Pleiotropic effects of trypsin modulating oostatic factor (Neb-TMOF) of the flyfly Neobelliera bullata (Diptera: Calliphoridae)

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Abstract. Trypsin Modulating Oostatic Factor (TMOF) of the grey flyfly Neobelliera bullata is a hexapeptide that has been isolated from vitellogenic ovaries. Its precursor is a 75 kDa protein that is abundantly present in oocytes but absent from hemolymph. TMOF inhibits trypsin biosynthesis in the gut, it lowers yolk polypeptide concentration in the hemolymph and strongly inhibits ecdysone biosynthesis by larval ring glands. Evidence is presented that a molecule with identical chromatographic behaviour and physiological effects is also present in the larvae of Neobelliera at the time when they stop feeding. Ecdysone inhibition does not suppress trypsin modulating activity of the hexapeptide. This, in combination with results that were obtained with four slightly different TMOF analogs, suggests that the inhibitory activity of trypsin biosynthesis is independent from that of ecdysone regulation. Several compounds that were tested for initiation or increase in trypsin biosynthesis in the gut were not active.

INTRODUCTION

In Neobelliera bullata, an anautogenous flyfly, oogenesis is cyclic and ovarian development of the follicles is synchronous. This means that initiation and cessation of vitellogenesis has to be regulated. We recently isolated from 10,000 vitellogenic ovaries of Neobelliera a hexapeptide (H-NPTNLH-0H) that inhibits the de novo biosynthesis of trypsin-like enzymes in the midgut (Bylemans et al., 1994). These proteases are synthesized in the midgut shortly after the intake of a protein meal. They release sufficient amounts of amino acids that are used by the fat body to massively synthesize yolk polypeptides in a short time. When digestion stops, the vitellogenin titer in the hemolymph drops to low levels and egg development is arrested. A peptide with trypsin modulating activity was isolated first from the mosquito Aedes aegypti by Borovsky et al. (1990, 1994) and was named Aea-TMOF. The amino acid sequences of Neb-TMOF and of Aea-TMOF are different. Recently, we found that Neb-TMOF also inhibits in vitro and in vivo ecdysone biosynthesis by larval ring glands of both Calliphora vicina (Hua et al., 1994a,b) and Neobelliera bullata (De Loof et al., 1995). The inhibition was reversible and reached a maximum at 5.10^{-7} M with an EC_{50} of 5.10^{-6} M, and we called it prothoracostatic activity (PTSH), opposite of the activity of the prothoracotropic hormone (PTTH) of Bombyx mori (Nagashawa et al., 1984, 1986) and Manduca sexta (Bollenbacher et al., 1984). In this paper we present evidence that Neb-TMOF is also present in larvae of Neobelliera

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bullata. Because of the well documented inducing role of ecdysone in vitellogenesis of Neobellieria bullata (Huybrechts & De Loof, 1977, 1981), we investigated the possibility that the inhibition of trypsin synthesis by Neb-TMOF could be an indirect consequence of a primary inhibition of ecdysone biosynthesis. The possible role for Neb-TMOF in controlling ecdysteroid titer and molting in larvae is discussed.

MATERIALS AND METHODS

Chemicals

Juvenile hormone III (JH) was from Fluka, 20-OH-ecdysone was from Simes, forskolin and cAMP from Sigma. 20-OH-ecdysone used for injections was dissolved in 10% ethanol in water. JH was dissolved in acetone and applied on the neck membrane with a fine tip. Solid phase 9-fluorenylmethoxycarbonyl (Fmoc)-technology was used to synthesize peptide analogs. Neobellieria Ringer solution was as described in Bylemans et al. (1994).

Experimental animals

Flies were reared as described by Huybrechts & De Loof (1977). During the first 2–3 days of adult life, N. bullata feeds on a sugar-water diet. On day 4, when the first batch of ovarian follicles completes previtellogenic development, flies were offered slices of beef liver as a source of protein required for the synthesis of their egg yolk polypeptides. The liver that was fed to the experimental animals was coated with sugar crystals. The liver-sugar combination attracted the flies resulting in a faster food intake and improved synchronization of ovarian development. Larvae stay on liver for 5 to 6 days. Then they move into dry peat where they start pupariation around day 10. The larval extract was made from 5 days old larvae.

