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Research Article

Angiotensin converting enzyme, a negative moderator of the innate immune response in both mammals and insects

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Abstract

In contrast to mammals, insects only display an innate immune response. Following immune challenge, antimicrobial peptides support the rapid cellular clearance of the pathogens from their hemolymph. Simultaneously, a phenol oxidation reaction, intended to deliver reactive oxidative intermediates starts up and results in melanisation. In *Locusta migratoria*, infection elicits a drastic increase in circulating functional angiotensin converting enzyme orthologue. This circulating Locmi-ACE enzyme in turn is needed for the appearance into circulation of a pleithora of peptides. One of these immune induced ACE processed peptides, AVVPHSEAGKELLE, originates from a known immune active hexamerin II precursor. Prolonged hydrolysis by rabbit ACE gave the C-terminal truncated Hex-12mer AVVPHSEAGKEL being ACE resistant. Hex-12mer, devoid of antimicrobial activity, resulted in inhibition of the immune melanisation reaction. Using L-DOPA substrate and alcohol activated hemolymph Prophenoloxidase-derived Phenoloxidase a dose dependent inhibition was evidenced. The mode of inhibition is mainly uncompetitive and a Ki of 558µM was calculated. Hitherto we name this Hex-12mer peptide "Locmi-antimelanin-I". Human ACE, apart from its function in blood pressure regulation has a well-described role in minimizing the risk of the immune evoked cytokine burst. Combined with the herein reported finding of an ACE depending peptide being down regulator of immune activated Phenoloxidase, we claim an evolutionary conserved role of ACE in prohibiting over-activation of the immune response. Functionally, "Locmi-antimelanin-I" represents an unconventional anti-inflammatory cytokine.

Keywords: ACE, Locmi-Anti-melanin-I, Locusta migratoria, phenoloxidase, melanisation, inhibitor

Introduction

In human, within the RAAS endocrine pathway somatic two domain angiotensin converting enzyme (s-ACE) primarily function in processing Angiotensin-I into Angiotensin-II that causes vasoconstriction. Simultaneously s-ACE enzymatically inactivates the vasodilator bradykinin. Hitherto, s-ACE functions in instant blood pressure upregulation. As Angiotensin-II evokes release of the mineralocorticoid aldosterone from the adrenal cortex, ACE turned out to be an excellent target for the therapeutic regulation of blood pressure [1,2]. However, worldwide use of ACE inhibitors such as "Captopril" soon highlighted side effects including a reduced inflammatory response mainly by down regulation of pro-inflammatory cytokines as IL-6 and upregulation of anti-inflammatory TGF-β [3].

Already in 1994 the occurrence in circulation of a Captopril/Lisinopril sensitive Angiotensin converting enzyme activity was described in insects as flies and

moths [4]. cDNA cloning combined with functtional assays further illustrated the wide spread occurrence of one or multiple single domain ACE variants in insects and other Protostomians such as leeches [4-7]. In some model organisms, as the nematode *C.elegans*, no ACE orthologue is described but lack of a given, overall common gene set in one or more branches of the animal kingdom is not exceptional in Evolution.

Insects, in contrast to mammals and human, do have an open circulatory system and the angiotensinogen precursor seems missing in insects. As such, ACE will most probably not have its main function in vascular homeostasis. As in vertebrates, insect ACE is present and functions within the testes where this testis ACE assures a still obscure but seemingly evolutionary conserved role in male fertility. Indeed functional sperm is only produced provided testicular ACE is active [9-11]. Regarding the function of somatic insect ACE the co-occurrence in neurosecretory cells of ACE and particular neuropeptide precursors was demonstrated [12]. In accord, a first role of somatic insect ACE within the intracellular secretory pathway in fine-tuning of peptides following the liberation from their precursor was suggested [13]. Indeed Furin-like prohormone convertase cleavage ends up in liberating peptides still having a C-terminal dibasic extension. As zinc dependent Carboxypeptidase, insect ACE is believed to cleave off these two residues. Evidently, this action is only possible for mature peptides that are ACE resistant, which opens the way for cerebral ACE being additionally involved in clearance of neuropeptides [14]. Apart from the function of ACE in neuropeptide modulation within the neuronal system multiple functionalities of insect angiotensin converting enzyme have been reported: in the fleshfly ACE from the eggs helps in making amino-acids available for the embryo by cleaving yolk proteins [15,16]. Additionally circulating insect ACE is believed to clear secreted neuropeptides and peptide hormones from circulation. Ample evidence illustrates the regulatory role of circulating fleshfly ACE in regulating ovarian derived Neb-TMOF and its subsequent role in regulation of post protein meal digestion initiation and termination within the gut [17,18]. More recently, the involvement of ACE in insect growth regulation and more directly into the modulation of the ecdysteroid pathway received attention [19-22]. In addition, the relation between ACE activity and ecdysteroid titers has been highlighted [23,24]. Presently it is becoming clear that the number of ACE isoforms varies drastically in insects. In the genome of the mosquito Anopheles gambiae no less than 9 ACE encoding genes are present [25]. Also in the silk moth Bombyx mori 4 isoforms are identified [26]. In the pea aphid, Pisum sativum, 3 ACE genes have been cloned [27]. It now becomes more and more evident that each functional enzyme variant has its own role as their temporal and tissue specific distribution is distinct [26]. In Bombyx ACER 1 is predominantly present in the hemolymph along all developmental stages and synthesis in fat body, epidermis and wing discs has been reported [26].

