

Characterization of digestive proteolytic activity in *Lygus hesperus* Knight (Hemiptera: Miridae)[☆]

M. Knop Wright^a, S.L. Brandt^a, T.A. Coudron^{a,*}, R.M. Wagner^b, J. Habibi^c,
E.A. Backus^d, J.E. Huesing^e

^aUSDA-Agricultural Research Service, Biological Control of Insects Research Laboratory, 1503 S. Providence Road, Columbia, MO 65203, USA

^bOffice of Technology Transfer, USDA-ARS, Peoria, IL, USA

^cDepartment of Entomology, University of Missouri, Columbia, MO 65211, USA

^dUSDA-ARS, Parlier, CA, USA

^eMonsanto Co., 700 Chesterfield Parkway North, Chesterfield, MO 63198, USA

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Abstract

The tarnished plant bug, *Lygus hesperus* Knight, is a pest that causes considerable economic losses to vegetables, cotton, canola, and alfalfa. Detailed knowledge of its digestive physiology will provide new opportunities for a sustainable pest management approach to control this insect. Little is known about the different protease class contributions to the overall digestion of a specific protein. To this end, the proteolytic activities in female adult *L. hesperus* salivary gland and midgut homogenates were quantified over a range of pH's and time points, and the contribution of different classes of proteases to the degradation of FITC-casein was determined. In the salivary gland, serine proteases were the predominant class responsible for caseinolytic activity, with the rate of activity increasing with increasing pH. In contrast, both aspartic and serine proteases contributed to caseinolytic activity in the midgut. Aspartic protease activity predominated at pH 5.0 and occurred immediately after incubation, whereas serine protease activity predominated at pH 7.5 after a 9 h delay and was resistant to aprotinin. The salivary serine proteases were distinctly different from midgut serine proteases, based on the tissue-specific differential susceptibility to aprotinin and differing pH optima. Collectively, the caseinolytic activities complement one another, expanding the location and pH range over which digestion can occur.

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1. Introduction

Digestive proteases play two critical roles in an insect's physiology—breaking down proteins into amino acids essential for growth and development and inactivating protein toxins ingested as a consequence of feeding (Terra et al., 1996). Disruption of these processes has the potential to suppress populations of phytophagous insect pests by

limiting nutrient availability or by increasing the pests' vulnerability to toxic proteins.

Lygus hesperus Knight is a major pest of cotton in the US (Nordlund, 2000). Its lacerate and flush feeding strategy (Miles, 1972; Backus, 1988; Cline and Backus, 2002) suggests that the introduction of plant defensive proteins i.e., lectins (Down et al., 2003; Habibi et al., 1993, 2000), α -amylase inhibitors (Amirhusin et al., 2004), polygalacturonase-inhibiting proteins (D'Ovidio et al., 2004), or protease inhibitors (Hilder et al. 1987; Gatehouse et al., 1993; Lecardonnell et al., 1999; Lee et al., 1999) may interfere with its normal digestive physiology sufficiently to suppress the pest in the field. The efficacies of plant defensive proteins, however, are dependent upon the fates of those proteins in the digestive systems of insects. Recent

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*Corresponding author. Tel.: +1 573 875 5361x225.

E-mail address: coudront@missouri.edu (T.A. Coudron).

studies have shown that insects can compensate for the inhibition of major proteolytic activities by increasing production or synthesizing alternate isoforms of the same enzymes, by upregulating the expression of alternate classes of proteases, or by changing the microenvironment of the gut to enhance an enzyme's activity (Broadway, 1995; Chougule et al., 2005; Cooper and Vulcano, 1997; Fujita, 2004; Jongsma and Bolter, 1997). A thorough characterization of the types and activities of digestive proteases is crucial to understanding the degradation of nutritional proteins and to determining the effects that xenobiotic proteins have on an insect's digestive system.

In the case of *L. hesperus*, residence times of ingested substances in the gut are ~12 h, with some proteins remaining in the gut 24 h after ingestion (Brandt et al., 2004; Habibi et al., 2002). This suggests that even minor or delayed proteolytic activities associated with the salivary glands and midgut may be sufficient to significantly degrade ingested proteins, and could impact the effectiveness of nutritive and toxic proteins. Although alkaline and acidic proteolytic activities have been detected, and the serine proteases have been partially characterized in both the gut and salivary glands of *Lygus* spp. (Agusti and Cohen, 2000; Laurema et al., 1985; Zeng et al., 2002a, b; Zhu et al., 2003), little is known about the different protease class contributions to the overall digestion of a specific protein. Here we present a thorough characterization of *L. hesperus* salivary and midgut proteases based on study of a single substrate, FITC-labeled casein.

2. Methods and materials

2.1. Chemicals

FITC-casein, cystatin, Penn-Strep, pronase and trichloroacetic acid were purchased from Sigma-Aldrich (St. Louis, MO); aprotinin and 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF) were purchased from Calbiochem (La Jolla, CA); aprotinin, bestatin, E-64 and pepstatin A were purchased from Roche Diagnostics Corp. (Indianapolis, IN); Acetyl- β -endorphin (A β e) was purchased from Bachem Bioscience Inc. (King of Prussia, PA). All dissection buffers were prepared using purified water (Milli-RO 2-10 system, Millipore Corp., Billerica, MA).

2.2. Insect rearing and dissections

L. hesperus were reared continuously on artificial diet (*L. hesperus* diet, Bioserv, Frenchtown, NJ) (16L:8D, $27 \pm 2^\circ\text{C}$, 70% humidity) according to Habibi et al. (2000). Adult diet-fed females, 7–10 days old with mature eggs, were chosen for these studies in order to ensure that sex, stage and diet-specific differences in protease activity would not confound the results (Terra et al., 1996). Prior to dissection, adults were starved for 16–20 h to purge gut contents. For dissection, insects were anaesthetized with CO₂ and mounted dorsally in paraffin. The abdomens were

covered with 200 μl of ice-cold dissection buffer (0.1 M NH₄COOH, pH 6.4; 0.1 M Tris-HCl, pH 7.0 or 10 mM NaCl). The ovipositor was pulled into a droplet of ice-cold dissection buffer, separating the apical segments from the rest of the abdomen. Insects were decapitated, and an incision was made along the lateral edges of the abdomen. Ventral abdominal sclerites and ovaries were removed, and the midgut was excised from the hemocoel. The hindgut and extraneous fat body were detached. Removal of the midgut exposed the dorsally located salivary glands, which were also excised. Midguts and salivary glands were rinsed three times in fresh buffer, transferred to separate 1.5 ml microcentrifuge tubes (Safe-Lock, Eppendorf, AG) containing 1 μl of dissection buffer for each insect equivalent (IE) of tissue, flash frozen and stored at -80°C for future use.

2.3. Preparation of insect homogenates

The tissues were thawed on ice and homogenized using plastic pestles. The volume was adjusted using dissection buffer to achieve a final concentration of 1 IE of midgut per 4 μl , or 1 IE of salivary gland tissue per 2 μl . Homogenates were kept on ice until used.

2.4. Determination of pH of midgut and salivary gland tissues

The pH of salivary and midgut tissues was measured in starved adult females, so that the artificial diet (Brandt et al., 2004) would not influence the result. Fresh midguts and salivary glands dissected into 10 mM NaCl were crushed onto pH test strips (colorPhast pH test strips, EM Science, Cherry Hill, NJ), and the color change was compared with a scale supplied by the manufacturer. Three samples of each tissue were analyzed.

2.5. Determination of protein concentration

Tissues were thawed on ice, homogenized and protein analysis was done using the Micro BCA Protease Assay Kit (Pierce, Rockford, IL). Three replicates of each tissue were analyzed.

2.6. Determination of caseinolytic activity (FITC-casein assay)

A fluorescent conjugate of casein, fluorescein isothiocyanate (FITC)-casein was used as a substrate for quantifying proteolytic activity. Previous studies indicated that digestion of FITC-casein and casein are similar, and that the labeled protein is an appropriate model substrate for characterizing the activity of *L. hesperus* digestive proteases (Habibi et al., 2002).

