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Captopril, a Specific Inhibitor of Angiotensin Converting Enzyme, Enhances Both Trypsin and Vitellogenin Titers in the Grey Fleshfly *Neobellieria bullata*

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A strong and constitutive angiotensin converting enzyme- or ACE-like activity was demonstrated in the hemolymph of the adult grey fleshfly *Neobellieria bullata*. In a competition assay, the *N. bullata* trypsin modulating oostatic factor (*Neb*-TMOF) was confirmed to be an in vitro substrate for this circulating *Neb*-ACE. Oral uptake of captopril, a selective and specific inhibitor of ACE, resulted in a complete phenotypic knockout of circulating ACE activity. When compared with control animals, captopril-fed female flies showed an increase in the liver meal–induced trypsin peak in the midgut and elevated levels of protein meal–induced yolk polypeptides in the hemolymph. The latter effect was not due to a slower vitellogenin uptake by the ovaries, because oocyte growth was not affected by the captopril treatment. The apparent synergism between the demonstrated ACE functionality and the previously reported effects of the oostatic peptide *Neb*-TMOF are discussed in the context of our recent finding that *Neb*-TMOF represents a prime candidate for being the first known in vivo substrate for circulating insect ACE. Arch. Insect Biochem. Physiol. 47:161–167, 2001.

Key words: angiotensin converting enzyme; trypsin modulating oostatic factor; captopril; vitellogenesis

INTRODUCTION

In mammals, the angiotensin converting enzyme or ACE (dipeptidyl carboxypeptidase I, kininase II, EC 3.4.15.1) plays an important function in the renin-angiotensin system (RAS), where it regulates blood pressure and water and salt retention by converting angiotensin I into angiotensin II, a potent vasoconstrictor (Corvol et al., 1995). Bradykinin, a vasodilator (Erdös and Skidgel, 1987) and the haemoregulatory peptide N-acetyl-Ser-Asp-Lys-Pro (Azizi et al., 1996) are the only other in vivo substrates known for ACE. Comparative studies showed that in several insect species, in particular in Diptera, ACE activ-

Abbreviations used: ACE = angiotensin converting enzyme; *Aea*-TMOF, *Aedes aegypti* trypsin modulating oostatic factor; *Neb*-TMOF; *Neobellieria bullata* trypsin modulating oostatic factor.

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ity is present and gene sequences encoding the active domain of the enzyme are highly conserved (Lamango and Isaac, 1994; Cornell et al., 1995; Wijffels et al., 1996; Schoofs et al., 1998). Enzymes of either origin are capable of hydrolysing identical substrates in vitro (Isaac et al., 1998, 2000).

The physiological importance of ACE in insects is still enigmatic. A major problem is that very little is known about the endogenous substrates for insect ACE. Recently, Zhu and co-workers (Zhu et al., 2001) demonstrated that in *Neobellieria bullata* the hexapeptide trypsin modulating oostatic factor or *Neb*-TMOF (Bylemans et al., 1994) is selectively hydrolysed by ACE, which is present in the fly's hemolymph. This hydrolysis is extremely fast (half-life for TMOF is 30 s) and occurs in the hemolymph of different adult ages.

TMOF was originally discovered in the mosquito *Aedes aegypti* by Borovsky et al. (1990). After its release by the ovaries and transportation through the hemolymph, the decapeptidic *Aea*-TMOF terminates protein meal–induced trypsin biosynthesis in the midgut, thereby impairing blood digestion. This results in a lack of free amino acids for further vitellogenin biosynthesis by the fatbody and so its uptake by the ovaries will stop (Borovsky et al., 1993; Borovsky and Mahmood, 1995). The hexapeptidic *Neb*-TMOF, though unrelated in sequence to *Aea*-TMOF, was shown to exert a similar physiological function in the grey fleshfly (Bylemans et al., 1994, 1995). In addition, *Neb*-TMOF inhibits in vitro and in vivo ecdysone biosynthesis by larval ring glands of *N. bullata* (De Loof et al., 1995) and *Aea*-TMOF was shown to modulate ecdysteroid production by the prothoracic glands of *Lymantria dispar* (Gelman and Borovsky, 2000). Since vitellogenin synthesis in this species depends upon 20-OH ecdysone stimulation (Huybrechts and De Loof, 1977, 1981) and the effect of *Neb*-TMOF on trypsin biosynthesis occurs independently of ecdysteroids (Bylemans et al., 1995), *Neb*-TMOF possibly downregulates vitellogenin synthesis by two independent pathways, one through inhibition of trypsin biosynthesis, and the other through ecdysteroid regulation.

