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Bioorganic Chemistry 32 (2004) 263–273
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Activity and mechanism of action of insect oostatic peptides in flesh fly

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Received 13 January 2004

Abstract

The relationship between structure and activity of insect oostatic decapeptide (Aed-TMOF) analogues in flesh fly was analyzed. The highest oostatic activity was exhibited by the pentapetide and tetrapeptide analogues, H–Tyr–Asp–Pro–Ala–Pro–OH and H–Tyr–Asp–Pro–Ala–OH, respectively. The tetrapeptide, either native or tritiated, was used to study its metabolism in the ovaries and hemolymph and to detect putative binding sites in the flesh fly ovaries and head. A high metabolism of the tetrapeptide with a half-life in the hemolymph and ovaries less than 1 h was determined. The initial limiting step in the degradation is tyrosine[1] cleavage. Other degradation products were detected only transiently in low quantities. Using tritiated tetrapeptide, we found that only very low specific binding was detected in the homogenates of ovaries and in the rough membrane preparation in the presence and absence of protease inhibitors.

Keywords: Insect oostatic peptide; *Neobellieria bullata*; Oostatic activity; Binding experiments; Degradation

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doi:10.1016/j.bioorg.2004.05.003

1. Introduction

During the last two decades, the number of studies on insect peptides and their analogues has significantly increased [1,2]. Special attention has been paid to the decapeptide H–Tyr–Asp–Pro–Ala–Pro–Pro–Pro–Pro–Pro–Pro–OH (10P) isolated from the mosquito *Aedes aegypti* and described by Borovsky et al. [3–5] as a trypsin modulating oostatic factor (Aed-TMOF). Aed-TMOF is released from the ovary 20–30 h after the blood meal and stops trypsin biosynthesis in the midgut of female mosquito. If it is injected immediately after the blood meal, it blocks the biosynthesis of trypsin, prevents blood degradation and stops oocyte growth, which is dependent on the degradation of the blood meal. Similarly, a hexapeptide isolated from flesh fly *Neobellieria bullata* was characterized as trypsin modulating oostatic factor (Neb-TMOF) and its proteolytic breakdown in the hemolymph of different insects and its gut epithelial transport was studied [6].

Our investigation [7–12] of the decapeptide 10P showed an effect on ovarian development of *N. bullata* as well. It caused a decrease in hatching by alteration of the development of the egg chamber. Synthesis of C-terminal truncated analogues showed in preliminary experiments that the penta (5P)—or even tetrapeptide (4P) sequences exhibited a high deterioration effect on egg development. This finding is in contrast to the reduced activity of the truncated peptides on trypsin biosynthesis [4]. The alternative way for expression of the effect on oocytes was suggested.

Due to these discrepancies, we performed detailed analysis of the oostatic activity of a series of truncated analogues of the oostatic decapeptide (Scheme 1) and tried to elucidate their mechanism of action. For the latter studies we used the peptide 4P (H–Tyr–Asp–Pro–Ala–OH) and LC/MS method or 4P tritiated in position 3 ([^{3}H]4P) and radio-HPLC, respectively. The tritiated peptide was also used for binding studies using flesh fly tissues. The peptide 4P was chosen for these studies because, besides

Structure of peptides used and their abbreviations

H-Tyr-Asp-Pro-Ala-Pro$_n$-OH

n=6	decapeptide		10P
n=5	nonapeptide		9P
n=4	octapeptide		8P
n=3	heptapeptide		7P
n=2	hexapeptide		6P
n=1	pentapeptide		5P
n=0	tetrapeptide		4P
	tripeptide	**H-Tyr-Asp-Pro-OH**	3P

Scheme 1. Structure of peptides used and their abbreviations.

the peptide 5P, it represents the most active analogue and its degradation represents the simplest model.

2. Materials and methods

2.1. Insect

Female *N. bullata*, Diptera (approximate weight 90 mg), raised at 25 °C, with 12 h light/dark cycle, and 60% RH, were used in the experiments. Larvae were raised on beef liver covered with sawdust (pupariation medium). Pupae were separated from the medium and placed into nylon cages before emergence. Flies were fed sugar and water for 3 days and day 4 on flies were fed beef liver that later served as an oviposition medium.

