

โครงการ การศึกษาคุณลักษณะและการกระจายตัวของเลปตินและเปปไทด์ในกุ้งก้ามกราม

Characterization and localization of leptin peptide and its encoding gene in the giant freshwater prawn, *Macrobrachium rosenbergii*

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สัญญาเลขที่ MRG/55/80018

รายงานวิจัยฉบับสมบูรณ์

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

Acknowledgement

I would like to acknowledge the support of many people, without whom this project would not have been accomplished. First, I would like to deeply express my gratefulness and deep appreciation to my mentor, Prof. Prasert Sobhon for his kindness, encouragement, unlimited advices, extensive supports, and for the revision of this research project and my manuscripts. I am deeply grateful to Prof. Peter J. Hanna for his valuable advices and research directions.

I am extremely grateful to Asst. Prof. Yotsawan Tinikul and Dr. Ruchanok Tinikul, for their help, valuable advices and companionship. My special thank is also expressed to Asst. Prof. Panat Anurachpreeda for his helps and suggestions, that help to create a friendly atmosphere for working in the Electron Microscopy and Cell Biology Lab in the Faculty of Science, Mahidol University, during my experiment.

I wish to extend special thanks to members in the Electron Microscopy and Cell Biology Laboratory, Department of Anatomy, Faculty of Science, Mahidol University for their kind assistance in my work and technical supports. I would also like to thank all the staffs of Department of Anatomy, Faculty of Science, Mahidol University, for their help and kindness.

I would like to acknowledge the financial support from the Thailand Research Fund (TRF), the Commission on Higher Education (CHE), and Mahidol University) to Jaruwan Poljaroen for support my research.

Jaruwan Poljaroen

Abstract

Project Code: MRG/55/80018

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Project Period: 2555-2557 เป็นเวลา **2** ปี

Abstract

Leptin, a highly conserved adipocyte-derived hormone, indicates the status of fat storage which is linked to food intake, energy homeostasis as well as gonadotropin release, gonadal maturation in both mammals and vertebrates. In *Macrobrachium rosenbergii*, we hypothesize that leptin is also present and may also act as link between nutrition and lipid status and gonadal development. In this study, the existence of leptin peptide in prawn tissues was verified by using Western blotting and immunohistochemical technique. The immunoreactivity (ir) of leptin-like peptide was detected in the adipocytes surrounding brain, and thoracic ganglion. In gastrointestinal tract, there was intense ir of leptin-like peptide in apical surface of the epithelial cells of the cardiac and pyloric stomach. In hind gut, the leptin-like peptide ir was found only in the cytoplasm of adipocytes and lamina propria. In hepatopancreas, there was no ir of leptin-like peptide in the hepatocytes when compared with the controls. In ovary, the intense leptin-like peptide ir was particularly found in the cytoplasm of oocyte stage III and IV, whereas, no positive staining detected in early stage of oocyte as well as in the follicular cell. For western blot analysis, the leptin-like peptide was mainly detected in brain, thoracic ganglia, and abdominal ganglia, the gastrointestinal tract, and also in the gonad.

In summary, it was suggested that leptin-like peptide was present in the prawn species. However, this is the first report for existence of this peptide in the prawn. It was also need to characterize the leptin encoding gene for the further study. The result of this study could improve the basic knowledge of hormonal regulation in the prawn reproduction in relation to nutrition and lipid status, and in helping to find the way to stimulate the gonadal maturation, increased fertilization rate, and the production of the prawn larvae for aquaculture.

Keywords: leptin, central nervous system, gonad, *Macrobrachium rosenbergii*

บทคัดย่อ

รหัสโครงการ: MRG/55/80018

ชื่อโครงการ: การศึกษาคุณลักษณะและการกระจายตัวของเลปตินและเปปไทด์ในกุ้งก้ามกราม

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บทคัดย่อ

เลปติน (Leptin) เป็นฮอร์โมนที่สร้างจากเซลล์ไขมัน (adipocyte-derived hormone) ซึ่งเป็นตัวบ่งชี้การกักเก็บไขมันในร่างกายและมีความเกี่ยวข้องกับการกินอาหาร ภาวะสมดุลทางพลังงานในร่างกาย รวมถึงการหลั่งฮอร์โมนที่เกี่ยวข้องกับการเจริญของระบบสืบพันธุ์ทั้งในสัตว์เลี้ยงลูกด้วยนมและสัตว์ที่ไม่มีกระดูกสันหลังได้แก่ การหลั่ง Gonadotropin เป็นต้น อย่างไรก็ตาม ในสัตว์ประเภท crustacean ซึ่งก็คือกุ้งก้ามกรามนั้นยังไม่มีหลักฐานการศึกษาวิจัยที่ยืนยันการมีอยู่ของฮอร์โมนชนิดนี้ ดังนั้น ผู้วิจัยจึงได้ตั้งสมมติฐานว่าเลปตินสามารถพบได้ในกุ้งชนิดนี้ และอาจมีความเกี่ยวข้องระหว่างภาวะโภชนาการ สภาวะของไขมันในร่างกาย และการเจริญของระบบสืบพันธุ์กุ้ง ในการศึกษาครั้งนี้ผู้วิจัยได้ทดสอบสมมติฐานที่กล่าวมาข้างต้นด้วยการตรวจสอบการมีอยู่ของเลปตินในเนื้อเยื่อประสาท เนื้อเยื่อทางเดินอาหาร และระบบสืบพันธุ์ของกุ้งด้วยวิธี Western blotting และ immunohistochemistry ผลการศึกษาพบว่า มี immunoreactivity (ir) ของเลปตินในเซลล์ไขมันที่ล้อมรอบสมองและปมประสาทอก และพบ ir ของเลปตินในเซลล์ประสาทขนาดกลางภายในปมประสาทอก สำหรับระบบทางเดินอาหารส่วนต้น (Foregut) พบ ir ของเลปตินที่บริเวณยอดของเซลล์เยื่อบุผิวของกระเพาะอาหารส่วน cardiac และ pyloric สำหรับในระบบทางเดินอาหารส่วนปลาย (hind gut) พบเลปตินอยู่ภายในไซโตปลาสมของเซลล์ไขมันที่บริเวณชั้นนอกสุดของท่อทางเดินอาหาร (lamina propria) นอกจากนี้ไม่พบ ir ของเลปตินในระบบทางเดินอาหารส่วนกลาง (Midgut) ได้แก่ hepatopancreas สำหรับการย้อม immunohistochemistry ของเลปตินในระบบสืบพันธุ์ พบว่ามีเลปตินจำนวนมากในไซโตปลาสมของเซลล์ไข่ระยะที่ 3 และ 4 แต่ไม่พบในไข่ระยะต้นรวมทั้งใน follicular cell ด้วย เมื่อทำการวิเคราะห์การมีอยู่ของเลปตินในกุ้งด้วยวิธี western blot analysis สามารถตรวจพบเลปตินอยู่ในระบบประสาทกลาง ได้แก่ สมอง ปมประสาทอก ปมประสาทท้อง ระบบทางเดินอาหาร และอวัยวะสืบพันธุ์.