Caseinolytic activities of midgut and salivary gland homogenates were analyzed using a derivation of the method of Twining (1984). Each IE of homogenate was

diluted to 56 μl with ice-cold reaction buffer of a selected pH. A stock solution of FITC-casein (100 μg in 4 μl) in purified water was added to make a final reaction volume of 60 μl /IE in a siliconized 500 μl Eppendorf tube. Tubes were incubated up to 27 h in a heating block at 30 °C, with mixing every 4 h. Ammonium formate and Tris buffers were used to span the pH range for this study and to be compatible with mass spec analyses. Reaction buffers were prepared by adjusting 0.1 M NH_4COOH with 1 N HCOOH or 1 M NH_4OH to achieve a pH range of 4.5–7.0 \pm 0.05 or by adjusting 0.1 M Tris–HCl with 1 N HCl and 1 M NaOH to achieve a pH range of 7.0–9.0 \pm 0.05.

At each time point, all tubes were vortexed, and 20 μl aliquots of each reaction were removed and added to 40 μl of 5% trichloroacetic acid (TCA) in a second set of tubes for quantification of caseinolytic activity. After vortexing, the second set of tubes was incubated for another 10 min at 30 °C, and then centrifuged at 4 °C for 5 min at 12,000*g* to precipitate undigested proteins. A 40 μl aliquot of the supernatant was then transferred to the well of a 96-well microplate (Costar 3912) containing 160 μl of assay buffer (0.5 M Tris–HCl, pH 8.8). Caseinolytic activity was expressed in terms of the relative fluorescence units (RFU) of the unprecipitated FITC-labeled peptides using a microtiter plate spectrophotometer (HTS 7000 Plus Bio-Assay reader, Perkin-Elmer, Foster City, CA) with excitation and emission filters of 485 and 535 nm, respectively. At least three replicates were performed per treatment. Additions of 100 $\mu\text{g}/\text{ml}$ penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin to the reactions had no effect on caseinolytic activity, confirming that the activity originated from the insect rather than from bacterial contamination.

2.7. Determination of the pH optima and rates of caseinolytic activity

The optimal pH values for caseinolytic activity in midgut and salivary gland homogenates were determined using the FITC-casein assay previously described. Three IE of tissue homogenate were incubated with 300 μg of FITC-casein, in either 0.1 M NH_4COOH or 0.1 M Tris–HCl buffers that were adjusted to 11 pH values over a range of 4.5–9.0. This range was chosen because it encompassed the estimated pH range of *L. hesperus* digestive tissues (6.0–7.0), the pH range of most plant or insect tissues that make up the insect's diet (4.0–8.5), and a pH range wide enough to detect the activity of the major classes of digestive proteases (Beynon and Bond, 2001; Harrison, 2001; Marschner, 1994). Aliquots of the salivary gland reaction mixture were removed at 0, $\frac{1}{2}$, 1, 2, 4, 6, and 18 h. Aliquots of the midgut reaction mixture were removed at 0, 2, 4, 6, 8, 12, 16, 20, and 24 h. These time points were chosen for each tissue based on preliminary experiments that identified both the time interval during which the rate of caseinolytic activity (RFU/h) was linear and the time

period over which activity reached a maximum (total RFU). Three replications were conducted.

The rates of caseinolytic activity in salivary and midgut homogenates over the pH range of 4.5–9.0 were calculated from the data on pH-dependent caseinolytic activity. The experimental maximum rate was defined as the amount of product formed (RFU) per unit time and was calculated as the mean of at least three rates measured in the linear region of each activity plot unless otherwise stated (Robyt and White, 1987). The substrate was shown not to be limiting, because doubling FITC-casein from 100 to 200 $\mu\text{g}/\text{IE}$ did not increase the rate of activity. Therefore, the initial velocity, v_i , was equal to the maximum velocity, v_{max} (Robyt and White, 1987; Tinoco et al., 1985), which was used as the maximum rate of caseinolytic activity at each pH.

2.8. Determination of the contribution of specific protease classes to caseinolytic activity

Combinations of protease inhibitors were chosen such that only one of the four mechanistic classes of proteolytic activity (metallo-, serine, cysteine, and aspartic) remained active during each reaction. Table 1 shows the protease inhibitors used: pepstatin A, bestatin, E64, cystatin, aprotinin and AEBSF. Because of the potential ability of the cysteine protease inhibitor, E64, to inhibit serine proteases (Novillo et al., 1997), serine proteolytic activity was measured twice, once using a combination of inhibitors that included E64 and once replacing E64 with cystatin, a more specific cysteine protease inhibitor (Calbiochem, 2002). Preliminary studies were performed to determine the optimal concentration for each protease inhibitor. The lowest concentration of inhibitor that blocked at least 90% of the activity during a 27-h incubation and over a pH range of 4.5–9.0 was used in subsequent experiments.

To quantify class-specific activity, combinations of protease inhibitors were pre-incubated with tissue homogenates for 30 min at 30 °C prior to the addition of FITC-casein. Aliquots of 20 μl were removed at 0, 9, 18, and 27 h after the addition of the substrate, and the activity of each protease class was quantified as described previously. Experiments were conducted at pH 5.0, 6.5, 7.5, and 8.5. At least three replications were conducted.

2.9. Matrix-assisted laser desorption and ionization–time of flight mass (MALDI–TOF) spectrometer analysis of the digestion of acetyl β -endorphin (*A β e*)

To further verify the presence of each protease class and determine potential cleavage sites, we conducted a parallel series of experiments in which FITC-casein was replaced with *A β e*. *A β e* has a sequence of 31 amino acids that provides specific sites suitable for proteolytic cleavage by serine, aspartic, and cysteine proteases (Beynon and Bond, 2001). The acetylated form was used to block initial

Table 1
Combinations of protease inhibitors used to determine the classes of proteolytic activity contributing to the caseinolytic activity of midgut and salivary gland homogenates (X indicates the presence of the inhibitor)

Class of proteolytic activity	Pepstatin A (Aspartic) 2.5 μ M	Bestatin (Aminopeptidase) 2 mM	E64 (Cysteine) 420 μ M	Cystatin (Cysteine) 16.4 μ M	Aprotinin ^a (Serine) 4.6 μ M	AEBSF ^a (Serine) 10 mM
All proteases						
Aspartic proteases		X	X		X	X
Aminopeptidases	X		X		X	X
Cysteine proteases/ +cys ^b	X	X			X	X
Serine proteases/E64 ^c	X	X	X			
Serine proteases/cystatin ^c	X	X		X		
Unknown proteases	X	X	X		X	X

Inhibitors used are in bold, followed by the specific class of proteases inhibited (in parentheses), and the concentration used.

^aA combination of both aprotinin and AEBSF were used to inhibit serine proteases because aprotinin-resistant serine protease activity is associated with the midgut.

^bA 2.5 mM cysteine was added during the incubation to enhance cysteine protease activity. Because cysteine interfered with the activity of other classes of proteases, it was not added to other incubations.

^cSerine proteases were measured twice using two different cysteine protease inhibitors, E64 or cystatin, since E64 decreased serine proteolytic activity in preliminary studies.

digestion by aminopeptidases. A β e (solubilized in 1% trifluoroacetic acid) was dried under argon or helium gas and resolubilized in an amount of acetonitrile equivalent to 1.25% of the final reaction volume. All reactions consisted of 87.7 μ g/ml A β e in 0.1 M NH₄COOH (pHs 5.0 and 6.5) or NH₄CH₂COOH (pH 8.0) and were preincubated for 30 min at 30 °C alone or in combination with one or more of the protease inhibitors pepstatin, aprotinin, or AEBSF at final concentrations of 0.25, 5, or 250 μ g/ml, respectively. Tissue homogenates were then added at a concentration equivalent to 2.5 IE/ml, and reactions were incubated for 0 or 6 h at 30 °C at pH 6.5 and 8.0 (salivary gland) or 5.0 and 6.5 (midgut). Reactions were stored at –80 °C until the peptide fragments were identified by matrix-assisted laser desorption and ionization–time of flight (MALDI–TOF) Mass Spectroscopy (Applied Biosystems Voyager DE Pro Biospectrometry Workstation). Background, consisting of inhibitors and unidentified peptides, was subtracted from the MALDI–TOF analyses.

