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Midgut exopeptidase activities in *Aedes aegypti* are induced by blood feeding

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Abstract

Midgut extracts from *Aedes aegypti* females exhibited hydrolytic activities against synthetic substrates for carboxypeptidase A, carboxypeptidase B and leucine-aminopeptidase. The three activities showed a broad pH optimum, with maximum activities at pH between 6.5 and 8.5. Enzymatic activities were further characterized by testing the effects of a variety of protease inhibitors. Captopril and 1-10-phenantroline inhibited the activities of carboxypeptidases A and B, while leuhistin, amastatin and bestatin inhibited aminopeptidase activity. Exopeptidase activities were induced by a blood meal and the highest activities were found during the peak of trypsin activity, about 20–24 h after feeding. An amino acid meal failed to induce significant increases in any of the three exopeptidase activities. The amounts of exopeptidase activities induced were proportional to the protein concentration of the meal. The addition of soy-trypsin inhibitor to the protein meal blocked the post-feeding induction of exopeptidases. The features of the induction of synthesis of the three exopeptidase activities resembled the induction of synthesis of late trypsin during the second phase of digestion. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Anautogenic female mosquitoes require a blood meal to develop eggs. Blood proteins are digested in the midgut by the combined action of endo- and exo-peptidases. The synthesis of endopeptidases in *Aedes aegypti* has been extensively studied at the biochemical and molecular level (Graf and Briegel, 1989; Noriega and Wells, 1999). Ingestion of a blood meal induces two phases of trypsin synthesis in the midgut of *Ae. aegypti* females. The first phase, which encompasses the first 4–6 h following a blood meal, is characterized by the presence of small amounts of early trypsin. The second phase, which occurs between 8 and 36 h after blood feeding, is characterized by the presence of large amounts of late trypsin. The enzymatic activity of early trypsin plays a unique and critical role in the regulation of late trypsin synthesis. Early trypsin acts like a 'sensor'. Blocking early trypsin activity using soy trypsin inhibitor (STI) prevents transcription of the late trypsin gene, digestion of blood meal proteins and egg development (Barillas-Mury et al., 1995).

Little is known however, about the activity of the exopeptidases during digestion. Graf and Briegel (1982) described the presence of constitutive and induced aminopeptidase activity in the midgut of *Ae. aegypti*. Two reports demonstrated inducible carboxypeptidase activity in mosquito midguts, carboxypeptidase B in *Anopheles gambiae* and *Ae. aegypti* (Moskalyk, 1998) and carboxypeptidase A in *Anopheles stephensi* (Jahan et al., 1999).

In this study we characterized leucine-aminopeptidase, carboxypeptidase A and carboxypeptidase B activities in the midgut of female *Ae. aegypti*. Exopeptidase activities were induced by a blood meal, with a maximum of activity coincident with the peak of late trypsin. We showed that the levels of enzymes induced are proportional to the protein concentration in the meal, and are affected by the presence of trypsin inhibitor in the meal.

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2. Materials and methods

2.1. Insects

Ae. aegypti of the Rockefeller strain were reared at 30°C and 80% relative humidity under a 16 h light:8 h dark photoperiod regime. Adults were offered a cotton wool pad soaked in a 10% sucrose solution until 12–16 h before experimental feeding. Sucrose-fed-only females will be referred to as 'unfed'.

2.2. Mosquito meals

Mosquitoes were fed using an artificial feeder as described by Kogan (1990). All meals were prepared as 100 mM NaHCO₃ and 100 mM NaCl, pH 7.0 solutions, equilibrated to 37°C, and ATP was added to a final concentration of 1 mM immediately before use. Protein meals were prepared as described by Kogan (1990), they contain a mixture of albumin (100 mg/ml), γ -globulin (15 mg/ml) and hemoglobin (8 mg/ml). Grace's amino acid solution (a 20 natural amino acid mix) was from Sigma. Pig blood was supplemented with ATP. All meals were readily ingested. Only fully engorged insects were used.

