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REVIEW ARTICLE

Oostatic peptides

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Abstract Oostatic peptides are organic molecules, which influence an insect reproduction due to a regulation of the eggs development. It was proved that decapeptide—H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-OH (YDPAPPPPPP)—isolated from mosquito *Aedes aegypti*, inhibits trypsin activity in the midgut of the mosquito. Therefore, it was named trypsin-modulating oostatic factor (*Aea*-TMOF). Feeding the recombinant cells with cloned and expressed TMOF on the coat protein of tobacco mosaic virus (TMV) to mosquito larvae, caused larval mortality. The TMOF was therefore designed for usage as a new biorational insecticide against mosquito. Similarly, a hexapeptide—H-Asn-Pro-Thr-Asn-Leu-His-OH (NPTNLH)—was isolated from the grey flesh fly *Neobellieria bullata*. This peptide and some of its analogs inhibited trypsin-like synthesis by the midgut in female flies and was therefore entitled *Neb*-TMOF. Interestingly, the synthetic *Aea*-TMOF and mainly its C-terminus shorten analogs, including those containing D-amino acids or methylene-oxy isosteric bond, quickly and strongly inhibited the hatchability and egg development in the flesh fly *N. bullata*.

Keywords Insect development regulation · Oostatic activity · TMOF and analogs · Genetic approach · Degradation in ovaries

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Introduction

Neuropeptides represent the largest single class of signal compounds and are involved in the regulation of development, growth, reproduction, metabolism and behavior of insect. Over the last three decades, there has been a tremendous increase in our knowledge of neuropeptide signaling due to genome sequencing, peptidomics, gene microarrays, receptor characterization and targeted gene interference combined with physiological and behavior analysis (Altstein and Nässel 2010). A search for alternative agents interfering with endocrine system of insects was stimulated by evident hazards of the use of conventional insecticides. The existence of peptides that inhibit egg development has been manifested in different insects like cockroach *Blattella germanica* (Iwanov and Mescherskaya 1935), decapods crustaceans (Carlisle and Knowles 1959) and sucking buck *Rhodnius prolixus* (Davey 1978; Davey and Kunster 1981). In mosquitoes (Else and Judson 1972; Meola and Lea 1972), an ovary-produced humoral factor secreted during oogenesis was demonstrated that inhibited yolk deposition in less developed follicles (King 1970). In the house fly *Musca domestica* (Adams et al. 1968; Adams 1981; Kelly et al. 1984, 1986; DeMilo et al. 1991), however, oostatic hormone inhibited the release or synthesis of egg developmental neurosecretory hormone (EDNH). For example, when semi-purified oostatic hormone, extracted from mature ovary of house fly *M. domestica vicina*, was injected into flies 12 h after emergence with a dosage of one pair ovary/fly, an oocyte development was obviously inhibited. Thus, this oostatic hormone regulates cyclical egg maturation in this housefly. Twenty-four hours after the injection, the revitellogenic development of oocyte was inhibited. When vitellogenin synthesis was initiated, the injected oostatic hormone inhibited vitellogenin synthesis

in the fat body, lowered vitellogenin titer in the hemolymph, and thus caused the delay of vitellin deposition in the oocyte. Vitellogenin uptake was not inhibited by the oostatic hormone. EDNH could restore the development of oocyte, inhibited by oostatic hormone. It is concluded that this oostatic hormone is not species-specific (He 1995).

Aedes aegypti-trypsin modulating oostatic factor (*Aea*-TMOF)

Borovsky (1985, 1988) isolated and partially purified peptide sequence, named oostatic hormone, from the ovaries of the mosquito *Aedes aegypti* and demonstrated that an injection of this oostatic hormone (9 ng) into female mosquitoes inhibited yolk deposition and biosynthesis of vitellogenin. The activity of this hormone in the mosquito ovary increased rapidly following blood feeding and reached a maximum after 48 h. Injected into *A. taeniorhynchus*, this hormone inhibited egg development, so that repeated injections at 24 h intervals resulted in 60 % reduction in the number of eggs laid. The hormone appeared to act on the ovary only, while no effect on release of egg development neurosecretory hormone (EDNH) was observed. The hormone regulated biosynthesis of alkaline proteases and trypsin-like enzymes in the midgut of *A. aegypti* (Borovsky and Schlein 1988), however, it was not found to be specific since its injection into *Culex quinquefasciatus*, *Culex nigripalpus*, and *Anopheles albimanus* caused inhibition of egg development, blood digestion, and synthesis of trypsin-like enzymes, too.

