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Host hemolymph proteins and protein digestion in larval *Habrobracon hebetor* (Hymenoptera: Braconidae)[1]

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Abstract

Host plasma proteins and protein digestion in larval parasitoids were studied during trophic interactions of the ectoparasitoid *Habrobracon hebetor* Say (Hymenoptera: Braconidae), with a host, larvae of the Indianmeal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae). We could detect no apparent differences in host hemolymph protein patterns up to 72 h after paralysation and/or parasitization by *H. hebetor*. A 190 kDa putative apolipophorin I present in host hemolymph could not be detected in the midguts of feeding *H. hebetor* larvae indicating that it is rapidly digested. The major 60 kDa storage proteins (putative hexamerins) in host hemolymph were detected in the parasitoid midgut and were completely digested 24 h after cessation of feeding and the beginning of cocoon formation. Host hemolymph had a pH of about 6.4. The pH optima of the midgut proteinases in the larval parasitoid were in the alkaline region, but midgut fluid in feeding parasitoid larvae was about pH 6.8. Based on enzyme activity against selected artificial proteinase substrates including azocasein, *N*-α-benzoyl-L-Arg *p*-nitroanilide (BA*p*NA), succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SAAPF*p*NA), succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide (SAAPL*p*NA), and inhibition by selected proteinase inhibitors, serine proteinases appear to be the predominant class of enzymes involved in protein digestion in the midguts of *H. hebetor*. There is also an active aminopeptidase (L*p*NA) associated with the microsomal fraction of midgut preparations. There was no evidence for preoral digestion or ingestion of proteinases from host hemolymph by the parasitoid larva. There was a very active BA*p*NAase in the soluble fraction of midgut extracts. This activity increased on a per midgut basis up to 24 h after the beginning of cocoon formation but decreased rapidly by 48 h. Two major (P1 and P3) and several minor proteinases were detected in midgut extracts of *H. hebetor* analysed with gelatin zymograms. The apparent molecular mass of P1 varied from 95 to 49 kDa depending on protein loading. P3 had an apparent molecular mass of 39 kDa that was independent of protein loading. In summary, electrophoretic evidence indicates that host hemolymph protein patterns do not change significantly for at least 72 h after paralysation by *H. hebetor*. The role, if any, of envenomization in preventing breakdown of hemolymph proteins during this time remains to be determined. Because the predominant host hemolymph proteins, a putative apolipophorin I and the putative hexamerins, are readily digested by the serine proteinases present in the midguts of this parasitoid larva, these or similar proteins would provide an easily digested source of dietary amino acids that could be used for development of artificial diets for this beneficial insect. Published by Elsevier Science Ltd.

Keywords: Parasitoid; Host; Hemolymph proteins; Larvae; Midgut; Digestion; Proteinase; Trypsin

1. Introduction

Habrobracon [=*Bracon*] *hebetor* Say (Hymenoptera: Braconidae) is a gregarious ectoparasitoid that is an important natural control agent of many lepidopterous pests. This wasp occurs in the stored grain ecosystem (Keever et al., 1985; Antolin and Strand, 1992) where it attacks several pyralid moths, including the Indianmeal moth, *Plodia interpunctella* (Hübner), a destructive pest of stored products. One factor that contributes to *H. hebetor*'s success as a natural enemy is a rapid growth rate and short development time. Grosch (1949) found that *H. hebetor* larvae parasitizing larvae of *Ephestia kuehniella* (Zeller) had a 5-fold increase in length and a 7-fold

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increase in width during a 40 h feeding period. With larvae of the pink bollworm, *Pectinophora gossypiella* (Saunders) as host, development from egg to adult at 30°C by *H. hebetor* took only 8.6 days (Jackson and Butler, 1984). Growth was faster at 35°C. Much of this rapid development can be attributed to morphological and physiological adaptations to a highly nutritious diet. Many hymenopterous larvae have a blind midgut with no direct connection to the hindgut, and in *H. hebetor* the pouch-like midgut makes up nearly the entire larval body cavity (Grosch, 1949). This morphological adaptation in larval parasitoids, in addition to storage of nitrogenous wastes as urate granules in the hemocoel (a mode of storage excretion), is thought to allow a rapid consumption of food which is then digested in the prepupal or pharate pupal stage (Salkeld, 1967).

Host feeding and allocation of food resources by adult parasitoids has been extensively studied (Jervis and Kidd, 1986; Heimpel and Collier, 1996). However, there is very little detailed information on trophic interactions, including possible host-associated changes induced by the paralysing toxin injected during stinging by the adult parasitoid, larval feeding mechanisms, and larval digestive processes between parasitoids and their hosts (Quicke, 1997). In one of the few detailed studies on feeding behavior and digestion in larval parasitoids, Jarjees et al. (1998) observed histological changes in the larval midguts of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae) that were thought to represent physical changes in properties of ingested food as a result of digestion. Their results had direct application to development of artificial diets and mass rearing protocols for this egg parasite. To provide a basis for development of mass rearing protocols for *H. hebetor* on artificial diets, we have examined the effect of both paralysation and parasitization by *H. hebetor* on hemolymph proteins of the host *P. interpunctella* and have initiated studies on the digestion of proteins in this parasitoid by partially characterizing larval midgut proteinases with respect to pH optima, substrate and inhibitor specificity, and activity during selected developmental stages.

2. Materials and methods

2.1. Insects

A laboratory strain of *P. interpunctella* used for this study was reared on a diet of cracked wheat, wheat shorts, wheat germ, brewer's yeast, honey, glycerin, and water. A strain (Miller) of *H. hebetor*, collected from wheat infested with *P. interpunctella* in Dickinson Co., Kansas, in October 1998, was cultured at 27°C and 50–55% RH on wandering larvae of *P. interpunctella*.