By exploiting the here described phenotypic knockout of ACE activity by its selective inhibitor captopril, we used the fleshfly model to collect evidence for *Neb*-TMOF being an in vivo ACE substrate. If this is indeed the case, the captopril treatment is due to affect both trypsin and vitellogenin biosynthesis.

MATERIALS AND METHODS

Insects

N. bullata were reared as described (Huybrechts and De Loof, 1981). Staging of ovarian development was according to Pappas and Fraenkel (1978). Oocyte length was measured with an ocular micrometer under a dissecting microscope, as described by Bylemans et al. (1994). For oral administration of captopril, sugar cubes were impregnated with ethanol containing captopril (0.01 g/ml). After evaporation of the ethanol, the sugar was fed to the flies. For collection of hemolymph, flies were anaesthetised with CO_2, a leg was amputated with a forceps, and hemolymph was collected from the bleeding wound with a glass capillary.

ACE Activity Assay

ACE activity was measured as proposed by Ryan et al. (1977). Briefly, samples (100 µl of total reaction volume) were incubated for 2 h at 37°C in borate buffer (0.05 M Borax, 0.3 M NaCl, 0.2 M $(NH_4)_2SO_4$, pH 7.5) with the ACE substrate p-[^{3}H](benzoylglycylglycylglycine (Sigma, St. Louis, MO) at a final concentration of 1 µM (1). The reaction was stopped by adding 1 ml of 0.1 M HCl. The reaction product (^{3}H-Hippurate) was separated from the unhydrolysed substrate (^{3}H-Hip-Gly-Gly) by extraction with 1 ml of ethyl acetate. Half a milliliter of the organic phase, containing the ^{3}H-Hippurate, was added to 4 ml of scintillation fluid and counted for 2 min in a liquid scintillation counter. Adding captopril (Sigma) to the samples at a final concentration of 10 µM completely inhibited ACE and served as a negative control (2). To determine the total amount of radioactivity added, the total amount of substrate added was also counted (3). ACE activity was expressed as % hydrolysis of the radioactive substrate: % hydrolysis = [cpm(1) – cpm(2)] × 2 × 100/cpm(3). In this calculation, only the enzymatic activity that could be fully inhibited by captopril was defined as ACE activity. The factor × 2 was added because only half of the total volume of the organic phase was added to the scintillation fluid.

Synthetic *Neb*-TMOF

Neb-TMOF:H-Asn-Pro-Thr-Asn-Leu-His-OH was synthesized by solid phase synthesis on a 2-chlorotrityl-chloride resin (Barlos et al., 1991) using Fmoc/t-Bu strategy and purified by RP-HPLC as described by Zhu et al. (2001).

Trypsin Assay

Flies were fed on sugar and water. On day 2 after adult eclosion, experimental flies were fed captopril/ethanol impregnated sugar cubes, whereas control animals were fed ethanol impregnated sugar. On day 4, flies were fed a liver meal and 6 h later, when trypsin activity reached its maximum level (Bylemans et al., 1994), midguts were dissected out of female flies and rinsed with *Neobellieria* Ringer (121.5 mM NaCl, 10 mM KCl, 2.1 mM NaH_2PO_4, 10 mM $NaHCO_3$, 0.7 mM $MgCl_2$, 2.2 mM $CaCl_2$, pH 6.8), to remove the gut contents. Trypsin biosynthesis was measured as described by Bylemans et al. (1994). Briefly, individual guts were homogenised, the homogenate was centrifuged, and the supernatant was collected. An aliquot of the supernatant (0.1 gut equivalent) was incubated with ^{3}H-diisopropylfluorophosphate (DFP) (NEN, specific activity 10 Ci/mmol) for 18 h at 4°C, in the presence of 5 mM tosyl-L-phenylalanine chloromethyl ketone (TPCK), which is a chymotrypsin inhibitor. Control and experimental animals (20 flies each) were statistically analysed using Microstat software (*t*-test for unpaired observations).

Vitellogenin Titers

Flies were fed on sugar and water. On the morning of day 4, sugar containing captopril was presented and 12 h later they received a liver meal. Twelve hours after the liver feeding, hemolymph of 5 females was pooled, diluted 1:5 in sample buffer, and 5 μl was run by SDS-PAGE (Laemmli, 1970). Hemolymph of sugar-fed and liver-fed females (also 12 h after a liver meal) served as controls.

