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Free amino acids are important for the retention of protein and non-protein meals by the midgut of *Aedes aegypti* females

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Abstract

There is a relationship between the normal progress of digestion and the retention or elimination of the proteins ingested with the meal by *Aedes aegyti* females. The addition of soybean trypsin inhibitor (STI) to a protein meal prevented digestion and resulted in a rapid elimination of the undigested proteins. The addition of a mix of free amino acids to a protein meal together with STI resulted in a significant increase in the retention of the undigested proteins during the first 10–15 hrs after feeding. The effect of the free amino acids on the retention of non-protein meals. When females were fed a meal containing FITC-dextran (20 kD), most of this compound was eliminated into the feces by 10 hrs; the addition of free amino acid resulted in a significant increase in the midgut during the first 15 hrs after feeding. The presence of free amino acids in the midgut lumen seems to be an important signal used by the mosquito to regulate the retention of the meal. © 2003 Elsevier Ltd. All rights reserved.

Keywords: Mosquito; Amino acid; Blood meal; Midgut

1. Introduction

Female mosquitoes use the blood of their vertebrate hosts as a protein source for egg production. Digestion of the blood proteins takes place in the midgut, nutrients are absorbed and the non-absorbable end-products of blood digestion, such as hematin together with proteolytic enzymes and undigested protein are passed directly from the midgut to the hindgut and eliminated during the second day after blood feeding (Van Handel and Klowden, 1996; Briegel, 1975; Briegel, 1980). Blood meal proteins need to be retained in the midgut long enough for proteases to act upon them; if the meal is not adequate or digestion is not proceeding normally, a rapid elimination of the meal is necessary before a new blood meal can be acquired. Little is known about the signals and mechanisms that the midgut of the mosquito uses to sense the progress of digestion and decide to retain or to void the lumen contents. In this research we investigate factors that are important for the retention of protein and non-protein meals in the alimentary canal. Our results suggest that an increase in the size of the free amino acid pool in the lumen is critical for retention of the meal in the midgut.

2. Materials and methods

2.1. Chemicals

Pig albumin, pig γ -globulin, pig hemoglobin, FITCdextran and Grace's amino acid solution were purchased from Sigma (St. Louis, MO, USA). Soy trypsin inhibitor and bovine serum albumin were from Calbiochem (San Diego, CA, USA).

2.2. Insects

Aedes aegypti unmated females of the Rockefeller strain were reared at 30 °C and 80% relative humidity under a 16 hr light: 8 hr dark photoperiod regime. Adults

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were offered a cotton pad soaked in 10% w/v of sucrose solution until 12 hr before experimental feeding.

2.3. Mosquito meals

Mosquitoes were fed using an artificial feeder as described by Kogan (1990). All the meals were prepared using a saline solution containing 100 mM NaHCO₃ and 100 mM NaCl, pH 7.4 solutions, equilibrated at 37 °C, and ATP was added to a final concentration of 1 mM immediately before use. The protein meal was prepared as described by Kogan (1990), and was a mix of pig albumin (102 mg/ml), pig y-globulin (15 mg/ml) and pig hemoglobin (8 mg/ml). Grace's amino acid solution is a mixture of the 20 natural amino acids. It was dried using a SpeedVac Concentrator (Savant Instruments Inc., Holbrook, NY, USA) and resuspended with the desired meal (protein or dextran). Blood meals were pig blood. For all experiments STI was used at a concentration of 2 mg/ml. FITC-dextran meals were made in saline solution. Only fully engorged insects were used. Insects were kept individually or in groups of five in plastic scintillation vials; at different times after feeding, insects were transferred to a new vial, and the old vial was washed with 1 ml of double distilled water to collect the feces.

2.4. Protein analysis

Protein concentration was evaluated using the Lowry protocol (Lowry et al., 1951). Standard curves were made using bovine serum albumin.

2.5. Fluorescein Isothiocyante-Dextran (FITC-DEXTRAN) assays

The presence of FITC-dextran in feces was detected by measuring the absorbance at a wavelength of 600 nm. Standard curves were made using different concentrations of FITC-dextran (5–320 μ g).

2.6. SDS-PAGE

SDS-PAGE analyses were carried out as described by Laemmli (1970). The equivalent of one mosquito (feces or midgut contents) was loaded per lane of 10% polyacrylamide gel. The proteins were stained by Coomassie Brilliant Blue R-250 (Sigma Chemical Co., St. Louis, MO, USA). Protein molecular weight standards were from BioRad (Richmond, CA, USA).