Already in 2003 our group reported the immune stimulation and drastically enhanced gene expression of ACE by hemocytes of *Locusta migratoria* [28]. Over the past years, this result was in house validated by a multitude of independent experiments and independent researchers. Although our observation is in line with the in vertebrates reported expression of s-ACE by activated monocytes/macrophages and endothelial cells and ACE titers correlated with cell growth and proliferation [29,30], our report was not yet picked up by the scientific insect community. In the crustacean crayfish *Astacus leptodactylus* also 4 ACE isoforms were cloned from different tissues and a hemocyte specific variant occurs for which in agreement with our Locusta data, a putative role in defense regulation has been postulated [31]. In contrast, hemocytes of most insect species only display basal expression under healthy condition as is not only the case in our *Locusta migratoria* but as well in lepidopteran models as *Spodoptera* and *Bombyx* [26,32,33]. Even following a selective fungal challenge using the entomopathogenic fungus *Metharhizium acridum* this ACE expression enhancement did not pop up in a *Locusta migratoria* transcriptome screen [34]. Fortunately, our finding about ACE, needed for the appearance in the hemolymph of a pleithora of LPS inducible peptides, revitalized our efforts to pinpoint the role of Lom-ACE in immunity [35]. In this paper we focus towards the role of a hexamerin II derived peptide that conditionally appears in circulation provided an immune challenge by LPS and the presence of unattenuated circulating ACE activity. This Hex-12mer turned out to be depleted of apparent antimicrobial activity but displayed melanisation regulating activity. The herein named Locmi-antimelanin-I inhibits phenoloxidase in an uncompetitive fashion.

Materials and Methods

Gregarious *Locusta migratoria* were reared under controlled conditions regarding relative humidity (40-60%), photoperiod (13 hours light) and temperature (32 +/-1°C). They were housed in ventilated cages (0.5 m x 0.5 m x 0.5 m) at high density (100-200 animals per cage) to assure crowded conditions. We obtained the locusts from Sprinkhanenwinkel.com in the Netherlands using express mailing. Upon arrival, the adult locust aged 3 days (+/-24hrs) and was used during 2 subsequent weeks. Only males were used in the experiments and within each experiment all animals had similar age.

Following CO2 anesthesia and distal amputation of a leg from the mesothorax 10µl hemolymph was with

drawn using micropipette. To avoid coagulation hemoymph was diluted by mixing with 10 fold excess of cold anticoagulants buffer (17mM EDTA, 41mM citric acid, 186mM NaCl and 98Mm NaOH, pH 4,5).

