J. Insect Physiol., 1975, Vol. 21, pp. 1887 to 1892. Pergamon Press. Printed in Great Britain.

ACTIVITY OF FAECAL FLUID OF A LEAF-CUTTING ANT TOWARD PLANT CELL WALL POLYSACCHARIDES

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(*Received* 15 *April* 1975)

Abstract—The faecal fluid of the leaf-cutting ant, *Atta colombica tonsipes*, has been shown to contain enzymes active in the degradation of pectin, sodium polypectate, xylan, and carboxymethylcellulose. In addition, glycosidase activity has been detected in the faecal fluid using various naturally occurring disaccharides and synthetic *p*-nitrophenyl glycosides as substrates. The importance of these enzymes in the symbiosis between *A. c. tonsipes* and its food fungus is discussed, with particular emphasis on the rôle of the pectin-degrading enzymes.

INTRODUCTION

ANTS of the genus *Atta* culture a fungus on fresh plant material collected from the surrounding forest. In common with other attine species, *Atta* workers characteristically defaecate on substrate which they are preparing for incorporation into their fungus gardens (WEBER, 1972). In our previous studies of the biochemical basis for this spectacular symbiosis, we established that the faecal fluid of the attine ants contains high levels of enzymes active in the degradation of protein, chitin, and starch (MARTIN and MARTIN, 1970a, b, 1971; MARTIN *et al.*, 1973). We have also demonstrated that the fungus cultured by *Atta* grows rapidly in a synthetic culture medium only if a mixture of amino acids is provided as the nitrogen source, but very slowly when proteins or polypeptides are the only available sources (MARTIN and MARTIN, 1970b). We have further shown that growth is greatly accelerated in a medium containing polypeptides if the ants' faecal fluid, or the proteinases present in the faecal fluid, are added to the culture medium (BOYD and MARTIN, 1975). We were thus led to suggest that the faecal enzymes serve a crucial adaptive function in the ants' fungus-culturing activities by catalysing the degradation of proteins present in the leaf tissue on which the fungus is cultured, thus facilitating rapid fungal growth.

The biochemical basis for this symbiosis is further examined in this paper by determining the activity of the faecal fluid of *Atta colombica tonsipes* toward the principal structural polysaccharides present in the tissues of higher plants.

MATERIALS AND METHODS

Faecal fluid

Faecal fluid was obtained either by dissection (MARTIN and MARTIN, 1970a) or by inducing the ants to defaecate using the ether immersion technique developed by BOYD and MARTIN (1975).

Activity toward polymeric substrates

Activity of faecal fluid toward citrus pectin, sodium polypectate (NaPP), soluble starch, larchwood xylan, and carboxymethylcellulose (CMC) was determined by measuring the liberation of reducing groups by the use of the 3,5-dinitrosalicylic acid reagent (BERNFELD, 1955). Samples of faecal fluid were incubated for 30 min at 37°C with 0·5 ml of a substrate solution (0·5%) in 0·1 M acetate buffer (pH 5·5). The incubation was terminated by adding 1 ml of the 3,5-dinitrosalicylic acid reagent and heating in a boiling water-bath for 5 min. Then 1·5 ml of water was added, the mixture centrifuged if necessary, and the O.D. at 540 nm determined. Controls were run with boiled, inactivated samples of faecal fluid.

Activity toward pectin and NaPP was also determined by measuring the reduction in viscosity of 5 ml of a solution of substrate (pectin, 2·4%; NaPP, 4%) in 0·01 M citrate (pH 5·2) following the addition of an aliquot of faecal fluid. Viscosity was determined at 30°C using a Cannon–Fenske viscosimeter (size 300).

Pectin methylesterase activity was assayed at 25°C by a modification of the continuous titration method of KERTESZ (1951). A quantity of faecal fluid derived from 10 to 15 ants was added to 4 ml of a 0·5% solution of citrus pectin in 0·1 N KCl which had been adjusted to pH 6·6 with 0·1 N NaOH. The pH of the incubation mixture was maintained at 6·6 by the automatic addition of 0·01 N NaOH over a period of 10 min. The rate of addition of base was linear during this time interval. The initial rate of the enzymatic hydrolysis was calculated from this linear portion. The initial

rates were proportional to the amount of faecal fluid assayed.

Activity toward low molecular weight substrates

Glycosidase activity of the faecal fluid was determined at 30°C by following the release of *p*-nitrophenol from *p*-nitrophenyl-α-D-glucoside, *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-α-D-galactoside, and *p*-nitrophenyl-β-D-galactoside. Assay mixtures consisted of 0·75 ml of substrate solution (3·32 mM), 0·75 ml of 0·2 M acetate buffer (pH 5·2), and an aliquot of rectal fluid derived from a known number of ants. The reaction was terminated by the addition of 0·5 ml of 1·0 M NH_4OH–NH_4Cl buffer (pH 9·8), and the absorbance read at 420 nm.