In contrast to common inhibitors (e.g. soybean trypsin inhibitor, diisopropylfluoro phosphate, phenylmethylsulfonyl fluoride), which bind to enzyme active site and prevent proteolysis, this hormone showed rather a modulatory than inhibitory effect. Later, this substance was well purified and analyzed using mass spectroscopy, to be characterized as a decapeptide with primary structure H-Tyr-Asp-Pro-Ala-Pro-Pro-Pro-Pro-Pro-Pro-OH (YDPAPPPPPP; molecular mass 1047.6). (Borovsky et al. 1990, 1991, 1993; Kochansky and Wagner 1992). The synthetic decapeptide was injected into mosquitoes, biting midges, flies and fleas, where it caused an inhibition of biosynthesis of most abundant proteolytic enzyme—trypsin—in the midgut. Therefore, this molecule was named trypsin-modulating oostatic factor—TMOF. The *Aea*-TMOF was suggested to be a physiological signal that terminates trypsin biosynthesis in mosquitoes. Binding of [^{3}H] TMOF to midgut receptor was demonstrated in vivo and the decapeptide that signals the termination of trypsin-like enzyme biosynthesis in mosquito midgut was in a parallel study covalently bound to Keyhole Limpet Hemocyanin using *N*-hydroxysuccinimide and dicyclohexylcarbodiimide. Polyclonal antibodies raised in rabbits against this conjugate were used to develop-specific RIA and enzyme-linked immunosorbent assay (ELISA) to detect the peptide hormone in female. *Aea*-TMOF and its C-terminus shortened analogs—octa- and pentapeptide, and polyproline, as well, were tested with antiserum, which fully recognized *Aea*-TMOF and partially recognized octapeptide. Thus, the amount of this decapeptide in the mosquito ovary was estimated to be thousand-fold higher in comparison with the brain—this decapeptide is probably not neural but ovarian in origin (Borovsky et al. 1992). Experiments with *Aea*-TMOF tritiated on Pro residue indicated this compound to be a secretory peptide, synthesized by the ovarian follicular epithelium (Borovsky et al. 1994a). It was confirmed, that specific binding sites of *Aea*-TMOF are located on the mosquito midgut and after hormone binding to its receptor a signal is transmitted that initiates the termination of the trypsin biosynthesis in the midgut (Borovsky et al. 1994b). Similar results were obtained when C-terminus shortened analogs of TMOF were used (Borovsky and Mahmood 1995). To determine the shortest sequence of TMOF that binds its receptor, a lethal concentrations at 50 % mortality (LC_{50}, mmol L^{-1}) of *Aea*-TMOF and 25 of its analogs were obtained as means of three determinations (Table 1). Although a removal of the three to five Pro residues from the C-terminus of *Aea*-TMOF lowered the activity to 45–31 %, a removal of the six Pro residues at the C-terminus again increased the activity to 95 % of that of original molecule.

It was hypothesized that the smallest size of *Aea*-TMOF that binds to corresponding gut receptor and maintains biological activity of the original decapeptide is the tetrapeptide H-Tyr-Asp-Pro-Ala-OH (YDPA). The six Pro residues at the C-terminus form a left-handed helix (Borovsky et al. 1993; Curto et al. 1993). When proline residues are partially removed, the truncated molecule cannot form a stable helix and thus the binding of TMOF analogs to corresponding receptor and the biological activity of the truncated analogs is reduced. After all, the Pro residues are removed from the C-terminus, the tetrapeptide assumes a more stable conformation, and the binding to the receptor is again enhanced. These findings could be partially supported by CD spectra of the oostatic peptides series (Maloň et al. 2003). The authors suggested that the two parts of the *Aea*-TMOF molecule are quite conformationally independent. The N-terminal tetrapeptide part is probably responsible for the biological activity, while the C-terminal oligoproline part could serve as an inert carrier or aid in some hormonogen-like function. The C-terminal oligoproline part assumes the polyPro II-type conformation, which is most frequent spatial arrangement of oligoPro sequences in aqueous media.

