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Cuticle-Degrading Enzymes of Entomopathogenic Fungi: Mechanisms of Interaction between Pathogen Enzymes and Insect Cuticle

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Extracellular enzymes of *Metarhizium anisopliae* had considerable affinity for insect cuticle. Binding of proteases immobilized over 70% of soluble enzyme activity, which in vivo could have a significant influence on the extent and nature of cuticle degradation. Adsorbed protease, carboxypeptidase, and *N*-acetylglucosaminidase activities were recoverable with 0.2 M buffer suggesting nonspecific ionic binding. Chitinase bound irreversibly as a specific enzyme–substrate complex. Cuticle degradation by an alkaline (optimum *p*H 9) basic (*p*I 9.5) protease was inhibited by increasing salt concentrations while anilide hydrolysis was unaffected. Inhibition arose from interference with essential electrostatic adsorption of the enzyme on to the cuticle. An anionic detergent enhanced enzymic solubilization of cuticle proteins (probably due to increased electronegativity of cuticle) at the expense of continued proteolysis of released peptides, clearly distinguishing between the two processes. A cationic detergent inhibited cuticle degradation, indicating that salt labile bonds form between the negative (probably carboxyl) groups of cuticle and the positively charged groups of the protease. The significance of these results in understanding the mechanism of cuticle degradation are discussed.

KEY WORDS: *Schistocerca gregaria;* Locust cuticle; Entomopathogenic fungi; *Metarhizium anisopliae;* Cuticle-degrading enzymes; Protease; Chitinase; Carboxypeptidase; *N*-acetylglucosaminidase; Binding of enzymes; Ionic binding; Effect of salts; Effect of detergents; Carboxyl groups on cuticle.

INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* produces a variety of extracellular insect cuticle-degrading enzymes in culture (St. Leger et al., 1986a). An "elastase" type protease (optimum *p*H 9, *p*I 9.5, and MW 25,000) and chitinase (optimum *p*H 5.3, MW 33,000) isolated from *M. anisopliae* culture fluids sequentially degraded locust cuticle releasing peptides (mean chain length, 5) and *N*-acetylglucosamine (NAG), respectively (St. Leger et al., 1986b).

To date the mechanism of interaction between pathogen enzymes and insect cuticle has not been studied but other related enzymes including pancreatic elastase (Gertler, 1971) and insect or bacterial chitinases (Bade and Stinson, 1981; Skujins et al., 1973) degrade their substrates via a complex process involving an adsorption step. Elastase (and other basic proteins) adsorb electrostatically to elastin via the carboxyl groups of the latter (Hall and Czerkawski, 1961; Gertler, 1971); *Streptomyces* chitinase binds irreversibly with chitin in a more specific enzyme–substrate bond (Skujins et al., 1973). Chitinases from some *Aphanomyces* spp. (crayfish pathogens) also bind to chitin (Unestam, 1966).

The nature and specificity of any enzyme adsorption to cuticle would have important implications in pathogenesis; an enzyme without the ability to be adsorbed may lack activity irrespective of its bond specificity. Conversely, a strong adsorption to cuticle components could immobilize enzyme activity completely or limit enzymic degradation to the vicinity of fungal structures; a similar mechanism to account for localized

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host wall breakdown has been proposed for enzymes of some phytopathogens (Cooper et al., 1981).

The aim of the present work was to determine the extent, if any, of enzyme adsorption to cuticle components, its nature, and specificity.

MATERIALS AND METHODS

The isolate of *Metarhizium anisopliae* (ME1), culture medium, and preparation of locust cuticle and chitin were as described previously (St. Leger et al., 1986b). The elastase type protease (Prl) was purified by ammonium sulfate precipitation and isoelectric focusing (St. Leger et al., 1986b); additional purification was achieved by column chromatography (Sephadex G-100 fine (Pharmacia), 60 × 2.5 cm) as described for chitinase (St. Leger et al., 1986b) and by recrystallizing twice. The resulting preparation had a specific activity vs hide protein azure of 142 optical density units/hr/μg Prl protein (compared with 124 optical density units/hr/μg elastase (type 1V, Sigma) against the same substrate). Chitinase and *N*-acetylglucosaminidase were partially purified by ammonium sulfate precipitation and column chromatography (St. Leger et al., 1986b).