The activity of the faecal fluid toward the disaccharides, maltose, cellobiose, turanose, trehalose, and sucrose was determined by measuring the rate of disappearance of these substances when incubated with faecal fluid. The assay mixture consisted of 10 μl of a substrate solution (73 mM in substrate and 139 mM in inositol) plus 10 μl of a solution of faecal fluid (from 10 ants) in 0·1 M phosphate buffer (pH 6·0). Samples were incubated for 2 hr at 37°C in capped Mini-Aktor tubes (Applied Science Laboratories). The reaction was terminated by immersing the reaction tube in boiling water for several minutes. Samples were prepared for GLC analysis by placing them in a desiccator over phosphorus pentoxide at reduced pressure (to remove water), then silylating them with 250 μl of Sil-Prep (Applied Sciences Laboratories) at 65°C for 1·5 hr. The amount of substrate present initially and after incubation was determined by gas–liquid chromatography on a 3% SE–30 column (175 to 290°C; 8°C/min; lower temperature interval, 7 min; upper temperature interval, 2 min). The per cent of substrate remaining after incubation was calculated from the ratios of the areas of the peaks due to substrate and inositol before and after incubation. The identity of the products of hydrolysis (glucose and fructose) was confirmed by comparison of the observed retention times with those of silylated derivatives of authentic samples.

Activity toward intact leaves

Aliquots of faecal fluid or a solution of pectinase derived from *Aspergillus niger* (Sigma) were applied to the surface of a lilac leaf, and the effects on the appearance of the leaf noted by microscopic examination.

Fungal growth studies

The fungus normally grown by *A. c. tonsipes* was cultured in cotton-plugged flasks at 25°C (rotated at 180 rev/min) in the synthetic medium described by ROBBINS and HERVEY (1960) and modified by MARTIN and WEBER (1969). The nitrogen source was enzymatic casein hydrolysate (Calbiochem), 10 g/l., and the carbon source was either soluble starch, CMC, or pectin (20 gm/l. for growth studies, 30 g/l. for studies of viscosity effects). The initial pH was adjusted to 6·4 to 6·6 by the dropwise addition of 0·5 N KOH. Flasks were inoculated with 1 ml of a seed culture of the fungus homogenized for 10 sec in a sterilized Waring blender. After inoculation, aliquots of faecal fluid derived from approximately 50 workers of *A. c. tonsipes* were added through Millex filter units. At the completion of the growth period, the cultures containing starch and CMC were filtered, and the recovered mycelial mats dried for 6 hr at 60°C, and weighed. Aliquots of medium were periodically removed from the cultures containing pectin, and the viscosities determined at 30°C using a Cannon–Fenske viscosimeter (size 300).

RESULTS

The enzymatic activity of the faecal fluid

The faecal fluid of *A. c. tonsipes* was found to catalyze the degradation of pectin, NaPP, starch, xylan, and CMC (Table 1). By far the highest activity was exhibited toward pectin and NaPP, from which reducing groups were liberated at rates thirty to forty-five times greater than from the other substrates. It was also established that the activity of the faecal fluid toward these polysaccharides is undiminished even after the isolated faecal fluid had been held for 60 hr at 25°C and pH 5·65. This finding reveals the high level of stability of the enzymes under the physiological conditions encountered in the ants' rectum, where a pH of 5·8 has been recorded (BOYD and MARTIN, 1975), and under the conditions likely to be encountered in the plant tissue onto which they are excreted.

Activity toward pectin and NaPP was also detected by noting the rapid decrease in the viscosities of solutions of these two substrates upon incubation with faecal fluid (Table 2). In sharp contrast to the faecal fluid of *A. c. tonsipes*, digestive fluid obtained by dissection of the midguts of workers of the three non-attine species, *Formica ulkei*, *F. dakotensis*, and *F. pallidefulva nitidiventris*, did not bring about the depolymerization of pectin. No reduction in the viscosity of a 2·4% pectin solution was observed after an incubation time of 1 hr with the midgut fluid derived from 10 workers of the three *Formica* species examined.

