it is present in the larvae but is absent in adults. Two types of peritrophic membranes are formed in insects. The so called discontinuous membrane, is found in L. dispar larvae and is synthesized from the ventricular epithelial cells, producing concentric lamellae independent or loosely attached to one another and giving the appearance of a multilayered structure. The other type of peritrophic membrane, called the continuous peritrophic membrane, is present in Diptera and consists of a single uniform layer extending from the stomodeal valve as a tubular structure surrounding the food inside the mesenteron. This type of peritrophic membrane is produced by a band of specialized cells in the anterior end of the mesenteron encircling the base of the stomodaeal valve.

The function of the peritrophic membrane is somewhat conjectural. The fore and hind guts of Lepidoptera larvae have a cuticular lining on the border of their cells to protect them. The midgut does not, but the delicate epithelial cells

of the midgut are still separated from food and other particulates by the peritrophic membrane. The membrane is however, permeable to digestive juices, enzymes and products of digestion, (47). Day (23) suggested that the peritrophic membrane mainly protects the midgut epithelial cells from abrasion by particulates. It may act however, as an ultrafilter allowing smaller molecules to filter through. As such it could function in preventing ingested microorganisms from penetrating through and injure the underlying delicate epithelial cells. Because of this selective permeability for only small molecules, microorganisms are prevented from penetrating into the hemolymph where they can readily grow and cause septicemia. Podgwaite and Cosenza (86) working with a chitinase inducible strain of S. marcescens pathogenic to L. dispar larvae, found that the cause of death was septicemia and the peritrophic membrane of infected larvae was disrupted. They proposed that the peritrophic membrane is the first physical barrier that must be breached if invasion by the bacteria into the hemolymph is to take place. Jeuniaux (53) reported that the removal of the free chitin by chitinase does not alter the ultrastructural pattern of the peritrophic membrane while removal of protein by alkali causes a more pronounced dissociation of the strands into separate microfibrils. Successive treatment by alkali and chitinase completely destroys the structure. Undoubtedly chitin plays a fundamental role in the structural organization of the microfibers of the peritrophic membrane.

Lehane (56) demonstrated that the peritrophic membrane of the stable-fly was multilayered, composed of five layers. He determined that the second innermost layer was composed of chitin-protein in a non-fibrous form. The inner most as well as the third and fourth layers were periodate-sensitive Schiff-positive material but he did not identify them as containing chitin. Since there is no histochemical test specific for chitin (44), chitin may conceivably be present in those other layers and not be limited to the second layer. Indeed Mustafa and Kamat (77) claim that periodate acid Schiff-positive materials in the peritrophic membrane of Musca domestica, another Diptera, is chitin.

Regardless of the structural organizations of the chitinous peritrophic membranes in larvae such as L. dispar, it is a formidable barrier preventing large particulates such as bacteria from penetrating into the hemolymph. The breaching of this barrier can conceivably be accomplished in several ways. First, underlying chitin synthesizing cells could be destroyed by low molecular weight toxins which can permeate through the membrane and prevent the resynthesis of new chitin tissue. Second, harsh abrasives could physically tear and rupture the thin membrane. Third, chitinolytic microorganisms or an active chitinase alone could internally digest and perforate the peritrophic membrane. It is with this last possibility that this study is concerned.

Chitinolytic Microorganisms

As mentioned above, it has been estimated that crustaceans contribute to the deposition of several billion tons of chitin in the marine environment annually. Probably several times that amount is deposited annually by fungi and arthropods including terrestial types. Yet no great accumulation of chitin results, nor does chitin decompose spontaneously. Several investigators (cf. 92) have identified chitin in fossil remains. The decomposition of crustacean cuticle in water was first reported by Schlossberger (102) in 1856. That chitin must be decomposed relatively rapidly is self-evident, otherwise its accumulation and the resulting depletion of available carbon and nitrogen would present serious problems.

Benecke (2) in 1905 isolated from rotting plankton, an aerobic gram-negative asporogenous motile rod-shaped bacterium which decomposed chitin. He called it Bacillus chitinovorous. Three years later Stromer (118) reported the decomposition of chitin by a streptomycete. The early realization that chitinovorous microorganisms were present in a variety of environments is largely due to the work of Benton (3). In a survey published in 1935, she isolated two hundred and fifty chitinovorous bacteria from the intestines of frogs, bats, snipes, speckled trout, and from mud, stagnant pools, sand under running water, soil, soil compost heaps, water from different lakes, crayfish and mayfly nymph shells. At about the same time, several other investigators isolated and described

microorganisms from manured garden soil and lake water (112) which decomposed chitin anaerobically as well as aerobically. In a study reported in 1938, Zobell and Rittenberg (125) isolated 31 chitinoclastic bacteria from marine sediment, animals and sea water off the coast of Southern California. Common features among these isolates were their need for sea water for growth and the loss of their chitinoclastic ability when they were removed from chitin for a period of time. Colonization on crustacean cadavers and decomposition of the chitin in these environments occurred at temperatures of 0-4C on the ocean floor. In pure culture studies these isolates expressed their chitinoclastic ability at 21C. It was estimated that between 0.1 and 1.0% of the bacteria found in the sea were to some degree chitinoclastic and were most numerous in the topmost layers of mud. Variation among sampling sites strongly indicated that rather than evenly distributed in the sea these bacteria were primarily colonizers on crustaceans. In a more recent study, Campbell and Williams (11) isolated from marine mud several chitinase positive species of Achromobacter, Pseudomonas, Flavobacterium and Micrococcus.

Bucherer (10) in 1935 isolated from soil a sporeforming bacillus which he named Bacillus chitinobacter. He also identified several actinomycetes as Streptomyces griseolus, S. exofoliatus, S. fradiae, S. aureus and S. griseus as strong chitinolytic microorganisms. Skinner and Dravis (105) isolated and identified from soils forty two strains of fungi

capable of decomposing chitin. These included species of Aspergillus, Mucor, Penicillium, Absidia, Trichoderma, Fusarium, Gliocladium and Thannidium.

Veldkamp (119) during a study of the microflora of different soils reported in 1955 the isolation of chitinase positive species of Achromobacter, Flavobacterium, Chromobacterium, Bacillus, Cytophaga and Pseudomonas. He also isolated chitinolytic Actinomycetes (Streptomyces, Micromonospora and Norcardia) and fungi (Aspergillus and Mortierella species).

Gray and Baxby in 1968 (39) in a study of the ecology of chitinoclastic microorganisms in forest soil concluded that decomposition of chitin was brought about by fungi, bacteria and actinomycetes. In acid horizons the predominant fungal chitin decomposers were Verticillum spp., Mortierella marburgensis and Trichoderma viride, whereas in alkaline horizons Mortierella alpina, Paecilomyces carneus, Gleomastix mucorum and to some extent Verticilium spp. were the predominant types. Bacteria and actinomycetes, mainly species of Pseudomonas, Bacillus and Streptomyces, were found in all horizons.

Okafor (81) studying the association of chitinolytic microorganisms in temperate and tropical soils isolated and identified both fungi and bacteria. In temperate soils the chitinolytic fungi were Chaetonium piluliferum, Penicillium lilacinum, Trichoderma koningii and Verticillium lateritium.

From tropical soils, he isolated Aspergillus fischeri, Emericella rugulosa, Malustella aeria, and Thielavia terricola. Chitinolytic bacteria from temperate soils included a pseudomonad (which he did not describe) Beneckia cantabria and two sporeforming rods one of which he named Bacillus chitinosporus. Actinomycetes were isolated from both tropical and temperate soils. Two of these (one from each soil) he identified as Streptomyces alboflavus (from temperate soil) and Streptomyces anulatus (from tropical soils). Okafor also noted that a number of microorganisms (which he did not describe) were capable of growing on chitin strips, however they failed to clear chitin agar. Quite possibly such microorganisms could be utilizing the protein associated (99) with chitin as a substrate or were stripping off the N-acetyl group from the glucose backbone of chitin as had been suggested previously by Zobell and Rittenberg (125). The clearing of chitin agar is assumed to be the result of one enzyme system. However, Okafor demonstrated protocooperation activity where, individually some isolates were unable to clear chitin but did clear chitin when they were cross-streaked on chitin agar. These included mixtures of Cytophaga sp. and Achromobacter sp. cross-streaked with Arthrobacter sp. A similar phenomenom had already been reported with bacteria of marine origin by Zobell and Rittenberg (125).

Jeuniaux (48) had also shown that intestinal chitinolytic activity in some snails may have been due to gut resident

Eubacteriales.

From the brief review of the literature, it is evident that representatives of many genera of microorganisms including fungi and bacteria, aerobes and anaerobes, gram positive rods, non-sporeformers and sporeformers and gram negative bacterial species possess exocellular chitinase enzyme systems. Recently (73), the suggestion has been made that the chitinolytic property of Serratia species be used for taxonomic purposes.

Known entomopathogenic microorganisms have been shown to be inducible for chitinase. These included several species of bacteria including strains of Bacillus thuringiensis (24), S. marcescens, (74, 85), also several entomophthorous fungi including several species of Entomophtora (i.e. E. apiculata, E. thaxteriana, E. virulenta and E. coronata) (34), Beauvaria bassiana (17, 59) and Cordyceps militaris (54) to mention a few.

Chitinase Systems in Bacteria and Actinomycetes

Much of the early work on chitin decomposition, particularly microbial decomposition, was primarily concerned with physiological studies rather than the enzymatic mechanism involved. To date studies on the chitinase systems of fungi (82, 17), bacteria (74), bean and other seeds (88), snails (41), insects (121), and birds (51) have been reported. Jeuniaux (52, part III) reported extensively on the distribution of

chitinolytic enzyme systems in animals. The most thoroughly studied chitinase system has been the exocellular chitinase of Actinomycetes (4, 49, 50, 91, 106, 107).

Reynolds (91) in a study on the chitinase of Streptomyces sp. demonstrated that chitinase was not only exocellular (present in the supernatant) but that the enzyme was adaptive (inducible). Only in the presence of the chitin substrate as the sole source of carbon was the enzyme found in cell-free supernatants. Also the supernatant enzyme activity was substrate concentration dependent. Reynolds and others (74) have shown that in media containing chitin plus more readily utilizable sugars (glucose, glucosamine, NAG sucrose, ribose), exocellular chitinase synthesis is inhibited until after these other carbohydrates are consumed. The products of the hydrolysis of chitin by the crude enzyme preparation were identified as NAG and chitobiose, the dissaccharide of NAG. This observation indicated that the hydrolysis of chitin was at least a two step mechanism. In a later publication Reynolds in collaboration with Berger (4) further elucidated the mechanism of supernatant chitinase of Streptomyces griseus. By using zone electrophoresis and absorption techniques on Bauxite they separated three chitinase active proteins. Incubation of these three proteins separately on various substrates including di-, tri and tetra-saccharides of NAG and chitodextrin resulted in the following: two of the proteins (Chitinase C_1 and C_2)

hydrolysed all the substrated to the lower molecular weight polysacchride and released NAG; these two chitinases however were not active on the disaccharide (chitobiose). The third active protein, chitobiase, was able to cleave only those saccharides of less than four sugar residues and release only NAG. The crude supernatant chitinase had an optimal pH at 6.3.

Working with a different Streptomycete sp. isolated from garden soil, Skujin and his colleagues (107) showed that although two chitinase active proteins could be eluted from hydroxyapatite columns, only one product, NAG, could be identified chromotographically. Preliminary separation on polyacrylamide gel columns resulted in the separation of one chitinase peak from a proteinase also present in the supernatant. Unlike the chitinase studied by Berger and Reynolds, Skujin found that his isolated enzyme had an optimal pH at around pH 4.2 when it was incubated in Na-PO_4 buffer and an optimal pH at 4.8 when it was incubated in Na-acetate buffer. From these observations it became suspect that cations may affect the chitinase activity of their purified enzyme. At 0.2M concentration Mg^{++}, Co^{++}, and Zn^{++} were inhibitory and below 0.001M concentration Co^{++} and Zn^{++} had a slight stimulating effect. At concentrations above 0.05M both sodium (as NaCl) and calcium (as $CaCl_2$) were inhibitory but below a 0.04M concentration calcium had a stabilizing (activating) effect.

Aluminum and copper (50) have strong deleterious effect on the chitinase activity at concentrations as low as 0.01M. They estimated the molecular weight of their first peak chitinase to be around 29,000 a value close to the value of 30,000 reported by Jeuniaux (51).

In a third extensive study on the chitinase system of Streptomyces sp., Jeuniaux (50) isolated three protein fractions each of which could hydrolyse chitin and release NAG. When the three fractions were recombined they had a synergistic effect on chitin. He estimated the optimal pH of his crude chitinase at pH 5.2. He also showed that some of his fractions were able to release NAG from chitin and others were capable of depolymerizing chitin to shorter chain polymers.

Further evidence that chitinase may be a multiple enzyme system with both a nonspecific hydrolase and a specific chitobiase activity comes from the study by Monreal and Reese (74). They found that crude chitinase from Serratia marcescens would release only NAG from chitin. However, after partial purification of the supernatant proteins they were able to show that some proteins would release both NAG and chitobiose and others would release only chitobiose after incubation of the various fractions with chitin. The optimal pH for S. marcescens crude chitinase was pH 6.6.

Chitinase systems in fungi appear to operate in the same fashion in that at least two (or more) enzyme proteins are necessary for the complete hydrolysis of chitin to NAG.

Otakara (82) using chitinase from Aspergillus niger showed by ammonium sulfate precipitation and hydroxyapatite column separation that one fraction could rapidly decrease the viscosity of glycol chitin and release NAG whereas the other fraction was specific for the hydrolysis of chitobiose.

Hackman (41) studied the chitinase from snails and showed the only product hydrolysed was NAG. The optimal pH of his crude preparation was pH 4.7. He did not attempt to purify the crude chitinase from snail guts. Dandrifosse and Schoffeniels (22) had shown that snail gut chitinase was inactivated by 0_2. The product of digestion of chitin was mainly NAG. Chitobiose and chitotriose may have been released also.

In a study of chitinases in monocotyledons and woody and herbaceous dicotyledons, Powning and his colleague (88) observed the following: three fractions extracted from bean seeds had chitinase activity; two of these appeared to hydrolyse chitin randomly releasing NAG and the third fraction was chitobiose specific. Of the plants studied bean seeds had the highest chitinase and chitobiase activity followed by wheat and cabbage. Chitobiase was also detected in almonds and waratah. Though these investigators did not determine the optimal pH of their crude enzymes, they observed high activity in .05M citrate buffer pH 4.5.

It is becoming increasingly clear that chitinase functions in a similar fashion to other hydrolases in that a portion of

the enzyme complex randomly hydrolyses the long polymer and the more specific disaccharases act exclusively on the lower molecular weight disaccharides produced by the random hydrolysis. Furthermore the pH optima for chitinase activity vary according to the source of the enzyme (or enzymes).

Biological Control

Chitinase has been implicated in the biological control of fungi pathogenic to plants (71, 72). The evidence comes from the observation that in chitin amended soils the disease incidence decreased as the Actinomycete, Bacillus, and Pseudomonas species populations as well as the chitinase levels in the soil increased. Secondly, fungal cell walls containing both chitin and laminarin were readily lysed by exocellular enzymes produced by a Streptomycete sp. with the concomitant release of glucose and NAG (69, 124) from the fungal walls. Moore and his colleagues (75), have demonstrated that chitinase will lyse the mycelial walls of Trichophyton rubrum and release glucose and NAG. Their source of chitinase was produced from microorganisms isolated from healthy L. dispar larvae.

Until recently the use of microorganisms for the biological control of pest insects had been largely limited to those microbes which produced known and identifiable toxins such as the B. thuringiensis delta-endotoxins or to species specific viruses such as the nuclear polyhedrosis virus. Yet before the time of Pasteur, bacteria and fungi were known to be the

aetiological agents of insect disease of epidemic proportions often resulting in the almost total decimation of an insect population. Excellent reviews have been published by Steinhause (113, 114).

Very little experimentation has been reported on the use of chitinase as a method to overcome an insect's defenses against bacterial infection. This is surprising since the cuticle, foregut, hindgut and peritrophic membrane all contain large amounts of chitin. Historically, Serratia marcescens (115) and species of Escherichia, Aerobacter and Klebsiella (100) have all been recognized as insect pathogens. The mechanism of infection has never been defined. Very few instances of bacteria attacking the epidermis of live insects have been reported. The epidermis of the squash bug (Anasa tristis) was attacked by a non-sporeforming rod (improperly named Bacillus entomotoxican). A soil isolate, Micrococcus nigrofaciens, specifically attacks the epidermis of the June beetle larvae (Phytophaga) (Cf. 102). Recently, Zacharuk (123) demonstrated the penetration of the cuticular layer of the Elaterid larva by Metarrhizium anisopliae. The mechanism of penetration was attributed to physical means rather than by chemical digestion of the cuticle. Zacharuk did not investigate the possibility of the presence of chitinase in his fungal preparations. Samsinakova et.al. (101) demonstrated the lytic activity of several exoenzymes particularly chitinase on the

cuticle of the Greater Wax moth (Galleria mellonella). Gabriel (35) demonstrated histologically the presence of chitinolytic activity around fungal hyphae of E. coronata as they penetrated through the insect cuticle. Others, notably Robinson (94) and Lipke and Geoghegan (62) also strongly suggest the implication of chitinase in the penetration of insect cuticle by fungi.

