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CHARACTERIZATION OF CUTINOLYTIC ENZYMES FROM *COLLETOTRICHUM LAGENARIUM* AND THEIR ROLE IN PENETRATION OF *CUCUMIS SATIVUS*

by

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ABSTRACT

CHARACTERIZATION OF CUTINOLYTIC ENZYMES FROM *COLLETOTRICHUM LAGENARIUM* AND THEIR ROLE IN PENETRATION OF *CUCUMIS SATIVUS*

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Five major components of cucumber fruit cutin were identified by combined gas chromatography-mass spectrometry. The components identified were: 16-hydroxyhexadecanoic acid, 8,16-dihydroxhexadecanoic acid, isomeric mixture of dihydroxypentadecenoic acid, 9,18- and 10,18-dihydroxyoctadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid. In addition, two minor components were identified, 8-hydroxypentadecanoic acid and 8-hydroxyhexadecane-1,16-dioic acid. Chemical tests indicated the presence of a carbonyl function (most likely a mid-chain ketone)in the 8,16-dihydroxyhexadecanoic acid.

Colletotrichum lagenarium, the causal agent of anthracnose of cucurbits, was shown to produce several cutin degrading enzymes. One of these, cutinase-a, was purified to homogeneity and characterized biochemically. This enzyme was found to be acidic with a molecular weight of 60kd. These properties make cutinase-a considerably different from previously isolated cutinases which are basic with approximate molecular weights of 25kd. In addition to cutin degrading activity, these enzymes

exhibit general esterase or p-nitrophenyl butyrate hydrolase activity, as does cutinase-a.

Additional cutinolytic enzymes were identified in the DEAE cellulose column 'void' fraction and in the spore matrix. These enzyme activities and levels of activity differ from those exhibited by cutinase-a and other previously reported cutinases. The 'void' fraction has cutinolytic activity but not PNB hydrolase activity, whereas the matrix fraction has both activities.

Paraoxon, an organophosphate compound and inhibitor of serine hydrolases, inhibited the enzyme activities in all 3 fractions, indicating the presence of an active serine residue in their active sites. Microscopic and visual observations of penetration and colonization in the presence of paraoxon suggest that cutinolytic enzymes do not have a major role in penetration. This possibility was supported by data with mutants of C. lagenarium, having increased and decreased levels of cutin degrading activity; there was a poor correlation between penetration and cutinase production. Thus, C. lagenarium differs from some other plant pathogenic fungi, where cutinase is thought to play a role in penetration.

To my parents, Sarah and James

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CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

In order to understand how a pathogen might penetrate the plant surface or cuticle, the nature of the cuticle must first be understood. The term cuticle was first used by Brogniart in 1885 (10,82) to describe the 'thin membrane covering the aerial surfaces of plants'. He considered the cuticle to be non-cellular and amorphous.

The structure and chemistry of the cuticle has been studied most intensely during the last 30 years. One of the first reviews of the literature concerning the plant cuticle was written in 1924 by Lee and Priestley (61). At this point little was known about the composition or structure of the cuticle, other than it consisted of fatty substances which were deposited over the surface of exposed plant parts. It had already been established that the roots did not have a cuticle <u>per se</u>. It was believed at this time that cutin was the result of oxidation at the air-surface interface of fatty acids produced by the epidermal protoplasts, and of fatty acids produced deeper within the tissues. The belief that the cuticle was formed by oxidation of fatty acids that had migrated to the surface was held well in to the 1940's, when Priestley (77) described the formation of the cuticle as "a continuous film of oil or fat" that eventually became a film of more "varnish-like consistency." This

varnish-like film was thought to be the result of air-oxidation and "a gradual linking together with the elimination of water."

In 1943 Priestley (77) noted that the main advance in the determination of cuticle structure since the 1920's was based on the use of the polarising microscope; this confirmed that the outer epidermal wall consisted of a layered or lamellar structure. The cuticle had long been considered distinct from the epidermal cellulose layer which it covered, but the exact structure of the outer epidermal wall remained obscure until the polarising microscope was used. The outer epidermal wall was thus defined as: "the cellulose wall of the epidermal protoplast extending into layers containing varying quantities of pectic substances and fatty acids to an outermost sheet of cuticle free from cellulose and usually free from pectic compounds". Priestly conceded that the main contribution to the cuticle was from the epidermal cells; however he still maintained that there was not sufficient difference between epidermal cells and those deeper within the tissue to conclude that the deeper cells had no role in the production of cuticular constituents.

The chemical composition of cutin was still uncertain in the 1950's. In 1925, Lee (60) reported cuticles from chrysanthemum and rose petioles and those from rhubarb leaves consisted of both free and esterefied fatty acids, 'soaps' of fatty acids, free higher alcohols and tannins.

Several classes of compounds were reported in cutin from Agave sp. in 1925 and 1929 by Legg and Wheeler (82). These included : 1) a semi-liquid acid $C_{26}H_{50}O_6$; 2) semi-liquid acid $C_{13}H_{22}O_3$ and 3) two other acids, one with a melting point of 107-108°C and one with a melting point of 88-90°C. Earlier reports by Fremy in 1881 and 1885 (68,82) showed Agave cutin to contain only two acids; one solid (stereo-cutic acid) and one semi-liquid (oleo-cutic acid). The cutin composition reported by Matic in 1956 (68) is probably the most reliable of the early reports. In the 1950's, cutin purification techniques utilizing strong acids had given way to milder reagents such as ammonium oxalate, oxalic acid (38) and enzymes (75). Using these milder techniques, Matic isolated five monomers from Agave sp. cutin (after hydrolysis), representing the bulk of the cutin from the ether soluble fraction:

1) 9,10,18-trihydroxyoctadecanoic acid (phloionolic acid)
2) 10,18-dihydroxyoctadecanoic acid
3) 18-hydroxyoctadecanoic acid
4) 18-hydroxyoctadec-cis-9-enoic acid
5) 10,16-dihydroxyhexadecanoic acid

This was the first record of isolation of 10,16-hydroxyhexadecanoic and 10,18-dihydroxyoctadecanoic acid. Matic concluded that conflicting results presented in

earlier work on Agave cutin composition were due to the analysis of mixtures rather than pure compounds. With Matic's work, as he himself pointed out, information was available as to the intermolecular structure of the cutin. The finding that a high proportion of the monomers carried more than one hydroxyl function per carboxyl function suggested an interesterification and cross-linking of these monomers into a polymeric compound. This was the first suggestion of the polyester nature of cutin. Matic noted the early references to the similarities of cutin with suberin, a plant polymer containing aromatics and hydroxy fatty acids. He pointed out some differences, such as a greater phenolic content in suberin and that suberin also contained some much longer chain fatty acids. Matic agreed that there were indeed some striking similarities in the C-16 and C-18 fatty acid components of the two plant polymers such as position of hydroxyl substitution.

Through the 1960's and 1970's the cutin composition of many more plant species was determined (6,12). By the early 1970's gas-liquid chromatography and mass spectral analysis were being utilized (21,93). These techniques greatly facilitated identification of cutin constituents. Since its first isolation in 1956, 10,16-dihydroxyhexadecanoic acid, along with its positional isomers, was determined to be the most common constituent of angiosperm cutin (7,35,41). The information gathered

from these studies allowed a more precise definition of cutin as a high molecular weight polyester consisting predominantly of two families of hydroxylated fatty acid monomers. These two families differed in carbon chain length (C-16 vs C-18) and substitutions on the carbon backbone. The major members of each family are as follows (47):

C-16 family -10,16-dihydroxyhexadecanoic acid and or its positional isomers with mid-chain hydroxylations at C-7, C-8 or C-9
-small amounts of 16-hydroxyhexadecanoic acid
-small amounts of hexadecanoic acid

C-18 family -mainly 18-hydroxyoctadecanoic
-18-hydroxy-9,10-epoxyoctadecenoate
-threo-9,10,18-trihydroxyoctadecanoic acid and its C-12 saturated analog

The monomeric composition of cutin can vary with species. Some cutins consist largely of the C-16 family while others contain a mixture of monomers from both the C-16 and C-18 families. Walton and Kolattukudy (93) suggested that plant tissues which grow and expand rapidly consist primarily of the C-16 monomers, whereas those which grow more slowly and have thicker cuticles contain a mixture of the C-16 and C-18 monomers. However, Holloway (34) objected to this conclusion by stating that the physiological and morphological evidence on which the conclusion was based was poorly defined.

In addition to the main constituents, monomers with more novel functions have been identified; those with aldehyde(43), epoxy (36,44) and ketone functions (16,23). There is evidence for the presence of small amounts of phenolic acids in cutin such as p-coumaric and ferulic acid (46,81), as well as chlorogenic acid and quercitin-3-glycosides (25). Whether these phenolic acids are covalently bound to the cutin is still unproven (33).

Differences in cutin composition were studied not only among plant species (15,37,39,93) but in different plant organs (11,23,24,51), abaxial and adaxial leaf surfaces (32) and between different developmental stages (5,42,45). These differences have included variations in thickness, proportion of C-16 and C-18 monomers and changes in saturations and substitutions on the monomer carbon chains.

As stated earlier, the general belief was that synthesis of cutin was a spontaneous oxidation and polymerization of fatty acids secreted onto the plant surface. By 1973 (41,67), there was general agreement that this process was dependent on specific enzymic steps. Evidence to support enzymatic involvement has accumulated through the use of radio-labelled precursors and intermediates such as C-18[1-C^{14}]-octadecanoic acid (46,53) and [C^{14}]-hexadecanoic acid (51). The first of such studies was carried out by P.E. Kolattukudy (51) who monitored [C^{14}]-hexadecanoic acid metabolism in Vicia

faba. The biosynthetic pathways (for the cutin monomers) determined by utilization of radiolabelled precusors are shown in Figure 1.1 (47).

In work with the incorporation of labelled fatty acids into cutins, Kolattukudy (48) determined that the only the epidermal cells have the capacity for cutin synthesis. How the precusors might move through the cell to the plant surface is unknown (33,48). Kolattukudy (48) has suggested that this might occur via vesicles.

The use of x-ray diffraction and polarising and electron microscopies has confirmed that aliphatic compounds alone comprise cutin (33). Other than the involvement of ester linkages, knowledge of the intermolecular structure of cutin has been limited mainly to the monomeric level. This is the result of the fact that as a polyester all the linkages present in it are equally susceptable to cleavage(46). Kolattukudy (46) suggested the use of enzymes for depolymerizaton, rather than the typical harsh degradative and reductive reagents commonly used. Enzymes should be capable of producing oligomers rather than monomers thus allowing an investigation into the nature of the intermolecular bonds. Oligomers which have been studied show free hydroxyls mainly in secondary positions and few free carboxyl functions (46) are found, indicating the ester linkages involve the primary hydroxyls.

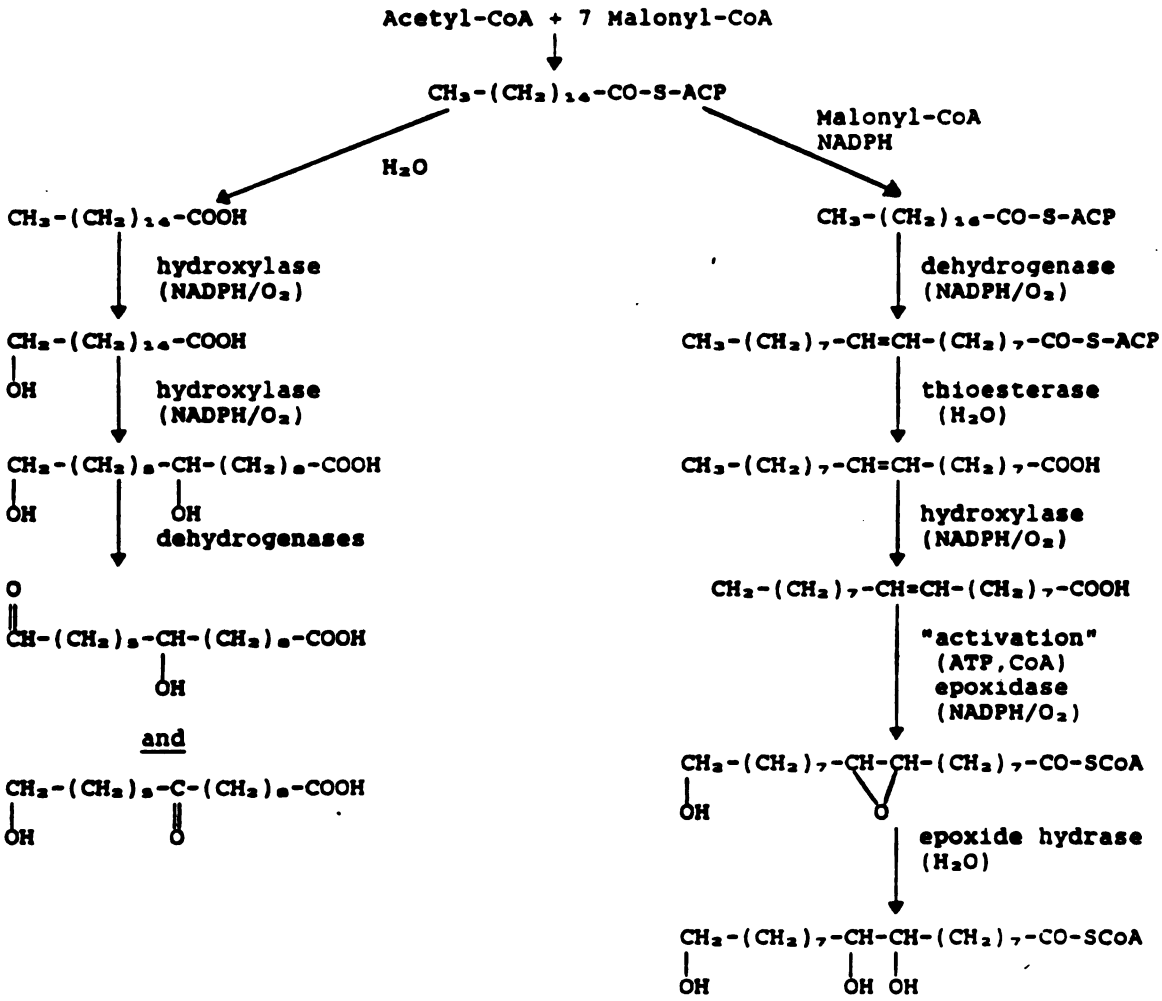

Figure 1.1. Biosynthetic pathway for cutin monomers (from ref. 47)

It is accepted that the plant cuticle and associated waxes prevent excessive water loss due to transpiration and control gas exchange (42). However, arguments have continued for over a century as to whether the cuticle constitutes a barrier to pathogens. In 1887, de Bary (17) concluded the "thickness or other structural characters of the membranes of the host are in most cases of little moment." Wiltshire (95), in 1919, stated "it appears probable the cuticle has a structure of its own and this may be an important factor in penetration of infection hyphae thru it." Higgens (31) and Corner (14) also considered the cuticle to be a barrier to infection. That younger leaves of certain plant species are more susceptible to infection than older leaves, with thicker cuticle, is also taken as evidence for a cuticular barrier (66,76,96). In a study often cited (97) in support for the importance of the cuticle as a barrier, Melander and Craigie (70) compared resistance to mechanical puncture in Berberis sp. with resistance to infection by Puccinia graminis. Results indicated that those species resistant to mechanical puncture were usually resistant to rust. However, it was observed that the inverse did not necessarily hold true. In addition, there were no histological studies showing that the hyphae of P. graminis could penetrate the resistant Berberis sp. without colonization. Stalder(90) was unable to correlate resistance to mechanical puncture with that of resistance

to fungi. J.T. Martin (67) took a firm stance against the idea of a cuticular barrier as did Verhoeff (92). However, Kolattukudy (46,48) took a firm position in favor of the cuticle as a barrier to invading organisms.

Histological studies of the relationship between pathogenic fungi and resistant, susceptible and non-host plants have ruled out the cuticle as a factor determining specificity; even in resistant and non-host interactions the pathogen can (and more often than not will) penetrate the plant cuticle (2,13,20,74,78). The observation that so many fungi can penetrate the cuticle directly would seem to rule out the concept of the cuticle as an impenetrable barrier.

Even though the role of the cuticle as a barrier may be in question, there is still a concern with the mechanism by which fungi might breach the cuticular layer. Several possibilities have been suggested. Penetration is believed to occur by one of several ways in fungi which penetrate directly (i.e. not through wounds or stomates): 1) mechanical force, 2) chemical or enzymatic degradation or 3) a combination of 1 and 2.

There are many examples in the literature supporting one view or the other. Many different host-pathogen combinations have been studied, some in extreme detail in attempts to determine mode of penetration. Definite problems are associated with these studies. Conclusions were often made with no supportive evidence or were based

on conclusions presented in other unrelated papers. Results often used in support of the mechanical force theory include: 1) fungi are capable of penetrating materials such as collodion, paraffin wax and gold foil, in which it is assumed chemical degradation could play no role (1,8,71,97); 2) flaccid leaves of Eucharis were penetrated more easily by Botrytis cinerea than turgid leaves. This was thought to be unexplainable except in terms of mechanical penetration (1,97); 3) studies correlating cuticle thickness with susceptibility and resistance have also been used as support for the mechanical theory (8,14,40). Included in this are reports such as etiolated tissue being more susceptible to infection than is green tissue (8) and demonstrations that younger leaves can be more easily penetrated than older leaves (31,66,76,97). In these cases both etiolated tissues and younger leaves are assumed to have thinner cuticles; 4) a study by Melander and Craigie (70), often cited as definitive evidence for mechanical penetration, correlated the resistance in Berberis species to puncture by a mechanical device to resistance to infection by Puccinia graminis. However, in a 1953 study, Stalder (90) was unable to find any correlation between susceptibility to a fungus with the ability to withstand mechanical pressure; 5) Wood (97) stated as evidence for support for mechanical penetration that infection structures appeared well adapted for the mehanical mode of penetration in that

they had the ability to firmly attach to the plant surface with this structure often modified to increase the surface in contact with the plant cuticle. In addition, the fineness of the infection hyphae allows the thrust on the hypha itself to be greatly reduced; 6) histological evidence given as proof for forceful penetration through the cuticle include such observations as inward bending or depressions in the cuticle itself (1,72), disorientation of the cuticle (72), rough edges and tearing around the penetration hole (91), and enlargement of the penetration peg after passage through the cuticle.

Prior to 1970, supporters of enzymatic dissolution of the cuticle cited histological evidence almost exclusively in support for their theory. This has included: 1) no inward bending at the point of penetration and smooth, clean edged penetration holes (69,83,92); 2) penetration holes larger than the diameter of the penetration peg (22,27); 3) disorganization of the cuticle resulting in a loss of structural integrity giving an appearance of amorphous densely staining material (1,72,83); 4) the presence of esterase activity at the penetration site (69.73); 5) non-staining halo surrounding the penetration peg (1,58,59); and 6) demonstration of the capacity to produce cutinolytic enzymes (22).

The main argument against the enzymic theory was that there had been no demonstation of the capability of plant pathogenic fungi to produce the needed enzymes. Wood (97)

stated that 'cutinases were not developed by plant pathogenic fungi, as they would be detrimental in that their action would not be limited to the point of entry. This would cause extensive degradation and allow desiccation of the leaf and entrance of secondary invaders'.

Although the strongest evidence supported a mechanical mode of penetration, Heinen's work (28) and the recognition that fungi were capable of production of cutinolytic enzymes, led many researchers to believe that cutinases were most probably involved in aiding direct penetration through the cuticle, either alone or in combination with force (22).

There were suggestions of cutin degrading enzymes as early as 1888 (94), but it was not until 1960 that supporting evidence became available. Heinen (28) demonstrated that Penicillium spinulosum had the ability to degrade cutin by growing the fungus on cutin as a sole carbon source. He illustrated the breakdown of the cutin particles by electron microscopy. An enzyme capable of releasing free fatty acids from the polymer was reported in the extracts of this fungus. In addition, Heinen presented evidence for a dehydrogenase in these extracts able to dehydrogenate the free fatty acids. The term proposed by Heinen for the cutin degrading enzyme was 'cutinase'. He pointed out the possibility that 'cutinase' may involve several enzymes and later in 1962

(30) reported the presence of multiple cutin degrading enzymes in the extract of Penicillium spinulosum. Partial purification (29) and characterization showed the cutinolytic enzymes to be stable (although inactivated by shaking) with no dialyzable co-factors and a pH optimum of 6.0.

In 1970, Shishiyama et al (85) reported cutinolytic activity in preparations from Botrytis cinerea. Although they found B. cinerea capable of growth on cutin as the sole carbon source, the enzyme was isolated from mycelia grown in sucrose medium. These workers determined the B. cinerea enzyme to be an exo-type as it split off only the minor components- the major components were not detected in the enzyme hydrolysate. The method of detection for these components was gas-liquid chromatography. Purdy and Kolattukudy (79) demonstrated Fusarium solani f. sp. pisi grew on cutin as a sole carbon source. They observed that esterase activity, using p-nitrophenyl palmitate (PNP) as the substrate, was greatly stimulated by growth on the cutin substrate over growth on sucrose, they believed this activity might reflect a cutin hydrolyzing enzyme. Unlike earlier reports these workers found that the enzyme hydrolysate of cutin, monomers identical to those found with chemical degradation and in the same proportions. Therefore this was not a peripheral release of fatty acids from the cutin as reported by Shishiyama et al (85). With the use of several synthetic model substrates it was

demonstrated that this enzyme was not a non-specific lipase. It had the capability of hydrolyzing simple wax esters but not tristearoyl glycerol.

A model substrate, p-nitrophenyl palmitate (PNP), was chosen to further characterize the cutinase activity of F. solani f. sp. pisi. With crude enzyme preparations, a broad pH optimum from 7.7-8.5 was observed. Beyond pH 8.5 the non-enzymatic hydrolysis was too high to allow accurate measurement of enzyme hydrolysis. Triton X-100 stimulated PNP hydrolase activity, supposedly through increased wetability of the substrate. Diisopropylfluorophosphate (DIFP), a potent inhibitor of serine hydrolases, severly inhibited the PNP hydrolase activity of this enzyme. Further characterization required purification. Along with the general esterase assay (PNP) a more specific assay was developed utilizing radio-labelled apple cutin as the substrate (double bonds of purified apple cutin were reduced with tritium gas) (80).

Following an initial ammonium sulfate precipitation, G-100 gel filtration resulted in a broad peak of cutinase activity that overlapped with PNP hydrolase activity. QAE sephadex (pH 9.0) bound the PNP hydrolase and the phenolics but not the cutinase. The PNP activity was therefore not associated with the cutinase. Cutinolytic activity did, however, correlate with p-nitrophenyl butyrate (PNB) activity (86). With the removal of the

phenolics, the cutinase eluted from the G-100 column in one major peak. A molecular weight of approximately 22,000 was estimated. By SDS polyacrylamide gel electrophoresis, it was shown that the PNP hydrolase contained a single protein while the cutinase contained two closely migrating proteins, both of which were shown to be capable of cutin hydrolysis. SP-sephadex resolved the two isozymes which were labelled cutinase I and cutinase II. Amino acid analysis showed cutinase I and II to be very similar; some differences were indicated in the amide groups. The PNP hydrolase, however, was quite different in amino acid composition. G-100 gel filtration gave molecular weights of 23,400 and 20,400 for cutinase I and II respectively. By amino acid analysis molecular weights were calculated to be 21,200 for cutinase I and 22,400 for cutinase II. SDS-PAGE gave a single molecular weight of 21,400. The determined molecular weight for the PNP hydrolase was 52,000 by gel filtration; 50,300 by amino acid composition and 54,00 by SDS-PAGE. All three enzyme activities were severely inhibited by DIFP. No similarity was found between the cutinase enzymes and other reported fungal lipases. These authors believed this to be the isolation of the first true fungal cutinase.

A cutinase isolated from another Fusarium species, Fusarium roseum f. sp. culmorum (86) compared quite closely to cutinase I and II from F. solani f.sp. pisi in

four ways; molecular weight (24,300), amino acid composition, pH optimum and inhibition by DIFP. Antibodies raised to cutinase I cross reacted with cutinase II, with spurs of non-identity. However, there was no cross reactivity with the enzyme from F. roseum f. sp. culmorum, although activity of this enzyme could be inhibited by addition of the antibodies indicating there were some common antigenic sites.

Further evidence for the presence of these enzymes in a variety of pathogenic fungi was reported by Baker and Bateman (3) who detected cutinolytic activity in Botrytis cinerea, B. squamosa, F. solani f. sp. phaseoli, Rhizoctonia solani, Helminthosporium carbonum, Pythium aphanidermatum, P. arrhenomanes, Gloecercospora sorghi, Cladosporium cucumerinum and Colletotrichum graminicola. Except for cutinase from B. cinerea and B. squamosa, the pH optimum for the various enzymes was in the alkaline range. Of those measured, cutinase from F. solani f. sp. pisi was by far the most active. Baker and Bateman also reported that Helminthosporiu maydis, Pythium ultimum and Sclerotium rolfsii were unable to utilize cutin as their sole carbon source.

By this time it was clear that many fungi are able to produce cutin degrading enzymes although few had actually been isolated and characterized. In 1982, Dickman, Patil and Kolattukudy (19) reported the isolation of a single, basic cutinolytic enzyme from Colletotrichum

gloeosporioides. Although this enzyme was much less active on the radio-labelled cutin than the enzyme from F. solani f. sp. pisi, it was similar in sevseral waysto this and other cutinases isolated by Kolattukudy's group. These similarities include a molecular weight of 24,000, pH optimum of 10 and inhibition by DIFP. Cutinases from C. gloeosporioides contained a greater percent carbohydrate (14-16% as compared to 3-5% for previously isolated cutinases). It also exhibited less selectivity concerning chain length of the p-nitrophenyl esters which it would cleave (ie. it cleaved equally well those from C_4-C_{16}). Antibodies from cutinase I did not cross react with the enzyme from C. gloeosporoiodes.