Enzyme substrates were purchased from Sigma. Other chemicals used were commercial products of analytical grade.

Enzyme Assays. *p*H optima of dialyzed enzymes were determined and used in assays as described previously (St. Leger et al., 1986a). Chitinase activity (vs. colloidal chitin) was assayed at *p*H 5.3 using Nelson's (1944) modification of the Somogyi method observing the precautions outlined by Marais et al. (1966). Results are expressed as micromoles amino sugar released per milliliter per hour. *N*-acetyl-β-D-glucosaminidase activity was determined by measuring the rate of hydrolysis of *p*-nitrophenyl-β-*N*-acetylglucosamine at *p*H 5. Activity is expressed as micromoles of *p*-nitrophenol released per milliliter per hour. Carboxypeptidase A activity was determined by measuring the release of phenylalanine [determined with ninhydrin (Moore and Stein, 1948)] from carbobenzoxyglycyl-L-phenylalanine. Activities are expressed as micromoles phenylalanine released per milliliter per hour. Proteolytic activity was assayed with hide protein azure. Activities are expressed as Δ_{OD} 595 nm/hr.

Protease activity was also measured by anilide hydrolysis (termed "anilidase" to distinguish it from the proteolysis of hide protein azure and cuticle) in 10-mm cuvettes containing 0.2 ml of substrate (1 mM succinyl L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (Suc $(Ala)_2$ Pro Phen NA) in dimethyl sulfoxide), 0.2 ml enzyme, and 2.5 ml Tris buffer (2 mM, pH 8) containing KCl (final concentration 0–0.3 M). The reaction was followed at 410 nm. Results are expressed as micromoles nitroanilide released per minute per milligram.

In some experiments anilidase activity was determined in the standard reaction mixture supplemented with detergents (final concentration of 0.2%). The enzyme was preincubated for 10 min at 20°C in the buffer before adding substrate. Control experiments were performed without detergents.

The effect of ionic conditions on cuticle degradation by Prl was usually determined in reaction mixtures containing 20 mg cuticle, 2.5 ml of Tris buffer (2 mM, *p*H 8.5) with KCl (0–0.3 M final concentration), and 0.2 ml enzyme (5 μ g ml^{-1}). The reaction mixtures were incubated with or without shaking at 30°C for 1 hr and centrifuged (4°C at 2000*g* for 20 min). The residue of undigested cuticle was removed by filtration through Whatman No. 1 filter paper and the absorbance of the filtrate was read at 280 nm. Enzyme activities are expressed as micrograms tyrosine equivalents per hour per milliliter. Free α-amino groups in the filtrate were measured by the ninhydrin method (Moore and Stein, 1948) using L-alanine as the standard.

Control reaction mixtures containing denatured enzyme (heated at 100°C, 5 min) or cuticle alone and enzyme in the absence of substrate were included in each experiment.

Cuticle degradation in the presence of detergents. Sodium dodecyl sulfate (SDS) or cetyltrimethylammonium bromide (CTAB) were added at a final concentration of 0.2% to a reaction mixture containing 10 mg cuticle in 3 ml of Tris buffer (2 mM, *p*H 8.5) and the mixture was incubated for 30 min at 37°C. The mixture was cooled to 4°C and 0.5 ml of enzyme solution (10 μg protein/ml) was added. The temperature was maintained at 4°C for 10 min to prevent enzyme denaturation by the detergents while adsorption took place. Subsequent incubation was conducted for 1 hr at 35°C. The experimental procedure was then identical to that described for measuring the effect of KCl on cuticle degradation. In control reaction mixtures, the detergents or enzyme were omitted. To quantify free α-amino groups present in low-molecular-weight degradation products, 2 ml of potassium phosphate buffer (0.7 M, *p*H 6) was added to cuticle reaction mixtures. Precipitated detergent was filtered off and 2 ml of the filtrate was mixed with 2 ml of trichloroacetic acid (TCA, 30% w/v) to precipitate solubilized proteins. After 30 min at room temperature the mixture was centrifuged (5000 *g*, 15 min) and amino groups in the supernatant were measured with ninhydrin (Moore and Stein, 1948).

