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Properties and compartmentalization of digestive carbohydrases and proteases in *Scaptotrigona bipunctata* (Apidae: Meliponinae) larvae

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Summary — Aminopeptidase (pH optimum, pH_o, 7.5; enzyme relative molecular weights, M_r values: 1, 110 000; 2, 190 000; 3, 300 000), amylase (pH_o 5.5, M_r values: 1, 21 000; 2, 68 000); cellobiase (pH_o 5.5) and maltase (pH_o 5.0, M_r values: 1, 75 000; 2, 110 000; 3, 200 000) are found in the anterior (60–80%) and posterior (20–35%) midgut contents, with minor amounts occurring in midgut cells (2–5%). Trypsin (pH_o 7.0, M_r 38 000) occurs mainly in the posterior (62%) rather than in the anterior (37%) midgut contents. Maltase 1 is more active on sucrose than on maltose, the reverse being true for the other maltases. A cysteine–proteinase (pH_o 5.6, M_r 79 000) was found in major amounts in the pollen grains ingested by the larvae. The results suggest that, except for a cysteine–proteinase derived from ingested pollen, all digestive enzymes originate in the midgut tissue and are most active in the luminal contents. Evidence is presented supporting the hypothesis that enzymes and nutrients diffusing through the peritrophic membrane are translocated forward by a countercurrent flux. The absence of a midgut differentiation of midgut luminal pH in *S bipunctata* larvae is though to be derived from putative Hymenopteran ancestors.

***Scaptotrigona bipunctata* / digestion / enzyme activity / Meliponinae**

INTRODUCTION

In spite of being a major insect order, the Hymenoptera have been the subject of few studies regarding digestive physiology (Terra, 1988, 1990). Among the Hymenoptera, Apidae is no exception. The properties of some adult *Apis mellifera* digestive enzymes, such as maltase (Huber, 1975), trypsin, chymotrypsin and 2 other endopeptidases (Giebel *et al,* 1971; Dahlman *et al,* 1978), have been studied in some detail, whereas others such as lactase (Peng, 1981), have been

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poorly characterized. Only the midgut compartmentalization of trypsin has been studied. This enzyme occurs in midgut cells and in luminal spaces inside (endoperitrophic) and outside (ectoperitrophic) the peritrophic membrane (Moritz and Crailsheim, 1987; Jimenez and Gilliam, 1989).

The absorption sites for leucine and glucose have been found in the anterior two-thirds of the honeybee ventriculus (Crailsheim, 1988a, b). These data, together with a detailed ultrastructural and cytochemical study of adult *Apis mellifera* midgut, led Jimenez and Gilliam (1990) to propose that in these bees enzymes and nutrients diffusing through the peritrophic membrane are translocated forward by a counter-current flux (endo-ectoperitrophic circulation), as previously described for other insects (see review in Terra, 1990). In spite of these reports, a detailed model for the digestion by adult bees of the material extruded from or present on the walls of pollen grains is not available. Moreover, digestive physiology in larval bees is still being investigated, and only the properties of trypsin and chymotrypsin have been determined to some extent in larval bees (Dahlman *et al*, 1978).

In this paper we describe the distribution and properties of several hydrolases occurring in different midgut regions of *Scaptotrigona bipunctata* (Apidae, Meliponinae) larvae. The results suggest that except for a cysteine–proteinase derived from ingested pollen, all digestive enzymes originate in the midgut tissue and are most active in the luminal contents. In addition, evidence is presented suggesting the existence of an endo-ectoperitrophic circulation of digestive enzymes and nutrients, and that the larval bees have lost a midgut differentiation of midgut luminal pH hypothetically present in Hymenopteran ancestors.

MATERIALS AND METHODS

Animals

Scaptotrigona bipunctata (Lepeletier, 1836) (Hymenoptera: Apidae: Meliponinae) combs with 5th instar larvae were collected from free-flying colonies. The 5th instar is the last larval instar stage (Cruz-Landim and Mello, 1981). Only larvae with midgut containing ample food and showing no connection between the midgut and the hindgut (hindgut lacking food) were used in the determinations.

pH of gut contents

S bipunctata larvae were immobilized by placing them on crushed ice and were then dissected in cold 231 mM NaCl. The rinsed midguts were transferred to a glass slide and sectioned in 3 parts of approximately the same length. To the contents of each section was added 20 µl of a 10-fold dilution of a universal pH indicator (pH 4–10) or 20 µl of 0.04% methyl red. The resulting colored solutions were compared with suitable standards.

Preparation of samples of pollen and of gut sections

Samples (67 mg) of pollen collected by *S bipunctata* were suspended in 1 ml of 5 mM citrate–sodium phosphate buffer pH 5.6 containing 3 mM EDTA (for abbreviations, see table I) and 1.5 mM DTT. The suspensions were ruptured with a sonicator semimicroprobe (Branson 250), with output set at 3 using 3 pulses of 30-s each at 10-s intervals. The sonicates were then homogenized in a Potter–Elvehjem homogenizer, passed through a 100-µm pore size nylon mesh and centrifuged at 100 000 *g* for 60 min at 4 °C. The resulting supernatants were used as pollen enzyme sources.

Larvae were dissected as described above. After the removal of the midgut, the tissue and the peritrophic membrane with contents were pulled apart and sectioned in an anterior and a posterior region. After being thoroughly rinsed

Table I. Assay conditions and methods used in the determination of hydrolases in *S bipunctata* larvae.