RESULTS

ACE Activity in the Hemolymph

To find out if the regulation of *Neb*-TMOF can be correlated with temporal changes in ACE activity during vitellogenesis, ACE activity in the hemolymph was followed during the vitellogenic cycle. At different time intervals after a liver meal, hemolymph was removed from 5 staged females, pooled and diluted 1/20 in borate buffer and tested for ACE activity, expressed as % hydrolysis of ^{3}H-Hip-Gly-Gly. At the moment of the liver meal on day 4 (time 0), 36 ± 3% hydrolysis was measured. This value at the previtellogenic stage is referred to as 100% ACE activity. Other measurements are expressed relative to this value (Fig. 1). Each point on the graph was an average of 5 independent experiments. Although standard deviations were quite large at some time intervals, it was apparent that a high and constitutive ACE activity can be detected in the fly's hemolymph throughout the entire vitellogenic cycle.

In Vitro Hydrolysis of *Neb*-TMOF by *Neb*-ACE

To confirm the earlier reported HPLC data of the in vitro hydrolysis of *Neb*-TMOF by ACE (Zhu et al., 2001), hemolymph of 5-day-old liver-fed females was pooled and diluted 1/20 in borate buffer. These samples were tested in competitive ACE activity assays in which increasing concentrations of synthetic *Neb*-TMOF were added (Fig. 2). When no *Neb*-TMOF (control) was added (0 μM final [*Neb*-TMOF]), 34% hydrolysis of the radiolabeled substrate was measured. Adding equal amounts of radiolabeled substrate and *Neb*-TMOF in the assay (1 μM final [*Neb*-TMOF]), resulted in 29% hydrolysis and was slightly lower than the control. At concentrations of 100 μM and 1 mM, the hydrolysis of Hip-Gly-Gly was inhibited by 53 and 88%, respectively. As increasing the concentration of *Neb*-TMOF leads to decreasing hydrolysis of the radiolabeled substrate, it is apparent that *Neb*-TMOF successfully competes with Hip-Gly-Gly for ACE binding.

Phenotypic Knockout of ACE Activity by Captopril Feeding

To assure that orally administered captopril can pass the gut epithelium and still exert its ACE inhibitory activity, flies were fed from day 2 on with captopril-saturated sugar. On day 5 (24 h after the liver meal), hemolymph from 5 flies was pooled and diluted 1/20 in borate buffer. Control flies were fed on ethanol-impregnated sugar. ACE activity was measured in 5 independent ex-