RESULTS

Binding or Inactivation of Fungal Enzymes by Cuticle Components

Protease, carboxypeptidase, chitinase, and *N*-acetylglucosaminidase activities were compared before and after incubation with dry or hydrated locust cuticle (presoaked in water for 4 hr) to determine any loss of activity. The results with cuticle in either condition were essentially similar (Fig. 1).

The presence of cuticle invariably re-

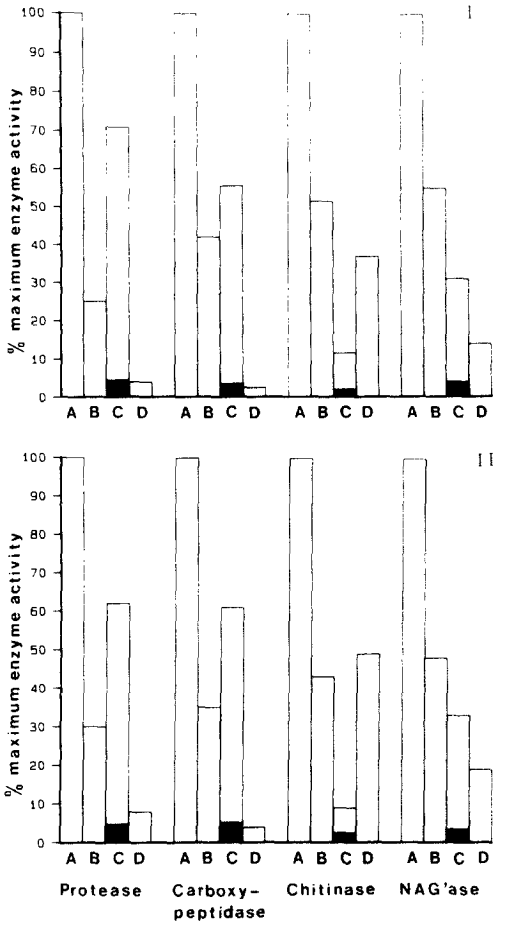

FIG. 1. Binding of four extracellular enzymes from *Metarhizium anisopliae* by freeze-dried (I) and hydrated (presoaked in water for 4 hr) (II) locust cuticle. Ordinate: Column (A); activity of control enzyme solutions (100%). Column (B); percentage soluble enzymatic activity remaining after 10 min incubation at 25°C of 3 ml culture filtrate with 15 mg (dry wt) cuticle. The lower shaded section of column (C) shows the activity of enzyme released from cuticle by washing in water, the upper section of the column represents activity of ionically bound enzyme released from cuticle by incubation for 15 min with potassium phosphate buffer (0.2 M, *p*H 7). Column D; column (A) minus combined activities of (B) and (C) represents inactivation or nonionic binding. Five day culture filtrates obtained from *M. anisopliae* (ME1) grown on 1% cuticle/basal salts medium were dialyzed and diluted so that activities used were: protease (700 optical density units/hr, equivalent to ca. 5 μg Prl protein); Carboxypeptidase A (2.97 units); chitinase (0.5 units) and *N*-acetylglucosaminidase (NAGase) (1.25 units). Units as described under Materials and Methods. Each result is the mean of at least three replicates. The experiment was repeated three times with similar results. Fresh enzyme and cuticle preparations were used on each occasion.

duced (>50%) both soluble enzyme activities (at 23°C) and protein levels (determined at 4°C by change in optical density at 280 nm) indicating adsorption onto the cuticle. Adsorption at 4°C occurred without measurable degradation of cuticle components indicating that enzymolysis is not involved in binding. The time for adsorption with culture filtrates, purified protease, or a semipurified preparation of chitinase was very rapid, being complete within the shortest time of measurement (<3 min). Adsorbed enzymes were firmly bound as very little (<5%) could be eluted by repeated water washings.

The proteolytic enzymes, however, were almost completely desorbed by washing in potassium phosphate buffer (0.2 M, *p*H 7) which indicates attachment by nonspecific ionic bonds. In contrast, chitinolytic activity was not recoverable which suggests either inactivation by inhibitors or more specific binding to the cuticle.