For preparation of the phenoloxidase stock, freshly collected hemolymph was immediately diluted (5:95 v:v) in ice cold 10µM Potassium Phosphate buffer pH 5,9 and kept on ice until pelleting the hemocytes (4°C, 10K rpm, 5 minutes). Equal volumes of cell free diluted hemolymph and absolute methanol were mixed in order to maximally activate the Prophenoloxidase (PO) [36]. L-DOPA (Sigma, 3mg/ml 10µM Potassium Phosphate buffer, pH 5.9) was dissolved freshly and kept protected from light. Using polystyrene microtiter plates in individual wells 100 µl peptide solution in a serial dilution series and 20 µl of activated PO were preïncubated for 30 minutes after which 180µl of L-Dopa substrate was added. Absorbance change was measured at 90 seconds intervals for 33 cycles at 495 nm using Tecan Infinite 200 multiplate reader. Dopachrome concentrations were calculated using extinction coefficient of 3600 M-1cm-1 and path length of 6,22mm. As Dopachrome concentrations and absorbance changes intercorrelate we used absorbance data for the dose-response illustrations. For Kinetics analysis and calculation of inhibition constant of Locmi-antimelanin-I the assay was performed using variable substrate concentrations ranging from 2,5 mM up to 15 mM whereas now the peptide concentration was kept constant at 250µM. All enzyme reactions were done in triplicate and repeated by independent researchers. Averaged numbers obtained within single experiments were used to calculate the graphics. GraphPad library of nonlinear regression models was used to fit data sets and the software included Michaelis-Menten enzyme kinetics model was used. For Lineweaver-Burk plots linear regression with extrapolation to the x-axis was used.

Peptides were synthetized at GL Biochem (Shangai) Ltd. Both Hex-14mer, AVVPHSEAGKELLE and Hex-12mer, AVVPHSEAGKEL were ordered. For ACE resistance assay both peptides were incubated with rabbit lung derived angiotensin converting enzyme in a 100µl reaction mix containing 20µl HEPES-NaCl (40mM-300mM) pH8,3, 10µl rabbit lung derived ACE (40mU), 10µl 1mM HPLC purified peptide and 10µl 1mM Captopril). All chemicals were obtained from Sigma. Finally newly ordered, now called Locmi-antimelanin-I (AVVPHSEAGKEL) was used as crude extract in both the control experiments and the dose response curve. In all other experiments initiated for calculating enzyme kinetics 98% HPLC purified peptide was used.

Pre and post co-incubation with rACE, the Duressa et al predicted ACE dependent LPS induced Hexamerin-II precursor derived peptides were Ziptip purified (desalted) before analysis using in house Maldi-Ms (cour-tesy Kurt Boonen). The solubilized ACE enzyme preparation was submitted to Maldi-Ms analysis to exclude ghost peaks or peak masking due to interfering contaminants.

Hex-12mer peptide AVVPHSEAGKEL was tested for antibacterial/antifungal activity by incubating a 2 steps dilution series starting at 1mM in LB containing 0.1% (v./v) of overnight stock of either E.Coli, M.aureus and C.albicans in the presence of Resasurine [37]. In absence of microbial growth the microtiter plate should remain blue whereas in presence of bacterial growth the color changes to red-yellowish due to acidification/oxidation. To avoid misinterpretation it is important to keep DMSO, used to initially dissolve the peptides, below 2.5% at all times.

Results

To investigate further interaction and/or resistance to ACE, the hexamerin-II derived peptides Hex-14mer and Hex 12-mer were exposed to prolonged action of mammalian ACE (40mU final). Pre and post co-incubation with ACE, the Duressa et al. [35] predicted ACE dependent LPS induced Hexamerin-II precursor derived peptides were analysed using in house Maldi-Ms (courtesy Kurt Boonen). To avoid ghost peaks and peak masking by contaminants of the enzyme preparation, the latter was analyzed in a similar way (Figure 1).

After 24 hours incubation of HPLC purified Hex-12mer in the presence of an excess of rabbit lung ACE the original 12-mer peptide was still intact and no truncated dipeptides were tracable in the incubation mix (Data not shown). As such, we continued working with Hex-12mer only.

As explained in materials and methods we screened the Hex-12mer for bacteriostatic and bacteriocidal activity against both Gram negative and Gram positive bacteria as well as against the fungus *Candida albicans* using Resasurine to access microbial growth. In none of the experiments inhibition or retardation of microbial growth was observed, even not in the presence of a dose as high as 1mM, As such, these negative experiments are not shown.

Testing the Hex-12mer for interference in the Phenoloxidase assay a strong enzymatic inhibition was noticed. As this effect was hoped for but rather unexpected, we initially performed distinct control experiments. To assure the observed effect is due to the synthetized peptide moiety and not simply the result of interfering contaminants that resulted from Fmoc peptide synthesis (e.g. acidification) the initial experiments were repeated in the presence of comparable doses of similar grade C-terminal and N-terminal truncated Hex-10mer peptide.