2.10. Statistical analyses

Data was analyzed using PROC MIXED (SAS Institute Inc., 1999). Differences between treatment means were evaluated using Tukey's Highly Significant Difference test with a Tukey–Kramer adjustment for unequal sample sizes (Tukey) and were considered significant when the probability (P) ≤ 0.05 . Treatment means were reported to 'show a trend toward significance' when the $P > 0.05$ that two treatment means were the same when using the Tukey procedure, but $P \leq 0.05$ when using the less-stringent Fisher's LSD procedure. Analyses were performed using the model for subjects (sub) with repeated measures in which compound symmetry (cs) was found to best describe covariance matrices. For pH optima studies, the program

parameters were: Class id pH hr; Model rfu = pH/hr; repeated hr/sub = id(pH) type = cs (SAS Institute Inc., 1999). For the contribution of specific protease classes, the program parameters were: Class id pH trt hr; Model rfu = pH|trt/hr; repeated hr/sub = id(trt pH) type = cs (SAS Institute Inc., 1999).

3. Results

3.1. pH and protein content of salivary glands and midgut

The pH of salivary tissue was 6.75 ± 0.25 , while the pH of midgut lumen contents or midgut homogenates was 6.25 ± 0.25 . The average protein content of one IE of salivary gland homogenate was 4.93 ± 0.32 μ g, while one IE of midgut homogenate consisted of 30.51 ± 0.13 μ g protein.

3.2. Effect of pH on caseinolytic activity

The upper and lower limits of fluorescence from undigested (buffer only) and digested (pronase-treated) FITC-casein were 471 ± 28 RFU and 13, 540 \pm 179 RFU, respectively (Fig. 1). Although autohydrolysis of FITC-casein over the pH range 4.5–9.0 was detectable (fluorescence averaged over all pH values increased only from 471 to 728 ± 28 RFU ($N = 33$) over 0–16 h at 30 °C), this activity did not significantly impact the total caseinolytic activities measured in this study.

The extent of caseinolytic activity in salivary gland homogenates (Fig. 1) increased with increasing pH ($F_{10,22} = 114.9$, $P < 0.0001$) and time ($F_{7,142} = 736.88$, $P < 0.0001$), where the increase over time was significantly affected by pH ($F_{70,142} = 25.0$, $P < 0.0001$). At pH ≥ 8.5 , caseinolytic activity in salivary gland homogenates was rapid, with over 75% of the activity occurring within 2 h at

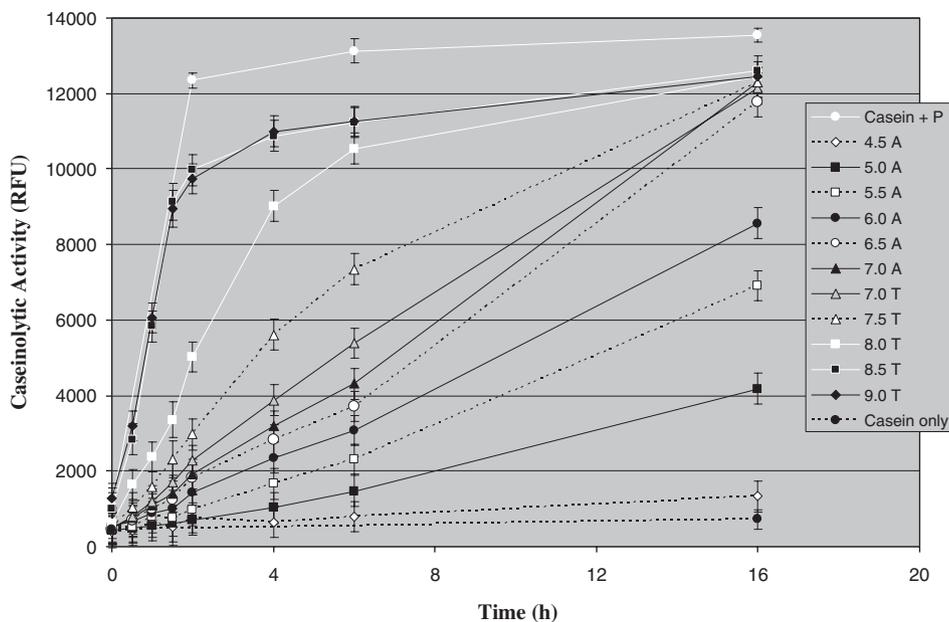


Fig. 1. Effect of pH on the caseinolytic activity of *L. hesperus* salivary gland homogenates. pH values are followed by an 'A' (0.1 M NH_4COOH) or 'T' (0.1 M Tris-HCl) to indicate the reaction buffer used. Activities without insect proteases (Casein only) and in the presence of pronase (Casein + P) are included for comparison. Means and standard errors were determined using PROC MIXED (SAS Institute Inc., 1999).

30 °C. The extent of activity after 16 h at pH 8.5 was $12,593 \pm 405$ RFU and was comparable to that measured in the presence of pronase. Caseinolytic activity over the pH range 6.5–8.0 was not as rapid, but the extent after 16 h was similar to that observed at pH 8.5. Caseinolytic activity was significantly reduced at each pH from 6.5 to 4.5, at which point no activity was detected.

The rate of caseinolytic activity in salivary gland homogenates increased with increasing pH ($F_{10,22} = 36.8$, $P < 0.0001$) and decreased with time ($F_{6,121} = 23.9$, $P < 0.0001$), as substrate became limiting. The timing of the maximum rate was also affected by pH ($F_{60,121} = 8.9$, $P < 0.0001$). Maximum rates of caseinolytic activity by salivary proteases were 4798 ± 630 (pH 8.5) and 4676 ± 525 (pH 9.0) RFU per hour and occurred within 1.5 h (Fig. 3). These rates were significantly greater than the rates of caseinolytic activity at pH's below 8.5 (Tukey, $P < 0.05$) and were at least 6X greater than the rates measured at pH's ≤ 7.0 .

The extent of caseinolytic activity in midgut homogenates (Fig. 2) also increased with time ($F_{8,164} = 342.5$, $P < 0.0001$) and varied with pH ($F_{10,22} = 10.8$, $P < 0.0001$), with a significant interaction observed between time and pH ($F_{80,164} = 5.11$, $P < 0.0001$). At acidic pH's, caseinolytic activity was immediate, with significant activity at pH 4.5 (Tukey, $P < 0.0034$). At pH's 5.5–8.0, significant caseinolytic activity occurred after a delay of 8 h (Tukey, $P < 0.05$). Above a pH of 8.0, significant activity was observed only after 20 h (Tukey, $P < 0.05$). Caseinolytic activity after 24 h at pH 4.5 and pH's 5.5–8.0 was at least 44% and 18% lower than that recorded with pronase after 16 h.

The rate of caseinolytic activity in midgut homogenates was affected by time ($F_{5,88} = 7.05$, $P < 0.0001$) and by pH ($F_{10,22} = 3.02$, $P = 0.0148$). The interaction between time and pH was not significant. The maximum rate of acidic caseinolytic activity in the midgut was 783 ± 106 RFU/h and occurred during the first 4 h at pH 4.5 (Fig. 3). The maximum rates of neutral to alkaline caseinolytic activity occurred over the pH range 5.5–8.0 after 12–20 h and ranged from 495 ± 157 to 643 ± 132 RFU/h (Fig. 3). Two peaks of caseinolytic activity separated by pH and time suggested that at least two separate proteolytic activities were present in the midgut.

