



Increase in the size of the amino acid pool is sufficient to activate translation of early trypsin mRNA in *Aedes aegypti* midgut

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Abstract

Early trypsin is a female-specific protease present in the midgut of the yellow fever mosquito *Aedes aegypti* during the first 4–6 h after ingestion of a blood meal. Transcription of the early trypsin gene occurs after adult emergence under control of juvenile hormone, but the transcript remains untranslated before feeding. Early trypsin was *in vitro* translated using mRNA extracted from midguts of unfed and fed females, indicating that there are not structural features in the early trypsin mRNA that impede translation *in vitro*. Eight single protein meals exhibiting different molecular weights and amino acid composition, as well as ingestion of several amino acid mixtures of different complexity, had the ability to prompt early trypsin translation. In contrast, ingestion of saline, latex or midgut-filling sugars were unable to induce early trypsin mRNA translation. In addition intra-thoracic injection of an amino acid solution induced early trypsin translation, while injection of saline or albumin failed. In summary an increase in the size of the midgut amino acid pool by feeding or injection of an amino acid solution was sufficient to induce translation of early trypsin mRNA; ³⁵S-labeled amino acids, fed with a protein meal, were incorporated into newly synthesized early trypsin; the first phase of trypsin synthesis is likely induced by an initial rapid increase in the concentration of amino acids in the midgut cells after ingestion of a blood meal. © 1999 Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

Post-feeding induction of trypsin synthesis and secretion in *Aedes aegypti* females is separable into an early and a late phase, each characterized by the presence in the midgut of a particular group of trypsin forms (Graf and Briegel, 1989; Felix et al., 1991; Barillas-Mury et al., 1995). A two-phase digestive system allows the mosquito to test the meal during the first phase, and to adjust the levels of late trypsin with unusual adaptability in response to a particular meal in the second phase (Briegel and Lea, 1975; Noriega et al., 1994). Early trypsin is an adult female-specific protease detected in the midgut during the first 4–6 hours following a blood meal (Noriega et al., 1996a). Transcription of the gene begins shortly after adult emergence under control of juvenile hormone (Noriega et al., 1997). Early trypsin mRNA levels increase dramatically during the first day follow-

ing emergence; despite the high levels of early trypsin mRNA present in the midgut of unfed female, translation of the mRNA occurs only after a blood meal (Noriega et al., 1996b). In this paper we describe experiments designed to explore the nature of the signals involved in the activation of early trypsin mRNA translation.

2. Materials and methods

2.1. Insects

A. aegypti of the Rockefeller strain were reared at 27°C and 80% relative humidity under a 12 h light: 12 h dark photoperiod. Adults were offered a cotton wool pad soaked in a 10% sucrose solution until 12–16 h before experimental feeding. In this paper we will refer to the cotton wool pad sucrose-fed females as ‘unfed’.

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2.2. Mosquito meals

Mosquitoes were fed 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.

The proteins fed were: pig γ -globulin (Cohn fraction II, III), pig albumin (fraction V), pig hemoglobin, lysozyme, ribonuclease and insulin from Sigma (St. Louis, Mo), cytochrome C from Boehringer (Mannheim, Germany) and gelatin from Matheson, Coleman and Bell (Norwood, OH). The amino acid mixes were: Grace's amino acid solution (10 \times) and casein acid hydrolysate from Sigma, and amino acid concentrate MEM (50 \times) from ICN (Costa Mesa, CA). Individual amino acids were from GIBCO (Rockville, MD). Latex beads (0.460 μ diameter), sucrose, lactose and trehalose were from Sigma.

2.3. Immunoblotting

The presence of early trypsin protein was determined by Western blot analysis. Midguts were homogenized in PBS pH 7.4, in the presence of protease inhibitors, diisopropylfluorophosphate (DFP) 25 mM and phenylmethylsulfonyl fluoride (PMSF) 5 mM. Homogenates were sonicated for 2 minutes and centrifuged at 10 000 *g* for 15 min. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Early trypsin was detected with a specific rabbit primary polyclonal antibody (Noriega et al., 1996a) and a secondary goat anti-rabbit IgG conjugated with peroxidase (Bio-Rad, Richmond, Ca).

