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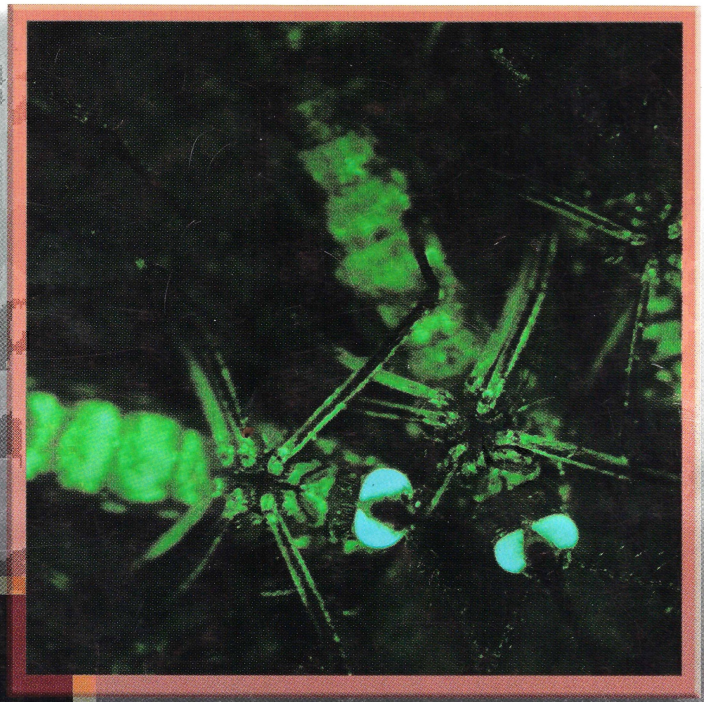

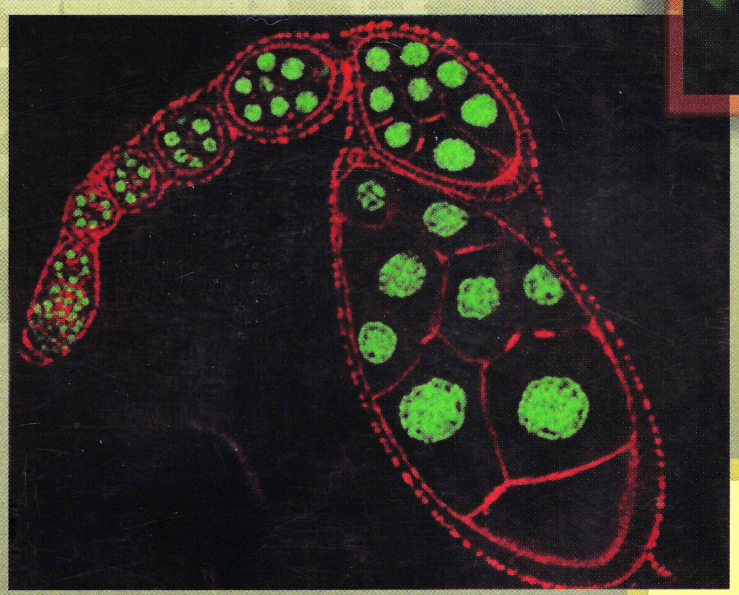

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Reevaluation of the role of early trypsin activity in the transcriptional activation of the late trypsin gene in the mosquito *Aedes aegypti*

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Abstract

In the female mosquito *Aedes aegypti*, trypsin expression is largely biphasic. Early trypsin synthesis, which is regulated at the translational level relative to feeding, peaks in the first few hours post-blood meal. Late trypsin expression is regulated at the transcriptional level, and peaks 18–24 h post-blood meal. It was proposed that early trypsin activity released unknown factors during digestion of a meal that caused activation of transcription of the late trypsin gene. This connection between early trypsin activity and late trypsin expression was dependent on the fact that feeding a single trypsin inhibitor, soybean trypsin inhibitor (STI), which blocked early trypsin activity, also blocked late trypsin expression. We show in this study that feeding different trypsin inhibitors which effectively blocked early trypsin activity did not result in reduced late trypsin expression. We also found that a different lot of STI failed to cause inhibition of late trypsin transcription, although it was effective in inhibiting early trypsin activity. In addition, using RNAi methodology to reduce the level of early trypsin expression had no effect on the level of late trypsin expression. We conclude that early trypsin activity is not necessary for the transcriptional activation of late trypsin and that the previous results were due to the effect of a cytotoxic agent present in some, but not all preparations of STI.

Keywords: RNAi; Trypsin inhibitor; Regulation; Transcription

1. Introduction

Female *Aedes aegypti* mosquitoes require the ingestion of a blood meal to complete oogenesis. Blood is mainly comprised of protein, which constitutes almost all of the dry weight. From this exogenous nutrient source, the mosquito is able to acquire a sufficient amount of amino acids for numerous functions, such as energy production, providing precursors to complete egg maturation and to replenish maternal reserves (Zhou et al., 2004). The principal luminal enzymes that break down blood proteins into amino acids are the endoprotease trypsin and the exopeptidases aminopeptidase and carboxypeptidase (Noriega and Wells, 1999; Pennington and Wells, 2004).