Enzyme	*Substrate*	*Concentration*	*pH*	*Substance or group determined*	*Ref*
Alkaline phosphatase	NPP	5.6 mM	10.4	Nitrophenolate	Terra *et al* (1979)
Aminopeptidase	LpNA	1 mM	7.5	Nitroaniline	Erlanger *et al* (1961)
Amylase	Starch	0.5%	5.0	Reducing groups	Noelting and Bernfeld (1948)
Cellobiase	Cellobiose	7 mM	5.5	Glucose	Dahlqvist (1968)
Glutamyl transferase	GpNA, Gly-Gly	1 mM, 10 mM	8.8	Nitroaniline	Erlanger *et al* (1961)
Maltase	Maltose	7 mM	5.0	Glucose	Dahlqvist (1968)
Proteinase (Cysteine)	BANA	0.5 mM	5.6	Naphthylamine	Hopsu *et al* (1966)
Proteinase (total)	Azocasein	0.5%	5.5–7.5	Dye released	Charney and Tomarelli (1947)
Sucrase	Sucrose	7 mM	5.0	Glucose	Dahlqvist (1968)
Trypsin	BAPA	0.83 mM	7.5	Nitroaniline	Erlanger *et al* (1961)

Assays were performed at 30 °C at the indicated pH values. The buffers (0.05 M) used were: citrate/phosphate (pH 5–7.5, except for trypsin and aminopeptidase), phosphate (7.5 for trypsin and aminopeptidase), Tris–HCl (pH 8.8) and glycine/NaOH (pH 10.4). The reaction medium with starch contained in addition to buffer 10 mM NaCl; with NPP, 1 mM $MgSO_4$ and with BANA, 3 mM EDTA and 1.5 mM DTT. Incubations were carried out for at least 4 different periods of time and the initial rates of hydrolysis were calculated. All assays were performed under conditions such that activity was proportional to protein concentration and to time. 1 U of enzyme is defined as the amount that catalyzes the cleavage of 1 µmol of substrate (or bond) per min. Abbreviations used in this table and in the text: BANA, *N*-benzoyl-L-arginine-β-naphthylamide; BAPA, α-N-benzoyl-DL-arginine-*p*-nitroanilide; DTT, dithiothreitol; EDTA, ethylenediamine – tetraacetic acid; GpNA, L-γ-glutamyl-*p*-nitroanilide; LpNA, L-leucine-*p*-nitroanilide; M_r, relative molecular weight, NPP, *p*-nitrophenyl phosphate; pHMB, *p*-hydroxymercuribenzoate; SBTI, soybean trypsin inhibitor.

with 231 mM NaCl, midgut tissue was homogenized in double-distilled water using a Potter–Elvehjem homogenizer, and then passed through a 100-μm pore size nylon mesh. Peritrophic membranes and contents were homogenized in the same manner as midgut preparations without previously rinsing in saline solution. Larval bodies from which the guts had been removed were rinsed, homogenized and passed through a nylon mesh as described for midgut tissue. The resulting filtrates from larval body homogenates were than centrifuged at 10 000 *g* for 10 min at 4 °C and the supernatants used as an enzyme source. Membrane-bound and soluble enzymes were determined in midgut tissue by homogenizing in water with the aid of a Potter–Elvehjem homogenizer and, after centrifugation of the homogenates at 100 000 *g* for 60 min at 4 °C, the resulting supernatants (soluble proteins) and pellets (membrane-bound proteins) were assayed for several enzymes. All enzymes assayed could be stored for at least 1 month at –20 °C without a noticeable change in their activities.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in gels of different concentrations as described by Hedrick and Smith (1968), using the system of Davis (1964), in glass tubes of 5-mm id and 100 mm length. Other details have been described elsewhere (Terra and Ferreira, 1983). Recovery of the aminopeptidase activities applied to the gels was in the range of 30–40%.

Density-gradient ultracentrifugation

Samples (0.2 ml) of preparations containing 1.5 mg bovine hemoglobin and 50 μg bovine liver catalase were layered on top of 10-ml linear glycerol gradients (10–30%, W/v) made up in 50 mM citrate–sodium phosphate pH 5.6, unless otherwise specified. Centrifugation and collection of fractions were performed as described previously (Terra and Ferreira, 1983). Molecular relative weight (M_r) values of enzymes assayed in the fractions were calculated by the method of Martin and Ames (1961), using sedimentation rates of bovine hemoglobin (M_r 64 500) and bovine liver catalase (M_r 232 000) as reference standards. Recoveries (%) of the activities applied to the gradients were: aminopeptidase, 30–35; amylase, 30–50; maltase, 8–15; cysteine–proteinase, 50–60; total proteinase, 40–60; trypsin, 40–60.

Hydrolase assays and protein determination

Protein was determined according to Bradford (1976) using ovalbumin as a standard. Enzymatic assays were carried out as described in table I.

RESULTS

Luminal pH and distribution of hydrolases in midgut

The pH of *S bipunctata* larval midgut contents was found to decrease slightly along the midgut as follows (mean ± SEM; N = 10): anterior midgut, 6.0 ± 0.1; middle midgut, 5.7 ± 0.2; posterior midgut, 5.6 ± 0.1. However, the differences found were small, and may not be significant.

