Toxicon 141 (2018) 88–93

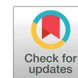
Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

Venomics reveals novel ion transport peptide-likes (ITPLs) from the parasitoid wasp *Tetrastichus brontispae*



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ARTICLE INFO

Article history:
Received 5 September 2017
Received in revised form
2 November 2017
Accepted 18 November 2017

Keywords:
Venom
Parasitoid
Neurohormone
Proteome
Evolution

ABSTRACT

Despite substantial advances in uncovering constituents of parasitoid venoms due to their potential applications as insecticides and pharmaceuticals, most of these studies are primarily restricted to braconid and ichneumonid wasps. Little information is available regarding virulent factors from venom of Eulophidae. In order to provide insight into the venom components of this family and parasitoid venom evolution, a venom protein repertoire (venomics) of the endoparasitoid wasp, *Tetrastichus brontispae* was deciphered using a proteomic approach. A large number of diverse venom proteins/peptides were identified, including novel proteins and those proteins commonly found in the venoms of other parasitoids such as serine protease, esterase, dipeptidyl peptidase IV, acid phosphatase, major royal jelly protein, superoxide dismutase, and venom allergen 3/5. Three ion transport peptide-likes (ITPLs) were abundantly detected in *T. brontispae* venom. Of these, two of them are reported as a novel form for the first time, with the characteristics of lengthened amino acid sequences and additional cysteine residues. These venom ITPLs are obviously apart from other general members within the crustacean hyperglycemic hormone/ion transport peptide (CHH/ITP) family. It implies that they would evolve unique functions essential for parasitism success.

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Venom, being a mixture of diverse compounds, is used by parasitoid wasps to manipulate host's physiology and development to support the growth of their progenies (Moreau and Asgari, 2015). Due to the unique biological function, parasitoid venoms are prospective recourses with potential as molecules in agricultural and pharmacological settings (Beckage and Gelman, 2004; Moreau and Guillot, 2005). Deciphering of components is of great value to facilitate the utilization of parasitoid venoms. Although parasitoids are one of the largest groups of venomous animals, there are only a small amount of data available for their venom compositions, most of which are restricted to families of Braconidae and Ichneumonidae (Moreau and Asgari, 2015). In addition to the common venom proteins across species, comparative analysis of unraveled venomics of parasitoids revealed that there is a remarkable inter- or intra-specific divergence (Colinet et al., 2013; Mathé-Hubert et al., 2016; Martinson et al., 2017). This invites investigations to explore venom composition of more different parasitoids, which will reveal novel toxins and provide insights into the routes of venom protein evolution across species.

Tetrastichus brontispae belonging to the family of Eulophidae (Hymenoptera) is a gregarious and koinobiont pupal endoparasitoid parasitizing the beetles of Chrysomeloidea, which was originally reported from Java, Indonesia (Meng et al., 2016). It has been introduced to many countries to efficiently control the seriously invasive coconut leaf beetle, *Brontispa longissimae* (Chen et al., 2010; Nguyen et al., 2012). Compared to the biological and ecological characterizations of this parasitoid that are relatively well known (Chen et al., 2010; Nguyen et al., 2012; Liu et al., 2014, 2016), there is still little information on the role of venom in the successful parasitism of its hosts. Only two recent studies demonstrated that parasitizaton by *T. brontispae* can alter the immune response and expression of immune-related genes of the nipa palm hispid beetle, *Octodonta nipae*, implying that venom of this parasitoid might have immunosuppressive function (Tang et al., 2014; Meng et al., 2016). Additionally, there are only limited literature recorded for venoms of several other eulophidae wasps with the

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https://doi.org/10.1016/j.toxicon.2017.11.008
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ability to mediate hosts physiological state and development (Coudron et al., 1999; Nakamatsu and Tanaka, 2003; Edwards et al., 2006). Besides a protein with native molecular weight of 66 kDa capable of arresting the host isolated from the venom of *Euplectrus comstockii* (Coudron and Brandt, 1996) and venom components recently revealed from *Chouioia cunea* (Xin et al., 2017), none of other toxins have been reported from the venoms of eulophid parasitoids. Development of knowledge regarding venom protein repertoire of *T. brontispae* will contribute to uncover strategies it used to suppress hosts immune system and development, and possibly provides an insight into the clear picture of eulophid venoms.

The crustacean hyperglycemic hormone/ion transport peptide (CHH/ITP) family peptides known as neurohormones are ubiquitous in ecdysozoans including both the arthropods (insects, chelicerata, crustaceans, and myriapods) and the nematodes, as well as lesser groups such as rotifers, cephalorhynchs, and onychophorans. This multi-gene family is well known in decapod crustaceans, where it contains neuropeptides with multifunction such as energetic metabolism, moulting, ionic and osmotic regulation, and reproduction (Fanjul-Moles, 2006; Webster et al., 2012). Based on structural and functional properties, it can be divided into two subfamilies, i.e. types I including CHH *sensu stricto* and CHH-related peptides, and type II consisting of moult-inhibiting hormone (MIH), gonad/vitellogenic-inhibiting hormone (GIH/VIH), and mandibular organ-inhibiting hormone (MOIH) (Lacombe et al., 1999; Webster et al., 2012). In insects, CHH family exists as ITPs and their homologs (often alternatively spliced products), ion transport peptide-likes (ITPLs), since the first member isolated from corpora cardiaca of the locust *Schistocerca gregaria* was able to stimulate chloride ion (Cl^-) transport causing enhanced fluid reabsorption (Audsley et al., 1992). Except for ion transport function, it is just known that silencing of ITP and ITPL can led to lethality during moulting, reductions in egg numbers and reduced survival of the offspring in the red flour beetle, *Tribolium castaneum*, and ITP can regulate wing expansion and cuticle melanisation in the brown planthopper, *Nilaparvata lugens* (Begum et al., 2009; Yu et al., 2016). Typically, insect ITPs and ITPLs were arranged in a monophyletic sub-group, designated as type III subfamily (Montagné et al., 2010). CHH/ITP peptides are widespread in venoms of spiders and centipedes (McCowan and Garb, 2014; Undheim et al., 2015). Regarding parasitoids, only one ITPL (venom protein 10) has been so far described in venom of *Microctonus hyperodae* by sequencing cDNA library of its venom gland (Crawford et al., 2008).