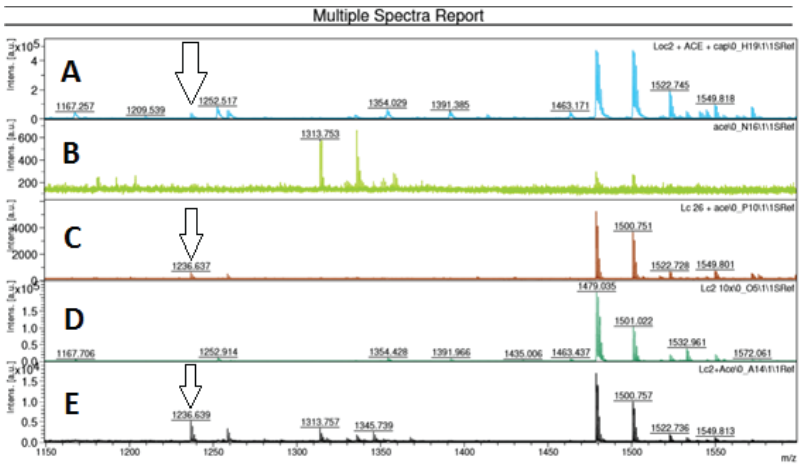

Figure1. Maldi-Ms analysis mammalian ACE affecting Hex-14-mer. (A) 1hour ACE treated Hex -14. (B) ACE control. (C) 8 hrs treated Hex-14. (D) untreated Hex-14. (E) 24 hrs treated Hex -14. Except for the controls (B) and (D) all samples displayed both the initial 14-mer peak at 1479 Dalton as well as the Hex-12 mer peak at 1236 Dalton (Arrow).

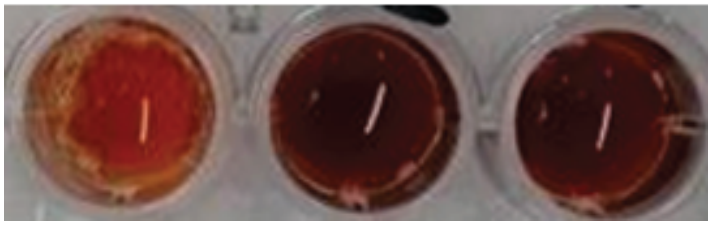

Figure 2. Assay validation: Prolonged phenoloxidase-melanisation assay in the presence of (1) Locmi-antimelanin-I (250µM), (2) 250 µM NAOH degraded and pH neutralized Locmi-antimelanin-I and (3) control without inhibiting peptide.

competitor (Data not shown). Secondly, alkali degradation and subsequent pH neutralization of our Hex-12mer peptide prior to incubation with the Phenoloxidase completely abolished inhibition of melanisation (Figure 2). As Hex-12mer definitely showed inhibition of melanisation by phenoloxidase interaction we called this peptide Locmi-antmelanin-I.

By use of a dilution series of Locmi-antimelanin-I ranging from 1mM, 500 µM, 250µM, 125µM, 62,2µM, 31,25 µM, 15,6µM, 7,3 µM a clear dose response inhibition of Phenoloxidase activity became evident. Compared to the control, in which the peptidic solution was replaced by an equal volume of phosphate buffer, each inhibitor concentration reduced the production of Dopachrome within the time frame of 25 minutes during which changes in absorbance at 495 nm of the reaction mix were followed (Figure 3).

Estimation of Michaelis Menten kinetics, determination of the inhibition constant (=dissociation constant (=dissociation constant in the presence of inhibitor) and conclusions regarding the mechanism of inhibition were done as explained in M&M. Hereto, phenoloxidase inhibition assays were performed in the presence of a fixed amount of enzyme and a fixed amount of inhibitor (250µM). The concentration of L-DOPA substrate varied between 2.5mM and 20 mM. Absorbance data used in the calculations were measured after 270 seconds reaction time. Same data were used to set out Lineweaver-Burk plots (Figure 4 and 5; Table 1).