Trypsin is of particular interest. *Ae. aegypti* possesses an interesting pattern of trypsin expression: it is biphasic. In the unfed mosquito, trypsin zymogen is absent (Graf and Briegel, 1989). In the first, early phase after feeding, trypsin is expressed rapidly, and diminishes within 8 h of meal ingestion. Late phase trypsin replaces early phase trypsin in greater quantities, peaking 18–24 h after ingestion of a blood meal. As such, the late phase trypsin appears to act as the main digestive enzyme (Noriega and Wells, 1999). Two trypsins have been cloned and characterized from each phase of digestion and are known as early trypsin and late trypsin (Kalhok et al., 1993; Barillas-Mury et al., 1991; Noriega and Wells, 1999).

Extensive studies on this pattern of expression have shown two different methods of control. Early trypsin transcript is absent in newly emerged females, and increases in expression, reaching maximal levels at 72 h after eclosion. After ingestion of a blood meal, early trypsin transcript levels are rapidly reduced. Early trypsin protein, however, is absent before blood meal ingestion, and only appears after feeding (Noriega et al., 1996). Late trypsin transcript and protein are both absent in non-blood fed mosquitoes and only appear about eight hours after

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feeding (Barillas-Mury et al., 1991). Therefore, relative to blood feeding, early trypsin is considered to be regulated at the translational level and late trypsin is regulated at the transcriptional level. The late trypsin gene also appears to be expressed in proportion to the quantity and quality of the protein meal present in the midgut during digestion (Noriega et al., 1994).

However, the mechanism for the transcriptional regulation of the late trypsin gene is not completely known. Studies on this regulatory mechanism have led to the hypothesis of a feedback mechanism. When early trypsin activity was inhibited by feeding soybean trypsin inhibitor (STI) in a meal, transcription of the late trypsin gene was blocked (Barillas-Mury et al., 1995). In addition, a follow-up experiment in which a protein meal was pre-digested with bovine trypsin ex vivo and then fed to mosquitoes in the presence of STI restored transcription of late trypsin. In light of these results, Barillas-Mury et al. (1995) suggested that the transcriptional control of late trypsin is regulated downstream of the proteolytic activity of early trypsin acting on meal proteins to produce some unknown factor. Caroci and Noriega (2003) showed, recently, that feeding STI results in early meal expulsion. Furthermore, supplementing the meal with free amino acids slowed down meal expulsion. This lead the authors to conclude that amino acids provide some signal that results in the nervous system repressing midgut muscle contractions and keeping the meal in the lumen.

Attempts to repeat the results from the previous study (Barillas-Mury et al., 1995) proved to be inconsistent. Feeding STI from different sources produced different effects on late trypsin transcription, even though each source had equivalent levels of trypsin inhibition. Because of these discrepancies, other inhibitors were investigated, as well as utilizing RNAi to determine the effect of early trypsin activity on late trypsin expression. We describe the results of those experiments in this paper and conclude that the previous results were due to an impurity in the preparation of STI used and not due to the inhibition of early trypsin activity.

2. Materials and methods

2.1. Mosquito rearing

Ae. aegypti (Rockefeller) were raised under photoperiod of 16 h light, 8 h dark. Environmental conditions were kept at 28 °C and at 80% humidity. Female adults were maintained with cotton pads soaked in a 3% sucrose solution. Blood-fed mosquitoes were maintained in these same environmental conditions after feeding.

2.2. Blood meals and trypsin inhibitors

Blood meals included 1 ml of porcine blood and contained a final concentration of 5 mM ATP. The trypsin inhibitors in these experiments are Soybean Trypsin Inhibitor (Sigma, St. Louis, MO), 4-(2-Aminoethyl) benzenesulfonylfluoride (AEBSF, EMD Biosciences, San Diego, CA), and Egg White Trypsin Inhibitor (EWTI, EMD Biosciences). Sodium citrate treated pig blood was obtained from The University of Arizona animal farm. Gamma-globulin meals were made as a 10% solution of porcine γ-globulin (Sigma) in 100 mM $NaHCO_3$ pH 7.4 with 150 mM NaCl. Experimental blood or γ-globulin meals containing these inhibitors were prepared to have an inhibitor concentration of 95 μM.

