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POLLEN PROTEASES: THEIR POTENTIAL ROLE IN INSECT DIGESTION

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Abstract—Pollens of plant taxa known to be visited by bees were analyzed *in vitro* for the presence of four enzymic activities: trypsin-like, chymotrypsin-like, carboxypeptidase A-like, and carboxypeptidase B-like. All pollens examined contained at least chymotryptic activity; other activities were irregular in occurrence. Quantities of the enzymes found are tabulated and are compared with levels of the same four enzymic activities of the midguts of *Apis mellifera* L. Honeybee midgut activity levels exceed those of the pollens, but the pollen enzymes seem sufficient in some cases for probable physiological activity.

Key Word Index: *Apis mellifera*, carboxypeptidase A and B, chymotrypsin, nutrition, trypsin

INTRODUCTION

POLLEN is a major nutrition source in the diet of many insects. Its role in the nutrition of the honeybee, *Apis mellifera*, is well established. A similar role in the nutrition of other bees and of pollen eating wasps (Masariade) may be confidently inferred. It also serves as an important nutritional source in some species of *Heliconius* butterflies (GILBERT, 1972) and syrphid flies (STURKEN, 1964). In all instances it has been assumed that pollen serves as a nutrient source. Little consideration seems to have been given to the possible role that pollen enzymes may play in the degradation of protein and other substances in the gut of insects that ingest pollen. PATON (1921) documented the presence of a variety of enzymes in pollen grains. More recent workers (HESLOP-HARRISON, 1971; STANLEY and LINSKENS, 1974) have demonstrated the localization of several enzymes in the microstructure of pollen walls and have outlined the role of some of these enzymes in compatibility recognition phenomena preceding pollination.

In order for an insect to obtain nutrition from pollen it must enzymatically digest the contents of the pollen grains. BARKER and LEHNER (1972) report that adult worker *Apis mellifera* enzymatically degrade pollen grain contents in their midguts, but they state that it is not known whether the enzymes originate in the bee midgut, in the gut microflora, or in the pollen itself. In a detailed study of enzymes, GIEBEL *et al.* (1971) characterized the midgut endopeptidase activity of *Apis mellifera*. They documented the presence of two enzymes that resembled the trypsin and chymotrypsin found in higher animal systems. In addition, they found two endopeptidases that possessed properties different from enzymes found in the mammalian gut. They did not consider the possibility that pollen proteases have similar cleavage specificities and occur in sufficient quantity to contribute to the total midgut protease activity of pollen-eating insects. The present investigation was therefore initiated to test this possibility. Several pollens were collected and examined using specific synthetic substrates to test for the presence of endopeptidase-like and exopeptidase-like enzymes which could be used by insects for digestive purposes. Quantities of these enzymes found in pollen were compared with levels of similar enzymic activities found in midguts of *Apis mellifera*.

MATERIALS AND METHODS

Fresh pollen was collected from a variety of flowers known to be visited by bees. The flowers were removed from the plants and the pollen carefully removed from the anthers with a camel hair brush. The pollen was collected, weighed, and transferred to a test tube.

Four enzymic activities were assayed for: trypsin-like, chymotrypsin-like, carboxypeptidase A-like, and carboxypeptidase B-like.

Trypsin-like

For an assay at least 15 mg of pollen was placed in 0.4 ml of 0.001 M HCl and leached for 15 min at 37 C. The extract was then centrifuged at 2500 ***g*** for 10 min at room temperature; the supernatant was then used as the enzyme source. The assay was conducted for 4 min in a 4 ml cuvette, using the change in absorbance at 247 nm as an an index of enzyme concentration. The final volume of the reaction mixture was 3 ml. It contained 40 mM Tris–HCl buffer (pH 8.1), 10 mM $CaCl_2$, and 0.1 ml of pollen extract. The reaction was initiated by the addition of *p*-toluenesulphonyl-L-arginine methyl ester (TAME) to a final concentration of 1 mM (HUMMEL, 1959).

