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Functional characterization of the adipokinetic hormone in the pea aphid, *Acyrthosiphon pisum*

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ABSTRACT

Aphids are important plant phloem-sucking pests and detailed knowledge about the hormonal control of their metabolism can potentially contribute to the development of methods for their management. The insect metabolism is predominantly controlled by neuropeptides belonging to the adipokinetic hormone/red pigment-concentrating hormone family (AKH/RPCH). The main goal of this study was to obtain the sequence of AKH transcripts and analyze its expression in all polyphenic female forms of the pea aphid, *Acyrthosiphon pisum*. The neuropeptide is expressed in the brain of all female forms and in the ovaries of the both (wingless and winged) parthenogenetic forms. The form of active *Acypi*-AKH decapeptide was confirmed by the LC/MS and + ESI tandem mass spectrometry. The highest relative amount of *Acypi*-AKH was recorded in winged virginoparae. Furthermore, a potential role of this hormone when directly applied to the aphid was studied as well. Interestingly, no significant increase of trehalose in the wingless virginoparae after application of synthetic *Acypi-AKH* was detected. Yet this treatment did affect the level of protective polyol (mannitol) and furthermore led to increased activity of the detoxification enzyme glutathione S-transferase. The possible physiological function of AKH in *A. pisum* under the stress conditions is discussed.

1. Introduction

Aphids (Hemiptera, Aphidoidea) are a well-known group of plant sap-sucking insect pests, and the pea aphid, *Acyrthosiphon pisum*, is the only representative from this clade whose whole genome sequence is so far available (The International Aphid Genomics Consortium, 2010). This species has been the subject of a number of studies concerning the molecular aspects of its peculiar life cycle, i.e. alternation of sexual and parthenogenetic generations (e.g. Le Trionnaire et al., 2008), and also the identification of candidate neuropeptide hormones (Christie, 2008; Huybrechts et al., 2010). The latter area involved predictions based on certain characters being present in *in silico* translated preprohormones. Nevertheless, as predicted, most of them are presented by calculated masses only and verifying data from analysis of the *A. pisum* nervous tissue are missing (Huybrechts et al., 2010).

One group of these putative neuropeptides belongs to the adipokinetic hormone/red pigment-concentrating hormone family (AKH/RPCH). AKHs are synthesized and released by neurosecretory cells in the paired glands called the *corpora cardiaca* (CC). The presence and level of AKH preprohormone transcripts were determined for example in different tissues of the fall armyworm, *Spodoptera frugiperda* (Abdel-latief and Hoffmann, 2007) and the mosquitoes *Anopheles gambiae*, *Aedes aegypti* and *Culex pipiens* (Kaufmann and Brown, 2006; Kaufmann et al., 2009).

AKHs have been established as mobilizing factors for carbohydrates, lipids and proline in insects (summarized in Gäde and Auerswald, 2003). Thus, AKHs control the release of carbohydrates as the main "fuel" for pollen feeding Hymenoptera (Lorenz et al., 1999; Lorenz et al., 2001) and the fruitfly *Drosophila melanogaster* (Lee and Park, 2004; Isabel et al., 2005). Further two forms of AKH (*Peram*-CAH I and II) mobilize both carbohydrates and lipids in the cockroach *Periplaneta americana* (Michitsch and Steele, 2008). In locusts, these neuropeptides play a role in the switch from the initial use of the hemolymph carbohydrate pool as the fuel for flight to lipids (Goldsworthy, 1983). The red firebug, *Pyrrhocoris apterus* utilizes lipids mobilized by AKH as their only source of energy (e.g. Socha et al., 2004). A third major fuel for flight in insect, proline, has been shown to be regulated by AKHs in several beetle species (e.g. Gäde and Auerswald, 2003).

In addition, to energy mobilizing function, the AKHs control also a number of further actions on the physiological/biochemical levels e.g. stimulation of locomotion, regulation of foraging behavior and/or the activation of antioxidative defense mechanisms (summarized by Kodrík, 2008).

The metabolism of aphids is based on the intake of mono- and disaccharides from the plant phloem. Therefore they have to cope with the osmotic pressure from these simple sugars in the hemolymph by their polymerization into oligosaccharides in the gut. The aphids

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utilize phloem sugars, predominantly sucrose, as the main substrate for energy metabolism (Rhodes et al., 1996). Tilley et al. (2000) have reported the energy mobilization effect of purified peptide fraction from the aphid, *Megoura viciae* in heterologous asssay with *L. migratoria* (hyperlipaemic action) and cockroach, *Blaberus discoidalis* (hypertrehalosemic action). Apart from this study and the predicted sequence of the AKH ortholog in *A. pisum* (*Acypi*-AKH), pEVNFTPTWGQamide (Huybrechts et al., 2010), there is still little known about AKH in the aphids at present.

Therefore the main goal of the present paper was to clone the *Acypi*-AKH cDNA, analyze its transcription in different polyphenic forms of *A. pisum*, confirm and determine the structure and level of the bioactive peptide in CNSs of these forms, and investigate the effect of *Acypi*-AKH the carbohydrate metabolism and activities of selected antioxidative enzymes.

2. Materials and methods

2.1. Experimental animals

The *A. pisum* colony was established from aphids collected in České Budějovice (Czech Republic) in 1985 and maintained on broad bean plants grown on moist sawdust. All developmental stages were kept at a constant 22 ± 0.5 °C temperature under a 18:6 h light: dark photoperiod. For production of sexual morphs, ten reproductive apterous virginoparae were transferred to a box with a constant temperature of 19 °C and a 12:12 h light:dark photoperiod. The first sexuals were collected after five weeks from the third generation reared under these conditions. Three to seven day old females of three forms —wingless (apterous), winged (alatae) virginoparae, and sexual females (oviparae) were used in studies detecting the *Acypi*-AKH transcript, its relative expression and the subsequent quantification of the bioactive peptide level. Because of their naturally high abundance, the reproductive apterous virginoparae were used as the only form for verification of the predicted *Acypi*-AKH structure (see Section 2.5) and also for extensive bioassays with synthetic *Acypi*-AKH (see Section 2.7).

