



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*

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Trypsin activity during the first hours after feeding is essential to induce late trypsin gene expression. These results are consistent with the idea that free amino acids or other products released during digestion might be the initial signal for transcriptional activation of late trypsin. Besides early trypsin, some other factor(s) have to be translated for induction of late trypsin. This is the first case in which the proteolytic activity of a digestive enzyme is part of the signal transduction system which regulates expression of a second gene. The presence of two trypsins allows the mosquito to assess the quality of the meal and adjust the levels of late trypsin for a particular meal with remarkable flexibility.

Mosquito Trypsin Signal transduction Transcription

INTRODUCTION

When the *Aedes aegypti* female mosquito takes a blood-meal, there is a large increase in proteolytic activity in the midgut (Fisk, 1950), and trypsin is the major endoproteolytic enzyme induced by feeding (Briegel and Lea, 1975). Studies by Graf and Briegel (1989) and Felix *et al.* (1991) support the suggestion that there are two groups of trypsin forms produced by the female mosquito midgut following the blood meal: a group of *early* forms which is produced in small amounts, appears in the midgut within 2 h of the blood-meal, and disappears by 8 h after the blood-meal; and a group of *late* forms which is produced in large amounts, begins to appear 12 h after the blood-meal, and is responsible for most of the endoproteolytic activity present in the midgut during blood-meal digestion.

Neither the early nor the late form were detected in homogenates of unfed females, suggesting that they are translated *de novo* after feeding (Graf and Briegel, 1989). This conclusion is supported by the observation that the administration of translational inhibitors, such as puromycin (Gooding, 1973) or cycloheximide (Felix *et al.*, 1991), completely inhibit the induction of trypsin activity after feeding.

The late trypsin protein was isolated and characterized by Graf and Briegel (1985) and Graf *et al.* (1988). This is the major endoprotease present in the midgut during blood-meal digestion, reaching, at the peak of protease activity, concentrations of approximately 4–6 µg/midgut (Graf *et al.*, 1988). Immunocytochemical studies localized this form in the midgut and described its secretory pathway (Graf *et al.*, 1986). A monoclonal antibody produced against this protein (Graf *et al.*, 1988) was used to clone the late trypsin cDNA. The amino-terminal 35 amino acids of the purified protein (Graf *et al.*, 1991) match the sequence of the cDNA (Barillas-Mury *et al.*, 1991). The late trypsin mRNA is not present in unfed females, and could first be detected 4 h after feeding, reaching a maximum level at 24 h. This increase in mRNA levels is followed by an increase in late trypsin protein, indicating that transcriptional activation plays an important role in the regulation of expression of this gene (Barillas-Mury *et al.*, 1991). Transcription of the gene is dependent on both the quality and quantity of protein in the meal (Noriega *et al.*, 1994).

Another trypsin gene induced by the blood-meal in *A. aegypti* is the 5g1 clone (Kalhok *et al.*, 1993). Although the pattern of expression of this gene resembles that of the late trypsin gene, no information is available regarding the translation of this mRNA. Because Graf *et al.* (1991) found only a single amino terminal sequence associated with the purified midgut trypsin from *A. aegypti*, which matched the cDNA sequence reported by Barillas-Mury *et al.* (1991), but not the cDNA sequence

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of 5g1 clone, it is not clear whether the protein translated from the 5g1 mRNA plays any role in blood digestion.

The early trypsin forms described by Graf and Briegel (1989) were never purified or characterized. *In vitro* translation experiments by Graf and Briegel (1989), using mRNA from unfed mosquitoes, showed that there was no translatable trypsin mRNA present in unfed mosquito midgut. However, studies by Felix *et al.* (1991) suggested that the first phase of trypsin synthesis can occur following a protein meal in the presence of transcriptional inhibitors. Such synthesis could only involve translation of mRNA present in the midgut cells at the time the blood-meal was taken. Kalkok *et al.* (1993) have reported the cDNA sequence for a midgut trypsin, termed clone 3a1, and shown that the mRNA for this trypsin is present in unfed midgut, and they proposed that this clone codes for an early trypsin protein.

