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THE PRESENCE OF PROTEASE ACTIVITY IN THE RECTAL FLUID OF PRIMITIVE ATTINE ANTS

MICHAEL M. MARTIN and JOAN STADLER MARTIN

The Departments of Chemistry and Zoology, University of Michigan, Ann Arbor, Michigan 48104

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Abstract—The excretion of proteolytic enzymes by representative species of the attine genera *Atta*, *Acromyrmex*, *Sericomyrmex*, *Trachymyrmex*, *Myrmicocrypta*, *Apterostigma*, and *Cyphomyrmex* has been established. The significance of protease excretion by the primitive attines is discussed in light of their use of materials as substrates in their fungus gardens which contain very little polypeptide. In addition, 20 more species of non-attine ants have been examined, and none has been found to concentrate proteolytic enzymes in its rectal fluid in the manner characteristic of the attines.

INTRODUCTION

THE OBLIGATE symbiosis between the attine ants and the fungi which grow in their nests is a spectacular example of a mutually advantageous association of very different types of organisms. Ants of the New World tribe Attini actively cultivate fungi in their nests and exploit this material as their primary and probably sole food source (WHEELER, 1907; WEBER, 1958, 1966). It has been repeatedly demonstrated that the viability of the fungus gardens depends directly upon the activities of the ants. We have recently clarified the biochemical basis for this symbiosis in studies of the attine species, *Atta colombica tonsipes*, and its food fungus (MARTIN and MARTIN, 1970b). The faecal material of this species, which is regularly applied to the gardens and to substrate being prepared for incorporation into the gardens, contains proteolytic enzymes, whereas the fungus which grows in its nest lacks the full complement of such enzymes necessary to make effective use of polypeptide nitrogen. Since the substrate on which this fungus must grow is fresh leaf material, in which alpha-amino nitrogen is present largely as polypeptide, the deposition of faecal material benefits the fungus by compensating for an ecologically critical deficiency in its metabolic machinery. Presumably, the faecal material serves the same function in attine species from two other genera, *Acromyrmex* and *Sericomyrmex*, which also excrete proteolytic enzymes (MARTIN and MARTIN, 1970a) and utilize fresh plant material as a substrate in their fungus gardens.

The use of fresh plant material in the fungus cultures is a practice restricted to the so-called advanced or specialized attine species from the genera *Atta*, *Acromyrmex*, and *Sericomyrmex* (WEBER, 1958). The primitive attines culture their fungi on substrates such as insect faeces, insect carcasses, rotting wood, and plant debris.

These substrates are very different biochemically from fresh plant material, since they have already been subjected to catabolic processes, such as digestion, microbial decay, or autolytic decomposition, before incorporation into the ants' gardens. These processes would reduce the level of polypeptide nitrogen relative to non-polymeric forms of reduced nitrogen, such as ammonia, amino acids, uric acid, allantoin, allantoic acid, and urea. Hence, there would appear to be no particular need for the primitive attines to add supplemental proteolytic enzymes to their fungus cultures. However (WEBER, 1958, 1966), the primitive attine species do defaecate on their gardens and substrates.

The present study was undertaken to determine whether proteolytic enzymes are excreted by all attine species or only those specialized, advanced forms which utilize substrates in which the alpha-amino nitrogen is present largely as polypeptide. Thus we have examined the rectal fluid of six attine species from the primitive genera *Cyphomyrmex*, *Apterostigma*, and *Myrmicocrypta*, and three from the transitional genus, *Trachymyrmex*. In addition we have examined two more species from the advanced genera, *Sericomyrex* and *Acromyrmex*, to supplement our previous study (MARTIN and MARTIN, 1970a). We have also established the polypeptide levels in two characteristic attine substrates, fresh leaves and caterpillar excrement, the former used by the advanced species, the latter by the primitive species.

In addition we have extended our survey of non-attine species to 35 in an attempt to determine whether protease excretion is characteristic of any group other than the attines.

