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*Full Length Research Paper*

# Comparative activity of three inhibitors of the angiotensin converting enzyme on growth, development and ecdysteroid contents of the mediterranean flour moth, *Ephestia kuehniella* Zeller

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**Three angiotensin converting enzyme (ACE) inhibitors, enalapril, lisinopril and captopril were tested *in vivo* by topical application on growth, development and whole body ecdysteroids in *Ephestia kuehniella* Zeller (Lepidoptera:Pyralidae), an important pest in stored products worldwide. The compounds were diluted in acetone and applied topically (10 µg/2 µl) on newly molted pupae. Results show that treatments delayed the pupal development and caused a large decrease in successful adult formation. Treatments had no significant effect on the pupal body weight at 1 and 3 days. Only enalapril at day 5 increases significantly the weight. The enzyme-immunoassay measurements of ecdysteroids in whole body extracts revealed that only lisinopril reduced significantly the hormonal contents at day 3.**

**Key words:** *Ephestia kuehniella*, development, growth, ecdysteroids, angiotensin converting enzyme, captopril, enalapril, lisinopril.

## INTRODUCTION

Angiotensin-converting enzyme (ACE) is a zinc metallopeptidase functioning primarily as a dipeptidyl-carboxypeptide (Macours et al., 2004). In mammals, ACE is involved in the homeostatic regulation of blood pressure and electrolyte balance and is strongly linked with a number of cardiovascular and renal diseases (Bernstein et al., 2005; Shen et al., 2008b). Moreover, it is well known that this enzyme is involved in the renin-angiotensin system through the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, and in the metabolic inactivation of bradykinin a vasodilatory peptide (Corvol et al., 2004). To date, synthetic ACE inhibitors like captopril, lisinopril and enalapril are widely used as antihypertensive drugs (Cushman et al., 1977, 1979).

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More recently, insect cell extracts were found to exhibit *in vivo* antihypertensive activity without an extra digestion requirement (Staljanssens et al., 2011).

High levels of ACE activity are found in the haemolymph and in reproductive tissues (ovaries, testes and accessory gland) of both male and female insects (Isaac et al*.,* 2007). Since the discovery of an ACE in *Musca domestica*, ACE- like activity has been detected in several insect species from different orders (Wijffels et al., 1996; Williams et al., 1996; Fernandez et al., 2001; Lemeire et al., 2007). Recently, in *Drosophila melanogaster* crystal structures, an ACE homologue, complex with a natural product-phosphonotripeptide K-26 (Akif et al., 2010) or with novel inhibitors and hypertensive drugs (Akif et al., 2011) were presented, and loss of ACE-related peptidase was reported to disrupt night-time sleep in adult (Carhan et al., 2011). Loeb et al. (1998) show that bovine ACE and bovine angiotensin II

elicit synthesis of ecdysteroid by testes and inhibit the action of testis ecdysiotropin in *Lymantria dispar*. The inhibition in ACE activity can interfere with the endocrine system and causes negative effects on growth, development and reproduction (Isaac et al*.,* 2007). Several ACE inhibitors were tested. Thus, Lemeire et al. (2008) reported that captopril and enalapril applied by injection into newly molted fifth instar larvae of *Spodoptera littoralis* stopped larval feeding and decreased their weight. Similarly, Isaac et al. (2007) found that lisinopril and captopril (2 µM) injected into *Manduca sexta* slowed larval growth rate over the first 3 days after administration.

Captopril treatment enhances both trypsin and vitellogenin titers in *Neobellieria bullata* without any effect on oocyte growth (Vandingenen et al., 2001) while in *Spodoptera littoralis*, it down regulates oviposition by two independent pathways, one through ecdysteroid biosynthesis regulation, and the other though regulation of trypsin activity (Vercruysse et al., 2004). In *Tenebrio molitor*, we have shown that this compound reduced the morphometric measurements of ovary and no significant effect was observed on both thickness and fine structure of chorion (Soltani-Mazouni et al., 2007).