Pollen is known to contain digestive enzymes (Grogan and Hunt, 1979). Enzymatic assays were therefore performed on pollen masses (taken from *S bipunctata* pollen pots) equivalent to the *S bipunctata* midgut fresh-weight (6.7 ± 0.2 mg/animal, mean ± SEM; N = 20). The activities found were < 5% of the activities displayed in table II. The role of pollen hydrolases in larval digestion was thus discounted, except for that a cysteine–proteinase (see below). Larval *S bipunctata* bodies from which the guts had been removed were homogenized instead of their salivary glands alone because of the difficulty in dissecting these glands. The amount of each hydrolase in these homogenates was always < 5% of the activity found in the midgut.

Digestive enzymes are found in high amounts in *S bipunctata* midgut contents, whereas only minor amounts are recovered from midgut cells (table II). Except for trypsin, which predominates in posterior midgut contents, all the other enzymes assayed occur mostly in the anterior midgut contents. In cells, amylase and maltase are mainly found in the anterior region; the other enzymes are uniformly distributed throughout the tissue (table II). Enzyme assays and protein determinations carried out in the different sections amounted to 75–85% of similar determinations carried out in whole midguts (data not shown). This suggests that small amounts of material were lost during dissections and that there seem to be no activators or inhibitors in the midgut cells or contents which affect *S bipunctata* digestive enzymes. This also suggests that there are no enzymes that are restricted to the ectoperitrophic space. The activity of all digestive enzymes studied in *S bipunctata* midgut homogenates was proportional to a time period of at least 12 h. This supports the assertion that *S bipunctata* digestive enzymes are remarkably stable in the presence of their own proteases.

The existence of soluble and membrane-bound activities for each cellular enzyme was investigated. Rinsed midgut tissues were homogenized in water and, after centrifuging the homogenates at 100 000 *g* for 60 min at 4 °C, the resulting supernatant and pellet was assayed for several enzymes. The following activities (% total activity) were found in the supernatant (the remaining activities were recovered in the pellets) (mean ± SEM; $N = 4$): aminopeptidase, 38 ± 4; amylase, 25.8 ± 0.5; cellobiase, 86 ± 8; maltase, 28 ± 5. Trypsin was not investigated due to its very low activity in tissue. The results suggest that, except for cellobiase, tissue enzymes are mostly membrane-bound, this being perhaps associated with the microvillar membranes. Attempts to confirm this by using known insect microvillar enzyme markers such as γ-glutamyl transferase (Espinoza-Fuentes and Terra, 1987) and alkaline phosphatase (Ferreira and Terra,

Table II. Hydrolases and protein present in midgut cells and contents of *S bipunctata* larvae.

Enzyme	*Midgut cells*		*Midgut contents*	
	Anterior	*Posterior*	*Anterior*	*Posterior*
Aminopeptidase	1.6 (65)	1.7 (46)	56.3 (330)	40.4 (100)
Amylase	3.5 (41)	1.3 (24)	61.9 (270)	33.3 (80)
Cellobiase	0.9 (0.7)	1.2 (0.84)	79.6 (12)	18.3 (1.3)
Maltase	1.4 (36)	0.3 (7.5)	61.3 (400)	37 (140)
Trypsin	0.5 (0.6)	0.5 (0.6)	37 (12.8)	62 (11.8)
Protein (μg/animal)	28	23	135	210

Enzyme results are relative activities displayed as percentage of the sum of activities found in the different sections of the midgut and specific activities (in parentheses) displayed as mUnits/mg protein. Figures are means based on determinations carried out in 7 different preparations obtained from 20 larvae each. SEM were found to be 10–15% (protein determinations), 10–25% (enzyme content determinations) or 20–45% (enzyme cell homogenate determinations) of the means.

1980; Santos and Terra, 1984; Ferreira *et al*, 1988) were unsuccessful. About 45% of the glutamyl transferase and 95% of alkaline phosphatase activities were found in the midgut luminal contents of *S bipunctata* (data not shown). Furthermore, most of these activities are in the soluble fraction. Thus, these enzymes cannot serve as midgut microvillar markers in the larval bee. *S bipunctata* membrane-bound enzymes were not investigated further due to their low activity.

Properties of midgut carbohydrases

The pH optima of *S bipunctata* larval midgut carbohydrases are: amylase, 5.0; cellobiase, 5.5 and maltase, 5.0 (data not shown). Amylase activities sediment as proteins of M_r 21 000 ± 800 (minor peak) and 68 000 ± 600 (major peak) (fig 1A). Amylase assays with and without chloride addition were carried out on midgut homogenates before and after ultracentrifugation in glycerol gradients, with identical results (data not shown). Thus *S bipunctata* larval amylase is not activated by chloride. The sedimentation profile of maltase activity displays peaks corresponding to the following M_r values: 1) 75 000 ± 7 000; 2) 110 000 ± 20 000; 3) 200 000 ± 30 000 (fig 1B). Sucrase displays a major peak, which sediments as peak 1 of maltase, and one shoulder which sediments with peak 2 of maltase (compare fig 1B,C). The results suggest that maltase 1 is more active on sucrose than on maltose, the reverse being true for maltases 2 and 3. It is possible that maltase 3 is a dimer of maltase 2. Recovery of amylase and maltase activities after electrophoretic separation in polyacrylamide gels was too low to permit further study with these techniques. Cellobiase was not studied by electrophoresis and ultracentrifugation due to its low activity.