Discussion

To study the role of circulating angiotensin converting enzyme, Lom-ACE, in Locusta migratoria we previously used both Captopril inhibition and RNAi knockdown under immune challenge conditions [35].This allowed the identification of ACE dependent LPS induced hemolymph peptides. Some of these peptides were traced back to their cognate precursor. Present study focused towards

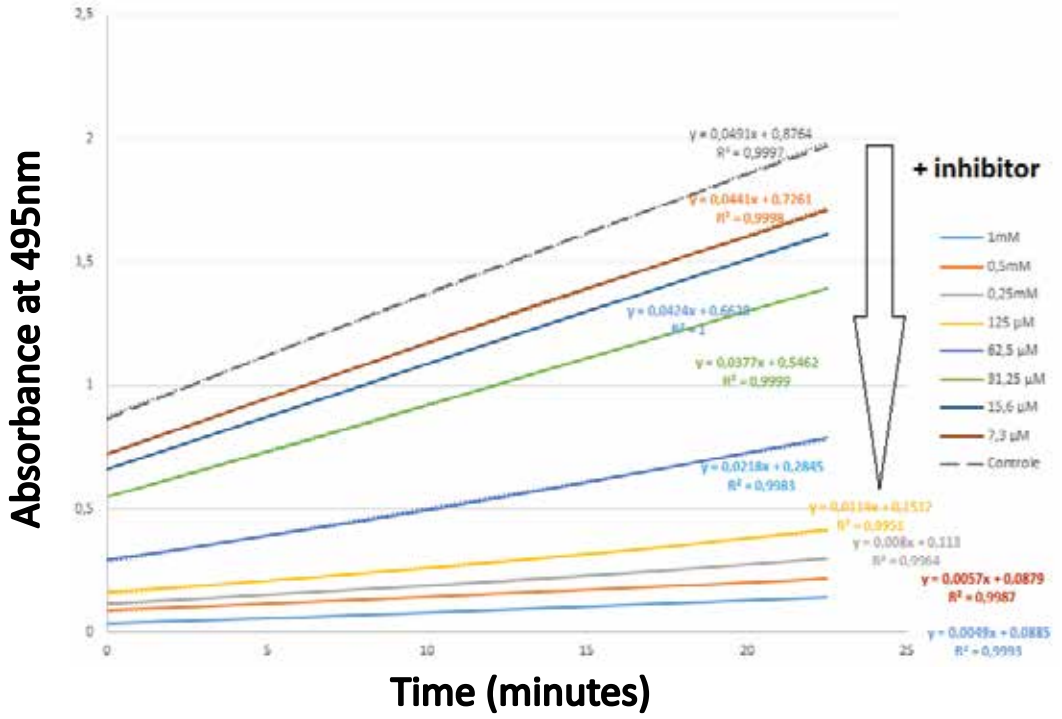

Figure 3. Dose dependent inhibition of Phenoloxidase by a dilution series of Locmi-Antimelanin-I starting at a maximal dose of 1mM. Changes in absorbance where measured over a 25 minutes time window. All data-points represent the average of 3 independent intra-assay reactions. Standard deviation was too small to be visualized graphically.

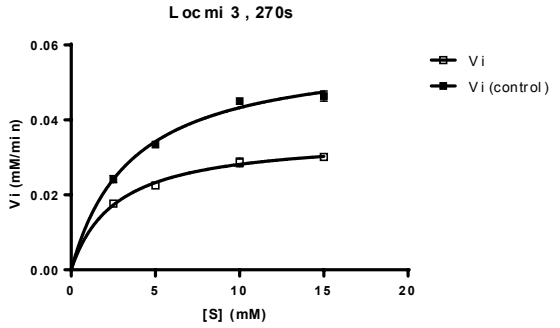

Figure 4. Michaelis Menten Kinetics using fixed amounts of phenoloxidase enzyme and inhibitor whilst changing L-DOPA substrate concentration. Notice the reduction in Vmax in the presence of Locmi-antimelanin-I inhibitor.

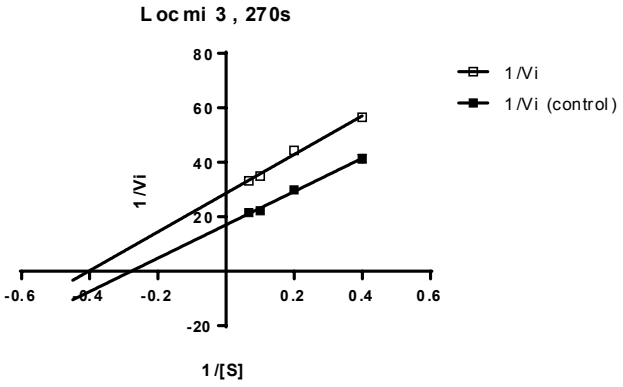

Figure 5. Lineweaver-Burk plots using similar data as used for Michaelis-Menten kinetics.