MATERIALS AND METHODS

The ants

Live ants taken directly from natural colonies were used in all of the experiments described in this paper. *Sericomyrmex amabalis*, *Trachymyrmex cornetzi*, *T. bugnioni*, *Myrmicocrypta ednaella*, *Apterostigma dentigerum*, *A. mayri*, *Apterostigma* sp., *Cyphomyrmex rimosus trinitatis*, and *C. costatus* were collected on Barro Colorado Island, Canal Zone, and were identified by Professor Neal Weber, Swarthmore College. *Eciton burchelli*, from Barro Colorado Island, was identified by Professor Edwin Willis, Oberlin College. *Camponotus abdominalis floridanus*, *Solenopsis saevissima richteri*, and *Pogonomyrmex badius*, from Tallahassee, Florida, were identified by Mr. Awinash Bhatkar, University of Florida. *Pheidole xerophila tucsonica*, *Pogonomyrmex pima*, *P. rugosus*, and *P. maricopa* from Tucson, Arizona, and *Solenopsis xyloni* from Portal, Arizona, were identified by Professor A. C. Cole, University of Tennessee. *Myrmecocystus depilis*, from Tucson, was identified by Mr. R. R. Snelling, Los Angeles County Museum of Natural History. *Paraponera clavata* from Barro Colorado Island, *Odontomachus haematoda* and *Leptogenys elongata* from Austin, Texas, *Acromyrmex versicolor* from Tucson, *Tapinoma sessile* from Portal, *Paratrechina longicornis* and *Dorymyrmex pyramicus flavopectus* from the Archbold Biological Station, Lake Placid, Florida, *Trachymyrmex septentrionalis*

from Tallahassee and the Archbold Station, and *Dolichoderus mariae* from the E. S. George Reserve, Livingston County, Michigan, were identified by the senior author using the work of CREIGHTON (1950).

Dissections and analytical procedures

Dissections of the midguts and rectums and the Azocoll assay for protease activity were conducted as described previously (MARTIN and MARTIN, 1970a). The assays were conducted at 37°C at a pH of 6·65 ± 0·03 on samples prepared by pooling and homogenizing the dissected midguts or rectums, or the contents thereof, from varying numbers of ants. With all of the attine species examined, homogenates of entire midguts and rectums were utilized in the assays. Among the non-attine species, the contents were removed with a capillary needle for all species except *Pheidole xerophila tucsonica*, *Solenopsis xyloni*, *Dolichoderus mariae*, *Dorymyrmex pyramicus flavopectus*, *Tapinoma sessile*, and *Paratrechina longicornis*, from which entire midguts and rectums were dissected. Previously we established that midgut and rectal tissue does not exhibit significant protease activity in this assay. For most of the species examined, assay samples derived from about 10 ants were sufficiently active to give acceptable spectrophotometer readings. For very small species, such as *Cyphomyrmex costatus*, *C. rimosus trinitatis*, *Apterostigma mayri*, *Myrmicocrypta ednaella*, and *Trachymyrmex bugnioni*, it was necessary to prepare samples by pooling midguts or rectums from 40 to 50 ants. Samples from *Trachymyrmex cornetzi*, *Sericomyrmex amabalis*, *Dorymyrmex pyramicus flavopectus*, and *Paratrechina longicornis* were derived from about 20 ants.

External dimensions of the midguts and rectums were estimated using an ocular micrometer, and volumes were calculated assuming cylindrical geometry. The volumes recorded in Tables 1 and 3, obtained from measurements on 4 or 5 ants of each species, should be regarded as rough approximations at best. Not only was the sample size small and the variability frequently high, but in many the shapes were irregular and did not approach the assumed cylindrical geometry very closely. In addition, since all measurements were of external dimensions, the calculated volumes include the volumes of the tissue in addition to the material contained within the structure. In the small species, this results in calculated volumes significantly greater than the actual volumes of the contents. Because of the approximate nature of these volume determinations, we have elected to present the protease activity data on a 'total activity per ant' basis rather than on a more conventional 'activity per unit volume' basis. While this mode of presentation does not permit convenient interspecific comparisons of relative midgut or rectal protease levels, it does provide an appropriate measure of the relative total amounts of protease present in the midgut and rectum of any given species, and permits useful and meaningful interspecific comparisons of ratios of total rectal to midgut protease levels.