Peptides were injected in Ringer solution (10 nmol in 5 μl/female) into the upper thorax of Et_2O-anesthesized 24–48 h old flies. After injection, females were placed into cages with untreated males and their ovaries were dissected 2, 4, 8, and 16 days later and examined for morphological and histological changes. Hatchability of eggs in the uterus and appearance of the eggs were evaluated in the first and second gonotrophic cycles. Each experimental group consisted of 50 flies.

2.2. Morphological assessment of the status of ovaries

Dissected ovaries were spread on a microscopic slide in physiological solution and microscopically examined for yolk deposition and oocyte growth. For further evaluation, oocyte development was scored as 1–5; with 1 being no yolk in the oocyte, 2, 3, and 4 being oocyte with yolk filling up a quarter of the egg chamber, half of the egg chamber and 3/4 of the egg chamber, respectively. Stage 5 indicates grown-up eggs ready to be deposited into the uterus. The thickness of the follicular cell layer and density of nutritive cell content were also recorded.

2.3. Hatchability assessment

Since *N. bullata* is partly a viviparous species, the eggs after being fertilized on the way to uterus from the spermathecae remain during whole embryogenesis in the uterus and when the larva is ready to penetrate the chorion the eggs are deposited. This enabled us to examine the uterus content before egg deposition and express the hatchability as the number of developed larvae in the uterus. Segmented larvae were counted. Untreated flies develop in the uterus 98–110 eggs, in which the segmented larvae are 95–98%. This corresponds to hatchability of 95–98%.

2.4. Histological approach, assessment of the changes

Selected ovaries, in which visible changes in the morphology of the egg chambers were detected, were fixed in Bouin's solution, embedded in Paraplast (Sigma–Aldrich

Table 1
Effects of oostatic peptides on hatchability and appearance of eggs in the varioles 8th day (end of the first ovarian gonotrophic cycle) and 16th day (end of the second gonotrophic ovarian cycle) after eclosion

Peptide (10 nmol/ female)	Number of eggs/female		Hatchability in %		Morphology[a]		Histology[b]	
	I cycle	II cycle	I cycle	II cycle	I cycle	II cycle	I cycle	II cycle
Control	110	98	95	95	0	0	0	0 (0)
3P	98	40	90	10	0	+	0+	+ (80)
4P	100	20	70	20	+	+++	0+	++ (50)
5P	95	30	80	20	+	+++	0+	++ (60)
6P	105–115	50	80	80	0	+	0+	+ (30)
7P	115	40	80	50	0	+	0+	+ (30)
8P	105	60	80	70	0	+	0+	+ (20)
9P	115	70	80	70	0	0	0+	+ (1)
10P	120	90	93	95	0	+	0	+ (5)

[a] A zero indicates no change. A "+" indicates some ovarioles resorbed (10–20%). A "+++" indicates massive resorption of ovarioles (>70%).

[b] A zero indicates no changes. A "+" indicates division of nuclei of follicular cells. A "++" indicates formation of multilayer epithelium and invaginations. The % of eggs with changes is indicated in parentheses. The 0+ indicates that the developed eggs have irregular shapes.

s.r.o. Czech Republic) and 5 μm sections were cut and stained with Mallory's or Mayer's hematoxyline. The sections were checked for changes in the follicular epithelium and the condition of its nuclei, yolk deposition, and its structure in the oocyte and appearance of nutritive cells and their nuclei. These changes and their frequency were correlated with intervals after each peptide application. The severity of changes was assessed as well as the percent of pathological changes in egg chambers of the ovariole (Table 1).

2.5. Peptide synthesis

Peptides used in this study were synthesized using solid phase methodology as described elsewhere [7,8]. The tetrapeptide having in position 3 dehydroproline was prepared using a polymer with a 2-chlorotritylchloride linker [12]. The synthesized peptides were purified by preparative HPLC and characterized using amino acid analysis, mass spectrometry, and analytical HPLC [7,8,12].

2.6. Preparation of tritiated radioligand

Tritiated tetrapeptide H–Tyr–Asp–[^{3}H]Pro–Ala–OH ([^{3}H]4P) was prepared by catalytic tritiation of the analogue containing dehydroproline and was purified by HPLC. The specific radioactivity of the radioactive labeled peptide was 49.2 Ci/mmol [12].