3.3. Contribution of specific protease classes to caseinolytic activity

Since preincubation with all inhibitors eliminated significant caseinolytic activity by salivary or midgut homogenates (non-significant activity labeled as "unknown activity" in Figs. 4 and 5), the activity observed in the absence of a class specific inhibitor was an accurate measure of the caseinolytic activity by that class in the absence of cooperative effects from other activities. The extent of caseinolytic activity in salivary gland homogenates was significantly affected by time ($F_{3,523} = 1091.3$, $P < 0.0001$), pH ($F_{3,177} = 213.1$, $P < 0.0001$) and the active class of proteases ($F_{7,177} = 524.9$, $P < 0.0001$). The interactions between time, pH, and protease class (PC) were also significant, indicating that the effects of time and class on caseinolytic activity were specific to the pH of the reaction (pH \times PC, $F_{21,177} = 55.9$, $P < 0.0001$; PC \times hr,

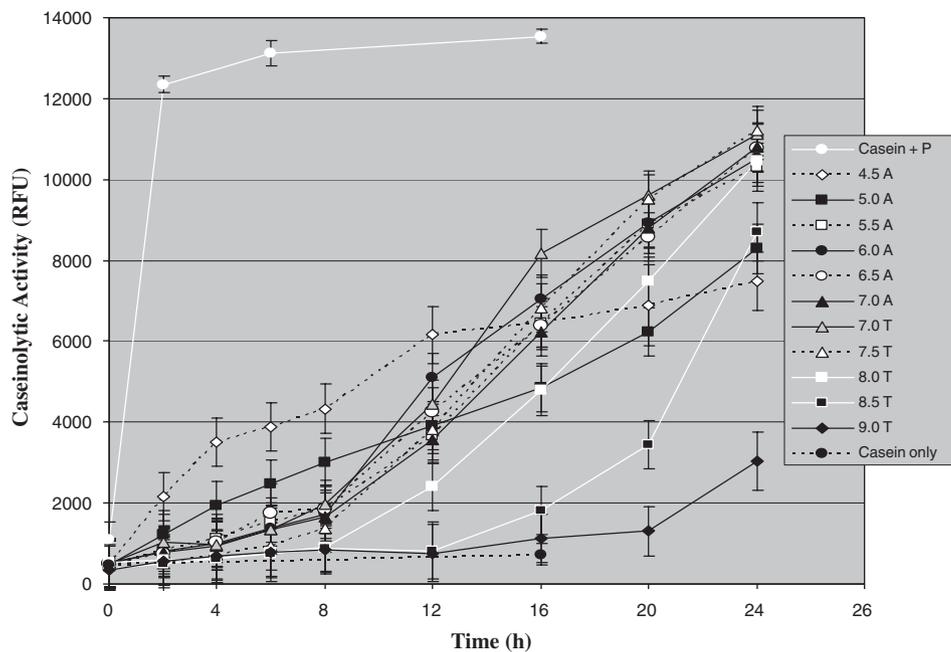


Fig. 2. Effect of pH on the caseinolytic activity of *L. hesperus* midgut homogenates. pH values are followed by an 'A' (0.1 M NH_4COOH) or 'T' (0.1 M Tris-HCl) to indicate the reaction buffer used. Activities without insect proteases (Casein only) and in the presence of pronase (Casein + P) are included for comparison. Means and standard errors were determined using PROC MIXED (SAS Institute Inc., 1999).

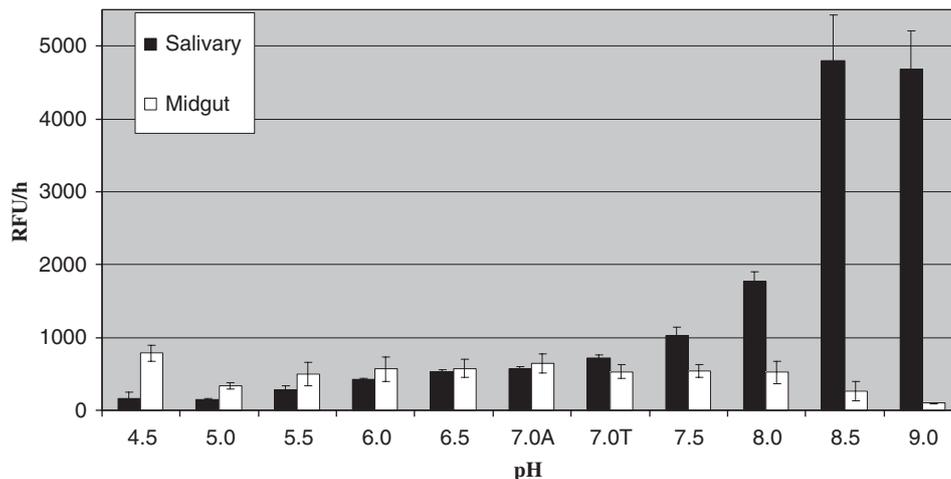


Fig. 3. Comparison of pH effects on the rate of caseinolytic activity in *L. hesperus* salivary gland and midgut homogenates. Experimental rates (RFU/h) were calculated from data taken at a minimum of three time points over which activity was linear, and therefore substrate was not limiting. This interval was 0–6 h for salivary activity at pH's 4.5–9.0, and 0–8 h for midgut activity at pH's 4.5–5.0, and 9.0, and 8–20 h for midgut activity at pH's 5.5–8.5. Means and standard errors were determined using PROC MIXED (SAS Institute Inc., 1999).

$F_{21,523} = 216.6$, $P < 0.0001$; $\text{pH} \times \text{hr}$, $F_{9,523} = 85.4$, $P < 0.0001$; $\text{pH} \times \text{PC} \times \text{hr}$, $F_{63,523} = 25.8$, $P < 0.0001$).

While caseinolytic activity in salivary homogenates was significant at pH 5.0 (Tukey $P < 0.0001$; Fig. 4A), it could not be attributed to any single class of proteases (Tukey $P > 0.05$). At pH 6.5 serine protease activity was significant (Tukey, $P < 0.0001$; Fig. 4B), however, this accounted for only 40% of the total activity observed. Aspartic protease was the only other class of proteases that showed a trend of increased activity at pH 6.5 after 27 h (Fisher's LSD,

$P < 0.05$). At a pH of 7.5 and 8.5, serine protease activity accounted for 80% and 95%, respectively, of the total caseinolytic activity (Fig. 4C and D). Salivary serine proteolytic activity measured in the presence of the cysteine protease inhibitor E64 was consistently lower than when measured in the presence of cystatin (Fisher's LSD, $P < 0.0222$ –0.0001), although this difference was highly significant only over the first 9 h at pH 8.5 (Tukey, $P < 0.0001$). In the case of midgut serine proteases, E64 did not have an effect at any pH (Tukey, $P > 0.05$; Fig. 5).