Chymotrypsin-like

Pollen was extracted as described above, and enzyme activity was determined in a 3.0 ml reaction mixture at 256 nm. The assay mix contained 0.1 ml pollen extract, 40 mM Tris–HCl buffer (pH 7.8) and 5 mM $CaCl_2$. The reaction was initiated by the addition of benzoyl-L-tyrosine ethyl ester (BTEE) in 50% methanol (w/w) to a final concentration of 0.5 mM (HUMMEL, 1959)

Carboxypeptidase A-like

Pollen was extracted with 10% (w/v) LiCl under the conditions described for trypsin above. A reaction mixture was formed by adding 2.9 ml freshly prepared 1 mM hippuryl-L-phenylalanine (HPA) dissolved in 25 mM Tris–HCl buffer (pH 7.5) and containing 500 mM NaCl to 0.1

Table 1. The endopeptidase-like and exopeptidase-like enzymes and the percent leachable protein of pollen of various plants visited by bees. The enzyme values are of two replications; protein values are the range of four replications. Trace = <0.001

Plant	Chymotrypsin		Trypsin		Carboxypeptidase A		Carboxypeptidase B		
	Units/mg protein	Units/mg pollen	Units/mg protein	Units/mg pollen	Units/mg protein	Units/mg pollen	Units/mg protein	Units/mg pollen	Protein wt/ pollen wt (%)
Liliaceae									
Hemerocallis fulva	0.178	0.021	0.000	0.000	0.000	0.000	0.000	0.000	8.4–11.8
(Day Lily)	0.104	0.012	0.000	0.000	0.000	0.000	0.000	0.000	
Saxafragaceae									
Philadelphus pubescens	0.218	0.016	0.110	0.005	0.000	0.000	0.028	0.001	5.7–9.1
(Mock Orange)	0.228	0.020	0.067	0.010	0.000	0.000	0.068	0.003	
Rosaceae									
Malus sp	0.186	0.016	0.000	0.000	0.072	0.003	0.082	0.004	5.5–11.0
(Pink Flowering Crab)	0.224	0.024	0.000	0.000	0.100	0.005	0.112	0.006	
(White Flowering Crab)	0.115	0.006	0.000	0.000	0.002	trace	0.055	0.002	4.1–6.1
	0.145	0.010	0.000	0.000	0.003	trace	0.085	0.003	
Leguminosae									
Robinia pseudo-acacia	0.253	0.016	0.000	0.000	0.000	0.000	0.000	0.000	3.3–7.2
(Black Locust)	0.094	0.006	0.000	0.000	0.000	0.000	0.000	0.000	
Cercis canadensis	0.205	0.010	0.103	0.006	0.152	0.003	0.032	0.001	3.2–7.2
(Redbud)	0.214	0.020	0.133	0.130	0.178	0.007	0.124	0.003	
Malvaceae									
Hibiscus syriacus	0.076	0.003	0.032	0.001	0.000	0.000	0.000	0.000	4.4–6.8
(Rose of Sharon)	0.078	0.003	0.026	0.001	0.000	0.000	0.000	0.000	
Solanaceae									
Peunia sp	0.083	0.009	0.000	0.000	0.000	0.000	0.000	0.000	6.7–12.8
(Petunia)	0.157	0.020	0.000	0.000	0.000	0.000	0.000	0.000	
Cornaceae									
Corunus florida	0.119	0.008	0.000	0.000	0.000	0.000	0.000	0.000	6.1–9.2
(Flowering Dogwood)	0.053	0.005	0.000	0.000	0.000	0.000	0.000	0.000	
Bignoniaceae									
Campsis radicans	0.110	0.009	0.123	0.010	0.000	0.000	0.000	0.000	6.8–9.2
(Trumpet Creeper)	0.123	0.011	0.234	0.021	0.000	0.000	0.000	0.000	
Caprifoliaceae									
Lonicera sp	0.163	0.011	0.000	trace	0.000	0.000	0.000	0.000	7.0–7.8
(Honeysuckle)	0.197	0.015	0.002	trace	0.000	0.000	0.000	0.000	
Compositae									
Taraxacum sp	0.114	0.008	0.000	0.000	0.033	0.002	0.120	0.008	6.7–7.4
(Dandelion)	0.213	0.015	0.035	0.002	0.042	0.002	0.104	0.007	
Cichorium intybus	0.190	0.019	0.000	0.000	0.012	0.001	0.038	0.003	7.8–10.3
(Chicory)	0.115	0.008	0.000	0.000	0.000	0.000	0.000	0.000	
Helianthus tuberosus	0.093	0.006	0.020	0.001	0.000	0.000	0.000	0.000	6.5–7.7
(Jerusalem Artichoke)	0.078	0.004	0.009	0.000	0.000	0.000	0.000	0.000	