While it is clear that late trypsin plays a major role in blood-meal protein digestion, the role of early trypsin is unclear. We have estimated that the amount of early trypsin produced in the midgut to be on the ng range (Pennington J. E. *et al.*, 1994, unpublished), and this protein is present in the midgut during a time where there is little blood-meal protein digestion (Graf and Briegel, 1989). In this paper we describe experiments designed to determine the role of early trypsin in blood-meal digestion.

MATERIALS AND METHODS

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 supplied with a cotton wool pad soaked in a 10% sucrose solution until 12–16 h before the experimental feeding. In this paper, we will refer to the sucrose-fed females as “unfed”.

Materials

Guanidinium isothiocyanate and sodium N-lauroylsarcosine were purchased from Fluka (Bucks, Switzerland); 2-mercaptoethanol from Sigma (St Louis, Mo.); phenol from Amresco (Solon, Ohio); Ultra pure agarose and RNA electrophoresis markers were obtained from GIBCO-BRL (Gaithersburg, Md); and RNA-binding glass powder (RNAid™ Kit) was purchased from BIO 101 (La Jolla, Calif.).

Protein meals

Solutions of pig γ -globulin (Cohn fraction II,III) were prepared in 100 mM NaHCO₃, 150 mM NaCl, pH 7.4, and dialyzed overnight at 4°C against the same buffer. Soybean Trypsin Inhibitor (STI) was added to the dialyzed protein solution at a final concentration of 2 mg/ml. For experiments using cycloheximide, a stock solution of 50 mg/ml in 50% ethanol was prepared and diluted 1:500 with the meal to a final concentration of 100 μ g/ml. *Ex vivo* digestion of the protein meal was carried by adding bovine trypsin at a concentration of

0.4 mg/ml and incubating at 37°C for 8 h. Proteolysis was stopped by adding 2 mg/ml of STI: at this concentration the inhibitor is in a 10-fold molar excess to the bovine trypsin. In some cases, the *ex vivo* digestion was carried as described, but instead of adding STI, the sample was frozen at -70°C after the 8 h incubation. The sample was then thawed and cycloheximide added immediately before feeding. All meals were equilibrated to 37°C and ATP was added to a final concentration of 1 mM before feeding the mosquitoes.

Anti-late trypsin polyclonal antibodies

The late trypsin cDNA was used to over-express late pro-trypsin, which includes the activation peptide, but not the signal sequence for secretion, in an *E. coli* system. The full length late trypsin cDNA (Barillas-Mury *et al.*, 1991) was used as template for a PCR reaction to introduce the appropriate restriction sites for cloning into the expression vector pET 11-a (Novagen, Madison, Wis.). The first primer had an NdeI restriction site and the initiating methionine at the 5' end, and 19 bases complementary to the sequence of the lower strand between positions 47 and 65. The second primer introduced a BamHI site and was complementary to the upper strand between positions 795 and 782. The PCR amplification was carried out using 200 ng of DNA as the template and 24 cycles under the following conditions: denaturation at 95°C for 45 s; annealing at 55°C for 45 s; and extension at 72°C for 1 min. The PCR product was digested with NdeI and BamHI and ligated into the plasmid pET 11-a. Following transformation and amplification in *E. coli* XL-1 Blue, the recombinant plasmid was used to transform *E. coli* strain BL21-(DE3) for expression according to the manufacturers protocol. The cells were homogenized after induction and the proteins were separated in a 12.5% SDS-PAGE gel. After staining with Coomassie Brilliant Blue, a prominent band of 30 kDa, absent in non-induced cells, was observed. The band of induced protein was the only protein in the whole cell homogenate which reacted on Western blots with a monoclonal antibody against late trypsin isolated from mosquitoes (Graf *et al.*, 1988). This band was cut from the gel and divided into three equal portions, each containing approximately 500 μ g of late trypsin protein. A New Zealand rabbit was injected with 500 μ g of protein, followed by two booster injections 6 and 8 weeks after the initial injection. For injection, each gel slice was neutralized in 100 mM Tris-HCl pH 7.4, containing 10% ethanol for 15 min, and then homogenized in 1 ml of adjuvant (RIBI; Hamilton, Mont.). The polyclonal antibodies could detect 0.4 mosquito/equivalents of late trypsin, even at 1:10,000 dilution, and there was no cross-reactivity with other mosquito proteins or between the pre-immune serum (1:1000) and the mosquito homogenate.

