DIGESTIVE PROTEASES OF TWO SPECIES OF WASPS OF THE GENUS VESPULA

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Abstract—Adults of *Vespula germanica* and *V. maculifrons* were examined for digestive protease activities. All caste members of both species that were analysed possessed trypsin, chymotrypsin, carboxypeptidase A, and carboxypeptidase B-like activities, with one exception: worker *V. maculifrons* lacked carboxypeptidase B activity. Adults of these two species can in all likelihood digest protein. The trypsin and chymotrypsin-like enzymes of the two species were also examined with specific inhibitors to determine their similarity to bovine enzymes. The wasp enzymes possess a serine and a histidine in their active centers; the trypsin-like enzyme has a specificity for basic amino acids, whereas the chymotrypsinlike enzyme specifies aromatic amino acids. These wasp enzymes are therefore similar to bovine trypsin and chymotrypsin in these respects. Methodological and interpretative differences are noted between the present study and an earlier publication on absence of digestive proteases in *Vespa orientalis*.

INTRODUCTION

IT HAS been shown that male Vespula receive part of their diet from the saliva of larvae through trophallaxis (MONTAGNER, 1963, 1964). The saliva is nutritious, containing sugars, proteins, and free amino acids (MASCHWITZ, 1966 in WILSON, 1971). There have been differences of opinion as to the importance of larval saliva as a food source for adult wasps. Montagner and Maschwitz consider the saliva to be only a colony food reserve. However, ISHAY and IKAN (1968) consider it to be the sole source of amino acid nutrition for adult Vespa orientalis. IKAN et al. (1968) say that V. orientalis lacks digestive proteases. That result, if indeed true, indicates that adult V. orientalis must receive all of their amino acid nutrition as free amino acids in solution. These reports took on new significance in light of the findings of BAKER and BAKER (1973, 1975), who found free amino acids to be regularly occurring constituents of floral nectar. Nectars of all flowering plant taxa that have been examined, including morphologically primitive species, have at least some free amino acids. BAKER and BAKER (1975) correlate amino acid presence and abundance patterns with the primary pollinators (i.e. bees, hawk moths, birds, etc.) of the plant taxa, and they suggest possible long-term co-evolutionary selective pressures on both plant and pollinator. It thus seems likely that adult wasps that visit flowers may have always had free amino acids available as a part of their diet. The possibility is further enhanced by noting that all liquid foods of adult Hymenoptera that have been examined contain free amino acids, including honeydew from Homoptera (WAY, 1963) and insect haemolymph (WYATT, 1961).

The consistency with which liquid foods of wasps

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contain free amino acids and the reported absence of digestive proteases in *Vespa orientalis* led us to initiate this study on the digestive proteases of adult *Vespula*. Freely available amino acids in natural liquid foods may have obviated the need for protein digestion capabilities in wasps in general. If adult *Vespa orientalis* lack digestive proteases, a similar condition may well be found in other wasp species. The research presented here was pursued as a test of this possibility.

MATERIALS AND METHODS

Removal of midguts

Wasps were removed under carbon dioxide anesthesia from the vacuum cleaner in which they were collected and transferred to several plastic containers. The live wasps were removed individually, sexed, and the midguts were removed. While holding a wasp with one pair of forceps, the apex of the abdomen was grasped firmly with another pair and the latter gently pulled. This movement usually removed the entire digestive tract from the abdomen. The midgut was severed from the remainder of the digestive tract and transferred to a test tube suspended in an acetone–dry ice mixture. This resulted in freezing of the midgut within 15 sec after removal from the living wasp. The test tube containing the midguts was stored at -60° C until use.

Midguts were collected from 88 workers and 58 males of *V. germanica* and from 365 workers, 26 males, and 28 queens of *V. maculifrons*. Also, the *V. maculifrons* nest contained capped queen cells from which 78 more queens eclosed. The midguts from these queens were also collected.