Table 1 Biological effects of oostatic peptides—*Aea*-TMOF and its analogs

Peptide	Effects on mosquito[a]		Effects on flesh fly[b]	
	LC_{50} (mM ± SEM)	Inhibition of larval growth, %	Hatchability[c] of larvae, %	Resorption[d] of egg, %
YDPAPPPPPP (*Aea*-TMOF)	0.2 ± 0.015	100	95	5
YDPAPPPPP($^{\Delta}$)P	–	–	95	10
YDAPPPPPPPR	>71.6 0	0	–	–
cyclo(YDPAPYDPAP)	–	–	100	0
$(H)_6$IEGRYDAPPPPPP	0.34 ± 0.032		59	–
DPAPPPPPP	0.44 ± 0.015	45	–	–
YDPAPPPPP	–	–	80	10
YDPAPPPP	0.44 ± 0.05	45	80	10
PAPPPPPP	0.58 ± 0.029	34	–	–
YDPAPPP	–	–	80	20
APPPPPP	1.18 ± 0.065	17	–	–
YDPAPP	0.64 ± 0.032	31	80	20
YDPAPR	0.24 ± 0.01	80	–	–
YDPAPK	≫2.9	0	–	–
PPPPPP	1.1 ± 0.025	18	–	–
YDPAP	0.64 ± 0.028	31	80	20
YD($^{\Delta}$)PAP	–	–	30	20
YDPA($^{\Delta}$)P	–	–	25	30
Y(d)D($^{\Delta}$)PAP[e]	–	–	40	70
YD($^{\Delta}$)P(d)AP[e]	–	–	30	
YDP($^{\Delta}$)AP	–	–	30	30
(d)YDPAP[e]	–	–	40	99
Y(d)DPAP[e]	1.2 ± 0.26	16	50	80
YD(d)PAP[e]	–	–	30	10
YDP(d)AP[e]	–	–	30	30
YDPA(d)P[e]	–	–	98	80
(d)Y(d)D(d)P(d)A(d)P[e]	–	–	20	88
cyclo(YDPAP)	–	–	100	0
YDPψ[CH_2O]AP	–	–	20	70
YDPψ[CH_2S]AP	–	–	40	60
YDPAR	0.12 ± 0.017	166	–	–
YDPA	0.21 ± 0.01	95	70	60
cyclo(YDPA)	–	–	100	0
$(YDPAR)_4$	0.095 ± 0.007	210	–	–
YD($^{\Delta}$)PA	–	–	95	70
YDPψ[CH_2O]A	–	–	25	75
YDPψ[CH_2S]A	–	–	45	55
YDPR	0.24 ± 0.02	80	–	–
DPAP	0.98 ± 0.017	20	70	50
DPAR	0.048 ± 0.011	43	–	–
VDPA	–	–	30	60
$(DPAR)_4$	0.048 ± 0.002	417	–	–
PPPP	1.5 ± 0.085	13	–	–
YDP	2.3 ± 0.36	9	80	5
DPA	0.4 ± 0.03	50	90	10
D($^{\Delta}$)PA	6.4 ± 0.23	3	90	10
(d)YDP[e]	0.51 ± 0.05	39	–	–

Table 1 continued

Peptide	Effects on mosquito[a]		Effects on flesh fly[b]	
	LC_{50} (mM ± SEM)	Inhibition of larval growth, %	Hatchability[c] of larvae, %	Resorption[d] of egg, %
Y(d)DP[e]	0.28 ± 0.015	71	–	–
(d)Y(d)DP[e]	1.7 ± 0.029	12	–	–
YD	1.2 ± 0.05	16	90	0
(d)YD[e]	1.4 ± 0.017	14	–	–
DP	–	–	100	0
D($^{\Delta}$)P	–	–	100	5
AP	–	–	100	0
PA	–	–	100	0
PP	1.83 ± 0.07	11	–	–

[a] *Aedes aegypti* (Borovsky 2007; Borovsky and Meola 2004); *Aea*-TMOF and its analogues were fed to first instar mosquito larvae and larval mortality was followed for 6 days. Lethal concentration (LC_{50}) at 50 % mortality was obtained by Probit analyse

[b] *Neobellieria bullata* (Slaninová et al. 2004; Hlaváček et al. 2007; Bennettová 2011 unpublished results; Hlaváček et al. 2012); peptides were injected in Ringer's solution (10 nmol in 5 μL/female) into the upper part of the thorax of the Et_2O—anesthetized 24–28 h old flies. After injection, females were placed into cages with untreated males and their ovaries were dissected 2–16 days later and examined for morphological and histological changes (Slaninová et al. 2004)

[c] Hatchability only in the 1st, no in the 2nd gonotrophic cycle was observed

[d] The difference to 100 % is equal to yolk content in the egg

[e] (d) denotes for D-configuration of amino acid in the three letter abbreviation

This decapeptide also modulated ecdysteroid production in the presence of extracts from brain of the gypsy moth *Lymantria dispar* and European corn borer *Ostrinia nubilalis*. The modulation was highly dependent on both the concentration of *Aea*-TMOF and brain extract. At lower concentrations—stimulatory, at higher concentrations—neutral or inhibitory (Gelman and Borovsky 2000) effects were found. In a search for trypsin biosynthesis regulation in the gut, the larvae of citrus weevil *Diaprepes abbreviatus* were treated topically, in a diet and by injection with *Aea*-TMOF in DMSO with a significant decrease in the growth rate and trypsin biosynthesis in larval gut (Yan et al. 1999; Nauen et al. 2001). In the assay on different mosquito species, also different potency of *Aea*-TMOF was recorded with LC_{50} 0.2–1.056 (mmol L^{-1}) (Borovsky 2003b). One study also reported the effects of ACE inhibitors on larval growth in the cotton leaf worm *Spodoptera littoralis*. A suggestion was made, that *S. littorals* ACE may influence trypsin biosynthesis in the larval gut by interacting with *Aea*-TMOF. Injecting third instars larvae with a combination of *Aea*-TMOF and the ACE inhibitor captopril, down-regulated trypsin biosynthesis in the larval gut indicating that an *Aea*-TMOF gut receptor analogue could be present (Lemeire et al. 2008).