Measurement of trypsin biosynthesis

Six hours after injecting TMOF or analogs, which was immediately followed by feeding the flies with liver, guts were removed and analyzed for trypsin biosynthesis (Borovsky & Schlein, 1988), or kept frozen at –20°C until use. Briefly, individual guts were homogenized, centrifuged, the supernatant was removed and an aliquot (0.1 gut equivalent) was incubated with [3H]DFF for 18 h at 5°C. In each experiment 2 control groups were used: (a) un.injected and (b) injected with Neobellieria Ringer solution. No significant differences were found among the 2 control groups.

In vitro effects on ecdysone biosynthesis

The in vitro assays for ecdysone biosynthesis were carried out according to Budd et al. (1993). Briefly, ring glands were explanted from Neobellieria bullata (late third instar, 7 days after oviposition). The brain hemispheres, suboesophageal ganglion, ring gland, thoracic and abdominal ganglia, which all form one complex, were dissected. The dissection has to be done with extreme care so that all neuronal connections between the parts of the CNS and the ring gland were left intact. The imaginal discs and any adhering tissue were removed. Ring glands were dissected from the complex, cut into left (control) and right (= experimental) halves and incubated in Ringer solution. After an incubation for 4 h, the ecdysteroid content of the medium was analyzed by RIA.

In vivo experiments

We have assumed that Neobellieria has 20 μl of hemolymph. The amount of injected compound was adjusted in such a way that following injection of a volume of 2 μl solution the in vivo concentrations of the compound would be the ones indicated.

Tissue extraction and High Performance Liquid Chromatography equipment

Tissues were dissected in Neobellieria Ringer solution and immediately placed in methanol/water/acetic acid (90 : 9 : 1) solution in an ice-bath. Tissues were homogenized, centrifuged for 30 minutes at 9,820 g at 4°C and sonicated for 2 minutes (MSE Soniprep 150 Sonicator). Methanol was evaporated and the aqueous extract was re-extracted with ethyl acetate and n-hexane. Organic solvents were decanted and the aqueous solution was dried in siliconized round bottom flasks. Extracts were then prepurified on MegaBond Elute C18 cartridges (Varian). Cartridges were activated with acetonitrile (ACN) : H2O : trifluoroacetic acid (TFA) (80 : 19.9 : 0.1) and afterwards rinsed with aqueous 0.1% TFA. Samples were redissolved in start solution and subsequently eluted with ACN : H2O : TFA
High Performance Liquid Chromatography (HPLC) was done with a Beckman Program-
nable Solvent Module 126 connected with a Diode Array Detector Module 168 (Gold system). A Super-
cosil LC-18 DB (4.6 × 250 mm) column was used, solvent A, 0.1% TFA in water; solvent B, 80%
acetonitrile in 0.1% aqueous TFA. Column elution conditions were 100% A for 8 minutes, linear gradient
to 75% B in 60 minutes, flow rate 1 ml/min. Absorbance was followed at 214 nm. An extract of 300 lar-
vae taken at the time that they just had stopped feeding was subjected to HPLC. The fractions of the larval
extract were collected every minute. For the assays the fraction, which was coeluting with synthetic Neb-
TMOF, was used.

Initiation of trypsin synthesis

For the in vivo assay, an acidic extract (Bylemans et al., 1994) of 5 brain equivalents of female liver-
fed flies (which was eluted with ACN : water (1 : 1) from prepurification on Sep-pak columns, was dried
and redissolved in Ringer solution), 200 ng 20-OH-ecdysone, 10 μg JH III, Lom-Sulfakinite (0.3 μg) (gift
from Dr L. Schoofs, K.U. Leuven), cAMP (30 μg) or forskolin (3 μg) were injected into or applied to 3
days old female sugar-fed flies. Immediately after injection, flies were fed liver. In the in vivo experi-
ments, guts were dissected 1 h after injection of the test compound. The positive control in this experi-
ment was feeding liver for 1 h followed by immediate dissection of the gut. For the in vitro assay one gut
of a 3-days old, sugar fed female fly was incubated at 25°C for 20 min. The same compounds were added
to the incubation medium (100 μl Ringer solution). Guts were then cooled and immediately assayed for
trypsin activity.

RESULTS

Evidence for the presence of Neb-TMOF in larvae

The finding that Neb-TMOF strongly inhibited biosynthesis of ecdysone by larval ring
glands raised the question whether this peptide, which is produced by vitellogenic ovaries
in the adult stage, might be present in the larvae. Five larval equivalents of the fraction
coeuluting with synthetic Neb-TMOF were injected into adult females. This resulted after 6
h in an inhibition of trypsin biosynthesis by 55 ± 39% (n = 8, p < 0.01) as compared to
the controls (Fig. 1b). Furthermore, this extract also significantly inhibited ecdysone biosyn-
thesis by larval ring glands (Fig. 1a).