3. Results

3.1. Effect of trypsin inhibitor on the elimination of protein meals

Females were fed a protein meal with or without trypsin inhibitor (soybean trypsin inhibitor (STI)). Fully engorged females were isolated in scintillation vials individually or in groups of 5. Midguts were dissected at different times after feeding and midgut contents were collected. Feces were collected over the same period of time. SDS-PAGE analysis of feces and midgut contents were used to evaluate the digestion of proteins in the midgut and the presence of undigested proteins in the feces. SDS-PAGE analysis showed that digestion of the protein meal proceeded normally in controls, and multiple bands corresponding to digestion products were visualized (Fig. 1(a)). Digestion of the meal proteins was negligible in midguts dissected from females fed protein + STI (Fig. 1(a)). The addition of STI to the protein meal resulted in the presence of large amount of undigested protein in the feces 9 hrs after feeding (Fig. 1(b)); in contrast undigested proteins were absent from the feces of females fed with a protein meal (Fig. 1(b)). Similar results were obtained when we fed a blood meal + STI; undigested blood proteins were present in large amounts in the feces (results not shown).

3.2. Effect of free amino acids on the retention of protein meals

Females were fed either a protein meal with STI, or a protein meal with STI + 20 mg/ml of a free amino acid mix (Grace's amino acid solution). Feces from individual females were collected at several times. SDS-PAGE analysis was used to evaluate the presence of undigested proteins in the feces. Feeding a protein meal + trypsin inhibitor resulted in the rapid elimination of



Fig. 1. Effect of trypsin inhibitor on the digestion and elimination of protein meals. SDS-PAGE analysis of (a) midgut contents, and (b) feces. Females were fed a protein meal (Kogan, 1990), or a protein meal plus STI. At different times after feeding (9, 23 and 30 hrs), feces were collected and midguts were dissected and contents analyzed. Protein: females fed a protein meal; Protein + STI: females fed a protein meal + STI; Meal: protein meal. For all the lines the contents of 1 midgut equivalent or the feces from 1 mosquito were loaded per lane.

undigested proteins into the feces. Fig. 2(a) shows that most of the undigested proteins were already present in the feces collected between 2 and 5 hrs after feeding. The addition of free amino acids to the protein meal + trypsin inhibitor, resulted in a significant shift in the peak of elimination of the meal (Fig. 2(b)). The undigested proteins were eventually eliminated, but they were retained in the midgut much longer. Most of the undigested proteins were detected in the feces collected between 15 and 24 hrs after feeding. In a similar experiment, feces were collected and the amount of protein was evaluated using the Lowry assay; the results confirmed what we observed using SDS-PAGE (Fig. 2(c)).

3.3. The effect of free amino acid on protein retention is concentration dependent

Females were fed protein meals + STI containing 7 different amino acid concentrations: 250 and 500 μ g/ml, 1, 2.5, 5, 10 and 20 mg/ml. Feces from individual females were collected at several periods of time. The amount of protein present in the feces was evaluated using the Lowry assay. Concentrations of free amino acids above 5 mg/ml had a significant effect on the retention of proteins. Concentration below 1 mg/ml did not increase retention of the meal when compared with controls without amino acids. An intermediate concentration (2.5 mg/ml), gave an intermediate pattern. To facilitate the visualization of the results, Fig. 3 shows only 3 representative amino acid concentrations. The effect of free amino acids in the retention of the meal was concentration-dependent between 250 μ g/ml and 5 mg/ml.

3.4. Free amino acids are important for the retention of non-protein meals

To test if free amino acids are also important for the retention of a non-protein meal, females were fed a 20 kD dextran molecule, with or without a free amino acid mix (20 mg/ml). Feces from individual females were collected at several periods of time. The amount of dextran in the feces was evaluated using the FITC-dextran assay. Free amino acids also appeared to be important for the retention of a non-protein meal. The profile of elimination of dextran was similar to that observed when we fed a protein + STI. The dextran meal was rapidly eliminated, but the addition of free amino acid resulted in a significant retention of the meal (Fig. 4).