The relative amounts of polypeptide and non-polypeptide alpha-amino nitrogen in the faecal pellets of two caterpillars, *Malacosoma americana* and *Hyalophora cecropia*, and in lilac leaves, *Syringa vulgaris*, were determined. The amount of

polypeptide present was calculated by subtracting the total alpha-amino nitrogen present in the sample before hydrolysis from the total alpha-amino nitrogen present in the sample after it had been subjected to the hydrolysing action of 6 N hydrochloric acid for 4 hr at 145°C. The sample for the determination of amino-nitrogen before hydrolysis was prepared by extracting the material for 3·5 hr with refluxing 80% ethanol. Solvent was removed by rotoevaporation and replaced by deionized water. In both cases, total alpha-amino nitrogen was determined colorimetrically after formation of the trinitrophenyl derivatives of the constituent amino acids in the sample. Glycine was used as the standard for the colorimetric reaction. These analyses were conducted by the Analytical Biochemistry Laboratories, Inc., Columbia, Missouri.

RESULTS

The midguts and rectums of a total of 17 species of attine ants from seven different genera have been assayed for protease activity in this (Table 1) and our previous study (MARTIN and MARTIN, 1970a). Every one of the 17 species was found to have significant activity in its rectum. While there clearly are differences in the absolute magnitudes of both rectal and midgut protease levels among various species, and even considerable variation between different colonies of the same species, significant rectal activity was detected in every species.

In 14 of the 17 species studied, there was significantly greater total protease activity in the rectum than in the midgut. In *Apterostigma dentigerum* and *A. mayri*, the total midgut activity was comparable to or slightly greater than the total rectal activity. However, since in these 2 species the midguts were distinctly larger than the rectums, there was actually a higher concentration of proteolytic enzymes in the rectum than in the midgut. Thus, in 16 of the 17 species, protease activity was accumulated and concentrated in the faecal fluid. Only in *Myrmicocrypta ednaella* was the midgut activity greater than the rectal activity on both a 'total activity per ant' and a concentration basis. In this species, the total rectal activity was 54 per cent of the total midgut activity.

Since it was the purpose of this study to establish whether there is a correlation between the presence of proteolytic enzymes in the faecal fluid of attine ant species and the level of polypeptide nitrogen in the substrates used in their fungus gardens, it was desirable to measure the polypeptide and non-polypeptide nitrogen levels in characteristic substrates of both advanced and primitive forms. Thus, the faecal pellets of 2 species of phytophagous lepidopterous larvae, a substrate employed by a number of primitive species, and lilac leaves, a substrate readily accepted by captive *Atta* colonies, were analysed (Table 2). Most of the alpha-amino nitrogen in the caterpillar faeces (81 and 100 per cent) was in the form of free amino acids. Very little (19 and 0 per cent) was present as polypeptide. These results are in agreement with the report of SRIVASTAVA (1962) that the faecal pellets of the caterpillar *Corycyra cephalonica* contained no protein. By contrast, in lilac leaves, 88 per cent of the alpha-amino nitrogen was present in the form of polypeptide.