Kolattukudy and co-workers have intensely studied cutinolytic enzymes over the last fifteen years since first isolating them in 1973. The results provide a detailed knowledge of the physical and biochemical nature of these proteins, of which I will give a brief overview.

Cutinases have been purified to homogeneity from F. solani f. sp. pisi, F. roseum f. sp. culmorum, F. roseum f. sp. sambucinum, Helminthosporium sativum, Ulocladium consortiales, C. gloeosporioides, Phytophthora cactorum and Streptomyces scabies (50). The enzymes from each of these organisms have all proven to be basic proteins with a general esterase activity expressed as an ability to cleave p-nitrophenyl esters. Preference for chain length appears species dependent, although in general the shorter

chain acids are cleaved at a higher rate . The optimum pH has been shown to be highly alkaline for both the esterase and cutinase activity, ranging from 8.0 to 10.0. The molecular weights range between 22,000 and 26,000. In certain cases along with the large full length protein two smaller peptides are found, their combined molecular weights is equal to that of the parent protein (64,80). The two smaller proteins are thought to result from proteolytic cleavage. Despite considerable similarity in amino acid composition very little immunological cross reactivity has been observed between the enzymes from different fungi. The features of major importance within the amino acid composition are: one tryptophan, one methionine, one or two histidines and two to four cysteines (involved in a disulphide bridge)(49,50).

Three pairs of enzymes have been isolated from three different strains of F. solani f.sp. pisi. Some immunological cross reactivity was detected within each pair (with spurs of non-identity) but little if any between the pairs. However, activity could be inhibited with antibodies raised to another enzyme suggesting some common antigenic sites do exist.

Cutinases have proven to be glycosylated, generally from 3.5-6.0% but as high as 14-16% for C. gloeosporioides (19). The carbohydrates are in most cases attached through O-glycosidic linkages, depending on the species, to serine, threonine and/or two novel amino acids B-

hydroxyphenylalanine and B-hydroxytyrosine (52,63,87). The N-terminal glycine is found to be in a unique amide linkage with glucuronic acid (64). Kolattukudy and co-workers do not feel there is any functional significance to the presence of the unusual amino acids and carbohydrates.

The mechanism of catalysis of cutinases is believed to involve an active serine residue, as shown by the severe inhibition of activity by diisopropylfluoro-phosphate (DIFP) and other organophosphates. The rate constant of the inhibition reaction is very similar to other serine esterases (57). Modification of a single serine residue per molecule of enzyme resulted in complete inhibition of both the PNB hydrolase and cutinase activity (57). Additional evidence for an active serine residue comes from the ability to reversibly inhibit the enzymes with organic boronic acid derivatives. The formation of a reversible complex with phenyl boronic acids protects from DIFP inhibition (57). Cutinases are also inhibited by alkyl isocyanates, another group of serine directed inhibitors (49). The amino acid sequence of an isolated portion of the protein containing the active serine residue showed little homology with active serine containing regions of other serine hydrolases (88).

Serine hydrolases contain what is called a catalytic triad. Consisting of one active serine residue, an essential histidine residue and an essential carboxyl

function. Through the use of specific inhibitors these two were also found to be present in cutinases (57). Reversible unfolding of the cutinase by sodium dodecyl sulfate (SDS) made it possible to chemically modify these two additional members of the triad (57). The catalytic activity of the cutinase was completely destroyed upon addition of diethylpyrocarbonate, a histidine specific inhibitor. The stoichiometry of the reaction was such to indicate the presence of a single essential histidine residue. An essential carboxyl group was identified with the inhibitor 1-ethyl-3-(3-[dimethylamino]propyl) carbodiimide. As with the histidine inhibition this was possible only in the presence of SDS. Using a [^{14}C]-labelled inhibitor three non-essential carboxyls were found and one essential carboxyl buried within the protein (available to the inhibitor only in the presence of SDS). An acyl enzyme intermediate was expected to be involved as it has been found in all other serine active hydrolases. Koller and Kolattukudy (57) found that the deacylation step was rate limiting and inhibited by a factor of 10^5 by carbethoxylation. By incubating the enzyme with diethylpyrocarbonate prior to the addition of [^{14}C]-labelled p-nitrophenyl acetate this fleeting intermediate was detected. This finalized the evidence for the presence of the serine hydrolase catalytic triad in cutinase. A disulfide bridge is also essential for the activity of cutinases. It is postulated to be important

for the stabilization of the secondary and tertiary structure of the protein and thus maintenance of catalytic activity through proper positioning of the members of the catalytic triad.

Evidence exists to suggest that an arginyl residue is involved in the binding of the cutin substrate to the enzyme through carboxyls present on the cutin. Modification of the enzyme with phenylglyoxal severely inhibits cutin hydrolysis. It was shown that the enzyme would bind to a column of cutin but not if the cutin had been first treated with phenylglyoxal. The arginine is not believed to be part of the active site as its modification has no effect on the hydrolysis of p-nitrophenyl butyrate (49).

Cutinase production by F. solani f. sp. pisi was found to be inducible. The fungus was first grown in culture on a glucose medium. Following depletion of the glucose cutin hydrolysate was added. This resulted in induction of cutinase. This cutinase, however, was not entirely immunologically identical to the enzyme obtained from growth on cutin (62). Inhibition of cutinase synthesis by cycloheximide showed protein synthesis to be necessary for cutinase production. Transcription was determined to be unnecessary because Actinomycin D had no effect on cutinase synthesis. It is of significance to note that this conclusion of no transcriptional involvement in cutin synthesis conflicts directly with

subsequent data from Kolattukudy and co-workers in which polyA$^+$ mRNA is utilized in obtaining cutinase protein and a cutinase cDNA clone.

Isolated poly A$^+$ mRNA, translated in cell free systems, produced a protein which was had a slightly higher molecular weight than the in vivo protein (26). This protein cross reacted with the cutinase antibody. Flurkey and Kolattukudy conclude this protein to be a precusor to the mature cutinase. Post-translational modifications such as removal of the signal sequence and glycosylation could account for the observed differences in molecular weight.

cDNA generated from the polyA$^+$ mRNA from induced cultures (89) and 75 putative cutinases clones were identified by screening for their ability to hybridize with [^{32}P]-DNA for mRNA unique to the induced culture. From these, 15 were identified by further selection through hybrid-selected translation; the products were examined with cutinase antibodies. Insert sizes of the 15 cDNA clones ranged from 279-950 nucleotides. The size of cutinase mRNA was determined to be 1050 nucleotides by Northern blot hybridization. Thus it was concluded the clone of 950 nucleotides represented nearly the entire cutinase mRNA. This clone was then sequenced and the sequence confirmed with two of the other larger and overlapping cloned fragments. An amino acid sequence of a large part of the protein showed agreement with the amino

acid sequence deduced from the nucleotide sequence. Previously determined amino acid sequence of the area around the active serine residue (thirty amino acids) was identical with that predicted by a portion from the nucleotide sequence. An NH_2-terminal signal sequence, thirty one residues long, was determined from the nucleotide sequence. Subtracting the signal sequence, Soliday et al concluded the cutinase enzyme to contain 199 residues, 40% of which have been confirmed by direct amino acid sequence of the protein. One difference found between the nucleotide sequence and the previous chemical data on the protein was in the number of cysteine residues present. Chemical data (dithiothreitol) indicates two SH groups but the nucleotide sequence identifies four cysteine codons. These researchers suggest the possibility of post-translational modification of two of the cysteines but the exact nature of these modifications was not mentioned.

Probing DNA restriction fragments from two F. solani f. sp. pisi isolates T-8 and T-30, virulent and avirulent respectively, showed one common fragment hybridizing to the cutinase cDNA. In addition, a restriction fragment unique to T-8, the virulent isolate, also hybridized to the probe. Kolattukudy (50) suggested that this gene, unique to the high cutinase producer, may be highly expressed.

Although the existence of cutin degrading enzymes

could no longer be disputed it was still necessary to demonstrate their involvement in the process of penetration. Spores of F. solani f. sp. pisi placed on a pea stem were shown by scanning electron microscopy to have penetrated by 18 hours. In an initial experiment to gain proof for the role of cutinase as a penetration enzyme, Shayk et al (84) labelled cutinase antibody, raised to cutinase I from F. solani f. sp. pisi, with ferritin. Adding the labelled antibody to the area of infection showed a high density of ferritin granules in close proximity to the penetration point. From this it was concluded that the enzyme was at least present at the point where it would be needed. Later it was determined infection by F. solani f. sp. pisi could be inhibited by the addition of the cutinase antibody (65). DIFP, a specific inhibitor of serine esterases, was also demonstrated to be inhibitory to infection at concentrations of 0.01mM to 1.0mM. Both the DIFP and the antibodies were effective inhibitors of infection only on intact cuticle. Although no histological data was presented, these authors stated there were no deleterious effects on the fungus by either the antibodies or DIFP. They reported profuse growth on the plant surface in the presence of DIFP. Other phospho organic compounds (insecticides and fungicides) known to be inhibitors of serine hydrolases were found to protect against infection but on intact cuticle only (55). Radial growth of the

fungus was unaffected by the insecticides and was affected only by the highest concentration of the fungicides used.

Benomyl and its breakdown product n-butyl-isocyanate (BIC), known inhibitors of acetylcholine esterase, another serine active hydrolase, were shown also to inactivate cutinase (54), with BIC being much more active of the two. Cutin hydrolysis is inhibited if the cutin is first treated with BIC. It is believed the BIC reacts with the free hydroxyls in the cutin. Protection against infection occurred on intact tissue and only if the benomyl or BIC is applied within 24 hours after inoculation. Benomyl provided highly effective protection at concentrations as low as 5uM - a point where mycelial growth was inhibited only 30%. This last result was unexpected as benomyl is toxic to the fungus in culture. These authors do not speculate as to how the fungus escapes the fungitoxic effect in tissues.

Using 4 isolates of F. solani f. sp. pisi differing in their production of cutinolytic enzymes, a correlation was observed between enzyme production and virulence (56). The most virulent isolate, T-8, produced the greatest amount of cutinase. Virulence roughly doubled in a less virulent isolate, T-30, upon addition of purified cutinase. Combining cutinase with two other enzymes, pectinase and cellulase gave an additional small increase in infectivity. The same was true when cutinase was combined with pectin methyl esterase. Addition of all

four enzymes resulted in infectivity almost equal to T-30 on tissue wounded prior to inoculation. This observation led these workers to conclude that it was a battery of enzymes, which they termed 'penetration enzymes' which are involved in penetration. This conclusion leads one to question whether these authors are looking at the ability to colonize, rather than the ability to penetrate. Especially when, in a 1985 review, Kolattukudy (50) reported that T-30, in fact did not increase virulence with the addition of any single enzyme but that all four were necessary. Without accompanying histological investigations it is difficult to ascertain the contribution of these enzymes, singly or in combination, to penetration by F. solani f. sp. pisi. Enhancement of infection by nutrients from the added enzymes was a possibilty, although considered highly unlikely due to the observation that T-8 and T-30 germinated and grew equally well in water alone. The conclusion from this was that the less virulent isolate, T-30, was missing all the 'penetration enzymes', although they only tested for the presence of cutinase. These authors postulated that the efficiency of penetration may be the degree of expression of genes involved in the production of the penetration enzymes.

Supporting evidence for the role of cutinases in virulence came from work with Mycosphaerella sp., a wound pathogen of papaya (19). Upon the addition of exogenous

cutinase, purified from Colletotrichum gloeosporioides, Mycosphaerella was able to penetrate intact papaya cuticle at a greatly increased frequency. Further supporting evidence came from cutinase-deficient mutants generated from C. gloeosporioides (18). These mutants were no longer capable of infecting papaya fruit having an intact cuticle but were competent pathogens on mechanically wounded tissue.

Baker et al (4) showed that cutinase purified from F. solani f. sp. pisi reduced the mechanical strength of cutin membranes and allowed passage of radiolabelled glucose as well as cell wall degrading enzymes through the membranes. The conclusion was that cutinase may facilitate fungal ingress of this barrier in vivo.

In order to show that cutinase is present at the time of penetration, Woloshuk and co-workers (96) looked at the induction of this enzyme in germinating spores. After the addition of cutin hydrolysate to a spore suspension, esterase activity was detected spectrophotometrically within one hour and increased rapidly over five hours. The cutinase mRNA was also shown by cDNA hybridization to increase rapidly over five hours in cultures grown in the presence of cutin. Because the observed changes in mRNA occurred prior to germination, cutinase induction by enhanced transcription of cutinase genes is considered to be an early event in the process of germination.

The early data and conclusions made concerning the

phenomenon of penetration relied heavily on assumptions made from histological studies. In the more recent literature, conclusions are made with little if any histological and physiological data. Obviously the question of the mode of fungal penetration is complex as it has yet to be satisfactorily answered after nearly a century of study. There is no doubt penetration and infection is a multi-functional process- which is well intimated by Kolattukudy's "penetration enzymes". In the past, questions concerning the nature and role of penetration in infection have not been precisely defined. Thus there is often confusion as to whether penetration or colonization is being studied. More specifically, does penetration of the plant surface involve only the cuticle or is the cell wall also included? Venturia inaequalis, for example, is quite a capable pathogen, although it remains subcuticular, apparently not penetrating the cell wall (74). In addition, there are numerous examples of pathogens on non-hosts which can penetrate but do not cause disease. We are now at a point technologically where it should be possible to discern between many of these stages of infection. In order to determine their level of importance in the overall fitness of a pathogen it seems necessary to discuss each step separately. Although the work from Kolattukudy's laboratory has added an immense amount to our knowledge of this subject, many questions are left unanswered. In the cutinase inhibitor

studies the ability to colonize was used as a measure of the ability to penetrate. The histology remains to be done to document actual penetration efficiency in these studies. Kolattukudy's group has rediscovered (56) that F. solani f. sp. pisi is a wound invading pathogen in nature. This creates doubt as to the broad significance of the results. The work by Dickman et al (18,19) with C. gloeosporioides, although a direct penetrator also disregards the histology leaving open the same question of penetration without further infection. It seems essential that before any conclusion can be made concerning the mechanism(s) by which fungi breach the host cuticle studies must be undertaken with direct penetrating fungi, combining biochemical, genetic, physiological and histological data.

The following work is restricted to cuticular penetration by a direct penetrating fungal pathogen of cucumber, Colletotrichum lagenarium, race 1. Biochemical studies are combined with physiological and histological observations in hopes of shedding further light on this area of study.

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CHAPTER 2

COMPOSITION OF THE CUTIN FROM THE FRUIT OF *CUCUMIS SATIVUS*

INTRODUCTION

Infection of cucumber (Cucumis sativus L.) plants by Colletotrichum lagenarium (Pass.) Ell. and Hals. race 1 involves the direct penetration of the cuticle (7). In order to fully understand the mechanism of penetration by this pathogen, the chemical nature of cutin, the stuctural polymer of the cuticle, must be known. Although cutin composition for a number of plant species has been determined (3,5), the composition of cucumber cutin has not been reported. The purpose of this study is to determine the chemical composition of cucumber cutin and to determine if the cutin has any functional groups that might be easily labelled for use in assays of cutinolytic enzymes produced by C. lagenarium. Even though it is known that fruit and foliage cutin of the same species can differ in composition (4), fruit cutin was chosen as the source of cutin due to the ease of preparing large amounts of cutin and because the fruit is as easily infected by C. lagenarium as is the foliage (2).

MATERIALS AND METHODS

Materials. Pectinase (Aspergillus niger) and cellulase (Aspergillus niger) were obtained from United States Biochemical Corp. Thin layer chromatography plates (silica gel G, 250u) were purchased from Fischer

Scientific. 2,4-dinitrophenylhydrazine (DNP) was purchased from Eastman Organic Chemicals. N,O-bis(Trimethylsilyl)- Trifluoroacetamide (BSTFA) was obtained from Pierce. All other chemicals were purchased from Sigma Chemical Company.

Purification of Cucumber Cutin.

Cucumber fruits (obtained from Michigan State University Botany and Plant Pathology and Horticulture Research Farms) were peeled and the peelings treated as follows to isolate cutin.

1. Boil peels in a mixture of 1.6% ammonium oxalate and 0.4% oxalic acid for several hours, followed by extensive rinsing in tap deionized water.

2. Shake peels in a mixture of 0.5% cellulase and 0.1% pectinase in 0.05M sodium acetate buffer pH 4.0, for 24 hours at 27°C.

3. Repeat steps 1 and 2.

4. Extract peels in chloroform:methanol (2:1) overnight.

5. Soxhlet extract peels in chloroform for 48 hours.

6. Repeat steps 2,4 and 5.

7. Dry peels and grind in a Wiley mill to pass a 60 mesh screen.

Depolymerization of Cucumber Cutin.

Reductive Depolymerization with Lithium Aluminum Hydride ($LiAlH_4$) (9). Redistilled tetrahydrofuran (THF) was added to 100 mg of cutin dried over P_2O_5. To this was added 300 mg dry $LiAlH_4$. This mixture was refluxed under nitrogen for 24 hours with stirring. The reaction was terminated by the successive addition of 0.2 ml dH_2O, 0.2 ml 15% (w/v) NaOH, and an additional 0.6 ml dH_2O per every 30 ml of reaction mixture. The resultant precipitate was removed by filtration through Whatman #4 filter paper. Twenty ml dH_2O were added to the liquid phase, and the liquid phase and the insoluble precipitate were each extracted two times with an equal volume of ethyl acetate. The ethyl acetate fractions were combined, dried with anhydrous magnesium sulfate, and concentrated to a small volume under reduced pressure.

Alkaline Hydrolysis with Potassium Hydroxide. One hundred mg of cutin were refluxed 72 hours in 6.0% KOH. Following refluxing, the reaction mixture was acidified to pH 3.0 with concentrated HCl. Two volumes of dH_2O were added to the sample, if needed, to increase the volume. Extraction was first done with ethyl ether (2 times,v/v) followed by chloroform (2 times, v/v). Fractions were combined and dried with anhydrous magnesium sulfate and the solvent removed under reduced pressure. The residue was resuspended in a small volume of ethyl acetate.

Thin Layer Chromatography (TLC).

Samples of depolymerized cutin were spotted onto TLC plates and the components were resolved by thin layer chromatography (ethyl ether: hexane: methanol: acetic acid [80:20:10:1.5]). Fatty acids were detected with iodine vapor (8). Carbonyl functions were detected by first spraying the plates with 2,4-dinitrophenylhydrazine followed by spraying with a 10% solution of NaOH. The fatty acids containing carbonyl functions stained red-brown (1).

Gas Chromatography-Mass Spectrometry.

Mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility which is supported, in part, by a grant (DRR-00480) from the Biotechnology Resources Branch, Division of Research Resources, National Institutes of Health. Each sample was dried under a stream of nitrogen gas and volatilized by the addition of N,O-bis(Trimethylsilyl) trifluoroacetamide (BSTFA) prior to injection into the gas chromatograph and mass spectrometer (HP 5890 GC, He carrier gas 10.7 ml/min.; JEOL HX110 HF, EI, ionizing potential 70eV, accel, 10kV voltage.)

RESULTS AND DISCUSSION

Identification of Cucumber Fruit Cutin Components. Utilizing comparative gas chromatography-mass spectrometry (GC-MS) techniques, five components of cucumber fruit cutin have been identified. The data shown are that using the silylated lithium aluminum hydride reduction products of purified cucumber cutin, except where noted.

Gas chromatography of the silylated lithium aluminum hydride reduction products revealed five peaks (Figure 2.1). Peak 1 (r.t. 5'20") (11% of the total) has been identified as 1,16-hexadecanediol. The mass spectrum (Figure 2.2) shows a molecular ion of m/z=402 with typical losses of M-15 (m/z=387), M-90 (m/z=312) and M-15-90 (m/z=297). Comparison with previously published mass spectra for 1,16-hexadecanediol (6,10) confirm the identity of this compound. The corresponding form of this compound found in the original cutin (i.e. non-degraded) would be 16-hydroxyhexadecanoic acid.

The mass spectrum for peak 2 (r.t. 7'24") (58% of the total) is shown in Figure 2.3. This compound has been identified as 1,8,16-hexadecanetriol with small amounts of the positional isomers 1,7,16-hexadecanetriol and 1,9,16-hexadecanetriol. In this case, the chromatogram (Figure 2.3) is that for the alkaline hydrolysis products of cucumber fruit cutin rather than the lithium aluminum hydride reduction products because this spectrum clearly

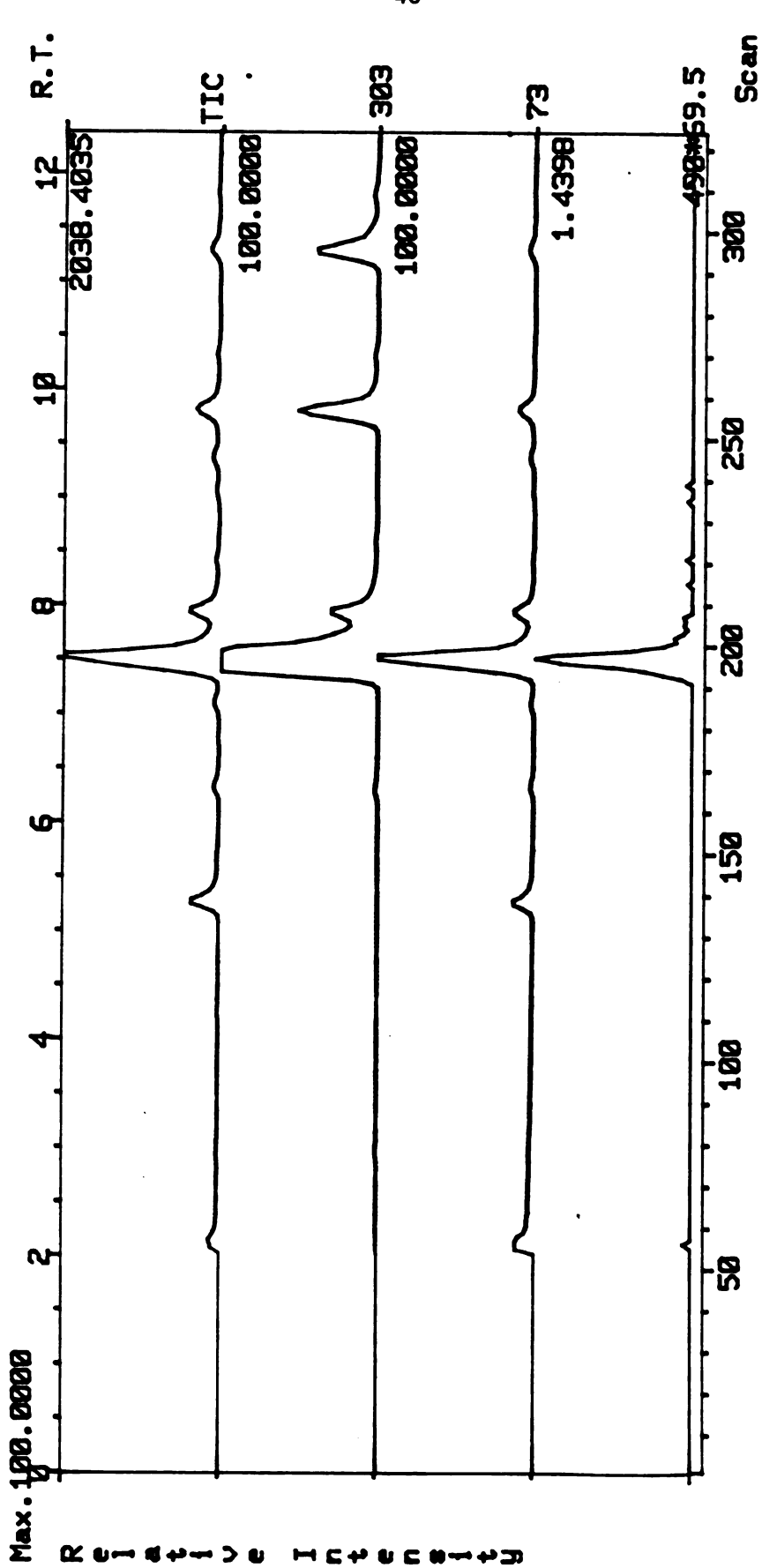


Figure 2.1. Gas chromatogram of products of the $LiAlH_4$ reduction of cucumber fruit cutin.