4. Discussion

Anautogenous female mosquitoes require blood for the nutrients needed for egg production. Proteins are the predominant components of blood, and 80% of the ingested protein has been digested 24 hrs after feeding (a)



Hours after feeding

(b) 10 MW 2.5 5 15 24 48 Hours after feeding (C) Protein in the feces (ug) 120 100 80 Protein + STI 60-Protein + STI + FAA 40 20 5 10 15 24 Hours after feeding

Fig. 2. Effect of free amino acids on the retention of protein meals. SDS-PAGE analysis of feces. (a) Females were fed a protein meal plus STI, or (b) Females were fed a protein meal plus STI and amino acids. Feces from the same females were collected at different times after feeding, 2.5: 0-2.5 hr, 5: 2.5-5 hr, 10: 5-10 hr, 15: 10-15 hr, 24: 15-24 hr, and 48: 24-48 hr. For all the lines, feces from 1 mosquito were loaded per lane. MW: molecular weight marker. (c) The amount of protein present in the feces was evaluated using the Lowry assay. Feces from the same females were collected at different times after feeding, 5: 0-5 hr, 10: 5-10 hr, 15: 10-15 hr and 24: 15-24 hr. Each point represents the mean \pm SD of the feces of at least 3 groups of 10 mosquitoes each.



Fig. 3. The effect of free amino acid on protein retention is concentration dependent. Females were fed a protein meal + STI and 7 different concentrations of free amino acid solution were added. Feces from the same females were collected at different times after feeding, 5: 0–5 hr, 10: 5–10 hr, 15: 10–15 hr and 24: 15–24 hr. Only 3 amino acid concentrations are represented in the figure, 0.25 mg/ml (\bullet), 2.5 mg/ml (\bullet) and 5 mg/ml (\bigcirc). The amount of protein present in the feces was evaluated using the Lowry assay. Each point represents the mean \pm SD of the feces of at least 3 groups of 10 mosquitoes each.



Fig. 4. Free amino acids are important for the retention of non-protein meals. Females were fed a FITC-dextran meal or a FITC-dextran meal + amino acids. Feces from the same females were collected at different times after feeding, 5: 0–5 hr, 10: 5–10 hr, 15: 10–15 hr, 24: 15–24 hr and 48: 24–48 hr. The amount of FITC-dextran present in the feces was evaluated as described in Materials and methods. Values are percentage of the total amount of FITC-dextran recovered from the feces during the experiment. Each bar represents the mean \pm SD of the feces of at least 3 groups of 10 mosquitoes each.

(Briegel and Lea, 1975). This is possible because the blood meal induces a large increase in midgut proteolytic activity (Fisk, 1950), with trypsin representing the main endoproteolytic enzyme (Briegel and Lea, 1975). Blocking trypsin activity using STI prevents transcription of the late trypsin gene (late trypsin is the most important endoprotease), digestion of blood meal proteins and egg development (Barillas-Mury et al., 1995). The synthesis of a large amount of trypsin in the absence of a blood meal could be deleterious to the mosquito. A two-phase digestive response allows the mosquito to assess the quality of the meal via early trypsin before committing to the synthesis of late trypsin (Noriega and Wells, 1999). If there is no protein in the meal, or if it has poor digestibility, no free amino acids, peptides or other "activators" will be released and the late trypsin gene will not be transcribed.

The amino acids produced by the digestion of the protein meal provide both a "stimulatory signal" and "building blocks" for synthesis of proteases. It has been shown that absorption of radioactive amino acids starts immediately after feeding in the midgut of *Anopheles stephensi* (Schneider et al., 1986) and *Aedes aegypti* (Noriega et al., 1999), and the labeled amino acids are incorporated into the proteases that are synthesized de novo.

Blood meal proteins must be retained in the midgut long enough for the proteases to act upon them; but if the meal is not adequate or digestion is not proceeding normally, a rapid elimination of the meal is necessary before a new blood meal could be acquired.

A series of three papers was published on the influence of a brain factor on retention of blood in the midgut of *Aedes aegypti* (Gillett et al., 1975; Cole and Gillett, 1978, 1979) describing a factor released from the brain that helped to retain the blood-meal in the midgut. Mosquitoes that had been fully fed, but decapitated, tended to excrete the blood-meal prematurely and ovary development was halted on an early stage of development. They also described that retention of the blood meal is modulated by the ovaries. Removal of the ovaries before a blood meal led to early haem-defecation, an effect mediated by ecdysterone. Mosquitoes decapitated immediately after a blood meal showed a delay in the onset of haem-defecation when injected with ecdysterone.