The chitinous components of the integuments of dead insects are undoubtedly depolymerized and degraded by chitinase of microbial origin. Chadwick (16) had shown that Enterobacteriaceae pathogenic to the wax moth could utilize the insect tissue as a sole source of carbon, whereas non-pathogens could not. She attributed this ability and the pathogenicity of these bacteria to strong production of unspecified proteinases acting on the hemolymph proteins. However, other strong proteolytic bacteria such as Bacillus licheniformis are not pathogenic to this insect. No attempt was made to determine what enzymes may have been present in these tissue media. More recently, Dubois and Gunner (26) reported that the supernatants of Bacillus sp. isolated from healthy gypsy moth larvae were highly toxic to healthy larvae after induction for chitinase. The chitinase inducible bacilli were able to grow in a basal medium using the exoskeleton as the sole source of carbon, whereas a chitinase negative Bacillus species failed to grow. Also, when the supernatant (crude chitinase) was incubated with the exoskeleton, NAG was released.

Lysenko (66) recently demonstrated that chitinase of Serratia marcescens was toxic to G. mellonella larvae when administered parenterally. Smirnoff (108) and his colleague Valero (109) reported that the addition of chitinase to commercial preparations of Bacillus thuringiensis significantly increased the effectiveness of this bacterium against the Spruce budworm. They presumed that chitinase facilitated the penetration of the pathogen through the chitin gut wall and peritrophic membrane of these caterpillars. In another field study on the same pest insect, Morris (76) also demonstrated the increased effectiveness of commercial preparations of B. thuringiensis when chitinase was included with the pathogen. In a non-storage rapid feeding insect such as the Spruce budworm or gypsy moth, the rapid penetration through the gut may be a very significant step for the successful intoxication of the insect.

In the study of the population dynamics of the gypsy moth larvae, Campbell (12) demonstrated the importance of a "disease complex factor" in controlling the natural pest population. The disease incidence was usually brought about by high population densities, wet site conditions and starvation. Shortly after this initial report, he, in collaboration with Podgwaite (14, 83) showed that along with nuclear polyhedrosis virus infection, the major component of the disease complex was attributed to bacteria including Bacillus sp., Streptococcus faecalis,

Enterobacter sp., Hafnia sp., Serratia marcescens, Proteus sp., unidentified Enterobacteriaceae, Pseudomonas sp., and Alcaligenes sp. In a separate study Podgwaite and Cosenza (84) compared the microflora of living and dead gypsy moth larvae and found essentially the same microbial types and that the Bacillus spp. were the predominant species present. Though they found a large number of microorganisms associated with dead insects very few of these were actually pathogenic when grown in an enriched medium and fed to healthy larvae. In their taxonomic scheme for identifying these isolates they did not consider the possibility these isolates may be inducible for chitinase and possibly become pathogenic to their host.

Gypsy moth as with other forest insects normally contain a microflora reflecting that of their environment (116). Generally few microorganisms are present in their alimentary track, for even though both the stomodaeum and proctodaeum have a pH around 7.25 to 8 the pH of the mesenteron is at or above pH 8.3 and feeding is rapid without food storage for later digestion. However, under conditions of stress such as starvation or when forced to feed on unfavorable host plants, the pH in the mesenteron usually drops to pH 7.0 or below. Under these conditions non-pathogenic microorganisms may become pathogenic. In past studies such isolates have been dismissed as contaminants on the basis of their failure to infect the host after cultivation on enriched media. Since chitin, a principal component of L. dispar larvae tissue, is susceptible

to enzyme degradation by environmentally native microbes, it is conceivable then that some microorganisms which are part of the resident flora of healthy larvae can become pathogenic to their host after induction for chitinase.

MATERIALS AND METHODS

An oak forest stand in Whateley Massachusetts was used as the site from which healthy L. dispar larvae were randomly collected for the isolation of resident chitinolytic microorganisms. The oak stand, approximately three acres, was surrounded by and mixed with other hardwood trees. The predominant tree species were red oak (Quercus borealis Michx. f.) and white oak (Quercus alba L.). The larval population density was light, causing only minor defoliation during the May-June larval feeding period. The insect population was free of noticeable diseases including nuclear polyhedrosis virus infections. However, some larval parasitism by Compsillura concinnata and Apanteles melanoscelus, was observed.

During the active larval feeding period in May and June, one hundred and twenty instar III, IV and V larvae were aseptically picked at random from the tree trunks between three and six feet above the forest floor. Each larva was individually placed in a sterile test tube, and immediately returned to the laboratory for the isolation of chitinolytic microorganisms.

Isolation of Chitinolytic Microorganisms

Chitinolytic bacteria, actinomycetes and fungi were isolated from whole larvae by an elective culture technique. Each larva was mascerated in 1.0 ml of sterile distilled water and 0.1 ml of the mascerated tissue was spread on chitin agar

plates. The chitin agar contained in part, the basal salts of Modified Morris Medium (40) which are per liter:

	K_2HPO_4	7.0 g	
	KH_2PO_4	3.0 g	
10.0% sol.	$MgSO_4$	2.0 ml	
1.0% sol.	$FeCl_3$	0.1 ml	
10.0% sol.	$CaCl_2$	0.01 ml	(autoclave separately)
0.2% sol.	H_2BO_3	0.2 ml	
1.86% sol.	$MnCl_2{\cdot}4H_2O$	0.2 ml	
0.2% sol.	$CuSO_4{\cdot}5H_2O$	0.2 ml	
0.75% sol.	$NaMoO_7{\cdot}2H_2O$	0.2 ml	
0.37% sol.	$CoCl_2{\cdot}6H_2O$	0.2 ml	
0.25% sol.	$ZnSO_4{\cdot}2H_2O$	0.2 ml	
	$(NH_4)_2SO_4$	3.0 g	

Also included in the chitin agar medium were: 0.2% Casamino Acids, 0.1% purified chitin and 1.5% agar. The purified chitin was prepared from commercially available chitin (initially obtained from Pfanstiehl Laboratories, 1219 Glen Rock Ave., Waukegan, Ill., later supplies were obtained from J.T. Baker Chemical Company, 1170 Clifton Ave., Clifton, N.J.) according to the method of Skujin et.al. (107) or Vessey and Pegg (120). The pH was adjusted to pH 7.2 and the medium was sterilized at 121C, 15 psi for 15 min. Both the purified chitin suspension and the $CaCl_2$ solution were autoclaved separately and added to the medium after cooling to 50C. The chitin agar was poured

into sterile petri dishes to a thickness of 3-4 mm giving an opaque appearance to the agar plates. The inoculated chitin agar plates were incubated at 28C and observed daily for growth. Incubation was continued for a total of 15 days.

Isolated colonies of bacteria, actinomycetes and fungi which formed a clear zone around the colonies were considered chitinolytic. All chitinolytic bacteria and actinomycetes were picked onto Trypticase Soy agar slants (BBL). Chitinolytic fungi were picked onto Sabouraud Dextrose agar slants (BBL). All isolates were incubated at 28C for 24 hrs and stored at 4C for later studies.

Isolate Selection for Further Studies

Four isolates, two bacillus species which were coded No. 97B and No. $138B_2$ and two actinomycete species coded No. 222 and No. 226B were selected for further studies. The selection of these four isolates was based primarily on the rapidity and size of the clear zone formed by the growing colonies on chitin agar. All four isolates formed a 4 mm-wide clear zone around the edge of the growing colonies in 24 hours when incubated at 28C, indicating substantial synthesis of exocellular chitinase. Isolate No. 97B was isolated from an instar III larva. All other isolates were from instar IV larvae.

Gram stains (104) were done on 20 hr old cultures of all the bacterial isolates to determine their gram reaction. Most bacterial isolates were gram positive Bacillus sp. Wet mounts, observed under phase contrast microscopy, were made of all actinomycete and fungal isolates to determine their mycelial morphology.

Preliminary characterization of the two bacillus species was done using the method described in the "Genus Bacillus" (38) and "A Guide to the Identification of the Genera of Bacteria" (104). The actinomycete isolates were classified generically primarily on the basis of their morphology (55). Bergey's Manual of Determinative Bacteriology (9) was also extensively consulted.

Chitinase Production

Growth conditions: Exocellular chitinase was routinely produced and harvested as follows: the selected isolates were grown in chitin broth which contains the same ingredients as chitin agar except that agar is omitted and purified chitin is replaced with 1.5% commercially prepared ground chitin. (Purified chitin was used where specified). The pH was adjusted to pH 7.2 and the medium was autoclaved as usual (only the $CaCl_2$ solution was autoclaved separately).

The chitin broth was seeded with a 10% (v/v) inoculation of a 4 h old active log phase TSB culture. The seeded flasks were incubated and shaken (90 RPM on a shaker-incubator, New

Brunswick Scientific Co., Model G-25) at 28C for 6 to 15 days. After appropriate incubation time the cell-free supernatant was harvested by centrifugation at 27,000 xg for 30 min. and assayed for chitinase activity. Where specified 2.0% glucose was used in place of chitin as the sole source of carbon.

Chitinase assay: The standard mixture for chitinase assay is prepared as follows: One ml of purified chitin suspension (320 mg/ml dry wt. to give a final concentration of 80 mg/ml) is mixed with 1 ml of 0.2 M citrate buffer (to give a final concentration of 0.05 M and a pH of 5.2) plus 1 ml of enzyme preparation and 1 ml of distilled water and gently mixed together (to avoid air entrapment). The 4 ml assay mixture is incubated at 32C for 1 hr (unless otherwise indicated) then immediately centrifuged at 27,000 xg at 10C for 15 min. 0.5 ml of the supernatant is then assayed for the presence of N-acetylglucosamine (NAG) using the method of Reissig et.al. (90). The release of NAG is quantitated by extrapolation from an NAG standard curve. For each assay, appropriate blank, substrate, and enzyme controls are included to assure the measurement of only the net release of NAG by the active enzyme. A unit of chitinase activity is defined as the release of 1 μM of NAG by 1 ml of enzyme preparation in 1 hr under the above described incubation conditions. All buffers used in the study were as described by Gomori (37). Protein concentration was determined by the procedure described by Lowry et.al. (65) using Bovine

Serum Albumin as a standard (NBCo.).

Enzyme harvest: The chitinase-active cell-free supernatant proteins were routinely harvested by centrifugation (27,000 xg for 30 min.) and concentrated by precipitation at pH 7.2 with $(NH_4)_2SO_4$ at 75% saturation. Using the nomogram described by Dixon (25) the required amount of salt (516 g per liter vol.) was added to the supernatant directly, stirred for 1 hr at room temperature (25C) and the formed precipitate was harvested by centrifugation at 27,000 xg for 30 min. at 10C. The precipitate, now considered stock enzyme preparations, was resuspended in minimal volume of 0.05 M pH 5.2 citrate buffer and stored in ice at 4C. Routine harvest from 1 liter of cell-free supernatant usually resulted in a 40 fold concentration of enzyme protein when the precipitate was resuspended in 25 ml of buffer. An enzyme assay of every freshly prepared stock, diluted 1:100 was done to determine the number of units harvested. Using this procedure approximately 200 units are usually harvested from 100 ml of media.

Optimal pH Activity

Stock chitinases of No. 97B, No. $138B_2$, No. 222 and No. 226B diluted 1:100 were assayed at a pH range from pH 3 to pH 7.5. Citrate buffer was used for the pH range for pH 3 to pH 6 and citrate-phosphate buffer was used for the pH range from pH 5 to pH 7.5. Buffer concentrations were 0.05 M. The enzyme assays were conducted as previously described.

IN VITRO STUDIES

Larval tissue as an inducer for chitinase: L. dispar larvae were reared to instar III and IV, and starved for 48 hr to clear the alimentary tract of ingested diet. The starved larvae were ground in a tissue homogenizer to small particulate tissue. The ground tissue was washed 5 times by centrifugation at 27,000 xg for 10 min with distilled water. After the final wash five grams of wet packed tissue per 100 ml volume were used in place of chitin in the chitinase production media. The tissue medium was autoclaved, inoculated with log phase cultures and incubated as described above for chitinase production. Every 24 hrs for 12 days, 5 ml samples of the growing cultures were withdrawn, centrifuged at 27,000 xg for 15 min and the clear supernatant assayed for chitinase activity. Where specified the viable cell concentration in the tissue medium was determined by the single drop count procedure of Reed and Reed (89) on Trypticase Soy Agar using a 0.01 ml drop volume.

Chitinase Activity on L. dispar Exoskeleton and Alimentary Tract Tissues: The alimentary tract was dissected from starved (48 hr) instar IV larvae. The whole gut and exoskeleton (with attached muscular, tracheal and neural tissues) were separately ground and washed as described above for ground tissue medium. 80 mg per ml, final concentration of the separated wet packed ground tissues were used in place of purified chitin as substrate for determination of enzyme

activity. Stock enzyme preparations diluted 1:100 were used for this assay. The mixture was then incubated at 32C and 1 ml samples removed at hourly intervals for 3 hours, the samples were centrifuged as described for chitinase assay and the net release of NAG from the tissue by the active enzyme was determined as previously described. Blanks, tissue and enzyme controls were included for each assay.

Effect of Chitinase on Isolated Peritrophic Membranes: The peritrophic membranes of instar III and IV larvae were carefully dissected and placed on small coverslips. The membranes were then bathed in 0.5 ml of either a) distilled water, b) 0.05 M citrate buffer pH 5.2 or c) o.8 units of each of the chitinase preparations in 0.05 M citrate buffer pH 5.2. The membranes were incubated in moist chambers at 32C for either 1/2 or 3 1/2 hours. After the incubation period the membranes were washed in a gentle stream of distilled water, quick frozen and lyophilized. The lyophilized peritrophic membranes were then prepared for scanning electron microscopic observations. The peritrophic membranes from five larvae were examined for each instar and each incubation condition.

Scanning Electron Microscopy: Peritrophic membranes selected for scanning electron microscopy were prepared as follows: The lyophilized peritrophic membranes were filmcoated with a gold-palladium alloy approximately 200Å thick using a sputter coater. The specimen were observed at several magnifications with an Eteck Autoscan Scanning Electron Microscope

at 20 Kv and at a 45^{o} tilt. The microscope was equipped with a 4 x 5 format polaroid camera attachment.

IN VIVO STUDIES

Bioassays

For all bioassays, field collected L. dispar eggs were hatched and reared on artificial diet according to the procedure described by ODell and Rollinson (79) except that antibiotics were omitted and the diet ingredients were purchased partially pre-mixed (Bio-Serv, Inc., Railroad Ave., Frenchtown, N.J. 08825). When the larvae reached the appropriate instar stage (specified for each bioassay), they were separated into groups of 10 per petri dish, and starved for 24 hrs before being fed the various test materials.

Test materials were presented to larvae by allowing them to feed freely on 1 cm^3 cube of diet coated with 0.1 ml of the test materials. The larvae were allowed to feed on the coated cubes (3 per petri dish) for 48 hours then returned to normal diet and observed for mortality (or other specified symptoms) for an additional 3-5 days. Throughout the bioassays the larvae were kept in environmental chambers at 26C, 70% RH and with 12 hrs of light and 12 hrs of dark.

Specific Bioassays: Isolates No. 97B and No. $138B_2$ were grown for 14 days in both glucose and chitin broth media. Cell free supernatants as well as whole cultures of both isolates grown in the two media were fed to instar III larvae (as

described above). Three replicates of 10 larvae each were used for each test material. The bioassays were terminated after 5 days.

Use of E-61 as a Marker: E-61, the international standard of B. thuringiensis (100 IU/mg) obtained from H. DeBarjac (Pasteur Institute, Paris, France) in 1972 and maintained at 4C, was used as a positive marker for the bioassay of the four chitinase preparations. A 0.05 mg/ml (sublethal dose) suspension of E-61 was prepared in sterile distilled water. The suspension was then fed to instar II larvae with various dilutions of the stock chitinase preparations (in 0.05 M citrate buffer pH 5.2) from the four isolates. Five replicates of 10 larvae each were used for each enzyme dilution used. Appropriate controls with only E-61 or the chitinase stocks diluted at 1/25 and 1/50 as well as blank controls were included. The bioassays were terminated after 5 days.

Larval Growth Retardation: Larvae were reared to instar II and fed for 48 hrs on diet containing either 0.005 mg of E-61 or the following units of the different chitinase preparations; 0.35 units of No. 97B, 0.33 units of No. $138B_2$, 0.58 units of No. 222 and 0.45 units of No. 226B. Combined E-61 and the different chitinase preparations were also assayed. Five replicates of 10 larvae each were used for each material (or combinations) tested. The larvae were weighed at day 0 (just prior to being fed the test materials), at day 2 (when

removed from the test material) and at day 7 (five days after being returned to normal diet).

In vivo Chitinase Activity on the Peritrophic Membrane: Instar II, III and IV larvae were fed several concentrations of the stock chitinases diluted in either citrate buffer or distilled water. E-61 alone (0.005 mg) and combined with the chitinase preparations was also fed to larvae. After 72-96 hours, the larvae were sacrificed and the peritrophic membranes were surgically removed intact and lyophilized. The lyophilized membranes were then prepared for scanning electron microscopy. Only live larvae that survived the doses used were examined.