To obtain *H. hebetor* larvae for dissection, 20–30 host larvae of *P. interpunctella* were placed in 15×100 mm plastic Petri dishes along with 5 female *H. hebetor* adults. At 27°C, mature *H. hebetor* larvae were usually available within 4–5 days.

2.2. Collection of hemolymph from P. interpunctella

Wandering stage (15-days-old) larvae of *P. interpunctella* were placed on ice for 10 min prior to collection of hemolymph. Larvae were surface sterilized by submersion in 95% ethanol. Hemolymph was collected by cutting the third pro-leg with micro-scissors and drawing hemolymph into a gel loading tip with a pipettor. Generally about 3 μl of hemolymph could be obtained from a single larva. Hemolymph was dispensed immediately into a few crystals of 1-phenyl-2-thiourea. When sufficient hemolymph was collected, the sample was centrifuged at 10,000 *g* for 5 min at 4°C to pellet hemocytes. Plasma was diluted 1:50 with distilled H_2O and protein concentration determined.

2.3. Effect of paralysation and parasitization on hemolymph proteins in P. interpunctella

Hemolymph proteins in larvae of *P. interpunctella* were monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at 0, 48, and 72 h after stinging by adult *H. hebetor*. Hemolymph from five larvae at each time period was combined and analysed. Because of the relaxing effect of the paralysing venom, hemolymph was easier to collect from the paralysed larvae than from control (non-paralysed) larvae.

To determine possible effects of parasitization on hemolymph proteins, hemolymph was also collected from *P. interpunctella* larvae that had been stung and parasitized. For this experiment, immediately after host larvae were paralysed by adult *H. hebetor*, two large parasitoid larvae were transferred from previously parasitized hosts onto each newly paralysed host. After 48 and 72 h, hemolymph was collected from 5 host larvae that were being fed upon by at least one *H. hebetor* larva and subsequently analysed by SDS-PAGE.

2.4. Dissection of parasitoid midguts and preparation of homogenates

Midguts of larvae of *H. hebetor* were dissected in cold 1% NaCl and placed in 50 mM Tris–chloride pH 7 in a 1.5 ml microcentrifuge tube. For most analyses, 10 midguts were dissected into 200 μl of buffer. Lumen contents were obtained by vortexing the midgut/buffer mixture until a homogeneous preparation was obtained. The tubes were centrifuged for 20 min at 10,000 *g* with a Beckman refrigerated table top centrifuge. Supernatant was drawn off and held at −20°C for 2–4 days until assayed. Enzyme aliquots were generally taken from a

1:4 dilution of the stock midgut preparation. Although the extraction procedure is mild, relative to homogenization, it is likely that soluble components from ruptured epithelial cells are also present in the preparation.

2.5. Effect of pH on proteinase activity

The universal buffer of Frugoni (1957), a buffer system that maintains a constant ionic strength over a wide pH range, was used to determine pH optima of proteinase activities in larval midguts of *H. hebetor*. Assays for total proteinase (azocasein), trypsin-like activity (BA*p*NA), chymotrypsin-like activity (SAAPF*p*NA), and aminopeptidase (L*p*NA) were conducted at eleven pH values ranging from pH 2 to pH 11.7. Substrate blanks were included at each pH. Details on the assays with these artificial substrates are given below. For each substrate, mean activities at each pH were obtained from assays of at least three separate midgut preparations.

2.6. pH of host hemolymph and larval parasitoid midgut fluid

pH of hemolymph of host larvae (*P. interpunctella*) and pH of midgut fluid of larval *H. hebetor* feeding on *P. interpunctella* larvae were estimated by color comparisons on pHydrion microfine pH paper (pH 6.0 to 8.0) (Aldrich Chemical Co., Milwaukee, WI). About 3 μl of hemolymph was streaked onto the paper. Parasitoid midguts were dissected, rinsed in distilled H_2O, blotted with tissue paper, and pressed onto the pH paper.

2.7. Distribution of proteinase activity

2.7.1. Preparation of soluble and microsomal fractions

Soluble and pellet (microsomal) fractions obtained by centrifugation of *H. hebetor* larval midgut homogenates at 100,000 *g* were assayed for total proteinase, trypsin, chymotrypsin, elastase, and aminopeptidase activities. Ten midguts were dissected into 200 μl 1% NaCl in 7×20 mm polyallomer microcentrifuge tubes and centrifuged for 60 min (4°C) at 28,000 rpm (100,000 *g*) in a 42.2 ti rotor in a Beckman L8-70M ultracentrifuge. Tubes were removed and 150 μl of supernatant was withdrawn and placed in a microcentrifuge tube. This was the soluble fraction and appropriate dilutions of this fraction were used for protein analysis and for enzyme assays. The remaining supernatant was removed and discarded. The pellets (microsomal fractions) were resuspended with a fine glass rod and washed 2× with 200 μl of 1% NaCl. Each wash was followed by a 60 min centrifugation at 100,000 *g* as above. The final pellet was resuspended in 200 μl 1% NaCl and appropriate dilutions used for enzyme assays. Four replicates of soluble and microsomal fractions of *H. hebetor* midguts were prepared.

2.7.2. Proteinase assays

Total proteinase activity was measured with azocasein. Enzyme (20 μl) was preincubated with 80 μl of buffer (50 mM Tris–chloride pH 7) for 10 min at 30°C. The reaction was started by adding 200 μl of 1.5% azocasein in buffer. Final azocasein concentration was 1% in a 300 μl reaction volume. After 3 h at 30°C, the reaction was stopped by adding 300 μl of 7.5% trichloroacetic acid. Tubes were centrifuged for 20 min at 10,000 *g* and 300 μl of supernatant transferred to a microtiter plate. Absorbance was determined at 415 nm. Enzyme activity was linear with these assay conditions and was expressed as change in absorbance at 415 nm/min/mg protein.

