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Identification of allatostatins in the brown-winged green bug *Plautia stali*

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ABSTRACT

Juvenile hormone (JH) biosynthesis is inhibited under short-day conditions in the brown-winged green bug *Plautia stali*. We investigated allatostatic molecules in the brain of *P. stali*. Methanol brain extracts strongly inhibited JH biosynthesis. The allatostatic activities of the brain extracts were heat stable but gently suppressed by trypsin treatment, indicating that the allatostatic molecules were peptides. Grybi-MIP1, found in *Gryllus bimaculatus* as an allatostatic molecule, inhibited JH biosynthesis in *P. stali*. In contrast, peptides such as Dippu-AST2, 8, and 9, found in *Diploptera punctata*, did not affect JH biosynthesis in *P. stali*. We found a cDNA sequence encoding a peptide precursor of myoinhibitory peptides (MIPs), which we named Plast-MIP. Three synthetic peptides, AWKDLSKAW-NH_2 (Plast-MIP1), GWSDLQSAGW-NH_2 (Plast-MIP5), and AADWGSFRGSW-NH_2 (Plast-MIP8), deduced from the precursor sequence, showed clear inhibition of JH biosynthesis in *P. stali*. Analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and tandem mass spectrometry showed that Plast-MIP8 resides in the brain. Expression of the Plast-MIP mRNA precursor was detected in the brain of insects reared under short- and long-day conditions. These results suggest that Plast-MIP is an allatostatic molecule and that MIPs are synthesized irrespective of photoperiod. To our knowledge, this is the first study to identify Plast-MIP as a functional allatostatin in hemipteran insects.

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1. Introduction

Juvenile hormones (JHs), synthesized and released by the corpus allatum (CA), are required for growth regulation and reproduction in insects. Many multivoltine insects living in temperate regions anticipate seasonal changes by photoperiod and adjust their growth and reproduction to occur when environmental conditions are favorable. It has been reported that seasonal growth and reproduction in insects are regulated by JHs, biosynthesis of which is affected by photoperiodic information (Denlinger et al., 2011). However, the neural mechanisms underlying the photoperiodic regulation of JH biosynthesis have yet to be elucidated.

Inhibitory and stimulatory factors of JHs have been reported in several species. Allatostatins (ASTs), short-neuropeptides F (sNPFs), and allatotropins (ATs), have been implicated in the regulation of JH biosynthesis (Bendena and Tobe, 2012). Bendena and Tobe (2012) have classified ASTs into three peptide families: (1) peptides with a consensus C-terminal sequence of Y/FXFGL-NH_2; (2) peptides with a C-terminal sequence of $W(X)_6W$-NH_2; and (3) peptides with a C-terminal sequence of PISCF, some of which are amidated at the C-terminus. The ASTs with a $W(X)_6W$-NH_2 sequence were first isolated from the migratory locust *Locusta migratoria* as myoinhibitory peptides (MIPs), which acted on the oviduct muscle and hindgut (Schoofs et al., 1991). Later, MIPs were reported as allatostatic molecules in the two-spotted cricket *Gryllus bimaculatus* (Lorenz et al., 1995). Although AST sequences have been found in many insect orders, their action on the inhibition of JH biosynthesis has been shown in few species, such as the cockroach *Diploptera punctata* (Woodhead et al., 1989), the tobacco hornworm *Manduca sexta* and *G. bimaculatus* (Kramer et al., 1991; Lorenz et al., 1995). Only in the silkworm *Bombyx mori* sNPFs and an AT have been shown to inhibit JH biosynthesis (Yamanaka et al., 2008; Kaneko and Hiruma, 2015); ATs are known stimulatory molecules in other insects (Bendena and Tobe, 2012). In addition,

Abbreviations: AST, allatostatin; AT, allatotropin; CA, corpus allatum; CC, corpus cardiacum; JH, juvenile hormone; mBr, median part of the brain excluding the optic lobe; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS/MS, tandem mass spectrometry; MIP, myoinhibitory peptide; PTTH, prothoracicotropic hormone; RpL32, ribosomal protein L32; sNPF, short-neuropeptide F.