In some experiments (see Section 2.7), the effect of *Acypi*-AKH on mobilization of lipids was tested in the firebug, *P. apterus* (Kodrík et al., 2000).

2.2. Nucleic acid isolation

Prior to nucleic acid extraction, whole aphids were frozen in liquid nitrogen and homogenized with a ceramic pestle into fine powder. Genomic DNA was isolated from 46.5 mg of the reproductive wingless virginoparae and total RNA from whole reproductive females of all the studied polyphenic forms (30–150 mg per isolation) or their dissected body parts (see Section 2.3.4). Both the nucleic acid forms were extracted using TRI reagent® following the manufacturer's protocol. RNA isolates were treated with TURBO DNase (Ambion) to remove traces of contaminant DNA.

2.3. Nucleic acid analysis

2.3.1. 3′ RACE PCR

Reverse transcription was carried out using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen) on 2 μg of template RNA with an oligo-dT primer. The resulting cDNA was amplified by a subsequent PCR reaction using a 1 μL aliquot of the RT reaction as a template, a forward primer (AKH-F1: 5′ ACC GCA CGA GTC ATC CAA CGA AG 3′) that was designed based on AphidBase transcript ACYPI56723 and a reverse primer from the oligo-dT adapter. The PCR profile included initial denaturation (2 min at 94 °C), 30 cycles of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The RT-PCR products were analyzed by agarose gel electrophoresis, cloned into pCR4-TOPO vector (Invitrogen) and sequenced.

2.3.2. 5′ RACE PCR

The 5′-end of the *Acypi*-AKH transcript was detected using the GeneRacer Kit (Invitrogen) with 2 μg of the template RNA according to manufacturer's instructions. Reverse transcription was carried out with a gene specific primer (AKH-R1: 5′ ACT GCA GCT GAC AGA GTG TCA TG 3′) derived from the previously sequenced 3′ RACE PCR products. The subsequent PCR reaction was performed with the GeneRacer 5′ forward primer and *Acypi*-AKH-specific reverse primer (AKH-R2: 5′ TCC GTT GCG CTT CAT TCT GGA CCA GC 3′). The PCR profile comprised an initial denaturation (2 min at 94 °C), 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The RT-PCR products were analysed as described above.

2.3.3. Genomic DNA analysis

Genomic sequence corresponding to *Acypi*-AKH transcript was cloned into pCR4-TOPO vector (Invitrogen) and it was analysed using the EZ-Tn5 Insertion Kit (Epicentre Biotechnologies) according to the manufacturer's recommendations.

2.3.4. Expression profile of Acypi-AKH gene

Thirty reproductive females of each polyphenic form were dissected in Ringer saline and their organs/body parts were divided into four groups: 1. brains—central nervous systems with suboesophageal and fused thoracic ganglia, and the head capsulae remains; 2. whole guts —from foregut to the hindgut; 3. ovaries—complete with oviducts and common accessory glands (in the case of oviparae); 4. bodies—the remains without the previously dissected organs. The samples were rinsed in 100 μL of ice-cold RNAlater® solution immediately after the dissection, frozen in liquid nitrogen and stored at −80 °C prior to RNA isolation.

Reverse transcription was carried out as above (see Section 2.3.1) using 0.5 μg of the template RNA. The subsequent PCR reaction was performed using either primers specific for *Acypi*-AKH (AKH-F1 and AKH-R1) or for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH-F: 5′ TGC TGC CCA AAA CAT CAT TCC T 3′ and GAPDH-R: 5′ CTT CTG CGG CTT CCT TGA CTT 3′). The PCR profile was an initial denaturation (2 min at 94 °C), 30 cycles of 94 °C for 1 min, 64 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The RT-PCR products of size 494 bp for *Acypi*-AKH and 200 bp for GAPDH were detected by agarose gel electrophoresis.

2.3.5. Quantitative RT-PCR analysis

The cDNA template for Q-RT-PCR was prepared as previously described (see Section 2.3.1) with 1 μg of the corresponding total RNA. Q-RT-PCR was performed using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) on a C1000™ Thermal Cycler (CFX96 Real-Time System, Bio-Rad Laboratories). PCR reactions (12 μL) contained 3 μL of cDNA, 6 μL of iQ SYBR Green Supermix, 0.25 μL of each primer and 2.5 μL of H_2O. The Q-PCR program was as follows: initial denaturation for 3 min at 94 °C, followed by 30 s at 94 °C, 35 s at 61 °C, 40 s at 72 °C, 40 cycles. A final melt-curve step was included post-PCR (ramping from 61 °C – 95 °C by 0.5 °C every 5 s) to confirm the absence of any non-specific amplification. The efficiency of PCR for each primer pair was assessed using a serial dilution of concentrated cDNA (10, 50, 100 and 200 times). Each qRT-PCR experiment consisted of three independent biological replicates with three technical replicates for each parallel group. The results were analyzed using the Bio-Rad CFX Manager software, version 1.0.1035.131 (Bio-Rad Laboratories). The obtained Ct values were used in subsequent calculations using Microsoft Excel 2007 with the geNORM plugin manual method (http://medgen.ugent.be/genorm/).