Protein analysis and detection of late trypsin

The effect of various meals on late trypsin translation was evaluated by Western blot analysis. For each time

point, two groups of five mosquitoes were analyzed. Each group was homogenized in 100 mM Tris-HCl buffer pH 8.0, containing 5 mM phenylmethylsulfonyl-fluoride (PMSF), using 50 μ l of buffer per insect. The proteins in the homogenate were separated by SDS-PAGE, loading either 0.4 or 0.6 mosquito equivalents per lane. The proteins were transferred to nitrocellulose and trypsin was detected with the polyclonal antibody described above at a 1:5,000 dilution.

RNA isolation and characterization

RNA was isolated from whole mosquitoes using RNA binding glass powder (Noriega and Wells, 1993). RNA was separated by electrophoresis on 1.5% agarose gels under denaturing conditions using the formaldehyde method (Fourney *et al.*, 1988). The RNA was transferred to Nytran (Schleicher & Schuell, Keene, N.H.) and hybridized with a late trypsin cDNA (Barillas-Mury *et al.*, 1991), or a ribosomal probe, which were labeled using a Random Primers Kit (BRL) and (α - 32 P)-dATP (ICN, Irvine, Calif.). Hybridizations were done under high stringency conditions, as previously described (Noriega and Wells, 1993). The amount of radioactivity bound to individual samples on the filter was quantified using a Betascope (Betagene, Waltham, Mass.). Under the high stringency conditions used, the late trypsin cDNA specifically recognizes the late trypsin DNA, and does not hybridize with the Kalkok *et al.* (1993) 3a1 or 5g1 clones (results not shown).

RESULTS

Effect of inhibition of early trypsin enzymatic activity on late trypsin synthesis

In order to assess the role of early trypsin in blood-meal digestion, we added a large excess of STI to a protein meal and then measured late trypsin mRNA and protein levels. The effect of inhibition of early trypsin activity on late trypsin mRNA levels is shown in Fig. 1. When STI was added to the protein meal, transcriptional activation of late trypsin was blocked. At 18 h, the late trypsin mRNA levels were less than 10% of the values found with the control meal. To insure that this inhibition of late trypsin mRNA transcription was due to the inhibition of early trypsin activity, and not to some other, unrelated inhibitory effect of STI, a control experiment was performed in which the meal was digested *ex vivo* with bovine trypsin before the addition of excess of STI. With this pre-digested meal, the transcriptional activation of the late trypsin gene was normal, even in the presence of STI (Fig. 1).

The effect of STI on translation of the late trypsin message was also evaluated, using Western blot analysis (Fig. 2). When a control meal was fed, trypsin protein could be detected at 12 h with greatly increased levels at 18 h. When the meal contained STI, no late trypsin protein was detected at 12 or 18 h, consistent with the very low levels of late trypsin mRNA. Although pre-

digestion of the meal with bovine trypsin restored normal levels of late trypsin mRNA, very little late trypsin protein was translated and only very small amounts of protein could be detected at 18 h (Fig. 2).