The extent of binding of enzymes to locust chitin was studied as a model for cuticular chitin unmasked by fungal proteases (Fig. 2). Semipurified chitinase had a very high affinity for the chitin. Thus 5 mg of chitin adsorbed more than 97% of the enzyme from 3 ml of solution (containing 8.4 units) and adsorption was completed (as judged by change in optical density at 280 nm) within 3 min. The chitinase was not recovered by washing in 0.2 M buffer indicating that binding is not a simple charge effect. It was found, however, that activity returned to a dialyzed supernatant after the chitin had been hydrolyzed by the enzyme. Furthermore, heat denaturation of the chitinase (60 min, 100°C) decreased the amount adsorbed from 97 to 43% and two-thirds of this could be desorbed with buffer. These results suggest that a specific enzyme substrate bond is formed which is dependent on the structure and activity of the chitinase. This affinity for chitin was utilized as a means of purification (St. Leger et al., 1986b).

The *N*-acetylglucosaminidase showed far less affinity than chitinase for the chitin (Fig. 2). Using 100 mg of chitin and 3 ml of partially purified *N*-acetyglucosaminidase, ca. 85% of soluble enzyme bound and about half of this was recovered by washing in buffer. That a substantial part of the activity was not recoverable indicates some degree of enzyme substrate binding even though activity against locust chitin is extremely low (unpubl.). The loss of activity is unlikely therefore to be due to reaction product inhibition; this is supported by the lack of increased activity following dialysis.

The alkaline protease, whether purified (Fig. 2) or in culture filtrate, showed some affinity for chitin. As with other substrate enzyme combinations, adsorption took place within 3 min. Almost complete recovery of enzyme was achieved with 0.2 M

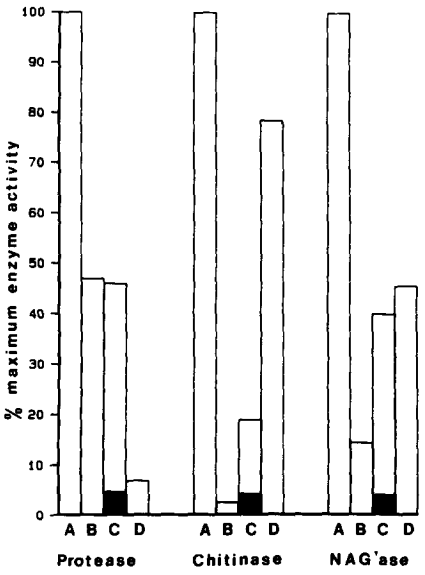

FIG. 2. Binding of three extracellular enzymes from *Metarhizium anisopliae* (ME1) by ground locust chitin. See Figure 1 for column designations. Enzyme activities and amount of chitin present are: Protease 2,115 optical density units/hr (17 μg protease protein), 100 mg chitin; chitinase 8.4 units, 5 mg chitin; *N*-acetyglucosaminidase (NAGase) 4.2 units, 100 mg chitin. Each result is the mean of at least three replicates. The experiment was repeated twice with similar results. Fresh enzyme and cuticle preparations were used on each occasion.

buffer indicating that binding was by ionic bonds.

Effect of Electrostatic Adsorption of Prl on Degradation of Cuticle Proteins

The effect of ionic conditions on cuticle degradation by Prl was examined to determine whether electrostatic adsorption is related to its enzymatic properties. Activities against ground locust cuticle and Suc(Ala)$_2$ Pro Phen NA were measured in the presence of various concentrations of KCl (Fig. 3). KCl (0.3 M) inhibited by 70% the release of degradation products from cuticle at *p*H 8.5 although anilidase activity was slightly increased; evidently therefore 0.3 m KCl does not prevent enzymatic activity per se. Furthermore, assay of supernatants from cuticle reaction mixtures with Suc(Ala)$_2$ Pro Phen NA demonstrated that levels of residual unbound enzyme were increased from 14 to 86% by 0.3 M KCl. These results indicate that KCl inhibits protease activity because of interference with adsorption onto the cuticle. The inhibitory effect of KCl on release of free amino groups was less, cf. decreased solubilization of total protein. This may be related to the continuing degradation of released peptides by the enzyme (see below).