2.7. LC/MS analysis

Peptides were resolved on Cromasil C18 HPLC column (250 × 2) using an isocratic mode with a mobile phase of MeCN–water (20:80) containing 0.04% TFA on a

LCQ apparatus with positive electrospray 4.2 kV for 20 min. A full scan in the range of 180–480 Da/0.5 s was performed. Samples were thawed, mixed with four volumes of mobile phase, centrifuged for 3 min and 2 μl injected onto the column. The column was calibrated using 4P, the tripeptides Tyr–Asp–Pro and Asp–Pro–Ala, and the dipeptide Asp–Pro.

2.8. Radio-HPLC

All HPLC measurements were performed using the Waters liquid chromatograph (Waters Milford, MA) with an on-line radioisotope flow through detection system and high efficiency mixer from Beckman Instruments (Irvine, CA) and injector model 7125 Rheodyne, Cotati, CA. After passing through the UV detector, the column eluate was mixed continuously with the liquid scintillator cocktail Ready Safe (Beckman) in ratio 1:2.5. The resulting mixture was passed through a 1 ml scintillation cell. The threshold detection was set at 0.03% and the residence time was 0.417 min. All analyses were performed on a stainless steel analytical column (250 × 4 mm) LiChroCart (Merck), packed 5 μm LiChrospher WP-300 protected by a guard column packed with LiChrospher 100 (4 × 4 mm). Acetonitrile containing 0.05% TFA (A) and doubly distilled water containing 0.035% TFA (B) were used as mobile phases as follows: 0–30% A in B, 12 min, 30–100% A in B 5 min. After thawing, samples were centrifuged for 5 min in an Eppendorf centrifuge and an aliquot (4 μl) of the supernatant was subjected to analysis using a flow rate of 0.8 ml/min and a continuous degassing with helium.

2.9. Incubation

Degradation of the peptides was followed after incubation of the peptides (10 nmol in 100 μl physiological solution) for different time intervals (1–90 min) at room temperature with hemolymph (2.5 μl) or ovarian homogenate in the absence or in the presence of protease inhibitor "CompleteMini" from Roche. The hemolymph and the ovaries were collected from 4-day-old females shortly before the experiment. Hemolymph was harvested from the fly leg using capillary and collected into small volume of physiological solution. Ovaries were removed under ether anesthesia from the fly, washed in physiological solution, and homogenized under such conditions that 10 μl of the homogenate corresponded to one ovary. The enzymatic reaction was stopped by boiling the samples for 5 min. For radio-HPLC,1 μCi of [^{3}H]4P was similarly incubated with hemolymph or an ovarian homogenate for 1, 30, and 60 min in the absence or presence of 10 nmol of 4P. Prior to analysis, samples were stored at −20 °C.

2.10. Binding experiments

In individual experiments, about 2 g of tissues (ovaries, heads, and larvae) or at most about 500 pairs of ovaries were employed. The samples were homogenized in 50 mM of Tris–HCl buffer, pH 7.4, with or without EGTA, or modified Hank's solution, in the presence or in the absence of protease inhibitors. To follow binding we

used raw homogenate, i.e. the supernatant after centrifugation (10 min at 600*g*), and also the raw membrane preparation, i.e., re-suspended pellet after ultracentrifugation (100,000*g*, 45 min).

Binding experiments were performed in buffer solution (250 μl) and the separation between bound and free peptide was done by rapid filtration (Brandel cell Harvester). Ligands were bound for 15–16 h at 4 °C. A radioactive labeled peptide 2.7–67.5 nCi/test tube was incubated with homogenate or membrane preparation (50–350 μg protein/tube) in the absence or presence of standard 4P (2 mM) to follow specific and non-specific binding.

3. Results

3.1. Oostatic effect

The oostatic potency of the substances tested is summarized in Table 1. The hatchability was generally slightly decreased in the first gonadotrophic cycle. However, in the second cycle, large differences between the groups were found (Table 1).

Ovaries of emerged flies are morphologically at stage 1. When the first gonadotrophic cycle starts, within 48 h, yolk deposition rapidly occurs (stage 2) and 4 days later the eggs pass stages 3 and 4 and by day 6, they are fully developed (stage 5). Chorion formation follows and the egg is ready to be deposited at day 10. At that time the second egg chamber starts its development. During the first gonotrophic cycle no morphological changes except some slight egg shape irregularities were observed. The majority of changes were observed during the second gonotrophic cycle. First, the nucleoli of the follicular cells started to divide. Changes in the follicular epithelium, proliferation of follicular cell nuclei, and formation of a polynucleated follicular epithelium were observed with all the peptides. At day 16 after application, degenerative changes occurred in most of the egg chambers and the resorption of the egg chambers started. During this period, changes in ovarian development were observed. Morphologically, affected egg chambers have irregular appearance and their shape is often distorted.