ml pollen extract. The change in absorbance at 254 nm was used as an index of enzyme activity (FOLK and SCHIRMER, 1963).

Carboxypeptidase B-like

The pollen was prepared as described for carboxypeptidase A. The assay mixture was prepared by adding 2.9 ml of freshly prepared 1 mM hippuryl-L-arginine (HA) in 25 mM Tris–HCl buffer (pH 7.65) and containing 100 mM NaCl to 0.1 ml of enzyme extract. The change of absorbance at 254 nm was measured (FOLK *et al.*, 1960).

Bee midgut analyses

Midguts of worker *Apis mellifera* from two hives were assayed for enzymic activities as described for pollen above. For an assay five midguts were removed from forager bees and the enzyme activities immediately determined, using the methodology described by GROGAN and HUNT (1977). Samples were taken from the hives semi-weekly beginning in March, 1977, and continuing for seven months.

Protein

Protein was determined as described by LOWRY *et al.* (1951) using bovine albumin as a standard.

RESULTS

The pollen results (Table 1) show that all pollens examined possessed one or more of the specific protease activities sought, and there was great variety in the presence of the different activities in different species. All pollens examined possessed chymotrypsin-like activity, but the presence of trypsin-like, carboxypeptidase A-like, and carboxypeptidase B-like activities was irregular.

Of the fourteen plant species examined only two, *Cercis canadersis* and *Taraxacum* sp. had measureable quantities of all four enzymic activities. Three of the species possessed at least trace quantities of three of the enzymes; five of the species possessed only two activities. Four of the species had only chymotryptic activity. The data of Table 1 do not reveal any apparent patterns of taxonomic representation of the four enzymic activities.

Results of the bee midgut analyses are presented in Table 2 and Fig. 1. Enzyme activities expressed as units/midgut (Table 2) clearly exceed the activities measured per mg pollen (Table 1). The chymotrypsin data of Fig. 1a show a strong tendency for direction of change (increase or decrease) in enzymic activity per mg midgut from one sampling period to the next to be the same in both hives [sign test (SIEGEL, 1956), $P = 0.194$]. The relative magnitude of change in enzymic activity shows no correlation between hives between consecutive sampling periods (sign test, $P = 0.613$). The trypsin data of Fig. 1b show no correlation between hives in direction of change in enzymic activity between consecutive sampling periods (sign test, $P = 0.613$). Carboxypeptidase activities in bee midguts (Table 2) are not sufficiently abundant to merit detailed analysis.

DISCUSSION

GIEBEL *et al.* (1971) state that in order for a honeybee to produce 4 mg of protein per day while brood rearing it must split 10 mg of pollen, assuming that 40% of the weight of pollen is protein. The assumption that pollen is 40% protein by weight is based on the work of TODD and BRETHERICK (1942) who document protein levels from 7.9% to 40% in pollens of different taxa. Todd and Bretherick do not