Effect of translational inhibitors on transcription of the late trypsin gene

When cycloheximide was added to the protein meal, transcriptional activation of the late trypsin gene was blocked (Fig. 3). In order to determine if early trypsin

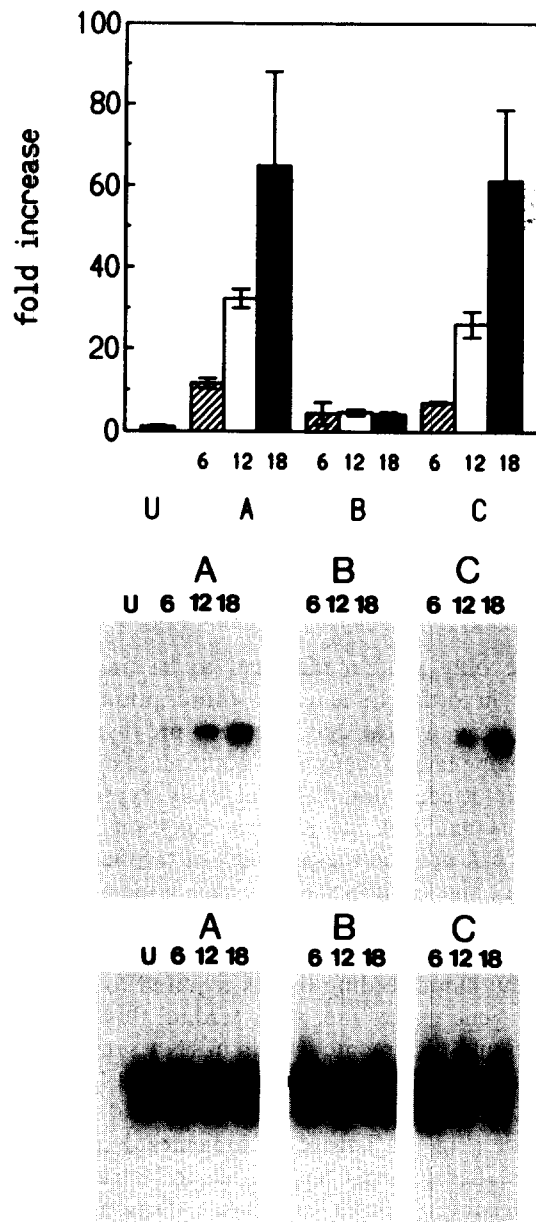


FIGURE 1. Late trypsin mRNA levels at different times after feeding (6, 12 and 18 h). A = pig γ -globulin (100 mg/ml), B = pig γ -globulin (100 mg/ml) + STI (2 mg/ml), C = pig γ -globulin (100 mg/ml) digested *ex vivo* with bovine trypsin (0.4 mg/ml) + STI (2 mg/ml) and U = unfed mosquito. Top: data from Betascope quantitation; values were calculated as cpm late trypsin mRNA/cpm ribosomal RNA. Fold increase is relative to the levels in unfed mosquitoes. Middle: photograph of Northern blot probed with a labeled, late trypsin cDNA. Bottom: photograph of the same Northern blot hybridized with a labeled ribosomal probe.