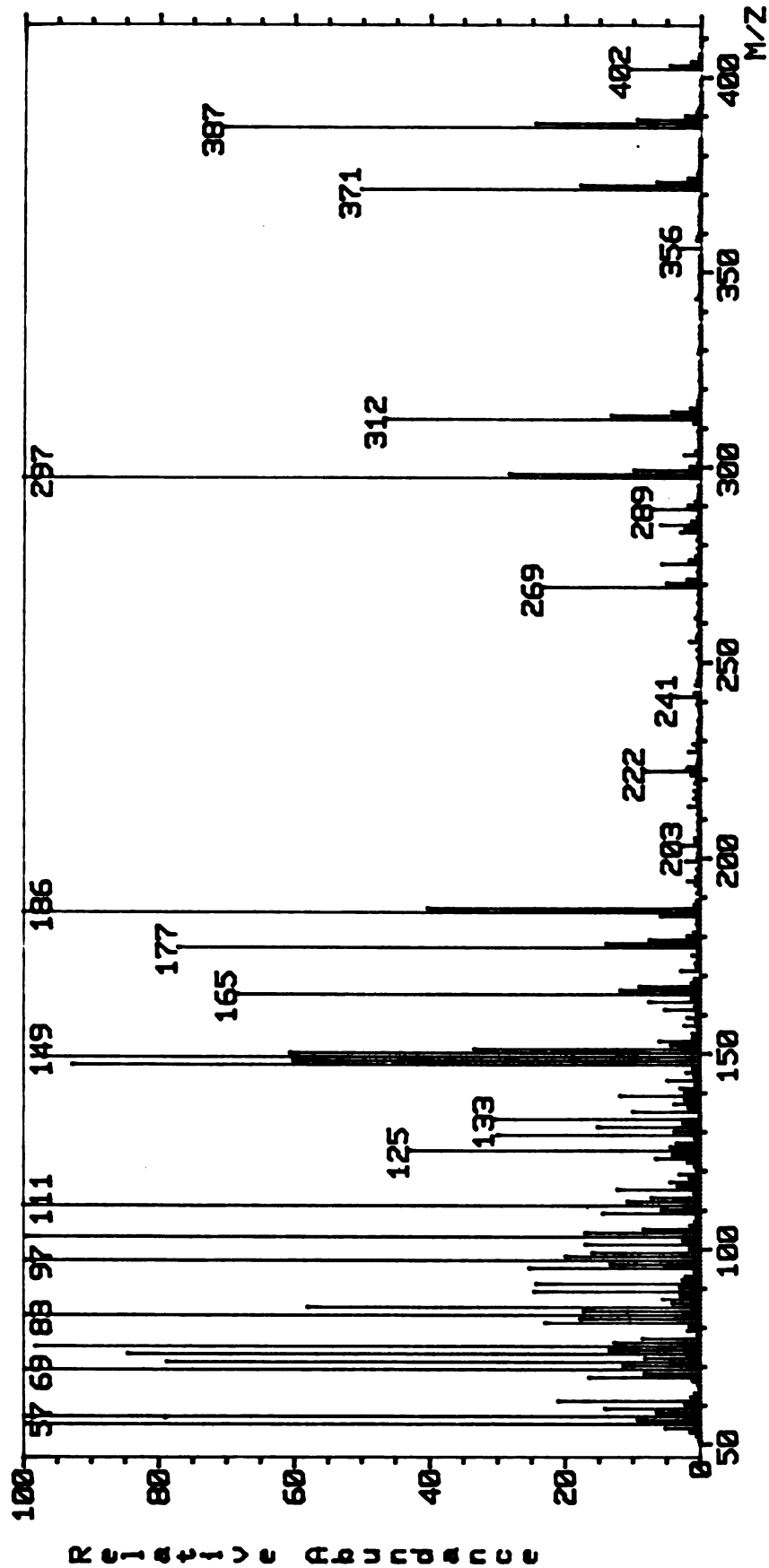


Figure 2.2. Mass spectrum of 1,16-hexadecanediol, $LiAlH_4$ reduction.

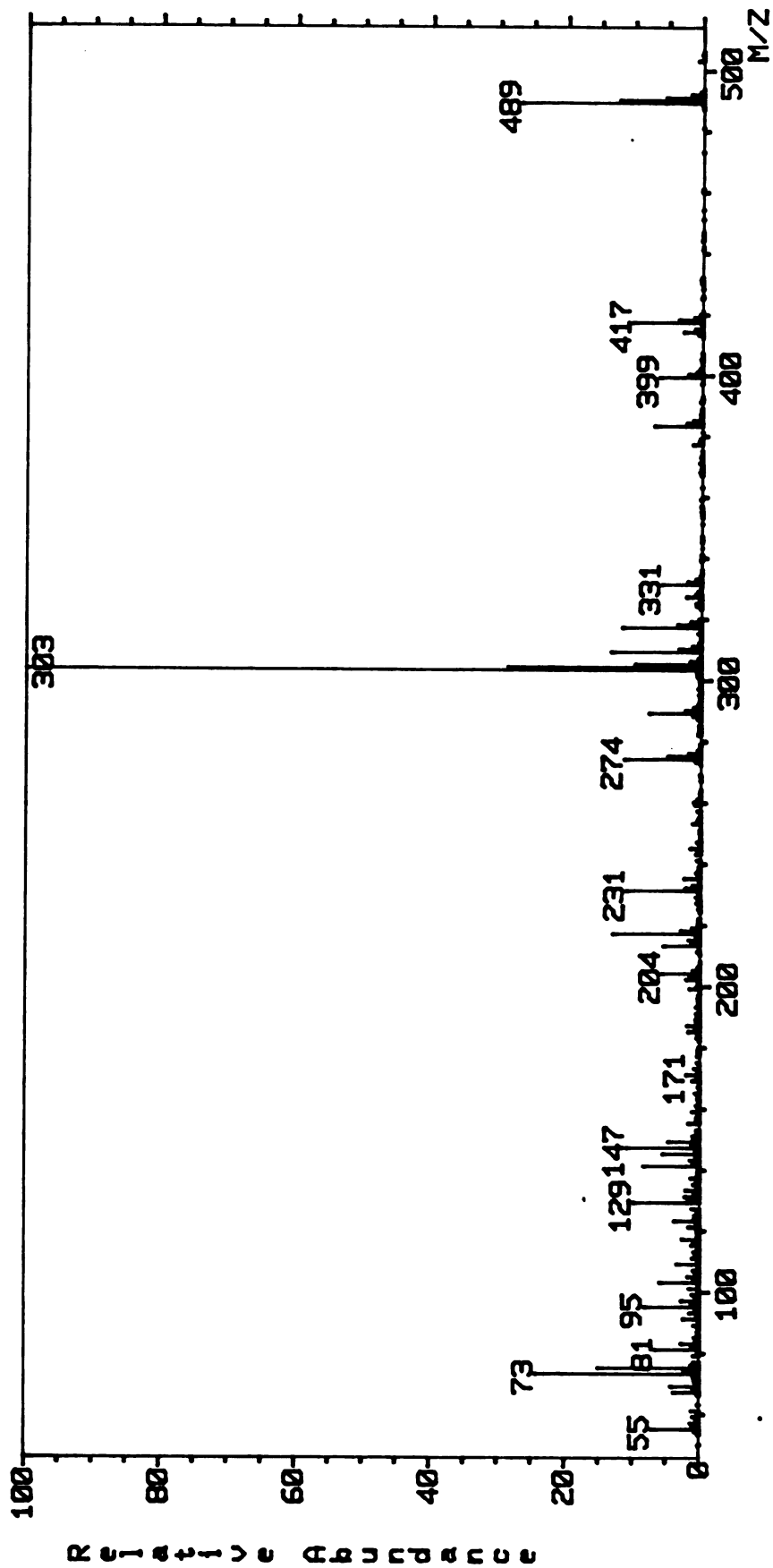
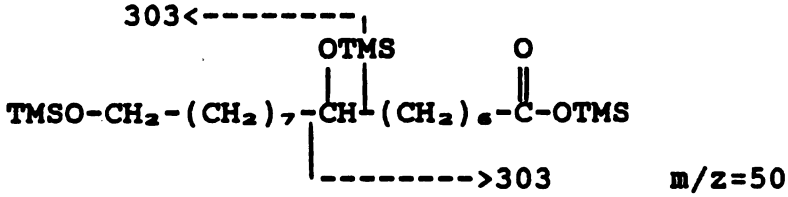


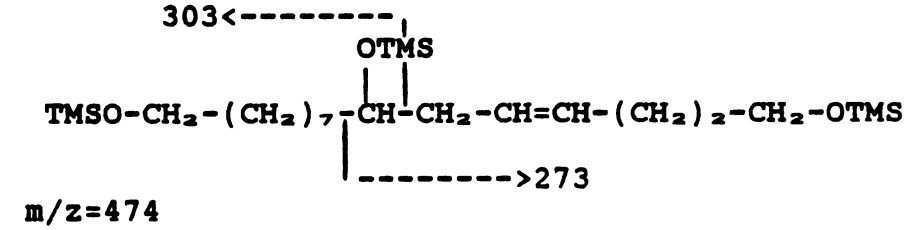
Figure 2.3. Mass spectrum of 1,8,16-hexadecanetriol and its positional isomers, KOH hydrolysis.

shows the position of the mid-chain hydroxyl by the distinct single α-cleavage ion of m/z=303. A molecular ion of m/z=504 with typical losses of M-15 (m/z=489), M-90 (m/z=414) and M-15-90 (m/z=399) are observed. The α-cleavage fragmentation pattern is illustrated below.



In the original cutin this compound corresponds to 8,16-dihydroxyhexadecanoic acid.

The mass spectrum corresponding to peak 3 (r.t. 7'56") (13.9% of the total) of Figure 2.1 is shown in Figure 2.4. This spectrum indicates a mixture of positional isomers of a trihydroxypentadecene with the typical M-15 (m/z=459) loss and the major α-cleavage ions of m/z=259, 273, 289 and 303. The exact positions of the mid-chain hydroxyl and the carbon-carbon double bond (C=C) are unknown at this point. Assuming the unsaturation site is located between the mid-chain hydroxyl and C-1 (the carboxyl end), then the position of the mid-chain hydroxyl could vary between C-6, C-7 or C-8 resulting in the α-cleavage patterns shown below.



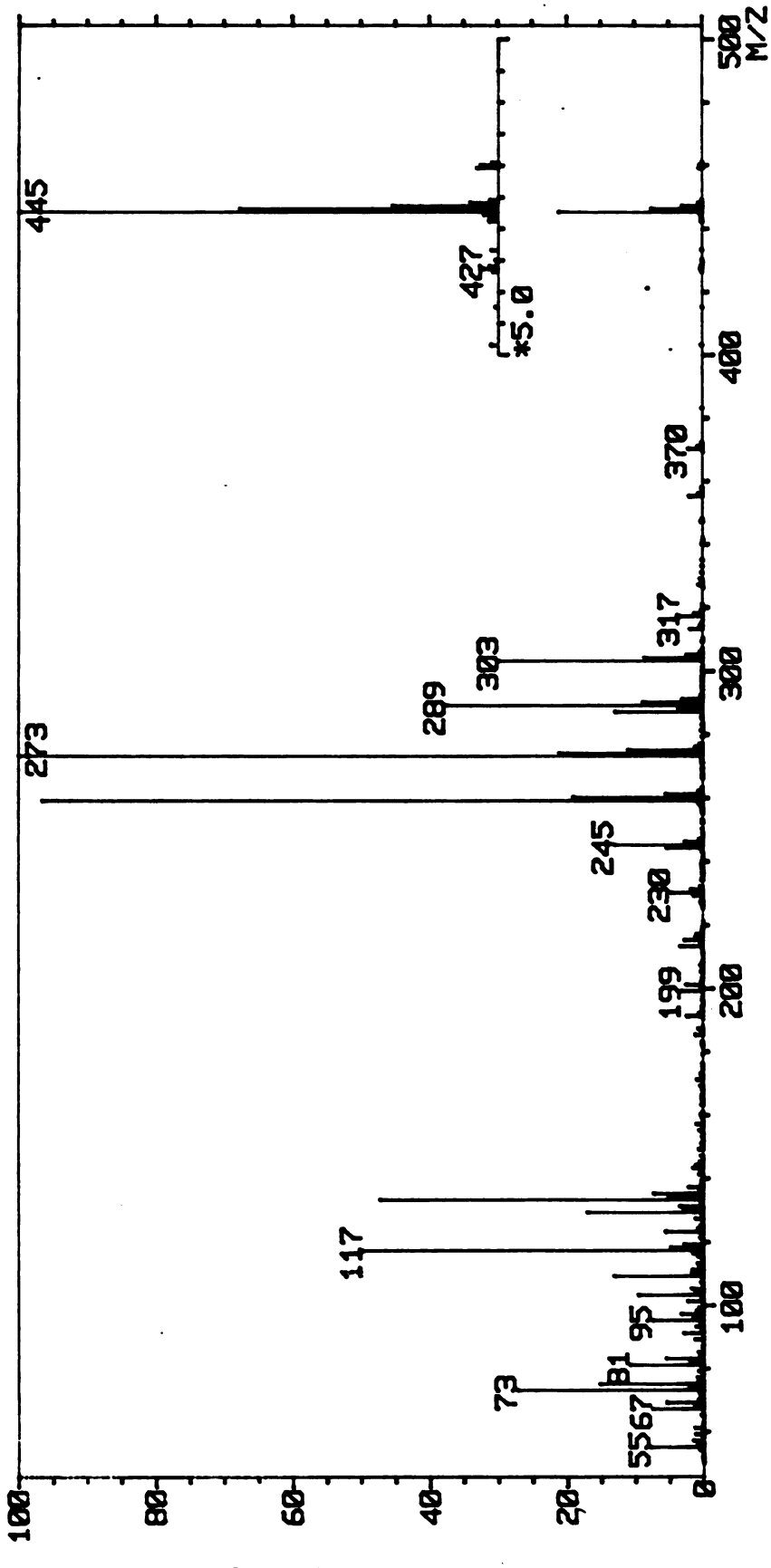


Figure 2.4. Mass spectrum of a mixture of positional isomers of trihydroxypentadecene, $LiAlH_4$ reduction.

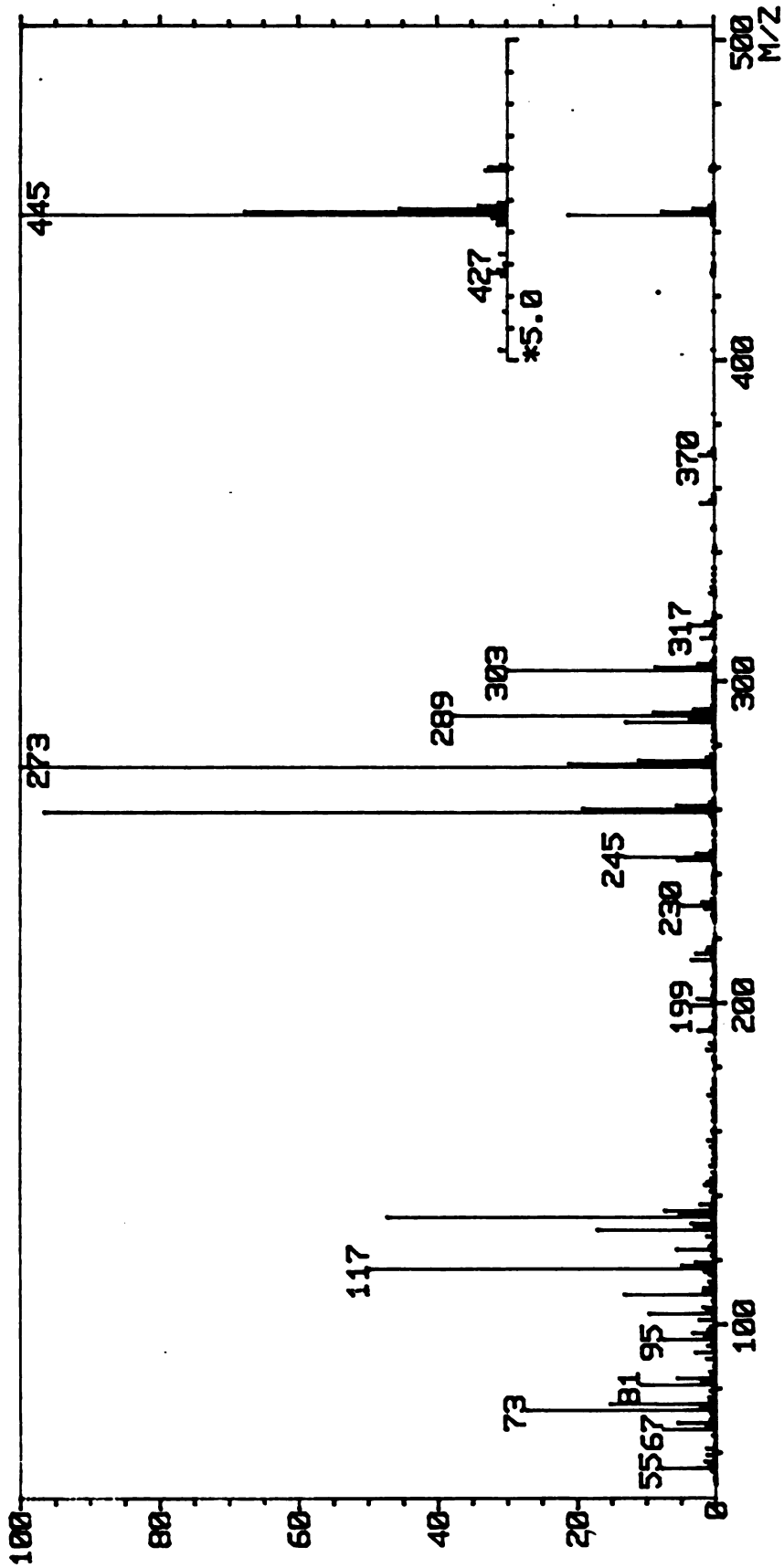
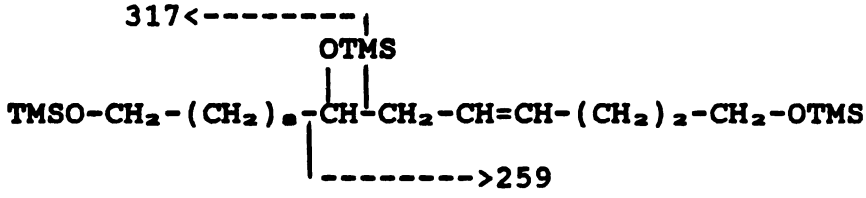
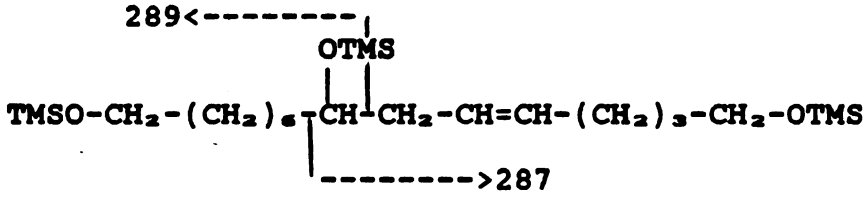
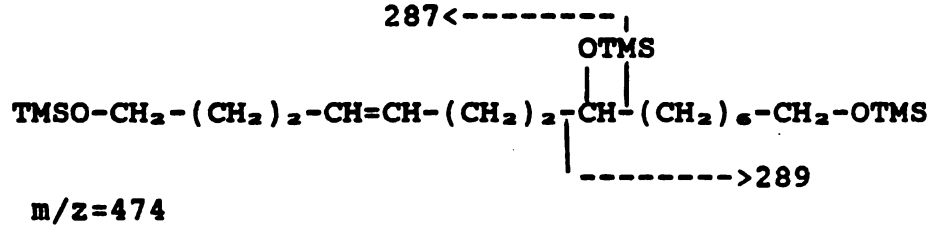


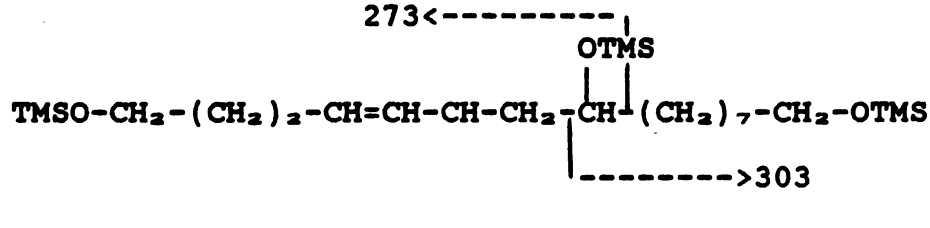
Figure 2.4. Mass spectrum of a mixture of positional isomers of trihydroxypentadecene, $LiAlH_4$ reduction.

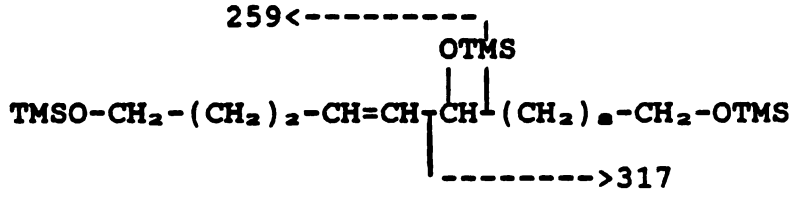




If the C=C should be between the mid-chain hydroxyl and C-15 then an *α*-cleavage pattern as shown below becomes possible.



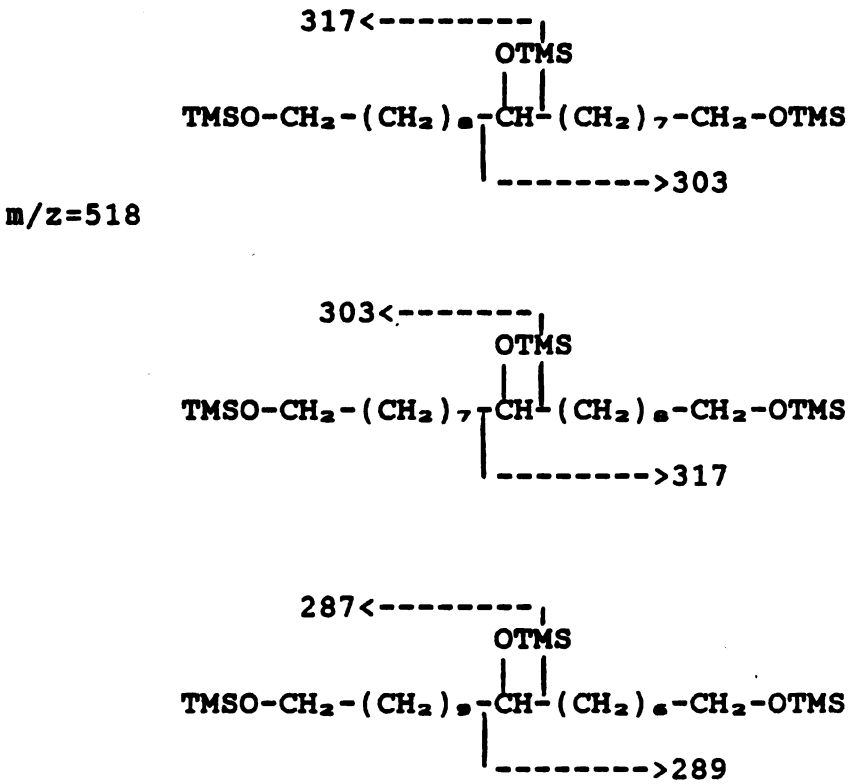




In the original cutin this compound most likely

corresponds to an isomeric mixture of (6 or 7 or 8),15-hydroxypentadecenoic acid or an isomeric mixture of (8 or 9 or 10),15-hydroxypentadecenoic acid.

Peak 4 (r.t. 9'46") of Figure 2.1 (10.6% of the total) was identified as an isomeric mixture of 1,9,18-trihydroxyoctadecane and 1,10,18-trihydroxyoctadecane with small amounts of the positional isomers 1,8,18-trihydroxyoctadecane and 1,11,18-trihydroxyoctadecane. Typical losses of M-15 (m/z=503) and M-90 (428) are illustrated in the mass spectrum for this compound (Figure 2.5). Below is illustrated the fragmentation pattern for the major α-cleavage ions m/z=303, 317, 289 and 331.