2.3. Expression of late trypsin in AD494 cells

Late trypsin protein used for antigen in antibody production was obtained from an AD494 *E. coli* expression system. The sequence coding for the mature peptide of the late trypsin cDNA was cloned into pET-32a (+) (Novagen, Madison, WI) plasmid as follows: PCR using 24 h post blood meal midgut cDNA synthesis as template, synthesized as described below, with primers ALT001F (5′-AGAGACCATGGCCTTCCCATCGTTGGACAAC GGTCGGGTAG-3′) and ALT001R (5′-AGAGAGAATT CACAGTCCAGTCTTCTGCTTGATCCAATC-3′) was performed to amplify a product with NcoI and EcoRI restriction sites at the 5′ and 3′ ends of the late trypsin sequence, respectively. The PCR product and pET-32a (+) plasmid were digested with the above enzymes (Invitrogen, Carlsbad, CA), gel purified (Qiaprep Spin gel purification kit, Qiagen, Valencia, CA) after digestion, and then ligated together. This cloning procedure resulted in late trypsin being expressed as an in-frame fusion protein at the C-terminus of thioredoxin. AD494 cells (Novagen, Madison, WI) were transformed with the expression-plasmid according to manufacturer's instructions. Cells were grown to mid-log phase in LB-broth containing ampicillin (Sigma) at 37 °C and then isopropyl-β-D-thiogalactopyranoside (Invitrogen, Carlsbad, CA) was added to a final concentration of 5 mM. Cells were incubated for 3 h, and then lysed according to the manufacturer's instructions. Late trypsin–thioredoxin fusion protein was purified by nickel affinity chromatography using the His–Bind system (Novagen) and then cleaved with enterokinase (Novagen).

2.4. Late trypsin antibodies

Enterokinase-cleaved late trypsin from the AD494 expression was resolved on a 12% SDS-PAGE gel and excised. This protein was used as antigen for antibody production in rabbits by Cocalico Biologicals (Reamstown, PA) according to the standard Cocalico Biologicals' protocols.

2.5. Assays for trypsin activity

Mosquito midgut samples in groups of ten were collected at 90 min and at 18 h post-blood meal. Dissection of the midguts, including the midgut contents, were performed in

phosphate buffered saline (PBS, 10 mM Sodium Phosphate buffer pH 7.2, 150 mM NaCl), being careful not to lose luminal contents by breaking the midguts. The midguts were quickly homogenized in PBS with a Kontes (Vineland, NJ) plastic pestle and microfuge tube, and then centrifuged at 16,000 × *g*. Trypsin activity in the supernatant was assayed using *N*-α-Benzoyl-DL-arginine-*p*-nitroanilide (BApNA, Sigma) as substrate. Samples were incubated with 10 mM BApNA for five minutes in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.0. The absorbance was read at 405 nm on a Cary 50 spectrophotometer (Varian, Palo Alto, CA). All experiments were performed in triplicate.

2.6. SDS-PAGE and Western blot analyses

The effects of the experimental meals on the expression of late trypsin were analyzed by Western Blot. Triplicate samples of ten midguts from mosquitoes 24 h after feeding blood containing no inhibitor, STI, AEBSF or EWTI were homogenized as described above in 50 mM Tris-HCl pH 7.0 containing 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 5 mM AEBSF, and 2.5 mM benzamidine (Sigma). Proteins were resolved on 12% SDS-PAGE, loading an equivalent of three midguts from each ten midgut sample per lane. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuel, Keene, NH) via electro-blotting in 25 mM Tris/200 mM Glycine pH 8.5 containing 20% methanol. Late trypsin was detected using the anti-late trypsin antibody described above at a dilution of 1:1000. Secondary antibody, goat-anti-rabbit-horseradish-peroxidase (Bio-Rad, Hercules, CA) was incubated at a dilution of 1:3000. The blot was developed using 4-chloronapthol (Sigma) as substrate.

2.7. Construction of dsRNA for RNAi

Early trypsin template for synthesis of dsRNA was constructed by using primers ET001f (5′-ATGAACCAA TTTCTCTTTG-3′) and ET001R (5′-TTATTCCATTTTC GAACAC-3′) using cDNA from unfed mosquitoes, synthesized as described below, for 30 cycles to generate a product comprising the entire open reading frame of early trypsin. This product was diluted 50 fold and 1 μl was used as template in a second reaction using primers ET001ft7 (5′-TAATACGACTCACTATAGGGATGAA CCAATTTCTCTTTG-3′) and ET001Rt7 (5′-TAATAC GACTCACTATAGGGTTATTCCATTTTCGAACAC-3′) for 20 cycles to generate a new product with T7 promoter sites at each end. The MegaScript RNAi kit (Ambion, Austin, TX) was used to generate early trypsin dsRNA using the second PCR product as template.

Luciferase was chosen as an exogenous injection control and a 400 bp luciferase dsRNA was synthesized as described above, using the pGL3 control vector (Promega, Madison, WI) as template. Primers Luc001f (5′-ATG GAAGACGCCAAAAAC-3′) and Luc001r (5′-TGCAA CCCCTTTTTGG-3′) were used for the initial amplification and Luc001fT7 (5′-TAATACGACTCACTATAGG GATGGAAGACGCCAAAAAC-3′) with Luc001rT7 (5′-TAATACGACTCACTATAGGGTGCAACCCCTTT TTGG-3′) were used to generate the second product with T7 promoters.

Injections were performed on newly emerged adult females using a Drummond Nanoject II (Broomall, PA) to inject 69 nl of a 5 mg/ml dsRNA solution into the anterior region of the thorax as described by Blandin et al. (2002).