จากผลการศึกษาสรุปได้ว่ามีฮอร์โมนเลปตินอยู่ในกุ้งก้ามกราม ซึ่งงานวิจัยนี้เป็นการศึกษาครั้งแรกที่พบฮอร์โมนชนิดนี้ในกุ้ง อย่างไรก็ตาม ข้อมูลเกี่ยวกับเลปตินยีนในกุ้งก้ามกรามยังต้องมีการศึกษาต่อไปในอนาคต ผลการศึกษาที่ได้จากงานวิจัยในโครงการนี้จะเพิ่มพูนองค์ความรู้และข้อมูลพื้นฐานเกี่ยวกับการควบคุมการหลั่งฮอร์โมนในระบบสืบพันธุ์ ซึ่งมีความเกี่ยวข้องกับภาวะทางโภชนาการ และอาจนำไปใช้พัฒนาต่อยอดไปสู่การกระตุ้นการเพิ่มผลผลิตกุ้งในเชิงพาณิชย์ได้

คำสำคัญ: leptin, ระบบประสาท, อวัยวะสืบพันธุ์, กุ้งก้ามกราม

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Introduction

Leptin is a hormone that was first detected in mammals (Zhang et al., 1994), and it was found to play a role in linking nutritional status with growth and reproduction (Budak et al., 2006). Leptin is a 167 amino acid secreted peptide (with molecular weight at 16 kDa) that is found mainly in adipose tissue. It informs the brain about the status of the metabolic store of fat in adipose tissue (Zhang et al., 1994; Halaas et al., 1995; Pelleymounter et al., 1995). Leptin acts as a major regulator for food intake and energy homeostasis. Leptin was synthesized in adipose tissue before being release to the blood circulation (Sinha et al., 1996; Casanueva and Dieguez, 1999). Leptin can bind to a membrane receptor (Ob-R) of arcuate neurons in the hypothalamus, possibly Kiss neurons, that control the release of GnRH and gonadotropins (Yu et al., 1997; Barb and Kraeling, 2004; Zieba et al., 2005; Smith et al., 2006). In the brain, leptin suppresses NPY expression in the arcuate nucleus with a consequent inhibition of food intake (Caprio et al., 2001; Moschos et al., 2002). In addition, the presence of leptin receptor in the peripheral organs of the reproductive system suggests that leptin may have also a direct effect on the downstream endocrine targets of the reproductive axis, such as anterior pituitary, ovary, testis, uterus, placenta, and adrenal glands (Caprio et al., 2001; Moschos et al., 2002; Cassy et al., 2004).

In vertebrates, leptin is the product of the obesity (ob) gene, which is produced from adipose tissue (Cunningham et al., 1999). Leptin can inhibit appetite and food intake (Budak et al., 2006). Leptin can stimulate Kiss-neurons in hypothalamus, and indirectly control the GnRH neuron (Smith et al., 2006). Ghrelin is the peptide hormone synthesized by gastric mucosa in the stomach and has function relate to appetite (Gallas et al., 2011), thus acting in opposite to leptin. It was suggested that both leptin and ghrelin may act on intermediate neurons (possibly Kiss and Neuropeptide Y, NPY) for controlling the release of GnRH (Fernández-Fernández et al., 2005; Budak et al., 2006; Smith et al., 2006; Lebrethon et al., 2007; Anubhuti, 2008). Through this circuit, leptin can control the GnRH neurons of hypothalamus in stimulating the release of gonadotropins, such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) and affect on the reproduction (Cunningham et al., 1999; Weil et al., 2003; Anubhuti, 2008). Similar pathway may be conserved in crustaceans which will be subjects of future studies.

At present, there was no evidence about the existence of leptin peptide in any invertebrates including decapod crustaceans. However, leptin receptor gene was first identified in the Chinese mitten crab, *Eriocheir sinensis* (Jiang et al., 2010). A partial sequence has been identified from the EST hepatopancreas library in this species which

shares high sequence identity to leptin receptor gene from vertebrate species (i.e. *Xenopus levis*, *Nasonia vitripennis* and *Macaca mulatta* etc.) (Jiang et al., 2009).

The leptin receptor gene expression had been detected in various tissues, including intestine, thoracic ganglia, cranial ganglia, hepatopancreas and gonad, indicating that leptin receptor may be involved in the regulation of metabolism and reproduction in Chinese mitten crabs (Jiang et al., 2010). In *Lymnaea stagnalis*, the leptin-like factor or Lymnaea storage feedback factor (LySFF) was produced by glycogen storage cell (Jong-Brink et al., 2001). This factor can inhibit food intake in this species. Moreover, it has been proposed that NPY neurons in Lymnaea may have receptors for LySFF for regulating energy homeostasis.

From the evidences in many of the above mentioned studies and in this prawn, *Macrobrachium rosenbergii*, we would like to hypothesize that leptin peptide is also present in decapods crustacean and that it plays important regulatory role in food intake, growth as well as reproduction as in fish and mammals. Thus, we aim to prove the existence of this hormone, and localize the peptide in the central nervous system, gastrointestinal tract, hepatopancreas, adipose tissue and gonad of the giant freshwater prawn using Western blotting and immunohistochemical analyses of the peptide.

Objectives

The goals of this project are to prove the existence of leptin in the giant freshwater prawn, *Macrobrachium rosenbergii*. In order to reach the goals as mentioned, the objective is to localize the leptin peptide in the central nervous system, gastrointestinal tract, adipose tissue and gonad of the prawn by immunohistochemistry and Western blotting.

Materials and methods

Animals

The mature male and female *M. rosenbergii* were purchased from the commercial farms at Ayutthaya province, Thailand.

Tissue preparation

For light microscope immunolocalization, tissues from adult prawn including brain/supraesophageal ganglion, thoracic ganglia, abdominal ganglia, hepatopancreas, adipose tissue, gastrointestinal tract, gonad (testis and ovary) and muscle were collected and preserved in a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) pH 7.4.