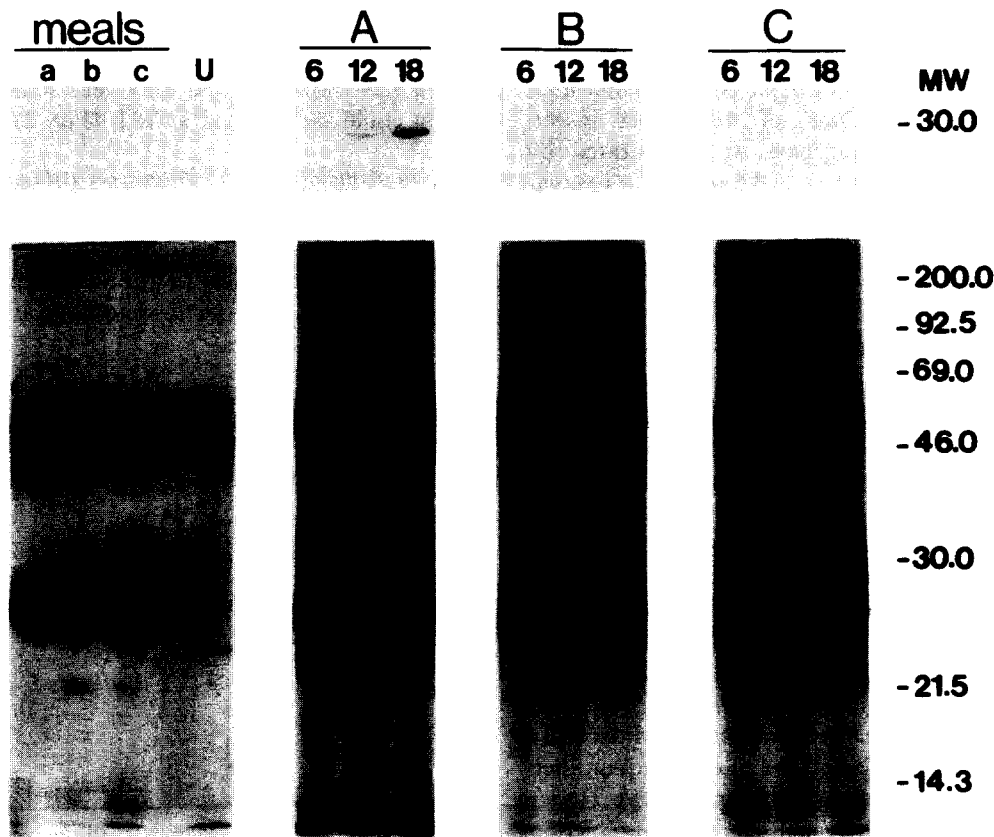


FIGURE 2. SDS-PAGE of whole mosquito homogenates at different times after feeding (6, 12 and 18 h). A = pig γ -globulin (100 mg/ml), B = pig γ -globulin (100 mg/ml) + STI (2 mg/ml), C = pig γ -globulin (100 mg/ml) digested *ex vivo* with bovine trypsin (0.4 mg/ml) + STI (2 mg/ml), U = unfed mosquito and a, b and c = meals alone. The lines indicate the molecular weight in kDa. For all mosquito homogenates, 0.4 mosquito equivalent were loaded per lane. The upper part of the figure shows the levels of late trypsin protein detected by western blot analysis for each of the samples shown in the SDS-PAGE below.

was the only protein that had to be translated after feeding in order to activate transcription of the late trypsin gene, a meal that had been pre-digested *ex vivo* with bovine trypsin before the addition of cycloheximide was also tested. Using this meal, early trypsin would not be translated, but the *ex vivo* digestion with bovine trypsin will provide the products of tryptic digestion of the meal, and as shown above, such a meal will activate transcription of the late trypsin gene. Thus, if early trypsin were the only protein whose translation is induced by blood-feeding that is required to activate late trypsin gene expression, then feeding this pre-digested meal should restore late trypsin mRNA synthesis. However, late trypsin mRNA levels remained low after the administration of the pre-digested meal with cycloheximide (Fig. 3). This result indicates that some other factor(s), besides early trypsin, has to be translated after blood-feeding in order to activate transcription of the late trypsin gene.

DISCUSSION

The results presented in this paper show that early trypsin activity is essential for inducing the synthesis of late trypsin. The results also show that another protein(s), presumably a transcriptional activator, must be translated in order for transcription of the late trypsin gene to

occur. It has been suggested that the late trypsin clone of Barillas-Mury *et al.* (1991) might not be the midgut trypsin induced by the blood-meal because of its unusual sequence around the specificity pocket (Kalhok *et al.*, 1993). If this were the case, the present results would have questionable significance. The following considerations provide strong proof that the cDNA cloned by Barillas-Mury encodes late trypsin: (1) a monoclonal antibody against late trypsin was used to isolate cDNA clones and all of the cDNA clones isolated had the same sequence; (2) the amino terminal sequence of the protein encoded by the cDNA clone matched exactly that of the late trypsin protein determined by amino terminal sequence analysis (Graf *et al.*, 1991); (3) the cDNA sequence has been confirmed by the gene sequence (Barillas-Mury and Wells, 1993); (4) the cDNA clone has been used to overexpress the protein and this protein reacts with the monoclonal antibody against late trypsin; (5) the overexpressed protein was used to produce polyclonal antibodies which detect a midgut trypsin whose pattern of expression is the same as the pattern of expression detected by the monoclonal antibody against late trypsin.