In recent studies, *Ephestia kuehniella* Zeller (Lepidoptera:Pyralidae) has been used as a model target lepidopteran to investigate normal development (El Ouar et al., 2010; Yezli-Touiker and Soltani-Mazouni, 2011) and to test several insect growth regulators (Hami et al*.,* 2005; Soltani-Mazouni et al*.,* 2012). As a consequence, these data provide a strong basis to better understand the mode of action of these xenobiotics such as IGRs and drugs like ACE inhibitors on development and reproduction of this pest. In order to complete previous results, in a first series of experiments we investigated the effect of three ACE inhibitors (lisinopril, captopril and enalapril) applied topically on growth and development, and in a second series of experiments we determined the free ecdysteroid contents in whole body of pupae under laboratory conditions in *E. kuehniella* an important pest in stored products worldwide. The latter results should help in better understanding the mode of action of these ACE inhibitors.

## MATERIALS AND METHODS

### Experimental animals

*Ephestia kuehniella* was reared on wheat flour at 27°C and 80% relative humidity in almost continuous darkness as reported by Soltani-Mazouni et al. (2012). Pupae were collected from a stock colony and classified according to their age from pupal ecdysis.

### Chemicals and treatments

The ACE inhibitors were captopril, lisinopril and enalapril (courtesy of Pr. G. Smagghe, Ghent University, Belgium). Captopril (D-3-mercapto-2-methyl-propionyl-L- proline), enalapril-maleate salt (N a-[(S)-1-ethox-ycarbonyl- ethox-ycarbonyl-3-phenylpropyl]-L-analyl-L-proline maleate salt) were purchased from Sigma (Bornem, Belgium). Lisinopril ($N^a$-[(S)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline) was provided by Merck and Co. Inc. (Whitehouse Station, NJ). Newly ecdysed (<8 h old) *E. kuehniella* pupae without cocoons were topically treated with each ACE inhibitor (10 µg per female) on the abdominal sternites with the use of a Gilson automatic micropipette. The compounds were diluted in acetone, allowing a better diffusion of the active ingredient throughout the cuticle. In the controls, pupae were dosed with 2 µl of acetone. Three groups of 20 pupae per compound were used. From the start of the experiment, the pupae were examined daily for aberrations and survival until adult emergence.

### Growth parameters

As aforementioned, newly moulted (<8 h) *E. kuehniella* pupae were treated topically with each drug dosed at 10 µg per female. The body weight of pupae was measured at different ages (1, 3 and 5 days) during the pupal development in control and treated series. The experiment was down with 20 individuals per series. The duration of pupal development was also determined for the different series using 15 pupae per series. To determine the comparative effectiveness of treatments on the inhibition of adult ecdysis, the percentage of successful adult formation was made.

### Enzyme immunoassay of free ecdysteroids in whole body

We used in this study whole body extracts because difficulties were encountered in the collection of haemolymph samples from pupae. Thus, female pupae were sampled from control and treated series at different times (1, 3 and 5 days) during the pupal development. Each pupa was individually extracted with 300 µl of methanol by sonication, and after centrifugation (5000 g, 10 min), the supernatants were taken and evaporated. The extracts were resuspended in phosphate buffer (0.1 M, pH 7.4). Each sample was analyzed in duplicate by enzyme immunoassay (EIA) as previously reported by Soltani-Mazouni et al. (2012) using a conjugate of 20 E coupled to peroxidase as the enzymatic tracer, tetramethyl benzidine as the colour reagent and a rabbit B polyclonal antibody. The antibody and the tracer were supplied by Dr. J. P. Delbecque (University of Bordeaux I, France). The number of repeats was four per treatment and each sample was analyzed in duplicate as previously reported (Soltani et al., 2002; Aribi et al., 2006). Data are expressed as pg 20-hydroxyecdysone equivalents per milligram of body weight.