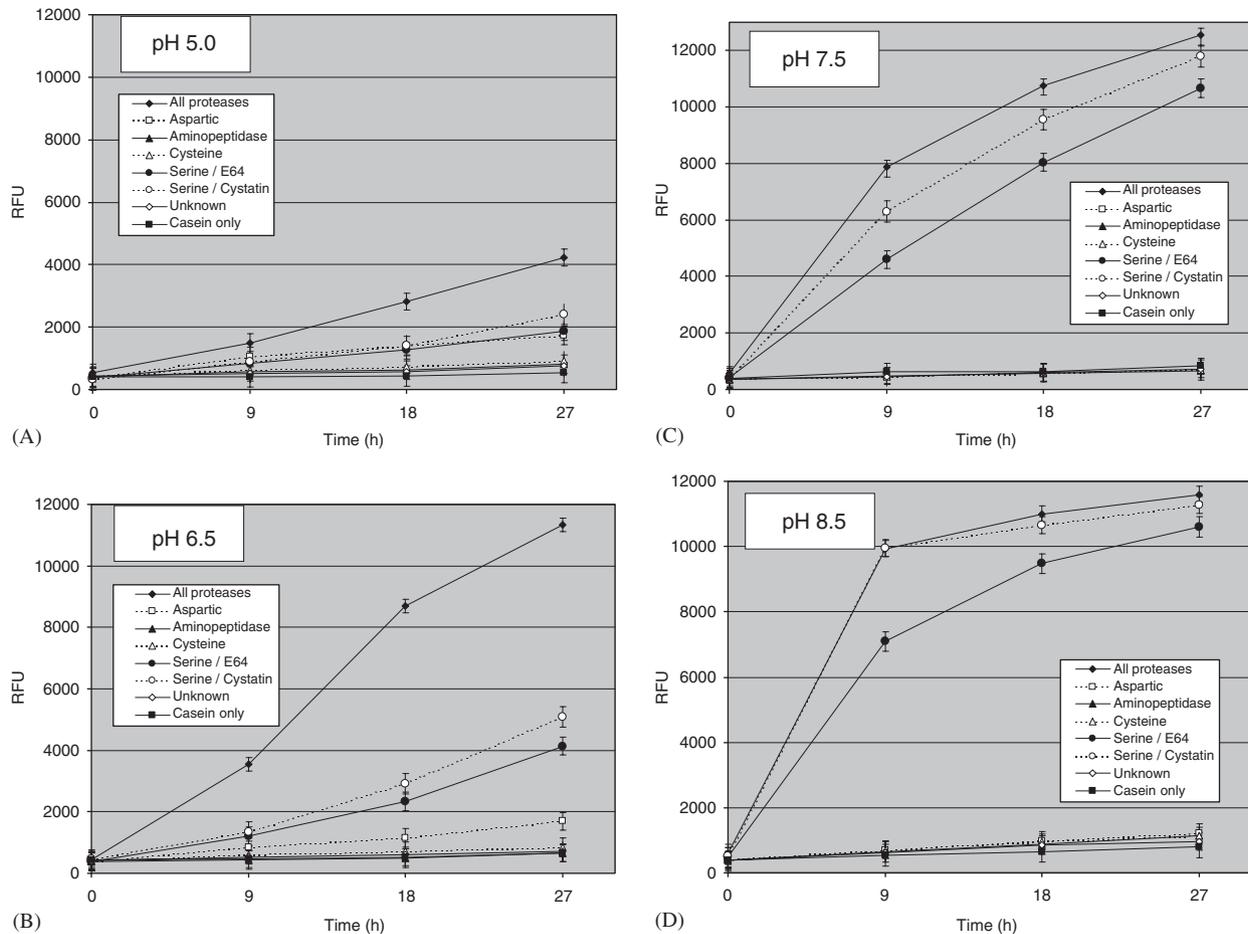


Fig. 4. Contributions of different protease classes to the caseinolytic activity of *L. hesperus* salivary gland homogenates over a pH range of 5.0–8.5. Means and standard errors were determined using PROC MIXED (SAS Institute Inc., 1999).

The extent of caseinolytic activity in midgut homogenates was also significantly affected by time ($F_{3,619} = 411.5$, $P < 0.0001$), pH ($F_{3,210} = 26.7$, $P < 0.0001$), and the active class of proteases ($F_{7,210} = 67.3$, $P < 0.0001$). The interactions between time, pH, and PC were also significant (pH \times PC, $F_{21,210} = 11.4$, $P < 0.0001$; PC \times hr, $F_{21,619} = 56.7$, $P < 0.0001$; pH \times hr, $F_{9,619} = 36.4$, $P < 0.0001$; pH \times PC \times hr, $F_{63,619} = 11.2$, $P < 0.0001$).

At pH 5.0, midgut aspartic activity was significant and accounted for 75–85% of the caseinolytic activity (Tukey, $P < 0.0001$; Fig. 5A). At pHs 6.5 and 7.5, serine caseinolytic activity in the midgut mirrored overall activity, with significant activity occurring after a 9 h delay (Tukey, $P < 0.0001$; Fig. 5B and C). At 18 h, midgut serine activity accounted for 63% of the total caseinolytic activity at pH 6.5 and 87% of the total activity at pH 7.5. No other class of proteases in the midgut homogenates contributed significantly to caseinolytic activity at a pH of 6.5 or 7.5. No detectable caseinolytic activity was measured in the midgut at pH ≥ 8.5 , (Fig. 5D).

A comparison of the rates of caseinolytic activity by aspartic and serine proteases in the salivary gland versus the midgut is shown in Fig. 6. The rate of salivary gland

serine protease activity was significantly greater at pH 8.5 than at lower pH values (Tukey, $P \leq 0.05$, Fig. 6), with most of the activity occurring during the first 9 h. In contrast, both aspartic and serine proteases contributed to the caseinolytic activity in the midgut. Aspartic proteolytic activity predominated at pH 5.0 within the first 9 h, whereas serine proteolytic activity predominated at pH 7.5 after 9 h. At the optimal pH for salivary serine proteases, pH 8.5, neither class of midgut proteases had significant caseinolytic activity.

3.4. Differential susceptibility of salivary and midgut serine proteases to aprotinin

The effect of aprotinin and AEBSF on caseinolytic activity by serine proteases after 27 h at 30 °C is shown in Fig. 7. Addition of AEBSF to salivary or midgut reactions similarly treated to inhibit all proteases except serine resulted in nearly complete inhibition of caseinolytic activity. Aprotinin inhibited nearly 80% of the salivary gland serine protease activity at its optimum pH 8.5. In contrast, aprotinin had no effect on the midgut serine protease activity at its optimum pH 7.5, indicating that

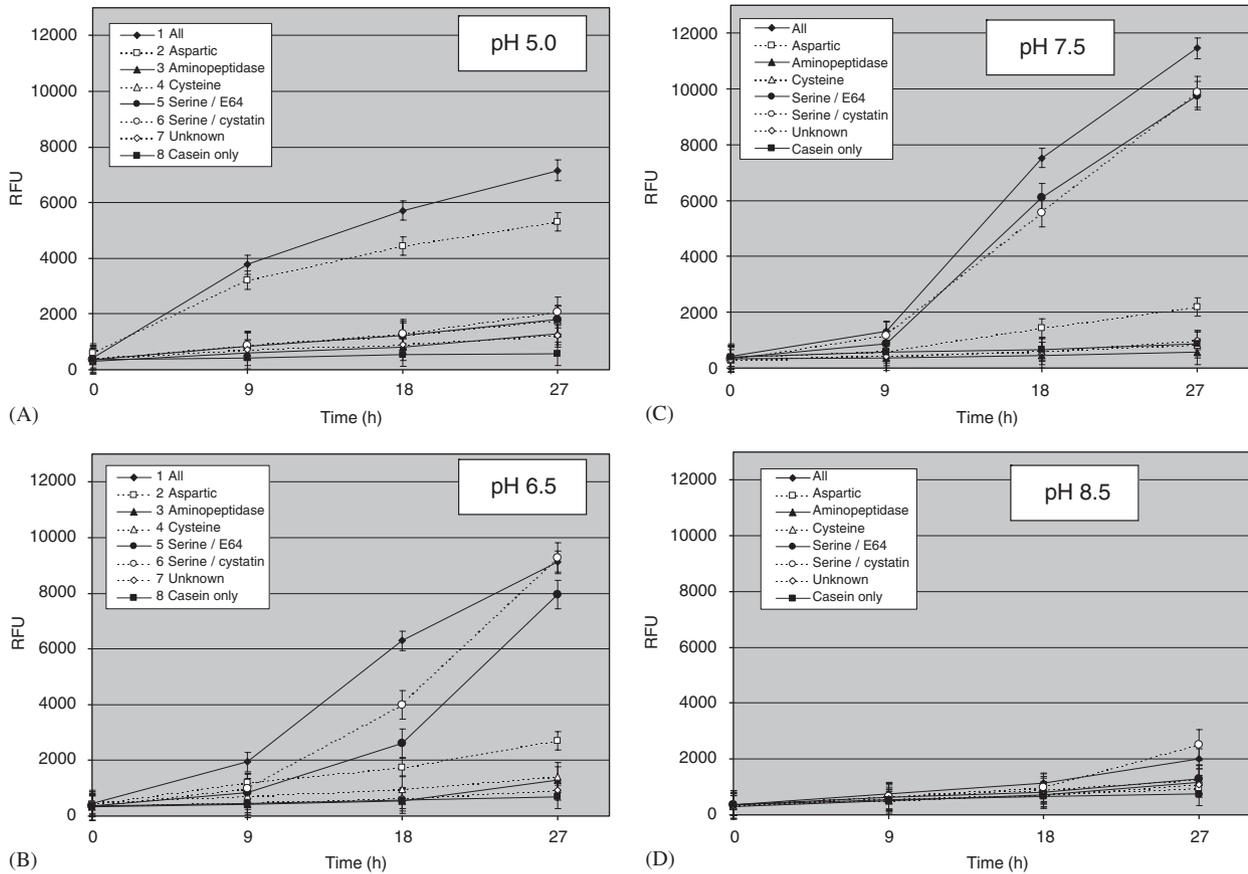


Fig. 5. Contributions of different protease classes to the caseinolytic activity of *L. hesperus* midgut homogenates over a pH range of 5.0–8.5. Means and standard errors were determined using PROC MIXED (SAS Institute Inc., 1999).