Determination of the pH of the Mesenteron: The pH of the mesenteron of larvae feeding on diet coated with E-61 (0.005 mg), citrate buffer (0.2 M pH 5.2) and the different chitinase preparations (in 0.05 M pH 5.2 buffer) was determined using the procedure described by Dadd (21). Phenol red or thymol blue pH indicators were incorporated into the diet at a concentration of 4 mg per 100 ml of diet. The indicator diets coated with the various preparations were fed to instar II and III larvae. A minimum of 10 larvae were dissected and the alimentary tract exposed for each material fed on each indicator diet for 24 to 48 hours. The approximate pH of the fore, mid and hindguts was determined according to the color of the indicator. Preliminary studies indicated that the pH within

each section of the alimentary tract was highly variable. For this reason each region of the alimentary tract had to be subdivided into regions. The stomodaeum (foregut), from the crop to the gastric cecum, was arbitrarily divided into 4 regions; the mesenteron (midgut), from the gastric cecum to the pyloric valve was divided into 6 regions; and the proctodaeum (hindgut), from the pyloric valve to the anal opening, was divided into 2 regions.

Ancillary Studies of the Chitinase from Isolates No. 97B and No. $138B_2$

Chitinase of both No. 97B and No. $138B_2$ were further investigated by ultrafiltration, fractional precipitation with $(NH_4)_2SO_4$, and by saturation kinetics.

Ultrafiltration: Three ml of stock chitinase was diluted with 27 ml of 0.05 M citrate buffer pH 5.2 and divided into three 10 ml aliquots. Each aliquot was filtered through Amicon ultrafilters (American Corp., Lexington, Ma.) under 80 psi pressure of N_2 gas at room temperature. One aliquot was filtered through a PM 30 ultrafilter (retains 30,000 mol, Wt), one filtered through a PM 10 ultrafilter (retains 10,000 mol, Wt), and one aliquot was filtered through a UM 2 ultrafilter (retains 1000 mol, Wt). Retentates on the filters were washed with 10 ml of buffer to resuspend them to original volume. Chitinase activity and protein concentrations were determined on both the

filtrates and retentates.

Fractional Precipitation: Isolates No. 97B and No. $138B_2$ were grown in 100 ml of chitin medium and the cell free supernatant was harvested after 15 days incubation. The supernatants were subjected to fractional precipitation with $(NH_4)_2SO_4$. The ammonium salt was added to the supernatants at room temperature (25C) at a pH of 7.2 in 15% saturation increments to 90% saturation where 8.7, 8.6, 9.4, 9.9, 10.5, and 11.5 grams of $(NH_4)_2SO_4$ were added successively to the supernatants (25). After each fractional addition, the supernatants were stirred for 1 hr and the precipitates formed were harvested by centrifugation at 27,000 xg for 30 min at 10C. Each fraction collected was redisolved in minimal volume buffer (0.05 M citrate, pH 5.2) and the chitinase activity and protein concentration determined.

Preliminary Enzyme Saturation Kinetics: A preliminary substrate saturation curve was done on the chitinase harvested from No. 97B and No. $138B_2$. The substrate (purified chitin) concentration ranged from 1 mg/ml to 80 mg per ml and stock enzyme concentrations were 0.34, 0.25 and 0.21 mg of enzyme protein per ml for the chitinase stock from No. 97B, and 0.32, 0.21, and 0.16 mg of enzyme protein per ml for the chitinase from isolate No. $138B_2$.

RESULTS

Isolation of Chitinolytic Microorganisms

Chitinolytic microorganisms were readily isolated from instars III, IV and V larvae (Figure 1) by using the elective culture technique described in Materials and Methods. The complete clearing of chitin around the edge of the growing colonies suggests that the exocellular chitinolytic enzyme system contains both chitinase and chitobiase enzyme activity necessary for total hydrolysis of chitin (52).

Though incubation of the inoculated chitin agar plates was continued for 15 days, chitin was usually cleared within a 5 to 7 day incubation period. Many of the isolates formed clear zones around the edge of the growing colonies within 24 hours after inoculation.

Non-chitinolytic microorganisms (those which fail to clear the chitin) were also observed growing on the chitin agar. Periodic inoculations of mascerated larval tissue on Trypticase Soy agar indicated that the total microbial populations present on individual larva was highly variable. Some larvae (at every instar examined) harbored high numbers of bacteria, actinomycetes and fungi. Other larvae were completely free of any aerobic microbial types.

Only 23% of the instar III larvae examined (120 larvae) harbored chitinolytic microorganisms. As the larvae matured and increased their migrating activity, a higher proportion of

the larvae contained a chitinolytic microflora such that about 87% and 82% of the instar IV and V larvae contained a mixed microflora of chitinolytic microorganisms. The various types and proportions of microorganisms (bacteria, actinomycetes and fungi) isolated from healthy L. dispar instar III, IV and V larvae are summarized in Table 1. The distribution of the microbial types found appears to correlate with the behavior patterns that accompany these maturing larvae. Younger larvae, i.e. instar I, II and III, are known to remain usually on the upper part of the trees, and since they are primarily nocturnal feeders they will rest on the underside of the leaves during the day (58). As the larvae mature to instar IV and V, they go through a behavior change were they migrate the length of the trees and through the decaying foliar matter on the forest floor. These older larvae will often rest during the day under the base of branches and under loose bark of dead or dying trees. Indeed many of the instar IV and V larval were picked from such natural niches when collections were made at midday. This behavior, called the diurnal cycle is expressed particularly in low density larvae populations (6) as were those used for this study.

Most of the chitinolytic bacteria isolated were gram positive sporeforming Bacillus sp. This was particularly true of isolates from instar III larvae where all bacterial

isolates were Bacillus species. Podgwaite and Cosenza (84), in a study of bacteria of living and dead L. dispar larvae, found that the predominant bacterial species associated with these insects were Bacillus species. From older instar larvae, all the actinomycetes isolated were Streptomyces species, one of the more common actinomycetes usually found in a forest environment (39, 46). Several species of chitinolytic fungi particularly Aspergillus species were also isolated from instar IV and V larvae.

FIGURE 1

Clearing of chitin agar by bacteria from a healthy L. dispar instar III larvae after incubation at 28C for 5 days. Arrow indicates the clear zone resulting from hydrolysis of the N-acetylglucosamine polymer at the β 1 $\longrightarrow$ 4 glycosidic bond.


689
6-8b

TABLE 1

Proportion of healthy Lymantria dispar (L.) larvae with chitinolytic microorganisms

	INSTAR					
	III		IV		V	
Total Examined	120		120		120	
Microbes Found	No. of larvae	Percent	No. of larvae	Percent	No. of larvae	Percent
Bacteria	27	22.5	24	20.0	56	47.0
Actinomycetes			13	10.8	2	1.7
Fungi	1	0.8	18	15.0	11	9.3
Bact. + Actino.			15	12.5	4	3.4
Bact. + Fungi			23	19.2	8	6.7
Actino. + Fungi			5	4.2	1	0.8
Bact. + Actino. + Fungi			6	5.0		
TOTALS	28	23.3	104	86.7	82	68.9

Isolate Selection for Further Studies

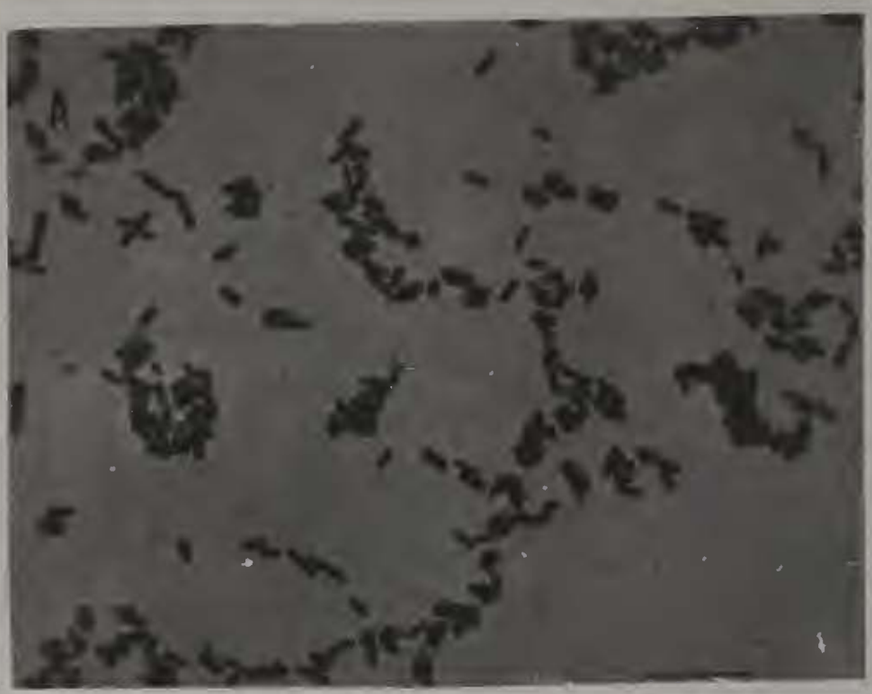
The selection of the four isolates for this study was essentially random. However, all four isolates coded No. 97B, No. $138B_2$, No. 222 and No. 226B grew rapidly on chitin agar and readily formed a clear zone (chitin hydrolysis) around the growing colonies within a 24 hour incubation period at 28C.

Both isolates No. 97B (isolated from instar III larvae) and No. $138B_2$ (isolated from instar IV larvae) have been tentatively identified as Bacillus coagulans. They are gram positive, sporeforming rods, (Figures 2 A and B) motile by peritrichous flagella and with dimensions of 0.6 - 1 x 2.5 - 5 μm. Preliminary physiological and biochemical characteristics are summarized in Table 2.

FIGURES 2

A. Gram stain of isolate No. 97B after incubation on Trypticase Soy agar at 28C for 24 hours. 1750 X, bar represents 10 µm.

B. Gram stains of isolate No. 138B$_2$ after incubation on Trypticase Soy agar at 28C for 24 hours. 1750 X, bar represents 10 µm.




B

TABLE 2

Physiological and biochemical characteristics of isolates No. 97B and No. $138B_2$ tentatively identified as Bacillus coagulans and compared to B. coagulans (38).

Characteristic	ISOLATES 97B	$138B_2$	B. coagulans (38)
Gram stain	+	+	+
spore position	c-pc, cyl	c-pc, cyl	variable
globules formed	-	-	-
atmosphere	f	f	f
catalase	+	+	+
V-P	+	+	+
pH in V-P	6	6	4.2 - 4.8
$NO_3 \rightarrow NO_2$	-	-	15-49% +, others-
propionate	+	+	15-49% +, others-
starch	+	+	+
gelatine	+ (slow)	+ (slow)	+ (slow)
growth in 7% NaCl	+	+	-
growth in pH 5.7	+	+	+
dihydroxyacetone	-	-	
litmus milk	A,R	A,R	A,R,C

Key: c = central, pc = paracentral, cyl = cylindrical, f = facultative, litmus milk, A = alkaline, R = reduction, C = curd formation.

Characteristics for both Bacillus species isolates (i.e. No. 97B and No. $138B_2$) agree very closely with those reported for B. coagulans except for growth in propionate broth. Only minor differences were observed for nitrate reduction, citrate utilization and growth in 7% NaCl. Characteristics reported for other Bacillus species did not correlate as closely with these two isolates.

The two actinomycetes selected, No. 222 and No. 226B, (both isolated from instar IV larvae) were tentatively identified on the basis of their morphological characteristics as described by Lechevalier and Lechevalier (55). Typical appearance of both isolates are presented in Figures 3 A, B, C, and D. Both isolates are typical Streptomyces species with aerial branching, non-septate mycelia, 1-3 micrometers in diameters. Agar surface growth was typical as described for this genus with growth into and adherance to the agar surface. Colonial appearances are pale white and dry.

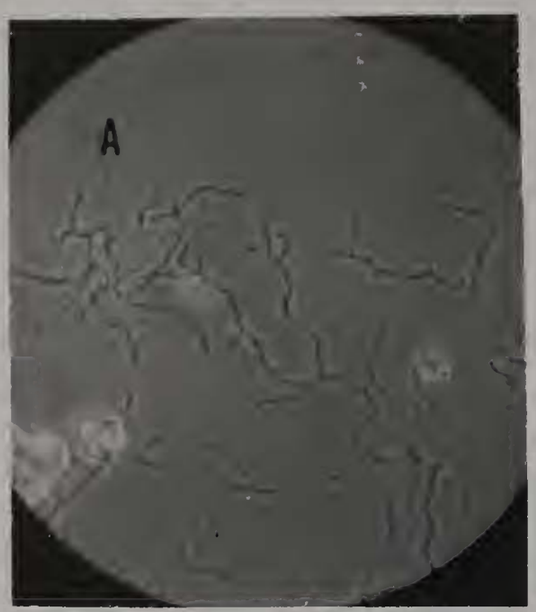
FIGURES 3

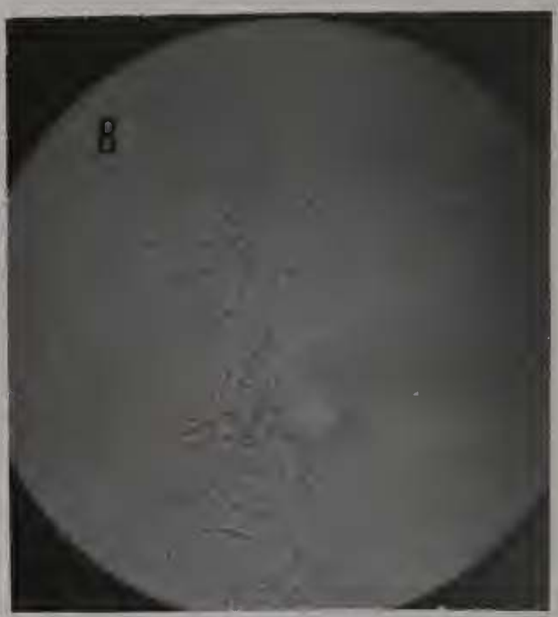
A. Mycelial arrangement of isolate No. 222 after growth at 28C for 24 hours in Trypticase Soy broth. Phase contrast, 1250 X.

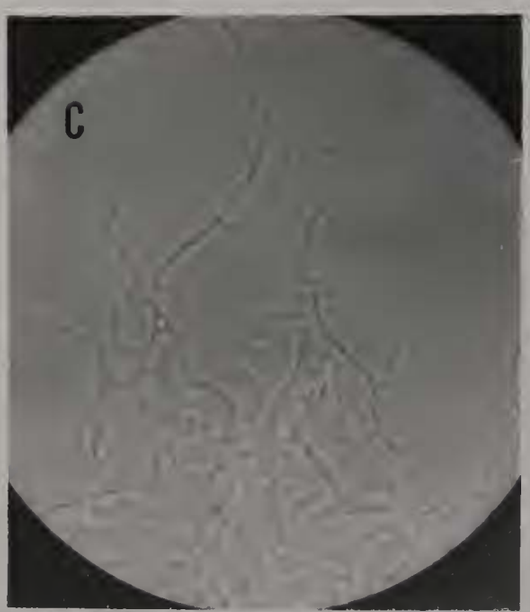
B. Colonial and aerial mycelial arrangement of isolate No. 222 on Trypticase Soy agar after growth at 28C for 120 hours. Phase contrast 675 X.

C. Mycelial arrangement of isolate No. 226B after growth at 28C for 24 hours in Trypticase Soy broth. Phase contrast, 1250 X.

D. Colonial and aerial mycelial arrangement of isolate No. 226B after growth at 28C for 120 hours on Trypticase Soy agar. Phase contrast, 675 X.


A


B


C


D

Chitinase Production

Routine production of exocellular chitinase by all four isolates in commercial grade chitin resulted in yields of 1.5 to 2.5 units of chitinase per ml of medium. Exocellular chitinase activity could not be detected in the supernatants of any of the isolates when grown in a glucose broth medium. For both Bacillus coagulans isolates (Figure 4), initiation of chitinase synthesis did not begin until after the first 48 hours of incubation and was usually complete in 14 days. Initiation of chitinase synthesis for both Streptomyces sp. isolates (Figure 5) on the other hand began within 24 hours and was complete in 6 to 10 days of incubation. These rates of enzyme synthesis are in agreement with the published reports for Streptomyces griseus (4) and Serratia marcescens (74). Routine harvest of 1 liter of the supernatant enzyme proteins by saturation with $(NH_4)_2SO_4$ to 75% (see appendix Tables A1 and A2) resulted in harvests of 25-30 ml of stock enzyme with 40-43 units of activity per ml.

FIGURE 4

Induction for chitinase by isolates No. 97B and No. $138B_2$ in commercial grade chitin.

