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Identification and localisation of selected myotropic neuropeptides in the ventral nerve cord of tenebrionid beetles

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ABSTRACT

MALDI-TOF MS and MS/MS techniques were used for the isolation and identification of neuropeptides from the ventral nerve cord (VNC) of two beetle species *Tenebrio molitor* and *Zophobas atratus*. Two peptides, proctolin and myosuppressin (Zopat-MS), with well-established myotropic properties were identified as well as Trica-NVPL-4trunc. The presence of proctolin and myosuppressin was confirmed by immunocytochemical studies in adults and larvae of both beetles. In addition, the myosuppressin gene in *Z. atratus* was sequenced and expression analyses showed that it is present in all parts of the beetle central nervous system. Results suggest that the identified peptides act as neurotransmitters/neuromodulators in beetles, regulate visceral muscle contractions and indirectly influence important physiological processes such as feeding and reproduction.

1. Introduction

Neuropeptides are important messenger molecules that influence developmental, reproductive and behavioural processes in insects (Nässel and Winther, 2010; Marciniak et al., 2011b). Over the last few years a huge number of peptides have been identified from several insect species aided by advances in chromatography and mass spectrometry techniques and the availability of genome databases (Audsley and Weaver, 2003; Clynen and Schoofs, 2009; Predel et al., 2010; Audsley et al., 2011; Predel et al., 2012). In beetles, which comprise the largest insect order, the neuropeptide complement of *Tribolium castaneum* has been identified from the genome database and by mass analyses of neuro-endocrine tissues (Li et al., 2008) and a few additional sequences have also been identified in other species (Weaver and Audsley, 2008).

Previously, the sequences of several neuropeptides were identified from two major neuro-endocrine organs of the beetle *Z. atratus* (Marciniak et al., 2010), and the majority of these were myotropic peptides with well-established activities (Sliwowska et al., 2001; Wasielewski and Skonieczna, 2008; Marciniak et al., 2011a; Marciniak et al., 2012). In this study, we have focused on the identification of myotropic neuropeptides from the ventral nerve cord (VNC) of two tenebrionid beetles *Zophobas atratus* and *Tenebrio molitor*.

The VNC together with brain and *corpora cardiaca*/*corpora allata* complex (CC/CA) form the neuro-hormonal system of insects. The insect VNC consists of metamerically repeated ganglia in the thorax and abdomen (Nassel, 1996). In beetles, there are eight abdominal ganglia and three thoracic ganglia. Thus far, except in *T. castaneum*, no neuropeptides have been structurally characterised in this tissue in beetles. Moreover, only a few immunocytochemical studies have been performed in beetles, using antisera against proctolin, FMRFamide, tachykinins and allatostatins (Breidbach and Dircksen, 1989; Breidbach and Wegerhoff, 1994; Sliwowska et al., 2001; Audsley et al., 2013).

Proctolin (RYLPT) was the first insect neuropeptide to be identified from a whole body extract of *Periplaneta americana* (Starratt and Brown, 1975). Subsequently it has been found in several other insect species (Konopinska and Rosinski, 1999). In beetles, this peptide, together with a natural bioanalogue (R to A substitution at the N-terminus) has been isolated from *Leptinotarsa decemlineata* (Spittaels et al., 1995). A precursor for this pentapeptide was also identified in *Tribolium castaneum* by genome analysis (Li et al., 2008).

Leucomyosuppressin (Leuma-MS) is a decapeptide (pEDVDHVFLRFa) first identified in the cockroach *Leucophea maderae* (Holman et al., 1986). This neuropeptide belongs to the FMRFamide related peptide family (FaRPs) and has since been identified from several other insects (Orchard and Lange, 2006). The insect myosuppressins are characterized by a conserved sequence $X^1DVX^4HX^6FLRFamide$ where X^1 can be pQ, P, T or A; X^4 can be D, G or V; and X^6 can be V, S or I. In beetles, Leuma-MS was found in CC/CA complex of *T. molitor* (Weaver and Audsley, 2008), *T. brevicornis* (Gäde et al., 2008) and neurohaemal tissues of *T. castaneum*

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(Li et al., 2008). It has also been identified in the brain and retrocerebral complex of *Z. atratus* (Marciniak et al., 2010). Furthermore in the brain of this species, a second myosuppressin (Zopat-MS-2 pEDVEHVFLRFa) with glutamic acid (E) substitution for aspartic acid (D) at position four, was also identified (Marciniak et al., 2010).

The NVP-like peptides were first found in the brain of *Apis mellifera* (Hummon et al., 2006), and later in *T. castaneum* (Li et al., 2008) and *Rhodnius prolixus* (Ons et al., 2011), but their roles as neuropeptides are unclear. Recent studies have shown that they possess myotropic activity in beetles (Marciniak et al., 2011a) and may be involved in the control of feeding in *R. prolixus* (Sterkel et al., 2011). NVP-like peptides were also present in the CC/CA of *Z. atratus* (Marciniak et al., 2010).

In this study, we report the identification of myotropic peptides in the ventral nerve cord of two beetle species *T. molitor* and *Z. atratus* using mass spectrometry and immunocytochemical techniques. In addition, to confirm the occurrence of myosuppressin in *Z. atratus*, its precursor gene fragment was sequenced and the expression levels in neuro-endocrine tissues determined.

2. Material and methods

2.1. Insects

Zophobas atratus Fab. adults were obtained from a culture maintained at the Department of Animal Physiology and Development (Adam Mickiewicz, University in Poznan) and reared according to Quennedy procedure (Quennedey et al., 1995). *Tenebrio molitor* L. adults were reared as described previously (Rosinski et al., 1978).