Model for regulation of trypsin synthesis following a blood-meal

Based on the studies reported in this paper and the results of others (Briegel and Lea, 1975; Graf and

Briegel, 1989; Felix *et al.*, 1991; Barillas-Mury *et al.*, 1991; Kalhok *et al.*, 1993; Noriega *et al.*, 1994), we propose the model shown in Fig. 4 for the regulation of trypsin synthesis following a blood-meal. In this figure, established facts are given in capital letters, whereas statements included in boxes indicate areas where more work is required to elucidate the mechanism.

The blood-meal induces secretion of early trypsin, either through stretching of the midgut or through an osmotic effect. It seems reasonable to assume that the endocrine cells in the midgut (Brown and Lea, 1989) are involved, but it is not known how. The mechanism whereby early trypsin secretion is induced is unclear. Studies by Graf and Briegel (1989) failed to show any stored zymogen or translatable mRNA in the midgut of unfed mosquitoes. However, studies by Felix *et al.* (1991) suggest that some trypsin synthesis can occur in the absence of transcription, which would suggest that a mRNA exists in the midgut before the blood-meal. More experiments will be needed to clarify this point.

It is clear that the activity of early trypsin is required in order for transcription of the late trypsin gene to occur. We presume that some digestion product(s), most

REGULATION OF MIDGUT TRYPsin PRODUCTION

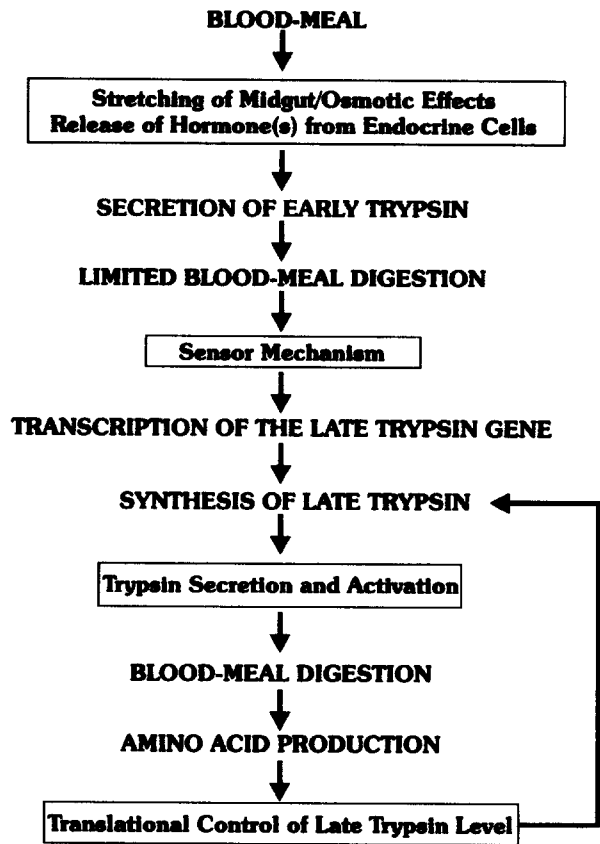


FIGURE 4. Model for regulation of midgut trypsin production. In this figure established facts are given in capital letters, whereas statements included in boxes indicate areas where more work is required to elucidate the mechanism.