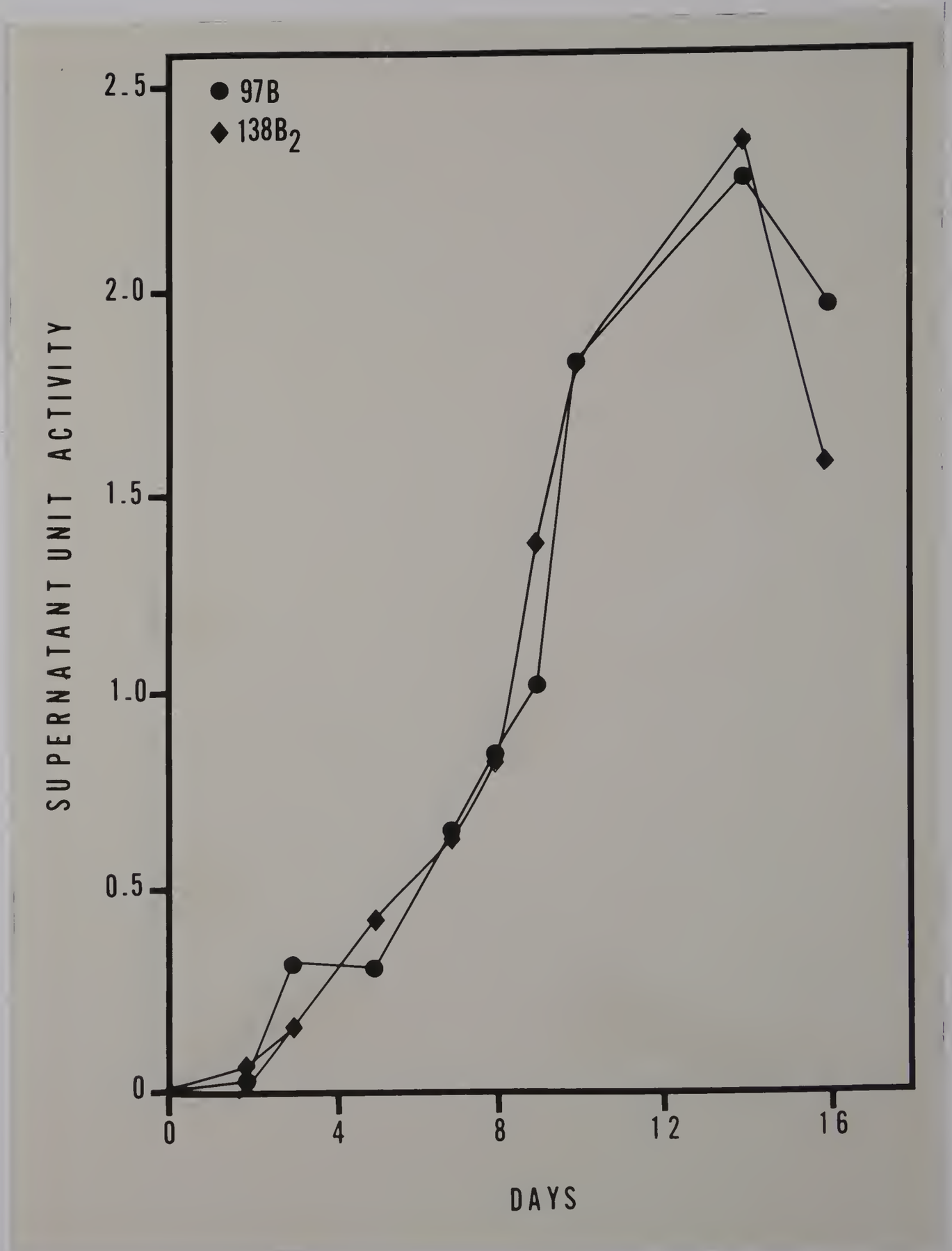
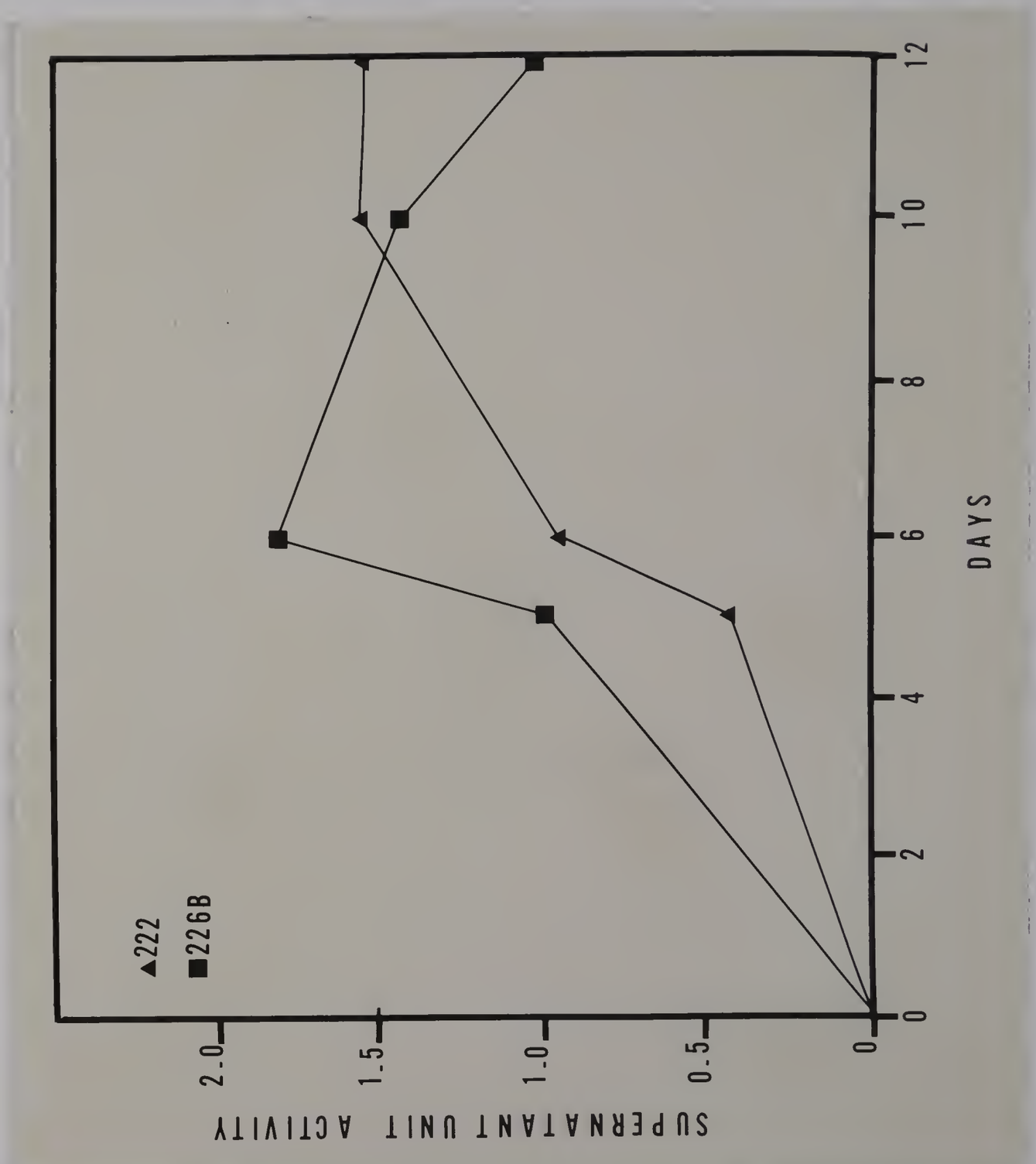

● 97B
◆ 138B2
SUPERNATANT UNIT ACTIVITY
2.5
2.0
1.5
1.0
0.5
0
0
4
8
12
16
DAYS

FIGURE 5

Induction for chitinase by the *Streptomyces sp.* isolates No. 222 and No. 226B in commercial grade chitin.

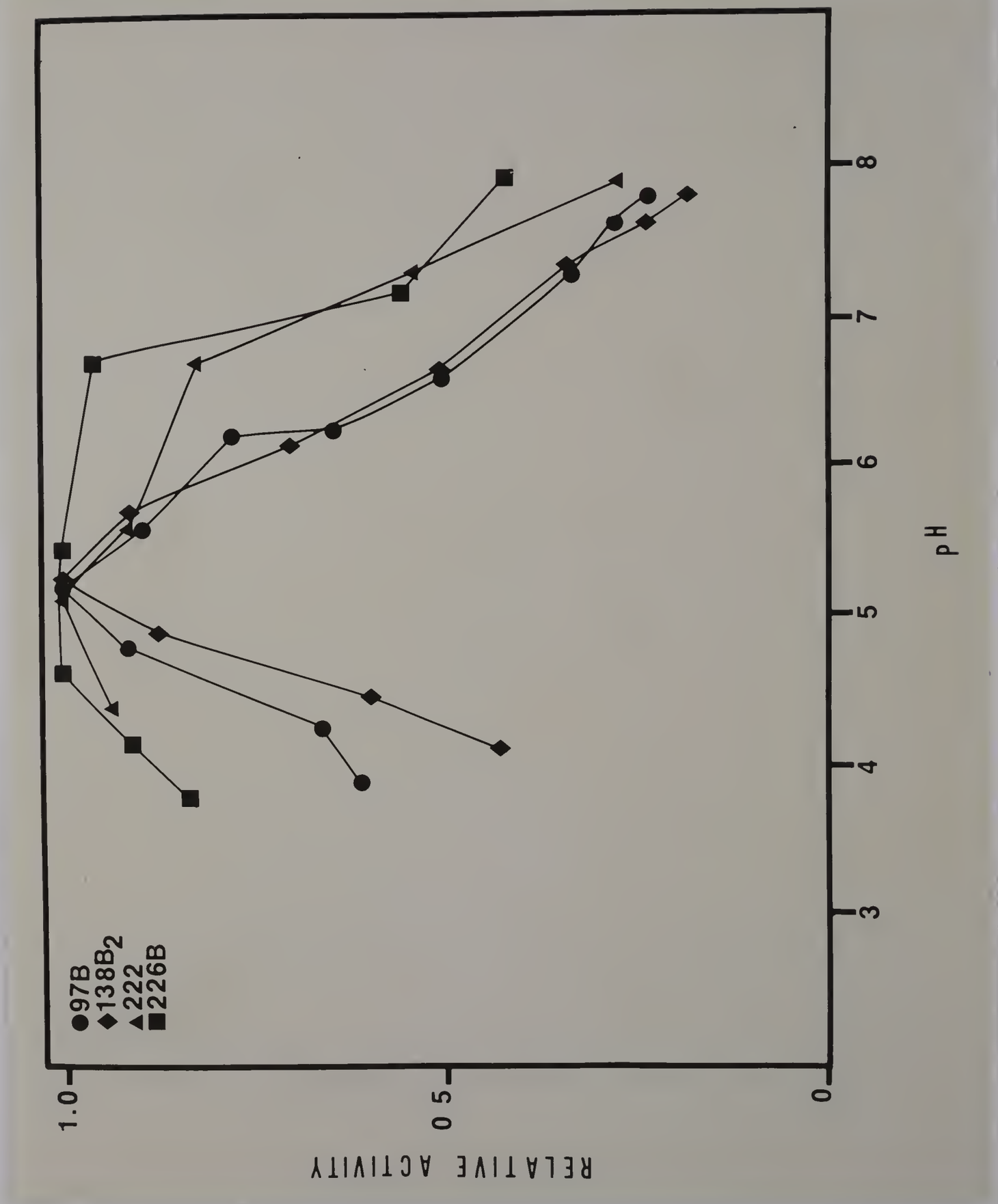

SUPERNATANT UNIT ACTIVITY
2.0
1.5
1.0
0.5
0
0
2
4
6
8
10
12
DAYS
▲222
■226B

Optimal pH

Optimal pH of the harvested chitinase (crude enzyme preparation) from all four isolates was at pH 5.2 (Figure 6). The activities of the crude enzymes from both Bacillus coagulans isolates decreased relatively rapidly when assayed at pH values removed from the optimum. However, the crude enzymes from both Streptomyces species exhibited a much broader optimal pH range particularly for isolate No. 226B where activity remained near optimum for a pH range from pH 4.6 to 6.8. At pH 8.0, a pH condition approaching that which is present in the mesenteron of L. dispar larvae, chitinase activity of the crude enzyme preparations of both Bacillus coagulans isolates (No. 97B and No. $138B_2$) was reduced to 25% of its optimal activity whereas enzyme activity for the crude enzyme preparations of both Streptomyces species (No. 222 and No. 226B) were still at 45 to 50% of their optimum.

FIGURE 6

Optimal pH of stock Chitinase from isolates No. 97B, No. $138B_2$, No. 222 and No. 226B.


RELATIVE ACTIVITY
1.0
0.5
0
3
4
5
6
7
8
pH
●97B
◆138B2
▲222
■226B

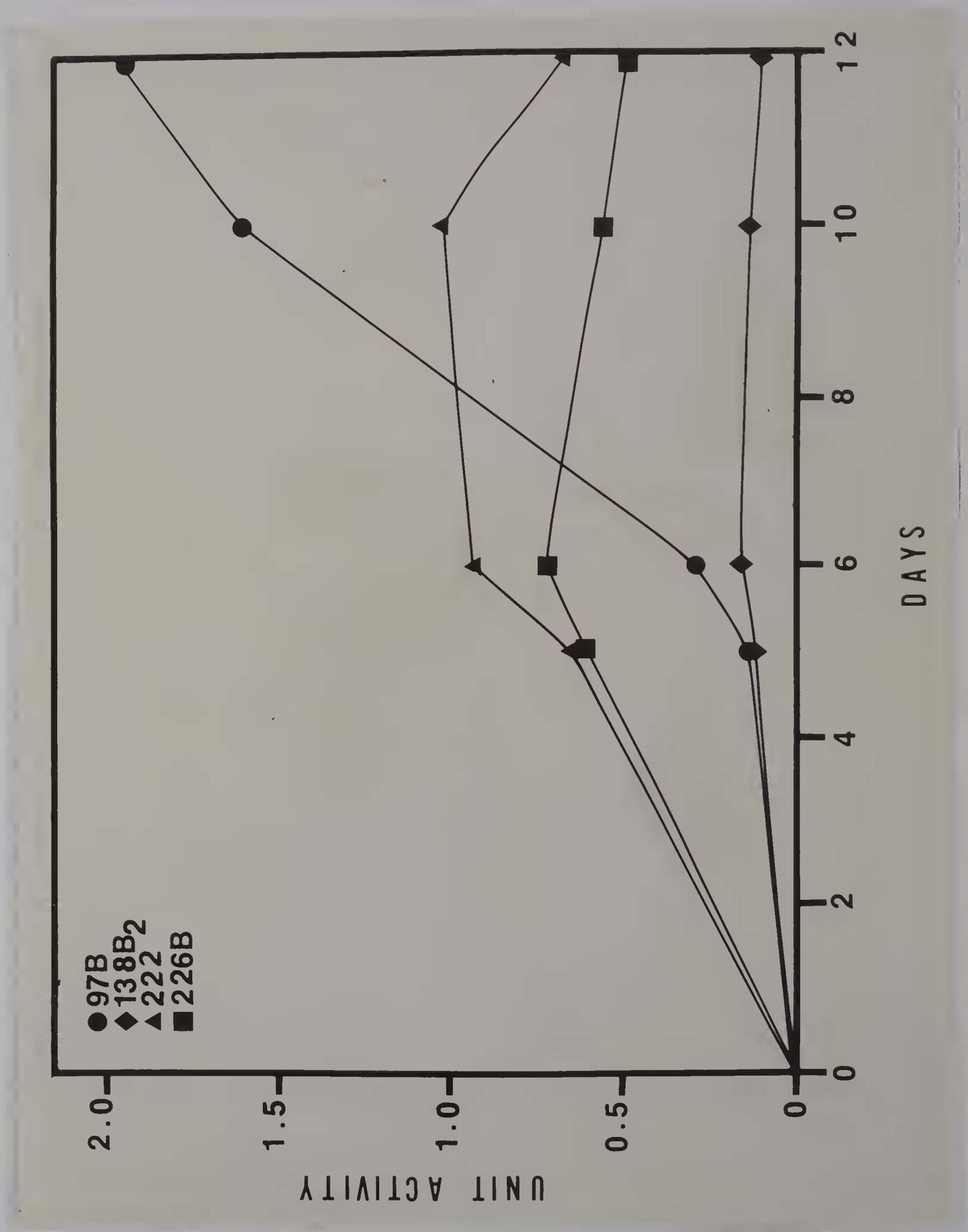
IN VITRO STUDIES

Induction for Chitinase by L. dispar Larval Tissue: L. dispar larval tissue which contains a substantial amount of chitin (20 to 50% of the exoskeleton, (52)) was examined as a possible inducer for exocellular chitinase by these four isolates. Results of replacing chitin with ground larval tissue in the chitinase production medium are presented in Figure 7. After an initial 5 day delay, B. coagulans isolate No. 97B synthesized approximately as many chitinase units in 12-14 days as when using chitin as substrate. But isolate No. $138B_2$ produced only low-levels of chitinase. Neither isolate appeared to substantially disintegrate the tissue further than that which was apparent from the initial grinding. Both isolates proliferated from an initial cell concentration of 1×10^6 viable cells per ml to approximately 1×10^9 viable cells (spores) per ml.

Both Streptomyces sp. isolates were readily induced for chitinase reaching a maximum enzyme activity in 6 to 10 days. Total enzyme production (units per ml) was however substantially less than that observed in chitin medium. Extensive growth was apparent, and noteworthy was the observation that after 12 days of incubation, the larval tissue was completely disintegrated and was unrecognizable as ground larval tissue.

FIGURE 7

Induction for Chitinase by isolates No. 97B, No. $138B_2$, No. 222 and No. 226B in ground *L. dispar* larvae tissue medium.