Table 1. Compared to the control (c) the presence of Locmi-antimelanin-I inhibitor (i) elicits a reduction in both Km and V-max. Calculations based upon Michaelis Menten plots and Lineweaver-Burk plots are comparable. Values for Km and Ki are in µM, values for Vmax are in µM/min.

Michaelis Menten				Lineweaver-Burk				
K_(m,i)	K_(m,c)	V_(max,i)	V_(max,c)	K_(m,i)	K_(m,c)	V_(max,i)	V_(max,c)	K_i
2650	3592	35,61	58,82	2481	3592	35	58	558

the physiological significance of one such described immune active and hexamerin-II derived hemolymph peptide AVVPHSEAGKELLE which we initially called HEX-14. Harsh treatment of Hex-14mer with rabbit derived ACE gave a C-truncated Hex-12mer peptide that was used in further experiments. Addition of this peptide in mM dose had no visible antimicrobial effect. Initial experiments using Captopril trying to prevent in vivo phagocytosis of fluorescently labeled heat killed bacteria by Locusta hemocytes were also ineffective (unpublished personal observation).

When testing the Hex-12mer for its modulating action towards the early innate response, in insects being melanisation, a substantial inhibition of Phenoloxidase was observed. This inhibiting activity disappeared following alkaline peptidic breakdown and neither the N or C- terminal truncated synthetic forms of this Hex-12mer/ Locmi_antimelanin-I peptide displayed similar inhibiting effects. A clear dose response inhibition was concluded (Figure 2). The calculated Ki of 558 µM seems reasonable high but more important is its mode of action: both Km and Vmax decrease indicating that our Locmi-antimelanin-I is an uncompetitive inhibitor. Accordingly, the Locmi-antimelanin-I inhibitor only binds the enzyme substrate complex E-S and changes it into an inactive enzyme-substrate-inhibitor complex E-S-I. Since the interaction between enzyme and its substrate starts almost instantly and product (in our case Dopachrome) is formed very fast our observation is in line with the expectations. E.g. a reported uncompetive inhibitor for adenosine deaminase (AD)has an LC50 value, of 350µM [38].

In addition to the first identified Phenoloxidase inhibitor isolated from housefly hemolymph [39] to our knowledge, this work represents the first report of an endogenous circulating immune induced, ACE dependent peptidic uncompetitive inhibitor of Phenoloxidase, the startup enzyme for melanisation. In insects, the PPO-PO cascade is recognized as one of the primary immune responses involved in both humoral and cellular defense against microbial attacks [40]. Importantly, in immune naïve insects, theoretically, solely an inactive Prophenolixidase (PPO) is traceable in the hemolymph which becomes activated almost instantly following immune challenge [41, 36]. In accord with earlier findings [35] our experiments strongly support the suggested role in the immune system of the circulating Locusta migratoria angiotensin converting enzyme orthologue (Lom-ACE). Earlier Lom-ACE had been demonstrated to be an LPS (and by extension bacteria) inducible zinc dependent carboxydipeptidase, originating in hemocytes [42,43] and fat body. Lom-ACE definitely plays key in the rapid appearance of peptides, herein illustrated by Locmi-antimelanin-I, which counteract the immune response overshoot. This demonstrated, post- inflammatory effect of Lom-ACE in no way excludes a pre-inflammatory role of Lom-ACE. Indeed, the functional characterization of immune induced ACE dependent regulatory peptides is initiated only recently. In mammals, ACE coordinates the inflammatory response mainly by clearing pre-inflammatory cytokines and stimulating post-inflammatory cytokines. Seen this similarity it might be tempting to suggest that Locmi-antimelanin-I, in addition to its evidenced role in downregulating PO, could display cytokine-chemokine activity in addition to previously identified cytokines as GBP [44-46], paralytic peptide, PP [47] and Diedel [48].

Conclusion

Angiotensin converting enzyme depending and LPS induced Locmi-antimelanin-I dose dependent downregulates circulating phenoloxidase and minimize melanisation. As melanisation in insects represents a primary innate immune response, Locmi-antimelanin-I has post inflammatory activity.

Abbreviations: HEX-14mer: AVVPHSEAGKELLE, Hex-12mer: AVVPHSEAGKEL, Locmi-antimelanin-I: *Locusta migratoria* anti-melanin-I peptide, Lom-ACE: Locusta migratoria angiotensin converting enzyme orthologue.

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Conflict of interest

The authors declare no conflict of interest.

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