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A quantitative peptidomics approach to unravel immunological functions of angiotensin converting enzyme in *Locusta migratoria*

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ABSTRACT

Locusta migratoria angiotensin converting enzyme (LmACE) is encoded by multiple exons displaying variable number of genomic duplications. Treatments of lipopolysaccharide (LPS) as well as peptidoglycan but not β-1-3 glucan resulted in enhanced expression of angiotensin converting enzyme in hemocytes of *Locusta migratoria*. No such effect was observed in fat body cells. Differential peptidomics using locust plasma samples post infection with LPS in combination with both an LmACE transcript knockdown by RNAi and a functional knockdown using captopril allowed the identification of 5 circulating LPS induced peptides which only appear in the hemolymph of locust having full LmACE functionality. As these peptides originate from larger precursor proteins such as locust hemocyanin-like protein, having known antimicrobial properties, the obtained results suggest a possible direct or indirect role of LmACE in the release of these peptides from their precursors. Additionally, this experimental setup confirmed the role of LmACE in the clearance of multiple peptides from the hemolymph.

1. Introduction

Angiotensin converting enzyme (ACE, EC 3.4.15.1) and its homologues are widely present in the animal kingdom and their vital roles in several physiological processes have been intensively investigated for several years (Bernstein et al., 2013;Fournier et al., 2012;Harrison and Acharya, 2014). The mammalian ACE, a Zn^{2+} dependent metalloprotease, is an important regulatory factor in the renin-angiotensin-aldosterone system (RAAS) for blood pressure and osmoregulation. ACE converts angiotensin I to the vasoconstrictor angiotensin II by cleaving a C-terminal dipeptide of angiotensin I (Bernstein et al., 2013). For such reasons, ACE inhibitors have been developed as drugs to counteract hypertension. The function of ACE, however, extends to processing of many other small hormonal peptides. ACE can readily hydrolyze the potent vasodilator bradykinin by sequential removal of dipeptides in the C-terminal region (Duncan et al., 2000;Ura et al., 1994). Apart from this, peptide substrates that are hydrolyzed by ACE include small hormones such as neurotensin and substance P (Skidgel et al., 1984), N-formyl-Met-Leu-Phe and enkephalins (Skidgel and Erdos, 1987), N-acetyl Ser-Asp-Lys-Pro (AcSDKP) (Azizi et al., 1996) and angiotensin 1–7 (Bernstein et al., 2013) which control physiological processes ranging from thermoregulation (Schwimmer et al., 2004) and hematopoiesis (Hubert et al., 2006) to nociception (Taniguchi et al., 1998;Zhang et al., 1982) and immune responses (Bernstein et al., 2013). Therefore, pharmaceutical use of ACE inhibitors has faced many limitations due to the diversity of ACE functions (Bernstein et al., 2013; Gonzalez-Villalobos et al., 2013;Harrison and Acharya, 2014).

The mammalian ACE exists in two isoforms: the somatic (sACE) and testicular (tACE) ACE which are integral membrane enzymatic proteins (Bernstein et al., 2013;Guang et al., 2012;Harrison and Acharya, 2014). The sACE contains two similar catalytic sites, each located separately on two homologous and tandem domains (N- and C-domain). The tACE consists of one functionally active domain which is highly similar to the sACE C-domain. While the tACE is solely expressed in spermatids with functional roles associated with male reproduction, the sACE expression is widespread in various tissues taking account of the diverse functions linked to ACE.

In contrary to mammals, insects have open circulation and lack all components, except ACE orthologues, of the RAAS system which are necessary for constriction/dilation of blood vessels and blood pressure enhancement (Fournier et al., 2012). Insect ACEs are single domain extracellular proteins similar to the tACE and the

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C-domain active site of sACE (Harrison and Acharya, 2014;Hens et al., 2002). In addition to expression in many tissues, the ACE orthologues in insects are presumed to act in peptide processing for many other physiological functions involving larval development (Isaac et al., 2007), heart and gut muscle contraction (Cornell et al., 1995;Tatei et al., 1995), neurotransmission (Isaac et al., 1998, 2009), digestion (Lemeire et al., 2008;Riviere et al., 2004), reproduction (Ekbote et al., 1999;Isaac et al., 1999;Schoofs et al., 1998;Vandingenen et al., 2002, 2001) and immune reactions (Aguilar et al., 2005; Macours et al., 2003). The main evidences suggesting the functional role of ACE in insects include premature death in *Drosophila* ACE (*Ance*) mutant larvae (Isaac et al., 2007), male *Drosophila* infertility due to homozygous hypomorphic *Ance* alleles (Isaac et al., 1999), and high ACE expression in various insect tissues including the neuronal tissues (Schoofs et al., 1998), gut epithelium and amniosera (Cornell et al., 1995;Tatei et al., 1995), reproductive tissues (Macours et al., 2003;Schoofs et al., 1998) and immune cells (Aguilar et al., 2005;Macours et al., 2003). Nevertheless, despite the empirical evidences, insect peptides subjected to ACE processing are still subjects of investigation.

The functional study of ACE in insects has wide implications, especially in our understanding of the multifunctional enzyme ACE in mammals. The diverse nature of ACE substrates implies that besides the convertase activity, the enzyme might indeed have a broad-range of enzymatic actions (Bernstein et al., 2013; Gonzalez-Villalobos et al., 2013;Harrison and Acharya, 2014; Macours et al., 2004). As a result, exploration of novel ACE peptide substrates along with structural studies might allow better understanding of pharmaceuticals targeting ACE and development of new substrate specific ACE inhibitors for therapeutic uses (Bernstein et al., 2013;Guang et al., 2012;Harrison and Acharya, 2014). Apart from this, characterizing such enzyme with potentially diverse roles in insects might as well be significant with regard to the possibility of designing new insecticides which target the enzyme itself (Harrison and Acharya, 2014;Wang et al., 2015).