Properties of midgut proteases

S bipunctata proteolytic activity with azocasein as substrate displays a peak ≈ pH 7.0, which is decreased in the presence of SBTI, and a shoulder ≈ pH 6, which is increased in the presence of EDTA plus DTT (fig 2).

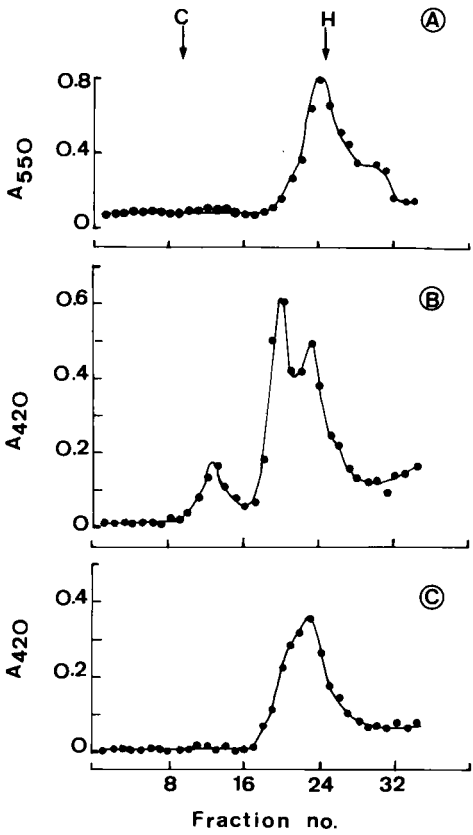

Fig 1. Sedimentation profiles of *S bipunctata* midgut carbohydrases in a linear glycerol gradient. Fractions were collected from the bottom of the tube. Samples are supernatants (100 000 g, 60 min) obtained from midgut homogenates. (**A**) amylase; (**B**) maltase; (**C**) sucrase. Profiles obtained from several other preparations are similar to those shown, which were obtained from the same experiment (gradient). **C**, bovine liver catalase (M_r 232 000); **H**, bovine hemoglobin (M_r 64 500). 1, 2 and 3 refer to hydrolases with different Mr values. Other details are given in *Materials and Methods.*

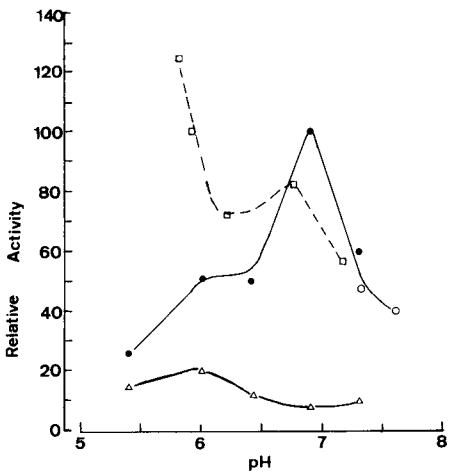

Fig 2. Effect of pH on *S bipunctata* midgut proteinase activity. The enzyme source was the supernatants (100 000 *g*, 60 min) from midgut homogenates and the buffers used were: 50 mM citrate–sodium phosphate (●, Δ, □) or sodium phosphate (O). The substrates used were: 0.5% azocasein (●), 0.5% azocasein with 17 μM SBTI (Δ); and 0.5% azocasein with 3 mM EDTA and 1.5 mM DTT (□). Profiles obtained from several other preparations are similar to those shown.

This suggests the existence of a major trypsin-like proteinase and a minor cysteine–proteinase. The occurrence of a major trypsin-like enzyme was confirmed with the use of the trypsin substrate BAPA. As shown in table III, SBTI abolishes BAPA hydrolysis and the presence of EDTA plus DTT do not affect this hydrolysis. The existence of a cysteine proteinase was supported by the finding that azocasein hydrolysis at pH 5.6 is not abolished by SBTI and that even in the presence of this compound EDTA plus DTT are able to increase azocasein hydrolysis (table III).

Figure 3A and B shows that the major azocasein hydrolase (peak 1) is active upon BAPA, is inhibited by SBTI and sediments as molecule with an M_r of 38 000 ± 2 000. Peak 2 in figure 3A sediments as a molecule with M_r 79 000 ± 5 000; it does not appear if the gradient and assay media does not contain EDTA and DTT (data not shown); peak 2 is able to hydrolyze BANA (compare figs 3A, C), and is inhibited by pHMB (fig 3C). These results, together with previous data, strongly suggest that peak 1 is a trypsin-like enzyme, whereas peak 2 is a cysteine–proteinase. Trypsin is absent (data not shown), whereas cysteine–proteinase is present in the pollen ingested by the larvae (fig 3C). Thus, only trypsin should be synthesized by *S bipunctata* larvae. Further study of *S bipunctata* proteinases using electrophoretic techniques was prevented due to their poor recoveries after the runs.

S bipunctata midgut aminopeptidase activity has a pH optimum of 7.5 (data not shown), sediments as a major enzyme (aminopeptidase 2) with M_r 190 000 ± 20 000 and a minor enzyme with M_r 300 000 ± 9 000 (aminopeptidase 3) (fig 4B). After electrophoresis, only one molecular form of aminopeptidase is detected (fig 4A) in each of several polyacrylamide gel concentrations (fig 4C). Aminopeptidase M_r value calculated from electropho-

Table III. Effect of several compounds on the BAPA and azocasein hydrolase activities from *S bipunctata* midguts.