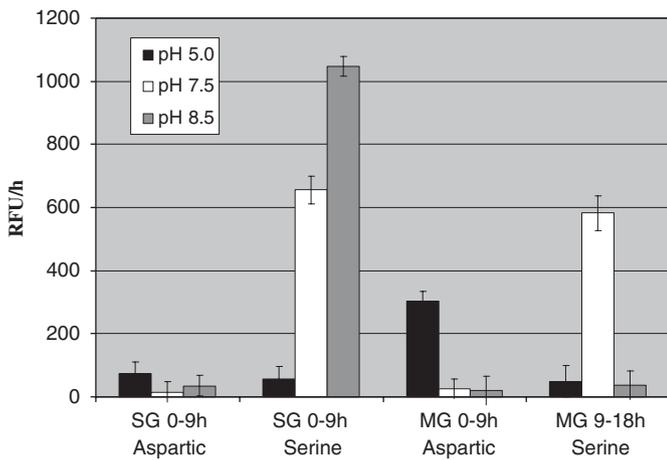


Fig. 6. Comparison of the average rates of caseinolytic activity (RFU/h) by aspartic and serine proteases in salivary gland (SG) and midgut (MG) homogenates of *L. hesperus*. Rates measured were averaged over the incubation period where greatest activity was detected. Means and standard errors were determined using PROC MIXED (SAS Institute Inc., 1999).

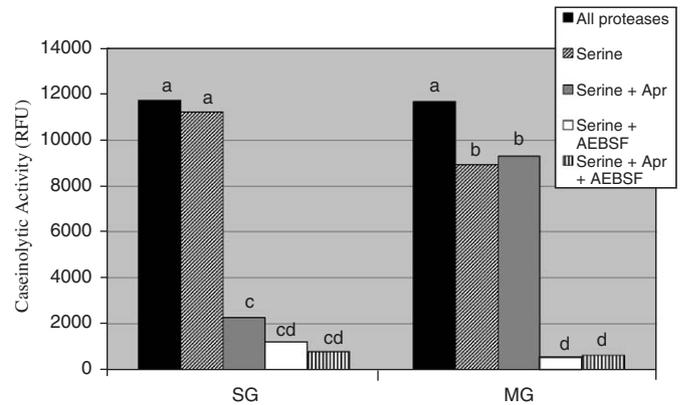


Fig. 7. Differences in the susceptibility of salivary gland (SG) and midgut (MG) serine proteases to the inhibitors aprotinin (Apr) and AEBSF. Bars with differing letters are statistically different from one another (Tukey, $P < 0.05$). Means and standard errors were determined using PROC MIXED (SAS Institute Inc., 1999).

midgut serine protease activity was resistant to aprotinin. Addition of antibiotics to the reactions ruled out bacterial contamination as an explanation for the observed resistance. Also, increasing the concentration of aprotinin by

20-fold or addition of more aprotinin after the first 8 h had no effect on the midgut serine proteolytic activity (data not shown). Therefore, it is unlikely that midgut homogenates contained a substance that inactivated or degraded aprotinin. In addition, midgut lumen extracts (lacking most of the structural proteins present in midgut homogenates) had the same characteristic delayed peak of

aprotinin-resistant serine protease activity (data not shown). The tissue-specific differential susceptibility to aprotinin and the differing pH optima indicate that the salivary serine proteases are distinctly different from midgut serine proteases.

3.5. MALDI–TOF Mass spectrometer analysis of the digestion of acetyl β -endorphin

Table 2 shows the major peptides between 1040 and 3506 daltons resulting from the reaction of A β e (MW 3507) with salivary gland or midgut homogenates and the indicated protease inhibitor treatments. In reactions with salivary gland proteases at pH 8.0, AEBSF completely inhibited digestion of A β e, corroborating the FITC-casein studies indicating that serine protease activity was predominant at this pH. In contrast, the aprotinin inhibition of salivary digestion of A β e at pH 8.0 was significant, but not complete. At pH 6.5, neither aprotinin nor AEBSF completely inhibited digestion of A β e, indicating that salivary serine proteases were not the only class of proteases active at this pH.

Digestion of A β e by midgut proteases at pH's 5.0 and 6.5 was significantly inhibited by pepstatin, in agreement with the FITC-casein studies. Peptides above a molecular weight of 1901 were only observed in the presence of pepstatin. Midgut serine proteases also contributed to the digestion of A β e at pH 6.5. Although aprotinin or AEBSF alone did not inhibit digestion at this pH, a combination of pepstatin and AEBSF blocked digestion of all but the two

C-terminal amino acids. This effect was not seen in reactions with both pepstatin and aprotinin, providing further evidence of aprotinin-resistant serine protease activity in midgut homogenates.

Cleavages consistent with trypsin-like activity (carboxy-terminal cleavage of lysine and arginine) were detected in both salivary and midgut reactions. Cleavages consistent with chymotrypsin-like activity (carboxyterminal cleavage of phenylalanine) were detected only in the salivary gland reactions and occurred in the presence of aprotinin, a weak chymotrypsin inhibitor, but not in the presence of AEBSF.

4. Discussion

Our findings support the concepts that digestive proteolytic activity in *L. hesperus* is dynamic and dependent on the interactions among tissue, pH, protease class and time. Our study has also discovered different protease classes active in the midgut and found evidence to suggest that the salivary gland and midgut activities complement one another, expanding the previously described range of protease activities found associated with *Lygus* spp.

Salivary caseinolytic activity in *L. hesperus* was due predominantly to serine proteases, with the highest rates of activity occurring within the first 9 h, at optimum pH 8.5. This is similar to the predominant trypsin-like serine protease activity found in *L. lineolaris* and *L. hesperus* based on hydrolysis of trypsin-specific substrates (BAPNA), 80–90% inhibition of these activities by aprotinin and the cloning of three trypsin-like cDNAs (Zeng et al.,

Table 2
MALDI–TOF Mass spectrometric analysis of the cleavage of acetyl β -endorphin by *L. hesperus* midgut and salivary gland proteases \pm inhibitors

Fragment	β -endorphin fragments	Mass (Da)	Salivary reaction						Midgut reaction							
			pH 6.5			pH 8.0			pH 5.0		pH 6.5					
			–I	+A	+F	–I	+A	+F	–I	+P	–I	+P	+A	+F	+PA	+PF
1–31	Ac-YGFMTSEKSTPLVTLFKNAIKNAYKGE	3506		X	X			X						X	X	X
1–29	Ac-YGFMTSEKSTPLVTLFKNAIKNAYK	3320								X				X	X	X
1–28	Ac-YGFMTSEKSTPLVTLFKNAIKNAYK	3192								X				X	X	X
1–25	Ac-YGFMTSEKSTPLVTLFKNAIKN	2829								X				X		X
1–24	Ac-YGFMTSEKSTPLVTLFKNAIK	2715								X						X
1–8	Ac-YGFMTSEKSTPLVTLF	2048	X	X			X									
15–31	VTLFKNAIKNAYKGE	1937					X									
1–7	Ac-YGFMTSEKSTPLVTL	1901	X	X							X		X	X		
14–29	LVTLFKNAIKNAYK	1864								X						
1–6	Ac-YGFMTSEKSTPLVT	1788	X							X	X	X	X	X	X	X
16–29	TLFKNAIKNAYK	1652			X										X	
18–31	FKNAIKNAYKGE	1624		X	X						X			X		
1–4	Ac-YGFMTSEKSTPL	1588	X				X									
19–31	KNAIKNAYKGE	1477	X	X	X		X									
1–3	Ac-YGFMTSEKSTP	1475												X		
18–29	FKNAIKNAYK	1438								X						
18–28	FKNAIKNAYK	1310									X		X			
20–29	NAIKNAYK	1163	X	X			X				X	X	X		X	
	Peptides <1040 Da (# fragments)	<1040	>3				>3			>3	1			1		

–I = absence of all protease inhibitors, P = pepstatin, A = aprotinin, F = AEBSF.