2.3. Enzymatic assays

Midguts were dissected and stored at -80° C. Samples were homogenized in PBS using a hand held plastic pestle, and sonicated for 1 min. After centrifugation at 15,000g, 4°C, for 15 min, the supernatants were used for enzymatic assays. For all assays, triplicate reactions were performed using separate pools of midguts. Aminopeptidase activity was measured using the synthetic substrate L-leucine-*p*-nitroanilide (LpNa)(Sigma). Samples contained 800 µl of substrate (2 mM LpNa — dissolved first in dimethyl sulfoxide (1 mg/60 µl) — in 100 mM Tris–HCl pH 8.5). Reactions were initiated by the addition of 10 µl of midgut extract (equivalent to 1/16 of a midgut), and carried out at room temperature for 5 min. The rate of formation of nitroaniline was measured at 410 nm in a Beckman DU-40 spectrophotometer.

Carboxypeptidase activities were measured as described by Ortego et al. (1996). The technique involves two steps, first the hydrolysis of a synthetic substrate, followed by the measurement of the increase of free amino acids (arginine or phenyalalnine) by the ninhydrin protocol (Moore, 1968). The synthetic substrates used were hippuril-arginine for carboxypeptidase B (HA)(Sigma), and hippuril-phenylalalnine for carboxypeptidase A (HPA)(Sigma). Carboxypeptidase Alike (HPA) and carboxypeptidase B-like (HA) activities were assayed in 1 ml of 100 mM Tris–HCl buffer pH 7.0 containing 20 µl of midgut extract (equivalent to 0.5 midgut) and 1 mM HPA (added in 100 µl methanol) or 1 mM HA (added in 100 μ l 0.15 M NaCl). The reaction was started by the addition of the substrate solution, incubated at 30°C for 1 h, and stopped by the addition of 500 μ l of 30% acetic acid. The rate of the reaction was measured estimating the liberated amino acids by the ninhydrin procedure (Moore, 1968).

Trypsin activity was measured using alpha-benzoyl-DL-arginine-*p*-nitroanilide (Ba*p*NA) as described by Erlanger et al. (1961).

2.4. Protease inhibitor studies

All Inhibitors were from Sigma. Leuhistin, captopril and bestatin were dissolved in water, 1-10-phenantroline in DMSO and amastatin in methanol. Midgut extracts were pre-incubated with the inhibitors at room temperature for 30 min and appropriate buffers were added to controls. Residual activity was determined by the spectrophotometric enzyme assays describe above. The results are expressed as a percentage of the activity in the control samples.

3. Results

3.1. Characterization of leucine-aminopeptidase and carboxypeptidase A and B activities in the midgut

Midgut extracts from *Ae. aegypti* females exhibited hydrolytic activities against synthetic substrates for carboxypeptidase A (HPA), carboxypeptidase B (HA), and leucine-aminopeptidase (LpNa). The activities against the three substrates showed a linear proportionality between the amount of enzyme (amount of tissue) and the rate of hydrolysis of substrate (results not shown).

The effect of pH on exopeptidase activities was determined using a discontinuous buffer system between 6.0 and 11.0 (Fig. 1). The three enzymes showed broad pH optima, with maximal activities between pH 6.5 and 8.5.

The enzymes present in midgut extracts were further characterized by the effect of inhibitors diagnostic for different types of protease activities (Fig. 2).

1-10-phenantroline was the best inhibitor of carboxypeptidases A and B, with IC_{50} (concentration of inhibitor to block 50% of the activity) of 100 µM for carboxypeptidase B and 500 µM for carboxypeptidase A. Captopril also inhibited the activities of carboxypeptidases A and B, but the IC_{50} were higher, 25 mM for carboxypeptidase B and 50 mM for carboxypeptidase A.

Leuhistin was a very effective and specific inhibitor of leucine-aminopeptidase activity (IC_{50} =50 nM); Amastatin (IC_{50} =1 μ M) and Bestatin (IC_{50} =50 μ M) were also effective inhibitors of aminopeptidase activity (Fig. 2).



Fig. 1. Effect of pH on exopeptidase activities. Each point represents the mean of three independent assays performed using separate pools of midguts extracts from five females. Activity levels are expressed as percentage of the maximum value observed.

