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FAECAL PROTEINASES OF THE FUNGUS-GROWING ANT, *ATTA TEXANA*: THEIR FUNGAL ORIGIN AND ECOLOGICAL SIGNIFICANCE

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Abstract—The fluid contained within the mycelium of the fungus cultured by the attine ant, *Atta texana*, contains three proteolytic enzymes. One enzyme is a DFP-sensitive alkaline proteinase; the other two are metal–chelator-sensitive neutral proteinases. These three enzymes are identical by all criteria examined to the three proteinases previously isolated from the faecal fluid of *A. texana*. It is concluded that the faecal enzymes of the fungus-growing ants are derived from the mycelial fluid upon which they feed. The basis for the symbiosis between the attine ants and the fungi which they cultivate in their nests is reinterpreted in the context of this finding.

INTRODUCTION

LEAF-CUTTING ants of the genus *Atta* culture a fungus on fresh plant material, and exploit this fungus as their primary and possible sole food source (WEBER, 1972). *Atta* workers characteristically defaecate on the plant material prior to incorporating it into their fungus gardens. In a series of publications we have reported the presence of a number of enzymes in the faecal material of the attine ants (MARTIN *et al.*, 1975, and references cited therein). The faecal fluid of workers of the genus *Atta* actively degrades protein, chitin, starch, pectin, sodium polypectate, xylan, and carboxymethylcellulose. We have also described studies which support our suggestion that these enzymes play a vital rôle in the ants' ability to maintain their fungus cultures in their nests (MARTIN *et al.*, 1975).

The question of the origin of these enzymes has largely been left to speculation. Recently, however, we reported the partial purification and characterization of the three enzymes responsible for the proteolytic activity of the faecal fluid of *Atta texana* (BOYD and MARTIN, 1975). The findings bear directly upon the question of the origin of the faecal enzymes. One of the proteinases was a DFP-sensitive alkaline proteinase; the other two were metal–chelator-sensitive neutral proteinases. The properties of these enzymes had little in common with the properties reported for other insect proteinases, but rather had a striking resemblance to those of proteinases isolated from microbial sources. This finding was rather unexpected, since it had been our bias that the faecal enzymes were probably digestive enzymes secreted in the midgut and concentrated in the rectum. There seemed to be but two plausible sources for the faecal enzymes. They might be produced by microbial endosymbionts living within the ants' gut, or they might be intracellular fungal enzymes which are ingested by the ants when they feed.

Preliminary experiments had revealed that there was proteolytic activity in an extract of a homogenate of the ants' fungus. The present study was undertaken with the objective of isolating the proteinases present in such an extract, and comparing them with the enzymes which earlier had been characterized from the ants' faecal material.

MATERIALS AND METHODS

Fungus culturing conditions

Pure cultures of the fungus cultivated by *Atta texana* were isolated from the ants' fungus gardens by standard techniques. The fungus was then cultured in the synthetic liquid medium described by ROBBINS and HERVEY (1960) and modified by MARTIN and WEBER (1969). The carbon source was soluble starch (20 g/l.), the nitrogen source was enzymatic casein hydrolysate (Calbiochem., 10 g/l.). Penicillin (500 units/l.) and streptomycin (100 μg/ml) were also included, and the initial pH was adjusted to 6·0. Ten litres of the medium in a 15 l. carboy equipped with a long magnetic stirrer and an inlet and outlet tube for aeration was autoclaved at 120°C for 45 min. After the medium had cooled it was inoculated with 200 ml of a seed culture of the fungus cultured in the same medium. Following a 14 day growth period at 25°C, a heavy mycelial growth developed which was separated from the medium by filtration through several layers of cheesecloth. After washing with water, the mycelial mat was suspended in 1 l. of calcium

acetate (0·02 M, pH 6·0) and sonicated batchwise (200 ml) at 4°C for 3 min in 30 sec intervals. The supernate obtained after centrifugation at 5000 *g* for 10 min exhibited proteolytic activity equivalent to 3·2 mg of bovine trypsin (Schwarz/Mann, lot No. 897) when assayed using Azocoll.