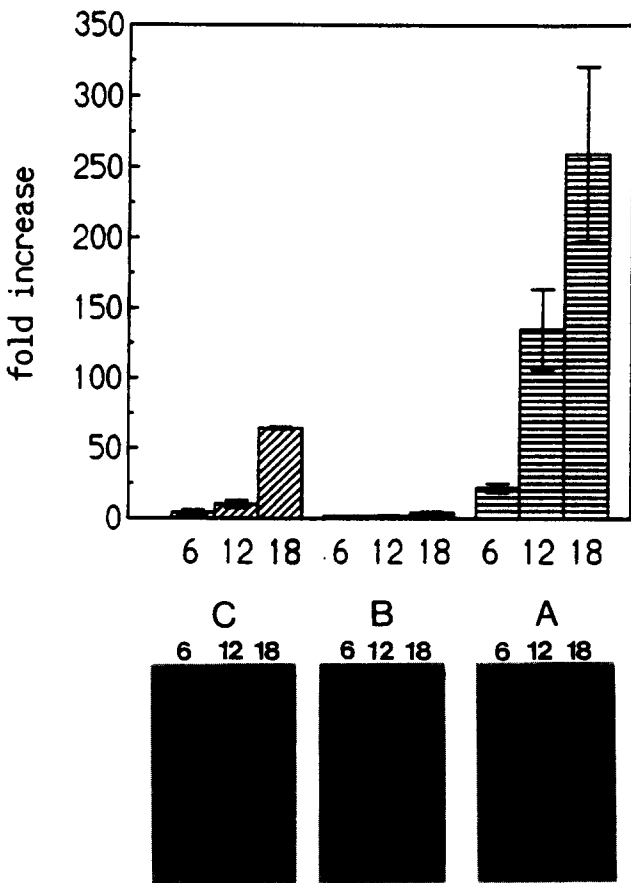


FIGURE 3. Late trypsin mRNA levels at different times after feeding (6, 12 and 18 h). A = pig γ -globulin (100 mg/ml), B = pig γ -globulin (100 mg/ml) + cycloheximide (100 μ g/ml), C = pig γ -globulin (100 mg/ml) digested *ex-vivo* with bovine trypsin (0.4 mg/ml) + cycloheximide (100 μ g/ml). Top: data from Betascope quantitation (as described in Fig. 1). Lower: photography of Northern blot probed with a labeled, late trypsin cDNA.

likely amino acids produced from the proteins in the meal are involved, but this remains to be proven. We also assume that other proteases, e.g. aminopeptidases or carboxypeptidases, already present in the midgut at the time of the blood-meal are involved (Graf and Briegel, 1982). We suggest that free amino acids or small peptides are the signal for transcriptional activation of the late trypsin gene, presumably causing activation of a regulatory protein (by inducing translation or some other post-translational modification), which in turn activates transcription of the late trypsin gene.

Some mechanism must also exist which regulates the amount of late trypsin translated in relation to the amount of protein in the meal. It was shown that, when a mixture of radiolabeled amino acids is added to the blood meal, these amino acids are incorporated into newly translated trypsin (Gooding, 1973). Thus, the amino acids released from protein digestion could be the rate-limiting step for late trypsin translation. This suggestion is supported by the observation that, when the meal was partially digested *ex vivo* with trypsin before adding STI, the products released during pre-digestion were able to restore transcriptional activation of the late

trypsin gene to control levels. However, the presence of STI, which prevents further digestion *in vivo*, did not allow production of normal amounts of amino acids, and therefore, only very limited translation of late trypsin took place. Regulation at both transcriptional and translational levels allows the mosquito to adjust the levels of late trypsin with remarkable flexibility in response to a particular meal. This tight regulation might be particularly important when most of the meal has already been digested and no more late trypsin is needed.

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 system allows the mosquito to assess the quality of the meal via early trypsin before committing to the synthesis of late trypsin. If there is no protein in the meal, or if it has poor digestibility, no free amino acids or peptides will be released and the late trypsin gene will not be transcribed.

To our knowledge, this is the first case in which the activity of a digestive protease has been shown to be part of the signal transduction system which regulates the expression of a second gene. It is possible that, in addition to late trypsin, other genes involved in digestion or midgut maturation may also be regulated by this mechanism. The pathway of signal transduction from the cell membrane of the midgut cell to its nucleus, which finally results in the transcriptional activation of the late trypsin gene is unknown. Our results with cycloheximide indicate that, besides the activity of early trypsin, some other factor(s) must be translated *de novo* in order for transcriptional activation of the late trypsin gene to occur. The nature of this factor is currently under investigation.

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