3.2. Changes in exopeptidase activities after feeding

Females were given a blood, protein or amino acid meal. Enzymatic activities were measured in crude midgut extracts at different times after feeding. Midgut of unfed females contained relatively high aminopeptid-

Inhibitors Aminopeptidase



Fig. 2. Effect of protease inhibitors on exopeptidase activities. Effect of increasing concentrations of six different protease inhibitors: leuhistin (\Box), bestatin (Δ), amastatin (\bigcirc), captopril (\blacktriangle), 1-10-phenantroline (•) and STI (\diamond) on aminopeptidase, carboxypeptidase A and carboxypeptidase B activities. Each point represents the mean of three independent assays performed using separate pools of midguts extracts from five females. Activity levels are expressed as percentage of the maximum value observed.

ase activity (Fig. 3), in contrast, carboxypeptidase A and carboxypeptidase B activities were almost undetectable in unfed females (Fig. 3). The activities of the three exopeptidases were strongly induced by a blood meal and the highest activities were found during the peak of late trypsin activity, about 20–24 h after feeding. Protein meals were also able to induce exopeptidase activities as effectively as a blood meal (results not shown).

We also evaluated the effect of an amino acid meal on the three enzymatic activities. The amino acid meal did not induce an increase of exopeptidase. While carboxypeptidase A and B activities were undetectable, the levels of aminopeptidase activity remained readily



Fig. 3. Changes in exopeptidase activities after feeding. Time-course of midgut exopeptidase activity levels after feeding mosquitoes on blood (•) or amino acids solution (21 mg/ml) (\triangle). Each point represents the mean ±SD of three independent assays performed using separate pools of midguts extracts from five females. Activity levels are expressed as percentage of the maximum value observed.

measurable during 48 h after feeding amino acids (Fig. 3).

3.3. Effect of meal protein concentration on the induction of exopeptidase activity

Females were fed equal volumes of different dilutions of a protein meal. Midguts were dissected 24 h after

feeding and exopeptidase activities were evaluated. The three exopeptidase activities showed a linear dependence between the amount of activity and the protein concentration on the meal (Fig. 4).

3.4. Effect of feeding proteases inhibitors on the activities of exopeptidases

A trypsin inhibitor (100 µM STI) or an aminopeptidase inhibitor (140 µM leuhistin) was mixed with a protein meal and fed to females. Midguts were dissected at different times after feeding. Trypsin, aminopeptidase, carboxypeptidase A and carboxypeptidase B activities were measured (Fig. 5). When leuhistin was added to the meal only aminopeptidase activity was affected. In contrast, the addition of STI caused a significant decrease in the activities of trypsin as well as the three exopeptidases tested. The addition of STI could have modified exopeptidase activities by causing a reduction in the synthesis of enzyme or by preventing the activation of exopeptidase zymogens. We attempted to activate potential zymogen molecules by adding 10 µg of bovine trypsin to the midgut extracts, incubating them at room temperature for one hour, and afterwards testing for changes in aminopeptidase, carboxypeptidases and trypsin activities. The addition of bovine trypsin resulted in an increase of trypsin activity in the extracts, however, the three exopeptidase activities were not modified (Fig. 6). A similar experiment with incubations for six hours gave an identical result. Carboxypeptidase assay results are not shown because all the values were below detection levels.

To test the effectiveness of inhibitors added to the meal, we used three physiological parameters known to be affected by STI: the induction of synthesis of late trypsin, the digestion of the protein meal and the development of the ovaries. Western blot analysis using specific antibodies showed the presence of late trypsin in controls and females fed with leuhistin, but not in STIfed females (Fig. 7). SDS-PAGE analysis of midgut extracts 24 h after feeding showed that digestion of the protein meal proceeded normally in controls and leuhistin-fed females (multiple bands corresponding to digestion products were visualized), but it was negligible in STI-fed females (results not shown). The incorporation of yolk into the developing oocytes was evident 24 h after feeding in controls and leuhistin-fed females; feeding STI prevented the maturation of the ovaries (results not shown).