Leptin immunohistochemistry

Immunoperoxidase

All fixed specimens as mentioned above were dehydrated through a graded series of ethanol (70%-100%) for 30 min, cleared with xylene, infiltrated and embedded in paraffin wax. For immunoperoxidase staining, a 5 µm thick paraffin sections of each specimen were deparaffinized with xylene, rehydrated through a graded series of ethanol (100%-80%), and finally in 70% ethanol containing 1% Lithium carbonate ($LiCO_3$) for 20 min. Subsequencely, the endogenous peroxidase was blocked by treating the sections with 3% H_2O_2 in methanol for 45 min. Then, the sections were covered with 4% normal goat serum (NGS) in 100 mM phosphate buffered saline, containing 0.4% Triton-X 100, pH 7.4 (PBST), for 2 h. Following the blocking step, the consecutive sections were incubated in polyclonal anti-human leptin antibody (Santa Cruz Biotechnology. Inc., CA, USA) for overnight, at room temperature. After incubation, the sections were washed two times with PBST and PBS, and then incubated in the HRP-conjugated goat anti-rabbit IgG as the secondary antibodies, for 3 h, at room temperature. Finally, the color reactions were developed by incubating the sections in Nova Red substrate (Vector Laboratory, Burlingame, Calif., USA), until a red color was observed, washed with tap water, counter-stained with Hematoxylin and mounted in the permount solution. The sections were observed and photographed by a Nikon microscope equipped with digital camera DXM 1200. The pattern of immunolabelling in each type of tissue was analyzed by comparing with the unstained histological structure of that tissue as observed under the light microscope.

Immunofluorescence

Immunofluorescence techniques were used for the detection of leptin immunoreactivity in various parts of the CNS, hepatopancreas, gastrointestinal tract, adipose tissue, gonad and muscle. Prior to dissection of the organs, the prawns were anesthetized on ice for 5 min. All tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at 4°C for overnight, for leptin detection. After fixation and paraffin-embedding, the tissue sections were cut at a thickness of 5 µm, mounted on slides coated with 3-aminopropyl triethoxy-silane solution (Sigma-Aldrich), and then were processed for immunofluorescence detection. The sections were cut from fixed tissue blocks as mentioned above. Non-specific binding was blocked by incubating fixed tissue sections in 4% BSA for 30 min. Then, sections were incubated in antibody against leptin (Santa Cruz Biotechnology. Inc., CA, USA) at room temperature for 1 h, washed 3 times

with 0.1M PBS for 5 min each, followed by incubation in secondary antibodies, Alexa488-conjugated goat anti-rabbit IgG, washed 3 times with 0.01 M PBS for 5 min each, and mounted in buffered glycerol solution. Finally, the sections were observed under a fluorescence microscope and a confocal laser-scanning microscope. Sections with omitted primary antibodies were used as the controls.

Detection of leptin by immunoblotting

Separation of proteins by SDS-PAGE

Tissue homogenates (10% w/v) were performed with 10 mM HEPES pH 7.4 using ground glass homogenizers. Immediately following homogenization 2 ml of PMSF were added. Proteins were estimated by Lowry protein assay. To prepare samples for SDS-PAGE, 6x sample loading buffer [final concentration 7.5% v/v glycerol, 62 mM Tris, 2.5 mM Na_2EDTA, 1% (w/v) SDS, 0.05% (w/v) bromophenol blue, 100 mM DTT (dithiothreitol)], protein, and distilled water were combined to a constant volume of sample per lane. Before loading, samples were boiled for 10 min at 100°C. Gels were run at 290 volts for 10 min before loading (which heated them to approximately 40°C). Samples were separated on a 15% gel with a 4% stacker at 290 volts for 2.5 h. All buffers and gel systems used were those of Laemmli (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R250 overnight and then destained with destained solution (100 ml Acetic acid, 100 ml Methanol, 800 ml dH_2O) for overnight.

Identification of leptin by Western blotting

The presence of leptin hormone was determined in each organ of the prawn by immunoblotting. Briefly, the protein bands in SDS-PAGE gel from each organ were transferred to polyvinylidene fluoride (PVDF) membrane. After air-drying, the prepared membrane were washed with distilled water and then incubated with 5% skimmed milk in 0.1% Triton X solution and PBS for blocking non-specific binding. After that, the membranes were incubated in 1:100 dilution of anti-human leptin antibody (Santa Cruz Biotechnology. Inc., CA, USA) on shaker for overnight, at room temperature. After washing, the membranes were incubated in goat anti-rabbit IgG conjugated to HRP (Sounthern Biotech) diluted 1:1000 for 2 h. The protein bands that contain leptin immunoreactivity was visualized by incubating with TMB substrate kit (Pierce, USA) for 5 min before exposing onto the film. The molecular weight of leptin band was estimated by comparing with standard marker proteins.

Results

Histological structure and Immunoperoxidase staining

The histological structure of the brain has been shown in Figure 1. It is composed of three main parts including protocerebrum, deutocerebrum and tritocerebrum. Each part composed of several neuropils and neuronal clusters that have an important role for controlling the motor and sensory neural pathway of the prawn. The segmental thoracic ganglia consist of five ganglia, which are named T1-T5 from cephalic to caudal, respectively (Fig. 3A). The fibers from thoracic ganglia innervate the walking legs, musculature of thorax, and heart. The abdominal ganglia are divided into 6 ganglia which are A1-A6 from cephalic to caudal part, respectively (data not shown). Each ganglion innervates swimmerets, the swimming legs (pleopods), extensor, and flexor muscle of the trunk. In addition, brain and thoracic ganglia play major role in controlling the sensory reception and motor functions of both somatic muscle (body wall, swimmeret muscles) and visceral muscle (heart, viscera). In addition, these two CNS structures may also be the major endocrine organ in this prawn. So, the presence of leptin in the central nervous system may be related to the hormonal regulation in this specie. For the immunoperoxidase staining, the immunoreactivity (ir) of leptin-like peptide was detected in the adipocytes surrounding brain (Fig. 2), and thoracic ganglion (Fig. 3). In the brain, the leptin-like peptide ir was found in adipose tissues that cover the dorsal surface of neuronal cluster 6 (Fig. 2A and 2B). However, there was no immunoreactivity in the neurons and nerve fiber of the brain. In thoracic ganglion, we found strong intensity of leptin-like ir in the adipocytes surrounding the subesophageal ganglion (SEG) (Fig. 3D and 3E) and the connective tissue sheet covering nerve root (Fig. 3F and 3G). In addition, the leptin-like peptide ir was also detected in the cytoplasm of adipocytes surrounding central artery (Fig. 3F). There was no positive leptin staining in the neuron and nerve fiber of abdominal ganglion (data not shown). In gastrointestinal tract, there was intense ir of leptin-like peptide in apical surface of the epithelial cells of the cardiac (Fig. 4C) and pyloric stomach (Fig. 4G and 4H) as well as in the lamina propria (Fig. 4F). In addition, the leptin-like peptide ir was also detected in the lumen of midgut (Fig. 5D). However, the leptin-like peptide ir was found only in the cytoplasm of adipocytes and lamina propria in the hindgut (Fig. 6C). In hepatopancreas, there was no ir of leptin-like peptide in the hepatocytes when compared with the controls (data not shown). In ovary, the intense leptin-like peptide ir was particularly found in the cytoplasm of oocyte stage III and IV (Fig. 8B, 8D and 8F), whereas, no positive staining

detected in early stage of oocyte (oogonia, oocyte stage I and II) as well as in the follicular cell (Fig. 8A, 8C and 8E).