Enzyme assays

Trypsin. For an assay 2 to 4 *V. germanica* midguts were placed in 1 ml of 0.001 N HCl and leached for 15 min at

Species caste	Weight of midgut (mg)	Trypsin specific activity (units/mg protein)	Trypsin (units/midgut)	Trypsin (units/mg midgut)
V. germanica				
Worker	6.6	0.87	0.46	0.070
Male	4.1	0.36	0.17	0.041
V. maculifrons				
Worker	2.7	0.14	0.055	0.021
Male	1.8	0.087	0.017	0.010
Queen	10.1	0.16	0.094	0.010

Table 1. Midgut trypsin activities in adult castes of two Vespula species. Midgut weights given are the averages over all assays; enzyme activity values are averages of 2-6 replicates

 37° C. For the smaller *V. maculifrons* worker, 10 to 20 midguts were used. The extract was then centrifuged at 2500 *g* for 10 min at room temperature, and the supernate was used as the enzyme source. The reaction mix had a final volume of 3.0 ml and contained 40 mM Tris-HCl buffer (pH 8.1), 10 mM CaCl₂, and 0.1 ml of midgut extract. The reaction was initiated by the addition of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) to a final concentration of 1 mM. The change of absorbance at 247 nm was measured as an index of enzyme activity (HUMMEL, 1959).

Chymotrypsin. Midguts were extracted as described above, and enzyme activity was determined in a 3.0 ml reaction mix containing 0.1 ml of midgut extract, 40 mM Tris–HCl buffer (pH 7.8), and 5 mM CaCl₂. 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. The change in absorbance at 256 nm was measured (HUMMEL, 1959).

Carboxypeptidase A. Midguts were extracted in 10% LiCl under the conditions described for trypsin above. A reaction mix was formed by adding 2.9 ml of 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 ml of enzyme extract. The change in absorbance at 254 nm was used as an index of enzyme activity (FOLK and SCHIRMER, 1963).

Carbox ypeptidase B. Midguts were prepared as described for carboxypeptidase A. A reaction mix was prepared by adding 2.9 ml of freshly prepared 1 mM hippuryl-Larginine (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). Inhibition experiments

Inhibitors of trypsin and chymotrypsin, N- α -p-tosyl-Llysine chloromethyl ketone (TLCK) (SCHOELLMAN and SHAW, 1963), phenyl-methyl-sulfonylfluoride (PMSF) (GOLD, 1965), and L-1-tosylamine-2-phenyl-ethylchloromethyl ketone (TPCK) (SHAW *et al.*, 1965) were added to the reactions. The final concentrations of the inhibitors were: TLCK, 0.2–0.4 mM; PMSF, 0.95–1.9 mM; TPCK, 0.2 mM. The sequence of addition of the components was: enzyme source to the buffer followed by the inhibitor. The enzyme was pre-incubated for 20 min with PMSF or for one hr with TLCK or TPCK before addition of the substrate. When inhibitors were added, the volume of Tris buffer was decreased by an equivalent amount in order to maintain a constant volume.

Protein analysis

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

RESULTS

Digestive protease activity of the V. germanica midgut

Both castes examined of *V. germanica* possessed the digestive proteases trypsin, chymotrypsin, carboxypeptidase A, and carboxypeptidase B. (We did not conclusively show that any of the enzymes we studied were identical to mammalian protease enzymes. They are, however, directly comparable, and for the sake of simplicity in presenting this report they are inferred to be the same.) Trypsin was the predominant

Table 2. Midgut chymotrypsin activities in adult castes of two Vespula species. Enzyme activity values are averages of 2–6 replicates

Species caste	Chymotrypsin specific activity (units/mg protein)	Chymotrypsin (units/midgut)	Chymotrypsin (units/mg midgut)
V. germanica			
Worker	0.130	0.086	0.013
Male	0.072	0.026	0.0064
V. maculifrons			
Worker	0.076	0.037	0.014
Male	0.081	0.016	0.0085
Queen	0.126	0.079	0.0079



Fig. 1. The trypsin-like midgut activities of male (a) and worker (b) and the chymotrypsin-like midgut activities of male (c) and worker (d) *Vespula germanica*. The response is shown of these enzymes $(\bullet - \bullet)$ to PMSF ($\circ - \circ$), TLCK ($\bullet - \bullet$), and TPCK ($\Box - - \Box$).

enzyme, with specific activity 6.7 and 5.0 times greater than the next most abundant enzyme, chymotrypsin, in workers and males respectively (Tables 1, 2). The reaction rates for trypsin were linear over a 7 min time period (Figs. 1(a), 1(b)).