4. Discussion

Endopeptidases have been extensively studied in mosquitoes, however, far less is known about exopeptidase activities. Graf and Briegel (1982) described measurable

100 Relative activity 80 60 40 20 0 0 25 50 75 100 125 Protein in the meal (µg/µl) Carboxypeptidase A Relative activity 100 75 50 25 0 75 25 50 100 125 0 Protein in the meal (µg/µl) Carboxypeptidase B Relative activity 100 80 60 40 20 0 25 50 75 125 0 100 Protein in the meal (µg/µl)

Aminopeptidase

Fig. 4. Effect of meal protein concentration on the induction of exopeptidase activity. Females were fed meals with different protein concentrations. Midguts were dissected 24 h after feeding. Each point represents the mean \pm SD of three independent assays performed using separate pools of midguts extracts from five females. Activity levels are expressed as percentage of the maximum value observed. Linear regressions are: aminopeptidase (r^2 =0.99, P<0.05), carboxypeptidase A (r^2 =0.96, P<0.05) and carboxypeptidase B (r^2 =0.96, P<0.05).

levels of constitutive aminopeptidase activity in the midgut of *Ae. aegypti* throughout adult life. However, after a blood meal activity rose by a factor of six within 24 h reaching a maximum coincident with trypsin activity. Using synthetic substrates, Graf and Briegel (1982) reported a highest affinity of midgut aminopeptidase for leucine (100%), medium affinity for alanine (76%), arginine (67%), methionine (55%) and lysine (49%), and a very low affinity (<10%) for the remaining amino acids tested (tyrosine, serine, glutamic acid and proline).

Hörler and Briegel (1995) reported a constitutive aminopeptidase activity in midguts of sugar-fed *Anopheles albimanus* females. In blood-fed females, maximal aminopeptidase activity was reached at 24 h. Similar constitutive and induced aminopeptidase activities were described in midguts of *An. stephensi* (Billingsley, 1990; Billingsley and Hecker, 1991; Hörler and Briegel, 1995), *Anopheles quadrimaculatus* (Hörler and Briegel, 1995) and *An. gambiae* (Lemos et al., 1996).

Little is known about carboxypeptidases activities in mosquitoes. Moskalyk (1998), using the hydrolysis of a radioactive substrate described the existence of carboxypeptidase B activity in the midgut of *An. gambiae* and *Ae. aegypti*. Activity levels in *An. gambiae* increased in response to blood feeding (Moskalyk, 1998). Jahan et al. (1999) described the presence of a blood-meal induced soluble carboxypeptidase A activity in the midgut of *An. stephensi*.

Brown et al. (1998) stated that demonstration of carboxypeptidase activity in insect digestive systems has been somewhat elusive, possibly due to the lack of a sensitive and specific assay system. We utilized the protocol described by Ortego et al. (1996), that employs ninhydrin to detect the amino acid released. Hydrolysis of the carboxypeptidase B substrate (HA) by *Ae. aegypti* midgut extracts was readily detectable in our studies, although activity was low compared to HPA hydrolysis. Similar results were described for the lepidoptera *Helicoverpa armigera* (Brown et al., 1998).

Using specific synthetic substrates we confirmed the presence of leucine-aminopeptidase-like activity (Graf and Briegel, 1982) and detected carboxypeptidases A- and B-like activities in the midgut of adult female *Ae. aegypti*. The three enzymes showed broad pH optima, with levels of activity remaining above 70% throughout ranges between 6.5 and 9.0. Similar exopeptidases with



Fig. 5. Effect of feeding proteases inhibitors on the activities of exopeptidases. Time-course of midgut exopeptidase activities levels after feeding mosquitoes on protein (125 mg/ml) (\bigcirc), protein plus trypsin inhibitor (100 μ M STI) (\square) or protein plus aminopeptidase inhibitor (140 μ M leuhistin) (\blacktriangle). Each point represents the mean ±SD of three independent assays performed using separate pools of midguts extracts from five females. Activity levels are expressed as percentage of the maximum value observed.

broad pH optima have been described for other insect species (Brown et al., 1998).

Chemical inhibitors are valuable in facilitating the characterization of an enzyme for the purpose of classification. The exopeptidase activities found in *Ae. aegypti* midgut extracts were very susceptible to well-known mammalian exopeptidase inhibitors. Phenantroline exhibited the greatest levels of inhibition of carboxypeptidase A and B activities in midgut extracts. Bestatin, amastatin and leuhistin are inhibitor of several classes of aminopeptidases in mammals, and they do not inhibit mammalian carboxypeptidases. These three inhibitors showed specificity for the aminopeptidase activity present in *Ae. aegypti* midgut extracts.