The effect of 0.3 M KCl on the *p*H activity curve of protease was studied (Fig. 4); KCl inhibited enzyme activity at any *p*H but the effect was greatest under optimal conditions (ca. *p*H 9).

Effects of Detergents on Cuticle Degrading Activity

Anionic and cationic detergents have been used to determine the nature of enzyme adsorption to elastin by blocking positively charged amino groups and negative carboxyl groups, respectively (Hall and Czerkawski, 1961). The effects of the anionic detergent SDS and the cationic detergent CTAB was determined on cuticle degradation and anilidase activity by Prl (Table 1).

Cuticle-degrading activity was greatly enhanced by SDS and inhibited by CTAB; both detergents, however, severely inhibited anilidase activity (vs Suc (Ala)$_2$ Pro Phen NA). The contrasting effect of SDS on cuticle degradation and anilidase activity suggests that solubilization of cuticle components and proteolysis may be distinct processes. To test this the high-molecular-weight degradation products solubilized from cuticle were precipitated by TCA and free amino acids in the superna-

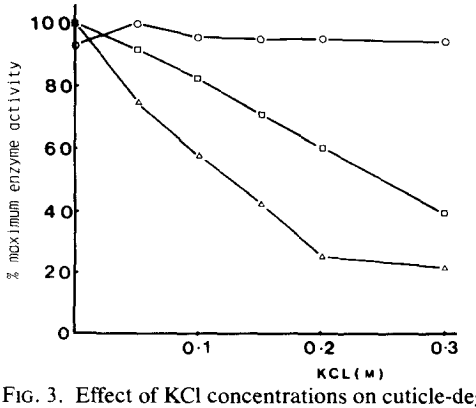

FIG. 3. Effect of KCl concentrations on cuticle-degradation optical density 280nm (△), release of a free α-amino groups (□), and anilidase activity (vs Suc(Ala)$_2$ Pro Phen NA) (○). Each result represents the mean of three replicates. The results are representative of two similar experiments.

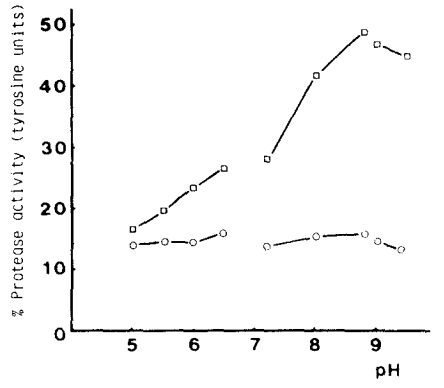

FIG. 4. *p*H activity curves of Prl against ground (0.2 mm) locust cuticle with (○) or without (□) added KCl (0.3 M). The experiment was performed in the following buffers: 2 mM citrate-phosphate buffer (*p*H 5–6); 2 mM Tris buffer (*p*H 7.2–8.8); and 5 mM glycine–NaOH buffer (*p*H 9–9.5). Each result represents the mean of three replicates. The results are representative of two similar experiments.

TABLE 1
CUTICLE DEGRADATION AND ANILIDASE ACTIVITY IN THE PRESENCE OF DETERGENTS

	Control	CTAB[b]	SDS[b]
Cuticle degradation[a]	31.2 ± 0.82	7.8 ± 2.66	89.2 ± 2.98
% Controls	100	25	286
Anilidase activity[a]	3.95 ± 0.14	0.60 ± 0.02	0.12 ± 0.01
% Controls	100	15	3

[a] Results are expressed in units ± SE (n = 5).
[b] CTAB = cetyltrimethyl ammonium bromide. SDS = sodium dodecyl sulfate.

tant measured (see Materials and Methods). The unprecipitated α-amino acids in low-molecular-weight peptides and as free amino acids comprised 0.18 ± 0.03 and 0.69 ± 0.08 μmol alanine equivalents/ml (n = 5), respectively, with or without SDS (0.2%). This result taken in conjunction with those shown in Table 1 indicates that the presence of the anionic detergent enhances solubilization of cuticle but inhibits further degradation of released products.