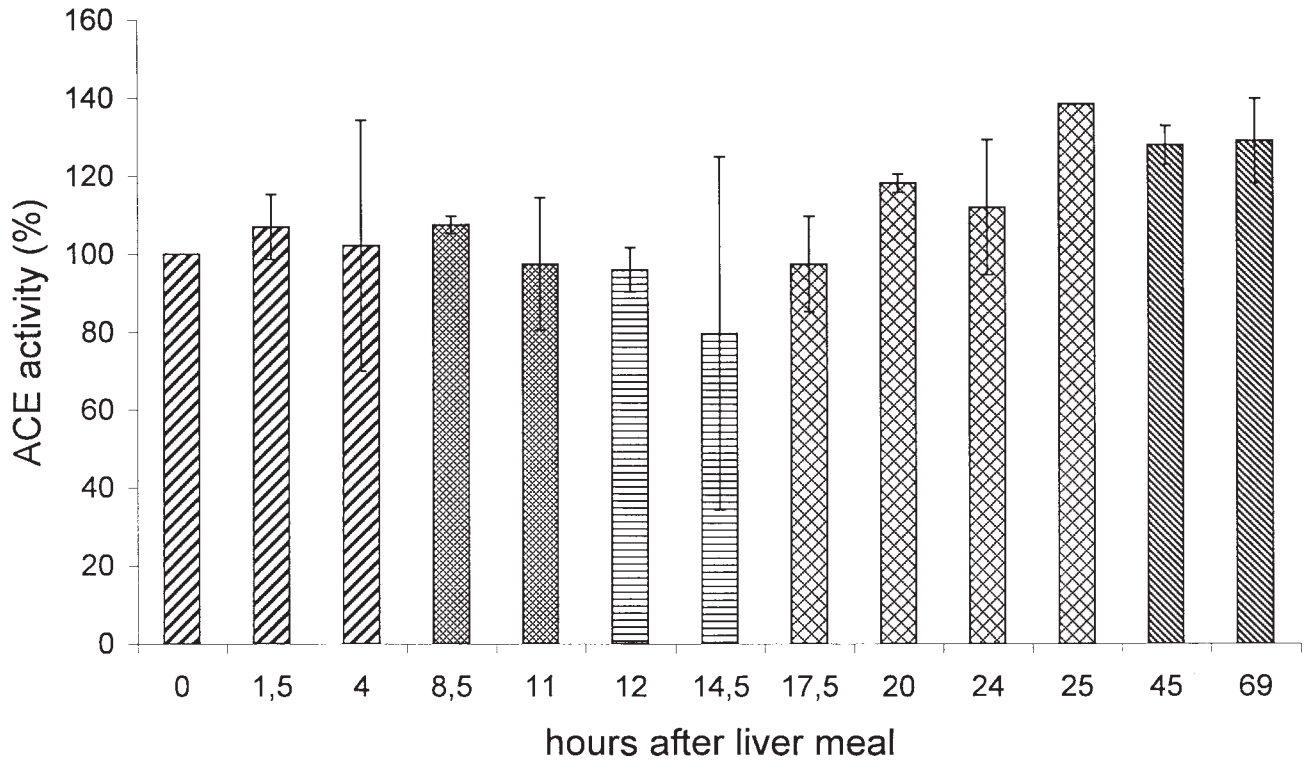

Fig. 1. ACE activity in hemolymph of female *N. bullata* during the vitellogenic cycle. Activity was measured at different time intervals after the liver meal and compared to the reference value before the liver meal (point 0). Values are a mean of 5 independent measurements. Vertical bars show standard deviation. Staging was according to Pappas and Fraenkel (1978); previtellogenic: early vitellogenic: mid vitellogenic: late vitellogenic: fully developed: .

periments and expressed as % hydrolysis of ^{3}H-Hip-Gly-Gly. While in the hemolymph of control animals 35 ± 2% hydrolysis was measured, captopril-treated flies showed a complete lack of ACE activity in their hemolymph. It can be concluded that the way of captopril feeding described here represents a reliable method for selectively shutting down ACE activity.

Trypsin in the Midgut

To find a possible role for ACE in the regulation of trypsin activity in the midgut, control- and captopril-fed females were assayed for trypsin biosynthesis (Fig. 3). Two independent experiments were executed: captopril (1) and (2). Values of control animals were referenced as 100% and experimental conditions were expressed relative to this value. Captopril feeding resulted in an increase of trypsin levels of 36% (1) and 19% (2), respectively, as compared to the control group. *P* values were always ≤ 0.01, indicating that the effect of captopril administration was significant.

Vitellogenin in the Hemolymph

To test for possible regulation of vitellogenin titers by ACE, captopril-fed and control females were compared. A typical SDS-PAGE run is shown in Figure 4. In *Neobellieria*, the three yolk polypeptide bands have a molecular weight of about 51 kDa (Huybrechts and De Loof, 1983). As expected, these bands are practically absent from hemolymph of sugar-fed females (Fig. 4, lanes 2 and 3) and appear only in liver-fed females (Fig. 4, lanes 4 and 5). In hemolymph of liver-fed females that were fed captopril (Fig. 4, lanes 6 and 7), the staining intensity of the yolk polypeptide bands was increased as compared to the controls that were not fed captopril (Fig. 4, lanes 4 and 5). These results indicate that the accumulation of vitellogenin in the hemolymph is due to the captopril treatment. To exclude that this effect was the result of an impaired uptake by the ovaries, oocyte length of control and captopril-fed females was compared (data not