### Statistical analysis

Results were presented as the mean ± standard deviation (SD). Comparisons were made between control and experimental groups. The ANOVA and the student t-test were applied. When the analysis of variance was significant ($p< 0.05$), mean values obtained were separated by Tukey test. All data were statistically analyzed by the MINITAB Software (version 13.31, PA State College, USA) and the significant level was at $p< 0.05$. The number of insects tested in each experiment is given in the results.

## RESULTS

### Effects of ACE inhibitors on pupal growth and development

ACE inhibitors (captopril, lisinopril and enalapril; 10

**Table 1.** Effect of converting enzyme inhibitors (10 µg/2 µl acetone) on body weight (mg) during the pupal stage of *Ephestia kuehniella*.

| Age (Day) | Treatment | | | |
|---|---|---|---|---|
| | Control | Enalapril | Captopril | Lisinopril |
| 1 | $15.12 \pm 2.90^{aA}$ | $18.62 \pm 0.62^{aA}$ | $17.40 \pm 1.48^{aA}$ | $17.42 \pm 1.56^{aAB}$ |
| 3 | $16.01 \pm 1.74^{aA}$ | $16.15 \pm 2.87^{aA}$ | $17.80 \pm 0.92^{aA}$ | $19.40 \pm 0.93^{aA}$ |
| 5 | $15.3 \pm 1.03^{aA}$ | $17.87 \pm 1.48^{bA}$ | $13.63 \pm 0.94^{acB}$ | $15.72 \pm 1.03^{adB}$ |

Values are expressed as mean ± SD (n = 20 individuals per series). For each age, mean values followed by different letters in minuscule are significantly different ($p < 0.05$), while for each treatment, and mean values affected by different letters in majuscule are significantly different ($p < 0.05$).

**Table 2.** Activity of converting enzyme inhibitors applied topically (10 µg/2µl acetone) on newly ecdysed pupae on the duration (days) of the pupal development of *E. kuehniella*.

| Treatment | Duration of pupal stage (Day) |
|---|---|
| Control | $10.00 \pm 1.09^{a}$ |
| Enalapril | $11.50 \pm 0.84^{b}$ |
| Captopril | $10.30 \pm 1.41^{a}$ |
| Lisinopril | $11.75 \pm 1.58^{c}$ |

Mean values followed by different letters are significantly different at $p<0.05$ (mean ± SD; n = 15).

µg/insect) were applied topically to newly ecdysed pupae. ANOVA indicates significant effects of treatment ($F_{3, 36}$ = 4.81; $p< 0.01$); age ($F_{2, 36}$ = 5.56; $p< 0.01$) and interaction treatment-age ($F_{6, 36}$ = 3.67; $p< 0.01$), respectively. In the control and treated series, the body weight do not change significantly ($p>0.05$) during the pupal development except for captopril and lisinopril (Table 1). Indeed, at day 5 following treatment we note a significant decrease in pupal weight in captopril and lisinopril series. In addition, there was no significant difference between all treatments at 1 and 3 days. However, at day 5, only enalapril increases significantly ($p<0.01$) the weight as compared with control pupae (Table 1). The duration of pupal development in the control was 10.00±1.09 days. After the application of captopril it was 9.52 ± 0.91 days, and did not differ from the control group (Table 2). Insects treated with enalapril and lisinopril present a significant increase in the duration of pupal development. It goes from 10.00±1.09 days in control to 11.5 ± 0.84 days ($p<0.01$) after treatment with enalapril and 11.75 ± 1.58 days with lisinopril ($p<0.01$). In addition to delayed pupal development, the percentage of successful adult formation was significantly reduced from 95.8±5.8% in the controls to 22.3 ± 9.2% with enalapril. Pupae treated with lisinopril and captopril was inhibed by 31.0 ± 7.8 and 42.7 ± 33.3%, respectively.