TABLE 1—PROTEOLYTIC ENZYME ACTIVITIES AND VOLUMES OF MIDGUTS AND RECTUMS OF ATTINE ANTS

Species	Protease activity/ant*		Volume of midgut		Volume of rectum	
	Midgut	Rectum	Av. (μl)	Range (μl)	Av. (μl)	Range (μl)
Cyphomyrmex costatus	3	6	0·02	0·01–0·05	0·01	0·01–0·02
Cyphomyrmex costatus	6	16	—	—	—	—
C. rimosus trinitatis	3	18	0·04	0·02–0·06	0·04	0·02–0·07
Apterostigma dentigerum	110	110	0·15	0·06–0·26	0·08	0·01–0·12
Apterostigma dentigerum	65	40	—	—	—	—
Apterostigma dentigerum	355	160	—	—	—	—
A. mayri	50	35	0·05	0·03–0·07	0·02	0·01–0·06
Apterostigma sp.†	35	150	0·10	0·04–0·20	0·15	0·08–0·24
Myrmicocrypta ednaella	15	8	0·02	0·01–0·05	0·02	0·01–0·05
Myrmicocrypta ednaella	24	13	—	—	—	—
Trachymyrmex bugnioni	6	22	0·03	0·02–0·03	0·03	0·01–0·05
T. cornetzi	⩽5	125	0·06	0·02–0·11	0·06	0·03–0·14
T. cornetzi	20	40	—	—	—	—
T. septentrionalis	⩽5	150	0·09	0·07–0·13	0·05	0·03–0·07
T. septentrionalis	10	100	—	—	—	—
T. septentrionalis	15	60	—	—	—	—
Sericomyrmex amabalis	⩽5	75	0·09	0·05–0·17	0·11	0·05–0·23
Sericomyrmex amabalis	25	40	—	—	—	—
Acromyrmex versicolor	395	3060	0·12	0·04–0·19	0·23	0·20–0·26

* Activity expressed in terms of the number of ng of fungal protease (Sigma, Type VI) which exhibited comparable activity in the Azocoll assay.

† Determined by N. A. Weber to be 'like a large *A. dorothea*, but not *A. auriculatum*'.

TABLE 2—ALPHA-AMINO NITROGEN IN THE FAECAL MATERIAL OF TWO LEPIDOPTERAN LARVAE AND IN FRESH LILAC LEAVES

Material	Alpha-amino nitrogen (wt. %)		Alpha-amino nitrogen present as polypeptide (%)
	Before hydrolysis	After hydrolysis	
Malacosoma americana pellets	3·02	3·75	19
Hyalophora cecropia pellets	1·60	1·51	0
Lilac* leaves	0·66	5·56	88

* *Syringa vulgaris.*

TABLE 3—PROTEOLYTIC ENZYME ACTIVITIES AND VOLUMES OF MIDGUTS AND RECTUMS OF NON-ATTINE ANTS

Species	Protease activity/ant*		Volume of midgut		Volume of rectum	
	Midgut	Rectum	Av. (μl)	Range (μl)	Av. (μl)	Range (μl)
A. Subfamily Ponerinae						
Paraponera clavata	2520	≤15	16·1	—	3·71	—
Leptogenys elongata	600	≤5	0·43	0·26–0·59	0·06	0·04–0·07
Odontomachus haematoda	1440	≤5	0·65	0·53–0·74	0·10	0·02–0·18
B. Subfamily Dorylinae						
Eciton burchelli	4100	120	0·68	0·55–0·90	0·36	0·10–0·77
C. Subfamily Myrmicinae						
Pogonomyrmex pima	70	≤5	0·15	0·09–0·20	0·09	0·04–0·12
P. rugosus	1070	5	0·94	0·83–1·31	1·87	1·53–2·31
P. maricopa	430	25	0·53	0·35–0·83	0·41	0·27–0·50
P. badius	1050	140	0·79	0·55–0·98	0·23	0·09–0·45
Novomessor albisetosus	1800	≤5	1·39	0·98–2·32	0·25	0·11–0·50
N. cockerelli	3300	≤5	2·21	1·61–2·59	2·43	1·94–3·65
Veromessor pergandei	1230	≤5	1·66	1·51–1·82	0·25	0·11–0·41
Pheidole xerophila tucsonica	100	≤5	0·13	0·06–0·17	0·05	0·04–0·06
Solenopsis xyloni	100	≤5	—	—	—	—
S. saevissima richteri	85	≤5	—	—	—	—
D. Subfamily Dolichoderinae						
Dolichoderus mariae	215	≤5	0·12	0·10–0·16	0·21	0·16–0·29
Dorymyrmex pyramicus flavopectus	10	≤1	0·14	0·08–0·26	0·03	0·02–0·04
Tapinoma sessile	20	≤5	—	—	—	—
E Subfamily Formicinae						
Componotus abdominalis floridanus	195	≤5	2·50	1·94–3·11	0·47	0·22–0·98
Paratrechina longicornis	45	≤5	0·06	0·03–0·11	0·02	0·01–0·02
Myrmecocystus depilis	120	≤5	0·49	0·32–0·93	0·27	0·01–0·65