N-α-benzoyl-L-Arg *p*-nitroanilide (BA*p*NA), *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide, (SAAPF*p*NA), *N*-succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide (SAAPL*p*NA), and *L*-Leucine *p*-nitroanilide (L*p*NA), all obtained from Sigma Chemical Co., St Louis, MO, were used to assay trypsin-like, chymotrypsin-like, elastase-like, and aminopeptidase activities, respectively. For each nitroanilide substrate, 20 μl of enzyme solution was preincubated with 80 μl of 50 mM Tris–chloride pH 7 for 10 min at room temperature in a microtiter plate well. Reactions were started by adding 200 μl of 1.5 mM substrate in 50 mM Tris–chloride pH 7 containing 5% dimethyl sulfoxide. Reaction rates at 30°C were followed in a BioTek microtiter plate reader for 30 min. Slopes of increase in absorbance at 405 nm, corrected for endogenous hydrolysis of substrate, were used to estimate velocities. A molar extinction of 8,800 $mole^{-1}$ L^{-1} cm^{-1} for released *p*-nitroaniline was used to calculate specific activity (Erlanger et al., 1961).

2.8. Effect of proteinase inhibitors on hydrolytic activity against BApNA and azocasein

BA*p*NAase activity and activity against azocasein were measured in larval midgut homogenates after preincubation with the following selected inhibitors obtained from Sigma Chemical Co.: phenylmethyl sulfonyl fluoride (PMSF), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), *p*-aminobenzamidine dihydrochloride, trypsin-chymotrypsin inhibitor (Bowman-Birk Inhibitor, BBI) from soybean, pepstatin A, and cystatin (egg white). Assays with BA*p*NA and azocasein were conducted as described above. Enzyme (20 μl) was preincubated with inhibitor for 15 min in a total of 100 μl buffer. Reactions were started by addition of 200 μl of substrate in 50 mM Tris–chloride pH 7 buffer. Final inhibitor concentrations are presented in the Results section. For evaluating the effects of cystatin, the final reaction solution also contained 1 mM dithiothreitol, an activator of cysteine proteinases. Inhibition values are means based on specific

activity values relative to controls with no inhibitor determined from 4 replicate groups of midgut homgenates prepared separately.

2.9. Stage related changes in BApNAase activity

Midguts from individual *H. hebetor* larvae that were (1) actively feeding on host larvae, (2) beginning to spin their cocoon, (3) enclosed in their cocoon for 24 h, and (4) enclosed in their cocoon for 48 h were dissected into 50 μl of 50 mM Tris–chloride pH 7 buffer. After vortexing and centrifugation for 20 min at 10,000 *g*, 3–15 μl aliquots (depending on stage) of supernatant were used for protein analysis and 20 μl aliquots were used to estimate BA*p*NAase activity. For each of the four stages, 10 individual midguts were analysed. The experiment was conducted twice with different cultures of *H. hebetor*. Data are mean values from 20 individuals for each stage.

2.10. Stage-related digestion of hemolymph proteins

Soluble proteins in midguts dissected from actively feeding larvae of *H. hebetor*, larvae that had ceased feeding and were beginning to spin their cocoon, and larvae (prepupa) that were enclosed in their cocoon for 24 h were compared with hemolymph proteins from paralysed host larvae by SDS-PAGE under reducing conditions. For these analyses, 5 midguts were dissected from larvae of each stage and placed in 150 μl of 5 mM Tris–chloride pH 7 containing 1 mM *p*-aminobenzamidine to reduce or eliminate *in vitro* protein digestion in the homogenate. After centrifugation at 10,000 *g*, 3 μl aliquots were analysed for protein and equal protein amounts (22.5 μg) were loaded onto a 10-well NuPAGE SDS gel (Novex). Hemolymph from *P. interpunctella* larvae was diluted 1:50 and 17.3 μl containing 22.5 μg protein was denatured and reduced as per manufacturers directions and loaded onto the gel. Mark12 molecular weight standards (200 to 2.5 kDa, Novex) were analysed in parallel. After electrophoresis, gels were stained in Coomassie Blue R250 (0.1% Coomassie, 40% ethanol, 10% acetic acid), destained, and scanned.

2.11. Gelatin zymograms

Novex 10% Tris–glycine 10-well, 1-mm-thick polyacrylamide zymogram gels containing 0.1% gelatin were used to detect proteinase bands in midguts from different larval stages of *H. hebetor*. For this study, 5 midguts from feeding larvae, spinning larvae, and larvae that were enclosed in their cocoons for 24 h were dissected into 150 μl 5 mM Tris–chloride pH 7. Samples were diluted 1:1 in non-reducing SDS sample buffer. Hemolymph from *P. interpunctella* and bovine pancreatic trypsin (1 μg/lane) (Sigma) were run as controls. Molecular sizes of proteinase bands were estimated by comparison of mobility with Novex MultiMark MW standards. After electrophoresis, gels were washed for 2 h in 2.5% Triton X-100 to remove SDS and incubated for 4 h at 37°C in 50 mM Tris–chloride pH 7. Activity bands were clear zones on a blue background after staining the gel with Coomassie Blue R250 as above.

2.12. Protein assay

Protein concentration was determined with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Assays were conducted in microtiter plates with bovine serum albumin as standard.

