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Molecular cloning and characterization of FGLamide allatostatin gene from the prawn, *Macrobrachium rosenbergii*

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ABSTRACT

Allatostatins are important regulatory neuropeptides that inhibit juvenile hormone (JH) biosynthesis by the corpora allata (CA) in insects. However, to date, the structure and expression of the gene encoding allatostatins have not been reported in any species other than insects. In this study, we used a combination of a semi-nested polymerase chain reaction (PCR) and screening of a central nervous system cDNA library of *Macrobrachium rosenbergii* to isolate and sequence a cDNA clone (2885 bp) encoding a 701 amino acid FGLamide allatostatin precursor polypeptide. This is the first reported allatostatin gene in crustacean. The deduced precursor was conceptually split into at least 35 FGLamide allatostatins at dibasic cleavage sites (Lys and Lys/Arg), far more than reported for any other known FGLamide allatostatin precursors from insects (13–14 allatostatins). Reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrated that the gene was expressed in the brain, gut, thoracic and abdominal ganglia, but not in the heart, muscle, ovary, gill, or hepatopancreas. Furthermore, developmentally-dependent expression of the gene was observed in the brain and thoracic ganglia of the prawn by using semi-quantitative RT-PCR analysis.

1. Introduction

Allatostatins are important regulatory neuropeptides that were initially identified by Woodhead et al. [50] and Pratt et al. [38] in 1989 from brain extracts of the cockroach, *Diploptera punctata*. The term allatostatin is derived from its specific function in inhibiting the biosynthesis of juvenile hormone (JH) by the corpora allata (CA) of insects [45]. JH plays a critical role in the regulation of larval development and control of reproduction in adult insects [20]. Structurally, these polypeptides can be subdivided into three different types: the type-A allatostatins (the FGLamide allatostatin superfamily) with a C-terminal Y/FXFGL-amide in common, first identified in cockroaches [38,50]; the type-B allatostatins (the W(X)$_6$W-allatostatins) with a common W(X)$_6$W-amide C-terminus, first identified in crickets [30]; and the type-C allatostatins (the Manduca sexta allatostatins) with the consensus sequences pEVRF/YRQCYFNPISCF-OH, first identified in the tobacco hornworm [25].

To date, more than 70 FGLamide allatostatins have been purified and sequenced from many insect species [5,6]. Although most of these allatostatins share a conserved C-terminal core sequence, Y/FXFGL/I/V-amide, which is essential for their inhibitory effect on JH biosynthesis [21,38,50], not all of them exhibit activity in the insect species from which they were isolated. The inhibitory action of the FGLamide allatostatins on JH-synthesis seems to be restricted to cockroaches, crickets and termites [4,30,37,49,50,51]. In addition, FGLamide allatostatins have been demonstrated to exhibit multiple functions in insects. Inhibition of spontaneous visceral muscle contraction has been demonstrated in the hindgut [27] and foregut [12] of cockroaches and the

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ileum of blowflies [16]. Restraint of the peristaltic movements of the oviduct was found in the locust *Schistocerca gregaria* [47], and in the cockroach *Blattella germanica*, FGLamide allatostatins affect egg development by inhibiting vitellogenin expression [32] and the release of vitellogenin from the periovarian fat body [31]. Moreover, immunocytochemical studies have revealed a wide distribution of FGLamide allatostatins in the nervous system and gut endocrine cells of various insects, which strongly suggests that these peptides act as neuro-transmitters and modulators that regulate physiological processes [8,15,41,44].

In addition to identification of the peptides, genes encoding FGLamide allatostatins have also been cloned and characterized in insects. In 1993, Donly first isolated and characterized a FGLamide allatostatin gene from the cockroach *D. punctata* [11]. Subsequently, many FGLamide allatostatin genes have been identified from Orthopterans [3,34,46], Dipterans [17,48], Lepidoterans [1,7,28,40], and Dictyopterans [3,9]. In nearly all investigated species, the allatostatins seem to be derived from a single polypeptide precursor except in the blowfly which has two FGLamide allatostatin genes [6,17]. This has enabled verification of the sequences of previously isolated allatostatins and facilitated identification of additional allatostatins. The precursors deduced from these FGLamide allatostatin genes are separated into several clusters by the acidic spacer regions. Each putative peptide sequence is flanked by G-K/R-R, an amide donor (Gly), and potential endoproteolytic cleavage sites [3,24].