It is difficult to compare the experiments of Cole and Gillett with those reported here; we looked at the presence of meal constituents in the midgut and faeces (protein and FITC-dextran), while they looked at elimination of the dark brown haem-containing faeces. These two processes are likely controlled by different pathways. In Aedes aegypti, 40% of the water from the blood meals is removed from the midgut within 2 hrs after feeding (Williams et al., 1983). Blood meal components and products of digestion are absorbed by midgut cells and transfered to the hemolymph (Boorman, 1960). The Malpighian tubules remove water, salts and uric acid from the hemolymph, and pass them through to the hindgut where some resorption occurs before the remains are passed out of the body through the anus (Boorman, 1960). The non-absorbed end-products of blood digestion, such as hematin, are passed directly from the midgut to the hindgut (Van Handel and Klowden, 1996). Consequently, parts of the material that end in the feces are coming directly from the midgut, and parts have been taken from the hemolymph by the Malpighian tubules. After a normal digestion of a protein or blood meal, the major excretory products of the female mosquito are water, colorless crystals of ammonium urate and the dark brown specks of the indigestible blood residue hematin (Van Handel, 1975). The defecation of uric acid, hematin and water are controlled by the terminal abdominal ganglion (TAG) (Van Handel

and Klowden, 1996), which innervates the hindgut. After removal of the TAG, the elimination of hematin, and uric acid was completely inhibited, and loss of water was partially inhibited (Van Handel and Klowden, 1996). Therefore, while we described the regulation of the rapid elimination of undigested proteins, Cole and Gillett looked at the last steps of excretion and defecation after digestion.

What controls the retention and elimination of the midgut contents? The contraction of the midgut circular and longitudinal muscles, and particularly the contraction of the strong muscles of the pyloric sphincter that regulate the passage of the meal from the midgut to the hindgut, may be points of regulation. A-type allatostatins are members of a family of peptides that have been described as regulator of muscle contraction for several parts of the digestive tract and for oviducts in several insects (Stay, 2000). Immunoreactivity against A-type allatostatin has also been described in a specific group of midgut cells of female Aedes aegypti (Veenstra et al., 1995; 1997; Noriega et al., unpublished). In addition, in situ hybridization showed the expression of allatostatin mRNA in the same group of cells (Noriega et al., unpublished results). These allatostatin endocrine cells are "open type" cells. They have a bottle shape and extend from the basal lamina to the midgut lumen. The apical part of the cell is in contact with the lumen and might be able to sense the lumen contents (Brown et al., 1985; Stracker et al., 2002). These allatostatin cells are located in the posterior part of the posterior midgut, very close to the muscles of the pyloric sphincter. These cells might directly or indirectly through the nervous system affect the activity of midgut muscles.

Our results suggest that an increase in the size of the free amino acid pool in the lumen is critical for retention of the meal in the midgut. Such a rapid increase in the concentration of amino acids in the midgut lumen after a blood meal is conceivable as a result of the presence of free amino acids in the blood (60–140 μ g/ml)(Everett, 1942; Forslund et al., 2000), combined with the enzymatic activity of exopeptidases and other proteases on the blood polypeptides (Noriega et al., 2002). The free amino acid concentration in the midgut lumen 2 hrs after the ingestion of a Kogan meal is 9.7 mg/ml (unpublished result). This quantity is in the range of values of free amino acids (2.5–5 mg/ml) that caused an increase in the retention of midgut lumen contents in our experiments.

The effect of free amino acid is concentration dependent, suggesting that the midgut has the ability to sense the amount of amino acid and adjust its physiological response. Similar feedbacks proportional to meal component concentrations have been previously shown for trypsin activity by Briegel and Lea (1975) and for the changes in the steady-state levels of late trypsin mRNA by Noriega et al. (1994). Free amino acids are also important for the retention of non-protein meals. The experiments with dextran suggest that it is not the quality of the meal that is critical, but rather the ability to generate free amino acids. Is interesting that we observed a reproducible biphasic pattern of elimination of proteins and dextran when mosquitoes were fed meals with amino acids (Figs. 2(a) and 4); there is a burst of elimination on the first 5 hrs, then a clear decrease by 10 hrs, followed by the major peak of elimination after 15 hrs.

Based on the results of these experiments, we propose that free amino acids play a key role as a signal used by the midgut to control the retention of its lumen contents. The presence of free amino acids in the midgut lumen might be sensed by digestive cells or midgut endocrine cells, such as the allatostatin cells, and these cells might directly or indirectly through the nervous system affect the activity of midgut muscles and consequently the retention of the meal.

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