Chymotrypsin was present in significant amounts in both worker and male V. germanica (Table 2). The specific activity of chymotrypsin was 0.130 and 0.072 for workers and males respectively. The reaction rates for this enzyme were not linear; the rate of the first $2 \min$ exceeded the rate of the last $5 \min$ (Figs. 1(c), 1(d)).

Both worker and male *V. germanica* possessed midgut carboxypeptidase A activity, but the amount of activity was less than either trypsin or chymotrypsin (Tables 1, 2, and 3). The specific activity of this enzyme was 7.3 times less than trypsin activity and 1.5 times less than the chymotrypsin specific activity in males and 15.0 and 2.2 times less than those enzymes in workers. The specific activity of carboxypeptidase A in workers exceeded that of males by a factor of 1.2 (Table 3).

Carboxypeptidase B activity was also found in the midguts of worker and male *V. germanica*, but its concentration was very low (Table 4). The specific activity in workers was approximately equal to that in males, but the workers contained approximately twice the amount of total enzyme (Table 4). In males, trypsin specific activity was 18 times as great and chymotrypsin activity was 3.6 times as great as carboxypeptidase

Table 3. Midgut carboxypeptidase A activities in adult castes of two Vespula species. Enzyme activity values are averages of 2-6 replicates

Species caste	Carboxypeptidase A specific activity (units/mg protein)	Carboxypeptidase A (units/midgut)	Carboxypeptidase A (units/mg midgut)
V. germanica			
Worker	0.058	0.030	0.0038
Male	0.049	0.014	0.0028
V maculifrons			
Worker	0.044	0.0088	0.0036
Male	0.044	0.011	0.0062
Queen	0.186	0.097	0.0091

Species caste	Carboxypeptidase B specific activity (units/mg protein)	Carboxypeptidase B (units/midgut)	Carboxypeptidase B (units/mg midgut)
V. aermanica			
Worker	0.021	0.011	0.0013
Male	0.020	0.0056	0.0012
V. maculifrons			
Worker	0.000	0.000	0.000
Male	0.0046	0.0012	0.0007
Queen	0.082	0.043	0.0043

 Table 4. Midgut carboxypeptidase B activities in adult castes of two Vespula species. Enzyme activity values are averages of 2-6 replicates

B; carboxypeptidase A specific activity exceeded carboxypeptidase B by a factor of 2.5. In workers the values were 41, 6.2, and 2.8 respectively.

Effects of inhibitors on V. germanica, trypsin and chymotrypsin

Several inhibitors were used in order to compare the *V. germanica* trypsin enzyme to bovine trypsin. PMSF was used to test the enzyme for the presence of a serine group in its active center. The trypsin in both worker and male *V. germanica* was inhibited by 1.9 mM PMSF (Figs. 1(a), 1(b)) and resembles bovine trypsin in this respect. The range of inhibition varied from 63% to 86% in extracts of both male and worker midguts.

TLCK will inhibit an enzyme that has a histidine in its active center and a specificity for basic amino acids. The trypsin in the worker and male wasps was inhibited by 0.4 mM TLCK (Figs. 1(a), 1(d)), indicating similarity between the *V. germanica* trypsin and bovine trypsin. The range of inhibition observed was 43% to 61%.

The effects of TPCK on the *V. germanica* trypsin are shown in Figs. 1(a) and 1(b). As can be seen, TPCK did not inhibit this enzyme. In order for TPCK to inhibit an enzyme it must possess a histidine in its active center and possess a specificity for aromatic amino acids. That TPCK did not inhibit the wasp trypsin further suggests a similarity between the trypsin of *V. germanica* and bovine trypsin.