Purification of the fungal proteinases

Acetone was added dropwise to the stirred supernate described above at 4°C until its concentration reached 60% (by volume). After standing for 1 hr at 4°C, the mixture was centrifuged at 15,000 *g* for 20 min. The precipitate, which contained 87 per cent of the original activity, was dissolved in 0·05 M phosphate buffer (pH 7·0). The proteolytic enzymes present in this fraction were then separated by the same sequence employed by BOYD and MARTIN (1975) for the purification of the enzymes present in the ants' faecal fluid. This sequence involved $(NH_4)_2SO_4$ fractionation, gel filtration on Sephadex G-75, and ion exchange chromatography on DEAE*–cellulose.

Characterization of proteolytic enzymes

All of the procedures listed below are described in detail by BOYD and MARTIN (1975). Molecular weights were determined by gel filtration on a calibrated Sephadex G-75 column, and by SDS-polyacrylamide gel electrophoresis. Proteolytic activity was routinely assayed using Azocoll, and inhibiting effects of DFP (10^{-3} M), EDTA (10^{-2} M), and 1,10-phenanthroline (10^{-3} M) were determined using this same assay. Amino acid esterase activity of proteinase I was assayed titrimetrically toward N-benzoyl-L-methionine methyl ester, N-acetyl-L-tyrosine methyl ester, and N-CBZ-L-serine methyl ester. The digestion of insulin A-chain (oxidized) by proteinases II and III was allowed to proceed until two labile peptide bonds had been cleaved, and the three resulting peptide fragments were located by electrophoresis on thin-layer cellulose sheets.

Determination of intracellular to extracellular proteolytic activity

The fungus cultivated by *Atta colombica tonsipes* was cultured in potato dextrose broth (Difco) in cotton-plugged culture flasks rotated at 250 rev/min at 25°C. In some cases this medium was supplemented with enzymatic casein hydrolysate (Difco), 1·5 mg/ml. The flasks were inoculated with small fragments of mycelium taken from an agar slant. At the conclusion of the growth period, the culture was filtered, and the mycelium was suspended in a volume of 0·1 M cacodylate buffer (pH 7·0) equal to the volume of the filtrate, and homogenized in a Waring blender. Proteolytic activity of the medium and the mycelial extract was determined toward Azocoll (30 min incubation at 37°C) using 2 or 3 ml aliquots of each solution, 1 ml of buffer, and 25 mg of Azocoll. Blanks were run in which aliquots from the medium and the extract were placed in boiling water for 30 min to inactivate the enzymes present.

Maintenance of ants on an artificial diet

A. c. tonsipes workers were isolated from their fungus gardens and placed in crystallizing dishes lined with filter paper. An autoclaved liquid diet consisting of a solution of 5% glucose, 1% bacteriological peptone (Nutritional Biochemicals Corp.), and 0·1% vitamin diet fortification mixture (Nutritional Biochemicals Corp.) was supplied to them in small crystallizing dishes packed with cotton. With the appearance of a growth of mould on the cotton the entire food container was replaced. After 1 week the ants were removed from their containers and held for 20 hr without food. Their faecal material was obtained by inducing them to defaecate using the ether immersion procedure described by BOYD and MARTIN (1975). Faecal fluid was assayed for activity toward Azocoll, chitin (MARTIN *et al.*, 1973), starch, xylan (by following the liberation of reducing groups), and sodium polypectate (using a viscosimetric procedure) as described by MARTIN *et al.* (1975).

RESULTS

Isolation and characterization of the fungal proteinases

An aqueous extract of the mycelium of the fungus cultivated by *A. texana* possesses significant proteolytic activity when assayed with Azocoll. The extract was subjected to a purification procedure involving acetone precipitation, ammonium sulphate fractionation, gel filtration on Sephadex G-75, and ion-exchange chromatography on DEAE–cellulose (BOYD and MARTIN, 1975). This procedure revealed that the proteolytic activity of the crude extract is due to the presence of three separate enzymes, hereafter designated proteinases I, II, and III in order of decreasing molecular weight. The first enzyme to be eluted from the Sephadex G-75 column (proteinase I) accounts for about 30 per cent of the total activity, the second (proteinase II) for 25 per cent of the total activity, and the third (proteinases III) for 45 per cent.