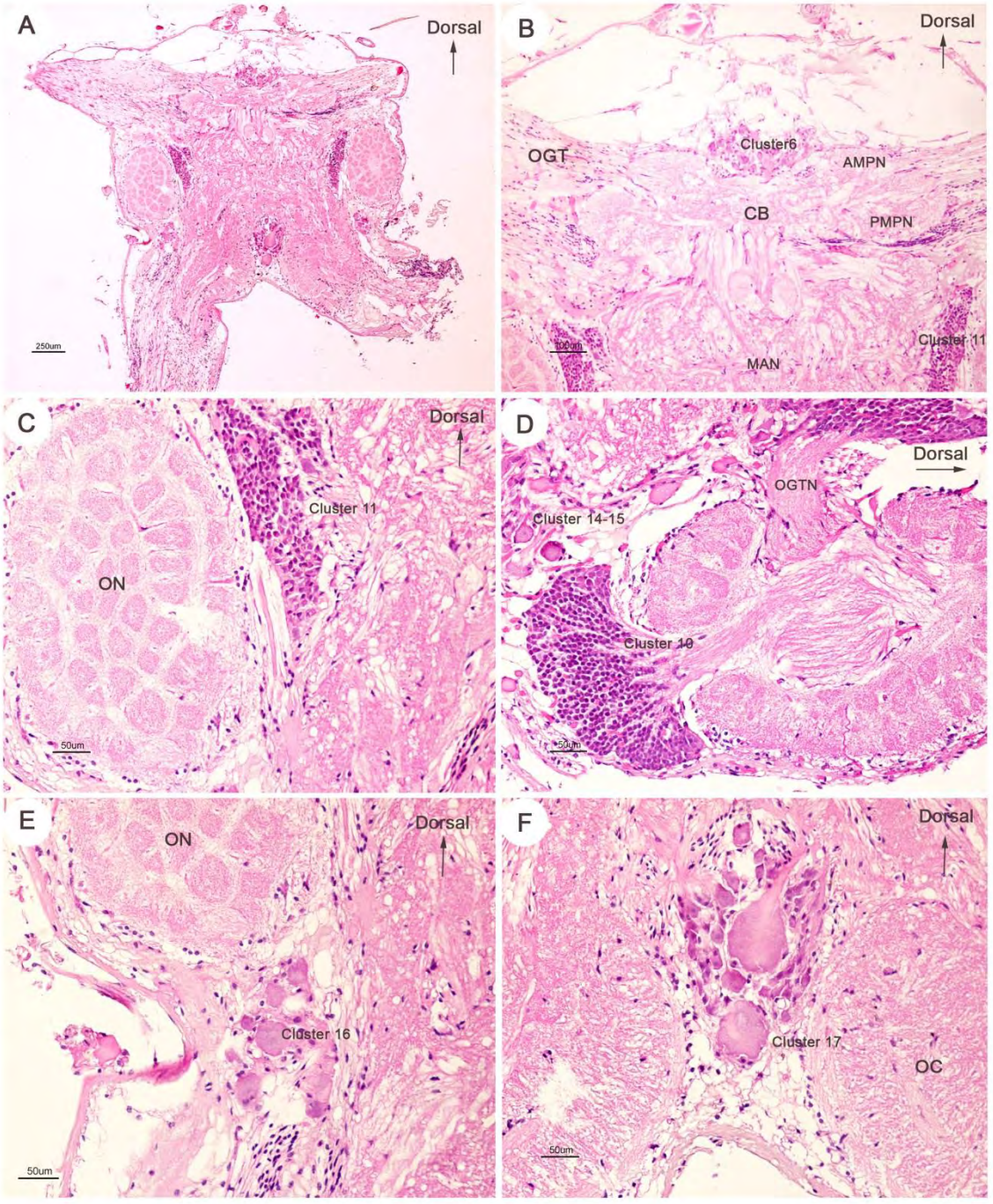

Fig. 1 The light micrographs showing the histological structure of the brain of *M. rosenbergii* stained with H&E. (A) Low micrograph showing the dorsal view of the brain. (B) Neuronal cluster 6 and the connective tissue covering the dorsal surface of the brain. (C) Olfactory lobe (ON) and neuronal cluster 11. (D) Olfactory globular tract neuropil (OGTN) and

neuronal cluster 10, 14 and 15. (E) ON and neuronal cluster 16. (F) Neuronal cluster 17 and Oesophageal connection (OC). OGT= olfactory globular tract, CB= central body, AMPN= anterior medial protocerebral neuropil, PMPN= posterior medial protocerebral neuropil, MAN=median antenna I neurophil

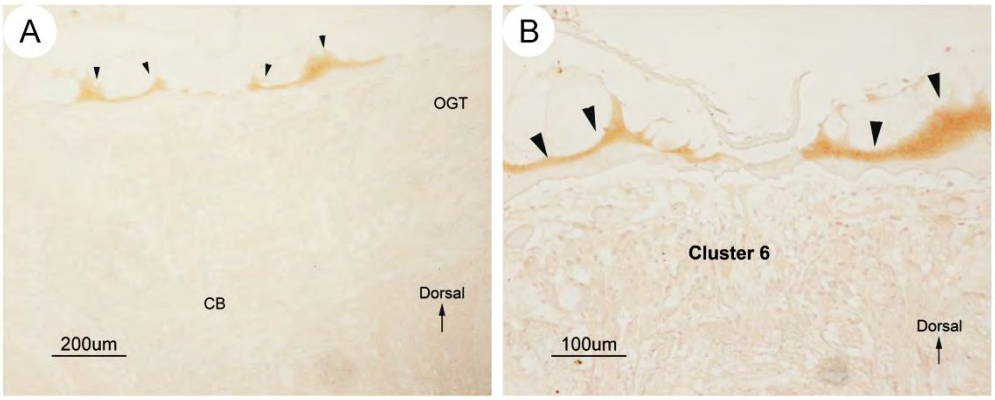

Fig. 2 Immunoperoxidase staining of leptin-like peptide in the brain of *M. rosenbergii*. (A) Low micrograph showing the leptin positive staining at the dorsal surface of the brain (arrowheads). (B) High micrograph showing the positive staining at the dorsal surface of the brain near the neuronal cluster 6 (arrowheads). CB: central body, OGT: olfactory globular tract.