3. Results

3.1. Host hemolymph proteins after paralysation

No significant qualitative or quantitative changes in hemolymph proteins in larvae of *P. interpunctella* were detected up to 72 h following paralysation and/or paralysation plus parastization by *H. hebetor* when plasma samples of equal protein concentration (20 μg/lane) were analysed by SDS-NuPAGE (Fig. 1). To determine if changes occurred among some of the minor components, samples that contained very high concentrations of protein (115 μg/lane) were also subjected to electrophoresis but no consistent changes in protein patterns were observed. Hemolymph plasma from *P. interpunctella* contained 2 predominant proteins: a 190 kDa protein,

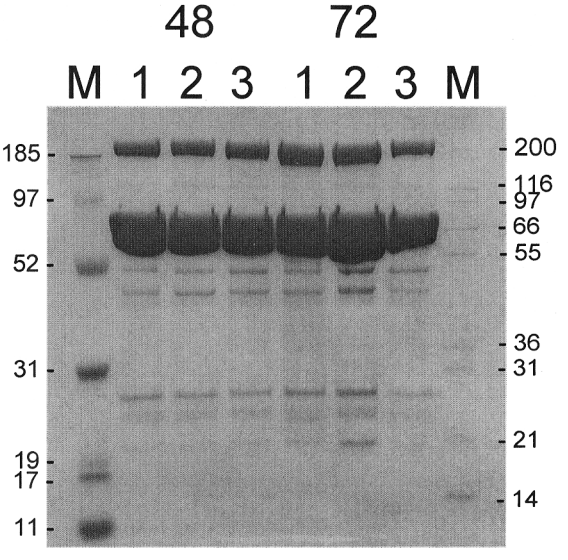

Fig. 1. SDS-PAGE analysis of hemolymph plasma proteins from *P. interpunctella* that were paralysed and parasitized by *H. hebetor*. Hemolymph was collected 48 and 72 h after paralyzation. M, marker proteins; 1, control larvae; 2, paralysed larvae; 3, paralysed and parasitized larvae. Equal protein amounts of 20 μg per lane were loaded. Gels were stained with Coomassie Blue R250.

thought to be apolipophorin I (Kanost et al., 1990; Sundermeyer et al., 1996), and a major 60 kDa band which probably includes storage proteins (hexamerins) (Haunerland, 1996) and apolipophorin II (Kanost et al., 1990). Other significant proteins had apparent molecular masses of 50 kDa and 42 kDa and there was a group of 4 proteins with molecular masses between 21 and 28 kDa. The presence of feeding *H. hebetor* larvae on the host larvae did not alter the host hemolymph protein patterns during the time intervals examined.

In separate assays, no BA*p*NAase activity was detected in aliquots of hemolymph plasma containing 20 μg protein from larvae that had been stung for 48 or 72 h (data not shown).

3.2. Host hemolymph pH and H. hebetor midgut pH

Hemolymph collected from *P. interpunctella* larvae was estimated to have a pH of 6.4–6.6 by color comparisons with the pH paper standards. The pH of midgut contents from *H. hebetor* larvae that were actively feeding on host larvae were not much different from that of host hemolymph. Based on color comparisons, *H. hebetor* midgut fluid pH was slightly acidic and estimated to be about pH 6.8.

3.3. Midgut morphology

In our Petri dish arenas, the adult *H. hebetor* could attack and paralyse their hosts, oviposit, and larvae complete the active feeding stage within 4 days at 27°C. The mature C-shaped parasitoid larvae weighed about 1.6 mg each with an estimated length of about 2.5–3 mm (Fig. 2A). Midguts in mature larvae of *H. hebetor* make up nearly the entire body cavity (Fig. 2B). A short, non-functional hindgut is attached to the posterior end of the midgut. However, the hindgut can be easily removed without tearing the midgut epithelium and with no loss of midgut fluid. A pair of thin, clear, Malphigian tubules arises from the junction of the midgut and hind gut. The tubules are apparently non-functional, lie closely appressed to the midgut, and extend anteriorly for about 1/3 the length of the midgut. The larval body cavity contains numerous, white granules, thought to be urate granules (Salkeld, 1967) that apparently result from a storage excretion mechanism. Midguts from larvae that had initiated spinning were the same size as those of feeding larvae. However, the larval midguts decreased in length and width after 24 h and particularly rapidly by 48 h after spinning. At 48 h, it was estimated that the midgut volume (assuming a cylinder) had decreased 88-fold compared with that in feeding larvae.

With our rearing conditions, a black, crystalline material is excreted from the posterior end of the larvae (prepupae) onto the cocoon between 24 and 48 h after cocoon formation begins. This material may be excreted as a fluid that subsequently undergoes an oxidation and hardening process when exposed to air, because no black, crystalline material was observed in larvae themselves. At this black-tip stage, eye pigments are forming on the anterior end, fat globules or clusters of fat body-type cells that are practically absent when the larvae are feeding become very prominent, head appendages can be seen in some individuals, and the small larval midgut that contains a brownish fluid appears to be unattached and free-floating in the body cavity.