These three fungal enzymes were then characterized by molecular weight determination, susceptibility to inhibition, dependence of activity upon pH, and by specificity toward a series of N-substituted amino acid esters. The results are summarized in Table 1. The comparable data for the three proteolytic enzymes previously isolated

* Abbreviations: CBZ, benzyloxycarbonyl; DEAE, diethylaminoethyl; DFP, diisopropylphosphofluoridate, EDTA, ethylenediamine tetraacetate.

Table 1. Comparison of proteinases I, II, and III derived from a fungal extract and from ants' faecal fluid.

Property	Proteinase I Origin		Proteinase II Origin		Proteinase III Origin	
	Ants*	Fungus	Ants*	Fungus	Ants*	Fungus
1. Estimated molecular weight						
(a) By SDS-electrophoresis†	----	------	41,700	41,700	14,900	14,900
(b) By Sephadex G-75 gel permeation	ca. 70,000	ca. 70,000	40,800	41,000	15,500	15,200
2. Percent inhibition of activity toward Azocoll						
(a) By DFP (10^{-3}M)	100	100	0	0	0	0
(b) By EDTA (10^{-2}M)	0	0	100	100	100	100
(c) By 1,10-Phenanthroline (10^{-3}M)	0	0	>95	>95	>95	>95
3. Ratio of activity toward Azocoll at pH 5.5 to activity at pH 7.5	0.40	0.42	0.81	0.81	0.80	0.82
4. Ratio of rate of hydrolysis of indicated substrate to rate of hydrolysis of N-benzoyl-L-methionine methyl ester						
(a) N-Acetyl-L-tyrosine methyl ester	0.69	0.65	No esterase activity in either enzyme		No esterase activity in either enzyme	
(b) N-CBZ-L-serine methyl ester	0.43	0.41				

*Data from Boyd and Martin (1975)

†The ant and fungal enzymes were run separately and as mixtures. No separation of the corresponding ant and fungal enzymes was observed.

from the ants' faecal fluid (Boyd and Martin, 1975) are also included in Table 1 to allow direct comparison of the enzymes. It is evident that for every characteristic measured the fungal proteinases I, II, and III are identical within experimental error to the corresponding enzymes previously isolated from the faecal fluid of *A. texana*, the ant which cultivates and feeds upon this fungus.

In addition to the studies summarized in Table 1, proteinases II and III were also incubated with the oxidized insulin A-chain. Both enzymes caused the rapid hydrolysis of two peptide bonds and the liberation of three peptide fragments, two of which migrate to the anode and one of which migrates to the cathode when subjected to thin-layer electrophoresis on cellulose sheets at pH 3·5. The peptide fragments are identical in their electrophoretic mobilities to the three peptides generated by the digestion of the oxidized insulin A-chain by proteinases II and III isolated from the ants' faecal fluid. This result indicates that the fungal enzymes exhibit the same specificity as the ant enzymes, cleaving the insulin A-chain preferentially at the same peptide bonds.

When the fungus cultivated by *A. c. tonsipes* was grown in potato dextrose broth, the ratio of proteolytic activity which could be extracted from the mycelium by homogenization to the proteolytic activity liberated into the culture medium during the growth period varied from a low value of 5 : 1 to a high value of 12 : 1. The ratio appears to depend upon a number of variables including the age of the culture, the stage of growth of the fungus, and the amount of mycelium present in the culture flask. We made no effort to clarify all of the factors which affect this ratio, but merely satisfied ourselves that the bulk of the proteolytic enzymes appears to be retained within the fungus, while relatively small quantities are secreted into the medium or are released into the medium upon the death and disintegration of the fungal cells. Activity was undetectable in the medium during the 2 or 3 days immediately following inoculation.

Enzyme activity in ants maintained on a synthetic diet

Approximately 350 workers of *A. c. tonsipes* were isolated from their gardens and maintained for 7 days on a synthetic diet of glucose, bacteriological peptone, and a vitamin supplement. At the end of this period, 272 ants remained alive. Deaths occurred chiefly among the smallest individuals. During the period of isolation, frequent feeding by the ants on the synthetic medium was observed. Ants provided only with water suffered over 75 per cent mortality after a period of 7 days. Finally, the appearance of faecal spots on the filter paper lining the containers revealed that the ants were defaecating during the period of isolation from their colony. From these observations it is clear that during isolation the ants are continuing to take in food and eliminate waste.