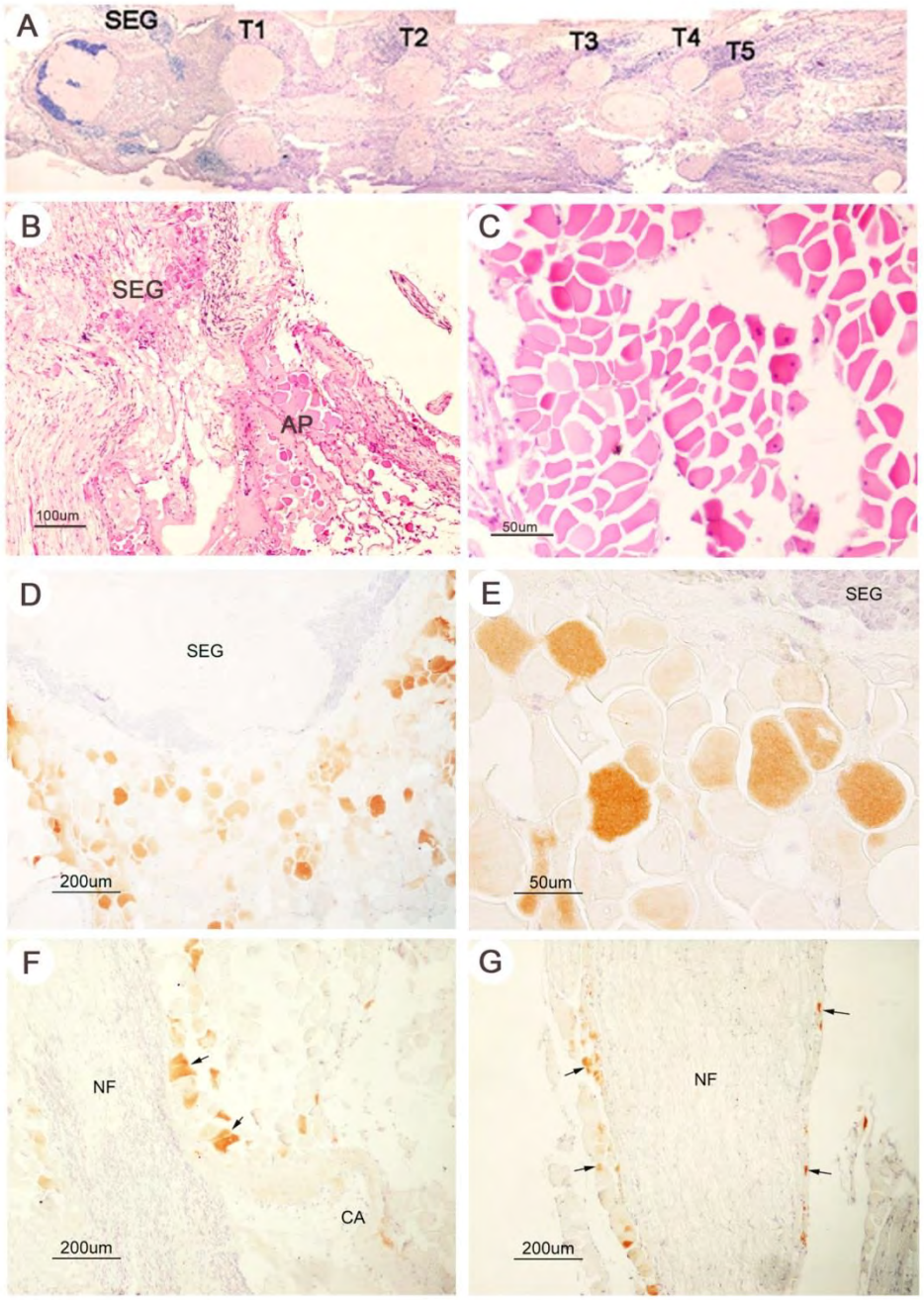

Fig. 3 (A) Light micrograph showing subesophageal ganglion (SEG) and 1st-5th thoracic ganglion (T1-T5) stained with H&E. (B) Low micrograph showing the group of adipocytes surrounding SEG stained with H&E. (C) High micrograph showing the group of adipocytes surrounding SEG stained with H&E. (D) The leptin positive staining was detected in cytoplasm of adipocytes that surround the subesophageal ganglion (SEG). (E) High micrograph showing intense immunoreactivity was found in the cytoplasm of adipocytes. (F) The leptin-like peptide immunoreactivity was detected in cytoplasm of adipocyte near the central artery (CA) of thoracic ganglion. (G) High micrograph showing the numerous adipocytes with connective tissue covering each level of thoracic ganglion.