In this study, we analyzed the venom components of *T. brontispae* by proteomic approach, resulting in the identification of a large number of diverse venom proteins/peptides. Interestingly, three ITPLs named ITPLVn1-3 were found as toxins abundant in venom of this parasitoid, two of which are a novel form.

T. brontispae was reared using one day old pupae of coconut leaf beetle as described by Chen et al. (2010). Adult wasps maintained in groups were fed on 10% honey solution soaked in a piece of tissue paper. Female adults of three to five days old were subjected to venom sample collection and RNA isolation. Total RNA extracted from 30 female adults using Trizol Reagent (Invitrogen) according to the user manual was used to prepare the transcriptome library following the method of Zhu (2016). Library was sequenced on Illumina HiSeq™ 2000 platform at the Novogene (Beijing, China). Generated raw data have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number of SRX3156757, which was subjected to bioinformatic analysis as described by Zhu (2016). Venom reservoirs were isolated from about 150 female adults anaesthetized on ice in phosphate-buffered saline (PBS) contained a protease inhibitor cocktail (Sigma) with dissecting needles under a light microscope. After at least three washes using the same buffer to discard debris and haemocytes, each reservoir was transferred to a 1.5 ml Eppendorf tube containing 50 μl PBS. Reservoirs were disrupted by freezing the tube in liquid nitrogen to release the venom. Tube was centrifuged for 15 min at 12,000 g to remove residual tissues. For analysis of the venom protein profile, 5 μg venom from the supernatant was run on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and silver stained. To identify venom proteins, 40 μg venom protein was firstly reduced with 10 mM dithiothreitol followed by alkylation with 55 mM iodoacetic acid ammonium in dark. They were digested with the treatment of 1 μg trypsin at 37°C overnight. Peptides purified using C18 column were freeze dried and dissolved into sample buffer (3% acetonitrile, 0.1% formic acid). LC-MS/MS was performed on the AB SCIEX Triple-TOF 5600^+ system coupled with Ekspert NanoLC 400 system (Shimadzu) as described by Liu et al. (2017). Protein identification was also followed the method of Liu et al. (2017) using a protein database derived from the above constructed transcriptome of female *T. brontispae*. The label-free quantification method-iBAQ (intensity based absolute quantification) was carried out as described elsewhere for the quantification of identified venom proteins (Schwanhausser et al., 2011). Identified proteins were assigned by manual annotation to the functional categories as described by Dorémus et al. (2013). Gene sequences of ITPLVn1-3 were derived from the transcriptome mentioned above. Multiple alignment of amino acid sequences and phylogenetic analysis were respectively conducted by ClustalX (Thompson et al., 1997) and MEGA version 7.0.18 (Kumar et al., 2016), of which used sequences were listed in Table S1. Phylogenetic tree was visualized by FigTree v1.4.2. With respect to quantitative real-time PCR (qPCR) analysis, total RNAs were extracted from venom apparatus and the remnant body (carcass) of female adults using Trizol Reagent (Invitrogen), respectively. After DNase I (TaKaRa) digestion, they were used to synthesize the cDNAs with the qPCR Reverse Transcription Kit (Zoonbio Biotechnology). qPCR was performed on LineGene K (Bioer) with 2 × SYBR Green qPCR Mix (Zoonbio Biotechnology) under the reaction conditions described by Liu et al. (2017). Primers were designed based on the sequences of ITPLVn1-3 derived from the transcriptome database (Table S2). The endogenous control was 60S ribosomal protein L13a gene. qPCR analyses were performed with three technical replicates on three independent biological pools. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to evaluate the quantitative variation. Then, relative expression ratio of ITPLVn1-3 genes in venom apparatus and the remnant bodies was calculated by comparison their logarithmic scale expression values in these two different tissues. Data were statistically analyzed on Data Processing System (DPS) software using a one-way ANOVA analysis (Tang and Zhang, 2012). Differences were considered as significant when $P < 0.05$.

Illumina sequencing generated 60,284,736 raw reads (Q20 = 95.2%) with the GC content of 46.32%. After eliminating low-quality reads, the remaining clean reads were approximated 5.79 million. Trinity assembly yielded 51,453 transcripts and 40,815 unigenes, respectively (Table S3). The mean length of unigenes was 973 bp with N50 of 2,139 bp. BlastX searches produced 16,799 hits using a cut-off E-value of 10^{-5}, which were accounted for 41.15% of all assembled unigenes (Table S4). The highest match percentage (55.67%) was to *Nasonia vitripennis* sequences, followed by *Ceratosolen solmsi* (17.75%), *Lasius niger* (2.17%), and *Microplitis demolitor* (1.61%).

SDS-PAGE analysis revealed numerous proteins over the size of 14.4 kDa (Fig. 1). There were several thick bands with high molecular weight proteins (above 116 kDa) characteristic of parasitoids venom extracts (Leluk et al., 1989). Eleven major bands

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