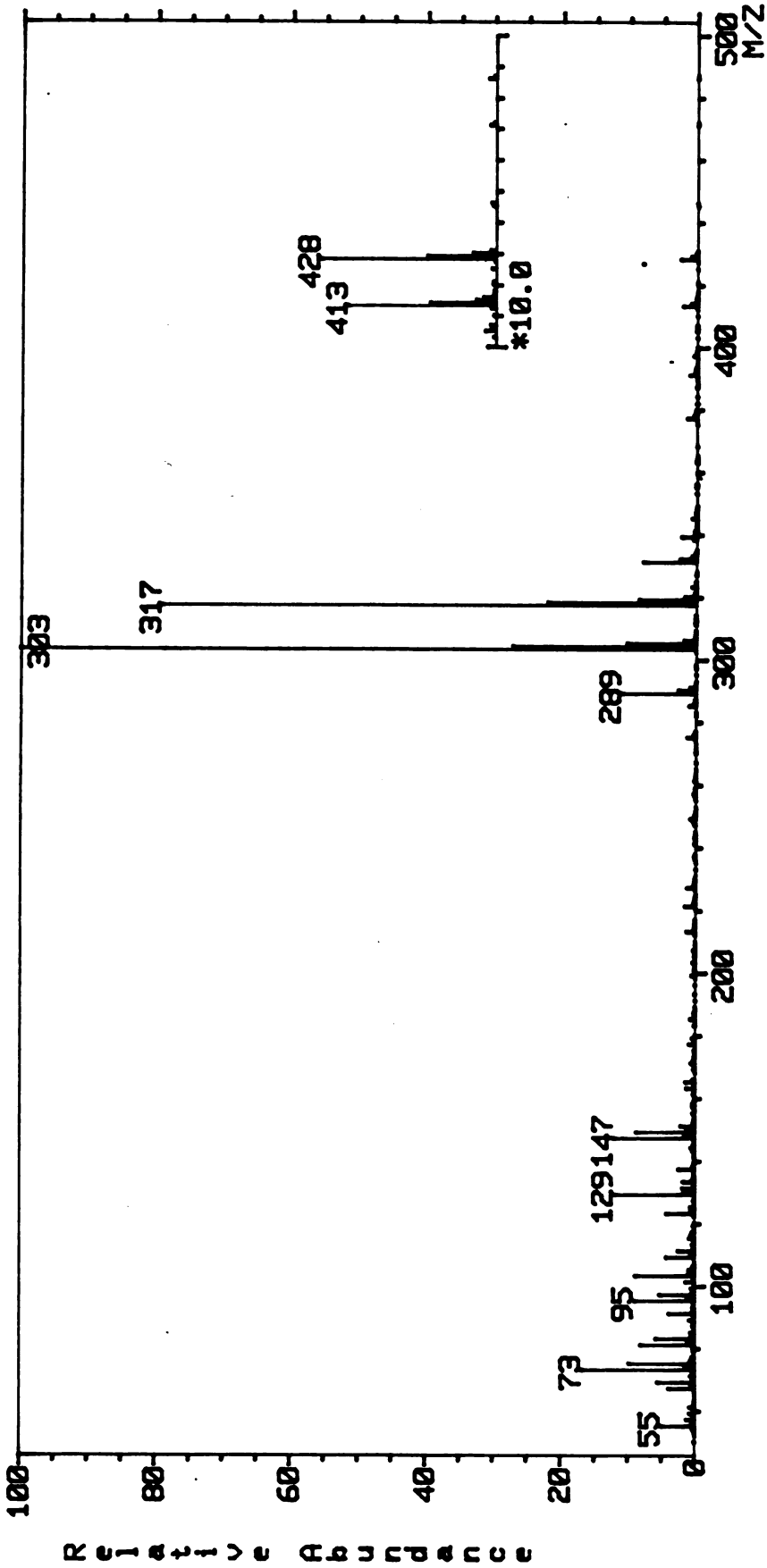
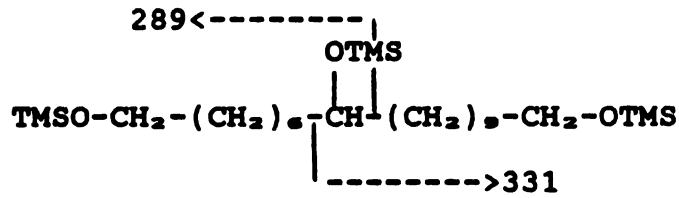
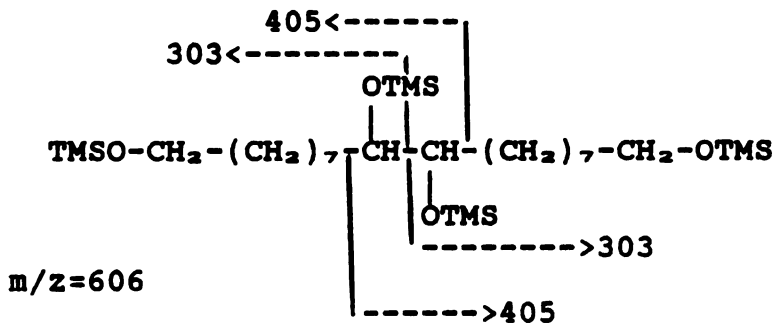


Figure 2.5. Mass spectrum of 1,9,18- and 1,10,18-octadecanediol, $LiAlH_4$ reduction.



Analysis of the alkaline hydrolysis products confirms the identity of this component. In the original cutin the 1,(9 or 10),18-trihydroxyoctadecanes correspond to 9,18- and 10,18-dihydroxyoctadecanoic acid.

The final component (r.t. 11'16") (6.7% of the total) identified from the cucumber fruit cutin was 1,9,10,18-octadecanetetraol. The mass spectrum for this compound is shown in Figure 2.6. No molecular ion was observed, but the typical losses of M-15 (m/z=591), M-90 (m/z=516) and M-15-90 (m/z=501) were found. Analysis of the alkaline hydrolysis products by GC-MS and comparison of this spectum with those previously published for this compound (4,10) confirms its identity as 1,9,10,18-octadecanetetraol. Below is illustrated the fragmentation pattern of the major α-cleavage ions m/z=303, 405.



In the original cutin the above compound corresponds to

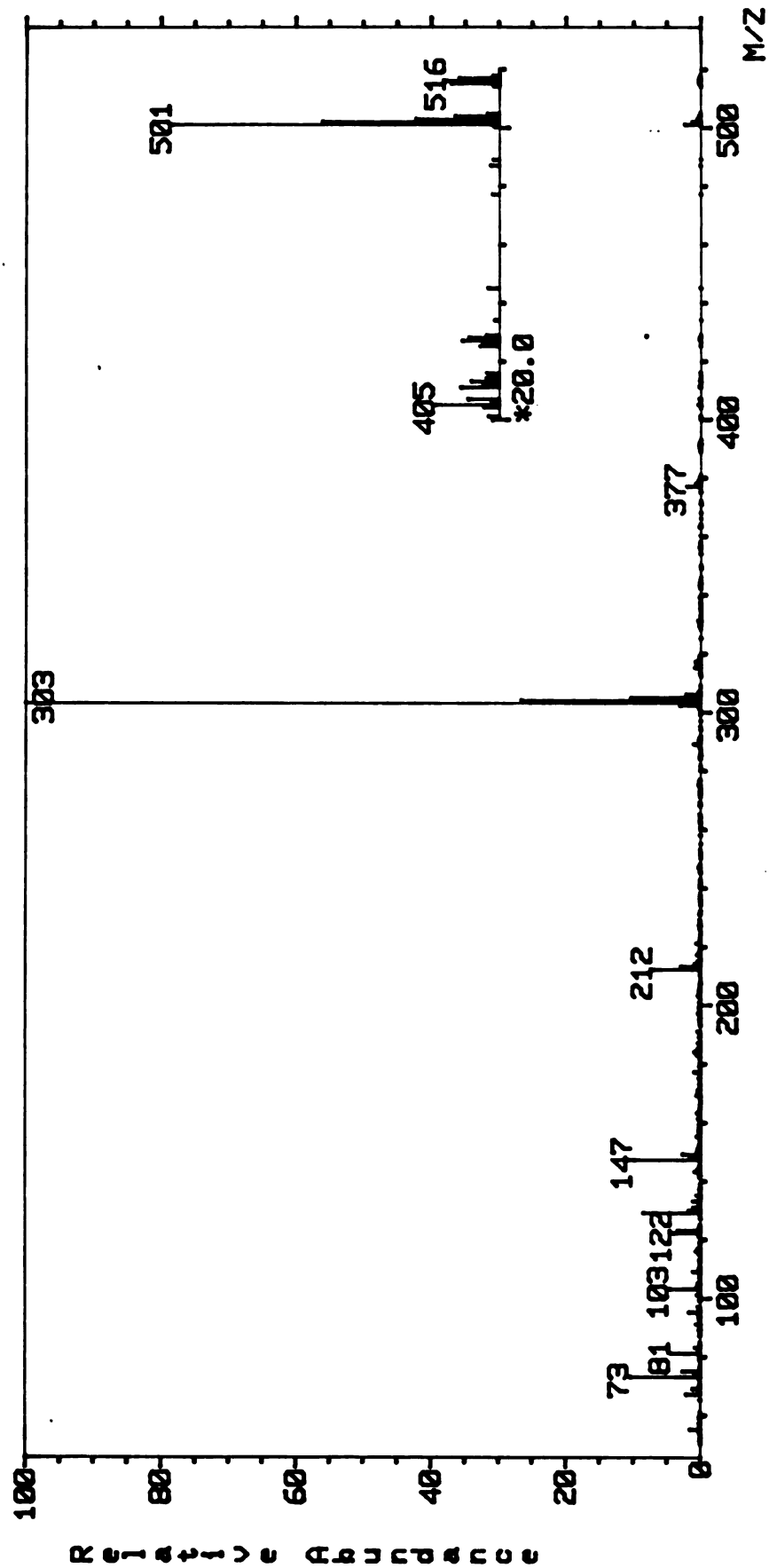


Figure 2.6. Mass spectrum of 1,9,10,18-octadecanetriol, $LiAlH_4$ reduction.

9,10,18-octadecanoic acid.

The techniques employed identified each component as a hydroxy alkane rather than as a fatty acid as it exists naturally in cutin prior to depolymerization. In addition to the loss of the carboxyl function as a result of depolymerization and volitilization, other possible functional groups such as epoxides and carbonyls are lost. The possibility exists that one or several of the mid-chain hydroxyls contained in the five identified cucumber fruit components could be ketones.

Determination of the Presence of Reducible Carbonyl Functions in the Cucumber Fruit Cutin.

One specific interest was in determining if any ketone function exists in cucumber fruit cutin so that it might be utilized in the radio-labelling of the cutin by $NaB[^3H]H_4$ reduction. Because of the highly reactive nature of carbonyl functions they were not detectable by the methods utilized. Both the $LiAlH_4$ and BSTFA made ketone functions indistinguishable from hydroxl functions. In order to determine the presence of carbonyl functions, the alkaline hydrolysis products of the cucumber fruit cutin were separated by TLC and then chemical tests were performed to detect the presence of these functions. Exposure of the TLC plates to iodine vapors indicated the presence of five components. The Rf values of each is listed below:

1) 0.95
2) 0.75
3) 0.60
4) 0.55
5) 0.36

The plate was then sprayed with 2,4-dinitrophenylhydrazine (DNP), dried and sprayed with 10% NaOH. DNP reacts with carbonyls to give a bright yellow band or spot which upon expose to alkali turns red-brown (1,4). Component 4 of the cucumber fruit cutin had a very strong positive reaction to the DNP. Component 2 also reacted positively with the DNP. Barely detectable reactions occurred with components 3 and 5 and no reaction with component 1.

Solid non-depolymerized cucumber cutin was also tested for the presence of carbonyl functions. Treatment with DNP followed by exposure to NaOH resulted in a strong positive reaction. Cutin first treated with $NaBH_4$, which reduces the carbonyl functions to hydroxyls, did not react with the DNP/NaOH.

These results indicate the presence of a reducible carbonyl function in cucumber fruit cutin. GC-MS of the TLC isolated alkaline hydrolysis products identifies component 4, the most DNP reactive component, as that fraction corresponding to 8,16-dihydroxyhexadecanoic acid. Thus it appears this compound or some fraction of it contains a carbonyl function. Most likely this is a mid-chain ketone at C-8. Although the possibility remains that there may be an aldehyde at C-16 maintaining the hydroxyl at C-8. However, this is much less likely as

these compounds are not commonly found in cutin (5). Component 2 from the TLC separation was identified as 16-hydroxyhexadecanoic acid. This fraction also contains minor contaminants tentatively identified as pentadecanoic acids with mid-chain hydroxyls. These contaminants could explain the positive DNP reaction which would not normally be expected with 16-hydroxyhexadecanoic acid. Another possibility, although less likely (4), is that this compound contains an aldehyde function rather than the carboxylic acid.

Minor Components. Two minor components were identified among the TLC separated constituents of the alkaline hydrolysate of cucumber fruit cutin. These represented less than 1% of the total. These were: 1) 8-hydroxypentadecanoic acid identified by a molecular ion m/z=402 and typical losses of M-15 (m/z=387) and M-90 (m/z=312) with major α-cleavage ions of m/z=303 and m/z=201, and 2) 8-hydroxyhexadecane-1,16-dioic acid identified by a molecular ion of m/z=518 and typical losses of M-15 (m/z=503) and M-90 (m/z=428). The major α-cleavage ions were m/z=317 and m/z=303.

In summary the identified components of cucumber fruit cutin include:

1) 8,16-dihydroxyhexadecanoic acid or 16-hydroxy-8-oxo-hexadecanoic acid, with small amounts of the positional isomers 7,16- and 9,16-hydroxyhexadecanoic acid (and possibly their corresponding 'oxo' forms) (Figure 2.3).

2) 16-hydroxyhexadecanoic acid (Figure 2.2)

3) isomeric mixture of 9,18- and 10,18-dihydroxyoctadecanoic acid (Figure 2.5)

4) isomeric mixture of trihydroxypentadecenoic acid (Figure 2.4)

5) 9,10,18-trihydroxyoctadecanoic acid with small amounts of the positional isomer 8,9,18-trihydroxyoctadecanoic acid (Figure 2.6)

6) 8-hydroxypentadecanoic acid

7) 8-hydroxyhexadecane-1,16-dioic acid

The components of cucumber cutin as identified are typical of those reported for other plant sources (4,5) with the exception of the dihyroxypentadecenoic acid. I have been unable to find any previous reports of a hydroxypentadecanoic acid which also contains an unsaturation site. In addition, this component was not found among the alkaline hydrolysis products. Thus I am not entirely convinced of its identification. Another difference from typical cutin constituents includes the C-8 position of the mid-chain hydroxyl on the dihydroxyhexadecanoic acid. The most common position for this hydroxyl function is on C-9 or C-10 , with minor positional isomers on C-8 and C-7 (4). The composition of cucumber fruit cutin is typical of other cutins by the presence of mostly C-16 and C-18 fatty acids. In the case of the cucumber fruit cutin, members of the C-16 family predominate.

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CHAPTER 3

CHARACTERIZATION OF CUTINOLYTIC ENZYMES FROM *COLLETOTRICHUM LAGENARIUM* AND THEIR ROLE IN PENETRATION OF CUCUMBER

INTRODUCTION

The ability of plant pathogenic fungi to produce cutin degrading enzymes has been known for more than 25 years. Heinen (8) first demonstrated the presence of these enzymes in cultures of Penicillium spinulosum using cutin as the sole carbon source. Since then many fungal pathogens have been shown to produce cutinases (1,23,25), although their purification has been accomplished in only a few systems (24).

The role of cutin degrading enzymes in the penetration of the host cuticle has been debated for over a century. Most conclusions made in favor of enzyme involvement, prior to the 1970's, were based on histological evidence such as lack of inward bending of the host cuticle at the point of penetration (21) and penetration holes larger than the penetration peg (6). In 1973, Purdy and Kolattukudy (23) demonstrated Fusarium solani f.sp. pisi to be capable of growing on cutinase as the sole carbon source. Biochemical and immunological data were presented as evidence for the requirement of these enzymes by F. solani f.sp. pisi in the penetration of its host, pea (Pisum sativum). However, because this fungus is naturally a wound invading pathogen, the significance of these findings are difficult to relate to direct penetrating fungi. Dickman et al (4) presented both immunological and histochemical evidence for the role

of cutinases in the penetration of papaya by Colletotrichum gloeosporioides. Mutants of C. gloeosporioides unable to produce cutinase were non-pathogenic (5) again suggesting a role for cutinase in penetration.

My interest was in purifying cutin degrading enzyme(s) from Colletotrichum lagenarium, a direct penetrating pathogen of cucurbits and to investigate the role of these enzymes in penetration.

MATERIALS AND METHODS

Materials. Bradford reagent was purchased from Bio-Rad. Scintillation vials and scintillation cocktail (Safety-Solve) were both obtained from Research Products International Corp. Ethylene glycol was obtained from J.T. Baker Chemical Co. Cucumber seeds (SMR-58) were obtained from Stokes Seeds Inc. Terra Coat L-205 was provided by Gustafson. All other chemicals were purchased from Sigma Chemical Co.

Culture Conditions.

Colletotrichum lagenarium (Pass.) Ell. and Hals. race 1 cultures were maintained on potato dextrose (PDA) or green bean agar (GBA) in the dark at 21°C. For production of cutinolytic enzymes, spores (2×10^6) from 8-10 day old cultures were used to inoculate 150 ml of a liquid mineral

medium modified from Hankin and Kolattukudy (8) by the elimination of calcium pantothenate. Finely ground cucumber cutin (2-3 g/liter) was added as the carbon source.

Preparation of Crude Culture Filtrate.

Still liquid cultures of C. lagenarium, grown with cutin as the sole carbon source for 21-27 days (room temperature), were filtered through several layers of cheesecloth to remove the mycelia and remaining cutin. Vacuum filtration through Whatman filter paper #4 removed the remaining particulates. The filtrate was chilled to 4°C and the protein precipitated by the addition of acetone (-20°C) to a final concentration of 50%. The precipitate was collected by vacuum filtration through Whatman filter paper #4, resuspended in distilled water dH_2O, dialyzed and lyophilized. The lyophilysate was resuspended in distilled water and dialyzed against 0.05M Tris-HCl buffer pH 8.9, in preparation for the first step in purification.

Protein Determination.

Protein concentration was determined by the method of Bradford (3) using a commercial preparation of the reagents and bovine albumin serum (BSA) as the standard.

Esterase Assay.

The general esterase assay utilizing p-nitrophenyl butyrate (PNB) as the substrate, was a modification of the method of Kolattukudy et al (11). This reaction mixture contained:

0.1-0.4 ug of protein

0.1 ml Triton X-100 (stock solution- 4mg/ml

0.01 ml PNB (stock solution-0.264 ml PNB per 15 ml acetonitrile)

in a total volume of 1.5 ml of 0.05M sodium phosphate buffer, pH 7.0, unless mentioned otherwise. Activity was followed spectrophotometrically at an absorbance of 405 nm. Specific activity was calculated using published extinction coefficient values for p-nitrophenyl (2).

Preparation of Radiolabelled Cutin.

Purified cucumber fruit cutin (chapter 2) was labelled with 100 mCi sodium borotritiide ($NaB[^3H]H_4$) by Amersham Corporation following the methods of Koller et al (13). The tritiated cutin was diluted 1:60 (w/w) with unlabelled cutin to give a final specific activity of 10^6 DPM/mg.

Cutinase Assay.

The following assay is a modification of a method of Kolattukudy et al. (11). The reaction mixture contained:

10-50 ug of enzyme

0.047ml of 2.0% thimerosal

0.025 ml of 1.0% Triton X-100

4.5 mg of [^{3}H]-cutin (approximately 10^6 DPM/mg) in a total volume of 1.0 ml. The reaction was carried out with a final concentration of 0.05M Tris-HCl pH 8.9, unless otherwise stated. The reaction mixture was incubated for 8.0 hours at room temperature without shaking. Termination of the reaction was accomplished by the addition of 2 drops of 6M HCl followed by filtration through glass wool to remove the particulate cutin. The filtrate was then extracted with ethyl acetate (1.5 ml, 2 times). The ethyl acetate was removed by air-drying and scintillant added (10 ml). Counting was done in a Beckman model LS 6800 scintillation counter.

Lipase Assay.

Lipase activity was determined by the method of Fukumoto et al. (7) using tributyrin and olive oil as substrates.

Purification.

Anion Exchange Chromatography (DEAE-Cellulose).

DEAE-Cellulose (1g Sephadex G-25 added per 3g DEAE-Cellulose) column (19 × 0.5 cm) was equilibrated in 0.05M Tris-HCl buffer, pH 8.9. The crude culture filtrate, previously dialyzed against the same buffer, was loaded onto the column. Protein was eluted with a linear gradient of 0.0-0.2M NaCl in a total volume of 100 ml.

The elution procedures were later revised to 0.0-0.1M in a total of 40 ml since the protein of interest was found to eluted at approximately 0.05M NaCl. Fractions (3.75ml) were collected and tested for PNB hydrolase activity and combined groups of fractions were tested for cutinolytic activity.

Hydrophobic Interaction Chromatography (Octyl Sepharose CL-4B) - Stepwise Gradient.

Fractions from the DEAE-Cellulose column exhibiting PNB hydrolase activity and cutinolytic activity were combined and dialyzed against 25% ammonium sulfate in 0.05M sodium phosphate buffer, pH 7.0. The dialyzed sample was then loaded onto an octyl sepharose CL-4B column (10 × 0.5 cm) equilibrated in the same buffer without the ammonium sulfate. Protein was eluted in a stepwise fashion beginning with a wash of 25% ammonium sulfate in 0.05M sodium phosphate buffer pH 7.0 (20 ml), followed by a wash with 10% ammonium sulfate in 0.05M phosphate buffer pH 7.0 (20 ml), then distilled water (20 ml) and a final wash with 25% ethylene glycol (20 ml). Fractions were collected (1.5ml) and tested for PNB hydrolase activity and combined groups of fractions were checked for both cutinolytic and PNB hydrolase activities.

Hydrophobic Interaction Chromatography (Octyl Sepharose CL-4B) - Linear Gradient.

Fractions from the stepwise elution which contained activity were dialyzed against 10% ammonium sulphate in 0.05M sodium phosphate buffer pH 7.0 and loaded onto an Octyl Sepharose column (10x0.5cm) equilibrated in the same buffer with out the ammonium sulfate. Protein was eluted with a linear gradient from 10% ammonium sulfate to 25% ethylene glycol (in a total of 30-40 ml) and 1.5 ml fractions were collected. Fractions were checked for PNB hydrolase activity and SDS-PAGE was used to determine the purity of each fraction exhibiting this activity. Fractions were then combined according to their relative purity and then checked for cutinolytic activity.

Partial Purification of 'Void' Fraction.

The protein fraction which did not bind to the DEAE cellulose column was collected, lyophilized, resuspended and dialyzed in 0.05M sodium citrate buffer, pH 4.0. SP-sephadex, a cation exchanger, equilibrated in the same buffer, was then used for batch separation of the 'void' fraction. After extensive washing with buffer, the protein was eluted from the exchanger with 0.5M NaCl. The eluted fraction was dialyzed extensively against dH_2O and lyophilized.

Preparation of the Crude Matrix Protein.
C. lagenarium was grown on PDA or GBA for 8-10 days at which time the plates were washed with dH_2O. The wash water was collected ,combined and the spores removed by centrifugation (10,000 g, Sorvall RC-5C, 4°C). The supernatant was dialyzed against dH_2O, lyophilized, resuspended and dialyzed again in dH_2O.

Concanavalin A Sepharose 4B (Con A Sepharose) Column.
Con A Sepharose (2ml) was equilibrated in 0.02M Tris-HCl buffer, pH 7.4, with 0.5M NaCl in a column, 5 × 0.5cm. Purified protein was added and the column was rinsed in the same buffer. Cutinase was eluted with 0.3M methyl α-D mannopyranoside in the same buffer. Fractions (1.0ml) were collected and activity was followed with the PNB hydrolase assay.

Polyacrylamide Gel Electrophoresis (PAGE).
Sodium Dodecyl Sulfate PAGE. 10% polyacrylamide slab gels (pH 8.8, 1.5 mm thick) were prepared according to the method of Laemmli (19).

Silver Stain for Proteins in Polyacrylamide Gels.
This procedure was modified from that of Morrissey (22). The gel was first placed overnight in a fixative (50% methanol, 10% acetic acid). After extensive rinsing in several changes of dH_2O for one hour, the gel was placed

in a solution of dithiothreitol (5mg/ml) for one hour. The gel was then incubated for one hour in 0.1% silver nitrate. The protein bands were visualized by placing the gel in 500 ml of 3.0% sodium carbonate with 0.1 ml formaldehyde. The reaction was terminated with the addition of 12 g citric acid per 500ml.

Amino Acid Analysis.

Determination of amino acid composition was carried out by the Macromolecular Structure Facility, Dept. of Biochemistry, Michigan State University. Separation was by HPLC (Waters Associates) on a Vydac C-18 column. The protein was eluted with a linear gradient (0-90min) from 0-90% acetonitrile:isopropyl alcohol:dH_2O (60:20:20 v/v/v) balanced with 0.1% trichloroacetic acid. A Beckman 890 M spinning cup sequencer was used for sequence analysis. Standard Edman degradation techniques were utilized (6N HCl,105-110°C) for determination of the amino acid composition. Data presented are based on a 24 hr hydrolysis. Amino acids were identified by HPLC using protein hydrolysate standards-Type H (Pierce).

Mutagenesis Procedure.

A spore suspension of 1.75×10^8 spores/ml were exposed to 1.0% ethyl methane sulfonate in 0.05M sodium phosphate buffer, pH 7.0 for 1.75 hr resulting in 99% kill (as calculated from a kill curve). The reaction was

terminated by a 100 fold dilution with sterile dH_2O. The spore suspension was plated onto PDA containing 0.0001% Terra Coat L-205 (included for maintenance of discrete colonies) and incubated at 21°C for 6 days. Surviving colonies were transferred to PDA plates (without Terra Coat L-205). The putative mutants were screened for growth on cutin as the sole carbon source, PNB hydrolase activity, pathogenicity and cutinolytic activity.

Pathogenicity Tests.

The surface of cotyledons of 8-10 day old cucumber plants (Cucumis sativus L.)were inoculated with 6-8 droplets (approximately 50 ul/droplet) of a 10^6 spores/ml suspension of C. lagenarium. The inoculated plants were covered with plastic bags for 24 hrs to maintain high humidity. When the cuticle was to be bypassed, a spore suspension of 10^6 spores/ml were infiltrated into the cotyledons with a syringe. Disease ratings were taken every 24 hrs beginning 48 hrs after inoculation. Isolates unable to cause disease when inoculated onto intact tissue were tested for pathogenicity by infiltration into the tissue.

Disease Rating System.

Numbers 0 to 6 were utilized in a disease rating system to indicate levels of disease observed on the cucumber cotyledons. This rating system is described in Figure 3.1.

0 = no symptoms

1 = barely visible water soaking at a few of the inoculation points (25%)

2 = water soaking at more inoculation points (50%)

3 = water soaking quite apparent at every inoculation point (100%)

4 = well developed lesions with browning beginning

5 = extensive browning of lesions but lesions still distinct from one another, some leaf curling

6 = lesions coalescing

Figure 3.1. Disease rating system.

Determination of Percent Penetration.

Cucumber hypocotyl segments, 2.0-2.5 cm in length from 4-6 day old etiolated seedlings, were surface inoculated with 4-8 droplets (approximately 50 ul/droplet)of 10^6 spores/ml. Epidermal peels were taken at 48 and stored in 100% ethanol. Prior to microscopic examination the peels were rinsed in dH_2O and stained with 0.1% toluidine blue (in 0.1M sodium phosphate buffer, pH 6.8).

Inhibition Assays with Paraoxon and Phenyl Boronate.

Stock solutions of 2.0mM and 2.0uM of paraoxon (O,O-diethyl-O-p-nitrophenylphosphate) and 0.01M and 0.1M of phenyl boronic acid were prepared in dH_2O and stored at 4°C. Appropriate dilutions were made of these stocks for each assay. Procedures for the enzyme assays and penetration and pathogenicity studies were identical to those previously described except that the spores for the pathogenicity tests were suspended in 0.05M sodium phosphate buffer, pH7.0 rather than dH_2O.