Bovine chymotrypsin contains a serine in its active center and is inhibited by PMSF. As can be seen in Figs. 1(c) and 1(d) the chymotrypsin of both male and worker V. germanica was inhibited by PMSF, but the degree of inhibition was neither immediate nor very obvious in a 7 min experiment. In fact, during the first 3 min of the experiment, the rate of the inhibited reaction equalled or in many cases exceeded the rate of the uninhibited reaction (e.g. Fig. 1(d)). In most cases the assay had to exceed 7 min before inhibition could be demonstrated. With an extended incubation time, however, PMSF always inhibited V. germanica chymotrypsin. Therefore, V. germanica chymotrypsin is similar to bovine chymotrypsin in that both enzymes are inhibited by PMSF. The wasp enzyme obviously differs in some respects from bovine chymotrypsin, however, as the latter is inhibited by PMSF from time zero.

TLCK did not significantly inhibit the male or worker *V. germanica* chymotrypsin (Figs. 1(c), 1(d)). In order for TLCK to have an inhibitory effect an enzyme must possess a substrate specificity for basic amino acids. Like bovine chymotrypsin, the wasp chymotrypsin does not.

TPCK does inhibit male and worker *V. germanica* chymotrypsin (Figs. 1(c), 1(d)). Therefore, this chymotrypsin and the bovine enzyme are similar in that both have a histidine group in their active center and both have specificities for aromatic amino acids. However, as was noted for PMSF, TPCK did not immediately cause inhibition. Ten minute incubations were usually required before inhibition could be demonstrated.

Midgut protease activity of V. maculifrons

The trypsin-like activities of V. maculifrons were similar to but less than those of V. germanica (Table 1). The reaction rates were linear over time (Figs. 2(a), 2(b), 2(c)), and the specific activity of the worker trypsin was approximately 1.6 times greater than that of males.

The trypsin activities for *V. maculifrons* queens are also shown in Table 1. Although the queens collected in the field and those that eclosed in the laboratory were analyzed separately, there were no significant differences in their activities, and therefore all replicates of both groups were averaged. The specific activity of queen trypsin exceeded the worker trypsin by a factor of 1.1 and was 3.7 times greater than in males.

Significant amounts of chymotrypsin were also found in the midguts of male, worker, and queen V. *maculifrons* (Table 2). As in V. *germanica* the reaction rates were not linear with time, with the first 2 to 3 min being greater than the last 4 to 5 min (Figs. 2(d), 2(e), 2(f)). The chymotrypsin activity was less than the trypsin-like activity in all castes. Trypsin specific activity exceeded that of chymotrypsin by a factor of 1.8 in workers, 1.1 in males, and 1.3 in queens. The specific activity of chymotrypsin in queens was



Fig. 2. The trypsin-like midgut activities of male (a), worker (b), and queen (c) and the chymotrypsinlike midgut activities of male (d), worker (e), and queen (f) *Vespula maculifrons.* The response is shown of these enzymes (\bullet — \bullet) to PMSF (\circ — \circ), TLCK (\blacktriangle — \bullet), and TPCK (\Box — $-\Box$).

1.6 times greater than in males and 1.7 times greater than in workers.

Carboxypeptidase A activity was found in the midguts of all castes of *V. maculifrons.* In queens carboxypeptidase A specific activity was greater than chymotrypsin; it was in fact about equal to that of trypsin. This result is the same when activity is expressed as units per midgut (Tables 1, 2, and 3). In caste comparisons of carboxypeptidase A specific activities, the queen enzyme had the highest activity, followed equally by workers and males (Table 3).

Carboxypeptidase B activity was found in queen and male V. maculifrons midguts but not in midguts of workers. Although males and queens possessed measurable amounts of carboxypeptidase B, the values were low (Table 4). Of all enzymes examined, carboxypeptidase B occurred in the lowest concentration. Of the castes that possessed carboxypeptidase B, the queens possessed more, with the specific activity exceeding that of males by a factor of 1.7.