Addition	*BAPA*	*Azocasein*
None	100	100
SBTI	5 ± 5	30 ± 7
EDTA + DTT	105 ± 8	170 ± 20
SBTI + EDTA + DTT	9 ± 9	110 ± 30

The enzyme samples were supernatants (100 000 *g* for 60 min) from midgut homogenates. When indicated, assay media contained 17 μM SBTI, 3 mM EDTA and 1.5 mM DTT. All assays were performed in 50 mM citrate–sodium phosphate, pH 5.6. Figures indicate relative activities (mean ± SEM; $N = 4$).

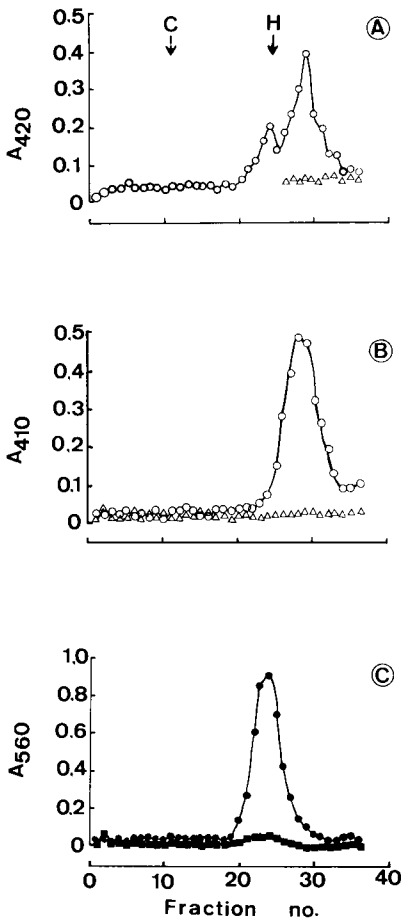

Fig 3. Sedimentation profiles in a linear glycerol gradient of proteinases from *S bipunctata* midgut (O, Δ) or from pollen (●, ■) in the presence (**A** and **C**) or in the absence (**B**) of 0.1% albumin, 3 mM EDTA and 1.5 mM DTT. (**A**) assays with azocasein as substrate in the absence (O) or in the presence of 17 μM SBTI (Δ). (**B**) Assays with BAPA as substrate in the presence (Δ) or absence (O) of 17 μM SBTI. (**C**) Assays with BANA as substrate in the presence (■) or absence (●) of 3 mM pHMB. Assays in (**A**) and (**C**) were performed in 50 mM citrate–sodium phosphate pH 5.6; in (**B**) as described for trypsin. Profiles obtained from several other preparations are similar to those shown. Other details as in the legend to figure 1.

retic data is 110 000 (aminopeptidase 1) (fig 4D). This value is ≈ 50% the M_r value of aminopeptidase 2, resolved by ultracentrifugation, if one takes into account the SEM found in these determinations. Aminopeptidase 2 has ≈ two-thirds the M_r value of aminopeptidase 3. These results suggest that there is only one molecular species of aminopeptidase in *S bipunctata* midgut with M_r 110 000, and that it is able to form dimers and trimers.

DISCUSSION

Origin and properties of S bipunctata *larval digestive enzymes*

Based on their morphological and histochemical data, Cruz-Landim and Mello (1981) argued that *Scaptotrigona postica* salivary glands were involved in lubricating the food to be ingested and, at the end of the larval stage, in secreting a silken cocoon. Our results lend support to their proposal, since digestive enzymes in salivary glands accounted for < 5% of the activity found in midguts. Thus it is possible that the activity found is a result of contamination during dissection. The only significant digestive enzyme found in pollen is a cysteine–proteinase (fig 3C), which accounts for ≈ 25% of the total proteolytic activity found in *S bipunctata* midgut, as determined at the midgut luminal pH (fig 3A). Our data thus showed that digestion is accomplished in *S bipunctata* midguts under the action of midgut enzymes, with the help of a pollen cysteine–proteinase.

The properties of amylase, trypsin and aminopeptidase from *S bipunctata* are similar to those from other insects (see references in Vonk and Western, 1984; Applebaum, 1985; Ferreira and Terra, 1986; Baker, 1989; Lemos and Terra, 1992). Nevertheless, *S bipunctata* amylase is not