* Activity expressed in terms of the number of ng of fungal protease (Sigma, Type VI) which exhibited comparable activity in the Azocoll assay.

A total of 35 non-attine species, representing 22 genera from 5 subfamilies have now been examined in this (Table 3) and our previous work (MARTIN and MARTIN, 1970a). The contrast between the attines and non-attines is striking. In 26 of the non-attine species examined it was not possible to detect any protease in the rectums. In 9 species there was a trace of rectal protease, but in none was rectal activity more than a small fraction of midgut activity. In 8 of the 9 species which did have detectable rectal protease, the rectal activity was less than 16 per cent of the midgut activity. In one species, *Myrmica brevinodis*, rectal activity was 36 per cent of midgut activity. Two other *Myrmica* species, *M. monticola* and *M. emeryana*, had no detectable rectal protease (MARTIN and MARTIN, 1970a). Thus to the extent that our survey is representative, it is apparent that the capacity to concentrated proteolytic enzymes in the faecal material is not widely held among ants, but rather is peculiar to the attines.

DISCUSSION

The main purpose of this study was to establish whether there is a correlation between the capacity of various species of attine ants to excrete proteolytic enzymes and their apparent need to do so. We have established that there is no correlation between the presence of proteolytic enzyme in the rectal fluid, and either the nature of the substrate on which the fungus is cultured or the evolutionary status of the species. The 17 species examined have varied and diverse substrate preferences, and are from genera which represent very different stages of evolutionary development. At one end of the scale are the advanced, specialized genera, *Atta*, *Acromyrmex* and *Sericomyrmex*, which grow their fungi on fresh leaves and other plant material. As noted earlier (MARTIN and MARTIN, 1970b), and substantiated in this study, ants from these genera all concentrate proteolytic enzymes in their rectal fluid. At the other end of the evolutionary scale is *Cyphomyrmex*, generally regarded as the most primitive attine genus (WEBER, 1958). We examined 2 species from this genus, *C. rimosus trinitatis*, which utilizes caterpillar excrement as its substrate, and *C. costatus*, which utilizes plant debris and fragments of insect exoskeletons as substrates. Ants of both species concentrate proteases in their rectal fluid. Ants from 3 species of *Apterostigma*, a morphologically aberrant but apparently primitive genus which utilizes rotting wood and the faeces of wood-boring beetle in its gardens, were found to concentrate proteases in their rectal fluid. *Myrmicocrypta ednaella*, a primitive species which utilizes plant debris of unknown origin along with varying amounts of insect faeces, produces faecal fluid which exhibits significantly higher protease activity than any of the non-attine species, although the extent to which proteolytic enzymes have been concentrated in the rectum appears to be less than in the other attine species examined. *Trachymyrmex* is a transitional genus, but is closer to the specialized leaf-cutting species than to the more primitive species. Ants of this genus utilize substrates characteristic of both primitive and specialized forms. All of the *Trachymyrmex* species examined were found to concentrate proteases in their rectums. Thus, all of the 17 species examined, from 7 different genera, representing forms of contrasting evolutionary status and

varied substrate preferences, produce faecal fluid with significant protease activity.