2.8. RNA isolation and RT–PCR analysis

After dissection, total RNA was isolated from five midguts per sample, done in triplicate, using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After RNA isolation, residual genomic DNA was digested with DNase I (Invitrogen), and RNA was quantitated by determining the absorbance at 260 and 280 nm using a Cary50 spectrophotometer. A portion of the total RNA extraction was run on denaturating agarose gel electrophoresis to determine the quality of the RNA and to compare ribosomal RNA quantity for samples taken at equivalent time points before or after feeding. First strand cDNA was synthesized from 1 μg of total RNA using the Superscript III system (Invitrogen). PCR was used to semi-quantitatively determine relative intensities of target products using procedures described by Blandin et al. (2002) and Hoa et al. (2003) with some modifications. PCR was performed using MasterTaq (Eppendorf, Hamburg, Germany) in 50 μl reactions. The number of PCR cycles was optimized for each target product, early trypsin, target of rapamycin (TOR), and late trypsin, using 0.5 μl of cDNA as template to assure that PCR does not become limiting, resulting in saturated products. Early trypsin was amplified for 26 cycles using primers ET002F (5′-ACACTCATCGCCATG-3′) and ET002R (5′-GGTAAATCTCGTCAGCT-3′). Late trypsin was amplified for 22 cycles using primers LT001F (5′-AT GTTCACTTCAACGG-3′) and LT001R (5′-TCACAGTC CAGTCTTC-3′). TOR was amplified for 32 cycles using primers that were designed based on the sequence reported by Hansen et al. (2004): ATOR001F (5′-GCCGTGC TGGAAGCTTTC-3′) and ATOR001R (5′-GATTTCCGG TGTCTACCAGAAAGG-3′). PCR products were resolved on 1.4% agarose gels, stained with ethidium bromide, and gel images were analyzed on Kodak 1-D gel analysis software (Kodak Digital Science, Rochester, NY).

2.9. Determination of meal retention and egg development

Mosquitoes were fed in groups of 20 on blood, blood containing STI, blood containing AEBSF, or blood containing EWTI. Eighteen hours after feeding mosquitoes were dissected. The presence of meal in the midgut and the formation of peritrophic matrix were recorded. Ovaries were also dissected and the number of blood meal induced developing eggs was recorded. Three follicles were removed

from the medial section of each ovary and their length measured using a Bausch and Lomb stage micrometer and eyepiece on a Leica Stereozoom 4 microscope (GTI Microsystems, Tempe, AZ).

2.10. Statistical analysis

Statistical analysis was performed using Graph Pad Instat (San Diego, CA).

3. Results

This study was undertaken to further investigate the role of early trypsin activity in late trypsin transcriptional regulation because attempts to repeat the results in the previous study (Barillas-Mury et al., 1995) proved to be inconsistent. Feeding different lot sources of STI from Sigma as well as STI from different manufacturers to mosquitoes produced different effects on late trypsin transcription, even though each source had equivalent levels of trypsin inhibition. For instance, Sigma STI (product number T-9128) from lot number 47H7095, the source of STI used in the experiments presented in this report, produced results similar to those reported earlier (Barillas-Mury et al., 1995), but feeding STI from Sigma lot number 051K7037 did not have an effect on late trypsin expression. Because of these discrepancies, other inhibitors were chosen to feed mosquitoes, as well as utilizing RNAi to determine the effect of early trypsin activity on late trypsin expression.

3.1. Confirming inhibition of trypsin-like activity with the addition of trypsin inhibitors

To ensure that the trypsin inhibitors consistently reduced the level of trypsin-like activity, colorimetric assays using BApNA were utilized (Fig. 1). Inhibitors were fed to mosquitoes at the same molar concentration (95 μM) as was calculated for the concentration of STI utilized by Barillas-Mury et al. (1995). Ninety minutes after blood feeding, all inhibitors significantly reduced hydrolysis of BApNA to similar levels, approximately 15% of the activity found in the non-inhibited control. The addition of 5 mM diisopropyl-fluorophosphate (DFP) to replicate assay mixtures from inhibitor fed midguts did not result in further repression of enzyme activity. At 18 h after blood feeding, all of the inhibitors significantly reduced the trypsin-like activity on BApNA to about half of the activity of non-inhibitor fed mosquitoes.

3.2. Late trypsin expression in the midgut 18 h after blood feeding

Western blot analysis was performed to demonstrate the effects of feeding the different trypsin inhibitors on the expression of late trypsin protein in the midgut (Fig. 2). The addition of AEBSF and EWTI resulted in a level of late trypsin expression similar to that of the control. The addition of STI however, resulted in a highly reduced level of late trypsin expression. Late trypsin transcript levels, as determined by RT–PCR after feeding on the inhibitors in pig blood or 10% γ-globulin showed the same pattern of expression as for the protein levels demonstrated by western blotting. AEBSF and EWTI fed in either blood or γ-globulin did not result in different late trypsin levels from the non-inhibited meals, although STI fed in either meal resulted in highly decreased late trypsin product (results not shown).