### Effects of ACE inhibitors on ecdysteroids

The ecdysteroid contents in the body were determined by EIA during pupal development in the control and treated pupae. Data are presented in Figure 1. The normal evolution of ecdysteroid contents during the pupal development presented a peak at day 3. Results show that the three ACE inhibitors had no effect on the value of peak compared to the controls. A significant difference ($p<0.05$) was observed only at day 3 between lisinopril treated and control series. ANOVA showed significant effects of treatments ($F_{3, 26}$ = 7, 13; $p<0.001$), in age ($F_{2, 26}$ = 21, 13; $p<0.0001$) and interaction treatment-age ($F_{6, 26}$ = 3, 013; $p<0.05$) on the rate of ecdysteroids during the pupal stage. A ranking of the various drugs used according to their effects on the content of ecdysteroids was made by Tukey test. The results reveal three groups of molecules: the first contain the control pupae, the second group contains the insects treated with enalapril and captopril, the third group is represented by the lisinopril.

## DISCUSSION

This study evaluates the effect of ACE inhibitors applied topically on growth and development of *E. kuehniella* an important pest in stored products worldwide. In addition, we report the effect of ACE inhibitors on ecdysteroid amounts in whole body of pupae. Changes in body weight during the pupal development following treatment with the tested ACE inhibitors show a significant decrease at day 5 only in captopril and lisinopril treated pupae. When comparison was made between all series for each time during pupal development, only enalapril was found to increase the body weight at day 5. Seinsche et al. (2000) tested the effect of ACE inhibitors in the development of *Heliothis virescens* larvae and found that larvae injected with captopril, enalapril maleate and lisinopril, three inhibitors of ACE, grew normally. On the other hand, the combined application of ACE-inhibitors and helicokinins caused a reduction in weight gain and

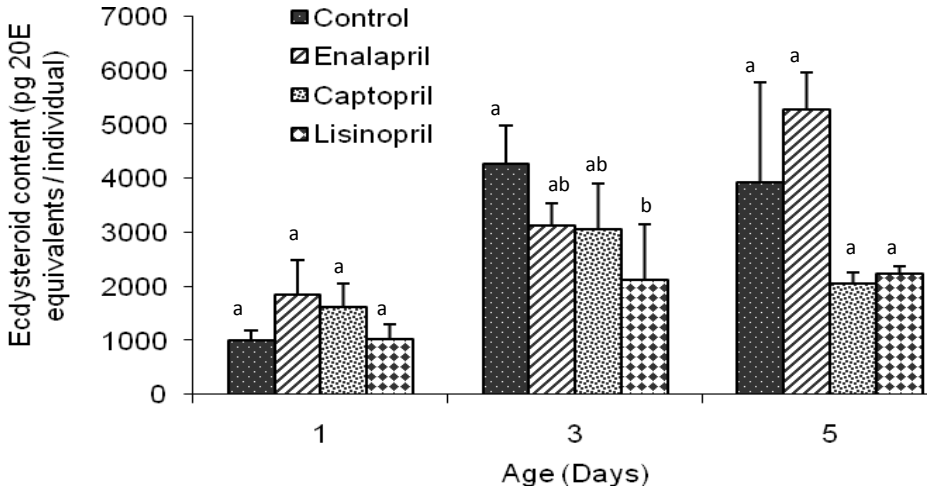


**Figure 1.** Effect of angiotensin converting enzyme inhibitors applied topically (10 µg/2 µl acetone) on newly ecdysed pupae on ecdysteroid contents (pg 20 E equivalent/individual) during the pupal stage of *E. Kuehniella*. Each value is the mean ± SD established on 4 different individuals per age each analyzed in duplicate by EIA. For each age, mean values affected by different letters in minuscule are significantly different ($p<0.05$).

higher mortality rates in last instar *H. virescens* larvae.