DISCUSSION

The data presented in this paper indicate that fungal cuticle-degrading enzymes have a considerable affinity for insect cuticle. Binding of protease by cuticle resulted in immobilization of over 70% of the enzyme activity; almost all the initial activity was recoverable with 0.2 M buffer suggesting that the enzyme was ionically bound to cuticle rather than inactivated. However, buffer solutions failed to desorb chitinase from either cuticle or deproteinized chitin. In view of the recovery of activity following solubilization of chitin, and that denatured chitinase no longer showed irreversible binding to substrate, it is apparent that specific enzyme–substrate complexes were formed. Irreversible binding of chitinase to substrate has been reported in other systems (Bade and Stinson, 1981; Skujin et al., 1973). In this study both protease and *N*-acetylglucosaminidase ionically bound when relatively large quantities of chitin were used; though in contrast to protease adsorption of *N*-acetylglucosaminidase was not completely reversible with salts. This is comparable with the binding of cuticular proteins to chitin described by Hackman (1955). However, in contrast to the present work, trypsin and chitobiase (Bade and Stinton, 1981) and ribonuclease (Skujins et al., 1973) have been found to lack affinity for chitin. The discrepancy may be due to a failure by Bade and Stinson (1978) and Skujins et al. (1973) to take into account the larger quantity of chitin required for maximal adsorption of enzyme proteins when ionic bonds only are involved.

The present results clearly indicate that adsorption is a prerequisite for cuticle degradation by the major protease of *M. anisopliae* (ME1). Adsorption and consequently cuticle degradation were inhibited by high levels of KCl and by blocking negatively charged carboxyl groups on cuticle with a cationic detergent. Increasing the net negative charge on cuticle with SDS resulted in increased solubilization of cuticle proteins at the expense of general proteolysis (appearance of free amino groups); the two processes are therefore distinct.

It has been suggested that ionic binding of degradative enzymes limits degradation of cell walls by certain plant pathogens (e.g., Cooper et al., 1981). Most ultrastructural and histological studies suggest that enzymic degradation of insect cuticle by invading fungi is also limited to the vicinity of fungal structures (see Charnley, 1984, for review). Our results indicate that adsorption may be one of the mechanisms responsible for localized cuticle degradation. This

could be beneficial to the fungus preventing premature dehydration of the insect host, while restriction of products of cuticle degradation should facilitate uptake and further enzyme induction. In addition binding to substrate stabilizes both protease and chitinase in nonoptimum conditions of temperature and *p*H (unpubl.).

We suggest that the mechanism of cuticle degradation by Prl is as follows: (1) the enzyme adsorbs via nonspecific electrostatic bonds which occur between positively charged groups on the basic protease molecule and carboxyl groups on the cuticle. Those available are either α-carboxyl groups or the side-chain carboxyl groups of glutamic acid and aspartic acid which form 5.2 and 4.0% of total amino acids in locust cuticle, respectively (St. Leger et al., 1986b). The importance of an adsorption step (involving carboxyl groups in elastin) on elastase activity has been emphasized by several workers (Hall and Czerkawski, 1961; Morihara and Tsuzuki, 1967; Gertler, 1971). (2) The active site comes into contact with any part of the convoluted cuticle protein chains and under appropriate conditions, e.g. temperature, splits susceptible peptide bonds thus loosening cuticle proteins. (3) Solubilized proteins are further degraded until a chain length ca. 5 is obtained (St. Leger et al., 1986b). The ability of the protease to degrade cuticle is obviously governed by its adsorption characteristics and its bond specificity; further degradation of solubilized proteins might be performed by a variety of exo- and endopeptidases which are produced by ME1 (St. Leger et al., 1986a).

Binding of the protease to negatively charged groups is presumably dictated by its basic nature. It follows that the acidic proteases also produced by ME1 (unpubl.) will bind preferentially to positively charged groups; proteins contained in cuticle will bear a positive or negative charge depending upon the ionization of carboxyl or amino groups. The possibility exists that cuticle may possess a nonuniform distribution of charge and thus regions may be favorable or unfavorable to binding (and consequently to degradation) by individual enzymes. This question and the modes of action and possible synergistic effects of acidic and basic proteases from *M. anisopliae* are currently under investigation.

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