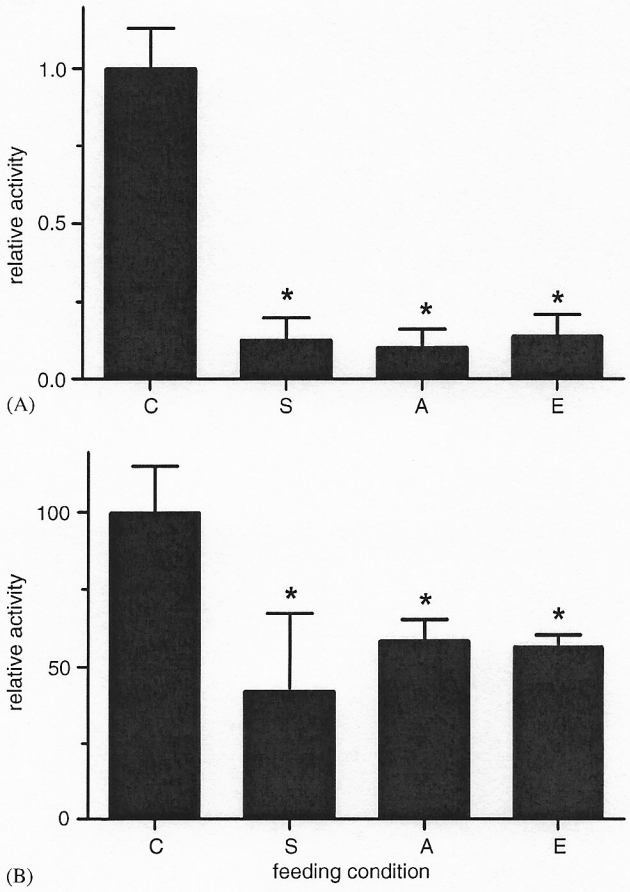

Fig. 1. Effect of feeding trypsin inhibitors with a blood meal on trypsin activity. Activity is reported as relative level of control (blood fed, no inhibitor) activity. (A) midgut trypsin activity 90 min after feeding. (B) midgut trypsin activity 24 h after feeding. (C) control, no inhibitor, S: soybean trypsin inhibitor, A: AEBSF, E: egg white trypsin inhibitor. Asterisks (*) indicate sample is significantly different ($p<0.05$) from the control (non-inhibitor fed sample) as determined by t-test.

3.3. Other effects of feeding trypsin inhibitors

One important aspect of feeding STI to mosquitoes mentioned by Caroci and Noriega (2003) but not in the original report (Barillas-Mury et al., 1995) is that feeding on STI results in early expulsion of the meal. In order to replicate this observation and to determine if whether there were other effects caused by feeding trypsin inhibitors,

Fig. 2. Western blot of 12% SDS-PAGE of late trypsin from midguts 24 h after feeding. Each lane represents loading 1/3 of an independent sample of protein extracted from a pool of ten mosquito midguts. Samples are processed in triplicate. (A) blood fed, B: blood fed with soybean trypsin inhibitor, (C) blood fed with AEBSF and (D) Blood fed with egg white trypsin inhibitor.

Table 1

Inhibitor added to meal	Meal retention (%)	Peritrophic matrix formation (%)	Egg count (mean ± SD)	Egg size in μm (mean ± SD)
No inhibitor	90	90	*57.2 ± 25.4*	177.8 ± 30.2
STI	*10*	*0*	*4.9 ± 13.9*	82.5 ± *23.3*
AEBSF	93	93	44.4 ± 15.2	*129.7 ± 27.4*
EWTI	90	90	52.1 ± 15.7	*151.1 ± 27.7*

Mosquitoes were fed blood meals with and without the inhibitors as listed. Mosquitoes were dissected 18 h after feeding and meal retention, peritrophic matrix formation, the number of eggs that that have initiated development and the follicle size determined. Meal retention and peritrophic matrix formation are listed as percentage of mosquitoes containing meal with visible peritrophic matrix in the midgut. Egg count is listed as the number of eggs that had initiated blood-meal induced development in a single ovary. Egg size was determined by measuring the length of three developing follicles from the medial section of the ovary of each mosquito. Italic type indicates samples that are significantly different ($p < 0.05$) from non-inhibitor fed samples as determined by *t*-test.

several other observations were made. Mosquitoes were fed blood or blood with each inhibitor in groups of 20 mosquitoes. At 18 h after feeding, meal retention, peritrophic matrix formation, the number of eggs undergoing meal-induced development in the ovary, and the average size of the follicles were all determined. The results are shown in Table 1. Almost all of the STI fed mosquitoes expelled the meal by 18 h after feeding, and those that did retain the meal had no detectable peritrophic matrix formation, as well as a low number of developing eggs and small follicle size. The AEBSF and EWTI fed mosquitoes retained the meal to a similar degree as non-inhibitor fed mosquitoes and all of the mosquitoes that retained the meal produced peritrophic matrix. They also produced similar numbers of eggs at this specific time point, but the size of the eggs produced were significantly smaller than the non-inhibitor fed mosquitoes.