We have further established that the polypeptide levels are very different in the preferred substrates of advanced and primitive forms. In fresh leaf material, a characteristic substrate employed by *Atta*, *Acromyrmex*, and *Trachymyrmex*, 88 per cent of the alpha-amino nitrogen was present as polypeptide, whereas in caterpillar excrement, a characteristic substrate of *Cyphomyrmex* and several other genera, very little (0 to 19 per cent) of the alpha-amino nitrogen was present in the form of polypeptide. Although we did not determine polypeptide levels of any types of plant debris, a substrate commonly utilized by several primitive and transitional species, autolytic processes known to occur (DOBY, 1965) in excised leaves, will certainly result in lower polypeptide levels than those found in fresh leaves. Thus we can reformulate our basic conclusion in somewhat more chemical terms: there is no correlation between the presence of proteolytic enzymes in the rectal fluid and the levels of polypeptide in the substrate. This is the single most important finding to emerge from this study.

Although there are proteolytic enzymes in the rectal fluid of all the attine species examined, it does appear that species from two primitive genera, *Myrmicocrypta* and *Apterostigma*, produce rectal fluid less concentrated in protease than that of the more advanced species. However, this probably reflects a behavioural rather than a physiological distinction between advanced and primitive forms. Ants from all attine species defaecate only in their fungus gardens. Thus, while an ant is engaged in activities distant from its nest, material would be passing through the hindgut and into the rectum, where it would be stored. Water resorption, which occurs in the rectum, would result in the concentration of soluble components. Thus, a decrease in the frequency of defaecation should result in the storage of larger volumes of more concentrated rectal fluid. Ants of the more advanced species of attines forage much further from their nests than ants of the more primitive species, and would, therefore, be expected to produce more concentrated rectal fluid. That is exactly what we have found. It is also interesting to note that in 6 of the 8 species examined from the genera *Atta*, *Acromyrmex*, and *Sericomyrmex*, the average size of the rectum was greater than the average size of the midgut, whereas in only 1 of the 6 primitive species was this the case. The greater engorgement of the rectums of ants which forage further from their nests is consistent with the suggestion that they defaecate less frequently. Thus the difference between the primitive and advanced attines is not in the quantities of faecal enzymes they apply to their fungus cultures, but rather in the frequency and concentration of the applications.

We can suggest no special rôle for the faecal proteases of the primitive attines. It is evident that they do not serve the same critical function in fungus-culturing activities as is the case in the advanced attines. Indeed, the significance of the defaecation behaviour of the primitive attines, and, in fact, the entire biochemical basis for the symbiosis between the primitive attines and their fungi, remains obscure at the present time. Hopefully, it can be deduced by further studies on the

metabolic attributes of their fungi and the chemical and enzymatic properties of their faeces. We are currently inclined toward the view that the excretion of proteolytic enzymes is inconsequential to the primitive attines, and that it is an unavoidable consequence of some other important physiological or biochemical adaptation. Accordingly, this capacity of the primitive attines to excrete proteolytically active faecal material may have been the pre-adaptation which permitted the evolution of fungus-growing species which could utilize fresh plant material in their cultures.

Although none of the non-attine species examined exhibits a capacity to concentrate proteolytic enzymes in its rectum, a few do produce faecal material in which the protease activity is far from negligible. *Eciton burchelli* and *Pogonomyrmex badius* are particularly noteworthy. It would be most interesting to know where these ants defaecate and whether the enzymatic activity of their excreta plays any rôle in their biology. It is interesting to note that when *P. barbatus* picks up a seed, it often touches it to the tip of its gaster (McCOOK, 1880). It has been presumed that this behaviour simply aids in positioning the seed for transport. *P. barbatus* has also been observed to touch its gaster to seeds while feeding, to press its mouthparts to the tip of its gaster while grooming, and to lick the apex of the gasters of dead ants (McCOOK, 1880). In none of these instances has a transfer of faecal material been suggested, but it is intriguing to entertain the possibility and to speculate upon possible implications.

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