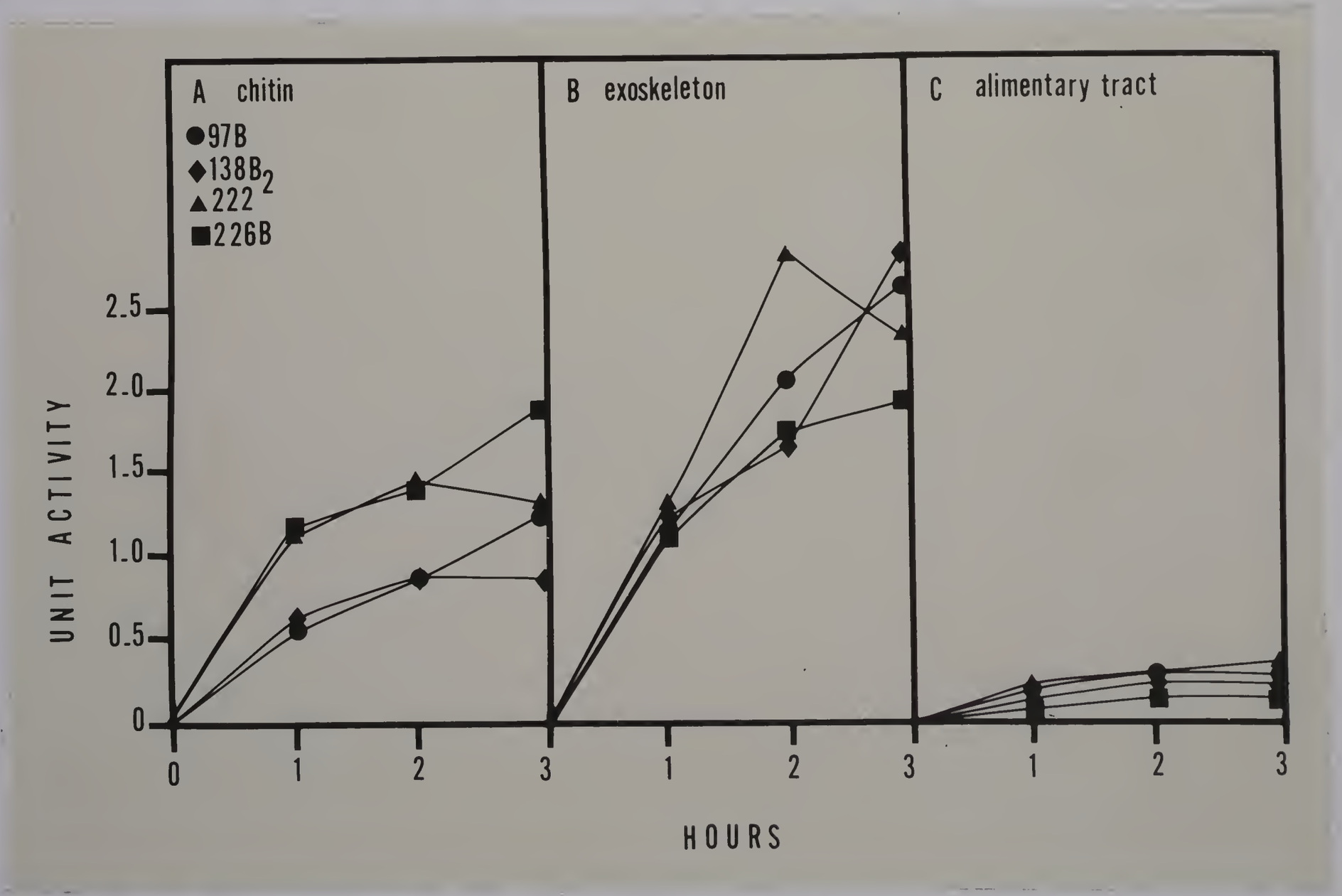

●97B
◆138B2
▲222
■226B
UNIT ACTIVITY
2.0
1.5
1.0
0.5
0
0
2
4
6
8
10
12
DAYS

Chitinase Activity on *L. dispar* Exoskeleton and Alimentary Tract Tissues

In vitro studies indicated that the crude chitinase harvests of all four isolates can readily attack and hydrolyse the chitinous integuments (i.e. native chitin) of these larvae and release the NAG monomer (Figures 8 A, B, C). Though all three substrates were at a final concentration of 80 mg per ml, the exoskeleton substrate contains approximately 45-50 mg of native chitin per ml and the alimentary tract tissue substrate contains 1.5 - 2 mg of native chitin per ml (i.e. peritrophic membrane (53)). Chitin substrate on the other hand contains 80 mg of purified chitin per ml and at this concentration saturation kinetics (maximum velocity (see Appendix Figures A1 and A2)) is usually achieved for the amount of enzyme protein used in this study (i.e. 0.4 mg of protein/ml for No. 97B and No. $138B_2$ and 0.3 mg of protein/ml for No. 222 and No. 226B).

FIGURE 8

Stock Chitinase activity of isolates No. 97B, No. $138B_2$, No. 222 and No. 226B on (A) purified chitin, (B) *L. dispar* exoskeleton and (C) alimentary tract tissue.


A chitin
B exoskeleton
C alimentary tract
●97B
◆138B2
▲222
■226B
UNIT ACTIVITY
2.5
2.0
1.5
1.0
0.5
0
0
1
2
3
1
2
3
1
2
3
HOURS

Effect of chitinase on isolated peritrophic membranes: Since the peritrophic membrane is the first physical barrier in the larval gut that must be breached before infecting microorganisms can penetrate into the hemolymph, studies were conducted to determine whether chitinase from these isolates can attack the peritrophic membrane, disrupting its physical integrity sufficiently to permit microorganisms to penetrate through into the hemolymph.

Peritrophic membranes from instar III larvae were carefully dissected and removed from the hemocoel cavity and incubated in distilled water or 0.2 M citrate buffer pH 5.2 with and without crude chitinase (0.8 units) for 1/2 and 3 1/2 hours. The results are presented in Figures 8 A, B, C, D, E, and F. Initial damage to the peritrophic membrane due to the preparation procedure can be seen in the form of small holes or tears perforating the membranes. The damage appears to be caused by the initial dissection or lyophilization and not to exposure to buffer or water for any length of time since the effect appears the same whether they are incubated (at 35C) for 1/2 hour or 3 1/2 hours, (Figures 9 A and B).

The *in vitro* dissolution of the peritrophic membranes was quick and dramatic. Stock chitinase from all four isolates (i.e. 0.8 units) very quickly destroyed the integrity of the membrane (Figures 9 C and D). However, when incubated with any of the four chitinase preparations (i.e. 0.8 units/

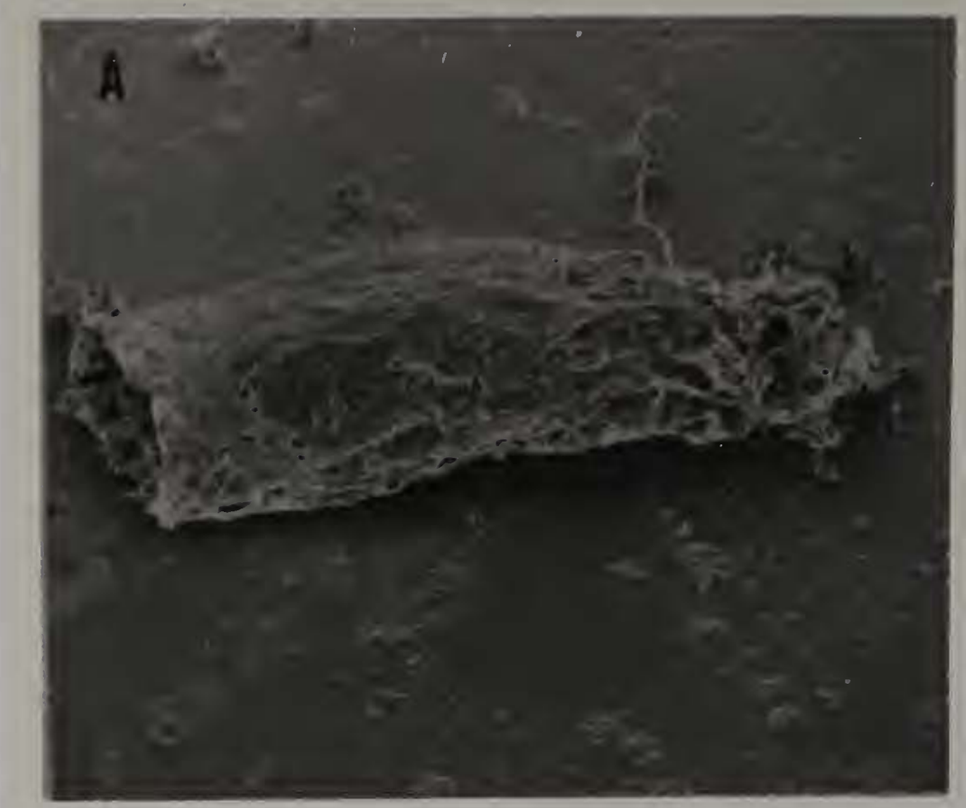
ml) massive ulceration and hydrolysis of the peritrophic membranes is readily observed (Figures 10 B, C, D and E).

The crude chitinase preparations from the four isolates (No. 97B, No. $138B_2$, No. 222 and No. 226B) readily attacked and hydrolized the peritrophic membranes of either instar III or instar IV larvae. These visual observations of *in vitro* digestion of the peritrophic membranes further supports the previous observations (Figures 8 B, and C) that the chitinous integuments of *L. dispar* larvae are very susceptible to extensive hydrolysis by these chitinase preparations.

FIGURE 9

A. Scanning electron micrographs of the isolated peritrophic membrane of an instar III L. dispar larva incubated in water at 35C for 0.5 hour. Magnification is 20 X.

B. Scanning electron micrographs of the isolated peritrophic membrane of an instar III L. dispar larva incubated in water at 35C for 3.75 hours. Magnification is 20 X.


A


B

FIGURES 9

C. Scanning electron micrographs of the peritrophic membranes of an instar III L. dispar larva incubated with 0.8 units of chitinase from isolate No. 222 for 3.5 hours. Magnification is 20 X.

D. Same as Figure 9C but incubated with chitinase from No. $138B_2$, for 0.5 hours.

E. Same as Figure 9C but incubated with chitinase from No. 97B, for 3.5 hours.

F. Same as Figure 9C but incubated with chitinase from No. $138B_2$, for 3.5 hours.

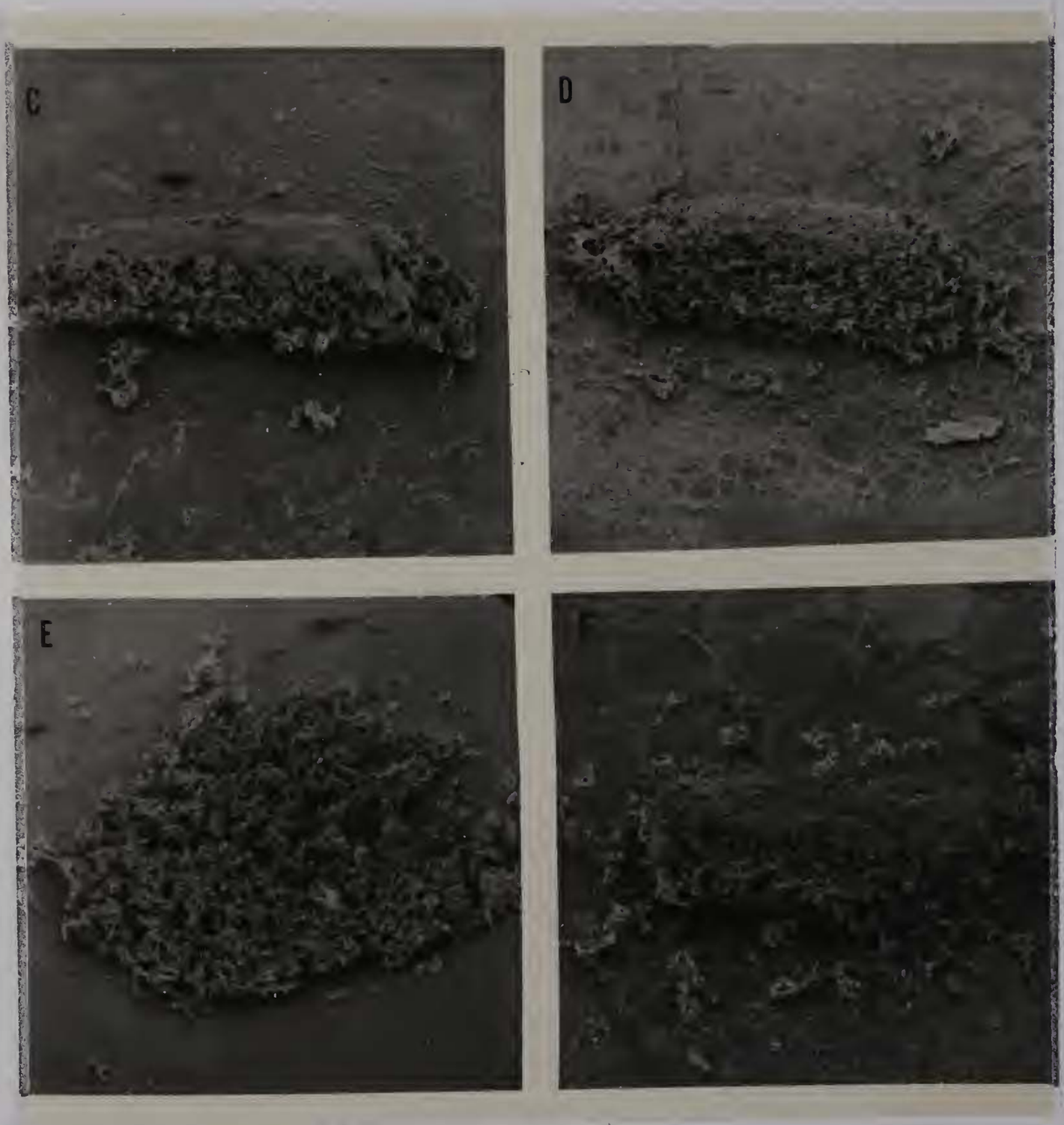

C
D
E
F

FIGURE 10

A. Scanning electron micrograph of an instar IV L. dispar peritrophic membrane incubated in water at 35C for 0.5 hours. Magnification is 20 X.


A

FIGURES 10

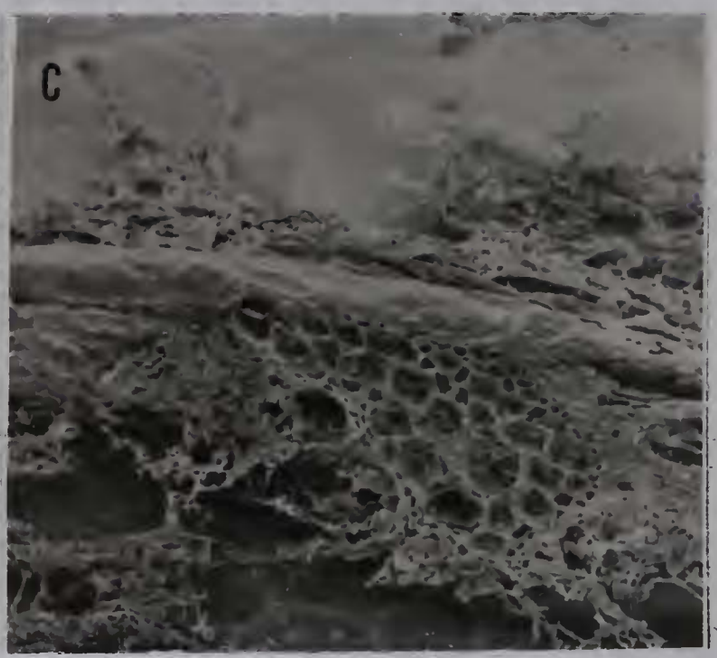
B. Scanning electron micrograph of the peritrophic membrane of an instar IV L. dispar larva incubated with 0.8 units of chitinase from No. 97B at 35C for 0.5 hrs. Magnification is 20 X.

C. Same as Figure 10B but incubated with chitinase from No. $138B_2$.

D. Same as Figure 10B but incubated with chitinase from No. 222.

E. Same as Figure 10B but incubated with chitinase from No. 226B.


B


C


D


E

IN VIVO STUDIES

Bioassays

Both No. 97B and No. $138B_2$ isolates were grown in chitin broth and in glucose broth media for 14 days and their cell-free supernatants were bioassayed for chitinase. Cell-free supernatants of the chitin grown cells were active for chitinase whereas cell-free supernatants of glucose grown cells were not. When whole cultures were fed to instar III larvae the chitinase induced cultures were lethal (death in 72-120 hrs) but glucose grown cultures were not (Table 3). Neither the cell-free supernatants nor the washed cells from any of the fermentations were lethal to their hosts when bioassayed separately. However, larval development appeared retarded when fed either the washed cells or the cell-free supernatants from the chitin medium. Apparently chitinase may play a significant role in facilitating the penetration of a microorganism through the gut (by disrupting the peritrophic membrane) and enter the hemolymph where it may cause a septicemia. Chitinase itself however, when introduced *per os* does not function as a toxin sufficiently potent by itself to kill these larvae.

TABLE 3

Insecticidal activity of No. 97B and No. $138B_2$ whole cultures against instar III Lymantria dispar (L.) after growth in either chitin or glucose media for 14 days.

Isolate No.	Growth Medium	Unit Activity[a/]	Percent Mortality[b/]
97B	chitin	0.36	96.0
	glucose	0.00	0.0
$138B_2$	chitin	0.61	100.0
	glucose	0.00	0.0

a/ 1 Unit = 1 µM NAG released from purified chitin by 1 ml of supernatant in 1 hr.

b/ 3 replicates of 10 larvae each were used for each assay. Mortality recorded after 5 days.

Other evidence which demonstrates that chitinase acts to facilitate the penetration of a pathogen through the peritrophic membrane comes from bioassay experiments where a sublethal concentration of a pathogen was combined with different concentrations of chitinase and then fed to instar II L. dispar larvae (Table 4). At a concentration of 0.05 mg/ml with 0.1 ml spread on diet cubes, E-61 (the International B. thuringiensis standard) effected very low mortality. When several dilutions of only the crude chitinase enzyme were fed to the larvae no mortality was observed after 5 days. However, when both E-61 and the chitinase were combined and fed to the larvae, extensive mortality was observed. In those bioassays where test mortality was low (those with ?) obvious retardation of larval development was observed when compared to the controls. Apparently these larvae failed to feed properly and their digestive mechanism was disrupted.