2.4. Affinity chromatography

Groups of 200 mosquitoes were homogenized in 5 ml of 300 mM imidazole buffer pH 7.4, 20 mM CaCl₂ and 0.1 mM phenylthiourea using a Brinkmann polytron. Homogenates were centrifuged at 10 000 *g* for 30 min. The soluble fraction was applied to a 2 ml benzamidine-Sepharose 6B affinity column (Pharmacia, Piscataway, NJ), and incubated in the column for 2 h at 4°C. The column was then washed with 6 column volumes of homogenization buffer. Trypsin fractions were eluted with 2 column volumes of 200 mM benzamidine in the same buffer, and applied to a PD10 column (Pharmacia) equilibrated with 100 mM Tris buffer pH 7.4, 150 mM NaCl, for buffer exchange. DFP was added to samples to a final concentration of 25 mM.

2.5. Trypsin activity

Trypsin activity was measured as described by Erlanger et al. (1961) using benzoyl-alanine-p-nitroanilide (BApNA) as substrate.

2.6. *In vitro* translation

Midguts were homogenized in 6 M guanidinium Isothiocyanate, Tris-HCl pH: 7.5, 25 mM EDTA and 0.1 M 2-mercaptoethanol, using a polytron homogenizer. Total RNA was isolated by cesium chloride gradient centrifugation as described in Current Protocols in Molecular Biology (Ausubel et al., 1998). Poly (A⁺) enriched RNA was isolated using an oligo(dT) spin column from New England Biolabs (Beverly, MA). Samples of 10 μ g of mRNA were translated using a FlexiTM rabbit reticulocyte lysate system (Promega) and ³⁵S-L-cysteine (ICN).

2.7. Immunoprecipitation

³⁵S-labeled proteins were immunoprecipitated using the early trypsin polyclonal antiserum described above. Rabbit early trypsin antiserum (250 μ l) was incubated overnight at 4°C with 200 μ l of protein A-Sepharose (Pharmacia). Protein A-sepharose was centrifugated, washed with 200 μ l of TNTE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.2% Bovine Serum Albumin, 0.05% Tween 20) and resuspended in 200 μ l of TNTE; 50 μ l of resuspended rabbit early trypsin IgG-Protein A conjugate were added to each *in vitro* translated sample, and incubated overnight at 4°C. Samples were centrifugated and the pellet washed three times with 80 μ l of TNTE buffer, resuspended in SDS-sample buffer and analyzed by PAGE and fluorography as previously described (Noriega and Wells, 1992).

3. Results

3.1. Effect of meal composition and injections of different solutions on early trypsin translation

Mosquitoes were fed on different protein and non-protein solutions. Three hours after feeding, midguts were dissected and homogenized. Early trypsin was detected by Western blot analysis using a specific polyclonal antibody (Fig. 1). Eight different protein meals were tested at a concentration of 100 mg/ml: gamma-globulin, albumin, hemoglobin, cytochrome C, gelatin, lysozyme, ribonuclease A and insulin. They all promoted trypsin synthesis to a similar extent.

Several different amino acid mixtures were also able to induce translation of early trypsin such as, a casein hydrolyzate (100 mg/ml), a mixture of the 20 natural amino acids (21 mg/ml, SIGMA), a mixture of 12 amino acids (arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine) (5mg/ml, ICN), mixtures of 8 amino acids (arginine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan and valine), and even 6