The techniques of molecular engineering enabled cloning the *Aea*-TMOF in cells and expressing it on the coat protein of tobacco mosaic virus (TMV) in *Chlorella* sp. and *Saccharomyces cerevisiae* cells (Carlson et al. 1994; Borovsky et al. 1998). Genetically modified TMV carries *Aea*-TMOF as a side chain of its viral coat. This virus can still infect tomatoes and other Solanaceae, therefore an insect (mosquito larvae) feeding on such TMV-TMOF-infected plants die while insect feeding on *Aea*-TMOF-infected control plants survive (Vanden Broeck et al. 1997; Borovsky et al. 2006a). The *Aea*-TMOF was therefore designed for usage as a new biorational insecticide against mosquito larvae (Borovsky 2003a, 2007; Gäde and Goldsworthy 2003; Borovsky et al. 2006b). Because of the insecticidal effects of *Aea*-TMOF and its potential to affect larval mosquito and several Lepidoptera larvae, the biochemical events that occur after binding of this factor to its gut receptor were studied in vitro. It was found that *Aea*-TMOF binding induces gut membrane phosphorylation and protease activation, which are probably the first steps in a signal transduction pathway that controls the translation of the trypsin message in the gut of many insects (Borovsky 2008; Borovsky and Hamdaoui 2008). The production and characterization of transgenic tobacco plants expressing a precursor of *Aea*-TMOF, which interferes with development of tobacco budworm larvae *Heliothis virescens*, was described. Tobacco plants were transformed with a synthetic gene containing six *Aea*-TMOF units spaced dibasic residues, Arg-Arg, as potential post-translational cleavage sites. Peptide extracts from transgenic plants had *Aea*-TMOF activity and inhibited in vitro biosynthesis of serine proteases. *H. virescens*, fed with transgenic leaves showed a reduced growth rate and an increased mortality of 20–33 % in *H. virescens* (Tortiglione et al. 2002). When *Aea*-TMOF was expressed in tobacco plants as a fusion with tomato prosystemin about 0.004 % of the total soluble proteins was

attributed to *Aea*-TMOF with low inhibition (4 %) of *H. virescens* larval growth (Tortiglione et al. 2003).

Attempts to formulate a new commercially acceptable insecticide yielded a product containing *Aea*-TMOF adsorbed onto yeast cells. This conjugate stopped synthesis of trypsin and growth of the mosquito larvae, as well (Borovsky and Meola 2004).

A commercial development of the *Aea*-TMOF as a new insecticide was further carried out to evaluate the various formulations of *Aea*-TMOF via 1—a recombinant *Aea*-TMOF yeast cell paste form, 2—*Aea*-TMOF yeast cell dried powder form, 3—combination of *Aea*-TMOF and *Bacillus thuringiensis* (*Bti*) rice husk, 4—*Aea*-TMOF and *Bti* wettable powder and 5—*Aea*-TMOF and *Bti* mosquito fudge cubes, against *Aedes aegypti* larvae, in the laboratory (Misni et al. 2010). Although the 1st and 2nd formulations caused mortality in larvae after 24—96 h exposure and prolonged residual effect for 4 weeks of observation, the 3rd to 5th formulations were effective in causing mortality within 1 h of treatment and gave prolonged residual effect for 4 weeks, too. The 5th formulation has shown high larvicidal activity for 3 months after treatment. Above formulations assayed, have the potential to be utilized for dengue vector control.

Even the *B. thuringiensis* (*Bti*) is not toxic to human and other mammals, its high cost, a need for frequent application and a possibility of resistance due to widespread application, have severally compromised its use. Thus, another attempts to formulate an optimal combination of this toxin with *Aea*-TMOF, resulted in the search for effective dose of Pichia-TMOF and the conjugate of Pichia-TMOF with *Bti* as larvicide on *A. aegypti* larvae (Lau et al. 2011). The Pichia-*Aea*-TMOF itself (400 ppm) was able to cause 100 % mortality in *A. aegypti* larvae on 8th day, and the combination of 400 ppm Pichia-TMOF with 0.1 ppm *Bti* moreover showed synergistic effect in the same insect.