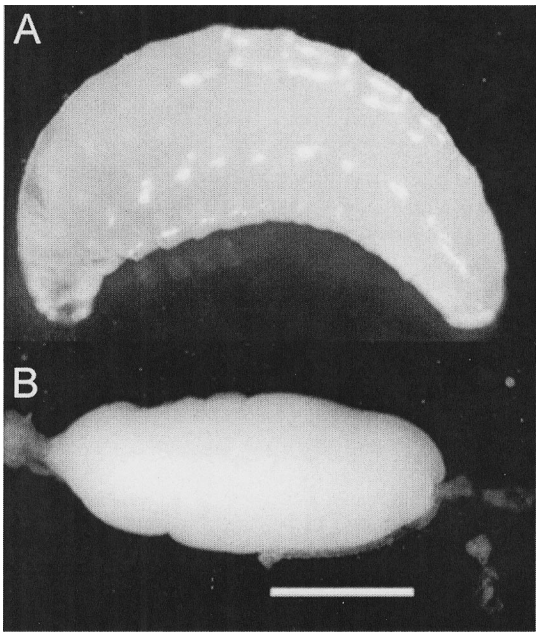

Fig. 2. (A) Mature larva of *H. hebetor* that was feeding upon a host *P. interpunctella*. The anterior end is to the left in this figure. (B) Blind midgut from mature larva of *H. hebetor*. One Malphigian tubule that is closely appressed to bottom right (posterior) region of the midgut is faintly visible. The hindgut attachment is also seen in the posterior region. Scale bar=1 mm.

Grosch (1949) observed that if a slit is made in the larval cuticle, the midgut would bulge out and occasionally would be completely expelled. We also observed this, but normally, after a slight tear in the cuticle near the posterior region, the midguts could be easily dissected intact with fine forceps. In occasional preparations, we also observed that the entire midgut lumen contents, surrounded by a clear, thin peritrophic membrane (PM), would also bulge out and emerge intact through a fine slit in the midgut wall. We did not determine if any proteinases were associated with the PM.

3.4. pH activity curves of proteinase substrates

Maximum activity against azocasein, BA*p*NA, and SAAPF*p*NA occurred in the alkaline range from pH 8–11, depending on substrate (Fig. 3). The pH optima for SAAPF*p*NA, a substrate for chymotrypsin-like activity, was the most alkaline and occurred at about pH 10.8.

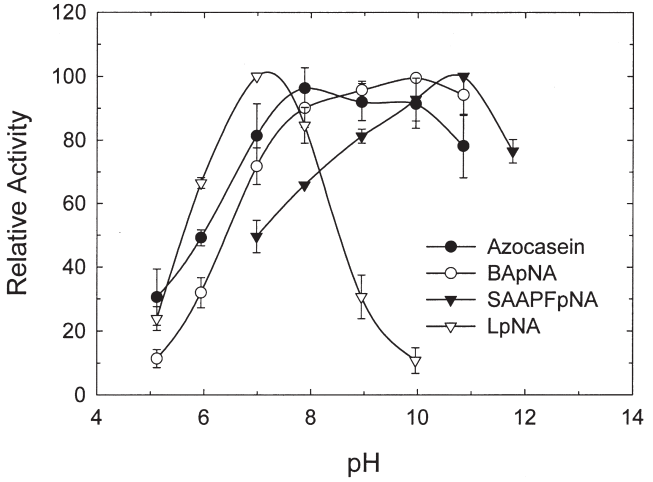

Fig. 3. Effect of buffer pH on relative specific activity of larval midgut preparations from *H. hebetor* against proteinase substrates. Results are means±S.E.M. of at least three separate midgut preparations for each substrate.

Aminopeptidase activity against L*p*NA was most active at about pH 7. Although activities against the proteinase substrates were in the alkaline range, we used buffers at pH 7 to compare activities with different substrates because midgut pH in feeding larvae of *H. hebetor* was just slightly below neutral.

3.5. Distribution of proteinase activities in midguts of *H. hebetor*

About 82% of the proteinase activity against azocasein was in the soluble fraction of *H. hebetor* midgut preparations (Table 1). Trypsin-like activity against BA*p*NA was much more predominant than chymotrypsin-like activity against SAAPF*p*NA. We tested BA*p*-NAase activity at both pH 7 and 8, and found no difference in the relative distribution between soluble and microsomal fractions. About 87 to 89% of the total activity was present in the soluble fraction.

There was very little activity against SAAPF*p*NA and less activity against SAAPL*p*NA, a substrate hydrolysed by both elastase and chymotrypsin. What activity was found against these two substrates was located in the soluble fraction. The presence of 1 mm DTT did not increase the activity against BA*p*NA, providing evidence that cysteine proteinases apparently do not contribute significantly to digestion.

There was a very active aminopeptidase activity against L*p*NA at both pH 7 and pH 8. About 70% of the total activity was found in the microsomal fraction.

3.6. Effect of inhibitors on proteinase activity

p-Aminobenzamidine, a low molecular weight inhibitor that interacts with the arginine active site residue in trypsin-like serine proteinases, was the most effective inhibitor, inhibiting 96% of the azocaseinase activity and giving complete inhibition of the BApNAase activity in midgut extracts of *H. hebetor* (Table 2). PMSF, a general serine proteinase inhibitor, and TLCK, a site-specific inhibitor of trypsin-like enzymes inhibited about 60% of the azocasein activity but about 95% of BA*p*NAase activity. Depending on concentration, Bowman–Birk trypsin-chymotrypsin inhibitor reduced the activity against azocasein by 20–50%. Pepstatin A, an inhibitor of aspartic proteinases, and cystatin, an inhibitor of cysteine proteinases, slightly activated azocaseinase activity and had little effect on BA*p*NAase activity.