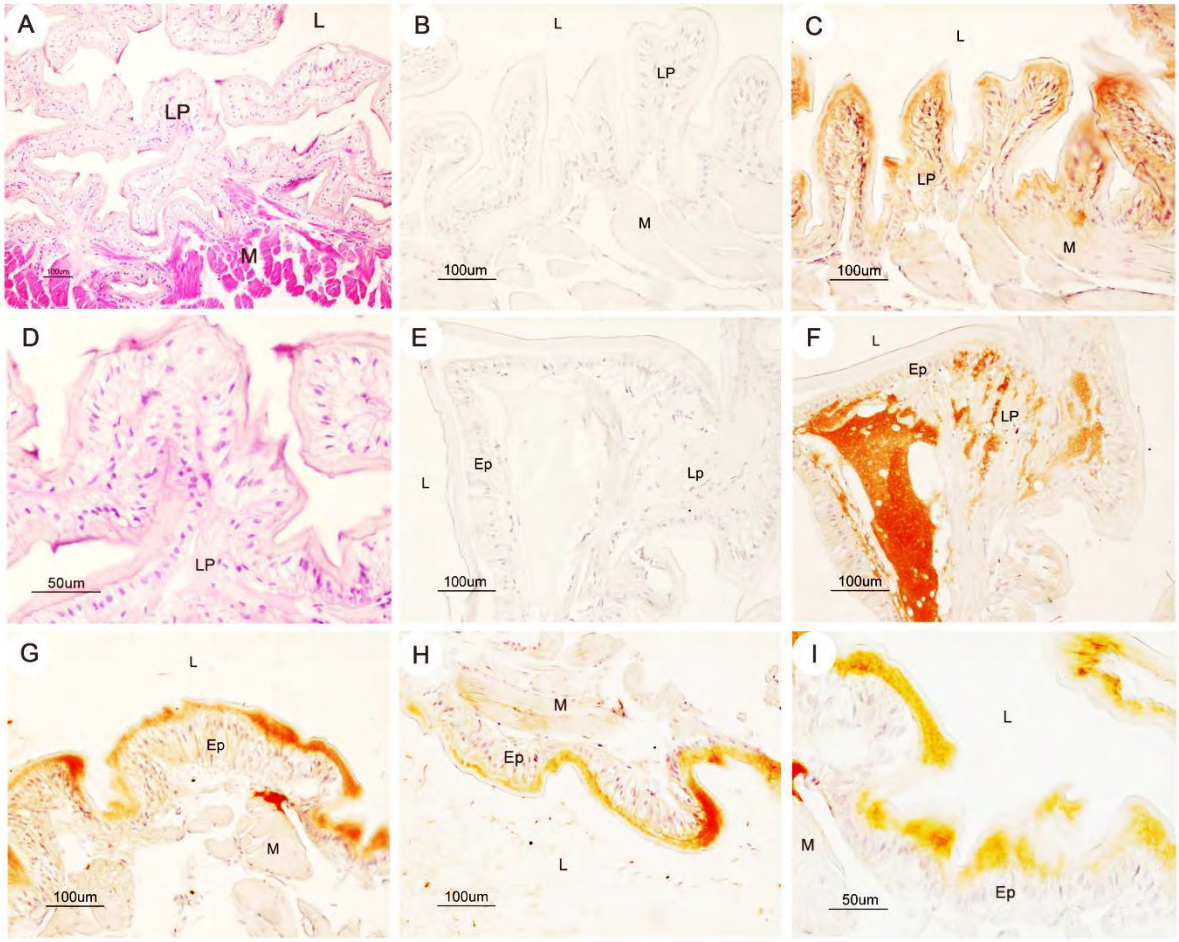

Fig. 4 Immunoperoxidase staining of leptin-like peptide in the foregut of *M. rosenbergii*. (A) Histological structure of stomach of *M. rosenbergii* stained with H&E. (B) No positive staining was found in the control section of mucosal and submucosal layer of the stomach. (C) The leptin-like peptide ir was detected in the apical surface of epithelial cells in the mucosal layer of cardiac stomach. (D) High micrograph showing the mucosal layer and lamina propria of the stomach. (E) No positive staining was found in the control section of mucosal and submucosal layer of cardiac stomach. (F) The leptin-like peptide ir was detected in the lamina propria of cardiac stomach. (G-I) The leptin-like peptide ir was detected in the apical surface of epithelial cell of pyloric stomach. L: lumen, Ep: epithelium, LP: lamina propria, M: muscular layer

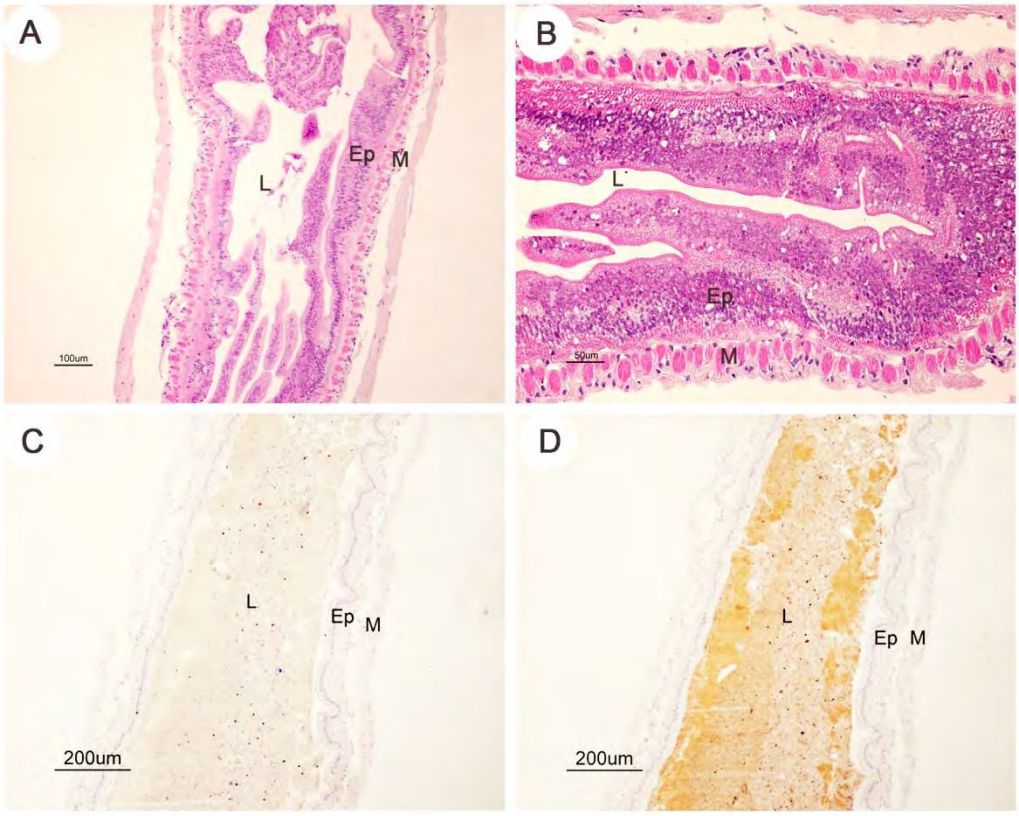

Fig. 5 Light micrograph showing the histological structure and immunoperoxidase staining of leptin in the midgut. (A) Low micrograph showing the histological structure of midgut. (B) High micrograph showing the histological structure of midgut. (C) No positive staining was found in the control section of the midgut. (D) The leptin-like peptide ir was detected in the lumen of midgut. L: lumen, Ep: epithelium, M: muscular layer