Vercruysse et al. (2004) reported no effect on larval growth when newly molted L1 larvae were fed captopril *ad libitum*. These authors applied captopril as a thin layer to the diet surface by distributing 75 ml of a 10 mg/ml captopril concentration in methanol and let the solvent evaporate. Isaac et al. (2007) found that ACE inhibitors (2 mmol) injected into *Manduca sexta* larvae ($4^{th}$ and $5^{th}$ instars) slowed down larval growth. Lisinopril and captopril reduced growth during 3 days post-injection. The effect of lisinopril, however, was irreversible for 8 days following treatment. According to Seinsche et al. (2000), ACE inhibitors could prohibit the hydrolysis of peptide causing weight loss probably by water loss. Kinin family peptides of insects from Orthoptera, Dictyoptera, Lepidoptera and Diptera are in general diuretic, but with different levels of efficacy (Gäde and Goldsworthy, 2003). Thus, the weight loss is probably due to a kinin-like peptide remaining active after application of ACE inhibitors, and consequently caused fluid loss and reduction in haemolymph volume.

In this study, enalapril and lisinopril applied topically on newly ecdysed pupae delayed the pupal development. The captopril did not affect duration of pupal stage (days). This is in consistent with the results of Lemiere et al. (2008), larvae fed enalapril were slow to develop but molted normally and the larval stage from L1 to pre-pupa took 23 ± 0.5 days. The duration of the larval period after feeding captopril and lisinopril was 17.8 ± 0.4 and 17.1 ± 0.2 days, respectively and did not differ from the control group 17.1 ± 0.2 days. The large decrease in successful adult formation of *E. kuehniella* after topical treatment of new pupae with ACE inhibitors shows that ACE has a role in metamorphosis of holometabolous insects. Our results agree with data from Vercruysse et al. (2004) who tested the effect of topical treatment of captopril on successful adult formation of *S. littoralis* pupae. Siviter et al. (2002) previously suggested such a role for ACE based on their findings that larval-pupal transition of *D. melanogaster* was accompanied by a 3-fold increase in ACE-activity. This increase was attributed to the strong induction of Ance expression in the imaginal cells by 20 E.

Houard et al. (1998) reported a 2-fold increase in ACE-activity during the early stages of *D. melanogaster* metamorphosis. Activity peaked between pupal stages P6 and P8, and 20 E increased the expression of an ACE-like gene in imaginal wing disc cells of *Bombix mori* (Quan et al., 2001). Ekbote et al. (2003b) also reported that lepidopteran insects display an increase in ACE activity during metamorphosis. ACE activity increased approximately 4-fold during the last larval instar and early pupal stages of *Lacanobia oleracea*. It is possible that during metamorphosis, ACE contributes to the generation of biologically active peptides and/or signal termination of already active peptides.

The enzyme immunoassay measurements show that lisinopril induced a significant decrease at 3 days on the content of ecdysteroids (pg 20 E equivalents/individual). Vercruysse et al. (2004) reported that the captopril treatment exerted an inhibitory effect on ecdysteroid levels in female but not in male adults of *S. littoralis.* ACE is not only thought to have a role during metamorphosis. Several studies suggest a physiological role for the enzyme in insect reproduction. When male *Anopheles stephensi* mosquitoes were treated with ACE-inhibitors

and allowed to mate with blood fed females, a drastic reduction in fecundity was observed (Isaac et al., 1999). In another study in which *A. stephensi* females were fed with a blood meal containing either captopril or lisinopril, the presence of the ACE-inhibitors did not affect feeding and mating behavior, but reduced fecundity in a dose-dependent manner (Ekbote et al., 2003a).

## Conclusion

The treatment with ACE inhibitors was found to disturb growth, development and ecdysteroid production. They caused a delay of development and a decrease in ecdysteroid amounts in *E. kuehniella* pupae. The effects seem more marked for lisinopril (delay in pupal development and lower ecdysteroid amounts) as compared with the two other compounds. This difference is probably related to their metabolism during the pupal stage. Further experiments are needed to better understand the mechanisms of action of these ACE inhibitors in insects. Thus, with *in vitro* experiments using ovaries and integument we can investigate whether these ACE inhibitors act directly on the ecdysteroidogenesis tissues or indirectly via the production of peptides. However, this first requires developing of culture conditions, including the culture medium, of tissues or organs from the insect model used.

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