3.4. RNAi mediated knock-down of early trypsin transcription

Early trypsin dsRNA was synthesized and injected initially according to the procedure of Blandin et al. (2002). In early experiments, 69 nl of 1 mg/ml early trypsin dsRNA were injected into 3-day post emergent females. Early trypsin mRNA levels were measured up to 96 h after injection, but no noticeable effect on early trypsin levels were found. Only after injection of newly emerged females with 69 nl of 5 mg/ml dsRNA was knock-down achieved consistently. At 96 h after injection, early trypsin levels were reduced by approximately 90% in unfed mosquitoes when compared to non-injected control mosquitoes (Fig. 3(A)). Injection of luciferase dsRNA as a control did not result in a significant reduction in early trypsin levels as well. In addition, midgut TOR levels were not significantly affected by injection of either dsRNA when compared to non-injected control mosquitoes (Fig. 3(B)), demonstrating that injection of dsRNA did not result in global reduction of transcripts.

Trypsin assays were chosen for phenotypic analysis of early trypsin knock-down after dsRNA injection. Mosquitoes were injected as described above and fed a blood meal. Midguts were dissected 90 min after feeding and trypsin activity was determined (Fig. 4). Early trypsin dsRNA injected mosquitoes had approximately 15% of the trypsin activity found in non-injected mosquitoes. There was no significant difference in trypsin activity between non-injected and luciferase dsRNA injected mosquitoes.

3.5. Late trypsin expression after dsRNA injection

Ninety-six hours after injection with early trypsin dsRNA or luciferase dsRNA, injected and non-injected mosquitoes were given a blood meal. Midguts were dissected and RNA extracted 24 h after feeding. RT-PCR was performed to semi-quantitatively determine the presence of late trypsin transcript. Late trypsin transcript levels were not found to be significantly affected by the injection of either dsRNA (Fig. 3(C)).

4. Discussion

The molar concentration of each inhibitor fed to the mosquitoes in a blood meal was 95 μM to assure that concentration variabilities are not responsible for different levels of trypsin inhibition. As it is possible that early

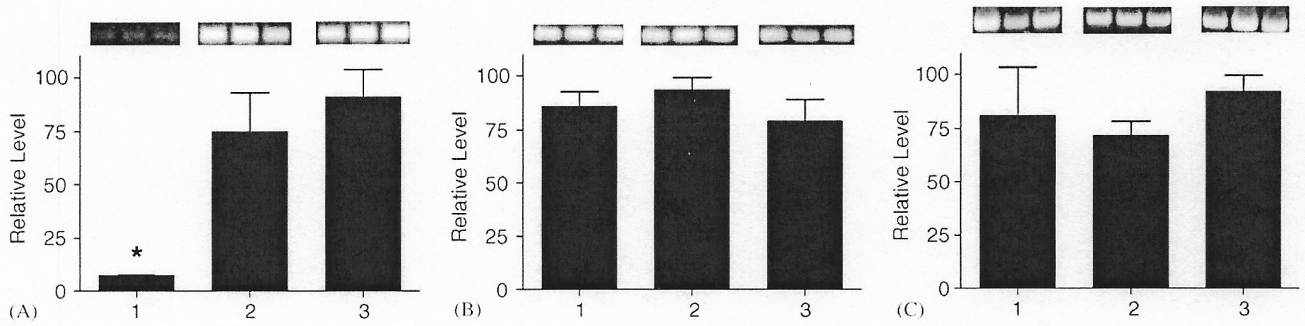

Fig. 3. Effect of dsRNA injection on transcript levels. Bars represent mean band intensity ± standard deviation of RT–PCR analysis of each transcript as determined by analysis using Kodak 1D software. RT–PCR was optimized for each sample to produce PCR reactions that are not exhausted and non-saturated bands on ethidium bromide stained agarose gels. Gel images are shown above graphs. (A) Early trypsin transcript levels in midguts from unfed mosquitoes injected with (1) early trypsin dsRNA, (2) luciferase dsRNA, or (3) non-injected mosquitoes. (B) TOR transcript levels in midguts from unfed mosquitoes injected with (1) early trypsin dsRNA, (2) luciferase dsRNA, or (3) non-injected mosquitoes. (C) Late trypsin transcript levels in midguts from 24 h blood-fed mosquitoes injected with (1) early trypsin dsRNA, (2) luciferase dsRNA, or (3) non-injected mosquitoes. Asterisks (*) indicate sample is significantly different ($p < 0.05$) from the control (non-injected sample) as determined by t-test.