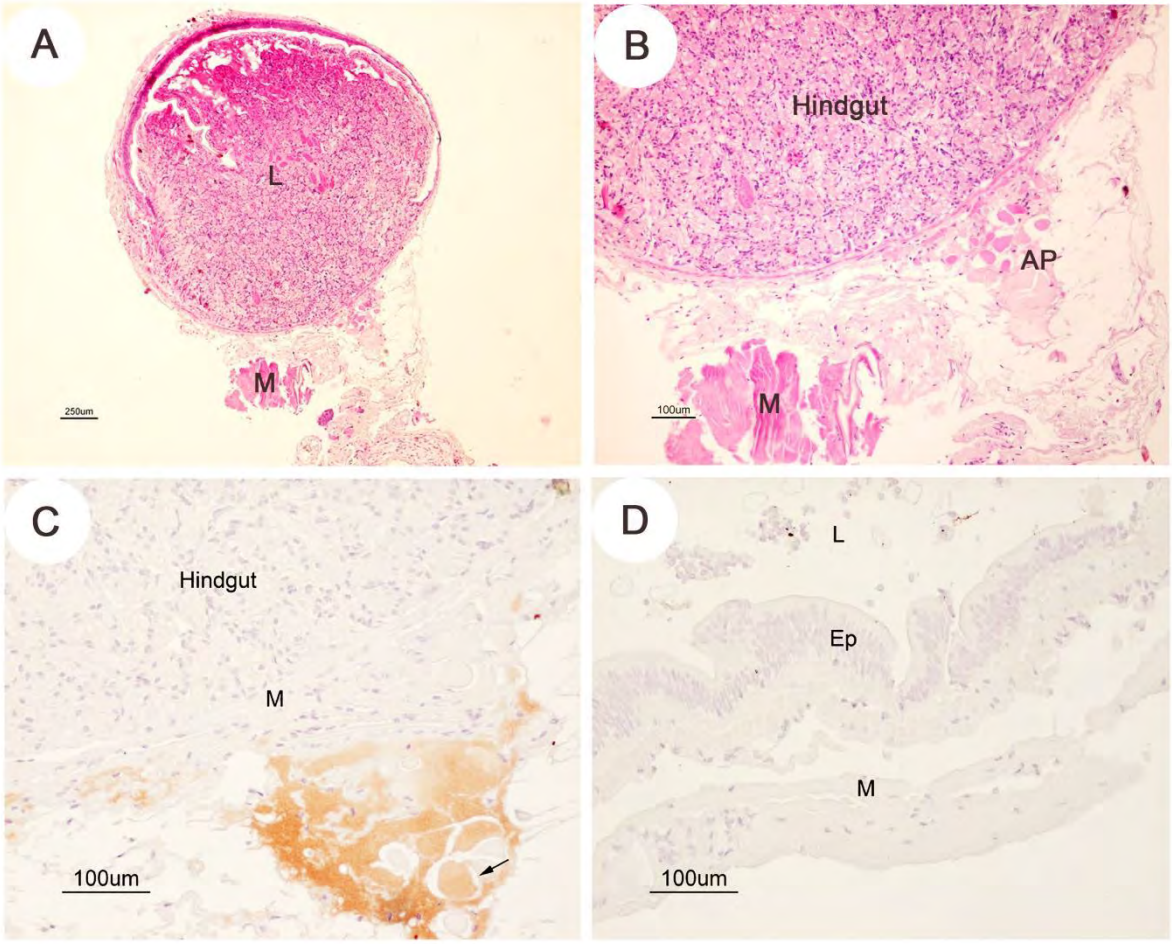

Fig. 6 (A) Low micrograph showing the histological structure of hindgut of *M. rosenbergii* stained with H&E. (B) High micrograph showing the wall of hindgut. Adipocytes (AP) were presented at the outer layer of the hindgut wall. (C) Immunoperoxidase staining of leptin-like peptide in the hindgut. The leptin-like peptide ir was detected in the cytoplasm of adipocytes (arrow). (D) There was no leptin-like peptide ir in the mucosal layer of hindgut. M: muscular layer, L: lumen, Ep: epithelium