The effectiveness and residual effects of *Aea*-TMOF-*Bti* in rice husk and *Aea*-TMOF-*Bti* in wettable powder formulations against *A. aegypti* on the larvae mortality were determined also in experiments performed outside laboratory. The results were recorded after 24 h and weekly for 5 weeks. All of the formulations were very effective on the first 2 weeks by giving 100 % larval mortality for all concentrations applied and for residual concentrations up to 4th week after application, with lesser effect (Saiful et al. 2012).

The *Aea*-TMOF resembles the primary structure of oligoproline-rich regions within the bacterial surface protein ActA, which is required for Listeria motility in host cells. It was shown that its injection into Listeria-infected PtK2 cells rapidly blocked Listeria-induced actin rocket tail assembly, as well as, intracellular locomotion of this pathogen (Southwick and Purich 1995).

Neobellieria bullata-trypsin modulating oostatic factor (*Neb*-TMOF)

Another TMOF, which was shown to control trypsin by the midgut in female flies through a translational control of the trypsin gene and also inhibits the ecdysone biosynthesis in the larval ring gland, was isolated from 10,000 vitellogenic ovaries of the grey flesh fly *Neobellieria bullata* and sequenced by mass spectrometry (Bylemans et al. 1994; De Loof et al. 1995a). *Neb*-TMOF is a hexapeptide H-Asn-Pro-Thr-Asn-Leu-His-OH (NPTNLH) and injection of this peptide at physiological concentrations (10^{-9} M) inhibits trypsin-like synthesis by the midgut of liver-fed female flies, and causes a reduction of the vitellogenin concentration in the hemolymph and of oocyte growth. This inhibition indirectly results in an arrest of oocyte growth. This oostatic peptide resists very well proteolytic breakdown by enzymes present in the lumen of the gut of pre-vitellogenic flesh flies in vivo, however, when incubated in hemolymph of adult flies, its half-life time is a mere 0.5 min. In hemolymph of last instar larvae, this value increases to about 1.5 min (Zhu et al. 2001a). Similarly, the *Neb*-TMOF degradation in vitro was monitored in the hemolymph of the lepidopteran *Spodoptera frugiperda*, the orthopteran *Schistocerca gregaria* and the dictyopteran *Leucophaea maderae* and half-life in the different species varied from 3 min to 25 min. The *Neb*-TMOF was cleaved in dipeptides starting from the C-terminus, which were the same in the hemolymph of all species. These observations suggest that a dipeptidase with very similar enzymatic properties as the mammalian ACE circulates in the hemolymph and, therefore, it could be involved in the breakdown of *Neb*-TMOF as a common, but not a universal

Table 2 Trypsin inhibitory activity of *Neb*-TMOF and the potent analogs[a]

Peptide	2.10^{-4}	2.10^{-3}	2.10^{-2}	0.2	2
NPTNLH (*Neb*-TMOF)	0	41 ± 12	55 ± 7	31 ± 11	29 ± 11
NPTNLH-*amide*	0	0	0	0	29 ± 7
Ac-NPTNLH	0	0	0	0	22 ± 14
NPSNLH	34 ± 11	45 ± 13	21 ± 7	16 ± 13	0
NPTNLK	14 ± 10	18 ± 11	18 ± 7	29 ± 10	46 ± 7
NSTNLH	0	21 ± 15	25 ± 19	32 ± 17 0	
NPTNLF (*p-NO*$_2$)	–	–	–	12 ± 5	–
NPTNLF (*p-NH*$_2$)	–	–	–	24 ± 10	–

[a] In pmoles injected per flesh fly *N. bullata* (Janssen et al. 1998)

enzyme in insect hemolymph (Zhu et al. 2001b). A study on oostatic activity of *Neb*-TMOF and some of its analogues suggested that the inhibitory activity of trypsin biosynthesis is independent from that of ecdysone regulation (Bylemans et al. 1995a).

With using polyclonal antisera raised against synthetic *Neb*-TMOF, an identification and immunolocalization of *Neb*-TMOF epitopes in different flesh fly tissues was attempted with a result, that in adult females the ovary appears to be the only site of synthesis of *Neb*-TMOF and of its precursor (Bylemans et al. 1996).

By the use of the *N. bullata* trypsin biosynthesis assay, a series of 17 analogs of *Neb*-TMOF was tested for their trypsin inhibitory activity. The following structural elements were found to be critical for this effect: the alcohol function at position 3 (Thr), a positively charged, basic group at the C-terminus (His) and the length of the side chain at positions 1 and 4 (Asn), as well as a spatial arrangement of the aromatic ring (Table 2). The threshold dose for oostatic activity was lowered by three orders of magnitude when *Neb*-TMOF tested was suspended in wheat germ oil. Comparison of the activity profiles of *Neb*-TMOF and its analogs in two bioassays might confirm the hypothesis that different receptors are involved in modulation of trypsin biosynthesis in the midgut and in inhibition of ecdysone production in the larval ring glands by *Neb*-TMOF (Janssen et al. 1998; Konopińska et al. 1998).