Carboxypeptidases have been generally described as luminal enzymes in Diptera (Terra et al., 1996). Leucineaminopeptidase is an integral component of the brush border membrane of insect midgut cells (Lemos and Terra, 1992). The distribution of activities within the lumen and epithelial fractions was not investigated in the present studies; but Graf and Briegel (1982) showed that almost 40% of the aminopeptidase activity was detected within the midgut epithelial cells of *Ae. aegypti*. We estimate that with the protocol we are using for homogenization we were able to recover most of the aminopeptidase activity; nevertheless it is possible that a small fraction of the enzyme activity was not assayed.

The activities of the three exopeptidases were strongly induced by a blood meal and the highest activities were found during the peak of late trypsin activity, about 20– 24 h after feeding. The sudden drop in aminopeptidase activity immediately after a blood meal surprised us. We have no clear explanation for this fact, but a similar drop has been observed in the midgut of *An. gambiae* for carboxypeptidase activity (Moskalyk, 1998) and aminopeptidase activity (Chege et al., 1996).

Mosquitoes can assess the amount of protein present in the meal and adjust trypsin activity accordingly (Briegel and Lea, 1975). We observed a significant linear relationship between the protein concentration in the meal and the amount of exopeptidase enzymatic activities induced. We noticed that the amount of activity detected 24 h after feeding the two lower protein concentrations is lower than the 'basal' level observed in sugarfed females. On the other hand even ingesting an amino acid meal resulted in a significant reduction in the amin-



Fig. 6. Tryptic activation of zymogens present in midgut extracts. Extracts from midguts 24 h after feeding protein plus trypsin inhibitor (100 μ M STI) were incubated at room temperature for one hour in the presence of 10 μ g of bovine trypsin. After one hour of incubation aminopeptidase and trypsin activities were assayed as described above. Results are expressed as activity in extracts before bovine trypsin was added (solid bars), activity in extracts after one hour incubation in the presence of bovine trypsin (open bars) and activity in midgut extracts 24 h after feeding protein without STI (controls)(hatched bars). Each point represents the mean ±SD of three independent assays performed using separate pools of midguts extracts from five females. Activity levels are expressed as percentage of the maximum value observed.



Fig. 7. Late trypsin protein expression after feeding proteases inhibitors. Western blot analysis of midgut homogenates dissected at different times after feeding protein meals + protease inhibitors (12, 18 and 24 h). Control = protein meal (125 mg/ml), Leuhistin = protein meal (125 mg/ml) + leuhistin (140 μ M), STI = protein meal (125 mg/ml) + STI (100 μ M). Extracts from five midguts were loaded on each lane. The figure shows the levels of late trypsin protein detected using a late trypsin polyclonal antibody as described by Barillas-Mury et al. (1995).

opeptidase values 24 h after feeding; we do not have at this time an explanation for this reproducible observation. In the case of late trypsin, Noriega et al. (1994) showed that the changes in the steady-state levels of late trypsin mRNA during the first 24 h post feeding are also directly proportional to the concentration of protein in the meal. A future study of the expression of exopeptidase genes might reveal a similar regulation at the transcriptional level.

STI at 100 mM concentration did not have any direct effect on the enzymatic activities of aminopeptidase, carboxypeptidase A or carboxypeptidase B (Fig. 2); however, when 100 µM were added to the protein meal, STI blocked the induction of exopeptidase activities observed after blood feeding. Barillas-Mury et al. (1995) showed that the addition of STI to a protein meal prevented transcriptional activation of the late trypsin gene and therefore protein digestion and oogenesis. Although in the experiments described in the present report we have not studied the effect of STI on the expression of exopeptidase genes, the reduction of exopeptidase enzymatic activities suggests a similar mechanism of regulation, in which early trypsin might play a role in the regulation of the expression of exopeptidase genes (Noriega and Wells, 1999).