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octopamine, dopamine, and serotonin have been shown to inhibit or stimulate JH biosynthesis, and their regulatory functions vary according to species or developmental stages within a species (Goodman and Granger, 2005).

Previous studies focusing the photoperiodic regulation of JH biosynthesis reported brain's regulatory role in several species. For example, in the linden bug *Pyrrhocoris apterus*, brain extracts stimulated *in vitro* JH biosynthesis of the CA and in *L. migratoria* brain extracts inhibited JH biosynthesis (Hodková et al., 1996; Okuda and Tanaka, 1997). However, the allatostatic molecules involved in the photoperiodic control of JH biosynthesis have not been identified in any species.

The brown-winged green bug *Plautia stali* has a novel JH, methyl (2R,3S,10R)-2,3;10,11-bisepoxyfarnesoate, also known as juvenile hormone III skipped bisepoxide ($JHSB_3$) (Kotaki et al., 2009, 2011). *P. stali* shows clear photoperiodic regulation of JH biosynthesis by the CA and reproductive diapause (Kotaki and Yagi, 1989; Kotaki, 1999; Matsumoto et al., 2013). In females reared under long-day conditions, JH production rates are higher than those reared under short-day conditions, and their ovaries are mature. The brain region including the pars lateralis and pars intercerebralis neurons, which innervate a complex composed of the corpus cardiacum (CC) and CA, inhibited *in vitro* JH biosynthetic activity by the CA (Matsumoto et al., 2013). These results suggested that allatostatic factors reside within the brain. Because no allatotropic activities are found in the brain, JH biosynthesis in *P. stali* is mainly regulated by allatostatic factors (Matsumoto et al., 2013). Moreover, starvation induces oosorption in *P. stali*, and this is caused by the brain's neural suppression of JH biosynthesis (Kotaki et al., 2016). The identification of allatostatic molecules within the brain is an important step needed to elucidate the neuroendocrine mechanisms underlying the photoperiodic or dietary regulation of JH biosynthesis. In the present study, we characterized and identified Plast-MIPs, which exert a strong inhibitory effect on JH biosynthesis in *P. stali*.

2. Materials and methods

2.1. Insects

The insects used in experiments were obtained from a stock culture of *P. stali* originally collected in Joso, Japan (36.1°N, 139.59°E) [described by Matsumoto et al. (2013)]. Insects were reared on raw peanuts and water supplemented with 0.05% sodium L-ascorbate and 0.025% L-cysteine. To prepare 80% methanol extracts of the brain, nymphs and adults were reared at 25 ± 1 °C under short-days (light: dark = 12: 12 h) (SD conditions) and long-days (light: dark = 16: 8 h) (LD conditions). A strain of *P. stali* that exhibits a more defined photoperiodic response than the Joso strain was used in quantitative PCR. This strain was collected using a synthetic pheromone derived from *P. stali* (Shin-Etsu Chemical, Tokyo, Japan) in Kawachinagano (34.27°N, 135.34°E), Japan in May, 2015. Kawachinagano strain was reared under the SD and LD conditions defined above.

2.2. Heat- and trypsin-treated brain extracts

To prepare the brain extracts, the median part of the brain excluding the optic lobe (mBr, Matsumoto et al., 2013) was dissected from females reared under SD conditions, 25 days after adult emergence (i.e., day 25). For the heat treatment, the mBr of 11 females was boiled in a 1.5 mL tube containing 40 μL distilled water for 15 min. Next, 160 μL methanol was added to the tube and homogenized. After centrifugation (10,000×*g* for 10 min at 4 °C), the supernatant was transferred to a new tube and freeze-dried under reduced pressure (FDU-1200, Rikakiki, Tokyo, Japan). The dried extract was stored at −80 °C.