Biological activity of synthetic analogs of Neb-TMOF

The effect of different analogs on the inhibition of the biosynthesis of trypsin (in vivo)
and ecdysone (in vitro) biosynthesis was tested (Table 1). In both assays Neb-TMOF was
the most active peptide. Physiological concentrations of NPTNLRT mimicked the effect of
Neb-TMOF activity only on trypsin biosynthesis whereas NPNLH inhibited only ecdy-
sone biosynthesis (Table 1). This suggested that different types of receptors may be

![Fig. 1. The presence of TMOF and PTSH activity in a fraction of the larval extract that is coeluting with synthetic TMOF on HPLC. (a) Incubation of a larval extract (Ex) of five larval equivalents in vitro with larval ring glands resulted in an inhibition of ecdysone biosynthesis (dotted) (n = 4) and (b) injection of the same extract reduced trypsin activity (crosshatched) in the midgut of adults by 55% compared to the respective controls (C) (n = 8).](image-url)
activated by Neb-TMOF. Replacement of asparagine by aspartic acid severely lowered the biological activity of this factor, indicating the specificity of the assay.

**TABLE 1. Effect of structural analogs of Neb-TMOF (NPTNLH) on biological activity. Trypsin inhibition was measured in vivo (n = 10) and ecdysone inhibition in vitro (n = 4). Primary structures of the different peptides are indicated by the single letter code.**

<table>
<thead>
<tr>
<th>Sequence of analogs</th>
<th>Inhibition of trypsin biosynthesis (%)</th>
<th>Inhibition of ecdysone biosynthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^{-8} \text{M}$</td>
<td>$10^{-9} \text{M}$</td>
</tr>
<tr>
<td>NPTNLH</td>
<td>28 ± 9</td>
<td>51 ± 12</td>
</tr>
<tr>
<td>NPNLH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DPTDLH</td>
<td>23 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>NPTNLK</td>
<td>42 ± 6</td>
<td>35 ± 11</td>
</tr>
</tbody>
</table>

Does 20-OH-ecdysone initiate trypsin biosynthesis?

We investigated the effect of ecdysone and other compounds on the initiation of trypsin synthesis after a protein meal, in vivo (Fig. 2) and in vitro (results not shown). Another goal of the latter experiments was to find out whether perhaps trypsin is already stored as a zymogen in the gut prior to ingestion of a protein meal. The cleavage of trypsin from this zymogen could then be activated by ecdysone or another compound. The following compounds were not effective in increasing trypsin biosynthesis in sugar-fed females: 20-OH-ecdysone, JH III, cAMP, forskolin, Lom-sulfakinine (a cholecystokinin/gastrin-like peptide isolated from *Locusta migratoria* (Schoofs et al., 1990) or a brain extract from liver-fed females. Only the uptake of liver increased trypsin activity in the midgut. If trypsin should be stored as a zymogen, none of the tested compounds is able to initiate the cleavage of trypsin from this zymogen. Unlike the mosquito *Aedes aegypti, Neobellieria bullata* needs to continuously feed on protein for a few hours in order for the trypsin activity to reach a maximum. When the liver meal was removed or when flies were ligated 10 min after liver feeding, trypsin activity which was measured 6 hrs later was only 30% of that found in flies fed ad libitum for 6 h (Fig. 2).

Fig. 2. 1–4. Trypsin activity depends on the continuity of feeding in *Neobellieria bullata*. Guts from (1) to (4) were assayed at 6 h after liver feeding. Continuous liver feeding for 6 h (1), removal of liver after 10 min of feeding (2), ligation of the head after 10 min (3), sugar fed for 6 h (4). 5–11. In vivo effect of 200 ng 20-OH-ecdysone (6), 10 μM sulfakinine (7), 5 brain equivalents of liver fed females (8), 10 μg JH III (9), 5 mM cAMP (10) and 40 μM forskolin (11) on trypsin activity in sugar fed female flies. None of these treatments differs from the sugar fed control flies (5) and were hence not comparable to the trypsin activity in guts of liver fed flies (12). Guts of (5) to (12) were assayed at 1 h after treatment of the flies and initiation of liver feeding. Five flies were used for each treatment.
20-OH-Ecdysone and the arrest of trypsin biosynthesis by Neb-TMOF