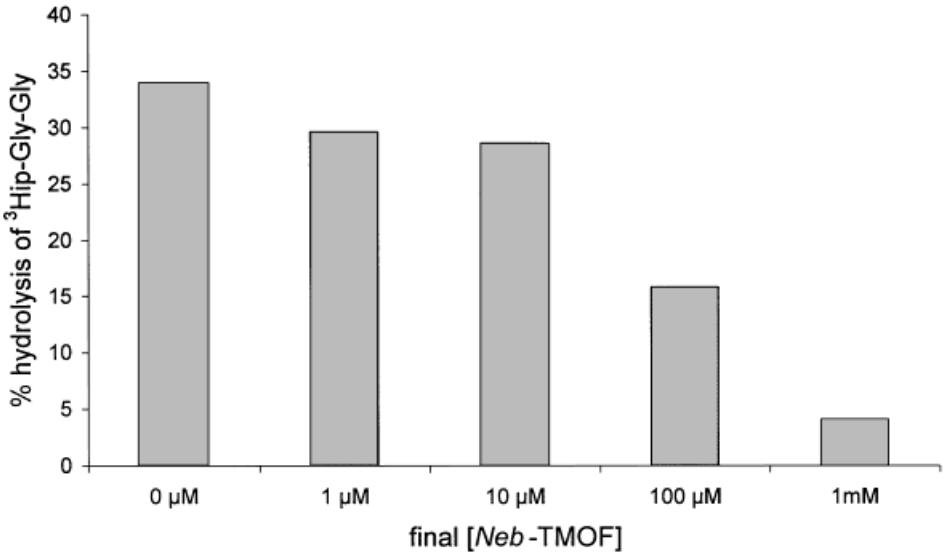

Fig. 2. Competition assay with *Neb*-TMOF. Increasing concentrations of synthetic *Neb*-TMOF were added in an ACE assay with hemolymph of females.

shown). No significant difference between ovaries of control and captopril treated animals was found, indicating that oocyte growth and vitellogenin uptake were not affected by the captopril treatment.

DISCUSSION

In this paper, we present evidence for an in vivo connection between *Neb*-ACE and *Neb*-TMOF in the grey fleshfly *Neobellieria bullata*. Recent in vitro studies by Zhu et al. (2001) showed that the degradation of synthetic *Neb*-TMOF was due to an ACE-like activity that is present in the hemolymph. A competition assay showed that TMOF can compete for ACE binding with Hip-Gly-Gly, a well-established synthetic ACE substrate (Ryan et al., 1977). The fact that *Neb*-TMOF can effectively compete with Hip-Gly-Gly (Fig. 2) strongly indicates that *Neb*-TMOF hydrolysis by ACE is the result of a specific physiological phenomenon. Hence, this hydrolysis will occur whenever *Neb*-TMOF is exposed to *Neb*-ACE in vivo.

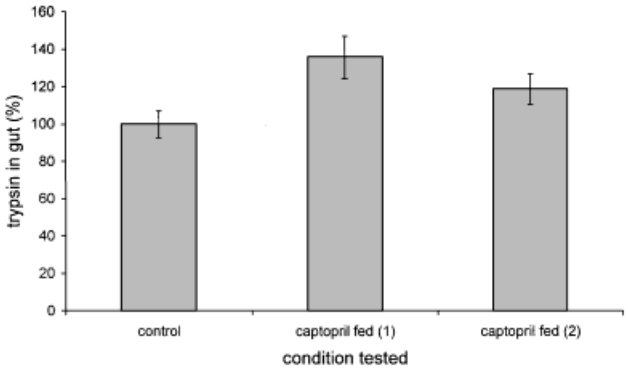

Fig. 3. Trypsin peaks in the midgut of captopril-fed females. (1) and (2) depict 2 independent experiments. Statistical analysis by Microstat software (*t*-test for unpaired observations) showed *P* values of, respectively, 0.001 (1) and 0.01 (2). Vertical bars show standard error.

Since *Neb*-TMOF is essential in terminating vitellogenesis (Bylemans et al., 1994, 1995), a drastic reduction in the circulating ACE activity is being expected when TMOF is released by the ovary, if one assumes that only the full-length hexameric TMOF is biologically active. Such a steep drop in ACE activity was, however, not detected (Fig. 1). Thus, it is possible that the TMOF hydrolysation products exert the real in vivo oostatic activity. In this light, further initiative should be taken in examining the oostatic potencies of these TMOF hydrolysation products.

To understand the in vivo relationship between *Neb*-TMOF and *Neb*-ACE, a phenotypic knockout of circulating ACE activity was realised by feeding the flies with captopril, a well-known ACE inhibitor that is used as an oral drug for the treatment of hypertension in humans. In agreement with data obtained in, e.g., the rat intestine (Zhou and Li Wan Po, 1994), we showed that orally administered captopril can also pass