Mammalian amino and carboxypeptidases are synthesized as zymogens, and require the proteolytic removal of an N-terminal activation peptide for maximum activity (Freisheim et al., 1967). The sequences of two carboxypeptidase A genes described in mosquitoes predicted putative pro-carboxypeptidases that could be activated by tryptic cleavage (Edwards et al. 1997, 2000), therefore, to clarify the effect of STI it was important to search for the presence of exopeptidase zymogens. The impossibility of detecting zymogens using bovine trypsin as a catalytic activator is an indirect evidence for the blocking of exopeptidase synthesis by STI.

In contrast to the effect of STI, the addition of leuhistin to the meal did not affect late trypsin synthesis, trypsin activity, carboxypeptidase A and B activities, digestion of the protein meal or egg development. We attempted to feed carboxypeptidase inhibitors (captopril and 1-10-phenantroline) with a protein meal, but the mosquitoes refused to engorge or died after feeding, preventing us to complete these experiments.

In summary we have described at least five attributes in common among the activities of late trypsin, leucineaminopeptidase, carboxypeptidase A and carboxypeptidase B in the midgut of *Ae. aegypti* females:

- 1. The enzymatic activities are stimulated by feeding a blood or protein meal.
- 2. The enzymatic activities are expressed during the second phase of digestion, with maxima that correlate well with the peak of digestion (20–24 h after feeding).
- 3. The enzymatic activities are not stimulated by feeding an amino acid meal.
- 4. The activities induced are proportional to the protein concentration of the meal.

5. The induction of enzymatic activities is blocked by the addition of STI to the meal.

These five features might be common to all the protease genes expressed during the second (late) phase of digestion previously described (Felix et al., 1991; Noriega and Wells, 1999). A gut-specific *Ae. aegypti* carboxypeptidase A gene has been cloned and characterized (Edwards et al., 2000) and its mRNA accumulates to high levels only late (\sim 16–24 h) after ingestion of a blood meal. The temporal profile of this carboxypeptidase A gene induction is similar to that of *Ae. aegypti* late trypsin (Barillas-Mury et al., 1991; Noriega et al., 1994), suggesting the existence of common regulatory elements.

These results give support to our working hypothesis that describes early trypsin as a master regulator of gene expression in the mosquito midgut (Barillas-Mury et al., 1995; Noriega and Wells, 1999). Its role as a 'sensor' of the meal goes beyond the regulation of late trypsin, and might be controlling the expression of a battery of protease genes that are transcribed during the second phase of digestion.

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References

- Barillas-Mury, C.V., Graf, R., Hagedorn, H.H., Wells, M., 1991. cDNA and deduced amino acid sequence of a blood meal-induced trypsin from the mosquito, *Aedes aegypti*. Insect Biochemistry 21, 825–831.
- Barillas-Mury, C.V., Noriega, F.G., Wells, M.A., 1995. Early trypsin activity is part of the signal transduction that activates transcription of the late trypsin gene in the midgut of the mosquito Aedes aegypti. Insect Biochemistry and Molecular Biology 25, 241–246.
- Billingsley, P.F., 1990. Blood digestion in the mosquito Anopheles stephensi Liston (Diptera: Culicidae): partial characterization and post-feeding activity of midgut aminopeptidases. Archives of Insect Biochemistry and Physiology 15, 149–163.
- Billingsley, P.F., Hecker, H., 1991. Blood digestion in the mosquito Anopheles stephensi Liston (Diptera: Culicidae): activity and distribution of trypsin, aminopeptidase, and α-glucosidase in the midgut. Journal of Medical Entomology 28, 865–871.
- Briegel, H., Lea, A.O., 1975. Relationship between protein and proteolytic activity in the midgut of mosquitoes. Journal of Insect Physiology 21, 1597–1604.
- Brown, D.P., Wilkinson, H.S., Gatehouse, J.A., 1998. Midgut carboxypeptidase from *Helicoverpa armigera* (Lepidoptera: Noctuidae) larvae: enzyme characterization, cDNA cloning and expression. Insect Biochemistry and Molecular Biology 28, 739–749.