Histological changes in the structure of the follicular epithelium of the egg chambers after peptide application are illustrated in Fig. 1. The follicular epithelium envelops oocytes and nutritive cells and normally is single-layered (Fig. 1A). After peptide treatment, nucleolus division of the follicular cell appeared and was followed by the nuclear and cell division (Fig. 1B). The migration of such modified cells into the yolk as well as into a region of nutritive cells was observed, causing their disintegration (Fig. 1C). The proliferating cells filled up the whole inner space of the egg chamber and caused resorption of the affected egg chamber (Fig. 1D). In the series of analogues studied, the occurrence of the changes in ovaries varied according to the length (number of the Pro residues) at the C-terminus. Deletion of prolines from the C-terminus reduced the time period for the effect to be observed. The highest activity was observed with the pentapeptide and tetrapeptide. Further shortening decreased the activity. However, the oostatic effect was still significant.

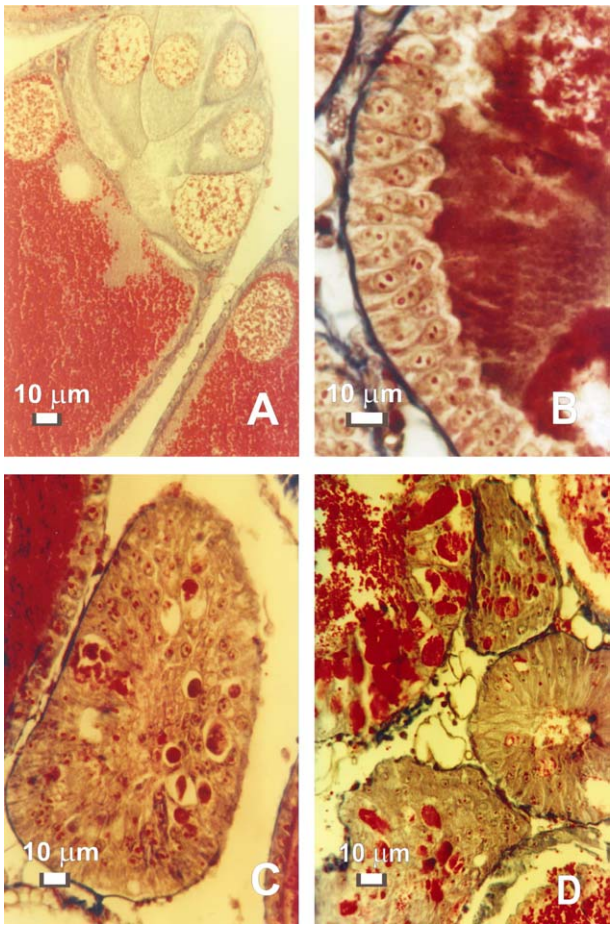

Fig. 1. Illustration of degenerative changes in the flesh fly *N. bullata* ovaries after application of oostatic tetrapeptide. (A) Normally developed egg chamber (control); (B) division of nucleolus of the follicular cells followed by cell division (after tetrapeptide application); (C) proliferation of follicular cells into yolk and a region of nutritive cells (after tetrapeptide application); and (D) resorption of the egg chamber (after tetrapeptide application).

In summary, changes in the egg development mainly appeared in the second gonotrophic cycle. The tetra- and pentapeptides were found to affect more ovarioles in comparison to the tri- and hepta- to decapeptides. In the case of the shorter peptides, the oostatic effects were detectable earlier than with the longer peptides.

3.2. Degradation experiments

Using both LC/MS and radio-HPLC, we showed that the peptides are rapidly degraded both by the ovaries and the hemolymph. The main degradation product is the des Tyr^1 peptide, i.e., H–Asp–Pro–Ala–OH (see Figs. 2A and B and 3A and B).