Effects of inhibitors on V. maculifrons trypsin and chymotrypsin

Vespula maculifrons trypsin-like enzyme of workers, males and queens was inhibited by PMSF (Figs. 2(a), 2(b), 2(c)). The results were similar to those obtained for trypsin in V. germanica. The range of inhibition obtained with 1.9 mM PMSF in all castes of V. maculifrons was from 43% to 100%. The trypsin of this species was also inhibited by TLCK (Figs. 2(a), 2(b), 2(c)). The range of inhibition observed in all castes was 66% to 100%. TPCK did not inhibit the trypsin enzyme of this species (Figs. 2(a), 2(b), 2(c)). Bovine trypsin and the trypsin enzyme of *V. maculifrons* therefore are similar in their response to inhibitors.

V. maculifrons chymotrypsin was also inhibited by PMSF (Figs. 2(d), 2(e), 2(f)), but as in *V. germanica* the degree of inhibition was negligible in the first few minutes of the assay. However, inhibition occurred if the enzyme reaction was continued. TLCK did not inhibit *V. maculifrons* chymotrypsin (Figs. 2(d), 2(e), 2(f)). TPCK did inhibit the chymotrypsin of male, worker, and queen *V. maculifrons* (Figs. 2(d), 2(e), 2(f)). However, the degree of inhibition was low in the initial minutes of the assay but increased with time. Ten minute incubations were sufficient to demonstrate inhibition. These results were similar to those noted in *V. germanica*.

DISCUSSION

Our results demonstrate that adult *Vespula* of the two species examined contain considerable digestive protease activity. We tested for the presence of four enzymes (trypsin, chymotrypsin, carboxypeptidase A, and carboxypeptidase B) that are normally associated with protein digestion in higher animals and found activities in *Vespula* midguts similar to the activities of these four mammalian enzymes. All caste members possessed all four activities, with one exception: *V. maculifrons* workers lacked carboxypeptidase B activity. Average total protease units per midgut can

be calculated, and although our survey is narrowly limited, it seems likely that intraspecific caste rankings are queen > worker > male. The values we obtained for average total protease units per midgut in *V. maculifrons* are queen = 0.31, worker = 0.10, male = 0.05, and in *V. germanica*, worker = 0.59, male = 0.22. Ecological or evolutionary correlates of these caste differences are not now apparent.

We assumed that proteases extracted from the *Vespula* midguts during the leaching process were from the midgut cavity. There is a possibility, however, that some of the enzyme activity was intracellular in origin, but we did not examine the midguts for the presence of lysozomes. The inhibition characteristics of the chymotrypsin-like enzyme that indicated differences from bovine chymotrypsin are, therefore, suspect in this regard. We further assumed, however, that regardless of the origin, the proteases that were present were capable of protein digestion.

Our results do not agree with results reported by IKAN et al. (1969) on Vespa orientalis. These workers concluded that adult V. orientalis do not possess digestive proteases. The discrepancy between their results and ours might be attributed to two factors other than the species involved: methodology and interpretation. All of our studies were conducted on isolated Vespula midguts. IKAN et al. obtained digestive juices by squeezing the abdomens of Vespa orien*talis* and aspirating the vomitus with a pipette. Due to the proventricular anatomy of wasps the vomitus that they examined was almost certainly of crop origin, not midgut. Also, IKAN et al. (1969) examined total body protease activity rather than midgut protease activity. The protease specific activity of total body protein would be lower than that of midgut protein. Interpretative differences may be noted by examining the tabular data of IKAN et al. (1969). These data are presented in an irregular manner; specific activity values for all enzymes in all castes are not given. Of the values presented, however, we feel that most do, in fact, indicate positive enzymic activity. The primary discrepancy between our results from Vespula and the reported results from Vespa orientalis may be largely one of data interpretation. In our assays on Vespula we found no instance in which any adult caste member was totally lacking in midgut protease activity.

In summary, we have documented the presence of digestive protease in midguts of all castes of the two *Vespula* species that were available for study. These insects are in all likelihood fully capable of protein digestion.

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