Faecal fluid obtained from the ants maintained on the synthetic diet was assayed for activity toward Azocoll, chitin, starch, xylan, and sodium polypectate. Toward each of these substrates the activity of the faecal fluid of ants isolated from their gardens and sustained on a synthetic diet for 7 days was considerably lower than the activity of the faecal fluid of ants taken directly from their gardens (Table 2). The greatest decline was observed for proteolytic activity.

The activity of the faecal fluid of the ants maintained on the synthetic diet was only 18 per cent of that of the faecal fluid of ants taken directly from their gardens. In a separate experiment, the proteolytic activity of the faecal fluid of ants kept on a synthetic diet for 10 days instead of 7 was observed to decline to 5 per cent of the value characteristic of ants collected from a fungus garden.

The proteolytic activities of the fungal extract, the faecal fluid of ants taken directly from their gardens, and the faecal fluid of ants maintained on a synthetic diet are all inhibited to approximately the same extent by 10^{-3} M DFP and 10^{-2} M EDTA (Table 3). This result suggests that the ants secrete little if any digestive proteinase of their own. Virtually all of the digestive endopeptidases of insect which have been studied are DFP-sensitive enzymes. If the ants were continuing to secrete digestive enzymes while being maintained on the synthetic diet, then the faecal fluid of ants which had been kept on the synthetic diet should have a higher proportion of DPF-sensitive enzymes than the faecal fluid of ants taken directly from fungus gardens. The demonstration that two groups of ants have the same proportion of DFP-sensitive and EDTA-sensitive proteinases implies that as the enzymes are eliminated from the rectum through the excretory activities of the ants, residual activity is not being augmented by the secretion of additional DPF-sensitive proteinases in the ants' gut.

DISCUSSION

The proteolytic activity of an extract of the mycelium of the fungus cultivated by *A. texana* is attributable to three enzymes. Proteinase I is a DFP-sensitive alkaline proteinase; the other two, proteinases II and III, are metal–chelator-sensitive neutral proteinases. These three enzymes are identical by every criterion examined to the three enzymes previously characterized in the faecal fluid of the ant, *A. texana*, which cultivates this fungus in its nests. From these results the conclusion follows that the fungal mycelial proteinases and the ant faecal proteinases are the same, and that the origin of the proteolytic enzymes in the ants' guts is the fluid within the fungal mycelium upon which the ants feed. It is also very likely that the numerous other enzymes previously identified in the ants' faecal fluid are similarly derived wholly or in part from the fungus. This conclusion is supported by the observation that the activity of the faecal fluid

Table 2. Enzymatic activity of faecal fluid of *A. colombica tonsipes* workers maintained for 1 week on a synthetic diet.

Substrate	Percentage of original activity remaining after one week
Azocoll	18
Starch	44
Xylan	30
Chitin	24
Sodium polypectate	30

Table 3. Effect of inhibitors on proteolytic activity of a fungal extract and of faecal fluid of *A. texana*.

	% Inhibition	
	10^{-3} M DFP	10^{-2} M EDTA
Fungal extract	30	70
Faecal fluid (ants from garden)	20	80
Faecal fluid (ants on diet)	20	80

toward Azocoll, chitin, starch, xylan, and sodium polypectate declines after the ants are removed from their fungus gardens and are forced to feed upon a synthetic medium devoid of fungal enzymes.

We have also shown that this medley of faecal enzymes is quite stable to the conditions encountered in the ant's gut (BOYD and MARTIN, 1975; MARTIN *et al.*, 1975). Thus, it is entirely feasible that enzymes present in the ants' food would survive passage through the digestive tract and ultimately be excreted.

The identification of the fungi cultured by the attines has proven to be a difficult undertaking (WEBER, 1972). Most investigators have placed these fungi in the Basidiomycetes (MOELLER, 1853; WHEELER, 1907; WEBER, 1972). However, in a recent report, Lehmann (1975) concludes that they are Ascomycetes, and places them in the *Aspergillus flavus* group. It is most intriguing that various species from this group have been extensively exploited by industry because of their copious production of diastatic and proteolytic enzymes (RAPER and FENNELL, 1965).