Therefore, we pursued an ongoing in-house research (Macours et al., 2003) which emphasized the potential role of ACE orthologue in the innate immune system of *Locusta migratoria*, a swarming locust pest known across the globe for notoriously devastating agricultural crops. By employing quantitative peptidomics along with locust ACE (LmACE) gene knockdown, we identified 27 novel LmACE regulated peptides in the locust hemolymph. In combination with an immune challenge 12 of these peptides absolutely required LmACE for induction/suppression. Therefore, we report here the novel LmACE regulated peptides we identified. We further discuss the possible regulatory actions of LmACE and the potential roles of the identified LmACE regulated peptides in the locust immune system.

2. Materials and methods

2.1. Locust breeding and injection materials

The locusts, *Locusta migratoria*, were kept in crowded conditions as previously described (Duressa et al., 2015;Wang et al., 2013) in transparent cages which were placed in a ventilated room with controlled temperature (32 ± 1 °C), daylight photoperiod (13 h) and humidity (40–60%). They were fed with grass and dried oat every day. Adult locusts 5–10 days old after final molt were used for the experiment. Injection materials include 2 μg/μL lipopolysaccharide (LPS, Sigma-Aldrich), 1 μg/μL peptidoglycan (PGN, Sigma-Aldrich), 10 μg/μL β-1,3-glucan (Sigma-Aldrich), 1 mM captopril (Sigma-Aldrich) and 15 ng/μL target gene-specific dsRNAs. Experimental injections were delivered in total volume of 10 μL saline buffer NaCl/Pi (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2).

2.2. RNA extraction and cDNA synthesis

After decapitation, the locusts were dissected in the saline buffer NaCl/Pi to collect fat body tissues. The fat body in the dorsal abdomen was scraped from the cuticle and immediately frozen in liquid nitrogen. For hemocytes, 20 μL of hemolymph was collected in 180 μL anti-coagulant solution (98 mM NaOH, 186 mM NaCl, 17 mM Na_2EDTA, 41 mM citric acid, pH 4.5) on ice and immediately centrifuged at 10,000*g* for 10 min at 4 °C to discard the supernatant. The fat body and hemocyte samples, which included 4 replicates each containing a pool of 6 adult locusts, were then stored at −80 °C until use.

RNeasy® mini kit (Qiagen) for hemocytes and RNeasy® Lipid tissue mini kit (Qiagen) for fat body tissues were employed to extract total RNA. Genomic DNA contaminants were removed by DNase treatment (Qiagen). NanoDrop® was used to determine RNA concentration. For cDNA synthesis, a 20 μL mix of 1 μg RNA, 0.5 mM dNTP (Invitrogen™) and 250 ng random primers (Invitrogen™) in RNAse-free water was preheated at 65 °C for 5 min. After brief cooling and centrifugation, 4 μL of 5× first-strand buffer (Invitrogen™), 1 μL of 0.1 M Dithiothreitol (DTT, Invitrogen™), 1 μL of 40 U/μL RNaseOUT (Invitrogen™) and 1 μL of 200 U/μL SuperScript® III (Invitrogen™) were added to the mix. The reaction was setup by incubating the final mixture at 25 °C for 5 min followed by 50 °C for 1 h. Afterwards, the reaction was stopped by heating at 70 °C for 15 min. The resulting cDNA was cooled to 4 °C, diluted 10× with QRT-PCR grade water and stored at −20 °C until use.

2.3. Quantitative real-time PCR (QRT-PCR)

A list of locust housekeeping genes (Spit et al., 2014;Van Hiel et al., 2009;Vandesompele et al., 2002) in fat body tissues and hemocytes was tested for stable expression. According to GeNorm values, best reference genes which were selected for fat body tissues were tubulin alpha 1 (*tuba1*) and ribosomal protein s13 (*rps13*) and for hemocytes tubulin alpha 1 (*tuba1*) and CG13220. Primer-express software (Applied Biosystems) and Step-One Plus software (Applied Biosystems) were used to design and validate synthetic LmACE QRT-PCR primers (Sigma-Aldrich), respectively. Standard and melt curves were used to confirm the validity of the primers. The list of QRT-PCR primers can be found in Table 1. Each PCR was run in duplicates and contained 1 μL each of forward and reverse primers (10 μM), 10 μL SYBR Green (Invitrogen), 3 μL sterile water and 5 μL cDNA. Blank sample, PCR mix containing all but cDNA, was included as negative control. Inter-run cDNA calibrating samples were used to normalize variations between PCR runs. The QPCR data (C_t values) were recorded in real-time (Step-One Plus program) and exported to qbase$^+$ software (Biogazelle) for analysis.

2.4. LmACE gene mapping

LmACE sequences were obtained from *Locusta migratoria* NCBI protein (GeneBank AAR85358), mRNA (GeneBank AY487174) and genome (locusta[orgn]) databases. The following online software tools were used for mapping LmACE gene: GeneWise (EMBL-EBI) and PromoterWise (EMBL-EBI) for determination of exon sites, McPromoter006® for prediction of promoter site, Prop 1.0 server (Duckert et al., 2004) for prediction of signal peptide and TMHMM Server v. 2.0 (CBS, Denmark) for prediction of transmembrane helices.