RESULTS

Tritium Labelling of Cucumber Fruit Cutin with $NaB[^3H]H_4$.

Previous work (Chapter 2) showed the presence of reducible carbonyl functions in the cucumber fruit cutin. This made possible the use of $NaB[^3H]H_4$ as a method for incorporation of tritium label into the cucumber cutin

(13) and thus the use of the cutin as a substrate in cutinolytic enzyme assays.

Associated with this assay was a slow non-enzymatic release of tritium into the assay fluid resulting in a very high background level of tritium counts (4000-6000dpm by 8hr). This created a significant problem due to the relatively low level of cutinolytic activity associated with Colletotrichum lagenarium. As a result it was not reliable to quantitatively compare different sets of assays. However, qualitative comparisons were possible, as well as quantitative comparisons within a set of assays. The problem with background was greatly reduced at higher levels of enzyme activity.

The specific activity of the crude culture filtrate prepared from cultures of C. lagenarium, grown on cutin as the sole carbon source, was compared to that grown on both cutin and sucrose (Table 3.1). Nearly six times more PNB hydrolase activity was observed in the cultures in which cutin served as the sole carbon source. In comparing the activity of crude culture filtrate from C. lagenarium to that from Fusarium solani f. sp. pisi (a pathogen known to produce cutinase with high specific activity [1,4]) (Table 3.2), C. lagenarium was observed to contain approximately 90 times less PNB hydrolase activity and 7 times less cutinolytic activity than that observed for F. solani f. sp. pisi.

Table 3.1

Comparison of PNB hydrolase activity of crude culture filtrate from C. lagenarium grown on sucrose and cutin (as the carbon source) to that grown on cutin alone.

	PNB hydrolase (ΔOD405/min·mg)
Cutin and sucrose	3.92
Cutin	22.50

Table 3.2

Comparison of PNB hydrolase and cutinolytic activity in the crude culture filtrate from C. lagenarium and F. solani f. sp. pisi grown on cutin as the sole carbon source.

	PNB hydrolase ΔOD405/min·mg	cutinolytic DPM/8hr·mg (x10⁴)
C. lagenarium	7.4	13.9
F. solani f. sp. pisi	780.6	104.0

No general lipase activity was detected in the crude culture filtrate utilizing tributyrin and olive oil as substrates.

Purification of Cutinase-a.

The scheme for purification of cutinolytic enzymes from Colletotrichum lagenarium is outlined in Figure 3.2. The crude culture filtrate was prepared as previously described. The first step in purification was application of the crude culture filtrate to a DEAE column equilibrated in 0.05M Tris-HCl buffer pH8.9. The protein was eluted with a gradient of 0.0-0.2M NaCl in the same buffer. The results are illustrated in Figure 3.3. Enzyme activity of each fraction was followed with a general esterase assay, utilizing p-nitrophenyl butyrate (PNB) as the substrate. Groups of fractions were then tested for their cutinolytic activity with tritiated cutin as the substrate. Although the majority of the protein (approximately 75%) bound to the column, approximately 25% came through with the void volume. Although the protein in the 'void' fraction did not exhibit significant PNB hydrolase activity, it did contain significant cutinolytic activity. Because of the difficulty asssociated with following the enzyme without a general esterase assay, the protein in the 'void' fraction was put aside for later investigations. The major peak of PNB hydrolase activity eluted at about 0.05M NaCl. This peak

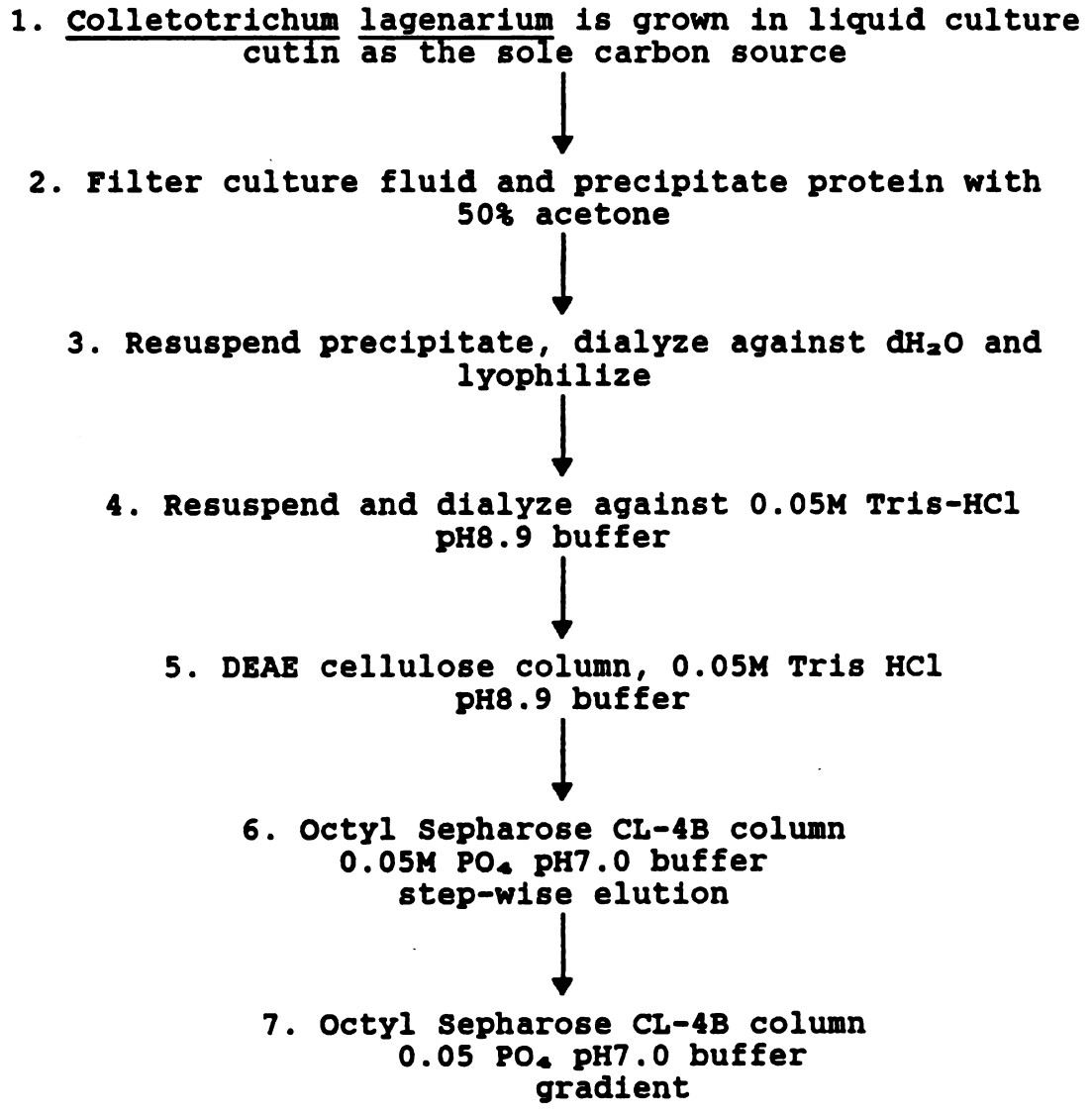

Figure 3.2. Outline for purification of cutinase-a from Colletotrichum lagenarium.

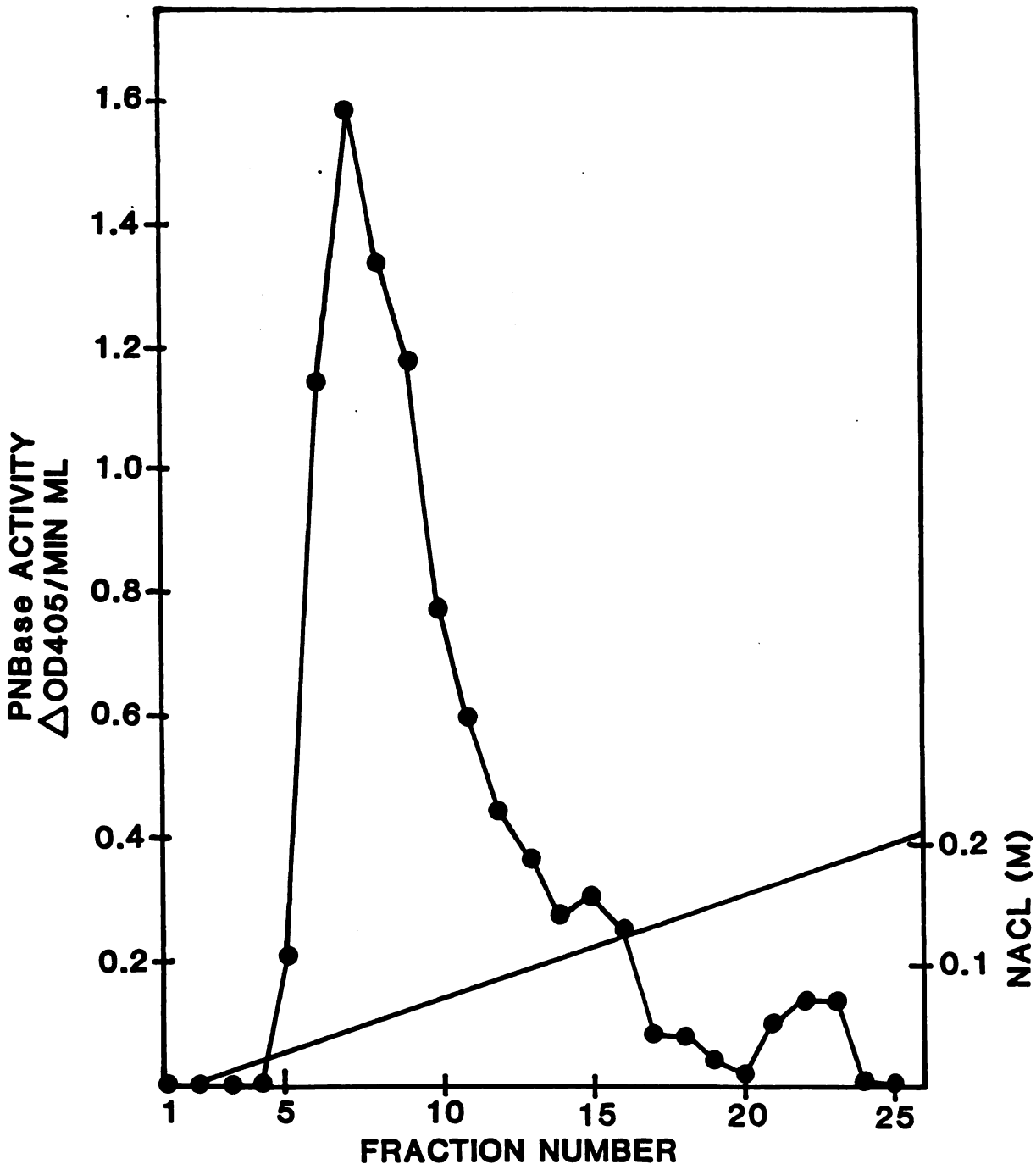

Figure 3.3. Elution profile from the DEAE cellulose column.

also contained fractions with the highest specific activity in terms of the tritiated cutin substrate. The fractions within the peak were collected and dialyzed against 25% ammonium sulphate [$(NH_4)_2SO_4$] in sodium phosphate buffer, pH7.0, in preparation for the first octyl sepharose column.

Following an initial wash with 25% $(NH_4)_2SO_4$ in buffer, a step-wise elution was carried out with the first octyl-sepharose column, starting with 10% $(NH_4)_2SO_4$, then dH_2O and finally 25% ethylene glycol(EG) in dH_2O. The elution profile of the protein and PNB hydrolase activity is shown in Figure 3.4. The bulk of the contaminating protein was removed with the 10% $(NH_4)_2SO_4$ and dH_2O steps. Table 3.3 shows the activity (both PNB hydrolase and cutinolytic) of the different groups of fractions collected from the step-wise elution of the octyl-sepharose column. Fractions with highest specific activity (both PNB hydrolase and cutinolytic) eluted with 25% EG and occasionally the end of the dH_2O wash. The dH_2O fraction contained a significant amount of cutinolytic and PNB hydrolase activity. When rerun on the octyl-sepharose column under the same conditions a similar profile was obtained with more hydrolase and cutinolytic activity appearing in the 25%EG wash. The fractions with the highest specific activity were collected and dialyzed against 10% $(NH_4)_2SO_4$ in 0.05M sodium phosphate buffer, pH7.0.

The final column was octyl-sepharose, equilibrated

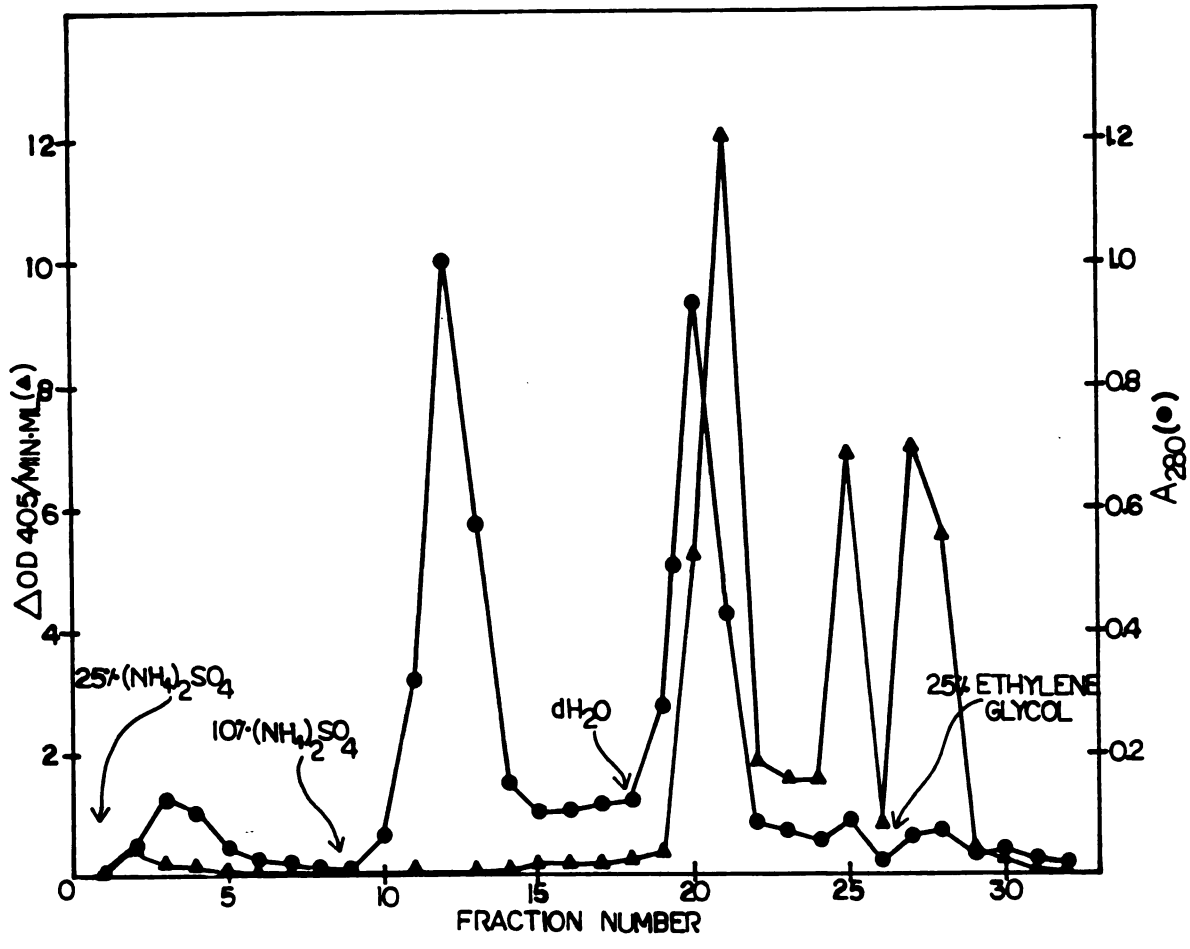

Figure 3.4. Elution profile from the step-wise elution of the Octyl Sepharose column.

Table 3.3

Groups of fractions from the step-wise elution of the Octyl Sepharose column.

Fraction #	1-9(a)	10-18(b)	19-25(c)	26-32(d)
PNB hydrolase activity(e)	0	0	34.9	170.4
Cutinase activity(f)	42.1	77.9	129.1	294.5

[a]Eluted with 25% $(NH_4)_2SO_4$
[b]Eluted with 10% $(NH_4)_2SO_4$
[c]Eluted with dH_2O
[d]Eluted with 25% ethylene glycol
[e]Expressed as ΔOD/min·mg
[f]Expressed as DPM/mg·8hr ($\times 10^3$)

under the same conditions as before except that a linear gradient was utilized from 10% $(NH_4)_2SO_4$ to 25% EG. The protein was applied and the column washed with 10% $(NH_4)_2SO_4$. Following the gradient was a wash with 25% EG. The elution profile is illustrated in Figure 3.5. The majority of the remaining protein contaminants were removed with the gradient. Monitoring protein content in fractions 27-40 by SDS-PAGE demonstrated that a single protein eluted with the final 25% EG wash (Figure 3.6). This protein is shown again in Figure 3.7, along with molecular weight standards (STDS). The calculated molecular weight is approximately 60kd. For ease of discussion this protein has been labelled 'cutinase-a'.

A summary of the specific activities, for both the PNB and tritiated cutin substrates, for each step in the purification of cutinase-a is given in Table 3.4. A steady increase in specific activity is observed with the PNB substrate in each step of the purification of cutinase-a. However, this is true for the tritiated cutin sustrate only after an initial drop in activity following passage of the crude culture filtrate through the first column (DEAE cellulose). This was postulated to be the result of the presence of cutinolytically active proteins present in the crude culture filtrate which do not exhibit PNB hydrolase activity and do not bind to the DEAE cellulose column. Evidence to support this is shown in Table 3.5.

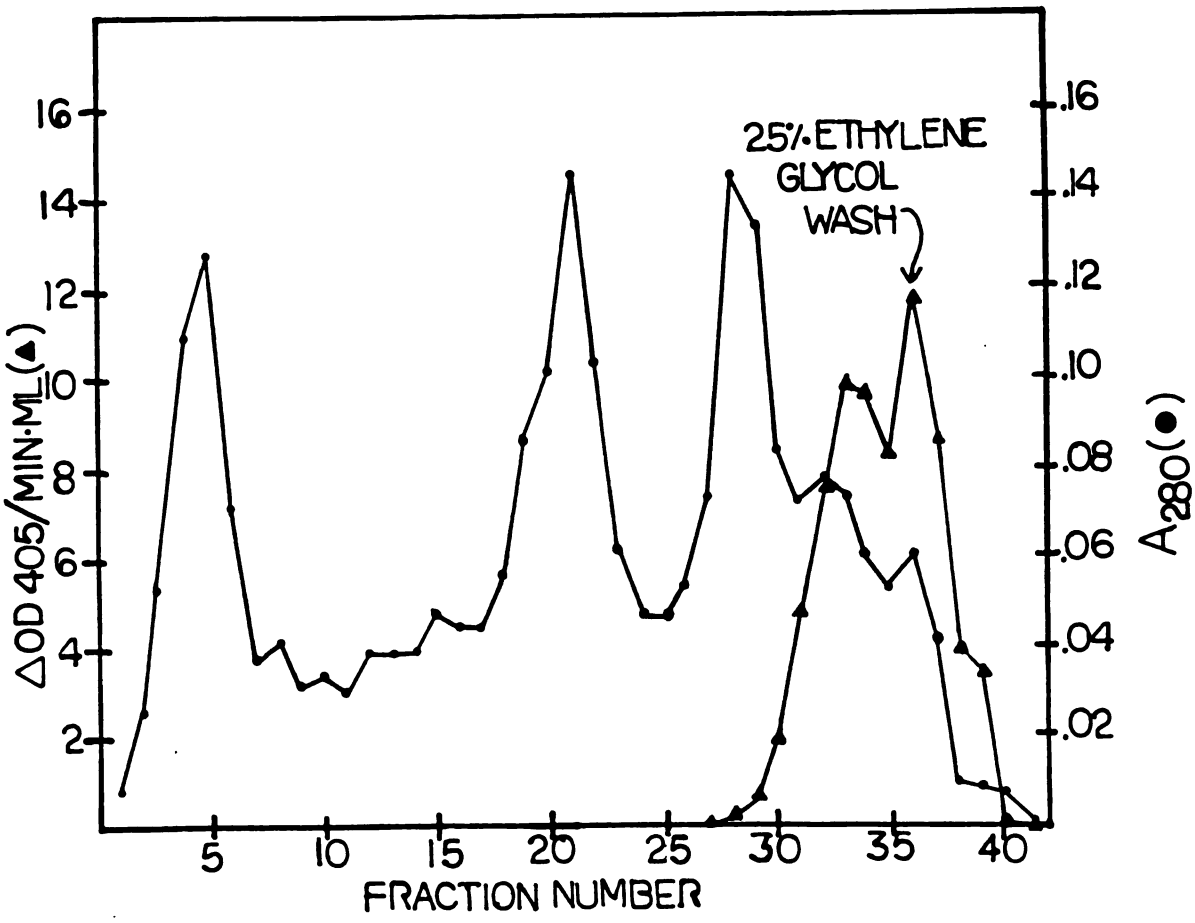

Figure 3.5. Elution profile from the linear gradient of the Octyl Sepharose column.

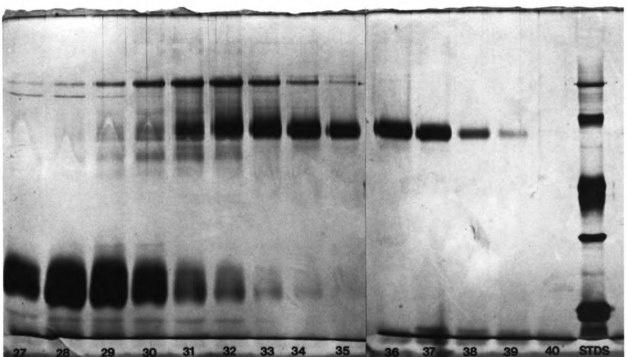

Figure 3.6. SDS-PAGE of fractions 27-40 from the linear gradient of the Octyl Sepharose column (see Figure 3.5).

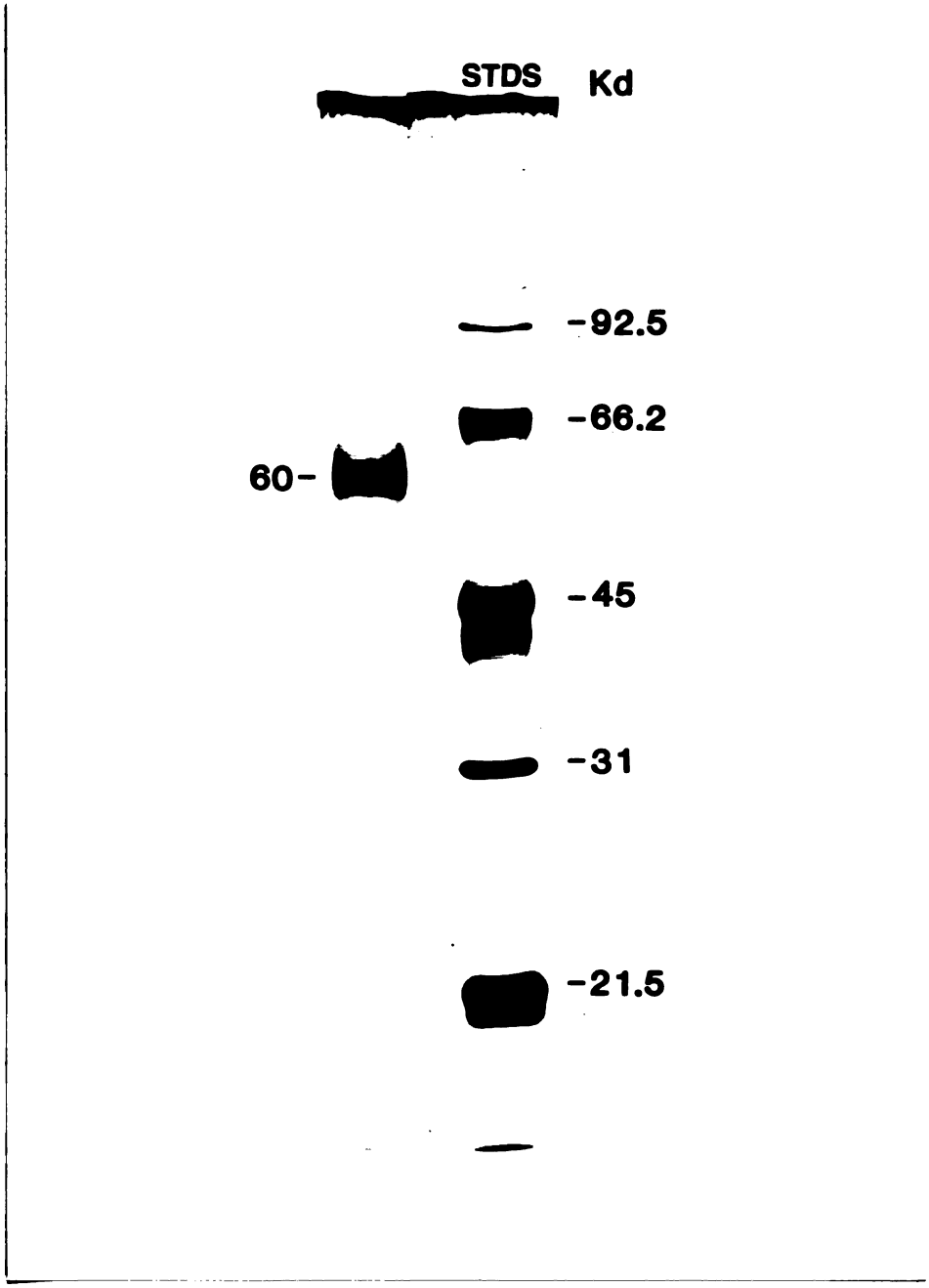

Figure 3.7. SDS-PAGE of purified cutinase-a and molecular weight standards.