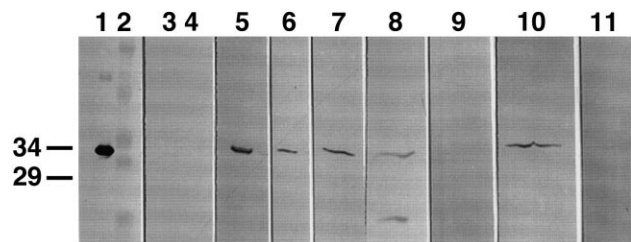


Fig. 1. Effect of meal composition and injections of different solutions on early trypsin translation. Western blot of *Aedes aegypti* midgut homogenates, three hours after feeding. Lanes: 1: recombinant early trypsin; 2: molecular weight markers (the position of two molecular weight markers (kDa) are shown at left); 3: saline fed (10 midguts); 4: saline injected (10 midguts); 5: 20 amino acid mixture fed (21 mg/ml; 10 midguts); 6: 20 amino acid mixture injected (20 mg/ml; 10 midguts); 7: 12 amino acid mixture injected (5 mg/ml; 10 midguts); 8: albumin fed (100 mg/ml; 3 midguts); 9: albumin injected (100 mg/ml; 5 midguts); 10: hemoglobin fed (25 mg/ml; 3 midguts); 11: hemoglobin injected (50 mg/ml; 5 midguts).

amino acids (arginine, isoleucine, lysine, methionine, threonine and tyrosine) (5 mg/ml, GIBCO).

Single amino acid solutions (isoleucine, lysine, glutamine, serine, and arginine) (5 mg/ml, GIBCO) induced early trypsin translation to a very limited extent. While saline, latex (1.8×10^8 particles/ml), sucrose (100 mg/ml), trehalose (100 mg/ml) and lactose (100 mg/ml) failed to induce early trypsin synthesis.

Mosquitoes were injected in the thorax with 0.3 μ l solutions using a glass needle. A 20 amino acid mixture (21 mg/ml, SIGMA) as well as a 12 amino acid mixture (5 mg/ml, ICN) induced significant levels of early trypsin synthesis. Injections of albumin (100 mg/ml), hemoglobin (100 mg/ml) or saline had no effect (Fig. 1).

Injection of amino acids were unable to induce early trypsin translation to levels seen when the same mixtures were fed. This was likely the result of the 'dilution' of the amino acids injected into the whole body amino acid pool, in detriment of the midgut amino acid pool. Injection of amino acids concentrations over 20 mg/ml killed most females; this limited the possibility of increasing the amino acid mixture concentrations injected to compensate the losses produced by this 'dilution'.

Trypsin activity in midguts homogenized three hours after feeding selected meals were measured using BApNA (Fig. 2). The activities found corresponded well with the presence of early trypsin protein detected by western blot.

3.2. Incorporation of ^{35}S -amino acids into early trypsin

Mosquitoes were fed on a 100 mg/ml gamma-globulin meal containing one mCi of ^{35}S -*E. coli* hydrolysate (labeled methionine and cysteine). Three hours later, ^{35}S -labeled trypsin was affinity-purified. Benzamidine-purified proteins were visualized by fluorography (Fig. 3);

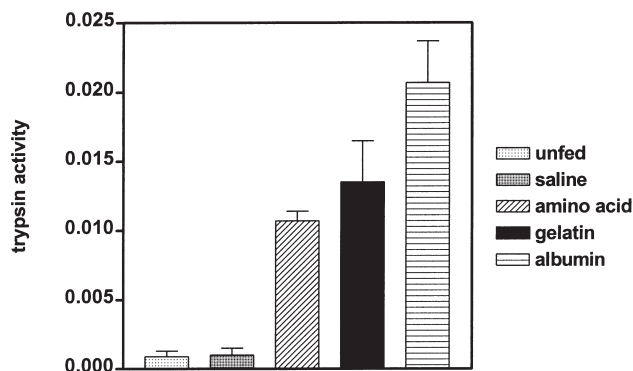


Fig. 2. Effect of feeding different meals on trypsin enzymatic activity. Amino acid mixture (21mg/ml). Gelatin (100 mg/ml). Albumin (100 mg/ml). Trypsin activity is expressed as increase in absorbance/min and was measured using BApNA as substrate. Each bar represents the mean + SD of a triplicate analysis of 10 midguts.