TABLE 4

Insecticidal activity of combined E-61 (0.05 mg/ml) and chitinase from No. 97B, No. $138B_2$, No. 222 and No. 226B against instar II *L. dispar* larvae.

Enzyme sources:		Percent Mortality[a/]			
		97B	$138B_2$	222	226B
Test Material	**Dilution**				
Control	-	0	0	0	2
E-61 only	.05 mg/ml	6	8	2	4
Enzyme only[b/]	1/25	0	0	0	0
	1/50	0	0	0	0
E-61 & Enzyme	1/25	34	42	20	34
	1/50	12	16	12	2 ?
	1/100	14	2 ?	18	2 ?
	1/500	52	8 ?	12	38
	1/1000	44	30	12	2 ?

a/ Average of 5 replicates of 10 larvae each. Mortality recorded after 5 days.

b/ At 1/100 dilution enzyme unit activity: 97B = 0.88 units, $138B_2$ = 0.83 units, 222 = 1.45 units, 226B = 1.12 units.

? Larvae in these tests did not appear to have been feeding and thus did not ingest a lethal dose.

Frequently larval mortality is not observed in a 5 to 7 day bioassay. However, symptoms of digestive or other physiological disturbance may be observed as delayed larval maturation, reduced growth (body weight) rate or death at a later stage of development. When larvae were fed sublethal doses of E-61 or the chitinase from the four isolates or combinations of E-61 plus chitinase, their growth rate was significantly reduced when compared to the controls (Table 5). When the larvae were fed only E-61, larval weight differences were significant from the controls within 48 hours after exposure to the test materials. Chitinase alone from isolate No. 222 also showed the same early symptoms of decrease of growth rate. After seven days, though all larvae had been returned to normal diet after the second day, the effect of the E-61, with or without the chitinases on the larvae was still very significant. The effect of the chitinases alone were also obvious particularly with isolates No. 97B and No. 226B chitinase preparations when their respective 7.83 and 7.90 fold increase in body weight were compared to the untreated controls where an increase of 10.93 fold was observed for the same growth period.

It is apparent from the data that even though no significant mortality is observed within the short term bioassay period, the chitinase retards the larval growth development, probably by disrupting the digestive mechanism of the larvae

by causing localized dissolution of the peritrophic membrane in the alimentary tract.

TABLE 5

L. dispar instar II larval weight differences in 2 and 7 days after being fed for 48 hours on diet coated with the chitinase from No. 97B, No. $138B_2$, No. 222 and No. 226B with or without E-61.

Sample (Enzyme)	2 DAYS			7 DAYS		
	Ave. Wt.[a/b/] (mg/larva)	± 95% Confidence limits	Proportional Wt. Increase from day 0	Ave. Wt.	± 95% Confidence limits	Proportional Wt. Increase from day 0
Controls	14.37	± 0.91	2.40 X	63.41	± 6.58	10.93 X
E-61	7.74	± 0.62	1.33 X	28.15	± 9.00	4.85 X
97B	13.68	± 1.11	2.36 X	45.39	± 5.53	7.83 X
$138B_2$	12.36	± 1.52	2.13 X	51.94	± 8.78	8.96 X
222	11.47	± 1.34	1.98 X	52.54	± 8.12	9.06 X
226B	12.99	± 0.88	2.24 X	45.82	± 2.33	7.90 X
E-61 + 97B	8.02	± 0.79	1.38 X	30.78	± 2.39	5.31 X
E-61 + $138B_2$	8.10	± 0.74	1.40 X	32.89	± 5.42	5.65 X
E-61 + 222	8.04	± 0.49	1.39 X	33.12	± 2.52	5.71 X
E-61 + 226B	7.92	± 1.14	1.37 X	33.21	± 5.29	5.73 X

a/ average of 5 replicates with 10 larvae per replicate

b/ day 0 ave. larval wt. = 5.72 mg/larvae ± 0.24 (95% confidence limits)

In vivo Chitinase Activity on the Peritrophic Membrane

Scanning electron microscopic observations of the peritrophic membranes of larvae after having fed on E-61 and or chitinase provides further evidence that the chitinase attacks the peritrophic membrane *in vivo*.

Peritrophic membranes from larvae fed on control diet or on diet coated with E-61 only are neither damaged nor show any dissolution or perforations other than that which is normally observed (Figures 11 A, B). But peritrophic membranes of larvae fed on diet coated with chitinase preparations diluted in buffer either with or without E-61 have been attacked by the enzyme (Figures 11 C, D, E, and F). They show large ulcerations and holes which resulted from localized dissolution. The deterioration of the peritrophic membrane was not as extensive as had been previously observed *in vitro* (Figures 9 C, D, E and F), probably because the highly alkaline pH in the mesenteron region of the alimentary tract limited chitinase activity. The buffering capacity of the citrate buffer was also reduced by a dilution effect during ingestion of the diet.

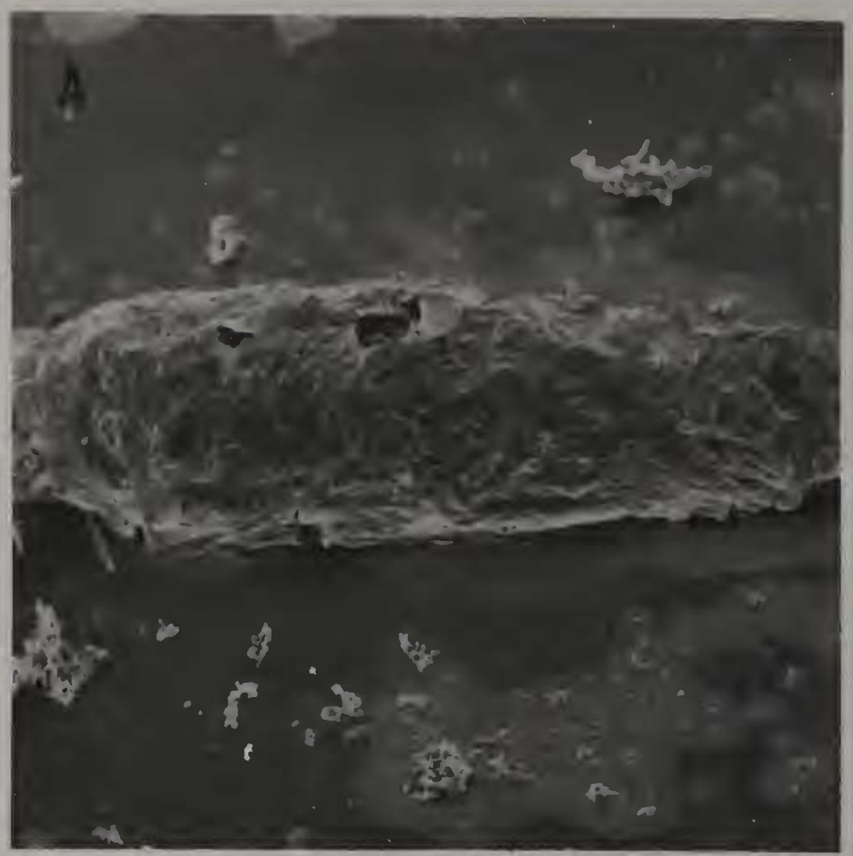
It is important to note that the peritrophic membrane does not show any sign of deterioration when the larvae are fed E-61 only. This tends to support the belief that the delta-endotoxins of *B. thuringiensis* act primarily on the underlying cellular tissue and not on the peritrophic membrane

of the infected larvae. Whereas the chitinase from these isolates primarily attacks the peritrophic membrane, which is the first physical barrier that must be breached before particulates such as bacteria can penetrate through into the hemolymph and cause septicemia.

FIGURES 11

A. Scanning electron micrograph of the peritrophic membrane of an instar II L. dispar larva after having fed on normal diet for 48 hours. Magnification 25 X.

B. Scanning electron micrograph of the peritrophic membrane of a newly molted instar IV L. dispar larva after feeding on diet coated with E-61 for 48 hours. Magnification is 25 X.


A


B

FIGURES 11

C. Scanning electron micrograph of the peritrophic membrane of an instar II L. dispar larva after feeding for 48 hours on diet coated with chitinase from No. 222 (0.8 units/ml, pH 5.2). Magnification is 50 X.

D. Scanning electron micrograph of the peritrophic membrane of an instar III L. dispar after feeding on diet coated with chitinase from No. 222 (0.8 units/ml) plus E-61 for 48 hours. Magnification is 25 X.

E. Scanning electron micrograph of the peritrophic membrane of instar III larvae after feeding for 48 hours on diet coated with chitinase from No. 222 (0.08 units/ml) plus E-61. Magnification is 25 X.

F. Scanning electron micrograph of the peritrophic membrane of an instar II L. dispar larva after feeding on diet coated with chitinase from No. 226B (0.16 units/ml) for 48 hours. Magnification is 50 X.


D


E

In vitro studies have indicated (Figure 6) that at pH 8, chitinase activity by the four enzyme preparations was dramtically reduced. Such a high pH approaches the pH 8.3 of the mesenteron reported for these larvae (60).

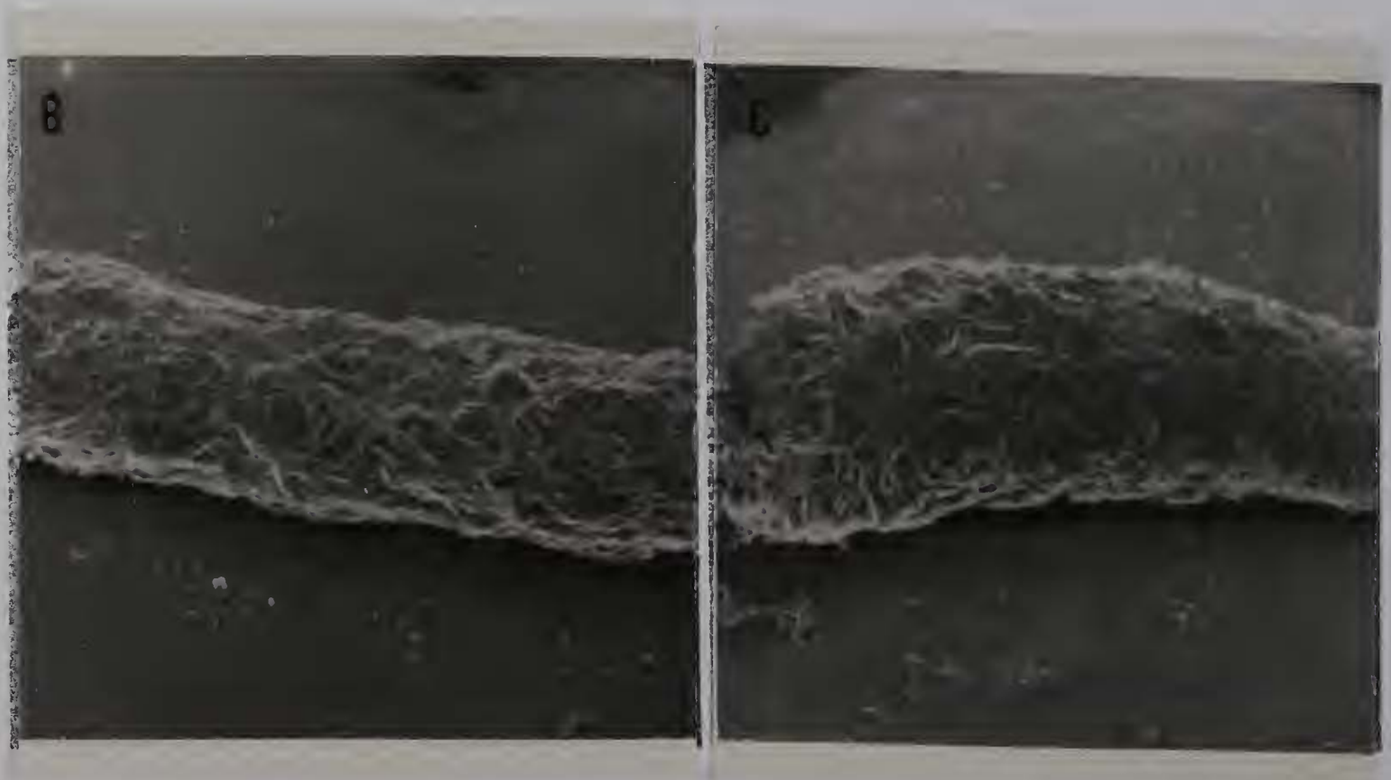
Instar IV larvae were fed unbuffered chitinase preparations (stock enzyme diluted in distilled water to 0.8 units per ml) according to the usual procedure. Under these conditions the pH of the mesenteron predominated. After feeding for 48 hours, the peritrophic membranes were dissected and examined by scanning electron microscopy (Figures 12 A, B, and C). Under these conditions (and unlike previous observations, Figures 11) the chitinase failed to attack the peritrophic membranes. There were no large perforations (holes) or other indication that dissolution or hydrolysis of the chitinous peritrophic membrane had taken place. Even the addition of E-61 (0.05 mg/ml conc.) to these preparations failed to disrupt the peritrophic membrane. These observations further indicate that *in vivo* dissolution of the peritrophic membrane is due solely to the presence of an active chitinase and E-61 intoxication does not involve the disruption of the peritrophic membrane. Furthermore the pH of the mesenteron may have a critical role in limiting *in vivo* chitinase hydrolysis of the peritrophic membrane of actively feeding *L. dispar* larvae.

FIGURES 12

A. Scanning electron micrograph of the peritrophic membrane of an instar IV L. dispar larva fed only on diet. Magnification is 20 X.

B. Scanning electron micrograph of the peritrophic membrane of an instar IV L. dispar larva after feeding for 48 hours on diet coated with 0.8 units of chitinase from No. 97B diluted in distilled water. Magnification is 20 X.

C. Scanning electron micrograph of the peritrophic membrane of an instar IV L. dispar larva after feeding for 48 hours on diet coated with 0.8 units of chitinase from No. 97B diluted in distilled water and 0.1 ml of a 0.05 mg/ml suspension of E-61. Magnification is 20 X.


B
C

Determination of the pH of the mesenteron: Because the pH of the mesenteron may have a critical role in limiting the chitinase activity on the peritrophic membrane, the following study was conducted to determine the *in vivo* pH changes and fluctuations that may occur in the mesenteron when larvae are feeding on indicator diet coated with E-61, the citrate buffer or the buffered chitinases. The indicator diet contained either phenol red (yellow at pH 6.8, red at pH 8.3) or thymol blue (gray at pH 8.3, and blue at pH 9.6) as described in Materials and Methods.

When phenol red indicator diet was fed to instar II larvae for 48 hours the following was observed: Both the stomodaeum and proctodaeum were almost always at or below pH 6.8. Periodically but infrequently small proportions of the mesenteron were below pH 6.8 particularly when the larvae were feeding on diet coated with E-61. In general the mesenteron was usually above pH 8.3 (red) with occasional proportions less than pH 8.3 but above pH 6.8 (orange).

A better estimate of the pH changes in the mesenteron around pH 8.3 could be made on indicator diet containing thymol blue rather than phenol red. When fed on indicator diet alone the pH was not uniform throughout the mesenteron region of the alimentary tract, nor was the change of pH gradual from the gastric cecum to the pylorus. Rather there were sharp lines of demarcations of pH differences in various

regions of the mesenteron in different larvae. Because of these variations, the mesenteron was arbitrarily divided into six equal proportions and both the proportion of the mesenteron above pH 8.3 and the percent larvae examined were recorded. Also proportional decrease of alkaline regions in the mesenteron almost always proceeded from the anterior (gastric secum) to the posterior (pylorus) regions of the mesenteron. After feeding on the indicator diet for 24 hours (Figure 13 A) 60% of the larvae had 2/3rd (0.67) of the mesenteron at or above pH 8.3 and the anterior third was below pH 8.3, while 30% of the larvae had 5/6th (0.83) of the mesenteron above pH 8.3. After 48 hours both the percent larvae and the proportions of the mesenteron above pH 8.3 did not change appreciably from the 24 hour observation.

When E-61 was fed with the thymol blue indicator diet (0.1 ml of a 0.05 mg/ml conc. as per usual) there was a noticeable shift (Figure 13 B) in the proportions of the mesenteron at or above pH 8.3. After feeding on this diet for 24 hours the majority of the larvae (60%) still had 2/3rd (0.67) of their mesenteron above pH 8.3 and 40% of the larvae had 1/3rd (0.33) or less of the mesenteron above pH 8.3. By the 48th hour, 50% of the larvae had lost complete alkalinity (i.e. pH below 8.3) in the mesenteron and few larvae retained a high proportion of the mesenteron above pH 8.3.