For the trypsin treatment, the mBr of 11 females was homogenized in 200 μL 80% methanol in a 1.5 mL tube. After centrifugation (10,000×*g* for 10 min at 4 °C), the supernatant was transferred to a new tube and dried under reduced pressure. The dried mBr extract was stored at −80 °C. We dissolved 1 mg trypsin (porcine pancreas, 4100 USP Trypsin Units/mg; Wako Pure Chemical Industries, Osaka, Japan) in 1 mL 50 mM Tris-HCl buffer (pH 7.8) with 10 mM $CaCl_2$. The mBr extract was dissolved in 11 μL of the trypsin solution, or Tris-HCl buffer mixed with 10 mM $CaCl_2$. We used the Tris-HCl buffer and $CaCl_2$ because trypsin is activated well in the buffer (Kilby and Youatt, 1954). The extract in the trypsin solution was incubated at 37 °C for 16 h, and then incubated at 100 °C for 30 min to inactivate trypsin. As controls, Tris-HCl buffer, trypsin solution, and brain extract in Tris-HCl were also incubated at 37 °C for 16 h, and then incubated at 100 °C for 30 min (Fig. 1A).

2.3. RNA sequencing

Total RNA was extracted from the whole body of adult females of the Joso strain, reared under LD conditions and short-day (light: dark = 12: 12 h) at 20 °C (SD-LT conditions), using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The mRNA was sequenced (RNA-seq) using paired-end 100 bp reads on an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA, USA) by Hokkaido System Science Co., Ltd. The number of raw reads obtained was 68,677,345 × 2 for LD and 57,580,750 × 2 for SD-LT females. Sequences obtained from LD and SD-LT females totaled about 25 GB, and were combined and assembled *de novo* by Trinity (Grabherr et al., 2011). The 95, 224 resulting contigs (average length: 1217 bp; longest contig: 21,573 bp; shortest contig: 201 bp; N50: 2671 bp) included 63,744 components.

2.4. BLAST search and structural analysis

Candidate neuropeptide sequences of *P. stali* were queried in the transcriptome database as described in Tanaka et al. (2014). Briefly, amino acid sequences of neuropeptides of other insects were used as queries in a basic local alignment search tool (BLAST) analysis using the tblastn search function. GENETYX version 11 (GENETYX Software Development, Tokyo, Japan) was used to perform a local BLAST search on the assembled contigs. For the identification of signal peptides, we used SignalP 4.0 Server, freely available at http://www.cbs.dtu.dk/services/SignalP/. The predicted peptide sequences were named as reported by Coast and Schooley (2011) and are listed in Supplementary Table 1.

2.5. Preparation of peptides and amines

Ten peptides were used to test potential allatoregulatory effects. Dippu-AST2 ($AYSYVSEYKRLPVYNFGL-NH_2$) (Pratt et al., 1990), Dippu-AST8 ($GGSLYSFGL-NH_2$), and Dippu-AST9 ($GDDRLYAFGL-NH_2$) (Woodhead et al., 1989) from *D. punctata* were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Grybi-MIP1 ($AWRDLSGGW-NH_2$) (Wang et al., 2004) of *G. bimaculatus* and Plast-MIP1 ($AWKDLSKAW-NH_2$), Plast-MIP5 ($GWSDLQSAGW-NH_2$), Plast-MIP8 ($AADWGSFRGS-NH_2$) (GenBank accession No. LC147082), Plast-AST-C ($SYWKQCAFNAVSCF-NH_2$) (GenBank accession No. LC146486), Plast-AT ($GFKNVALSTARGF-NH_2$) (GenBank accession No. LC146488), and Plast-sNPF ($NSNRSPQLRLRF-NH_2$) (GenBank accession No. LC146523) from *P. stali* were newly synthesized (Funakoshi Co. Ltd., Tokyo, Japan). Octopamine, dopamine, and serotonin were also purchased from Sigma-Aldrich Corporation. These peptides, or amines, were added

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