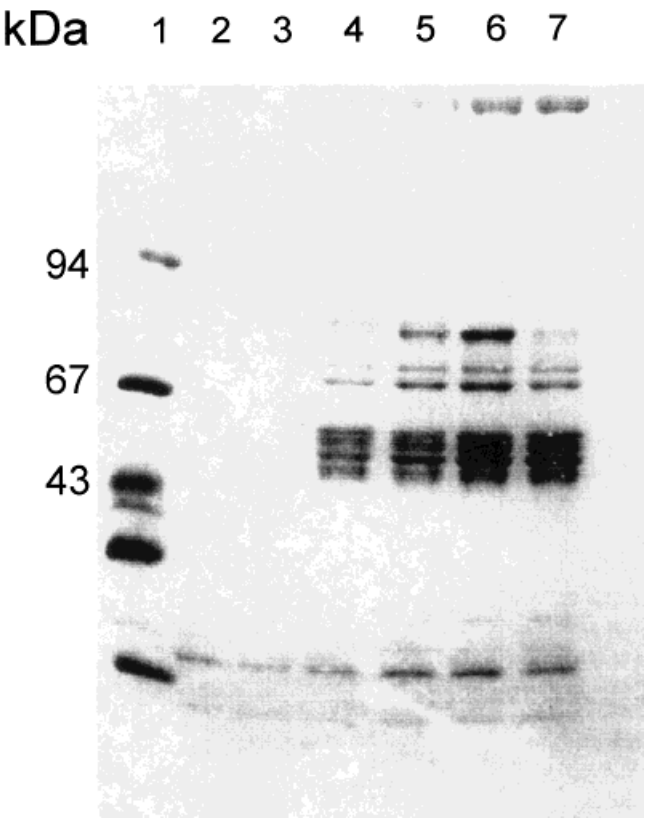

Fig. 4. SDS-PAGE of hemolymph of captopril-fed females. Each condition was done in duplicate. **Lane 1**, the molecular weight marker. **Lanes 2,3:** Sugar-fed females. **Lanes 4,5:** Liver-fed females. **Lanes 6,7:** Captopril treated liver-fed females. The 3 yolk polypeptides appear as bands at ± 51 kDa.

the gut epithelium of *N. bullata* and still exert its ACE inhibiting activity in circulation. If, indeed, *Neb*-TMOF is an in vivo substrate for *Neb*-ACE, the captopril treatment should have a direct effect on trypsin activity and vitellogenin concentration, since both are strongly regulated by TMOF. Indeed, captopril treatment resulted in an increase of the liver meal–induced trypsin peak in the midgut (Fig. 3) and it also enhanced the vitellogenin titer in the hemolymph (Fig. 4).

The captopril treatment effectively reversed the effect of TMOF on trypsin and vitellogenin biosynthesis. The enhancement of vitellogenins in the hemolymph was not the result of impaired sequestration by the ovaries (data not shown) but can be explained by an increase in trypsin levels, which makes more AA's available for the production of more vitellogenins by the fatbody. However, earlier data obtained by Huybrechts and De Loof (1977, 1981) clearly demonstrated that an increase in vitellogenin synthesis is observed in liver-fed female *N. bullata* by simple 20 OH-ecdysone injection. Since trypsin biosynthesis in the gut of *N. bullata* is not controlled by 20 OH-ecdysone (Bylemans et al., 1995), it is possible that captopril directly stimulates ecdysteroid production, with an increase in vitellogenin synthesis as a result. In addition, since *Neb*-TMOF has ecdysiostatic properties (Hua et al.,1994a,b; De Loof et al., 1995), the most likely explanation for the increase in vitellogenin titers in the hemolymph due to captopril feeding, is that captopril increases ecdysteriod titers by inhibiting the breakdown of *Neb*-TMOF. So *Neb*-TMOF may have an effect on ecdysteroid biosynthesis whereas breakdown product(s) of *Neb*-TMOF may have biological activity on trypsin biosynthesis. This means that captopril could exert its effect on vitellogenin synthesis in two independent pathways, one by increasing trypsin activity and the other by increasing ecdysteroid titers. Based on our experimental data, it is logical to assume that both pathways probably involve *Neb*-TMOF and its hydrolysis products.

Our results point towards a synergistic relationship between *Neb*-TMOF and *Neb*-ACE. This necessitates a rethinking of the hypothesis that *Neb*-ACE would be a donwregulator of *Neb*-TMOF activity. This view originated from the assumption that only the intact hexapeptidic *Neb*-TMOF is biologically active. We think our results favour an alternative view that states that ACE indeed cleaves *Neb*-TMOF but that, as is the case in mammals where ACE hydrolyses the inactive angiotensin I into the active angiotensin II, the biological activity of TMOF is rather due to a cleavage product. Hence, we think that *Neb*-ACE is an activator of *Neb*-TMOF.

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