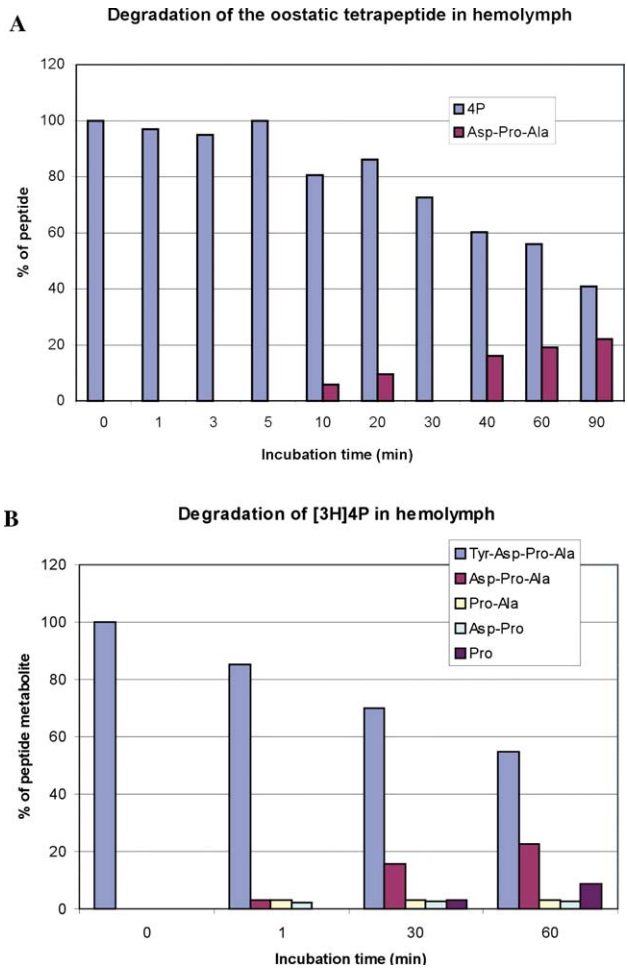

Fig. 2. Time course of the degradation of the tetrapeptide in the hemolymph. (A) Followed by LC/MS and (B) followed by radio-HPLC. A 100 µl—portion of tetrapeptide solution (5×10^{-2} mg/ml 4P or 1 µCi [^{3}H]4P) was incubated with 5 µl of diluted hemolymph (1:1). The hemolymph from about 50 fly females was pooled and diluted at different time intervals. The conditions for chromatography are described in Section 2.

Using tritiated peptide, it was possible to detect the dipeptides Asp–Pro, Pro–Ala, and also Pro. In addition, using radio-HPLC, a metabolic product of tyrosine was also detected in the incubation mixture [12]. One half of the peptide is degraded by one ovarian equivalent in 5–30 min and by 2.5 µl of undiluted hemolymph in 60 min. These results indicate that in the intact flesh fly, a pair of ovaries and about 10 µl of hemolymph will degrade the peptide very rapidly. The addition of a cocktail of protease inhibitors completely stopped the degradation. From the comparison of the time courses of degradation by LC/MS and radio-HPLC it follows that the latter achieved higher detection sensitivity so that more degradation products were detected.