However, this hexapeptide exhibited a zero effect on regulation of the hatchability and egg development in *N. bullata*, similarly to the corresponding pseudohexapeptide containing a methyleneoxy isosteric bond in place of Pro-Thr peptide bond (Bennettová 2003; Hlaváček et al. 2004).

A combined spectroscopic and potentiometric studies on the copper (II) complexes of *Neb*-TMOF and its analogs modified in amino-terminus were performed in order to examine the binding ability of *Neb*-TMOF and its analogs on the coordination to copper (II) ions, which are present in hemolymph of the insects (Steinkraus et al. 1973). The effect of Pro residue inducing β-turn conformation into peptide molecules, imidazole nitrogen of histidine residue, and the substituent in amino terminus, as well, are suggested to affect the *Neb*-TMOF coordination to Cu (II) (Kowalik-Jankowska et al. 2008).

During the purification of *Neb*-TMOF, a new factor with oostatic activity was discovered. Its amino acid sequence was determined as SIVPLGLPVPIGPIVVGPR. Owing to structural sequence similarities with parts of several known collagens and its oostatic activity, this nonadecapeptide was named *Neb*-colloostatin. It inhibits yolk uptake by previtellogenic oocytes and might have a role in the absence of yolk deposition in penultimate oocytes. However, this peptide does not inhibit trypsin biosynthesis in the gut or ecdysone biosynthesis by larval ring glands, but decreases vitellogenin concentration in the hemolymph (Bylemans et al. 1995b). The discovery of the dipteran folliculostatins, which do not show any resemblance to inhibins of vertebrates, may significantly contribute to a better understanding of the hormonal control of growth in insects. None of above folliculostatins is blocked at its N- or C-terminus. This, in combination with the pleiotropy of their effects and the narrow species specificity make such peptides significant for examination of their efficiency to control insect reproduction using methods of molecular biology (De Loof et al. 1995b).

Action of *Aea*-TMOF and its derivatives on flesh fly *Neobellieria bullata*

A series of peptides derived from the sequence of decapeptide *Aea*-TMOF was prepared, characterized and assayed on their oostatic activity in *N. bullata* (Table 1, Hlaváček et al. 1997, 1998; Šolínová et al. 2004; Roco et al. 2007; Koval et al. 2008), which was estimated using morphological assessment of the status of ovaries, hatchability assessment and histological assessment of the changes in the eggs development. Investigation of the decapeptide showed only a weak effect on ovarian development of *N. bullata*, observed as a decrease in hatching during the 2nd gonotrophic cycle, by alteration of the egg chamber development. Further studies on the C-terminus shortened analogs of this decapeptide—especially penta- and tetrapeptides (YDPAP and YDPA) revealed a strong oostatic effect inhibiting the egg development in this flesh fly with a significant decrease in hatching in the 2nd gonotrophic cycle (Slaninová et al. 2004). After oostatic peptide application, a monolayered follicular epithelium divides, forming a multilayered structure and filling the entire egg chamber, which degenerates and is finally resorbed (Figs. 1, 2, 3, 4). To study a mechanism of action, a distribution and degradation of these most active oostatic

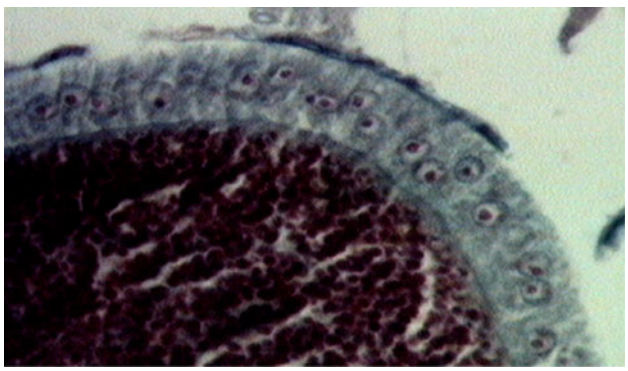

Fig. 1 Follicular epithelium; see yolk granules in oocytes and follicular cells with visible nucleolus in the nucleus

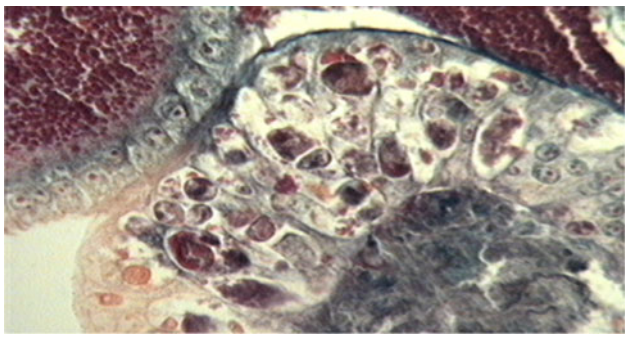

Fig. 2 Follicular epithelium proliferation towards the center of the egg chamber; see giant and pycnotic nuclei