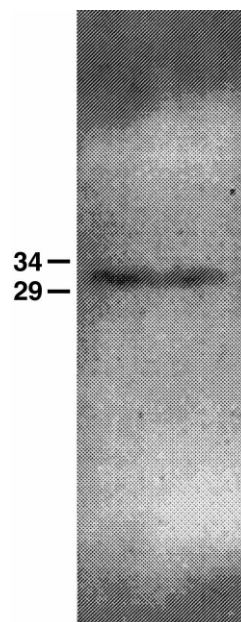


Fig. 3. SDS-PAGE analysis of mosquito labeled proteins after benzamidine affinity chromatography. Both lanes represent benzamidine-purified ^{35}S -proteins three hours after feeding. Lines show the positions of molecular weight markers (kDa).

one major protein with an apparent molecular weight of 30 kDa was detected. In a previous paper we reported that the most abundant polypeptide isolated by benzamidine-Sepharose affinity chromatography, under these conditions, is early trypsin (Noriega et al., 1996a). Labeled-amino acids were readily incorporated into newly synthesized early trypsin.

3.3. Translatability *in vitro* of the early trypsin mRNA

Early trypsin was *in vitro* translated using both mRNA extracted from midguts of unfed females and midguts from females 3 h after feeding albumin (100 mg/ml)

(Fig. 4). Immunoprecipitation was specific for early trypsin, since the control, luciferase mRNA, was translated but not immunoprecipitated.

4. Discussion

Early trypsin is post-transcriptionally regulated by blood feeding (Noriega et al., 1996b). Translation of early trypsin is one of the first 'events' in the digestion of blood's proteins (Noriega et al., 1996a). The enzymatic activity of early trypsin is essential for transcriptional activation of the late trypsin gene (Barillas-Mury et al., 1995). Therefore, the identification of the signals necessary for translation of early trypsin mRNA is critical to understand regulation of the digestive process.

In the present study we have demonstrated that no structural features in early trypsin mRNA prevent translation *in vitro* before feeding. The molecular mechanism silencing translation of the early trypsin mRNA *in vivo* is unknown at present. Identification of the signals in the meal that activate translation of the dormant early trypsin RNA is the aim of this paper.

Our results provide evidence that feeding *'per se'* or filling of the midgut is not sufficient to stimulate early trypsin translation. Meals such as saline, latex beads and several sugar solutions when ingested filled the midgut, however they did not stimulate early trypsin synthesis.

Because these solutions are not isotonic with mosquito hemolymph, we can conclude that osmotic stress or mechanical abdominal distention is not sufficient to induce early trypsin mRNA translation. These results contradict those reported by Graf and Briegel (1989), where the early phase of trypsin synthesis could be stimulated by enemas of either blood or sugar solution into isolated midguts. It was suggested that early trypsin synthesis was a result of mechanical/osmotic stimulation. Unfortunately, the early trypsin form they described was never characterized, so it is not possible to determine whether that trypsin protein is identical to the early trypsin we have identified. Immunological techniques, in combination with amino-terminal sequencing, have allowed us to show that the protease described in this manuscript is the major early trypsin form present in the midgut of *Aedes aegypti* during digestion (Noriega et al., 1996a).

Several reports show that midgut proteolytic activity increases in insects after feeding on protein (see review by Lehane et al., 1995). Soluble proteins have been shown to play an important role in the regulation of digestive enzyme levels (Blakemore et al., 1995). Felix et al. (1991) proposed that the first phase of trypsin synthesis in *Aedes aegypti* was stimulated by meals containing soluble proteins of variable molecular weights.

In our study, eight different proteins of variable molecular weights (5.7–66 kD) and disparate structures were able to induce early trypsin synthesis. Therefore, we can exclude the possibility that a particular peptide derived from digestion of a specific blood protein is essential for induction. Similar results were described by Blakemore et al. (1995) in relation to the ability of proteins to promote trypsin secretion in the hematophagous diptera *Stomoxys calcitrans*.

When proteins with different amino acid composition and polypeptides like ribonuclease A, gelatin and insulin, which lack specific amino acids (e.g.: tryptophan, cysteine or methionine) were fed, all were able to induce early trypsin translation. Therefore, amino acid composition of the protein, or the presence of all 20 natural amino acids in the sequence were not critical factors.