2002a,b; Zhu et al., 2003). We also found evidence for chymotrypsin-like activity in *L. hesperus* salivary glands, based on cleavage of A β e and differential inhibition with aprotinin and AEBSF. This contrasts with the findings of Agusti and Cohen, 2000, that detected no chymotrypsin activity in *L. hesperus* salivary homogenates. Researchers have suggested that the presence of salivary trypsin activity, and an absence of chymotrypsin activity, is characteristic of zoophagous heteropterans enabling the digestion of animal proteins (Boyd et al., 2002; Cohen, 1993; Colebatch et al., 2001). The presence of both trypsin-like and chymotrypsin-like activities in the salivary glands of *L. hesperus* may be a result of its zoophytophagous feeding strategy, or indicative of the digestive plasticity of this polyphagous insect.

We also found significant salivary caseinolytic activity between pH 5.0–7.0. Although none of the activity at pH 5.0 could be attributed to a single class of proteases, our finding that aspartic proteases may be a contributor differed from the report of cysteine-like protease activity found in *L. lineolaris* (Zeng et al., 2002b). No cysteine protease activity was detected in our studies. However, the cysteine protease inhibitor, E64, consistently lowered salivary serine caseinolytic activity, a phenomenon previously reported in other insects (Novillo et al., 1997) including *L. lineolaris* (Zhu et al., 2003). Consequently, caution should be taken when using BApNA or casein hydrolysis with E64 inhibition alone as an indicator of cysteine protease activity in insects without additional verification.

Acidic midgut caseinolytic activity in our studies had a pH optimum <4.5 and was attributed to aspartic proteases based on inhibition by pepstatin at pH 5.0. In contrast, gut proteolytic activity reported in *L. lineolaris*, had a pH optimum of 4.25 and was postulated to be a cysteine protease, based on inhibition by E64 and low inhibition by pepstatin at pH 8.5 (Zhu et al., 2003). It should be noted that because gut aspartic protease activity was assayed at pH's ≥ 8.0 in *L. lineolaris* (Zhu et al., 2003), very little activity would have been detected even if significant aspartic proteases had been present.

Alkaline midgut caseinolytic activity in our study had a pH optimum of 7.0–7.5, with no activity at pH's ≥ 8.5 . This activity occurred after 9 h and was aprotinin-resistant. In contrast, *L. lineolaris* gut homogenates had an optimum BApNAse activity at pH 8.5, with an active pH range of 5.3–10.6 and trypsin-like serine protease activity was inhibited by aprotinin (Zhu et al., 2003). The differences in pH optimum and aprotinin sensitivity suggest that alkaline midgut protease activity in *L. hesperus* is different from that associated with *L. lineolaris*. In addition, the serine caseinolytic activities in the salivary glands and midgut of *L. hesperus* in our studies were distinctly different from one another: salivary activity occurred immediately with a pH optimum 8.5 and was aprotinin-sensitive, while midgut activity was delayed 9 h with a pH optimum 7.5 and was aprotinin-resistant. This is evidence

that the observed alkaline midgut activity was not due to the ingestion of salivary proteases. This midgut-associated *L. hesperus* serine protease may be an inhibitor-resistant variant that enables the insect to digest proteins even in the presence of plant defensive serine protease inhibitors, a hypothesis supported by the fact that some *Lygus* populations are unaffected by plants engineered to have higher levels of trypsin inhibitors (Wu et al., 2002).

Previous researchers have suggested that salivary proteases make the greatest contribution to proteolytic activity in *Lygus* spp. (Laurema et al., 1985; Agusti and Cohen, 2000; Zeng et al., 2002a,b; Zhu et al., 2003). In our study, salivary proteases had 45-fold greater caseinolytic activity per IE than midgut proteases at high pH, however, this difference decreased to 4-fold at neutral to acidic pH (Fig. 3). The frequency that insects encounter a high-pH environment in the field is unknown, as is the identity and digestibility of the most commonly ingested protein substrates. Given our current knowledge, the contribution of midgut proteases to digestion should not be underestimated.

Our work points out the need for standardization of methods for the characterization of proteolytic activity in insects. Casein (azocasein, BODIPY-casein or FITC-casein) and hemoglobin are often used as protein substrates for measuring this activity. However, digestion of these different proteins by the same tissue homogenate often yields different pH optima and different class-specific activities (Colebatch et al., 2001; Laurema et al., 1985). We propose that a digestible plant protein soluble over a wide pH range (i.e. rubisco, legumin, zein) could possibly yield data more indicative of what the insect commonly encounters.

Class-specific inhibitors have proven to be useful tools in identifying types of proteases. However, the effect of inhibitors can also be substrate specific. Direct measurement of class specific proteolytic activities using combinations of inhibitors to inhibit all, but one class of protease may aid in the accurate classification and quantification of digestive proteases, especially against large protein substrates. This technique may also be more sensitive in detecting and quantifying minor proteolytic activities that could potentially have a significant biological effect on the digestive process.

In summary, an important finding of our research is that proteolytic activities in the salivary glands and midgut of *L. hesperus* complement one another, with salivary serine proteases active over a pH range of at least 6.5–8.5, midgut aspartic proteases active at pH <6.5 and midgut serine proteases active over a pH range of at least 6.5–7.5. Whether these proteases evolved in response to the different functionalities of the salivary gland and midgut, or to the diversity of plant, bacterial, and insect protein substrates encountered in nature is unknown. However, possession of digestive proteases that remain active over a wide pH range and which consist of multiple protease classes may give this polyphagous herbivore/facultative

entomophage an adaptive advantage in the field, because of its varied diet and utilization of extra-oral digestion to degrade food prior to ingestion (Cohen, 1995, 1998, 2000; Cohen and Wheeler, 1998). Our research has confirmed serine proteases as the predominant proteases in the salivary glands of *L. hesperus*, but presents new findings that aspartic and aprotinin-resistant serine proteases in the midgut are important to the digestion of proteins by *L. hesperus*. These complementing activities expand the pH range over which proteins can be digested, and perhaps provide a mechanism for circumventing plant defensive trypsin inhibitors.