Table 3.4.

Purification table. Specific activities of the collected fractions from each step in the purification of cutinase-a.

	Specific Activity	
	PNB hydrolase(a)	cutinolytic(b)
Crude Culture Filtrate	13.1	162.5
DEAE Cellulose	32.5	42.1
Octyl Sepharose Step-wise elution	185.0	74.6
Octyl Sepharose Gradient	548.6	335.4

[a]Expressed as ΔOD/min·mg
[b]Expressed as DPM/8hr·mg ($\times 10^3$)

Table 3.5

Specific activities of two fractions from the DEAE cellulose column.

DEAE cellulose	Specific Activity	
	PNB hydrolase	Cutinolytic
	ΔOD405/min·mg	DPM/8hr·mg ($\times 10^3$)
'Void'	0.82	125
'Bound'	10.90	98

Two fractions from the DEAE cellulose column are shown, that which does not bind under the conditions used to obtain cutinase-a (termed 'void') and that which does bind (termed 'bound') and from which cutinase-a is obtained. Both fractions exhibit high levels of cutin degrading activity, but only the fraction which binds to the DEAE cellulose contains significant PNB hydrolase activity. The low level of hydrolase activity associated with the 'void' fraction can be further reduced by passage through a cation exchanger.

Characterization of Cutinase a from C. lagenarium. Cutinase-a was shown to be a glycoprotein by its ability to bind to Con A Sepharose and by its elution from this column material with methyl α-D mannopyranoside.

The amino acid composition of cutinase-a was determined and is given in Table 3.6 along with the amino acid composition for three other cutinases; one from Colletotrichum gloeosporioides and two from Fusarium solani f. sp. pisi. No striking similarities were observed between the enzymes from different systems.

Effect of pH and Substrate.

The optimum pH for PNB hydrolase activity was shown to be 6.0 for both the crude culture filtrate and purified cutinase-a (Figure 3.8). The optimum pH for the tritiated cutin substrate for both cutinase-a and the crude culture filtrate was determined to be 9.0 (Figure 3.9).

The effect of substrate concentration on the PNB hydrolase activity of cutinase-a is shown in Figure 3.10. Substrate saturation occurred at approximately 1.0mM PNB. The V_{max}=46.7 uM/min·mg protein and the K_M=0.16mM as determined by Lineweaver-Berke plots. The rate of hydrolysis of PNB increased linearly in response to an increasing protein concentration.

Determination of the Presence of a Serine Active Residue.

Cutinases characterized thus far contain a catalytic triad having one active serine residue, one histidine

Table 3.6

Amino acid compositions of cutinase-a from C. lagenarium, cutinase from Colletotrichum gloeosporioides, and cutinase I and II from Fusarium solani f. sp. pisi.

	Mole %			
Amino Acid	Cutinase-a	C. gloeosporioides(a)	Cutinase I(b)	Cutinase II(b)
ASX	7.79	8.00	11.10	11.06
GLX	8.34	7.55	6.06	6.25
HIS	0.76	0.44	1.01	0.96
ARG	2.01	4.44	6.56	6.25
LYS	3.65	4.00	3.03	2.88
ALA	14.48	13.77	14.14	13.46
VAL	6.32	4.88	4.04	3.36
LEU	6.34	10.22	9.59	9.61
ILE	3.73	6.22	7.07	6.25
PRO	7.41	5.77	4.54	4.80
PHE	2.56	4.88	3.53	3.36
MET	0.41	0.88	0.51	0.48
GLY	15.93	10.22	12.62	13.90
SER	12.53	7.55	6.06	6.73
THR	6.26	6.66	6.56	8.17
TYR	1.47	4.88	3.53	2.48

[a]Recalculated from previously published data (24)
[b]Recalculated from previously published data (4)

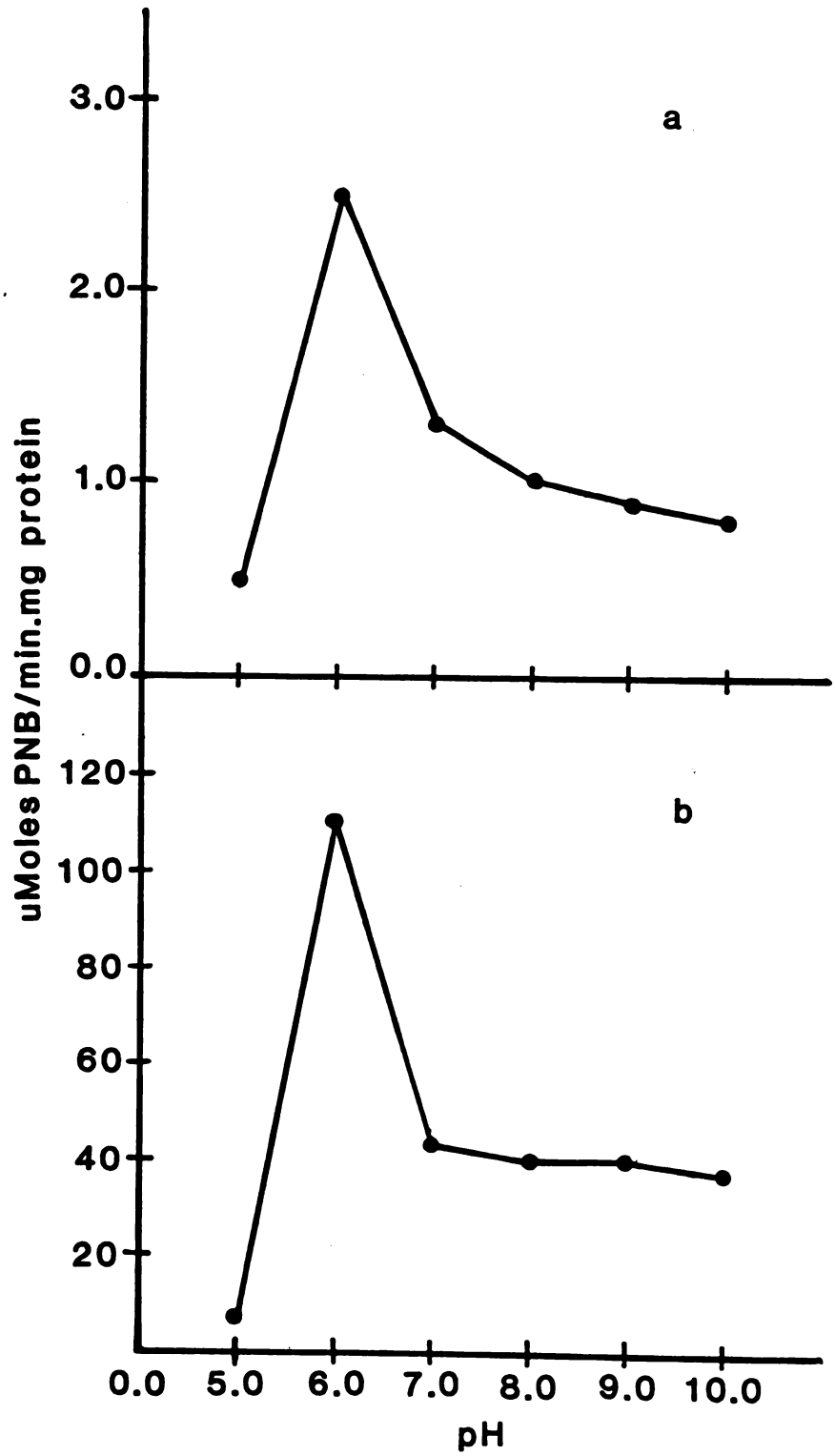

Figure 3.8. Effect of pH on the PNB hydrolase activity of a) crude culture filtrate and b) cutinase-a.

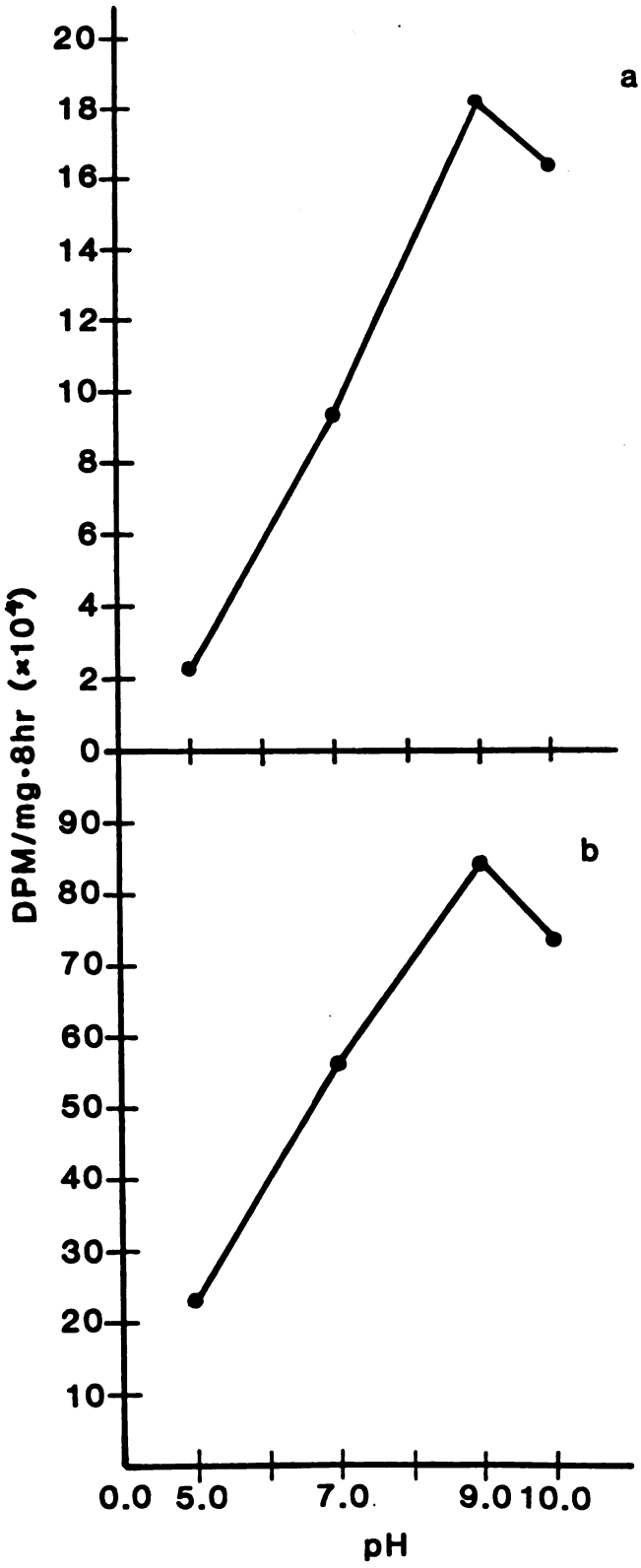

Figure 3.9. The effect of pH on the cutinolytic activity of a) crude culture filtrate and b) cutinase-a.

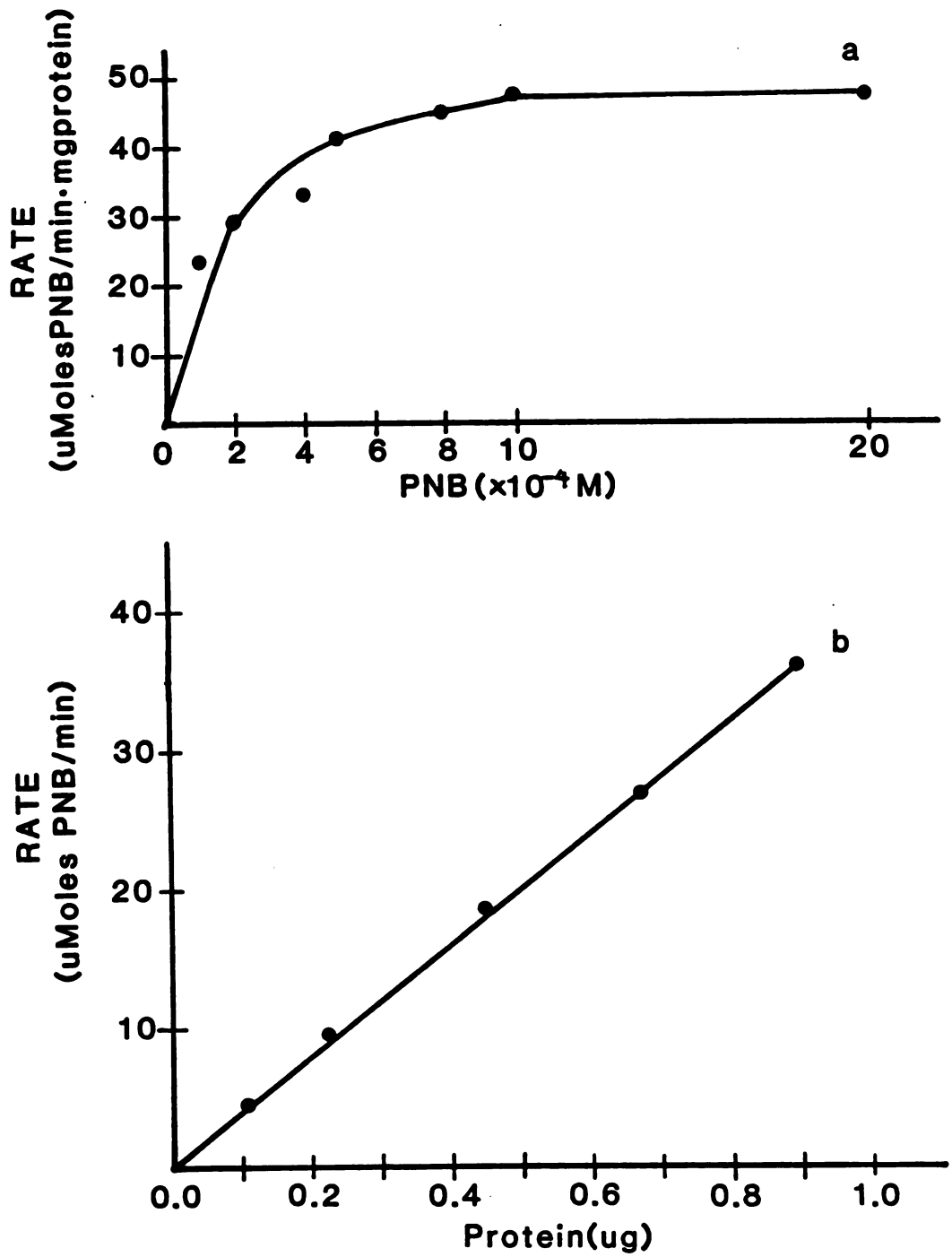

Figure 3.10. The effect of a) substrate concentration (0.3ug/ml protein) and b) protein concentration (1.0mM PNB) on the rate of PNB hydrolysis by cutinase-a (K_M=0.16mM; V_{MAX}=46.7 uM/min·mg protein).

residue and one carboxyl, all of which are essential in enzyme catalysis (12). The presence of this catalytic triad places cutinases into a category of enzymes termed serine hydrolases or serine esterases (10). The activity of serine hydrolases is known to be severely inhibited in the presence of organophosphate compounds (14,15) and organic boronic acid derivatives, both of which are reported to act specifically at the active serine residue. Both types of compounds (12,14) have been shown to be inhibitory to cutinases (4,12,14). In order to determine the presence of an active serine residue both crude culture filtrate and cutinase-a hydrolase and cutinolytic activities were observed in the presence of paraoxon, an organophosphate, and phenyl boronate, an organic boronic acid derivative.

Paraoxon was found to severely inhibit the PNB hydrolase activity of both the crude culture filtrate and cutinase-a (Figure 3.11a,b), reducing the hydrolytic activity of the purified protein to 0.0 with a concentration of 0.2uM. An inhibition of 80% was observed for the crude preparation, at this same concentration of paraoxon. Cutinolytic activity was also reduced but less so as compared to the PNB hydrolase activity (Figure 3.11c,d). Again the activity of the purified protein was the most severely affected, with 70% inhibition (at 2.0uM paraoxon) as compared to 50% inhibition of the crude preparation. A concentration of 50.0uM paraoxon did not

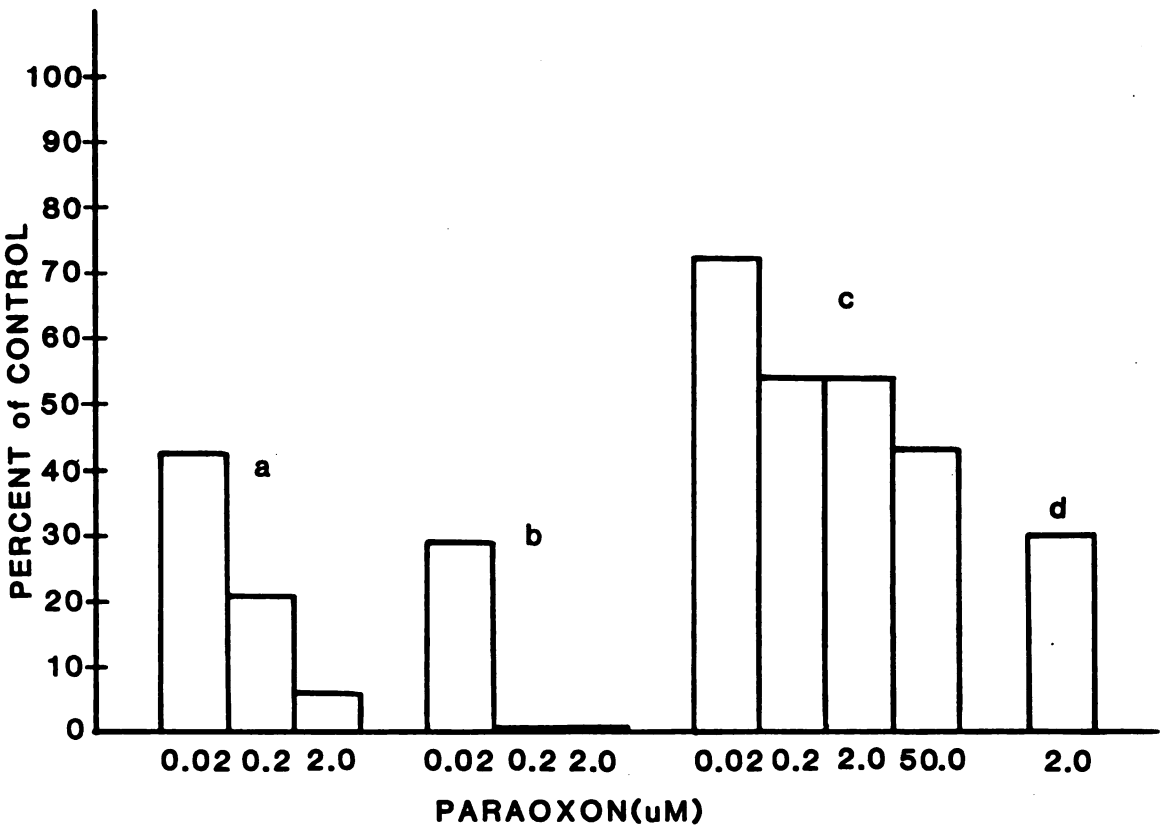

Figure 3.11. The effect of paraoxon on the PNB hydrolase (a and b) and cutinolytic activity (c and d) of the crude culture filtrate (a and c) and cutinase-a (b and d) from C. lagenarium.

significantly reduce the cutinolytic activity of the crude culture filtrate beyond that observed with 2.0uM.

At a concentration of 1.0mM, phenyl boronate was observed to inhibit the PNB hydrolase activity of the crude culture filtrate about 72% (Figure 3.12a) and the PNB hydrolase activity of cutinase-a about 66% (Figure 3.12b). The same concentration (1.0mM) of phenyl boronate inhibited cutin degrading activity of both cutinase-a and the crude culture filtrate 10-15% (Figure3.12c,d).

Inhihibitors and Pathogenicity of C. lagenarium.

The radial growth of C. lagenarium was unaffected by any of the concentrations of paraoxon used (0.02, 0.2 and 2.0uM)(Figure 3.13a). Paraoxon also had no effect on the ability of the fungus to cause disease (Figure 3.13b). At a concentration of 2.0uM symptom development was only slightly behind that observed for the control at 4 days (no paraoxon) and by six days was equal to the control. The ability of C. lagenarium to penetrate etiolated cucumber hypocotyls in the presence of paraoxon was also tested (Figure 3.13c). At the two highest concentrations, 0.2uM and 2.0uM, penetration was reduced by approximately 30%. The percentage of appressoria produced under each concentration (relative to the control) is represented by the hatched boxes in Figure 3.13c. Percent appressoria was utilized as control, in addition to radial growth, in order to determine the affect of the inhibitor on the

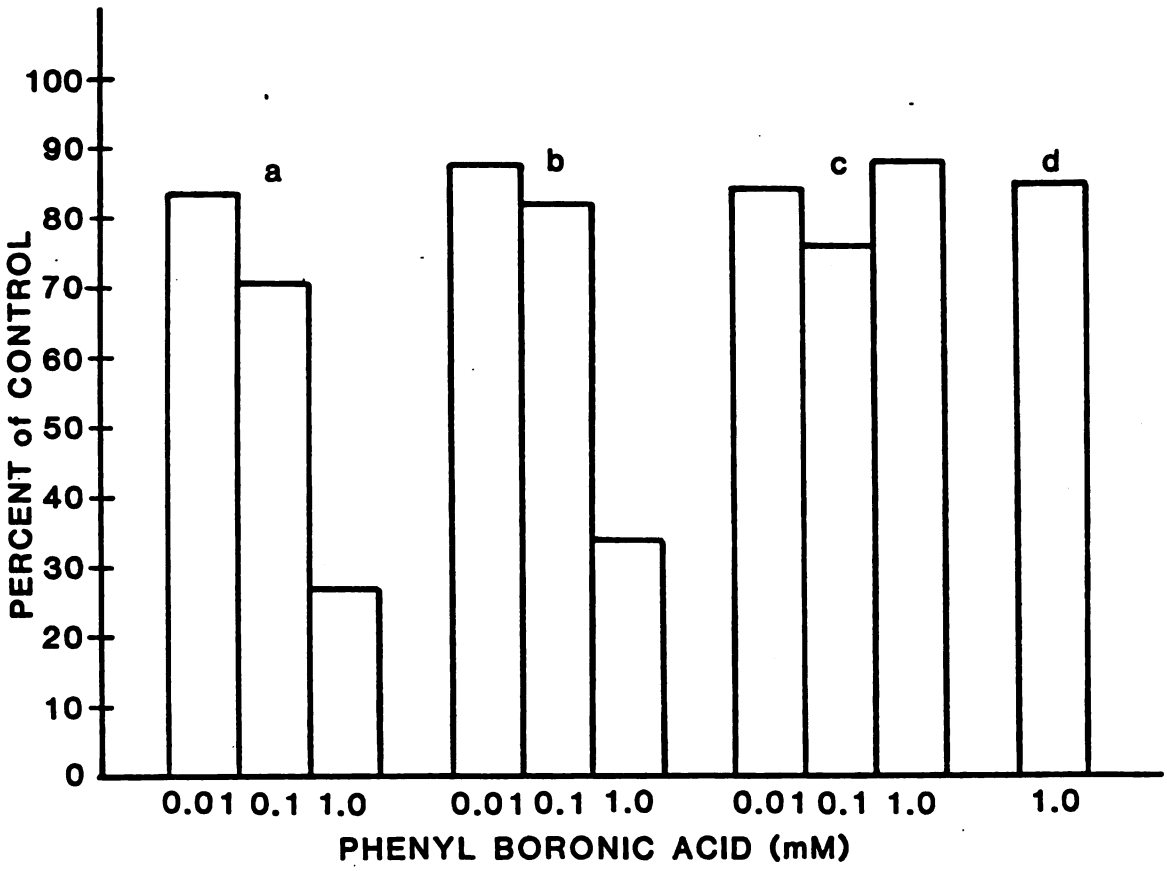

Figure 3.12. The effect of phenyl boronic acid on the PNB hydrolase (a and b) and cutinolytic activity (c and d) of the crude culture filtrate (a and c) and cutinase-a (b and d) from C. lagenarium.