We decided to explore the possibility that these proteins were simply a source of amino acids, and that these were the actual inducers of early trypsin synthesis.

Feeding amino acid mixtures containing 20 and 12 amino acids induced substantial early trypsin translation. Simple mixes of 6–8 essential amino acids were also good inducers; in contrast, single amino acid meals were able to induce translation of early trypsin to a very limited extent.

The amino acid meal is providing both a 'stimulatory signal' and 'building blocks' for synthesis of early trypsin. It has been shown that absorption of ^3H -amino acids starts immediately after feeding in the midgut of *Anopheles stephensi* (Schneider et al., 1986). We confirmed

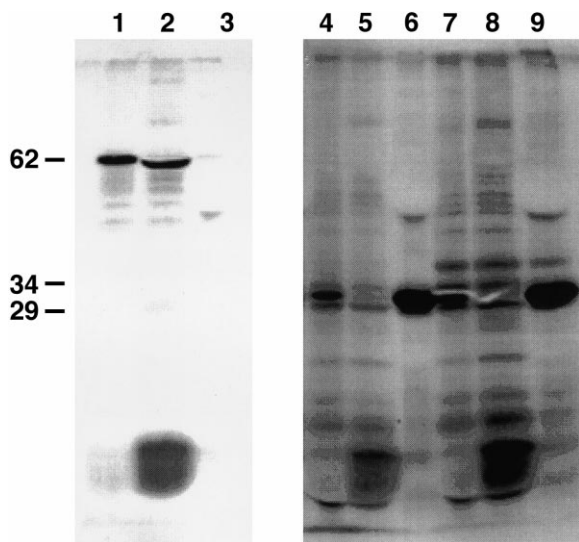


Fig. 4. *In vitro* translation of midgut mRNA. Midgut mRNA samples from unfed females and females 3 h after receiving a protein meal were *in vitro* translated. Aliquots (10%) of each reaction were saved (T), and the rest immunoprecipitated using an early trypsin antiserum. After spinning, the supernatant (S) and pellet (P) were isolated and analyzed by SDS-PAGE and autoradiography. Luciferase RNA was used as control. 1: luciferase RNA (T); 2: luciferase RNA (S); 3: luciferase RNA (P); 4: unfed midgut RNA (T); 5: unfed midgut RNA (S); 6: unfed midgut RNA (P); 7: fed midgut RNA (T); 8: fed midgut RNA (S); 9: fed midgut RNA (P). The positions of molecular weight markers (kDa) are shown by lines.

this observation, showing that when ^{35}S -labeled amino acids were added to a protein meal they were rapidly absorbed and incorporated into newly synthesized early trypsin.

In an attempt to separate these two distinct roles, we injected a mixture of amino acids into the hemocoel of the female and found that this was sufficient for translation of early trypsin mRNA. Saline or albumin injections failed. These results suggest that an increase in the size of the amino acid pool in midgut cells is critical for activation of early trypsin mRNA translation. 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, combined with the enzymatic activity of exopeptidases and other proteases on the blood polypeptides. Graf and Briegel (1982) reported that in *Aedes aegypti* a considerable, fairly constant residual aminopeptidase activity remains after adult emergence and it slowly declines in aging female mosquitoes. Rapid induction by a blood meal of a carboxypeptidase gene in the gut of the mosquito *Anopheles gambiae* has been recently described (Edwards et al., 1997; Moskalyk, 1998). A similar induction takes place in *Aedes aegypti* (Moskalyk, personal communication).

Other examples of regulation of gene expression by changes in the amino acid pool have been recognized (Kilberg et al., 1994). Mechanisms for control of transcription and translation in response to changes in amino acid availability have not yet been elucidated (Laine et al., 1996). The intimate relationship between amino acid supply and the fundamental process of protein synthesis, makes amino acid dependent control of gene expression particularly important. Study of post-transcriptional control of early trypsin synthesis should provide important insight into the mechanisms of amino acid control of gene expression.

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