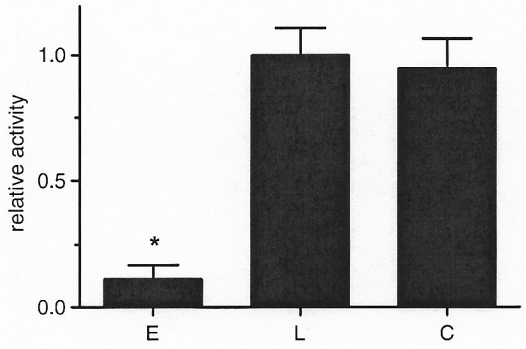

Fig. 4. Midgut trypsin activity 90 min after feeding from mosquitoes injected with dsRNA. Activity is reported as relative level of mean activity of luciferase injected mosquito samples, which had maximal mean activity. Bars are represented as mean ± standard deviation. E: early trypsin dsRNA injected mosquitoes, L: luciferase dsRNA injected mosquitoes, C: control, non-injected mosquitoes. Asterisks (*) indicate sample is significantly different ($p < 0.05$) from the control (non-injected sample) as determined by t-test.

trypsin may have different affinities for each inhibitor, trypsin activity was measured at 90 min after feeding to compare inhibitory effects on early trypsin. As shown in Fig. 1(A), there was no significant difference between the activities of the fed inhibitor, each reducing trypsin activities to similar levels. At 18 h after feeding, when late trypsin is active, trypsin activity also was reduced to similar levels for each inhibitor, eliminating the possibility that the inhibitors may not be stable for extended periods in the midgut. Taken together, these observations indicate that inhibitor specificity and/or stability are not sources of potential artifacts when determining the effects of early trypsin inhibition on late trypsin expression.

Each trypsin inhibitor was not capable of fully inhibiting the hydrolysis of BApNA in the assays from the 90 min fed midguts. In replicate assays 5 mM DFP was added to the reaction mixtures to determine if there was additional trypsin to be inhibited. Since DFP was incapable of further inhibiting BApNA hydrolysis, there may be other non-serine hydrolases in the midgut homogenate capable of hydrolyzing BApNA. It can be concluded though that at 90 min after feeding, there does not appear to be additional non-inhibited trypsin present.

Caroci and Noriega (2003) mentioned additional effects of feeding STI to mosquitoes, most notably early elimination of midgut contents. We determined how the additional inhibitors affected meal retention and peritrophic matrix formation, as well as their effect on egg maturation. After 24 h, it was found that only feeding STI resulted in meal expulsion, inhibited peritrophic matrix formation, and reduced egg count when compared to the non-inhibitor fed control (Table 1). Feeding any of the other inhibitors did result in significantly smaller follicle size. The fact that AEBSF and EWTI, despite having similar trypsin inhibition activities as STI at both 90 min and 18 h after feeding, did not result in premature meal expulsion would indicate that some factor other than trypsin inhibitor in the STI mixture is responsible for the meal expulsion reported by Caroci and Noriega (2003).

Midgut late trypsin levels after feeding on blood meals containing the various inhibitors were analyzed by western blot as shown in Fig. 2. Only feeding with STI resulted in decreased late trypsin expression. These results were verified by RT–PCR for the presence of late trypsin transcript as well. The results of RT–PCR matched the results of the western blot: highly reduced late trypsin expression only after feeding on a blood meal containing STI. To verify that experimental artifacts may not be present due to feeding on blood rather than on the 10% γ-globulin meal used in the original study (Barillas-Mury et al., 1995), the inhibitors were fed in a 10% γ-globulin meal and then late trypsin transcript levels were analyzed by RT–PCR. As no noticeable difference in the pattern of late trypsin expression was found after feeding the γ-globulin meal when compared to late trypsin expression after the blood meals, it is apparent that in this case meal type is not a source of potential artifacts.

Although elimination of early trypsin activity by feeding trypsin inhibitors has shown that inhibition of trypsin activity has no effect on trypsin expression, elimination of early trypsin expression would be a more conclusive method to determine how necessary it is in the regulation of late trypsin expression. The development of RNAi technology since the original study has made this type of experiment possible. Initial attempts to achieve RNAi mediated knock-down of early trypsin mRNA levels proved unsuccessful. Several variables were tested and it was found that injecting five-fold higher amounts than previously reported (Blandin et al., 2002) and in newly enclosed adult females resulted in highly decreased early trypsin transcript levels, as measured using RT–PCR methodologies (Fig. 3(A)). Although this semi-quantitative PCR procedure did demonstrate that the majority of early trypsin transcript was eliminated, it was also important to determine if in fact the smaller amount of expression present could produce an appreciable amount of trypsin activity in first hours after feeding. As displayed in Fig. 4, trypsin activity was reduced to similar levels as shown after feeding the three trypsin inhibitors seen in Fig. 2. Based on this phenotypic confirmation, it can be assumed that early trypsin expression is negligible in dsRNA injected mosquitoes.

Late trypsin transcript levels in early trypsin dsRNA injected mosquitoes were determined 24 h after feeding as shown in Fig. 3(C). Injection of early trypsin dsRNA, resulting in negligible amounts of early trypsin activity did not have a significant effect in reducing late trypsin transcript levels. This fact, in combination with the observed effects on late trypsin after feeding the various trypsin inhibitors, leads to the conclusion that early trypsin activity is not necessary for the transcriptional activation of the late trypsin gene.