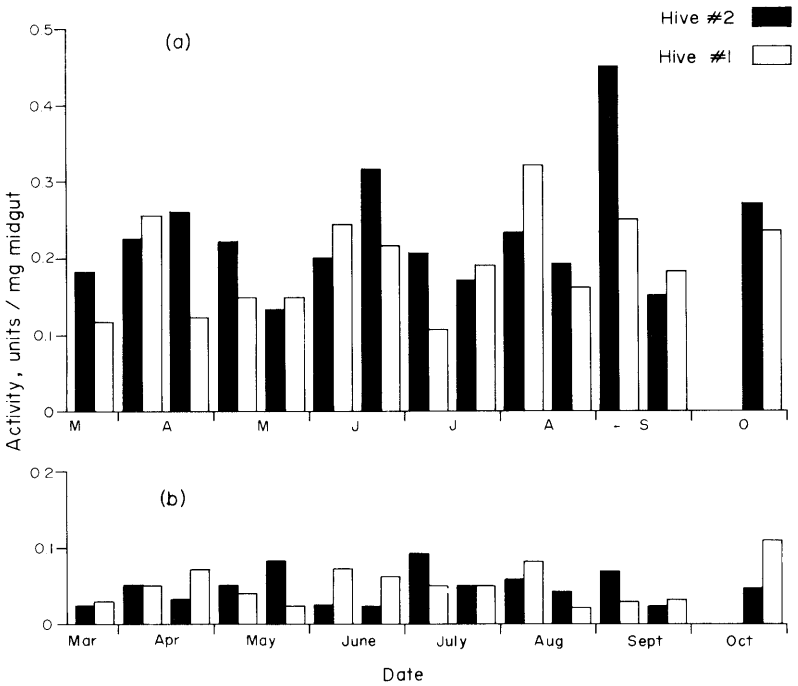

Fig. 1. The chymotrypsin (a) and trypsin (b) activities in midguts of bees from two hives of *Apis mellifera* L. Each value is based on a sample of five foragers and is an average of two determinations. Exact sampling dates are given in Table 2.

Table 2. Protease activities recorded in midguts of the honeybee, *Apis mellifera* L. Values are given for two determinations of samples of five foragers from each of two hives

Date	Mean midgut size (mg)		Enzyme activity (units/midgut) Chymotrypsin		Trypsin		Carboxypeptidase A		Carboxypeptidase B	
Hive	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
Mar. 25	12.5	10.4	1.32	1.99	0.33	0.18	0.26	0.07	0.01	0.00
			1.53	2.00	0.31	0.26	0.26	0.09	0.03	0.00
Apr. 5	9.7	9.8	2.68	2.09	0.38	0.47	0.27	0.18	0.09	0.01
			2.18	2.27	0.53	0.47	0.45	0.24	0.05	0.01
Apr. 21	9.0	9.8	1.32	2.44	0.60	0.30	0.12	0.16	0.00	0.00
			1.60	2.54	0.60	0.30	0.14	0.22	0.00	0.00
May 4	9.5	9.8	1.40	2.00	0.25	0.46	0.04	0.09	0.02	0.00
			1.44	2.30	0.35	0.50	0.06	0.09	0.02	0.00
May 23	8.0	10.1	1.22	1.30	0.14	0.70	0.04	0.18	0.00	0.00
			1.26	1.66	0.18	1.02	0.08	0.20	0.00	0.00
June 8	7.9	11.7	1.82	2.16	0.58	0.37	0.08	0.15	0.00	0.00
			1.92	2.28	0.58	0.39	0.11	0.15	0.00	0.00
June 20	9.8	9.6	2.00	2.80	0.61	0.13	0.09	0.20	0.00	0.00
			2.14	3.00	0.61	0.13	0.13	0.24	0.00	0.00
July 6	8.2	9.6	0.87	1.80	0.38	0.86	0.10	0.08	0.00	0.00
			0.87	2.20	0.42	0.92	0.16	0.12	0.00	0.00
July 25	8.1	11.7	1.40	1.86	0.38	0.49	0.06	0.07	0.00	0.00
			1.62	2.07	0.44	0.67	0.08	0.11	0.00	0.00
Aug. 11	9.2	9.9	2.97	2.20	0.65	0.50	0.09	0.09	0.25	0.02
			2.97	2.34	0.84	0.55	0.10	0.15	0.34	0.02
Aug. 25	8.5	9.7	1.35	1.80	0.11	0.29	0.05	0.04	0.02	0.02
			1.38	1.86	0.13	0.37	0.07	0.04	0.06	0.04
Sept. 8	8.7	8.7	2.04	3.26	0.15	0.52	0.05	0.09	0.07	0.01
			2.45	3.72	0.25	0.66	0.11	0.09	0.09	0.01
Sept. 29	10.6	10.1	1.83	1.20	0.26	0.17	0.02	0.12	0.02	0.01
			1.94	1.12	0.29	0.26	0.03	0.14	0.02	0.03
Oct. 20	9.9	9.5	2.30	2.49	1.09	0.38	0.01	0.09	0.01	0.01
			2.30	2.51	1.13	0.41	0.01	0.09	0.01	0.01