- Chege, G.M.M., Pumpuni, C.B., Beier, J.C., 1996. Proteolytic enzyme activity and *Plasmodium falciparum* sporogonic development in three species of *Anopheles* mosquitoes. Journal of Parasitology 82, 11–16.
- Edwards, M.J., Lemos, F.J.A., Donnely-Doman, M., Jacobs-Lorena, M., 1997. Rapid induction by a blood meal of a carboxypeptidase gene in the gut of the mosquito *Anopheles gambiae*. Insect Biochemistry and Molecular Biology 27, 1063–1072.
- Edwards, M.J., Moskalyk, L.A., Donelly-Doman, M., Vlaskova, M., Noriega, F.G., Walker, V.K., Jacobs-Lorena, M., 2000. Characterization of a carboxypeptidase A gene from the mosquito *Aedes aegypti*. Insect Molecular Biology 9, 33–38.
- Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two chromogenic substrates for trypsin. Archives of Biochemistry and Biophysics 95, 271–278.
- Felix, C.R., Betschart, B., Billingsley, P.F., Freyvogel, T.A., 1991. Post-feeding induction of trypsin in the midguts of *Aedes aegypti* L. (Diptera: Culicidae) is separable into two cellular phases. Insect Biochemistry 21, 197–203.
- Freisheim, J.H., Walsh, K.A., Neurath, H., 1967. The activation of bovine carboxypeptidase A. 1, Isolation and properties of the succinylated enzyme precursor. Biochemistry 6, 3010–3019.
- Graf, R., Briegel, H., 1982. Comparison between aminopeptidase and trypsin activity in blood-fed females of *Aedes aegypti*. Revue Suisse of Zoology 89, 845–850.
- Graf, R., Briegel, H., 1989. The synthetic pathway of trypsin in the mosquito Aedes aegypti L. (Diptera:Culicidae) and in vitro stimulation in isolated midguts. Insect Biochemistry 19, 129–137.
- Hörler, E., Briegel, H., 1995. Proteolytic enzymes of female Anopheles: biphasic synthesis, regulation, and multiple feeding. Archives of Insect Biochemistry and Physiology 28, 189–205.
- Jahan, N., Docherty, P.T., Billingsley, P.F., Hurd, H., 1999. Blood digestion in the mosquito, *Anopheles stephensi*: the effects of *Plasmodium yoelii nigeriensis* on midgut enzyme activities. Parasitology 119, 535–541.
- Kogan, P.H., 1990. Substitute blood meal for investigating and maintaining *Aedes aegypti* (Diptera:Culicidae). Journal of Medical Entomology 27, 709–712.
- Lemos, F.J.A., Terra, W.R., 1992. A high yield preparation of *Musca domestica* larval midgut microvilli and the subcellular distribution of amylase and trypsin. Insect Biochemistry and Molecular Biology 22, 433–438.
- Lemos, F.J.A., Cornel, A.J., Jacobs-Lorena, M., 1996. Trypsin and aminopeptidase gene expression is affected by age and food composition in *Anopheles gambiae*. Insect Biochemistry and Molecular Biology 26, 651–658.
- Moore, S., 1968. Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. Journal of Biological Chemistry 243, 6281–6283.
- Moskalyk, L.A., 1998. Carboxypeptidase B in Anopheles gambiae (Diptera: Culicidae): effects of abdominal distention and blood ingestion. Journal of Medical Entomology 35, 216–221.
- Noriega, F.G., Barillas-Mury, C.V., Wells, M.A., 1994. Dietary control of late-trypsin gene transcription in *Aedes aegypti*. Insect Biochemistry and Molecular Biology 24, 627–631.
- Noriega, F.G., Wells, M.A., 1999. Mini-review: a molecular view of blood-meal digestion in the yellow fever mosquito *Aedes aegypti*. Journal of Insect Physiology 45, 613–620.
- Ortego, F., Novillo, C., Castañera, P., 1996. Characterization and distribution of digestive proteases of the stalk corn borer, *Sesamia nonagrioides* Lef. (Lepidoptera: Noctuidae). Archives of Insect Biochemistry and Physiology 33, 163–180.
- Terra, W.R., Ferreira, C., Baker, J.E., 1996. Compartmentalization of digestion. In: Lehane, M.J., Billingsley, P.F. (Eds.) Biology of the Insect Midgut. Chapman and Hall, London, pp. 206–235.