Barillas-Mury et al. (1991, 1995) reported that predigesting a meal with bovine trypsin before feeding with STI resulted in restored late trypsin transcription. Although it is now clear that the trypsin inhibitory effects of STI are not the sole cause of the transcriptional repression of late trypsin, it is not clear how predigestion of the meal mitigates the effects of feeding the STI preparation on transcription. Predigestion may result in additional absorbable digestion products that may compete with the absorption of some unknown contaminating factor in the STI preparation that causes early expulsion of the meal and continued inactivation of the late trypsin gene.

Even though the exact factor in STI preparations that causes the effects described in this report and others (Barillas-Mury et al., 1995; Caroci and Noriega, 2003) is not known at this time, it is certain that these effects cannot be accounted for by trypsin inhibition alone. STI is a plant derived protease inhibitor, and may in fact be contaminated by naturally produced insecticidal agents. For instance, one possible contaminant is Soya toxin, purified and characterized by Vasconcelos et al. (1994). Soya toxin is a 21 kDa protein that has similar properties to another well characterized plant-derived toxin, canotoxin. In the report by Vasconcelos et al. (1994), STI co-purifies with Soya toxin, and is removed only after trypsin affinity chromatography. One reported method by which STI is routinely purified (Kassell, 1970) is similar to that of the purification of Soya toxin as well. Although there is no evidence that Soya toxin is responsible for the detrimental effects seen in mosquitoes when feeding on STI, it is important to note there are possible contaminants and that caution should be taken when utilizing plant derived sources of reagents for in vivo methods on insects that might be sensitive to these agents.

Ongoing studies are underway to identify the contaminating factors in the commercial STI preparations responsible for the effects described in this report and the mechanisms by which the effects occur. The conclusions of this study also have led us in new directions in investigating the transcriptional activation of late trypsin.

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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 Biochem. 21, 825–831.

Barillas-Mury, C.V., Noriega, F.G., Wells, M.A., 1995. Early trypsin activity is part of the signal transduction system that activates transcription of the late trypsin gene in the midgut of the mosquito *Aedes aegypti*. Insect Biochem. Mol. Biol. 25, 241–246.

Blandin, S., Moita, L.F., Kocher, T., Wilm, M., Kafatos, F.C., Levashina, E.A., 2002. Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *defensin* gene. EMBO Rep. 3, 852–856.

Caroci, A.S., Noriega, F.G., 2003. Free amino acids are important for the retention of protein and non-protein meals by the midgut of *Aedes aegypti* females. J. Insect Physiol. 49, 839–844.

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 Biochem. 19, 129–137.

Hansen, I.A., Attardo, G.M., Park, J.H., Peng, Q., Raikhel, A.S., 2004. Target of rapamycin-mediated amino acid signaling in mosquito anautogeny. PNAS 101, 10626–10631.

Hoa, N.T., Keene, K.M., Olson, K.E., Zheng, L., 2003. Characterization of RNA interference in an *Anopheles gambiae* cell line. Insect Biochem. Mol. Biol. 33, 949–957.

Kalhok, S.E., Tabak, L.M., Prosser, D.E., Brook, W., Downe, A.E.R., White, B.N., 1993. Isolation, sequencing and characterization of two cDNA clones coding for-trypsin-like enzymes from the midgut of *Aedes aegypti*. Insect Mol. Biol. 2, 71–79.

Kassell, B., 1970. Trypsin and chymotrypsin inhibitors from soybeans. In: Perlmann, G.E., Lorand, L. (Eds.), Methods in Enzymology. Elsevier, USA, pp. 853–862.

Noriega, F.G., Wells, M.A., 1999. A molecular view of Trypsin synthesis in the Midgut of *Aedes aegypti*. J. Insect Physiol. 45, 613–620.

Noriega, F.G., Barillas-Mury, C.V., Wells, M.A., 1994. Dietary control of late-trypsin gene transcription in *Aedes aegypti*. Insect Biochem. Mol. Biol. 24, 627–631.

Noriega, F.G., Pennington, J.E., Barillas-Mury, C., Wang, X.-Y., Wells, M.A., 1996. Early trypsin, an *Aedes aegypti* female specific protease, is post-transcriptionally regulated by the blood meal. Insect Mol. Biol. 5, 25–29.

Pennington, J.E., Wells, M.A., 2004. The midgut: structure and function. In: Marquardt, W.C., Black, W.C., Freier, J., Hagedorn, H., Hemingway, J., Higgs, S., James, A.A., Kondratieff, B.C. (Eds.), Biology of Disease Vectors, second ed. Elsevier, Amsterdam, in press.

Vasconcelos, I.M., Trentim, A., Guimares, J.A., Carlini, C.R., 1994. Purification and physicochemical characterization of soyatoxin, a novel toxic protein isolated from soybeans (*Glycine max*). Arch. Biochem. Biophys. 312, 357–366.

Zhou, G., Flowers, M., Friedrich, K., Horton, J., Pennington, J.E., Wells, M.A., 2004. Metabolic fate of [^{14}C]-labeled meal protein amino acids in *Aedes aegypti* mosquitoes. J. Insect Physiol. 50, 337–349.