The finding that the faecal enzymes of the leaf-cutting ants are derived from the fungus, and that they are not digestive enzymes produced by the ants, significantly clarifies the nature of this intriguing symbiosis. Contrary to our earlier suggestion (MARTIN, 1969; MARTIN *et al.*, 1973), it is not their biochemical machinery which the ants are contributing to this mutalistic association, but rather their capacity to serve as vehicles of transport. The ants are simply moving fungal enzymes from a region of the garden in which the fungus is in a state of rapid growth and the enzymes are in ample supply, to the site of inoculation where the enzymes are in short supply. The application of this supplement of active catabolic enzymes at the inoculation site would greatly facilitate the maceration of the leaf tissue and the degradation of potential nutrients in the plant material. These conditions would permit a rapid initiation of growth and a significant shortening of the lag phase during which time metabolic preparations for the rapid growth phase are occurring. A reduction in the length of the lag phase would, of course, reduce the interval of time during which the substrate would be susceptible to additional colonizations by other potentially competitive fungi. In the fungus garden the lag phase appears to be quite short indeed, since substrate is extensively covered by mycelium within 24 to 36 hr after incorporation and inoculation.

Our earlier studies on the growth of attine ant fungi in synthetic media has clearly indicated that very little proteolytic enzyme is secreted into the medium. The fungus grows fairly well in a medium in which a mixture of amino acids is provided as the nitrogen source, but very poorly when only polypeptides are provided. Furthermore, growth is greatly accelerated in a medium containing polypeptide if faecal proteinases are also included. For a number of fungi, the distribution of enzymes between the mycelium and the medium has been found to be dependent upon the age of the culture and details of the culturing procedure (COCHRANE, 1958). Although we have not determined this distribution for any of the enzymes produced by an attine ant fungus under natural conditions, we have established that when the fungus cultured by *A. c. tonsipes* is grown in a liquid medium, the proteolytic enzymes are retained largely within the mycelium. If this finding is indicative of the situation under natural growth conditions in the ants' gardens, then the activities of the ants are seen to be even more important, since they are effectively replacing the process of secretion.

It is interesting to note that we have been unable to detect any trace of digestive proteolytic enzymes produced by the ants. All three of the proteinases present in the ants' faecal material are also present in the fungus. Even the residual proteolytic activity in the faecal material of the ants maintained on the synthetic diet appears to be due to the same fungal enzymes, albeit in lower quantity. Thus, the interesting possibility exists that the chief biochemical adaptation of the attine ants to their fungus-growing habits has been the cessation of production of their own digestive proteinases. Since the ants' digestive enzymes might degrade the fungal enzymes, the deletion of these enzymes would clearly maximize the survival of the fungal enzymes during their passage through the digestive tract.

This interpretation presumes that it is not necessary for the ants to produce digestive enzymes capable of degrading proteinaceous components in the mycelial fluid in order to derive a sufficient supply of amino acids from their diet. In an earlier study (MARTIN *et al.*, 1969) it was shown that an extract of the mycelium of an attine fungus contains a rich mixture of carbohydrates and free amino acids. Thus it would appear that the ants' diet is probably adequate even if dietary proteins are not extensively degraded and subsequently utilized. It is also possible, of course, that fungal proteins which ordinarily do not encounter the fungal proteinases within the fungal cell are degraded by these enzymes when they mix with them in the ant's gut. In this case there would be some digestion of dietary protein, but still without the necessary involvement of any enzymes secreted by the ants.

In the literature describing the fungus-growing ants it has been commonplace to emphasize the domestication of the fungus by the ant. This reflects a rather anthropomorphic point of view which tends to obscure the extent to which the ants have been domesticated by the fungus. The ants forage for substrate, prepare the substrate for inoculation, inoculate and fertilize the new culture, weed the garden, carry out expended substrate, and transport solutions of enzymes from parts of the garden where the fungus has already entered a

rapid growth phase to the area of newly incorporated plant tissue where the initiation of rapid growth is of prime importance. In return the fungus donates to its benefactors some of its metabolic surpluses, low molecular weight nutrients which can be absorbed readily without further digestion.

However one wishes to view the symbiosis between the attine ants and their fungi, we believe that our studies on this subject illustrate how an analysis of the biochemical interaction of organisms can contribute to the clarification of some of the principles and mechanisms which underlie the incredible complexity of natural biological communities.

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