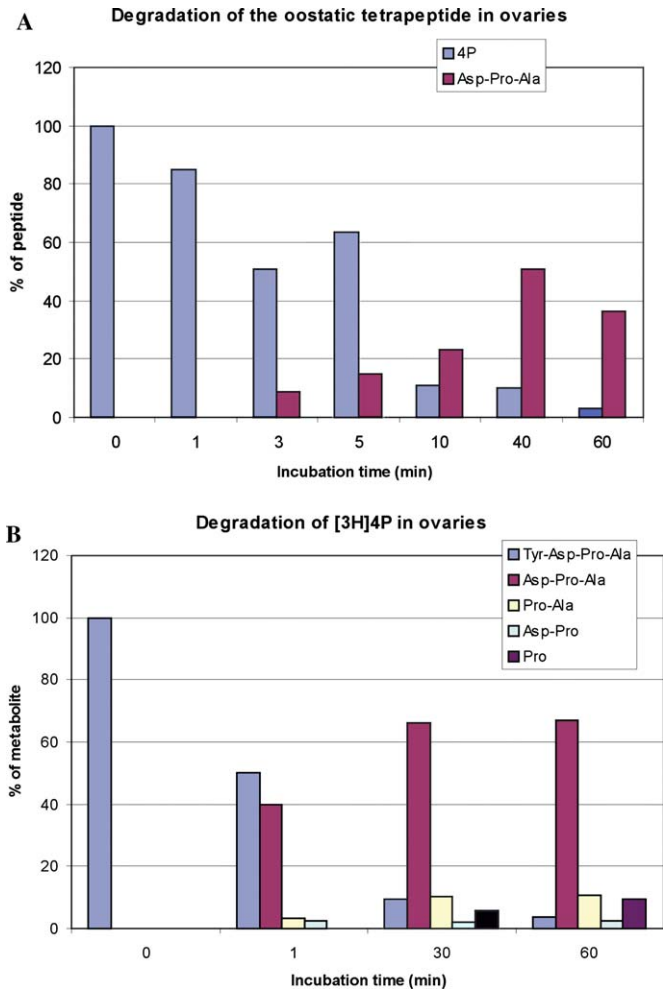

Fig. 3. Time course of the degradation of the tetrapeptide in ovaries. (A) Followed by LC/MS and (B) followed by radio-HPLC. A 100 µl—portion of tetrapeptide solution (5×10^{-2} mg/ml 4P or 1 µCi [^{3}H]4P) was homogenized with one ovary and incubated at different time intervals. The conditions for chromatography are described in Section 2.

3.3. Binding experiments

The calculated values for specific binding were very low. On average, they amounted to 50% of the total binding that was equivalent to about 2% of total radioactivity in the reaction. However, neither the total nor the nonspecific binding depended on the amount of protein in the reaction. The binding to preparations from individual isolations also did not depend on concentration of the radioactive ligand, incubation temperature, minor modifications in the buffer composition or incubation time. Thus, it appears either (i) there are no specific binding sites for the oostatic peptides on the

membranes of the ovaries, or (ii) the binding sites have an affinity constant lower than is the detection limit of the assay (higher than 10 mM) using tritiated peptide.

4. Discussion

Two analogues of the insect oostatic decapeptide, the tetrapeptide and pentapeptide, have strong deteriorating effect on oogenesis of *N. bullata* (Diptera) 16 days after applying these peptides (10 nmol). The single layer of follicular cells surrounding the second oocyte and the nutritive cells, which never proliferated during normal development, showed marked proliferation after peptide application to the inner part of egg chamber. The multi-nuclear formation 2 weeks after peptide application eliminated both oocytes and nutritive cells. Eggs in such ovarioles did not mature and were resorbed. The metabolism of the peptides is very fast, 2 h after application most of the peptide might be degraded inside the fly. No binding sites in the ovaries or in the fly heads were detected using [^{3}H]4P. The peptides also do not actively accumulate in the ovaries. Thus, it seems that the peptides do not act via receptors on the ovaries.

The peptides when administered 24–48 h after fly emergence cannot influence the first egg chamber development because it has already started. On the other hand, development of the second egg chamber starts much later and can be influenced by the peptide application. From this we hypothesize that the peptides act in a very short time interval, probably influencing protein synthesis. There is also a possibility of an effect via a metabolite of tyrosine [12] that may last longer. From our recent studies of the circular dichroism and conformation of the peptides under study, a carrier-like role for the C-terminal oligoproline sequence may be involved [13]. More work is needed to fully understand our results [7–13].

Using two insect species, two different mechanisms of oostatis caused by the same decapeptide and its analogues were proposed: (i) inhibition of trypsin synthesis in mosquito [5] and (ii) induction of proliferation of follicular cells causing resorption of eggs in the fly as described in this paper. Contrary to our results for the structure activity relationship in fly showing greater effects for 10P analogues truncated from the C-terminus [8] in comparison to 10P, Borovsky et al. [4] indicated that removal of two to five prolines from the C-terminus reduced the activity in the mosquito. Just recently, Borovsky showed that the peptide 4P is as effective as the peptide 10P [5].

A more detailed study could further elucidate the effects of insect oostatic peptides. In such a study, other possible effects should be considered. One possibility is peptide inhibition of ecdysteroid production in ovaries either by direct action or via the egg development neurosecretory hormone (EDNH) [14]. More work is needed to elucidate the insect oostatis by peptides.

Acknowledgment

The study was supported by Grant No. 203/02/0247 of the Grant Agency of the Czech Republic.

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