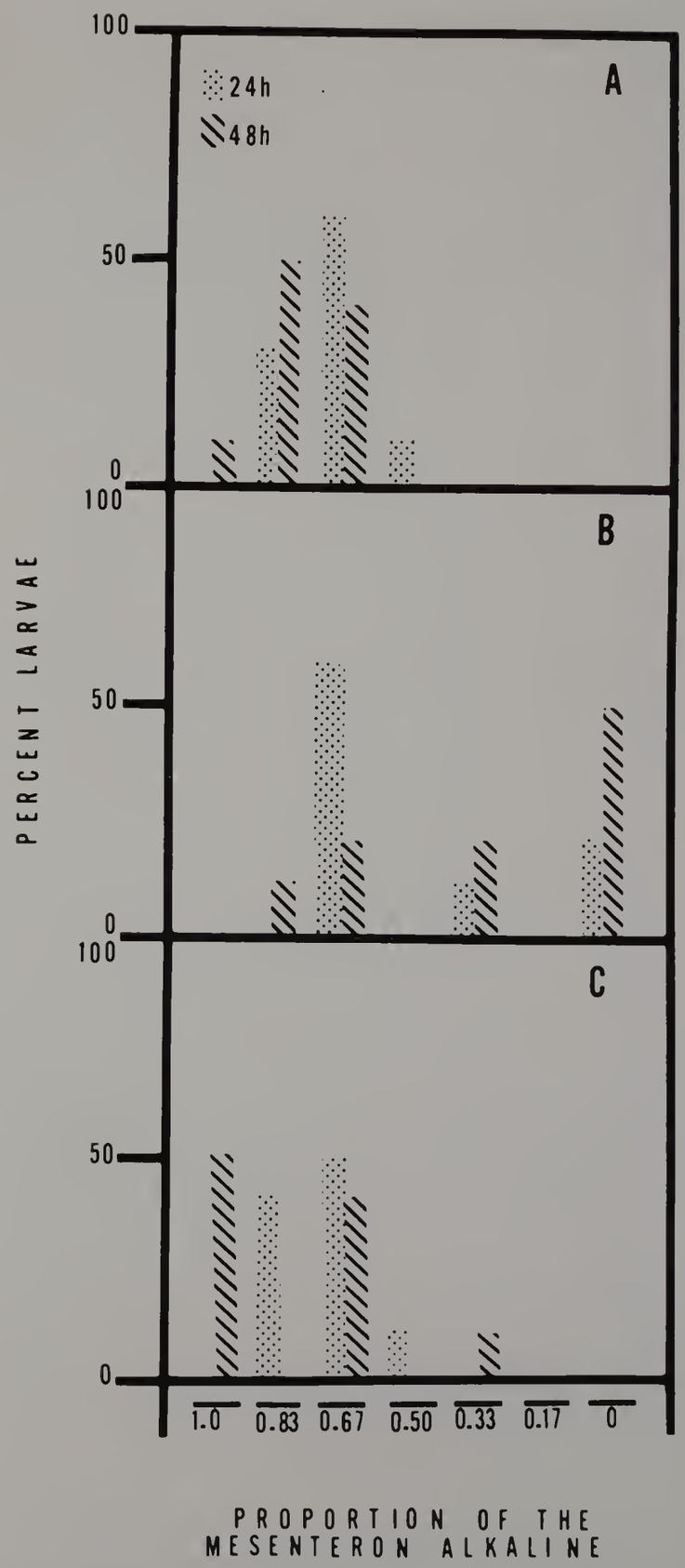
Citrate buffer (0.2 M pH 5.2) on the other hand seemed to stimulate the mesenteron buffering mechanism of the larvae (Figure 13C). The proportional distribution of regions above pH 8.3 in the mesenteron was similar to the controls after 24 hours. By the 48th hour there was a greater tendency for the entire mesenteron to be above pH 8.3 in that 50% of the larvae examined had all the mesenteron above 8.3 and 40% of the larvae had 5/6th (0.83) of the mesenteron above pH 8.3.

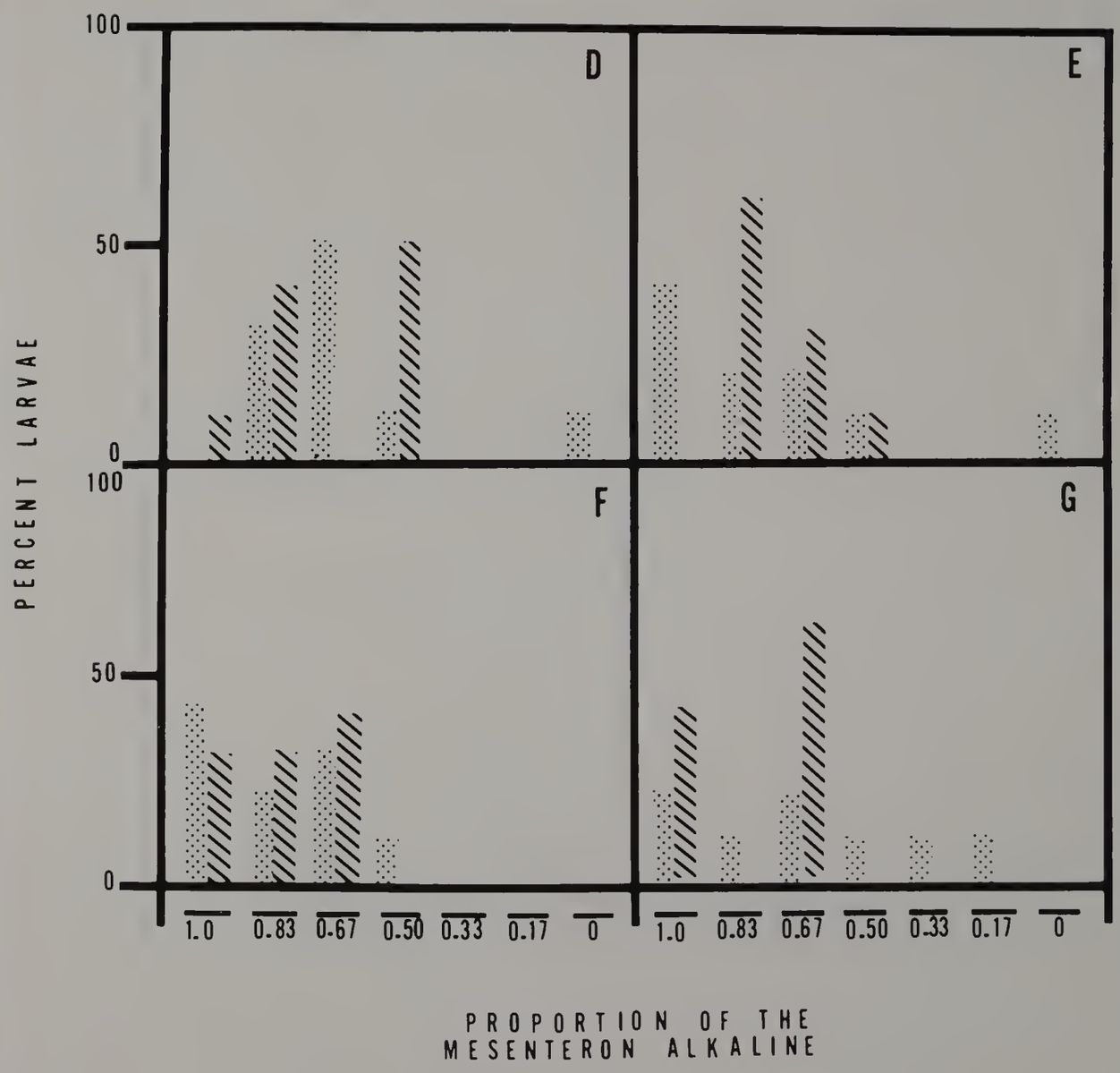
Buffered chitinase from both B. coagulans isolates (i.e. No. 97B, and No. $138B_2$ 0.1 ml of 8 units/ml preparation) tended to cause a shift to a lower proportion of the mesenteron above pH 8.3 (Figures 13 D and E). After feeding on the chitinase preparations for 24 hours, the pH distribution of the mesenteron was not appreciably different from the controls. However, by the 48th hour an increasing percentage of the larvae had a lower proportion of the mesenteron remaining at or above pH 8.3.

Buffered chitinase preparations from both Streptomyces sp. isolates (No. 222 and No. 226B, 0.1 ml of an 8 unit/ml preparation) did not appear to cause any changes in the pH of the mesenteron from those observed in the controls (Figures 13 F and G). With both preparations, an appreciably higher percentage of the larvae (30-40%) had the entire mesenteron above pH 8.3 and the remainder of the larvae examined had at least 2/3rd (0.67) of the mesenteron above pH 8.3

FIGURE 13

Percent of instar III L. dispar larvae with varying proportions of the mesenteron remaining alkaline after 48 hours of injesting (A) control diet, or diet coated with (B) E-61, (C) buffer, or stock chitinase from (D) No. 97B, (E) No. $138B_2$, (F) No. 222 or (G) 226B.


100
24h
48h
A
50
0
100
B
50
0
100
C
50
0
PERCENT LARVAE
1.0
0.83
0.67
0.50
0.33
0.17
0
PROPORTION OF THE
MESENTERON ALKALINE


100
50
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D
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PERCENT LARVAE
1.0
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0.67
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PROPORTION OF THE
MESENTERON ALKALINE

Attempts were made to feed combinations of E-61 with the chitinases but presumably because of the admixture of the E-61, chitinase and indicator the larvae failed to feed properly. Consequently there was no food or indicator in the mesenteron and a pH estimate could not be made. Because of the variability encountered and the small number of larvae used for each observation (10 larvae) these results must be interpreted as supportive and not conclusive in themselves. It can be stated however that even though the average pH of the mesenteron may be at levels which are prohibitive for chitinase activity, localized portions of the mesenteron may be of a sufficiently low enough pH to permit localized chitinase dissolution of the peritrophic membrane to take place. Too ingestion of a sublethal dose of E-61 appears to cause a lowering of the pH of the mesenteron and establish a pH environment favorable for chitinase activity on the chitinous peritrophic membrane. The observation that ingestion of the acid buffer alone had essentially the opposite effect on the pH of the mesenteron, was unexpected.

It appears then that in vivo combined chitinase and E-61 may act in a protocooperative fashion in that E-61 establishes a favorable pH environment in the mesenteron and in that environment the chitinase may act to facilitate the penetration of E-61 through the peritrophic membrane barrier.

DISCUSSION

The isolation procedure used in this study was necessarily restrictive in that only aerobic microorganisms capable of growing on chitin agar were observed. Of these, only those which were chitinolytic were actually noted and isolated. Nonetheless a substantial proportion of the randomly sampled healthy larvae harbored chitinolytic microorganisms. The complete clearing around the edge of the isolated colonies was considered as total hydrolysis to the soluble monomer, NAG. Jeuniaux (52) has shown by nephelometric methods that the total hydrolysis was due to both chitinase activity (hydrolysis to short poly-NAG chains and NAG) and chitobiase activity (hydrolysis of short poly-NAG). This does not necessarily mean that two different enzymes are always involved in the hydrolysis of chitin. Jeuniaux in a separate study (50) with a Streptomycese species isolate found that though three active proteins could be isolated from the supernatants, only one hydrolytic product, NAG, was found. The crude chitinase of S. marcescens also released only NAG as a hydrolytic product of chitin (74). However, after partial purification, some of the active proteins released both NAG and chitobiose and others released only chitobiose. Berger and Reynolds (4) have shown that the active supernatant of

another Streptomyces species isolate, contained active proteins of differing specificities depending on the length of the poly-NAG. Some proteins hydrolysed to NAG polymers of four or less NAG units, others were active only on longer polymers releasing NAG and shorter polymers of NAG. In preliminary studies with the active supernatants of isolates No. 97B and No. $138B_2$ (see Appendix Tables A1, A2, A3, and A4), two or three proteins active on purified chitin were separable by ammonium sulfate precipitation. Using ultrafiltration techniques, two proteins were found to be active on chitin; one of the proteins had a molecular weight greater than 30,000 and, the other had a molecular weight of less than 30,000 but greater than 10,000. Whether chiobiose was released, was not determined.

As was noted in the results several isolates were observed growing on chitin agar without clearing (lysing) the agar. This is not a new observation since several investigators (81, 119, 125) have reported on the ability of microbes to utilize chitin as a substrate for growth without clearing (lysing) the chitin. Such chitinoclastic isolates were suspected of utilizing the contaminating arthropodin present and of releasing and assimilating the acetate from the NAG rather than hydrolize the NAG polymer (chitin) at the β 1 $\longrightarrow$ 4 glycosidic bond. True chitinolytic microorganisms hydrolize the NAG polymer at the

β 1 $\longrightarrow$ 4 glycosidic bond and release the soluble NAG into the medium resulting in the formation of the clear zone in the otherwise opaque chitin agar. The soluble NAG is then readily assimilated by the actively growing isolate.

Of particular interest is the change in the proportion of larvae harboring chitinolytic microorganisms and the increasing complexity of the resident microbial types found between instar III and instar IV and V larvae (Table 1). L. dispar larvae like other Lepidoptera, are voracious non-storage feeders capable of passing food through the alimentary tract in a 1/2 to 1 hour period. Because of this rapid ingestion of food and the strongly buffered alkaline mesenteron portion of the alimentary tract, (47, 60) much of the microflora is probably from contamination of the integuments and reflects the microflora of the environment inhabited by the larvae (100, 117).

As instar III, these Lepidoptera normally remain in the upper crown of the trees feeding mostly on newly emerged foliage essentially free of microbial contamination. Instar IV larvae are usually more active and at this stage of development the larvae go through a migratory pattern called the diurnal cycle (58). During this cycle, the larvae tend to migrate the length of the trees as well as on the forest floor through dead and decaying foliar and wood matter. This behavior, expressed particularly in low

density larval populations (6) is very conducive to the acquisition of a microflora found in a forest environment where there are large numbers of different species of chitinolytic bacteria, actinomycets and fungi (39).

Both the proportion of larvae sampled and the complexity of the chitinolytic microflora found seems to reflect the migratory behavior of these larvae. Podgwaite and Cosenza (84) have reported that Bacillus species were the predominant microbial types isolated from living and dead instar IV and V L. dispar larvae. Here, the same appears to be true in that the largest proportion of larvae, in any instar stage, harbored chitinolytic Bacillus species. As the larvae molted to instar IV and V, their increased mobility exposed them to a larger forest environment including the forest floor, under the loose bark of decaying trees and near the base of the trees, areas favorable for the presence of fungi and actinomycetes. If these larvae harbored a consistent resident microflora, differences between individuals in a given instar and between instars would probably not be as extensive. These observations are consistent with other reports (100, 117) of a transient microflora in Lepidoptera.

Both Bacillus species isolates were tentatively identified as B. coagulans. Both isolates have similar physiological and biochemical characteristics to those

reported for numerous strains of this species by Gordon et.al. (38). Minor differences observed were also reported for some of the forty strains of B. coagulans investigated. Several chitinolytic Bacillus species i.e. B. pumilus, and B. circulans, have been reported by others (39). But neither these of two species produce a curd in litmus milk, nor hydrolysis of starch. They do grow well in 7.5% NaCl broth (variable for B. circulans) and B. pumilus does not reduce nitrate. B. chitinosporous (81) does not utilize citrate nor reduce nitrate to nitrite and produces acid in litmus milk. B. coagulans is normally found in silage, spoiled milk and cheese (9). Much of the area where these larvae were located was surrounded by dairy farms. Undoubtedly spores of this Bacillus species were present in the oak stands inhabited by these larvae.

As stated by Lechevalier and Lechevalier (55), "Actinomycetes, particularly those freshly isolated from their natural habitat, such as soil, can usually be readily classified generically on the basis of morphological features". Both species i.e. No. 222 and No. 226B, appeared as typical Streptomyces species and on the basis of their morphological characteristics they were identified as such. Numerous chitinolytic Streptomycese species isolated from soils have been reported (39, 81, 119) and this genus is most noted for chitinase production (4, 49,

50, 91, 106, 107). For this study, identification was limited to the genus.

In an earlier study Dubois and Gunner (26) reported that chitinase production by a Bacillus species, isolated from L. dispar larvae, was concentration dependent and maximal after 6 days of incubation. In the present study, using commercial grade chitin, maximal chitinase production by the two B. coagulans isolates required 14 days of incubation. Maximal chitinase production by S. marcescens required 6 days of incubation (74), however both the incubation time and yield was affected by the source and pretreatment of the chitin used as substrate. Maximum yield for Streptomyces sp. No. 226B after 6 days of incubation is in agreement with that reported in the literature for this genus (4, 74, 90). Maximum chitinase production for isolates No. 222 was however slower.

The crude chitinase from all four isolates had an optimal pH at pH 5.2. Noteworthy is the broad pH optimum for Streptomyces sp. No. 226B. Also both Streptomycese sp. isolates generally exhibited a greater tolerance of pH changes compared to the B. coagulans isolates. Reported pH optima for crude chitinase preparations from Streptomyces species range from pH 4.2 (107) to pH 6.3 (4). Jeuniaux reported that optimal pH for the crude chitinase

from a Streptomyces species to be at pH 5.2 (50), the same pH found for these isolates. S. marcescens was reported to have a pH optimum at pH 6.4 and more than a 50% loss of activity was observed below pH 4.8 and above pH 7.2 (75). At pH 7.2, the activity of the crude chitinase of both B. coagulans isolates was greater than 50% and also greater than 50% at pH 4.5. Jeuniaux (51) indicated that other than a few exceptions, chitinases have optimal activity around pH 5 and this ranges from pH 4.8 to pH 5.7. It can be reasonably concluded that the exocellular chitinases produced by the four randomly selected isolates are of similar characteristics and activity to those reported for this enzyme system by others.