give their methdology, however, and in our own studies we found protein that is water leachable over 15 min to be only 5–10% by weight of pollen. To achieve the production of 4 mg protein, therefore, a bee must split from 10 mg (assuming 40% protein by weight) to 80 mg (assuming 5% protein by weight) of pollen per day. By taking these estimated extremes and utilizing the data of Table 1, it can be seen that ingested pollens could yield from 0.04–1.60 units of chymotrypsin-like activity per day. This is compared to 0.87–4.24 units/midgut measured in the honeybee (Table 2). Similarly, the potential range for trypsin-like activity derived from pollen is 0.00–0.68 units/day compared to a measured range of 0.12–1.30 units/midgut in the bee (Table 2). Estimates can be made for lowest and highest possible percent contributions of pollen enzymic activities to the activities measured in midguts. The range for chymotrypsin is 0.94–183.9% potential contribution from ingested pollen; for trypsin the range of potential contribution from ingested pollen is from 0–566.7%. Actual activities in bee guts which are attributable possibly to pollen certainly lie well below these maxima, but we feel that the likelihood of some physiological activity being contributed by pollen enzymes is high.

Correlative evidence for the nutritional role we ascribe to pollen enzymes can be drawn from the rank order and relative abundances of the various enzymes we assayed. They are the same both in pollens as a group and in bees' guts: chymotrypsin > trypsin ≫ carboxypeptidases. The correlations between the two beehives assayed are also strongly suggestive. The tendency for direction of increase or decrease of chymotryptic activity between sample periods to be the same in both hives suggests that common factors

of weather, affecting foraging rates and flowering phenologies, similarly influence the two hives in their rate of pollen collection and, hence, rate of intake of the most common pollen protease, chymotrypsin. At the same time, the observed lack of correlation between hives in magnitude of increase or decrease in chymotryptic activity as well as the lack of any correlation in tryptic activity levels could reflect, we feel, the marked differences in abundances of these enzymes between plant taxa, as shown in Table 1. The surburban environment in which the assayed bees forage is very heterogeneous, containing only small patches of a large number of flowering species, and we feel it is highly unlikely that a single pollen type would regularly predominate in the foraging of both hives. We also feel that the bee samples that we assayed fairly represent the nutritional status of the entire hive from which they were collected, based on the finding of NIXON and RIBBANDS (1952) that forager bees have very high rates of food sharing among themselves.

Further evidence for the nutritional role we ascribe to these pollen enzymes may be taken from the observations of GILBERT (1972) on pollen feeding in some *Heliconius* butterflies. These insects are incapable of pollen ingestion. Instead, they take a slurry of pollen and nectar on their proboscis and then slowly coil and uncoil their proboscis for hours, gently agitating the pollen mix. Certain pollens are known to release most of their free amino acids and proteins within minutes of being incubated in a sugar solution, but with continued incubation the free amino acid and protein concentrations increase (STANLEY and LINSKENS, 1965; LINSKENS and SCHRAUWEN, 1969). The long time period used by the butterflies for pollen agitation may be analogous to the time required for internal pollen digestion, and analysis of the butterflies pollen loads by GILBERT (1972) showed that only pollen enzymes were present.

Pollens of different plant taxa are known to differ nutritionally. The TODD and BRETHERICK (1942) study plus our own data (Table 2) suggest that protein content may vary from 5% to 40% in pollens of different taxa. A greater range of variability between pollens, however, exists in the presence and abundance of pollen enzymes. If pollen enzymes are sufficient, at least in some cases, for physiological activity, then we suspect that enzyme variability could be a factor in the known nutritional variability between pollens of different taxa.

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