The capacity of the faecal fluid of *A. c. tonsipes* to degrade pectin was also examined in cultures of the ants' fungus growing in a medium containing pectin. If faecal fluid derived from approximately 50 ants was added to the culture flask (which contained

Table 1. Activity of faecal fluid (FF) of *A. c. tonsipes* toward pectin, NaPP, starch, xylan, and CMC*

Substrate	Number of ants from which FF was derived	ΔO.D./min./ant ($\times 10^3$)	Maltose equivalents liberated, mmoles/min./ant ($\times 10^3$)
Pectin	0.76	7.89	14.6
"	0.38	8.34	15.4
NaPP	0.76	11.62	21.5
"	0.38	11.84	21.8
Starch	26.8	0.243	0.45
"	13.4	0.248	0.46
Xylan	26.8	0.252	0.47
"	13.4	0.236	0.44
CMC	26.8	0.202	0.37
"	13.4	0.211	0.39

*Assay mixtures were incubated for 1 hour at 37°C. FF was obtained by the ether immersion procedure. Blanks consisted of the normal incubation mixture plus FF which had been inactivated by boiling.

Table 2. Effect of faecal fluid (FF) of *A. c. tonsipes* on the viscosities of solutions of pectin (2·4%) and NaPP (4%)*

Substrate	Number of ants from which FF was derived	Viscosity reduction after 6 minutes, (%)	Time for 50% reduction in viscosity, (minutes)
Pectin	Blank	0	-
"	5	18	30
"	10	43	13
"	20	66	4
NaPP	Blank	0	-
"	5	41	9
"	10	56	5
"	20	71	3

*FF was obtained by the ether immersion procedure. Viscosity measurements were conducted on 5-ml samples of substrate solution to which the FF was added. Blanks consisted of the substrate solution plus FF (from 10 ants) which had been deactivated by boiling.

Table 3. Pectin methylesterase activity of faecal fluid (FF) of *A. c. tonsipes* at 25°C*

Number of ants from which FF was derived	NaOH consumed (mmoles/min./ant) $\times 10^6$
10	2.53
15	2.46
15	2.87
Blank	0

*The first two entries describe results obtained with FF obtained by the ether immersion procedure; the third, with FF obtained by dissection. The blank consisted of the normal incubation mixture plus FF (from 15 ants) inactivated by boiling.

Table 4. Glycosidase activity of faecal fluid (FF) of *A. c. tonsipes**

p-Nitrophenyl-glycoside	Number of ants from which FF was derived	ΔO.D./min./ant ($\times 10^3$)	p-Nitrophenol liberated, mmole/min./ant ($\times 10^7$)
α-D-Glucose	2.25	10.60	13.1
β-D-Glucose	2.25	3.78	4.7
"	4.50	3.37	4.2
α-D-Galactose	2.25	4.04	5.0
β-D-Galactose	2.25	0.28	0.35
"	4.50	0.23	0.28

*Assay mixtures were incubated at 30°C for periods of time varying from 20 to 90 minutes. FF was obtained by the ether immersion procedure. Blanks consisted of the normal incubation mixture plus FF which had been inactivated by boiling.

1·5 g of pectin dissolved in 50 ml of medium) at the time of inoculation, an average reduction in viscosity of the medium of 63 per cent was observed after 18 hr. If the faecal fluid were omitted, no reduction in viscosity was detectable 18 hr after inoculation.

It was also noted that the application of the ants' faecal fluid to the surface of a lilac leaf brought about the rapid maceration of the leaf tissue. That this effect is due to the capacity of the faecal fluid to degrade pectin was supported by the observation that a purified fungal pectinase from *A. niger* (Sigma) brought about similar changes in the appearance of the leaf tissue.

The faecal fluid of *A. c. tonsipes* also contains a pectin methyl esterase (Table 3).

Glycosidase activity was detected in the faecal fluid of *A. c. tonsipes* using the synthetic *p*-nitrophenyl glycosides of glucose and galactose (Table 4), and also some naturally occurring disaccharides (Table 5). With the synthetic *p*-nitrophenyl glycosides as substrates, α-glucosidase activity was found to be significantly higher than either β-glucosidase or α-galactosidase activity, which were present at approximately comparable levels. β-Galactosidase activity was lower still, showing only 6 per cent of the activity of α-galactosidase and about 2 per cent of the α-glucosidase activity. The difference in α- and β-glucosidase activity is reflected in the relative tendencies of the disaccharides listed in Table 5 to undergo hydrolysis

when incubated with faecal fluid. Maltose, trehalose, sucrose and turanose, all α-glucosides, are hydrolysed much more rapidly than cellobiose, a β-glucoside. Turanose, an α(1→3) fructosylglucoside, appears to be somewhat less rapidly hydrolysed than the other three α-glucosides.

Effects of faecal fluid on fungal growth

The addition of sterile faecal fluid to a sterile shaken liquid culture of the ants' fungus increases the extent of growth when the carbon source in the medium is either starch or CMC (Table 6). The effects are small, a factor of 1·5 in the starch medium and 3 in the CMC medium, but significant (at the 95 per cent level in the starch medium and at the 99 per cent level in the CMC medium).