Table 1
Proteinase activity in soluble and microsomal fractions of *H. hebetor* larval midgut homogenates following centrifugation at 100,000 *g* for 60 min[a]

	Specific activity[b]		Percentage distribution[c]	
Substrate	Soluble	Microsomal	Soluble	Microsomal
Azocasein[d]	53.3±3.1	30.2±8.1	82.2±1.1	17.8±1.1
BA*p*NA (pH 8)	184.1±24.8	65.8±7.2	87.4±1.7	12.6±1.7
BA*p*NA (pH 7)	145.6±16.1	44.0±10.2	89.2±1.3	10.8±1.3
BA*p*NA (w/DTT, pH 7)	145.3±29.1	–	–	–
SAAPF*p*NA	9.3±3.4	<1.6	98.2±1.2	1.8±1.2
SAAPL*p*NA	4.9±2.1	0	100	0
L*p*NA (pH 8)	44.7±3.9	270.5±16.6	29.9±1.6	70.1±1.6
L*p*NA (pH 7)	55.2±3.5	334.0±16.3	30.0±1.9	69.9±1.9

[a] Values are means±S.E.M. based on 4 replicates.
[b] Specific activity=nmoles/min/mg protein.
[c] Distribution based on total units of activity for each substrate per midgut.
[d] Azocaseinase activity=mOD units 415 nm/min/mg protein.

Table 2
Effect of selected proteinase inhibitors on azocaseinase and BA*p*NAase activity in midgut extracts of *H. hebetor* larvae feeding on larvae of *P. interpunctella*[a]

		Inhibition (%)[c]	
Inhibitor	Concentration[b]	Azocaseinase	BA*p*NAase
PMSF	5 mM	61.7±4.3	92.9±2.9
TLCK	1 mM	44.2±2.8	96.8±0.3
	10 mM	64.2±2.0	–
p-Aminobenzamidine	2 mM	95.9±2.1	100±0
BBI	0.1 μM	21.9±1.7	93.0±0.5
	2 μM	24.5±1.1	–
	10 μM	47.1±3.1	–
Pepstatin A	2 μM	0	2.3±3.6
	20 μM	0	–
Cystatin	5 μM	0	0

[a] Values are means±S.E.M. based on 4 replicates per inhibitor concentration for each substrate.
[b] Concentration in final reaction volume of 300 μl.
[c] Inhibition based on specific activity relative to controls with no inhibitor.

3.7. Stage-specific changes in midgut protein and proteinase activity

After cessation of feeding, *H. hebetor* larvae immediately begin to spin their cocoon in the Petri dish rearing chambers. Protein levels in midguts of feeding larvae and larvae that had just initiated the spinning process were nearly identical (Fig. 4). Digestion continued after spinning began, and after 24 h the protein contents were reduced 6.5-fold from about 110 μg/midgut to about 17 μg/midgut. After 48 h, the midgut itself was much reduced in size (about an 88-fold reduction in volume compared with feeding larvae) and the protein content was reduced to about 4.6 μg/midgut.

After initiating the spinning process, BA*p*NAase activity increased on a per midgut basis after 24 h then declined precipitously. BA*p*NAase specific activity, increased 21-fold, from about 70 nmoles/min/mg protein in feeding larvae to 1,500 nmoles/min/mg protein in larvae 24 h after spinning primarily because of the large reduction in protein levels in these midguts. By 48 h BA*p*NAase activity decreased to about 11 nmoles/min/mg protein.

3.8. Stage-specific changes in midgut proteins resolvable by SDS-NuPAGE

The high molecular weight proteins that are present in *P. interpunctella* hemolymph were rapidly digested in the larval midgut of *H. hebetor* (Fig. 5). The 190 kDa putative apolipophorin I present in hemolymph was not detected in *H. hebetor* midgut homogenates from any aged larvae, even in the presence of 1 mM *p*-aminobenzamidine, which would prevent degradation of the protein in the homogenate. Assuming this protein was ingested, it was apparently rapidly cleaved to lower molecular weight fragments. The major hemolymph hexamerins at about 60 kDa were detected in midguts of feeding larvae and in larvae that have just begun to spin their cocoons. However, after 24 h these major hemolymph proteins were hydrolysed to lower molecular weight peptides. Based on protein patterns on the gels, there is some evidence that the group of proteins between 21 and 28 kDa in host hemolymph was less readily digested.

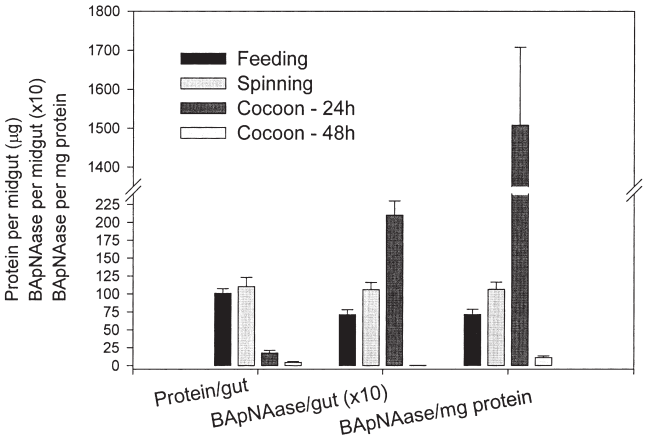

Fig. 4. Effect of *H. hebetor* larval stage on midgut protein contents (μg protein/midgut), and BA*p*NAase activity expressed on a midgut basis (nmoles/min/midgut) or on a per mg protein basis (nmoles/min/mg protein). Four stages of immature parasitoids reared at 27°C on larvae of *P. interpunctella* were analysed: actively feeding larvae, spinning larvae that had initiated cocoon formation, larvae (prepupae) that had been enclosed in their cocoon for 24 h, and larvae (prepupae) that had been enclosed in their cocoon for 48 h (black tip stage). Results are means±S.E.M. and are based on 20 individual larvae per stage.