The native chitin i.e. the host's tissue, proved to be an adequate inducer for chitinase by these isolates. (Figure 7) Other than a few reports of microbes found colonizing insect parts (3, 39, 81) no one has presented any evidence that the host's tissue itself might be an inducer for chitinase. Chadwick (16) reported that pathogens of Galleria mellonella could utilize this insect's tissue as a substrate for growth whereas non-pathogens could not. This, she attributed to the ability of these pathogens to produce proteinases which could break down and utilize the proteins present in the host's tissue. But non-pathogens such as Bacillus licheniformis, a strong

protease producer, is not pathogenic nor would it grow in the tissue medium. Dubois and Gunner (26) had reported that chitinolytic Bacillus species isolated from healthy L. dispar could utilize the insect tissue as a substrate for growth whereas a non-inducible Bacillus sp. could not. All four isolates not only prolifereated in the insect tissue medium but both Streptomycese sp. totally disrupted the insect tissue debris. Indeed with B. coagulans isolate No. 97B the production of chitinase was as extensive as had been previously observed in chitin medium.

In as much as the larval tissue was an inducer for chitinase it was, as anticipated, also susceptible to stock chitinase (Figure 8). It was noteworthy, however, that the native chitin i.e. the host tissue, was more amenable to enzyme attack and hydrolysis than purified chitin (Figure 8) at comparable concentrations of either 45-50 mg of chitin per ml or 1-2 mg of chitin per ml. A possible explanation for the higher activity on native chitin may come from the reported observation that chitin regardless of its original source, is converted to a dehydrated αform with a tight matrix structure when it is purified. In this form chitin is least amenable to enzymatic hydrolysis. However, the native chitin which may be in either the α form (hard cuticle) or β form (soft cuticle and peritrophic membrane) is not dehydrated, is interlaced with arthropodin

and is structurally organized in a looser matrix. This looser matrix structure permits the availablity of many more active sites on the substrate (53). This greater availability of active sites results in higher enzyme activity on the native chitin substrates when compared to purified chitin.

Visually the isolated peritrophic membranes of these insects were, *in vitro*, very susceptible to the crude chitinases so that the structural integrity of the membrane was completely disrupted (Figure 9). Jeuniaux (53) had stated that both alkali treatment to remove the protein, and chitinase were necessary for destruction of the structural integrity of the peritrophic membranes. It is apparent in this study that chitinase alone will destroy the integrity of the isolated *L. dispar* peritrophic membrane, regardless of the age of the larvae i.e. instar III or IV.

Both Smirnoff (108) and Morris (76) have shown that the effectiveness of *B. thuringiensis*, an insect pathogen, was significantly increased against *Choristoneura fumiferana* when combined with chitinase. Smirnoff and Valero (109) further showed that differences in enzyme levels in the hemolymph between healthy and *B. thuringiensis* infected *G. fumiferna* larvae were even more pronounced when chitinase was used with the pathogen. These investigators concluded that chitinase facilitated the penetration of

the pathogen through the peritrophic membrane.

In trials with L. dispar larvae, both B. coagulans isolates proved to be lethal to their hosts after induction for chitinase. This was true when the whole induced culture was fed to the larvae. Neither chitinase alone nor the cell cream were lethal when introduced per os. Lysenko (66) reported that chitinase from S. marcescens was toxic to G. mellonella when introduced by parentereal injection.

The stock chitinases used in this study also increased the effectiveness of B. thuringiensis (E-61, Table 4) such that ingestion of sublethal doses of E-61 with any of the chitinases in most cases caused significant mortality. Furthermore in instances where chitinase alone may not have been lethal to these larvae digestive disturbances affecting their growth rate were observed (Table 5) over a 7 day growth period.

The increased effectiveness of E-61 was undoubtedly due to the disruption of the peritrophic membrane by the chitinase. In vivo activity of the chitinase (Figures 11) was observed where localized dissolution and perforation of the membrane were observed. The effect on the peritrophic membrane was unquestionably due to the added chitinase alone since when E-61 was fed alone, no perforations (large holes) could be observed (Figure 1B) and when chitin-

ase alone was fed, large perforations were observed (Figure 11C). E-61 delta-endotoxin was never implicated in disruption of the peritrophic membrane in its mode of action but appeared rather to destroy the underlying gut wall cells responsible for the synthesis of new peritrophic membrane material. Chitinases on the other hand destroy this membrane barrier and facilitate the penetration of the bacteria into the hemolymph.

In vivo dissolution of the peritrophoric membrane was not as extensive as in vitro dissolution. The reason for this difference may be the high alkaline pH of the mesenteron (60). Contrary to assumptions hitherto advanced, the pH in the mesenteron appears to be rigidly localized so that even though the overall measured pH of the mesenteron may be high enough to limit chitinase activity it appears that regions within the mesenteron are of low enough pH to permit localized chitinase activty (Figure 13). It is perhaps because of these localized pH differences that discreet holes and perforations occur rather than a generalized attack on the entire peritrophic membrane. The delta-endotoxin of E-61 by destroying the underlying gut wall cells, probably also destroys the capacity of the mesenteron to maintain a high alkaline environment. In this way the combination of both, chitinase and E-61, act in a protocooperative manner where in one

case the buffering capacity of the mesenteron is destroyed and in the other the barrier preventing penetration of the bacteria into the hemolymph is destroyed. Without destruction of the buffer metabolizing mechanism it is not surprising then that when citrate buffer alone was fed to the larvae, the alkaline buffering mechanism of the host was stimulated rather than overcome by the ingested citrate buffer.

It may be concluded that healthy L. dispar larvae acquire a resident microflora of chitinolytic microorganisms which are inducible for chitinase not only by a separate source of chitin but also by the host's tissue itself. Further, the chitinase produced by these randomly selected isolates is similar in characteristics to other chitinases and can readily attack the integuments of the host, including the peritrophic membrane.

In vivo studies show that the chitinase will hydrolyse the peritrophic membrane causing large ulcerations and thus facilitate the penetration of a bacterial pathogen through this physical barrier. It has also been demonstrated that unlike previous conceptualizations, the alkalinity of the mesenteron is highly compartmentalized so that though the average pH of the mesenteron may be to high for effective chitinolysis, localized regions in the mesenteron are of sufficiently low pH to permit localized dissolution of the

peritrophic membrane. Furthermore, in conjunction with sublethal concentrations of delta-endotoxin of B. thuringiensis where the buffering capacity of the mesenteron is destroyed and its pH is lowered, a favorable pH environment is established whereby these chitinolytic microorganisms or their exocellular chitinases may function in destroying this physical barrier which must be breached if invasion into the hemolymph and septicemia is to occur.

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APPENDIX

Ancillary Studies of the Chitinase From Isolates No. 97B and No. $138B_2$

Preliminary to the development and resolution of the proposed research problem, a number of studies were conducted on the exocellular chitinases of isolates No. 97B and No. $138B_2$. The purpose of these studies was primarily to permit this researcher to familiarize himself with this enzyme system and produce the stock chitinases used in this study. Though the data presented in this appendix have no direct bearing on the resolution nor the conclusions of this study, they are nonetheless presented as ancillary information pertaining to these two isolates.

Ultrafiltration

The stock chitinase preparations precipitates from the 75% saturation with $(NH_4)_2SO_4$ were investigated by ultrafiltration using Amicon ultrafilters (Amicon Corp., Lexington, Mass.) to determine whether more than one chitinase active protein species differing in molecular weight could be present in the stock chitinases. The results presented in Tables A1 and A2 indicated that the stock enzymes contained at least two protein species: one with a molecular weight greater than 30,000 and one less than 30,000 but greater than 10,000.

Table A1 Ultrafiltration of stock Chitinase of Isolate No. 97B

	PROTEIN		CHITINASE ACTIVITY	
	Total Unit	Percent Recovery	Total Unit	Percent Recovery
Original	5.00 mg	100.00	6.36	100.00
PM30				
Retentate	2.50	50.00	3.71	58.30
filtrate	2.30	46.00	2.38	37.40
Totals	4.80	96.00	6.09	95.70
PM10				
Retentate	3.35	67.00	5.59	89.50
filtrate	1.65	33.00	0.24	3.80
Totals	5.00	100.00	5.93	93.00
UM2				
Retentate	4.29	85.80	5.19	81.60
filtrate	0.59	11.80	0	0
Totals	4.88	97.60	5.19	81.60

Table A2 Ultrafiltration of stock chitinase of isolate No. $138B_2$

	PROTEIN		CHITINASE ACTIVITY	
	Total	Percent Recovery	Total	Percent Recovery
Original	4.25	100.00	7.51	100.00
PM30				
Retentate	2.73	64.24	4.78	63.65
filtrate	1.40	34.35	1.81	24.10
Totals	4.19	98.59	6.59	84.75
PM10				
Retentate	3.00	70.59	6.38	85.00
filtrate	1.18	27.77	0.12	1.60
Totals	4.18	98.36	6.50	86.60
UM2				
Retentate	3.47	81.65	5.70	75.90
filtrate	0.63	14.82	0.11	1.47
Totals	4.10	96.47	5.81	77.37

Ammonium Sulfate Precipitation

The chitinase active supernatant proteins were harvested by precipitation with $(NH_4)_2SO_4$. Results of saturation in 15% increments are presented in Tables A3 and A4. The precipitated proteins harvested at 75% saturation were used as the source of stock enzymes from all four isolates used in this study. The results indicate that two and in the stock from isolate No. 97B possibly three chitinase active proteins with differing solubilities may be present in the supernatants.

Table A3 Precipitation of chitinase active proteins from cell-free supernatants of isolate No. 97B by saturation with $(NH_4)_2SO_4$.

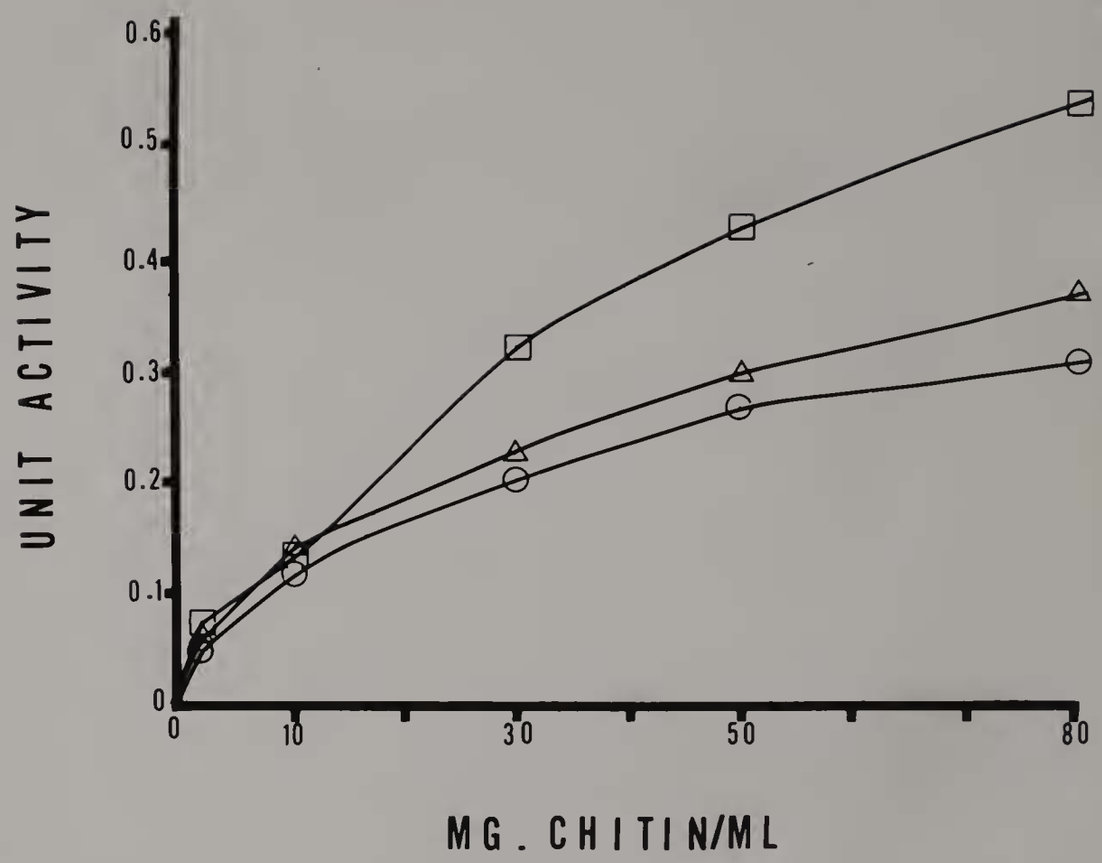
Sample	Total Protein		Total Activity	
	mg	%	Unit	%
Original Supern.	118.00	100.00	72.96	100.00
Percent Saturation				
15	7.14	3.51	1.77	2.45
30	5.36	4.54	0.87	1.19
45	14.29	12.11	12.03	16.49
60	30.36	25.73	15.66	21.46
75	39.29	33.29	8.07	11.88
Total Recovery	96.44	79.18	39.00	53.47

Table A4 Precipitation of chitinase active proteins from cell-free supernatants of isolate No. $138B_2$ by saturation with $(NH_4)_2SO_4$.

	Total Protein		Total Activity	
	mg	%	Unit	%
Original Supern.	118.00	100.00	51.84	100.00
Percent Saturation				
15	8.93	7.57	0.90	1.73
30	1.79	1.51	1.35	2.96
45	17.86	15.13	11.57	22.32
60	10.71	9.08	5.28	10.19
75	41.07	34.81	1.37	3.64
Total Recovery	80.36	68.10	20.47	39.74

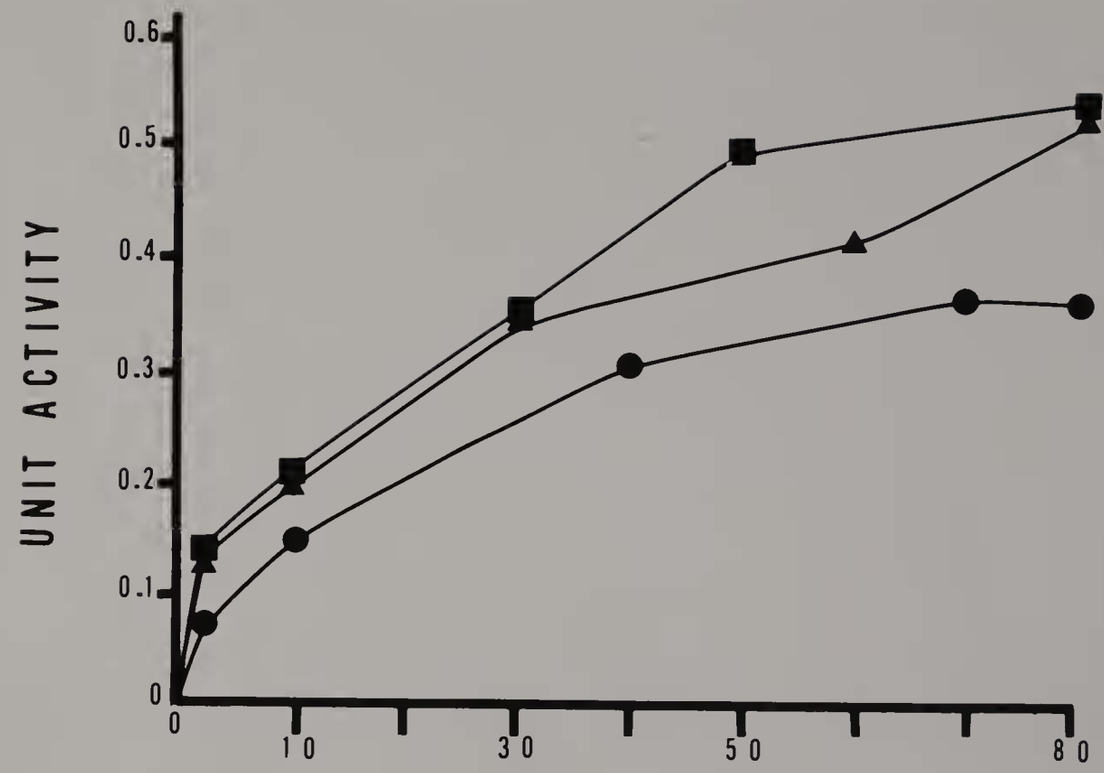
Saturation Kinetics

Three dilutions of the stock chitinases were prepared in buffer pH 5.2. They were reacted with nine concentrations of purified chitin ranging from 2 mg to 80 mg per ml for 1 hour at 32C. Unit chitinase activity was determined as described in the material and methods. The Km, Vmax and Vmax corrected to 1 mg of enzyme protein were calculated from a Lineweaver-Burke plot of the data. As expected the Km was the same for all three enzyme dilutions. Also the Vmax when corrected to 1 mg or enzyme protein calculated to the same value except for the one assay with the high concentration of chitinase protein for isolate No. $138B_2$. The results are presented in Figures A1 and A2.



KEY	PROTEIN CONCENTRATION	Km	Vmax	Vmax CORRECTED
□	0.34 mg/ml	68.97	1.00	2.94
△	0.25	68.97	0.71	2.88
○	0.21	68.97	0.61	2.88

Figure A1 Saturation kinetics and determination of Km, Vmax and Vmax corrected for 1 mg of stock chitinase protein of isolate No. 97B assayed at three concentrations.



KEY	PROTEIN CONCENTRATION	Km	Vmax	Vmax CORRECTED
■	0.32 mg/ml	40.00	0.81	2.60
▲	0.21	40.00	0.76	3.60
●	0.16	40.00	0.57	3.60

Figure A2 Saturation kinetics and determination of Km, Vmax and Vmax corrected for 1 mg of stock chitinase protein of isolate No. $138B_2$ assayed at three concentrations.