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References

- Agusti, N., Cohen, A.C., 2000. *Lygus hesperus* and *L. lineolaris* (Hemiptera: Miridae), phytophages, zoophages, or omnivores: evidence of feeding adaptations suggested by the salivary and midgut digestive enzymes. *Journal of Entomological Science* 35, 177–186.
- Amirhusin, B., Shade, R.E., Koiwa, H., Hasegawa, P.M., Bressan, R.A., Murdock, L.L., Zhu-Salzman, K., 2004. Soyacystatin N inhibits proteolysis of wheat α -amylase inhibitor and potentiates toxicity against cowpea weevil. *Journal of Economic Entomology* 97, 2095–2100.
- Backus, E.A., 1988. Sensory systems and behaviors which mediate hemipteran plant-feeding: a taxonomic overview. *Journal of Insect Physiology* 34, 151–165.
- Beynon, R., Bond, J.S. (Eds.), 2001. *Proteolytic Enzymes: A Practical Approach*, second ed. Oxford University Press, Inc., New York (340pp).
- Boyd, D.W., Cohen, A.C., Alverson, D.E., 2002. Digestive enzymes and stylet morphology of *Deraeocoris nebulosus* (Hemiptera: Miridae), a predaceous plant bug. *Annals of the Entomological Society of America* 95, 395–401.
- Brandt, S.L., Coudron, T.A., Habibi, J., Brown, G.R., Ilagan, O.M., Wagner, R.M., Wright, M.K., Backus, E.A., Huesing, J.E., 2004. Interaction of two *Bacillus thuringiensis* δ -endotoxins with the digestive system of *Lygus hesperus*. *Current Microbiology* 48, 1–9.
- Broadway, R.M., 1995. Are insects resistant to plant proteinase inhibitors? *Journal of Insect Physiology* 41, 107–116.
- Calbiochem, 2002. Inhibitor Source Book (www.emdbiosciences.com) pp. 85–86.
- Cline, A.R., Backus, E.A., 2002. Correlations among AC electronic monitoring waveforms, body postures, and stylet penetration behaviors of *Lygus hesperus* (Hemiptera: Miridae). *Environmental Entomology* 31, 538–549.
- Cohen, A.C., 1993. Organization of digestion and preliminary characterization of salivary trypsin-like enzymes in a predaceous heteropteran, *Zelus renardii*. *Journal of Insect Physiology* 39, 823–829.
- Cohen, A.C., 1995. Extra-oral digestion in predatory Arthropoda. *Annual Review of Entomology* 40, 85–103.
- Cohen, A.C., 1998. Solid-to-liquid feeding: the inside(s) story of extra-oral digestion in predaceous Arthropoda. *American Entomologist* 44, 103–117.
- Cohen, A.C., 2000. How carnivorous bugs feed. In: Schaefer, C.W., Panizzi, A.R. (Eds.), *The Economic Importance of Heteroptera*. CRC Press, Boca Raton, FL, pp. 563–570.
- Cohen, A.C., Wheeler Jr., A.G., 1998. Role of saliva in the highly destructive fourlined plant bug (Hemiptera: Miridae: Mirinae). *Annals of the Entomological Society of America* 91, 94–100.
- Colebatch, G., East, P., Cooper, P., 2001. Preliminary characterization of digestive proteases of the green mirid, *Creontiades dilutus* (Hemiptera: Miridae). *Insect Biochemistry and Molecular Biology* 31, 415–423.
- Cooper, P.D., Vulcano, R., 1997. Regulation of pH in the digestive system of the cricket, *Teleogryllus commodus* Walker. *Journal of Insect Physiology* 43, 495–499.
- Chougule, N.P., Giri, A.P., Sainani, M.N., Gupta, V.S., 2005. Gene expression patterns of *Helicoverpa armigera* gut proteases. *Insect Biochemistry and Molecular Biology* 35, 355–367.
- D'Ovidio, R., Raiola, A., Capodicasa, C., Devoto, A., Pontiggia, D., Roberti, S., Galletti, R., Conti, E., O'Sullivan, D., De Lorenzo, G., 2004. Characterization of the complex locus of bean encoding polygalacturonase-inhibiting proteins reveals subfunctionalization for defense against fungi and insects. *Plant Physiology* 135, 2424–2435.
- Down, R.E., Ford, L., Woodhouse, S.D., Davison, G.M., Majerus, M.E.N., Gatehouse, J.A., Gatehouse, A.M.R., 2003. Tritrophic interactions between transgenic potato expressing snowdrop lectin (GNA), an aphid pest (peach-potato aphid; *Myzus persicae* (Sulz.)) and a beneficial predator (2-spot ladybird; *Adalia bipunctata* L.). *Transgenic Research* 12, 229–241.
- Fujita, A.I., 2004. Lysozymes in insects: what role do they play in nitrogen metabolism? *Physiological Entomology* 29, 305–310.
- Gatehouse, A.M.R., Shi, Y., Powell, K.S., Brough, C., Hilder, V.A., Hamilton, W.D.O., Newell, C.A., Merryweather, A., Boulter, D., Gatehouse, J.A., 1993. Approaches to insect resistance using transgenic plants. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 342, 279–286.
- Habibi, J., Backus, E.A., Czaplá, T.H., 1993. Plant lectins affect survival of the potato leafhopper (Homoptera: Cicadellidae). *Journal of Economic Entomology* 86, 945–951.
- Habibi, J., Backus, E.A., Huesing, J.E., 2000. Effects of phytohemagglutinin (PHA) on the structure of midgut epithelial cells and localization of its binding sites in western tarnished plant bug, *Lygus hesperus* Knight. *Journal of Insect Physiology* 46, 611–619.
- Habibi, J., Brandt, S.L., Coudron, T.A., Wagner, R.M., Wright, M.K., Backus, E.A., Huesing, J.E., 2002. Uptake, flow, and digestion of casein and green fluorescent protein in the digestive system of *Lygus hesperus* Knight. *Archives of Insect Biochemistry and Physiology* 50, 62–74.
- Harrison, J.F., 2001. Insect acid-base physiology. *Annual Review of Entomology* 46, 221–250.
- Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F., Boulter, D., 1987. A novel mechanism of insect resistant engineered into tobacco. *Nature* 300, 160–163.
- Jongsma, M.A., Bolter, C., 1997. The adaptation of insects to plant protease inhibitors. *Journal of Insect Physiology* 43, 885–895.
- Laurema, S., Varis, A.-L., Miettinen, H., 1985. Studies on enzymes in the salivary glands of *Lygus rugulipennis* (Hemiptera: Miridae). *Insect Biochemistry* 15, 211–224.
- Lecardonnell, A., Chauvin, L., Jouanin, L., Beaujean, A., Prevost, G., Sangwan-Norree, B., 1999. Effects of rice cystatin I expression in transgenic potato on Colorado potato beetle larvae. *Plant Science* 140, 71–79.
- Lee, S.I., Lee, S.-H., Koo, J.C., Chun, H.J., Lim, C.O., Mun, J.H., Song, Y.H., Cho, M.J., 1999. Soybean Kunitz trypsin inhibitor (SKTI) confers resistance to the brown planthopper (*Nilaparvata lugens* Stal) in transgenic rice. *Molecular Breeding* 5, 1–9.

- Marschner, H., 1994. Mineral Nutrition of Higher Plants, second ed. Elsevier Publishing Inc., New York (889pp).
- Miles, P.W., 1972. The saliva of Hemiptera. *Advances in Insect Physiology* 9, 183–255.
- Nordlund, D.A., 2000. The *Lygus* problem. *Southwestern Entomologist* 23 (Suppl), 1–5.
- Novillo, C., Castanera, P., Ortego, F., 1997. Inhibition of digestive trypsin-like proteases from larvae of several lepidopteran species by the diagnostic cysteine protease inhibitor E-64. *Insect Biochemistry and Molecular Biology* 27, 247–254.
- Robyt, J.F., White, B.J., 1987. *Biochemical techniques: theory and practice*. Waveland Press, Inc., Prospect Heights, IL (407pp).
- SAS Institute Inc, 1999. PROC User's Manual, Version, fifth ed. SAS Institute, Cary, NC, USA.
- Terra, W.R., Ferreira, C., Jordao, B.P., Dillon, R.J., 1996. Digestive enzymes. In: Lehane, M.J., Billingsley, P.F. (Eds.), *Biology of the Insect Midgut*. Chapman & Hall, London, pp. 153–194.
- Tinoco, I., Sauer, K., Wang, J.C., 1985. *Physical chemistry: principles and applications in biological sciences*. Prentice-Hall Inc., Englewood Cliffs, NJ, USA (706pp).
- Twining, S.S., 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. *Analytical Biochemistry* 143, 30–34.
- Wu, K., Li, W., Feng, H., Guo, Y., 2002. Seasonal abundance of the mirids, *Lygus lucorum* and *Adelphocoris* spp. (Hemiptera: Miridae) on Bt cotton in northern China. *Crop Protection* 21, 997–1002.
- Zeng, F., Zhu, Y., Cohen, A., 2002a. Molecular cloning and partial characterization of a trypsin-like protein in salivary glands of *Lygus hesperus* (Hemiptera: Miridae). *Insect Biochemistry and Molecular Biology* 32, 455–464.
- Zeng, F., Zhu, Y., Cohen, A., 2002b. Partial characterization of trypsin-like protease and molecular cloning of a trypsin-like precursor cDNA in salivary glands of *Lygus lineolaris*. *Comparative Biochemistry and Physiology Part B—Biochemistry and Molecular Biology* 131, 453–462.
- Zhu, Y.C., Zeng, F.R., Oppert, B., 2003. Molecular cloning of trypsin-like cDNAs and comparison of proteinase activities in the salivary glands and gut of the tarnished plant bug *Lygus lineolaris* (Heteroptera: Miridae). *Insect Biochemistry and Molecular Biology* 33, 889–899.