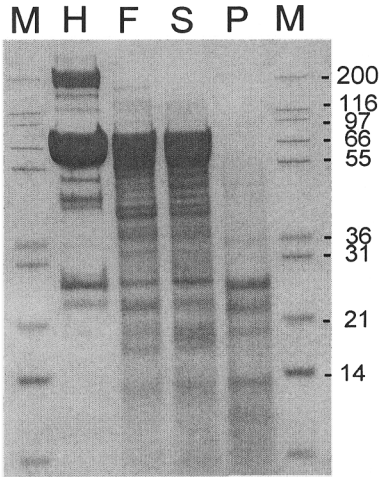

Fig. 5. SDS-PAGE analysis of the rate of digestion of ingested host hemolymph proteins in larval midguts of *H. hebetor*. H, hemolymph plasma from the host *P. interpunctella*; F, midgut proteins from feeding larvae; S, midgut proteins from spinning larvae; P, midgut proteins from larvae enclosed in their cocoon for 24 h; M, marker proteins. Protein loading was 22.5 μg/lane for each sample.

3.9. Gelatin zymograms

Two major and several minor proteinase bands with gelatinase activity were demonstrated in midguts from feeding larvae and larvae that were beginning to spin their cocoon (Fig. 6). The proteinase pattern in midguts from larvae at 24 h after spinning was slightly different. However, the high concentration of protein in midguts from feeding and spinning larvae made it impossible to load equal protein amounts in the different lanes. For example, protein loads for lanes 1 and 2 of feeding and spinning larvae had 10 and 30 μg/lane, respectively, whereas to get similar activity bands the protein loads for 24 h post-spinning larvae were 0.1 and 0.5 μg/lane, shown in lanes 1 and 2, respectively. These differences in protein loading may influence the proteinase patterns that are found in midguts of the 24 h post-spinning larvae. We could detect no proteinase activity in *P. interpunctella* hemolymph with the gelatin zymograms. Bovine pancreatic trypsin (24 kDa) traveled near the dye

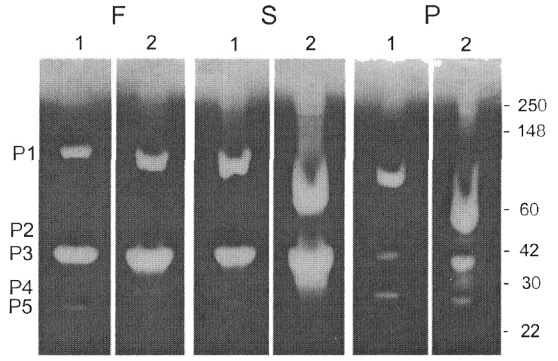

Fig. 6. Zymogram showing proteinase activity against gelatin following SDS-PAGE analysis of *H. hebetor* midgut preparations from (F) feeding larvae, (S) spinning larvae, and (P) larvae that had been enclosed in their cocoons for 24 h. Protein loads for F1 and F2 were 10 and 30 μg/lane, respectively. Protein loads for S1 and S2 were 10 and 30 μg/lane, respectively. Protein loads for P1 and P2 were 0.1 and 0.5 μg/lane, respectively.

front and had an estimated molecular mass of 22 kDa in this gel system.

P1 and P3 are the major proteinases with gelatinase activity. In samples from all three larval stages, the mobility of P1 increased with increasing protein loading in the 10% acrylamide gels. The change was not as dramatic in samples from the feeding larvae, but in midgut samples from larvae that had been enclosed in their cocoon for 24 h the apparent molecular mass of P1 decreased from 94 kDa to 42 kDa when the protein loading increased from 0.1 μg to 0.5 μg per lane. Migration of P3 in the *H. hebetor* extracts was not altered in a similar manner. P1 streaked extensively on these gels, perhaps indicating that SDS does not completely bind to P1 or that the SDS complex does not completely inactivate this proteinase. This may also explain its electrophoretic behavior. In addition, the composition of host hemolymph proteins in the parasitoid gut changes significantly as digestion proceeds. Whether P1 binds to or somehow interacts with undigested hemolymph proteins, or oligopeptides produced during digestion, in a manner that affects it's mobility remains to be determined.

In addition to P1 and P3, several minor proteinases were detected. P2 (50 kDa) was detected in 24 h post-spinning larvae although a weak band with this mobility was also occasionally seen in the other larval stages. P4 (29 kDa) and P5 (25 kDa) were present in feeding and spinning larvae. There were several minor proteinase bands with gelatinase activity in midguts from 24 h post-spinning larvae that had slightly different mobilities compared with those in feeding and spinning larvae. It is not known whether these proteinases are qualitatively different or whether their mobilities are influenced by protein loads.

4. Discussion

4.1. Host physiology after paralysation

H. hebetor is an ectoparasitic idiobiont, and larvae of *P. interpunctella* that are stung by *H. hebetor* undergo neuromuscular paralysis. Although the host's heart continues to beat for an extended time, development ceases and these larvae eventually die even if they are not parasitized. In contrast to the significant changes in host hemolymph proteins induced by endoparasitoids (Ferkovich et al., 1983), based on our SDS-PAGE analysis of host larvae, neither venom-induced paralysation nor parasitism by *H. hebetor* resulted in any electrophoretically-observable changes in plasma protein patterns up to at least 72 h after stinging and/or parasitization. As reviewed by Richards and Edwards (1999), parasitism-specific proteins (PSPs) have been documented extensively in associations of endoparasitoids and their hosts but less is known of the induction of PSPs by ectoparasitoids. In their studies they detected a 27 kDa protein that is present in plasma from larvae of *Lacanobia oleracea* (Lepidoptera: Noctuidae) that were being parasitized by the ectoparasite *Eulophus pennicornis*. The 27 kDa protein was not induced by venom or general injury but only by parasitism. They speculated that the origin of the parasitism-specific protein (PSP) in this association may be the parasitoid larva itself and that it's function may be to alter the host immune response. Fabrick et al. (unpublished data) have shown that the prophenoloxidase system of *P. interpunctella* remains competent for at least 7 days after paralysation but whether the complete immune system remains competent for an extended time period is not known. However, with our experimental conditions, we did not observe any induced PSPs of similar size (27 kDa) in hemolymph of paralysed and/or parasitized host *P. interpunctella* larvae.