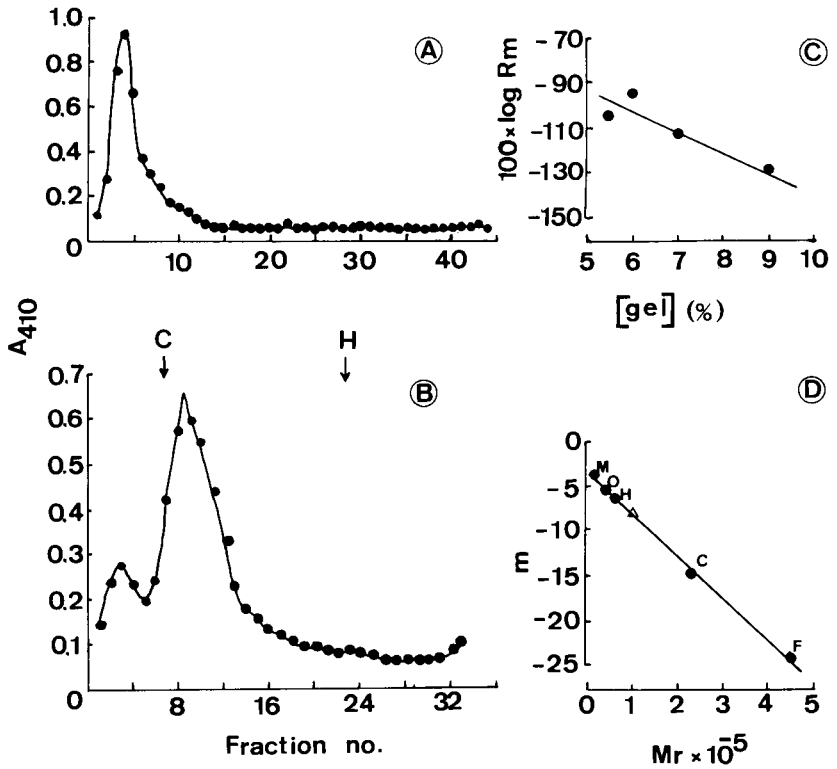

Fig 4. Properties of *S bipunctata* midgut aminopeptidase. Samples are supernatants (100 000 *g*, 60 min) obtained from midgut homogenates. (**A**) Electrophoretic run of aminopeptidase in 5.5% polyacrylamide gel. (**B**) Sedimentation profile of aminopeptidase in a linear glycerol gradient prepared in 50 mM phosphate buffer pH 6.2. (**C**) Effect of different polyacrylamide gel concentrations on the electrophoretic migration of aminopeptidase. R_m, electrophoretic migration of the enzyme in relation to the tracking dye. (**D**) Determination of the M_r of aminopeptidase (Δ) by the method of Hedrick and Smith (1968). The slopes of plots similar to that in figure 4C are m values. M_r markers: M, myoglobin (M_r 17 800); O, ovalbumin (M_r 43 000); H, bovine hemoglobin (M_r 64 500); C, catalase (M_r 232 000); and F, ferritin (M_r 450 000).

activated by chloride, like some other insect amylases (Baker, 1989; and references therein), and in contrast to most animal amylases (Vonk and Western, 1984). There are in *S bipunctata* at least 2 maltases (α-glucosidase, EC 3.2.1.20) with pH optima and M_r values similar to those found in other insects (see references in Baker, 1991; Jordão and Terra, 1991). One of the *S bipunctata* maltases hydrolyzes sucrose better than maltose. No β-fructosidase (EC 3.2.1.26) seems to occur in *S bipunctata*. It should be noted that sucrose is a major constituent of nectar (Percival, 1961), used by the worker bee to prepare honey (which still contains sucrose), and pollen (Todd and Bretherick, 1942). The absence of β-fructosidase contrasts with enzymological data obtained for other insects, such as the Lepidoptera, which, like bees, are major consumers of nectar at the adult stage. Lepidoptera hy-

drolyze sucrose from leaves (larvae) or from nectar (adults) with the aid of a β-fructosidase (Santos and Terra, 1986; Terra *et al,* 1987). This finding lends further support to the assertion (Terra, 1988, 1990) that studies regarding digestive morphology, physiology and enzymology should include phylogenetic in addition to dietary considerations.

Spatial organization of digestion in S bipunctata *larvae*

Meliponinae worker bees provision the brood cells with a mixture of pollen, honey and glandular secretion. After the queen has laid an egg inside the cell, the workers close it (Sakagami *et al,* 1965). The pollen and nectar used by *S bipunctata* workers come mostly from *Eucalyptus* flowers (Ramalho, 1990). The larvae feed on the cell food and at the end of the 5th larval instar, their midguts become enormously distended (mainly at their distal ends) with undigested food residues, which are not evacuated until just before pupation (Cruz-Landim and Mello, 1981). This is characteristic of these bees, as well as of all the other larvae of Hymenoptera Apocrita (Richards and Davies, 1977), wherein the midgut is closed at its posterior end, and remains unconnected to the hindgut until pupation. Once ingested by the larvae, the pollen grains probably burst and open at the pores, owing to the presumed low osmotic pressure of the midgut, thereby resulting in the extrusion of protoplasm as described in *Apis mellifera* adults (Kroon *et al,* 1974; Klungness and Peng, 1984).

Digestive enzymes, except for trypsin, occur mainly in *S bipunctata* anterior midgut contents. This is at first sight an unexpected finding. Even if digestive enzymes are secreted in the anterior midgut, they are expected to be translocated to the posterior midgut, as peristalsis moves the ingested food backward, thereby distending the posterior midgut. The possibility that digestive enzymes are inactivated at the posterior midgut has no experimental support. There appear to be no activators or inhibitors in midgut tissue or contents which affect *S bipunctata* digestive enzymes, and these enzymes are stable in the presence of *S bipunctata* midgut proteases. It is possible, then, that *S bipunctata* shows an endo-ectoperitrophic circulation of digestive enzymes similar to that described for other insects (for reviews see Terra, 1988; 1990). This circulation results from a countercurrent flux which depends on the secretion of fluid at posterior midgut and its absorption back at the anterior midgut. The countercurrent flux displaces to the anterior midgut the enzymes, and products of digestion, which are able to diffuse from the endoperitrophic space to the ectoperitrophic space. Although the composition of pollen varies widely, major components are protein, sugars (sucrose and monosaccharides), carbohydrate associated with the pollen wall and, among the minor components, there is starch (Todd and Bretherick, 1942). This it is probable that as the pollen food passes along the midgut, sucrose, starch and the resulting oligosaccharides are hydrolyzed, thus permitting the corresponding carbohydrases to diffuse through the peritrophic membrane. Protein, nevertheless, probably remains in substantial amounts associated with the pollen in the posterior midgut. The fact that the protein present in the posterior luminal contents is much higher than in the anterior agrees with this view (table II). As a consequence, it is reasonable to suppose that trypsin remains bound in substantial amounts to pollen protein in the posterior midgut and, as such is prevented from diffusing through the peritrophic membrane. Polypeptides, resulting from the action of trypsin on protein, are probably able to diffuse through the peritrophic membrane to-