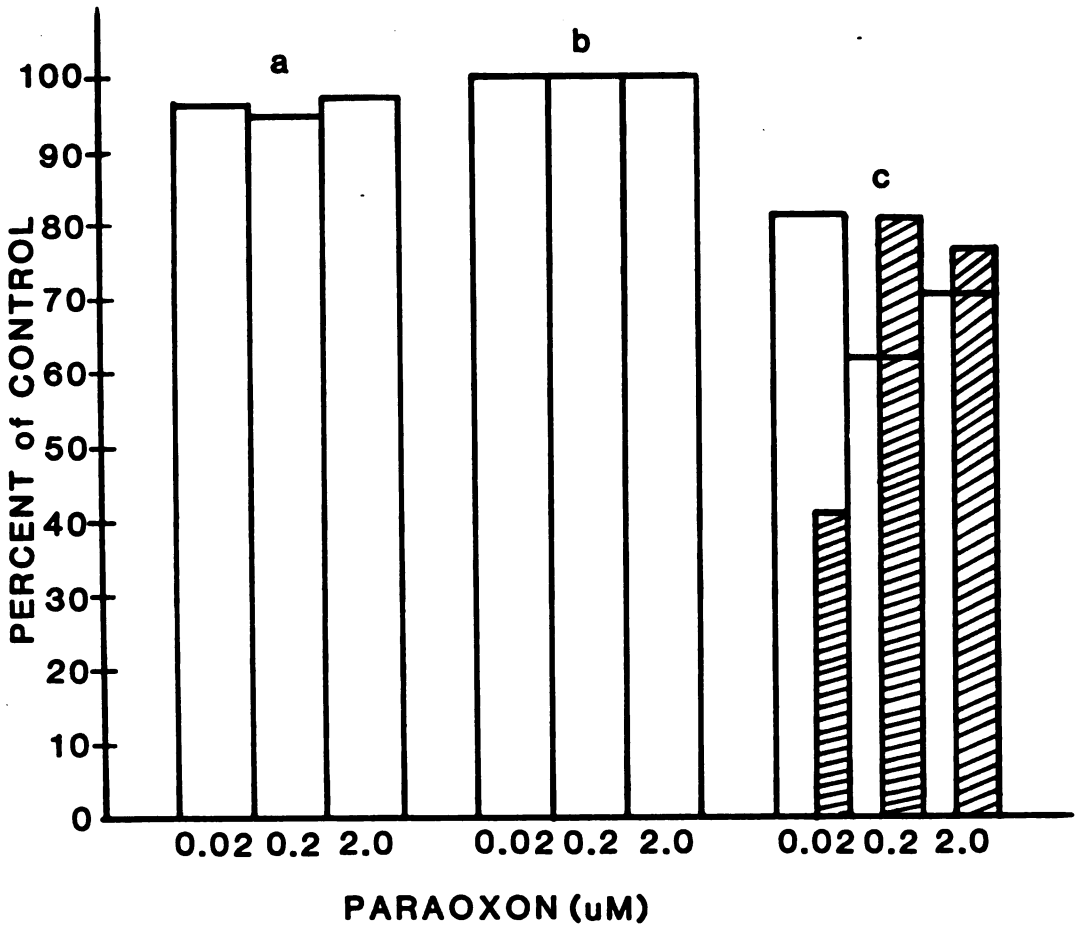

Figure 3.13. The effect of paraoxon on C. lagenarium: (a) radial growth on PDA, (b)infection of cucumber cotyledons, (c) penetration of etiolated cucumber hypocotyls (hatched boxes indicate percentage of appressoria produced).

general metabolism of the fungus. Although radial growth was unaffected the number of appressoria produced was significantly reduced. This result indicated that the inhibitor was not specific for the cutin degrading enzymes, and as such, any reduction in % penetration could not be taken as being strictly due to a loss in cutinolytic activity. The observation that the lowest concentration of paraoxon (0.02uM) resulted in the greatest reduction in number of appressoria produced was unexpected. The significance, if any, of this result is unknown.

Radial growth in the presence of phenyl boronate was increasingly reduced with increasing concentration of phenyl boronate (Figure 3.14a). At 1.0mM growth was reduced 80%. Growth was reduced 2.0% and 10% with 0.01 and 0.1mM, respectively. Infection of cucumber cotyledons (Figure 3.14b) was reduced by 40% with 0.1mM phenyl boronate and by 100.0% with 1.0mM. The percentage of penetrations by C. lagenarium of etiolated cucumber hypocotyls was also reduced in the presence of phenyl boronate (Figure 3.14c). At 1.0mM penetration was reduced by 60%. At the lower concentrations penetration was inhibited only 20%. The number of appressoria produced was reduced by more than 50% with 0.01mM and more than 80% with 0.1 and 1.0mM concentrations of phenyl boronate.

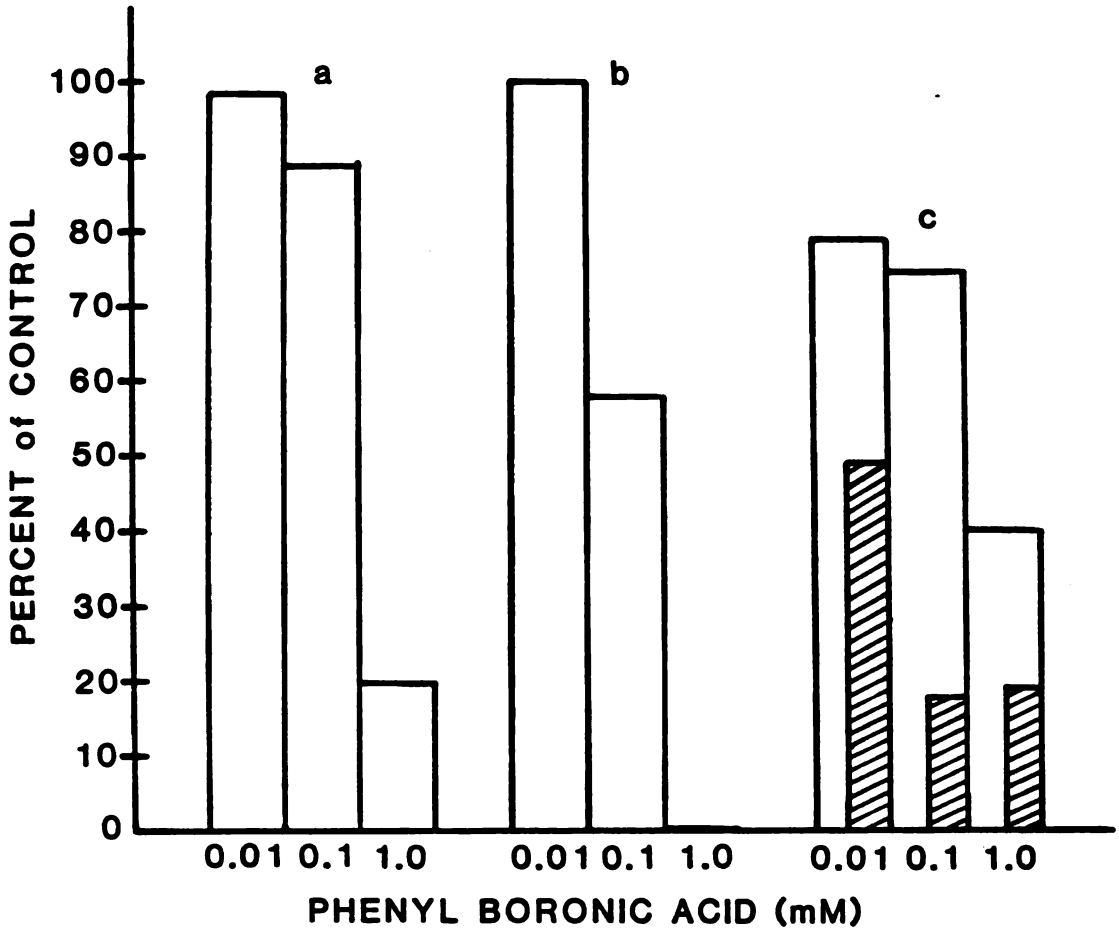

Figure 3.14. The effect of phenyl boronic acid on C. lagenarium: (a) radial growth on PDA, (b) infection of cucumber cotyledons, (c) penetration of etiolated cucumber hypocotyls (hatched boxes indicate the percentage of appressorria produced).

Other Cutin Degrading Enzymes from C. lagenarium. Cutinolytic activity was observed in several fractions in addition to that from which cutinase-a was obtained. One, already mentioned, is the void volume from the DEAE cellulose column. The other fraction, termed the matrix, is a water-soluble mucilagenous material produced in the acervuli during sporulation. The enzyme activities of these two fractions were characterized in crude or partially purified preparations.

The 'void' fraction did not exhibit general esterase activity, using PNB or p-nitrophenyl palmitate (PNP) as the substrate. Figure 3.15 illustrates the effect of pH on the cutinolytic activity of this fraction. Optimal activity with the tritiated cutin substrate occurred at a pH of 9.0. The two inhibitors, paraoxon and phenyl boronate, had less of an effect on the cutinolytic activity of the 'void' fraction as compared to cutinase-a. Phenyl boronate reduced activity by 10-15% maximum (Figure 3.16a) and paraoxon at 2.0uM, the highest concentration used, reduced activity almost 30% (Figure 3.16b).

The effect of pH on the PNB hydrolase and cutinolytic activities of the matrix is shown in Figure 3.17. Both activities were found to be optimal at a pH of 9.0. Paraoxon, at 2.0uM, reduced both the hydrolase activity and cutinase activity of the matrix to less than 20% of the control (Figure 3.18a,b). At the lower concentrations, 0.02 and 0.2uM, paraoxon had a slightly

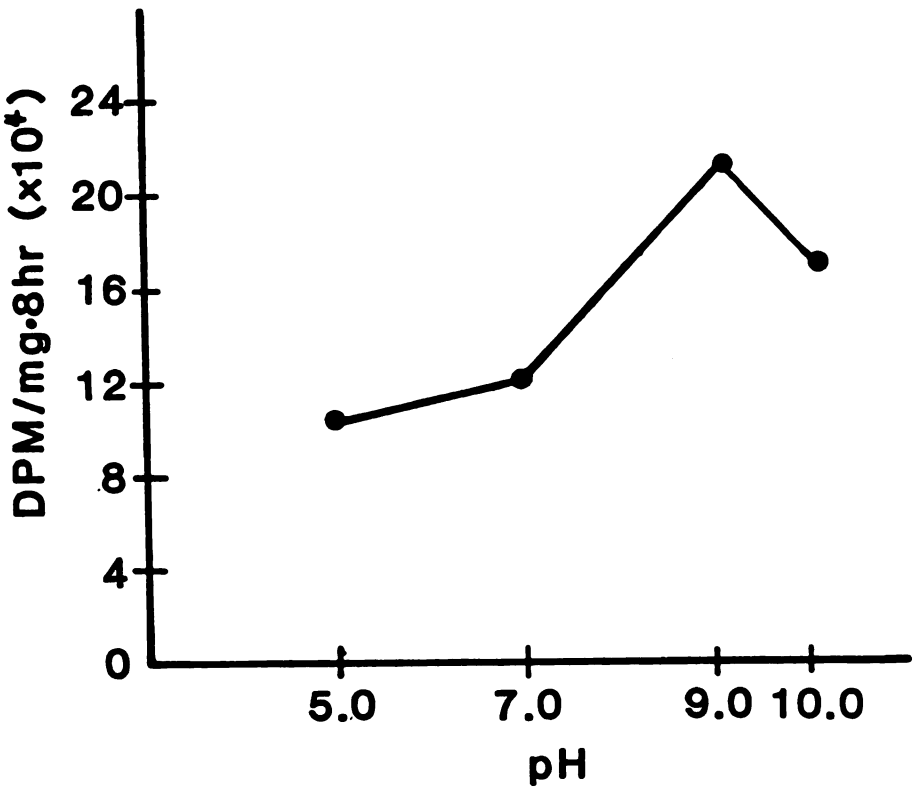

Figure 3.15. The effect of pH on the cutinolytic activity of the 'void' fraction from the DEAE cellulose column.

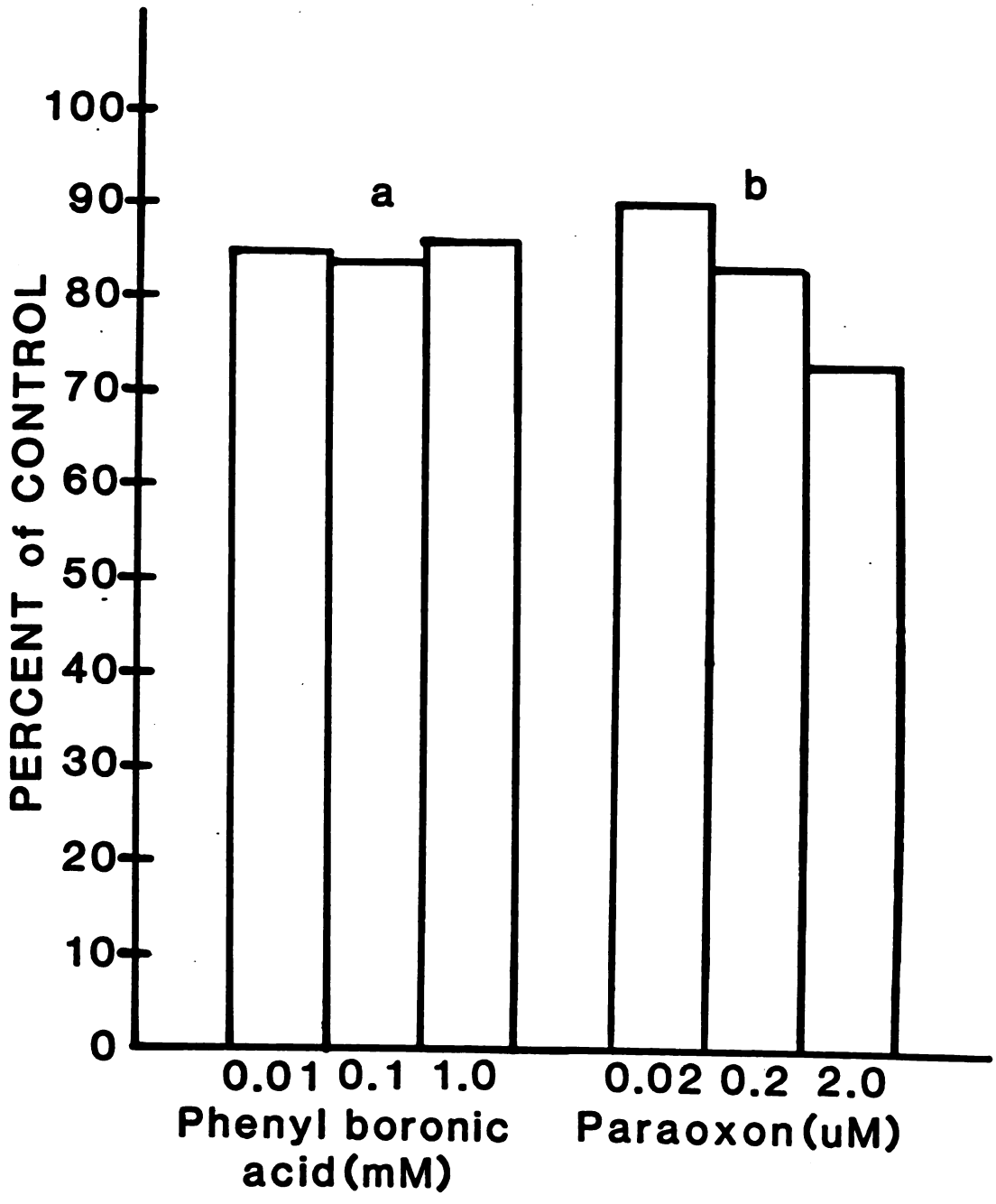

Figure 3.16. The effect of phenyl boronic acid (a) and paraoxon (b) on the cutinolytic activity of the 'void' fraction from the DEAE cellulose column.

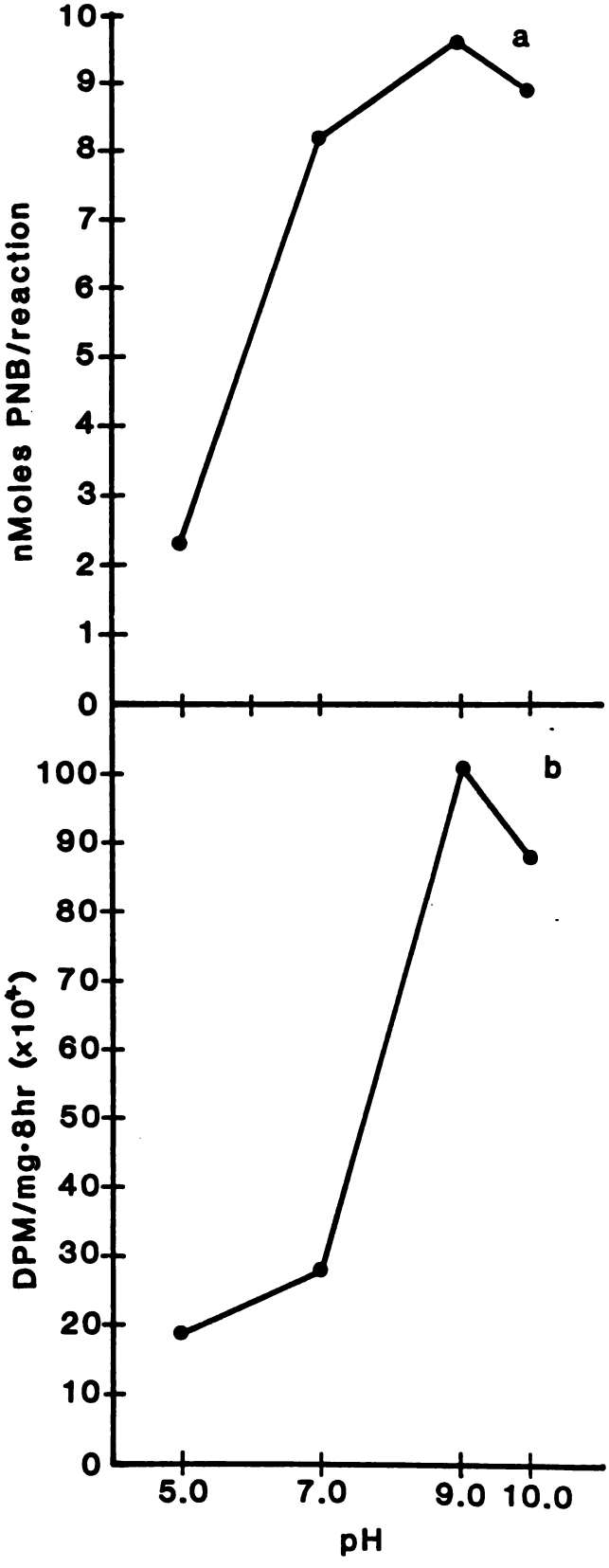

Figure 3.17. The effect of pH on the PNB hydrolase (a) and cutinolytic (b) activity of the crude matrix preparation.

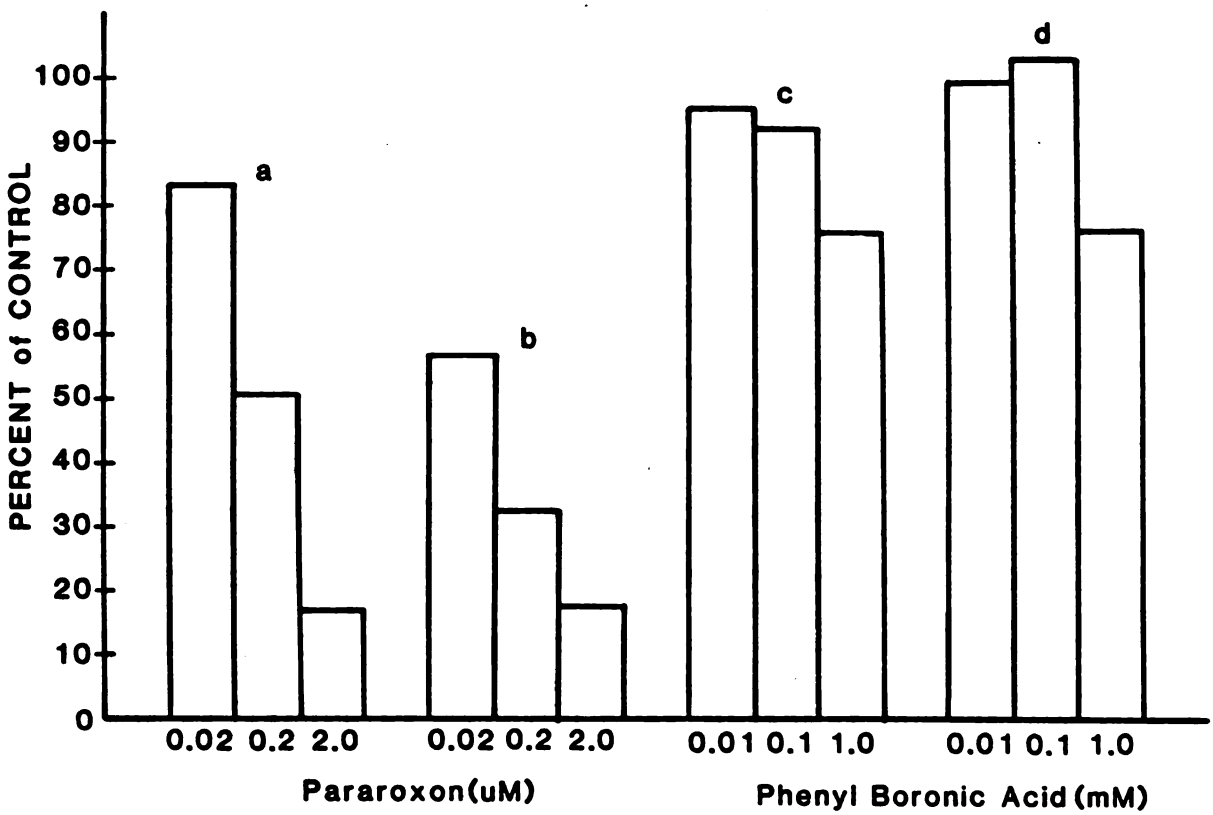

Figure 3.18. The effect of inhibitors on the enzyme activities of the crude matrix preparation from C. lagenarium; paraoxon and (a) PNB hydrolase activity, (b) cutinolytic activity, and phenyl boronic acid and (c) PNB hydrolase activity, (d) cutinolytic activity.

more inhibitory effect on the cutinolytic activity, as compared to hydrolase activity (Figure 3.18a,b) Phenyl boronate inhibited both the PNB hydrolase and cutinase activities by 25% at a concentration of 1.0mM (Figure 3.18c,d). Neither activity was inhibited by phenyl boronate at the lower concentrations.

Generation and Characterization of Mutants of C. lagenarium.

Mutants of C. lagenarium were generated in attempts to help define the role of cutinolytic enzymes in the penetration of cucumber by this fungus. The mutagenesis procedure is outlined in Figure 3.19. Following exposure to ethyl methane sulfonate (EMS) the spores were plated onto PDA plates containing 0.0001% Terra Coat L-250. Addition of this chemical increased the ease of single colony isolation by causing more discrete colony formation than PDA alone. The active ingredient is believed to be pentachloronitrobenzene (PCNB). Terra Coat L-250 was determined not to reduce the rate of germination of C. lagenarium. The first screens utilized were growth on cutin (in liquid culture medium) as the sole carbon source and levels of PNB hydrolase activity per ml of culture fluid. Individuals exhibiting either increased or decreased levels of hydrolase activity or growth on the cutin substrate, relative to the wild-type, were selected for further screening.

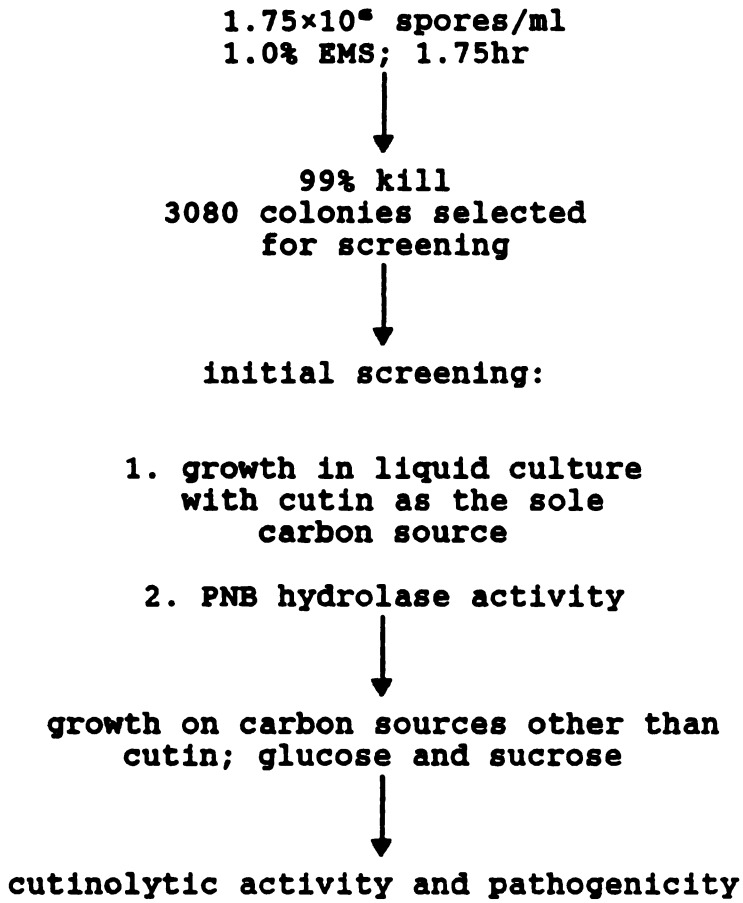

Figure 3.19. Outline for generation of mutants from *C. lagenarium*.

In order to determine if reduced growth on cutin was a result of a general inability to metabolize carbon, those colonies exhibiting reduced growth, relative to the wild-type, were grown on media containing either sucrose or glucose. Those with reduced growth on these media were discarded.

The remaining mutants were then checked for their cutinolytic activity and pathogenicity. In order to discern between those mutants which could not penetrate and those which were no longer able to colonize the tissue, all isolates unable to cause disease when surface inoculated were infiltrated into the tissue. Any isolates unable to cause disease when infiltrated were discarded.

The radial growth of the 13 remaining mutants, on PDA, was found to be equal to or greater than the wild-type, with the exception of M-9, M-24 and M-30, which had 70% the growth of the wild-type. Each of the mutants were checked for 1) PNB hydrolase activity per ml culture fluid, 2) cutinolytic activity per ml culture fluid, 3) their ability to penetrate etiolated cucumber hypocotyls (percent penetration) and 4) their ability to infect cucumber cotyledons (pathogenicity). Results are shown in Table 3.7.