DISCUSSION

The primary objective of this study was to determine whether the faecal material of a leaf-cutting ant catalyses the degradation of the structural polysaccharides present in plant tissue. Clearly it does. Pectin, sodium polypectate, xylan, and cellulose, or at least the soluble derivative carboxymethylcellulose, are all degraded by the faecal fluid of *A. c. tonsipes*.

It is easy to envisage several important functions for these faecal enzymes in the fungus-culturing activities of the ants. Obviously, the degradation of the structural polysaccharides of the leaf would provide a source of readily metabolized nutrients for the newly planted fungus. Thus, rapid initial growth would be favoured at a time prior to the secretion onto the substrate of significant quantities of catabolic enzymes by the fungus. Indeed, we have noted just such a growth-enhancing effect when the fungus is cultured in a synthetic medium containing either starch or CMC as the primary carbon source.

A second and probably even more important rôle for the ants' faecal enzymes involves their capacity to bring about the maceration of plant tissue. Enzymes which degrade pectin and protein have

Table 5. Activity of faecal fluid (FF) of *A. c. tonsipes* toward disaccharides*

Substrate	Percent substrate remaining after 2-hour incubation at 37°
Maltose	45
Trehalose	37
Sucrose	44
Turanose	62
Cellobiose	87

*Aliquots containing the FF of 10 ants were taken from a sample of FF obtained by the ether immersion procedure, and added to each substrate solution.

Table 6. The effect of faecal fluid (FF) from *A. c. tonsipes* on the growth of the fungus when cultured in synthetic media*

Source of carbon in medium	Faecal fluid	Dry weight of mycelia, (mg)	S.E.M.(N)
Starch	Absent	89.7	7.7(6)
"	Present	135.4	18.7(4)
CMC	Absent	23.3	2.7(5)
"	Present	66.3	9.1(3)

*Fungus was cultured in 50 ml of a synthetic liquid medium containing 20 gms/l of the carbon source. Growth was carried on for 10 days in the starch medium, 16 days in the CMC medium. When FF was included, the quantity added was derived from approximately 50 ants.

been implicated in the process of tissue maceration by pathogenic fungi as well as by some insects (BATEMAN and MILLAR, 1966; TALMADGE and ALBERSHEIM, 1969; KING, 1973). Both of these types of enzymes are present at high levels in the faecal material of leaf-cutting ants. During the initial stages of vegetative growth by a fungus on plant tissue, the maceration process is critical in permitting the initial invasion of the tissue by the hyphae and in facilitating the subsequent ramification of the fungus within the tissue. In addition, the maceration of the plant tissue would improve the access of other catabolic enzymes, particularly the proteinases (BOYD and MARTIN, 1975), to potential substrates contained within the leaf. That the faecal enzymes are performing such a function when leaf-cutting ants defaecate upon substrate prior to incorporating it into their gardens is supported by two observations. First, we have observed directly the maceration of leaf tissue by the ants' faecal material. Second, we have noted that the degradation of pectin in a synthetic culture medium proceeds very rapidly when some of the ants' faecal material is included, but very slowly when none is present.

Enzymes which degrade pectin are commonly produced by micro-organisms and herbivores which consume the tissues of higher plants. A pectinase has been reported in the seed-gathering ant, *Veromessor pergandei* (WENT *et al.*, 1972), but none has been detected in the three species of *Formica* which we examined. Since most ants are predators or scavengers and make little use of plant tissue in their diets, the secretion of enzymes active in the degradation of plant cell wall polysaccharides would not be expected to be a common trait of this family. The diet of the attine ants does not include pectin, xylan, or cellulose. It is doubtful that pectic substances occur in fungal cell walls. Furthermore, since the attine ants feed only upon the fluid material contained within the mycelium of the fungus which they cultivate (WEBER, 1972), structural polysaccharides would not be ingested by them in any event. The presence of enzymes active in the degradation of pectin, xylan and cellulose in the digestive fluids of the attine ants is, therefore, noteworthy, and raises anew the question of the origin of these enzymes. Are they digestive enzymes secreted by the ants, microbial enzymes produced by endosymbionts, or fungal enzymes present in the fluid within the hyphae upon which the ants feed?

Finally, it is interesting to note that many pectinases are inhibited by phenols and their oxidation products (BATEMAN and MILLAR, 1966). It would be interesting to establish whether the leaf-cutting ants avoid foraging for plants high in phenols or other potent enzyme inhibitors.

Acknowledgements—We wish to thank the National Science Foundation for a grant (No. GB-31581) which supported this work. We also wish to thank Dr. JOAN S. MARTIN and Mr. HOWARD E. LISS for their technical assistance.

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