We could detect no trypsin-like enzyme activity against the substrate BA*p*NA in *P. interpunctella* hemolymph from unparalysed or paralysed larvae, and there were no detectable proteinase bands when hemolymph was analysed on the gelatin zymograms. Insect hemolymph contains high concentrations of serine proteinase inhibitors. Although proteinase inhibitors may have specific regulatory functions in the hemolymph, primarily during the immune response (Kanost, 1999), it is possible that one secondary effect of the presence of these inhibitors could be to delay any specific or non-specific proteolytic degradation of hemolymph proteins in paralysed larvae of *P. interpunctella* and to indirectly maintain the quality of these proteins as a food source for the parasitoid. Inhibition of proteinases by plasma proteins may also help explain why we see no activity against BA*p*NA or activity bands in gelatin zymograms with host hemolymph.

4.2. Protein digestion in larval H. hebetor

Our studies provide no evidence for preoral digestion of hemolymph proteins by *H. hebetor* larvae and no evidence that significant breakdown of hemolymph proteins occurs in paralysed and parasitized larvae prior to ingestion of host fluid by the parasitoid larvae. In *H. hebetor* protein digestion occurs throughout larval growth and during feeding, but the titer of proteinases increases just after cocoon spinning is complete. When larvae have been in their cocoons for 48 h, protein digestion is nearly complete and the midgut itself, which in the feeding larvae composes almost the entire body cavity, becomes much reduced in size.

There have been very few studies of protein digestion in Hymenoptera (Terra and Ferreira, 1994). Trypsin-like and chymotrypsin-like enzyme activities were found in midguts of adult honey bees, *Apis mellifera* (Giebel et al., 1971; Malone et al., 1995). Jany et al. (1978) demonstrated trypsin-like enzyme activity in larval midguts of two species of Vespidae and later (Jany et al., 1983) documented the first amino acid sequence of an insect chymotrypsin from the hornet *Vespa orientalis.* In these studies, serine proteinases were predominant in both adult and larval stages. Serine proteinases in the trypsin family are also primarily responsible for the initial stages of protein digestion in *H. hebetor,* based on activity against the artificial substrate BA*p*NA and inhibition of activity against azocasein and BA*p*NA by several serine proteinase inhibitors, including PMSF, *p*-aminobenzamidine, TLCK, and Bowman–Birk trypsin-chymotrypsin inhibitor. There is a low level of activity against SAAPF*p*NA, indicating the possible presence of a chymotrypsin-like enzyme, and very weak activity against SAAPL*p*NA, a substrate for elastase-like enzymes. However, DTT did not enhance BA*p*NAase activity and cystatin had no effect on hydrolysis of azocasein, providing evidence that cysteine proteinases do not contribute significantly to protein digestion. In addition, pepstatin A, an inhibitor of aspartic proteinases, did not inhibit either BA*p*NAase activity or activity against azocasein, indicating that acid proteinases are relatively inactive in this species.

Midgut fluid of larval *H. hebetor* that are feeding on host tissues has a pH of approximately 6.6–6.8. This pH may be influenced by the rapid ingestion of host *P. interpunctella* hemolymph that has a pH of about 6.4, similar to the hemolymph pH in other insects (Mullins, 1985). It is not known whether the midgut pH changes as feeding stops and as digestion proceeds in the cocoon.

Most endoproteinase activity was found in the soluble fraction, however, the microsomal fraction of *H. hebetor* midgut extracts contained significant amounts of azocaseinase and BA*p*NAase activity, as well as most of the aminopeptidase activity. The microsomal fraction contains fragments of plasma membrane which are known to contain bound peptidases as well as other digestive enzymes (Terra and Ferreira, 1994).

Because the midgut samples used for the gelatin zymograms were analysed under non-reducing conditions and because the proteinases may interact with gelatin during electrophoresis, this procedure does not necessarily give accurate estimates of the molecular masses of the proteinases that are detected. However, with these conditions, the estimated molecular mass of bovine pancreatic trypsin was very similar to the actual mass of the enzyme. Currently, we have no direct evidence that P1 or P3 are serine proteinases. However, the estimated molecular mass of 39 kDa for P3 is similar to that for several trypsin-like enzymes in crude preparations of several other insects (Terra and Ferreira, 1994). We are attempting to purify these proteinases to obtain more information on their biochemical properties.

In summary, our results indicate that the composition of hemolymph proteins in paralysed hosts of *H. hebetor* remains unchanged for up to 72 h after stinging by the adult wasp. Directly or indirectly, envenomization does not alter the quality of these host proteins as nutrients for the immature parasitoid for an extended time period. Based on electrophoretic evidence, the putative apolipophorin I and hexamerins present in host hemolymph are readily digested by the serine proteinases present in the midguts of *H. hebetor* larvae. Digestion by active trypsin-like midgut enzyme(s) with activity against BA*p*NA occurs during the continuous feeding larval stage, reaches a peak in activity about 24 h after the initiation of cocoon formation, and is nearly completed after an additional 24 h. Proteins similar to the major storage proteins present in the host hemolymph would provide a readily digestible dietary source of amino acids when incorporated into artificial diets for these species.

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