gether with aminopeptidase. Diffusion through the peritrophic membrane depends on the size of the translocating molecules and the diameter of peritrophic membrane pores. The larger *S bipunctata* digestive enzymes (maltase 2 and monomeric aminopeptidase) have diameters of 8 nm, as interpolated in a plot of log (M_r) against Stoke's radius for 11 proteins (details in Terra and Ferreira, 1983). These proteins penetrate the *S bipunctata* endoperitrophic space, as judged by the finding (results not shown) that ultracentrifugation profiles of midgut contents are similar to those obtained from midgut homogenates (figs 1, 4A). Thus, *S bipunctata* larval peritrophic membrane has pores of at least 8 nm diameter. Pore sizes of the peritrophic membranes from well-studied insects vary from 7.0–8.0 nm (Terra, 1990).

Digestion by larval bees: evolutionary considerations

Based on the literature and following cladistic-like techniques (Cracraft, 1974), Terra (1990) proposed that in relation to their digestive physiology, Hymenopteran ancestors displayed the following characteristics: 1) polymer, oligomer and dimer hydrolases that were free and small (< 8 nm in diameter) and thus able to pass through the peritrophic membrane; 2) endo-ectoperitrophic circulation of digestive enzymes caused by the secretion of fluid in the posterior midgut and its absorption in the anterior midgut; 3) differentiation of an acidic anterior midgut (with high carbohydrase activity) and an alkaline posterior midgut (with high protease activity). Derived characters found among larval Apocrita include a midgut closed at its rear end. We may speculate that bees (Apidae) display as a derived character the loss of a clear midgut differentiation of luminal pH and enzyme distribution. Although trypsin is found mainly in posterior midgut, the same is not true for aminopeptidase. Hence, the observed enzyme distribution is derived from the hypothetical ancestral condition. Some Hymenoptera acquire digestive enzymes, with vital roles by ingesting fungi (Martin, 1987). Although *S bipunctata* larvae acquired a cysteine–proteinase by ingesting pollen grains, the role of this enzyme seems to be secondary in relation to the bee's proteinase. Thus, the acquisition of this proteinase does not appear to represent a significant phylogenetic character.

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Résumé — Propriétés et compartimentation de l'activité des carbohydrases et des protéases dans l'intestin moyen des larves de *Scaptotrigona bipunctata* (Apidae, Meliponinae). Le pH du contenu de l'intestin moyen des larves de *S bipunctata* a été déterminé à l'aide d'indicateurs de pH. Les résultats obtenus sont les suivants : partie antérieure de l'intestin moyen : 6,0; partie médiane : 5,7; partie postérieure : 5,6. Des dosages enzymatiques ont été faits sur des homogénats de tissus et de contenus de l'intestin moyen, sur des homogénats de corps de larves dont on avait extrait le tube digestif et sur des sonicats de grains de pollen récoltés par *S bipunctata*. Les enzymes testés et

les substrats utilisés sont les suivants : aminopeptidase sur L-leucine-*p*-nitroanilide; amylase sur amidon; cellobiase sur cellobiose; maltase sur maltose; protéinase totale sur azocaséine; cystéine-protéinase sur N-benzoyl-L-arginine-β-naphtylamide (testée également en mesurant l'augmentation de la protéinase totale après addition d'acide éthylènediaminetétraacétique et de dithiothreitol); trypsine sur N-benzoyl-DL-arginine-*p*-nitroanilide.

Les activités enzymatiques trouvées dans les corps dépourvus de tube digestif (y compris les glandes salivaires) et dans les grains de pollen (sauf pour une cystéine-protéinase) sont inférieures à 5% des mêmes activités mesurées dans les intestins moyens. Le rôle des hydrolases des glandes salivaires et du pollen dans la digestion larvaire (sauf pour la cystéine-protéinase du pollen) n'a donc pas été pris en compte. Les enzymes digestives sont présents en grandes quantités dans le contenu de l'intestin moyen de *S bipunctata*, alors qu'on ne les trouve qu'en quantités mineures dans les cellules de l'intestin moyen (tableau II). Mise à part la trypsine, prédominante dans le contenu de la partie postérieure de l'intestin moyen, toutes les autres enzymes testées sont principalement présentes dans le contenu de la partie antérieure. L'amylase et la maltase se trouvent principalement dans les cellules de la région antérieure, les autres enzymes étant distribuées uniformément le long du tissu (tableau II).

Les valeurs optimales de pH pour l'activité enzymatique (pH_O) et les poids moléculaires relatifs des enzymes (Mr), déterminés par ultracentrifugation avec gradient de densité, sont les suivants : aminopeptidase (pH_O : 7,5; M_r : 1, 110 000; 2, 190 000; 3, 300 000), amylase (pH_O 5,5; M_r : 1, 21 000; 2, 68 000), cellobiase (pH_O 5,5), maltase (pH_O 5,0; M_r : 1, 75 000; 2, 110 000; 3, 200 000), trypsine (pH_O 7,5; M_r: 38 000), et cystéine-protéinase du pollen (pH_O 5,6; M_r : 79 000). L'amylase n'est pas activée par le chlorure et la maltase 1 est plus active sur le saccharose que sur le maltose, l'inverse étant valable pour les autres maltoses.