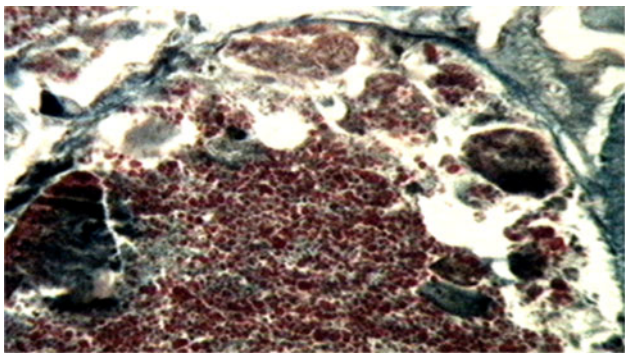

Fig. 3 Resorption appears in the yolk area

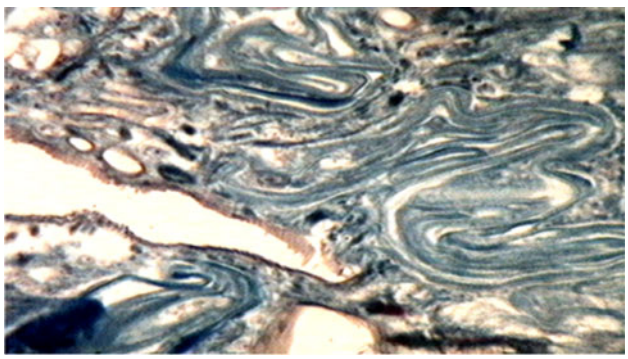

Fig. 4 Resorption of the egg chamber; note the folded vitelline membrane

peptides in selected organs of *N. bullata* was investigated using their derivatives with ^{3}H Pro and ^{3}H Tyr labels, respectively (Hlaváček et al. 2007). The dependencies of the radioactivity on the time after peptides application, on the location of tritium label in the molecules and on the target organs (head, ovaries) were observed. As a decisive step of the degradation, the very quick splitting of the C-terminal Pro residue from the pentapeptide to tetrapeptide was found (Tykva et al. 2007). Even if the pentapeptide is degraded quite quickly, the fully active tetrapeptide is formed, that is degraded much slower. The fate of YD(^{3}H)PAP after its in vivo injection was compared to its uptake after in vitro incubation of dissected ovaries, during selected stages of yolk deposition (King 1970). While the uptake of the applied radioactivity strongly depends on the stage of vitellogenesis (maximum was reached after 30 min in later oocyte vitellogenic stages 9 and 10), degradation of above pentapeptide is very quick (30 s) and independent of whether the label was injected or incubated with the ovaries, regardless of their developmental stage. It was hypothesized that an active intake of the analyzed peptide sequences into the intercellular spaces between follicular cells (Davey 1996) might be assumed (Bennettová et al. 2010). This idea could be supported by the suggestion that *N. bullata* midgut contains morphologically and functionally distinct segments that transport small peptides, and thus employment of active peptides for insect control may be feasible (Zlotkin et al. 1992).

The cyclization of the linear tetra- and pentapeptides decreased their effect in a reproduction of the flesh fly *N. bullata* by one order of magnitude. Comparative ^{1}H and ^{13}C NMR study on conformation of the cyclopeptides and their linear precursors revealed that a space structure of the cyclic analogs is too restricted to adopt an optimal biological conformation and therefore only minor oostatic activity is observed after their application (Hlaváček et al. 2001).

An insertion of some structures, isosteric with peptide bond Pro-Ala in the corresponding tetra- and pentapeptides affected a development of the eggs in *N. bullata*, too. An introduction of CH_2O substitution significantly accelerated and enhanced biological activity of corresponding oostatic peptides contrary to the CH_2S surrogate that evoked a moderate effect only (Mařík et al. 2001; Hlaváček 2004). Pseudotetrapeptide YDPψ[CH_2O]A showed devastating changes in ovarian development after its application to flesh fly *N. bullata*. Morphologically affected egg chambers had irregular appearance and their shape was distorted. Histological evaluation revealed very frequent changes throughout ovary. The changes appeared mostly in the development of the 2nd batch of eggs (the 2nd gonotrophic cycle) when no hatchability was observed; the changes in the 1st gonotrophic cycle were manifested by decreased hatchability of eggs in uterus.