2.2. RNA isolation and cDNA preparation

The brain, VNC, CC/CA, as well as the whole head and thorax of the adult *Z. atratus* beetles were microsurgically collected. Tissues were immediately placed in RNA stabilizing reagent (Novazym). Total RNA was isolated using RNeasy Plus Universal Mini Kit (Qiagen) according to the manufacturer's instructions. Prior to RNA isolation the tissues were homogenized in lysis buffer provided with the kit. Concentration and purity of RNA was assessed by spectrophotometric measurement of microdrops using a Synergy H1 Hybrid Reader (Biotek). RNA samples were then treated with DNase I (Thermo Scientific) and 500 ng of RNA was used as a template in a reverse transcription reaction using RevertAid™ Reverse Transcriptase (Thermo Scientific) and oligo-dT primers.

2.3. Primers design

In view of the lack of *Z. atraus* genome sequence, primers complementary to the myosuppressin precursor gene were designed based on the sequence of the gene encoding prepropeptide in *Tribolium castaneum*, which is a closely related species to *Z. atratus*. The *T. castaneum* peptide precursor gene sequence is available in the database Beetle Base (http://www.beetlebase.org/cgi-bin/report.cgi/BeetleBase3.gff3/?name=TC001469).

Three primers for the myosuppressin precursor gene were designed for amplification of a 271 bp fragment (used in identification of the sequence of myosupressin precursor gene) and a 156 bp fragament (used in Real-time PCR study). Primers for amplification of the gene for ribosomal protein RpS18, which was used as reference gene, were published by Toutges et al. (2010). Sequences of primers used in the study are presented in Table 1.

2.4. Identification of the myosuppressin precursor gene fragment

cDNA from the head and thorax of *Z. atratus* served as an template in the PCR reaction for amplification of the myosuppressin precursor gene fragment with two specific primer pairs M-Fa and M-R (Table 1).

Table 1
Sequences of primers used in the study and length of the amplified PCR reaction fragment of the gene.

Name	Sequence	Length of the fragment
M-Fa	5' CGTTCGTGGCTATTGTTTTTGG 3'	271 bp
M-R	5' TATAAACCGAAAGGTCTTCCGA 3'	
M-Fb	5' GACACCTTTGCTCTTTCATC 3'	156 bp
M-R	5' TATAAACCGAAAGGTCTTCCGA 3'	
RpS18-F	5' CGAAGAGGTCGAGAAAATCG 3'	235 bp
RPS18-R	5' CGTGGTCTTGGTGTGTTGAC 3'	

PCR reaction conditions were determined and optimized for amplification of the gene in *Z. atratus* cDNA. PCR reactions were performed in a total volume of 50 μL. The final mixture contained 1 μM primers, 200 μM dNTPs, 1X PCR buffer and 1 U/25 μL mixture of DreamTaq Polymerase (Thermo Scientific). The samples were amplified in 35 cycles. Each cycle consisted of the following steps: denaturing at 95 °C for 1 min (first cycle for 90 s), annealing at 56.9 °C for 1 min and extension at 72 °C for 1 min Reactions were performed in a DNA thermal cycler (Biometra). The amplification products were then separated in 2% agarose gel with the addition of ethidium bromide and visualized in a UV transilluminator. Products of the PCR reaction were cut and eluted from the agarose gel using MinElute Gel Extraction Kit (Qiagen), according the manufacturer's instructions. Purified fragments of DNA were then sequenced in the Laboratory of Molecular Biology Techniques, Faculty of Biology, Adam Mickiewicz University in Poznan. Obtained sequences were analyzed using Chromas Lite 2.01 and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) programs.

2.5. Real-time PCR

Real-time PCR was performed in the RotorGene 6000 (Corbett Research) using SYBR-Green I as the detection dye. Target cDNA was quantified using the relative quantification method. The amplification was performed with specific primers and their suitability of the reaction was tested by the melting curve analysis as well as gel electrophoresis of the PCR product. The quantity of the myosuppressin transcripts in each sample was standardized by ribosomal protein RpS18 transcript level. Real-time PCR reactions were performed separately for the investigated myosuppressin gene and for reference gene in a total volume of 20 μL. cDNA (2 μl) was added to 18 μl mixture of Power SYBR Green Master Mix (Applied Biosystems) and the primers M-Fb and M-R or RpS18-F and RpS18-R, which sequences are listed in Table 1. The conditions of the reaction were: hold at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing and elongation at 60 °C for 40 s, for 40 cycles. To establish the levels of given transcripts in the studied samples, standard curves were constructed with five subsequent ten-fold dilutions of linear DNA, which was the product of a given set of primers. Each reaction was conducted three times. Data were collected using the RotorGene 6000 software (Corbett Research) and statistically analyzed by one-way ANOVA (Graphpad Prism 6).

2.6. Nervous tissue extraction and liquid chromatography

A hundred abdominal ganglia from both beetles were separately dissected and placed into Eppendorf tubes containing 1 mL ice-cold 100% methanol. Tissues were infused at the room temperature for 15–30 min and then removed. Tissue extracts were diluted with ten volumes of 0.1% TFA for separation by reversed-phase high performance liquid chromatography (RP-HPLC). Separations were performed using a Beckman System Gold chromatographic system (Beckman Coulter Ltd., UK), comprising a dual pump programmable solvent module 126 and a System Gold UV detector module 166. Samples were loaded via a Rheodyne loop injector onto a Jupiter C_{18} 10 μm 300 Å narrow bore