À la lumière des travaux faits sur les autres insectes, les résultats soutiennent l'hypothèse suivante : la majeure partie de la digestion a lieu à l'intérieur de la membrane péritrophique, les enzymes et les aliments diffusent à travers elle et sont transportés vers l'avant par un flux à contre-courant. Si l'on retrouve la trypsine en plus grandes quantités dans le contenu de la partie postérieure de l'intestin moyen, c'est probablement parce qu'elle reste liée à la protéine du pollen en excès. On estime que l'absence d'une différentiation du pH du lumen dans l'intestin moyen chez les larves de *S bipunctata* serait dérivée des ancêtres supposés des hyménoptères.

***Scaptotrigona bipunctata* / digestion / activité enzymatique / Meliponinae**

Zusammenfassung — Eigenschaften und Aktivitätsverteilung von Carbohydrasen und Proteasen im Mitteldarm der Larven von *Scaptotrigona bipunctata* (Apidae, Meliponinae). Der pH Wert des larvalen Mitteldarms von *S bipunctata* wurde an verschiedenen Stellen mit folgendem Ergebnis bestimmt: Im vorderen Drittel wurde ein pH von 6,0 gemessen, im mittleren Teil 5,7 und im hinteren 5,6.

Enzymtests wurden mit Homogenaten aus Mitteldarmgewebe, Mitteldarminhalt und von Larven ohne Darm durchgeführt. Tests wurden auch an mit Ultraschall behandelten Pollenkörnern vorgenommen, die von diesen Bienen gesammelt worden waren. Folgende Enzyme wurden mit folgenden Substraten getestet: Aminopeptidase mit L-Leucin-*p*-Nitroanilid; Amylase mit Stärke; Zellobiase mit Zellobiose; Maltase mit Maltose (Malzzucker); Gesamt-

Proteinasen mit Azocasein; Cystein-Proteinase mit N-Benzoyl-L-Arginin-β-Naphthylamide (außerdem wurde die Zunahme an Gesamt-Proteinasen nach Zugabe von Ethylen-Diamin-tetra-Essigsäure und Dithiothreitol getestet); Trypsin mit N-Benzoyl-DL-Arginin-p-Nitroanilid.

Die Enzymaktivität im Larvenhomogenat ohne Darm (aber mit Speicheldrüse) und in Pollenkörnern (mit Ausnahme von Cystein-Proteinase) betrug weniger als 5% im Vergleich zur Aktivität des Mitteldarms. Demnach spielen die Hydrolasen der Speicheldrüse und des Pollen (mit Ausnahme der Cystein-Proteinase der Pollen) keine Rolle für die larvale Verdauung.

Größere Mengen von Verdauungsenzymen wurden im Mitteldarminhalt nachgewiesen, während nur kleinere Mengen im Mitteldarmgewebe gefunden wurden (Tabelle II). Außer Trypsin, das in erhöhten Mengen im hinteren Drittel des Mitteldarms vorkommt, verteilen sich die anderen getesteten Enzyme über den gesamten Mitteldarminhalt. Im Mitteldarmgewebe fanden sich Amylase und Maltase vor allem in der vorderen Region, die anderen Enzyme wurden gleichmäßig im gesamten Gewebe nachgewiesen (Tabelle II).

Das Optimum der Enzymaktivität in Abhängigkeit vom pH Wert (pH_o) und die relativen Molekulargewichte der Enzyme (Mr Wert) wurden bestimmt: Aminopeptidase – pH_o 7,5 / Mr Wert 1: 21 000; 2: 190 000; 3: 300 000. Amylase – pH_o 5,5 / Mr Wert 1: 21 000; 2: 68 000. Cellobiase – pH_o 5,5. Maltase – pH_o 5,0 / Mr Wert 1: 75 000; 2: 110 000; 3: 200 000. Cystein-Proteinase von Pollen – pH_o 5,6 / Mr Wert 79 000. Amylase wird nicht durch Chlorid aktiviert und Maltase 1 ist aktiver bei Saccharose (Rohrzucker) als bei Maltose (Malzzucker), im Gegensatz zu den anderen Maltasen.

Im Vergleich mit Untersuchungen bei anderen Insekten stützen diese Ergebnisse die Hypothese, daß die Verdauung hauptsächlich innerhalb der peritrophen Membran stattfindet, daß Enzyme und Nährstoffe durch die peritrophe Membran diffundieren und daß die Enzyme in einem Kreislauf vom hinteren Teil des Mitteldarms wieder nach vorne transportiert werden (endo-ectoperitrophische Zirkulation).

Trypsin wurde in größeren Mengen im hinteren Teil des Mitteldarminhalts nachgewiesen. Wahrscheinlich bleibt es an überschüssigem Polleneiweiß gebunden. Das Fehlen einer Differenzierung des pH Wertes innerhalb des Darmlumens bei der Larve von *S bipunctata* wird als Abstammung von angenommenen ursprünglichen Hymenopteren gedeutet.

***Scaptotrigona bipunctata* / Verdauung / Enzymaktivität / Meliponinae**

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