A series of oostatic pentapeptides containing D-amino acid residue, either in differing or in all of the positions of the sequence, was prepared and assayed on effect in *N. bullata* (Hlaváček et al. 2012). The D-amino acid residue containing analogs exhibited an equal or even higher oostatic effect. The change in a configuration also significantly delayed a radioactivity incorporation into ovaries and also degradation of the corresponding molecules proceeded at a significantly lower rate as compared to parent pentapeptide without D-isomer. The decreased intake of radioactivity, the lower degradation and finally the high

oostatic effect may be ascribed to the decreased enzymatic degradation of the peptide bonds neighboring the D-amino acid residue in the corresponding peptides. The introduction of the non-coded D-amino acids thus enhances the oostatic effect in *N. bullata* owing to the prolonged half life of the corresponding pentapeptides, which can thus affect more ovarian cells.

In addition to *N. bullata*, which has ovaries of polytrophic type, above tetra- and pentapeptides were also tested on the bugs, *Dysdercus cingulatus* and *Pyrrhocoris apterus*, which have telotrophic ovary type and on the locust, *Locusta migratoria*, which has panoistic type (Němec et al. 2003, 2007). These oostatic peptides induced in the bugs and locust some disturbances in egg development, as the lower number of laid eggs, decreased hatchability and also a malformation in the structure of egg chambers. The tritiated pentapeptide was cumulated in the ovaries of *P. apterus* and followed the rate of vitellogenesis.

The hypothesis of the delayed effects of the *Aea*-TMOF-like peptides on dipteran reproduction was tested also using the long-lived viviparous tsetse fly, *Glossina morsitans*. The effects of the pentapeptide analog YDPAP on blood-feeding, mortality and fecundity of mated *G. morsitans* females were monitored after an injection of this pentapeptide at the beginning of their 1st gonotrophic cycle (Žďárek et al. 2009). A decrease in larval production per female during later geotropic stages (after about 40 days post-injection of 0.3 nmol/fly) was observed, in comparison with saline injected controls. The decrease in fecundity was observed only in females injected with the higher dose (2 nmol/fly).

A study on the effect of the pentapeptide YDPAP with high oostatic activity on oogenesis of mammals represented by the mice was carried out (Slaninová et al. 2002). Such a study was important from two points of view. On one side, it made possible to compare the effects on oogenesis for selected models of insects and mammals, and on the other side, it made more complex evaluation of ecotoxicity/ecosafety of the investigated oostatic peptides. This approach could also be applied for other insect growth regulators (e.g. juvenile hormone analogs) in analyses of their ecotoxicity in addition to pollution of soil, sediments and ground water (Tykva 1998). Even at comparably high doses (0.5 mg/kg) either no changes in the ovaries as well as in the uterus at different time intervals after the peptide injection were found, nor any significant differences in the number of pregnant mice, as well as a weight of the mice and number of offsprings were observed. Thus, these experiments showed no effect on the reproduction of mice. With high probability, such group of oostatic peptides exerts no ecotoxicity and their biological activity is limited to invertebrates only.

Conclusion

It was found in the mosquito or in the flesh fly that the oostatic peptides from ovaries have anorexigenic effects by inhibiting trypsin synthesis in the gut of females. Hence, they were named trypsin-modulating oostatic factors (TMOF). This mechanism can be understood as a feedback loop safeguarding cyclic feeding behavior and the consequent oogenesis in the ovaries. Genetic manipulations, with the Borovsky *Aea*-TMOF from mosquito *A. aegypti*, enabled cloning this molecule and expressing it on the coat protein of tobacco mosaic virus (TMV). Then formulations containing the conjugates of TMOF with yeast or *Bti* were developed as the new commercially acceptable insecticides stopping the synthesis of trypsin and growth of the mosquito larvae, as well. The *Aea*-TMOF was therefore designed for usage as a new biorational insecticide against mosquito larvae. *Neb*-TMOF from flesh fly *N. bullata* was shown to control trypsin by the midgut in female flies through a translational control of the trypsin gene, and also to inhibit the ecdysone biosynthesis in the larval ring gland. Several C-terminus truncated analogs of the *Aea*-TMOF were shown to possess strong antigonadotropic activity in the flesh fly *N. bullata.* The oostatic tetra- and pentapeptides and even more their D-isomers and isosteric analogs caused degradation of ovarian functions, namely pathological proliferation of the follicular epithelium followed by the resorption of the egg chamber. Strangely enough, these effects were more pronounced during the 2nd than the 1st gonotrophic cycle following the peptide application.

In this review, a survey on oostatic peptides and their properties is submitted, which speaks in favor of suggestion that these compounds are entitled to be prime candidates for testing their potential in insect pest control by means of molecular biology methods.

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Conflict of interest None.

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