In order to investigate whether the effect of Neb-TMOF on trypsin biosynthesis in the midgut of adult females is a secondary event in which the primary effect is on ecdysone biosynthesis, we studied the effect of 20-OH-ecdysone on trypsin activity, and on the inhibition of trypsin biosynthesis after a liver meal by TMOF. Injection of 25 μg 20-OH-ecdysone did not increase trypsin activity. If ecdysone was injected together with $10^9$ M Neb-TMOF, trypsin activity was not significantly different from that in flies that had been injected only with Neb-TMOF (Fig. 3).

DISCUSSION

Indirect evidence indicated that Neb-TMOF is probably also present in larvae; larval extract was coeluted on HPLC with synthetic Neb-TMOF and inhibited ecdysone production by larval ring glands and trypsin biosynthesis by the adult midgut. In adults Neb-TMOF might be cleaved from a 75 kDa protein that might be part of the vitelline membrane (Bylemans, 1994). The question then is whether TMOF in larvae originates from the same precursor or from another one. Immunocytochemical methods will be used to obtain more data about the possible production site of Neb-TMOF in larvae. The presence of Neb-TMOF-like molecules in males should also be investigated since trypsin activity in males of Neobellieria is always low (Bylemans et al., 1993) and ecdysone titers in the hemolymph cannot be detected by RIA (Briers & De Loof, 1980).

As to the problem of which factor(s) initiates the production of trypsin after a protein meal, neither ecdysone nor any other compound we tested was able to do so in sugar-fed flies. Some authors reported that the phenomenon of increased protease activity after a blood meal is due to a secretagogue (Blakemore et al., 1993) or to stretching of the gut (Graf & Briegel, 1989). The active factor remains to be identified.

Ecdysone did not affect trypsin biosynthesis after the blood meal. Injection of 20-OH-ecdysone did not significantly increase the level of trypsin. De Clerck & De Loof (1983) demonstrated that daily feeding of 1 to 10 μg 20-OH-ecdysone to males increased the volume of protein solution ingested. However, the increase in ecdysone-fed males was less than 50% of that of females. The effect of ecdysone observed by De Clerck & De Loof (1983) might hence be a behavioral one.

The effect of Neb-TMOF on trypsin biosynthesis does not depend upon its effect on ecdysone biosynthesis. This follows from our observation that injection of ecdysone
together with Neb-TMOF did not significantly overrule the effect of the oostatic peptide on the inhibition of trypsin biosynthesis.

So far, we have not been able to demonstrate an effect of Neb-TMOF on ecdysone biosynthesis in adults. The major problem here is that the exact site(s) of synthesis in adult Neobellieria is not known. Ovariecromized Neobellieria females can still produce vitellogenin (Briers & De Loof, 1980). Since this requires ecdysone, the ovaries cannot be the only site that produces the hormone. The epidermis or other tissues might also be possible production sites. TMOF did not affect ecdysone synthesis by the ovaries which is low compared to that by larval ring glands (unpublished results). It is possible that (part of) the large amounts of ecdysteroids present in the ovary (mostly conjugated ecdysteroids) might be of extravarian origin. Delbecque et al. (1990) reviewed alternatives for ecdysone production.

In order to unequivocally prove the role of Neb-TMOF in larvae and adult males, a quantitative assay to measure TMOF concentrations during the different larval and pupal stages will have to be developed. The same assay can then be used to measure the amount of Neb-TMOF in female adults. We expect that production and secretion of Neb-TMOF in females starts shortly before ecdysone- and vitellogenin titers in the hemolymph reach their maximum (between 24 and 48 h after liver feeding) and that the titer decreases when choriogenesis starts.

We think that PTSH molecules, whatever their nature, could play an important role in controlling ecdysone titers. In the past, increases in ecdysone titers were usually thought to be the result of temporary secretion of PTTH-like molecules. We believe that ecdysone titers are probably regulated by an interplay of PTTH and PTSH. During intermolts, PTSH predominates while at the time of molts and during metamorphosis, the opposite situation prevails. This model is more extensively discussed by De Loof et al. (1995). At the end of the last larval instar, digestion in the gut falls to very low levels just like in late vitellogenic females. Whether TMOF plays a role in this process remains to be investigated.

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REFERENCES