In crustaceans, FGLamide allatostatins have been isolated and identified from three species: 20 from the crab, *Carcinus maenas* [13], 3 from the crayfish, *Orconectes limosus* [10], and 40 from the tiger prawn, *Penaeus monodon* [14]. Immunocytochemical studies have revealed FGLamide allatostatin abundance in the central and stomatogastric nervous system [10,14,42] of some crustacean species. Neurophysiological studies also have provided evidence of their role as inhibitors of the pyloric motor pattern [43] and stomatogastric neurotransmission [23] in the crab, *Cancer borealis*. In the crayfish *O. limosus*, three FGLamide allatostatins inhibit the frequency and amplitude of hindgut contractions [10]. Furthermore, in the crayfish *Procambarus clarkii*, allatostatins were shown to stimulate methyl farnesoate biosynthesis from the mandibular organs, which are homologous to the insect CA [26].

To further understand the functions of FGLamide allatostatins in crustaceans, it is necessary to characterize and study the spatial and temporal expression patterns of their genes. However, few such studies have been reported to date. In the present study, we isolated and identified the first FGLamide allatostatin gene from a crustacean, the prawn *Macrobrachium rosenbergii*, which contains at least 35 potential allatostatins. In both male and female prawns, the gene was differentially expressed in the brain and thoracic ganglia.

2. Materials and methods

2.1. Animals

The prawn, *M. rosenbergii* (7.2–35.0 g), of both sexes, was obtained from a market in Hangzhou, China, and reared in a 15 L glass aquarium with circulating freshwater at 25 °C. Prawns were acclimated for at least 2 weeks prior to experimentation and were fed twice daily. Prawns at different developmental stages were obtained from a commercial prawn farm in Hangzhou, China. The different developmental stages were determined according to previously reported criteria [35,52].

2.2. Nucleic acid preparation

The prawns were placed in an ice-bath for 1–2 min until they were lightly anesthetized. All tissues were removed and snap-frozen in liquid nitrogen. The resected tissues were homogenized in Trizol Reagent (Invitrogen, CA, USA) and total RNA was prepared according to the manufacturer's instructions. Genomic DNA for Southern blotting analysis was obtained using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions.

2.3. Preparation, polymerase chain reaction (PCR) screening, and hybridization screening of the cDNA library

PolyA RNA was purified from the total RNA of the prawn central nervous system (CNS, containing brain, thoracic ganglia and abdominal ganglia) using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). A λ-ZAP cDNA library of the CNS was subsequently constructed using the Uni-ZAP XR Library Construction Kit (Stratagene, La Jolla, CA).

Two degenerate primers (ASDP1 and ASDP2, Fig. 1) were based on the amino acids sequences (AGPYAFG and YSFGL) of known FGLamide allatostatins from the crayfish, *O. limosus* [10], the tiger prawn, *P. monodon* [14], and the crab, *C. maenas* [13] (Fig. 1). The vector-derived primer M13F (5′-CAGGAAACAGCTATGAC-3′) and the two degenerate primers were used in searching for the 3′ ends of the cDNA sequence. A 1 μl aliquot of the cDNA library was diluted to 100 μl with sterile water and, prior to addition to the reaction mixture, was incubated for 5 min at 95 °C. The reaction mixture (50 μl) contained the following: 1× reaction mixture buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of the vector primer M13F, 3 μM of the degenerate primer AS-DP1, and 5U Taq DNA polymerase (Promega, Shanghai, China). Following the initial reaction, semi-nested PCR was conducted using the first PCR products as the template and the degenerate primer ASDP2 and vector primer M13F. The obtained cDNA sequence was used to design gene-specific primers ASF1 and ASR1 (Fig. 1, Table 1).

For hybridization screening of the cDNA library, a fragment was amplified using the gene-specific primers ASF1 and ASR1, and labeled with digoxigenin–deoxyuridine triphosphate at 37 °C overnight using the DIG High Prime Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany). Hybridization was performed overnight at 42 °C using the DIG Easy Hyb system (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The blots were washed twice at room temperature at low stringency (2× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS)) for 5 min and then at 55 °C twice at high stringency (0.1× SSC, 0.1% SDS) for 15 min. After washing, hybridized probes were detected