It was thought that perhaps the non-pathogenic, low cutinase producers, inoculated onto plants in the presence of the crude culture filtrate (0.4ug/ul)(the crude was chosen over the purified cutinase-a because there was not

Table 3.7

Characterization of mutants of *C. lagenarium*.

Mutant	M-4	M-9	M-11	M-18	M-20	M-24	M-25	M-29	M-30	M-31	M-36	M-37	M-42	WT
Growth on cutin	+	+-	-	+-	+-	+-	-	++	+-	+-	+	+-	+-	+
PNB hydrolase activity(a)	0.38	0.14	0.37	0.24	0.19	0.04	0.23	0.53	0.05	0.21	0.15	0.25	0.22	0.36
Cutinolytic activity(b)	14.5	4.6	9.1	14.6	11.2	0.46	10.1	16.1	0.8	7.5	7.1	10.3	19.7	12.7
Percent penetration	96	79	81	91	92	14.6	94	15	8	75.5	3	90	79	86
Pathogen-icity	4.75	2.5	3.25	3.5	4.45	0.0	4.75	0.0	0.0	4.3	0.0	4.75	5.5	4.25

[a]Expressed as ΔOD405/min·ml
[b]Expressed as DPM/ml·8hr ($\times 10^3$)

enough of the purified preparation) might result in increased penetration and disease. Increased disease symptoms were observed without an increase in penetration. The crude culture filtrate alone (no fungus) was found to cause severe tissue maceration. Thus what appeared by gross observation to be an increase in disease caused by the fungus was simply tissue masceration due to the enzymes present in the crude culture filtrate.

Scatter graphs were drawn up in which hydrolase activity, cutinolytic activity and percent penetration of each mutant and one wild-type isolate are plotted against their pathogenicity (Figure 3.20a,b,c). In addition, a scatter graph comparing cutinolytic and hydrolase activity is included (Figure 3.20d). Correlation coefficients were calculated for each comparison and are shown below. The coefficients given in parenthesis are those recalculated leaving out those individuals not appearing to fit into the general trend. Individuals not included in a calculation are circled on the corresponding graph.

PNB hydrolase activity vs. pathogenicity: 0.25 (0.7)
(Fig. 3.20a)

Cutinolytic activity vs. pathogenicity: 0.56 (0.86)
(Fig. 3.20b)

Percent penetration vs. pathogenicity: 0.94
(Fig. 3.20c)

PNB hydrolase activity vs. cutinolytic activity: 0.71
(Fig. 3.20d)

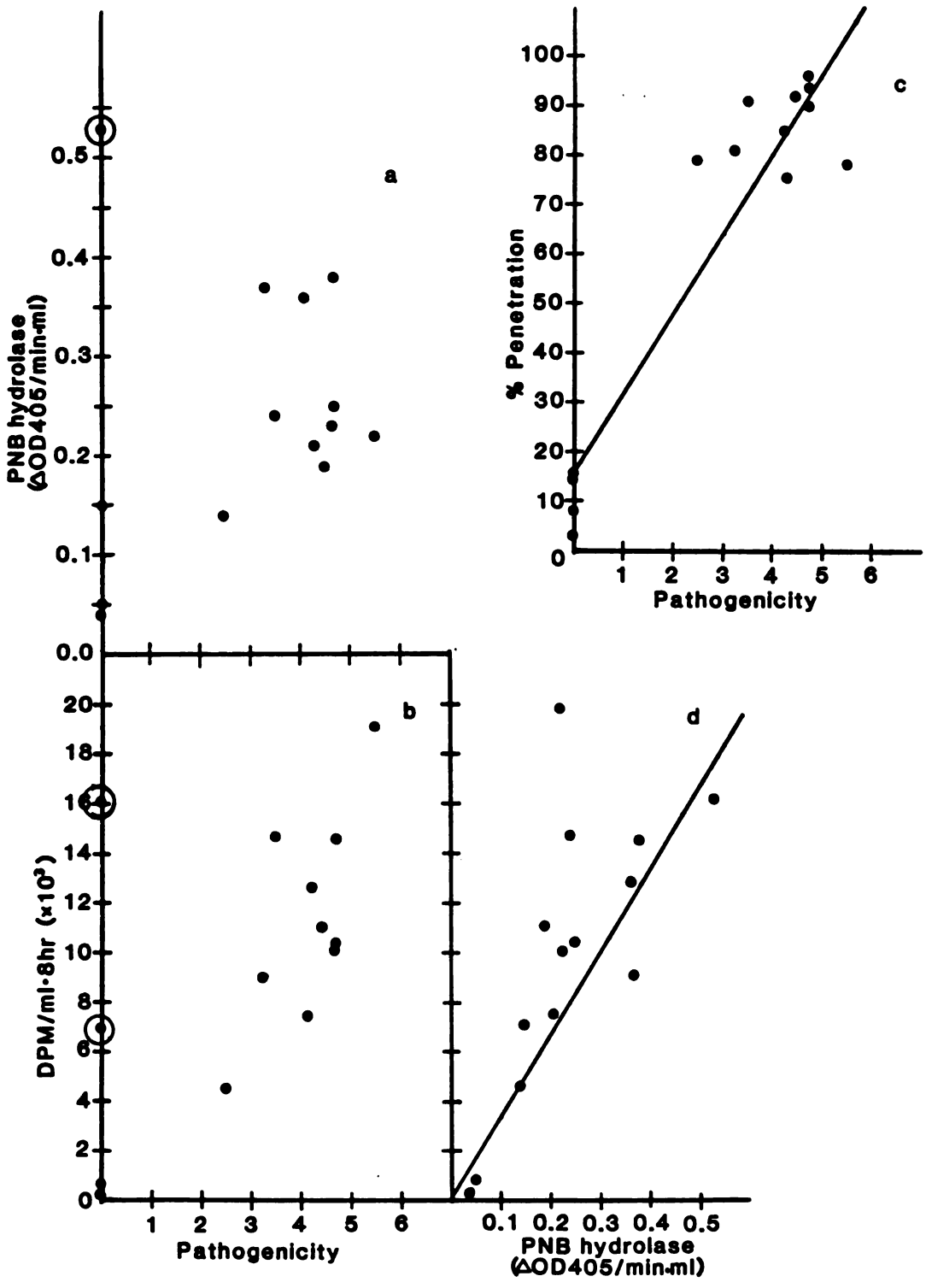

Figure 3.20. Scatter graphs of pathogenicity versus (a), PNB hydrolase activity, (b) cutinolytic activity, and (c) percent infection; and (d) PNB hydrolase activity versus cutinolytic activity, of 13 mutants and a wild-type isolate of C. lagenarium.

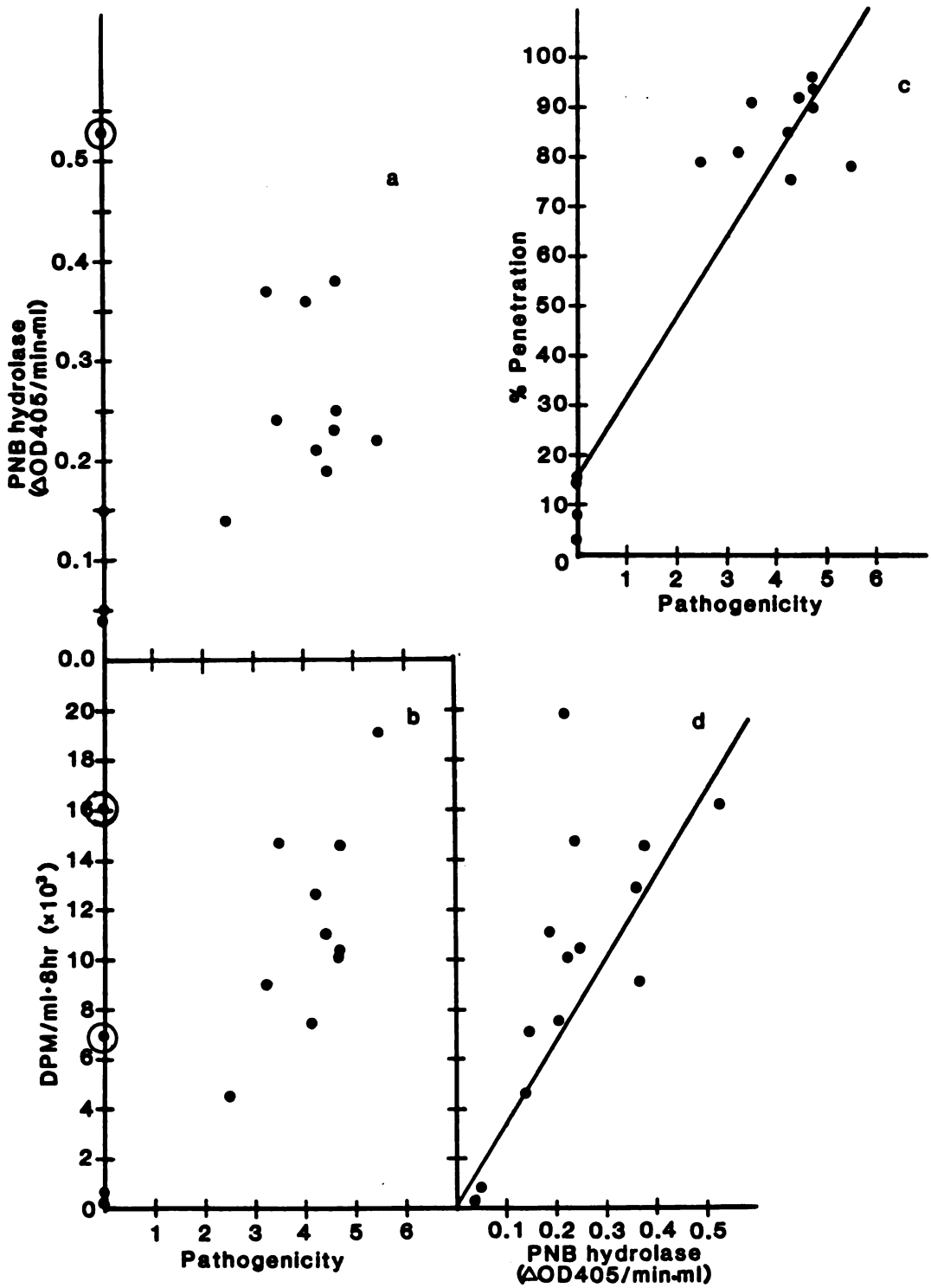

Figure 3.20. Scatter graphs of pathogenicity versus (a), PNB hydrolase activity, (b) cutinolytic activity, and (c) percent infection; and (d) PNB hydrolase activity versus cutinolytic activity, of 13 mutants and a wild-type isolate of C. lagenarium.

The correlation coefficient for PNB hydrolase activity versus pathogenicity was determined to be 0.25. This calculation included mutant M-29 which exhibited extremely low levels of pathogenicity with very high levels of PNB hydrolase activity. If M-29 was not included in the calculations then the coefficient rose to 0.70. When comparing cutinolytic activity with pathogenicity a correlation coefficient of 0.56 was determined. This calculation included two mutants, M-29 and M-36, both of which exhibited exteremely low levels of pathogenicity with either normal (M-36) or very high (M-29) levels of cutinolytic activity. If these two were not included in the calculation then the correlation coefficient rose to 0.86. A high correlation of 0.94 was determined for the percent penetration of the mutants as compared to pathogenicity and correlation coefficient of 0.71 was determined for the comparison of PNB hydrolase activity with cutinolytic activity.

DISCUSSION

Characterization of Cutinolytic Enzymes from *Colletotrichum lagenarium*.

Although cutinolytic enzymes have been reported to be produced by numerous plant pathogenic fungi, few have been purified and characterized. In systems where this has been done, the enzymes have been found to be quite similar

(negative charge, molecular weight of 25 kd and exhibiting both general esterase and cutinolytic activity). The fungi in which these enzymes have been studied most extensively are Fusarium solani f. sp. pisi (23,24), Fusarium roseum culmorum (26) and Colletotrichum gloeosporioides (4).

Cutinase-a isolated from Colletotrichum lagenarium has been shown to be an acidic rather than basic protein with a molecular weight of 60,000. Characteristics which cutinase-a shares with other cutin degrading enzymes include, its glycoprotein nature and its ablility to hydrolyze p-nitrophenyl esters. This enzyme shows a preference for the shorter chain ester, C-4, butyrate, over the C-16 palmitate ester as have all cutinases except that from C. gloeosporioides which does not appear to exhibit any preference (4). The K_m of cutinase-a for p-nitrophenyl butyrate (PNB) was determined to be the same as that reported for the enzyme from F. solani f. sp. pisi, 0.16mM (23).

The enzyme activity (both cutinase and PNB hydrolase) of cutinase-a is severly inhibited by paraoxon, an organophosphate compound. Organophosphates have been reported to be specific inhibitors of cutinases (10) and of serine hydrolases (15). This inhibition indicates cutinase-a may contain an active serine residue and therefore may belong to the class of enzymes referred to as serine hydrolases, to which cutinases, thus far

characterized, have been shown to belong. Phenyl boronic acid, a reversible inhibitor of serine hydrolases, also acts on the serine residue. Hydrolase activity of cutinase-a was reduced by 65% with 1.0mM phenyl boronate. However, cutinolytic activity was reduced by only 15% at this same concentration. It appears that considerably higher concentrations of phenyl boronate are required to significantly reduce this activity as compared to the hydrolase activity. The cutinase from F. solani f.sp. pisi required 0.2M phenyl boronate for a 30% reduction in cutin degrading activity and 0.6M for an inhibition of 75% (12).

Cutinase-a was determined to be N-terminal blocked as is cutinase from F. solani f. sp. pisi (20). In the case of F. solani f.sp. pisi the N-terminal glycine is in amide linkage with glucuronic acid. It is unknown if this is the case for the enzyme from C. lagenarium.

The pH optimum of cutinase-a using the tritiated cutin substrate was determined to be 9.0, thus differing slightly from the optimum pH of 10 reported for most other cutinases (10). Variations in pH optimum of cutinolytic activity have been reported before. For example the cutinase isolated from Penicilium spinulosum (8) has a pH optimum of 6.0 and cutinases from Botrytis cinerea and Botrytis squamosa (1,25) have pH optima of 5.0. The PNB hydrolase activity of cutinase-a exhibited a pH optimum of 6.0 - quite different from that reported for other

cutinases (4,23). C. gloeosporioides has a reported pH optimum of 10 with the PNB substrate and F. solani f.sp. pisi exhibited quite a broad optimal range from 7.7-8.5. It was not clear with the latter two if a correction was made for the increase in extinction coefficient with increasing pH that is associated with p-nitrophenol. Without this correction there might appear to be greater activity at the more alkaline pH levels.

The other cutinolytic enzymes produced by C. lagenarium also differ in several respects from those previously described from other systems as well as from cutinase-a. The cutin degrading enzyme associated with the void volume of the DEAE cellulose column (used in the first step of purification of cutinase-a) was similar to other cutinases (but different from cutinase-a) in its charge which was found to be negative. But this enzyme differed from all previously isolated cutinases by a lack of general hydrolase activity. Inhibition of cutinolytic activity by paraoxon was less severe in comparison to cutinase-a. At 2uM paraoxon a 28% reduction in activity was observed as compared to 70% for cutinase-a. Phenyl boronic acid (1.0mM) reduced activity 15%, as was seen for cutinase-a.

The matrix enzyme preparation showed an alkaline pH optimum (9.0) for both the PNB and the tritiated cutin substrates. Thus it is more like those cutinases isolated from other systems. This enzyme activity also appeared to

contain an active serine residue as both the PNB hydrolase activity and the cutinolytic activity were severely inhibited (85%) in the presence of 2.0uM paraoxon. The matrix enzyme(s) were slightly more sensitive to phenyl boronic acid than the two enzyme preparations previosly described with a 25% reduction in both activities in the presence of 1.0mM phenyl boronate.

The four enzyme preparations discussed and their corresponding activities on each of the substrates, PNB and tritiated cutin, are shown in Table 3.8. Since neither the matrix nor the 'void' proteins have been isolated it is difficult to make precise conclusions about differences or similarities among them and the purified cutinase-a. However, it is quite clear that several distinct enzymes (as shown by the variations in substrate specificity, pH optima and protein charge) are produced by C. lagenarium which are involved in the degradation of cutin. The matrix preparation exhibits relatively little PNB hydrolase activity and even in the crude preparation has considerably more activity on the tritiated cutin substrate than the purified cutinase-a or the 'void' preparation. As it has yet to be purified the activity of this preparation may represent more than a single protein. Initial work indicates the activity of the matrix to be associated with an acidic protein(s).

The 'void' protein preparation also exhibits exceptionally high cutinolytic activity relative to the

Table 3.8

Comparison of the enzyme activities of four protein preparations from C. lagenarium.

	Crude Culture Filtrate	Cutinase-a	DEAE 'Void'	Matrix
PNB hydrolase(a)	7.25	394.0	0.0	0.78
cutinase(b)	116.9	445.0	270.0	786.8

[a]Expressed as ΔOD405/min·mg
[b]Expressed as DPM/8hr·mg ($\times 10^3$)

purified cutinase-a. This activity has yet to be purified to homogeneity, and it is unkown whether this activity represents one or more proteins.

The activity in the crude culture filtrate likely represents the activity of all three enzyme preparations: cutinase-a, 'void' and matrix. The fact that at least 3 enzyme activities can be separated may explain some of the difficulties experienced in the early attempts to identify single fractions containing cutinolytic activity. Biologically it would make sense that more than a single enzyme would be necessary for the breakdown and metabolism of a complex carbon source such as cutin. However, more than one enzyme activity may not be needed for the penetration of cutin, if any is required at all. If an enzyme is used to facilitate penetration it would probably be an esterase because this is the most readily cleaved bond known to be in cutin.

The cutinolytic activity of the crude culture filtrate from C. lagenarium was found to be considerably lower than that found in crude preparations from F. solani f. sp. pisi. This is consistent with other reports of much lower activity in all other systems studied as compared with F. solani f.sp. pisi (1,4). Two possible explanations for this observation might be ralated to the fact that F. solani f. sp. pisi is a very efficient saprophyte; 1) the high level of cutinolytic activity associated with this organism may be most

important for the degradation and metabolism of cutin as a carbon source to support saprophytic growth, 2) alternatively, because it is by nature a wound invading pathogen which does not have any specifically developed mode of penetration such as appressoria and penetration pegs, F. solani f.sp. pisi may require enzymes with much higher specific activity than those fungi which are direct penetrators and would not necessarily require the help (or as much) of enzymes in penetration.

Cutinolytic Enzymes and their Role in Penetration of Cucumber by Colletotrichum lagenarium.

Several distinct proteins or protein fractions from C. lagenarium have been identified as containing cutinolytic activity. Whether any are involved in the penetration of cucumber by C. lagenarium is unknown. Studies utilizing specific inhibitors of cutinase, paraoxon and phenyl boronic acid (10), were undertaken in attempts to determine the role of cutinases in penetration.

Paraoxon, at all concentrations tested (0.02, 0.2 and 2.0uM), failed to affect the radial growth of C. lagenarium on PDA. This presumably indicates normal metabolic functioning of the fungus. However, an additional control, the number of appressoria produced in the presence of paraoxon, indicated that the inhibitor was not specific for the cutinolytic enzymes. So the observed reduction in percent penetration of etiolated cucumber

hypocotyls could not be attributed solely to the loss of cutin degrading activity. And despite the reduction in penetration efficiency no reduction in disease was observed. These results are quite different from that reported for F. solani f. sp. pisi. In that case paraoxon severly reduced infection on pea hypocotyls without affecting the growth of the fungus (12). This was taken to indicate the involvement of cutinolytic enzymes in penetration by F. solani f. sp. pisi. It must now be taken as an indication that cutin degrading enzymes are not involved (at least not as important) in penetration by C. lagenarium.

Phenyl boronic acid, on the other hand, was found to severly reduce the colonization of cucumber cotyledons by C. lagenarium. In the presence of 1.0mM phenyl boronate penetration was reduced 40% and colonization 100%. Unlike paraoxon, phenyl boronate also reduced radial growth of the fungus (80% reduction with a concentration of 1.0mM). In addition, the number of appressoria produced at this same concentration of phenyl boronate was reduced 80%. Together this information indicates that phenyl boronate, rather than being a specific inhibitor of cutinase, has a more broad deleterious effect on the general metabolism of the fungus.

Mutants of C. lagenarium were generated also in an attempt to determine the role of cutinase in the penetration of cucumber by this fungus. No isolates from

the mutagenesis treatment were found which could not grow at all on cutin as the sole carbon source, although there were individuals with significantly reduced growth on the cutin. Two of these (M-24 and M-30) also had reduced levels of cutin degrading activity (see Table 3.7). These two were non-pathogenic unless infiltrated into the leaf tissue (thus by-passing penetration of the cuticle). Two isolates, M-4 and M-42, with increased pathogenicity relative to the wild-type also showed increased cutinolytic activity. This information might imply that cutinolytic enzymes are important in penetration; however, two other isolates were found which had reduced pathogenicity (M-29 and M-36) but with significant to very high levels of cutinolytic activity. These were also pathogenic only when infiltrated into the tissue (see Figure 3.21a,b).

Correlation coefficients were determined for pathogenicity versus PNB hydrolase activity, cutinolytic activity and percent penetration for the 13 mutants and a wild type isolate. Of these three comparisons, only pathogenicity versus penetration was significantly correlated (0.94). The correlations between pathogenicity and cutinolytic activity and pathogenicity and PNB hydrolase activity could be improved if two of the mutants, M-29 and M-36, were disregarded. These data indicate that pathogenicity of C. lagenarium is not directly proportional to either of the enzyme activities

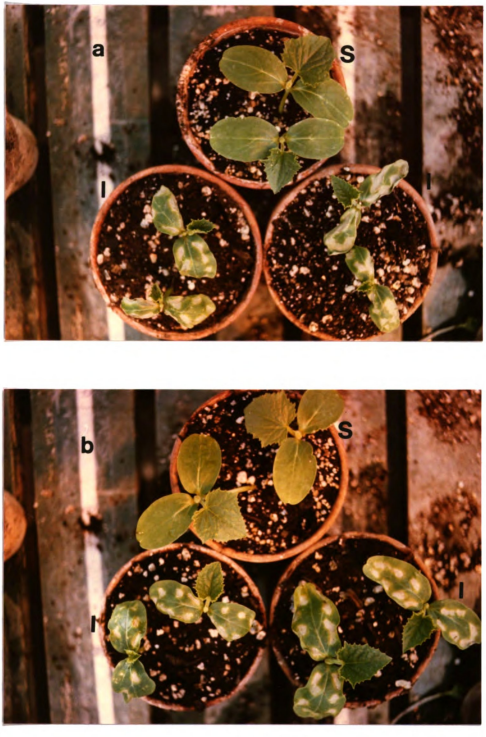

Figure 3.21. Cucumber cotyledons infiltrated (I) and surface inoculated (S) with mutants of C. lagenarium, M-29 (a) and M-36 (b).

observed, especially to PNB hydrolase. The correlation between cutinolytic activity and PNB hydrolase activity was also low (0.71) indicating, as was found during purification of cutinolytic enzymes from C. lagenarium, that there is not always a direct relationship between these two activities.

Colletotrichum lagenarium and cucumber as a system to study cutin degrading enzymes and their role in penetration has turned out to be more complicated than first suspected. Until all the enzymes involved are isolated and characterized, it will be difficult to assign a definitive role for them in penetration. However, from initial work it appears their role in this system is not as essential as that reported for F. solani f. sp. pisi in the penetration of pea and C. gloeosporioides in penetration of papaya. In the case of C. lagenarium, mechanical force may be a much more important factor, if not the singular factor, involved in penetration by this fungus. This is supported by evidence from several groups investigating the importance of melanization of the appressoria in mechanical penetration (16,17,18,27,28,29). Non-melanized appressoria of C. lagenarium, whether the lack of melanization was due to specific chemical inhibition (triacylazole) or mutation, resulted in a 90-95% reduction in the penetration of nitrocellulose membranes by this fungus (17,18,27). Microscopic examination showed that the in vivo situation on

cotyledons was similar to that on nitrocellulose filters. Thus the authors concluded that appressorial pigmentation is essential for penetration by C. lagenarium. Wolkow et al (28) and Woloshuk et al (29) determined the same importance for melanin in appressoria for Colletotrichum lindemuthianum and Pyricularia oryzae, respectively. As observed with C. lagenarium,an organophosphate, in this case diisopropylfluorophosphate (DIFP), had no affect on the disease caused by C. lindemuthianum an P. oryzae. As a result these authors discount the importance of cutinolytic enzymes in penetration and subsequent disease development for both C. lindemuthianum and P. oryzae.

The fact that cutinases are inducible by small amounts of cutin hydrolysate may give some significance to the presence of this activity in the spore matrix of C. lagenarium. As spores are dispersed by rain water, the cutinolytic activity present in the matrix may serve as a mode of creating cutin hydrolysate which could then induce cutinase production by the germinating spore. One would expect only small quantities of the matrix per spore. This may explain the exceptionally high specific activity of the matrix protein relative to the other cutinolytically active preparations from C. lagenarium.

Although the question yet remains as to whether cutinolytic enzymes are important for many fungal pathogens in the penetration of their hosts, the evidence accumulating in recent studies suggests at least for

direct penetrating fungi, these enzymes are less important than is mechanical force. It is possible cutin degrading enzymes play a minor role in reducing the force necessary for penetration by 'loosening' the cutin. This is an activity which may not be essential nor readily detectable.

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