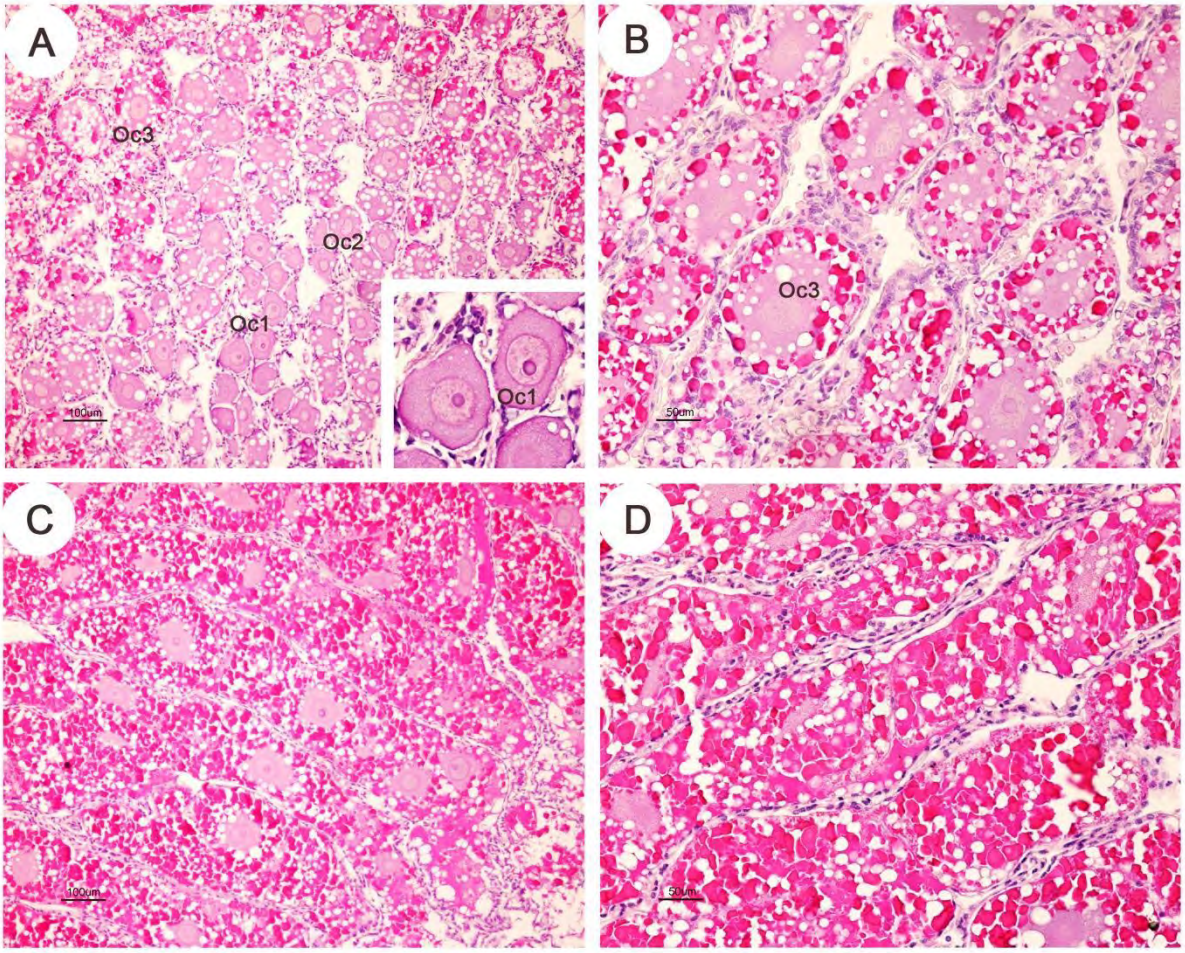

Fig. 7 Light micrographs of different steps of oocytes stained with H&E. (A) Oocyte stage 1-3 (inset=Oc1). (B) Oocyte stage 3. (C-D) Oocyte stage 4.

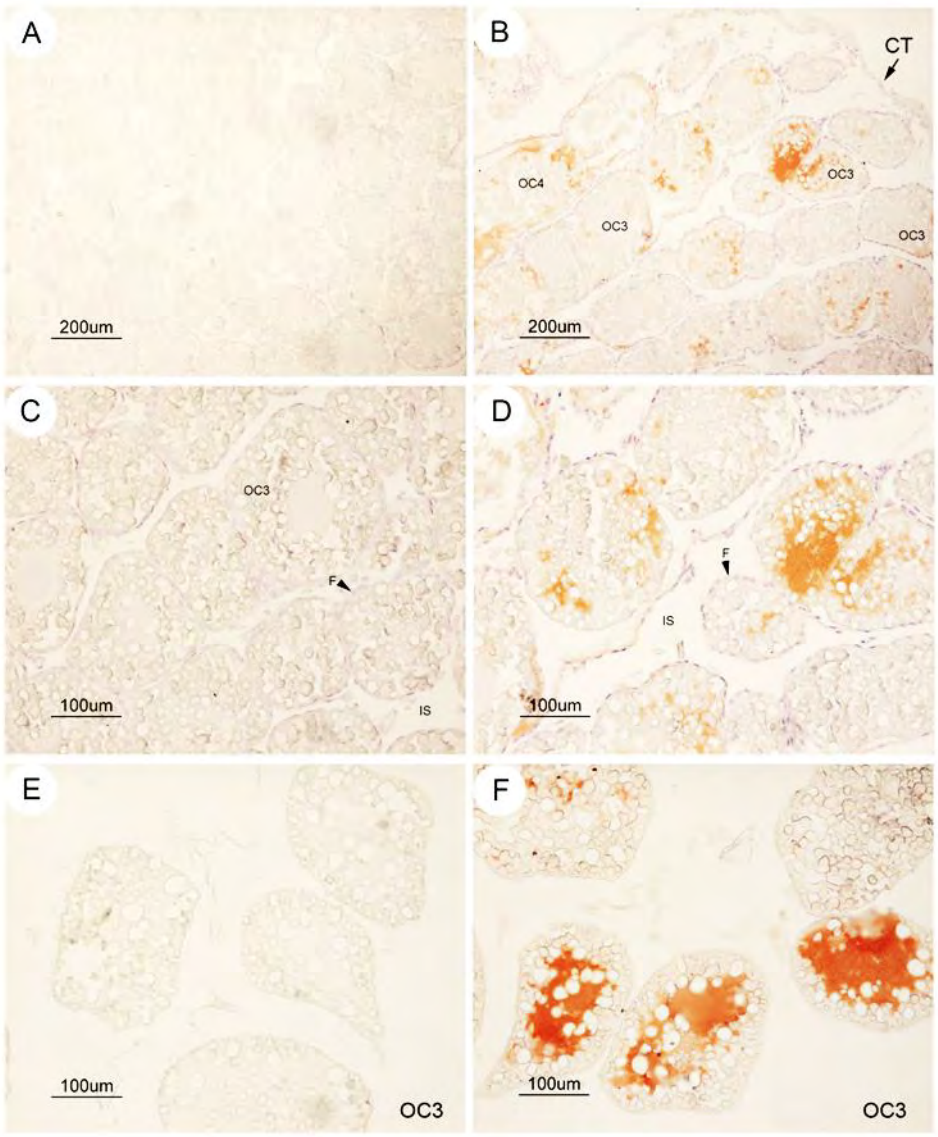

Fig. 8 Immunoperoxidase detection of leptin-like peptide in the ovary of *M. rosenbergii*. A, C, and E. No positive staining was found in the control sections. B. Low micrograph showing the leptin immunoreactivity in the cytoplasm of oocyte stage 3 and 4. D. and F. High micrograph of leptin immunoreactivity in the cytoplasm of oocyte stage 3 and 4. There was no leptin-like ir in the early stage of oocyte and follicular cell. CT: connective tissue, F: follicular cell, OC_3: oocyte stage 3, OC_4: oocyte stage 4.

Immunofluorescence staining

The immunoreactivity (ir) of leptin-like peptide was detected in the brain, thoracic ganglion (Fig. 9), ovary (Fig. 10) and testis (Fig. 11). In the brain, the leptin-like peptide ir was found in the olfactory neuropil (ON) (Fig. 9B). As well, we found strong intensity of leptin-like ir in the medium sized neurons in the thoracic ganglion (T1) (Fig. 9D, 9E) and subesophageal ganglion (SEG) (Fig. 9F). In addition, the leptin-like peptide ir was also detected in the adipocytes surrounding thoracic ganglion (Fig. 9C) and abdominal ganglion (data not shown). In hepatopancreas, there was no ir of leptin-like peptide in the hepatocytes when compared with the controls (data not shown). In ovary, the ir of leptin-like peptide was detected in the cytoplasm of oocyte stage 1 (Fig. 10B), 3 (Fig. 10C) and 4 (Fig. 10D). Particularly, the strong intensity of leptin staining was observed in oocyte stage 3. In testis, the ir of leptin-like peptide was found in primary spermatocytes (Fig. 11D, 11E). There was no positive leptin staining in the spermatozoa and spermatic duct (Fig. 11F). No leptin-like peptide staining was observed in the control sections (Fig 9A, 10A and 11C).

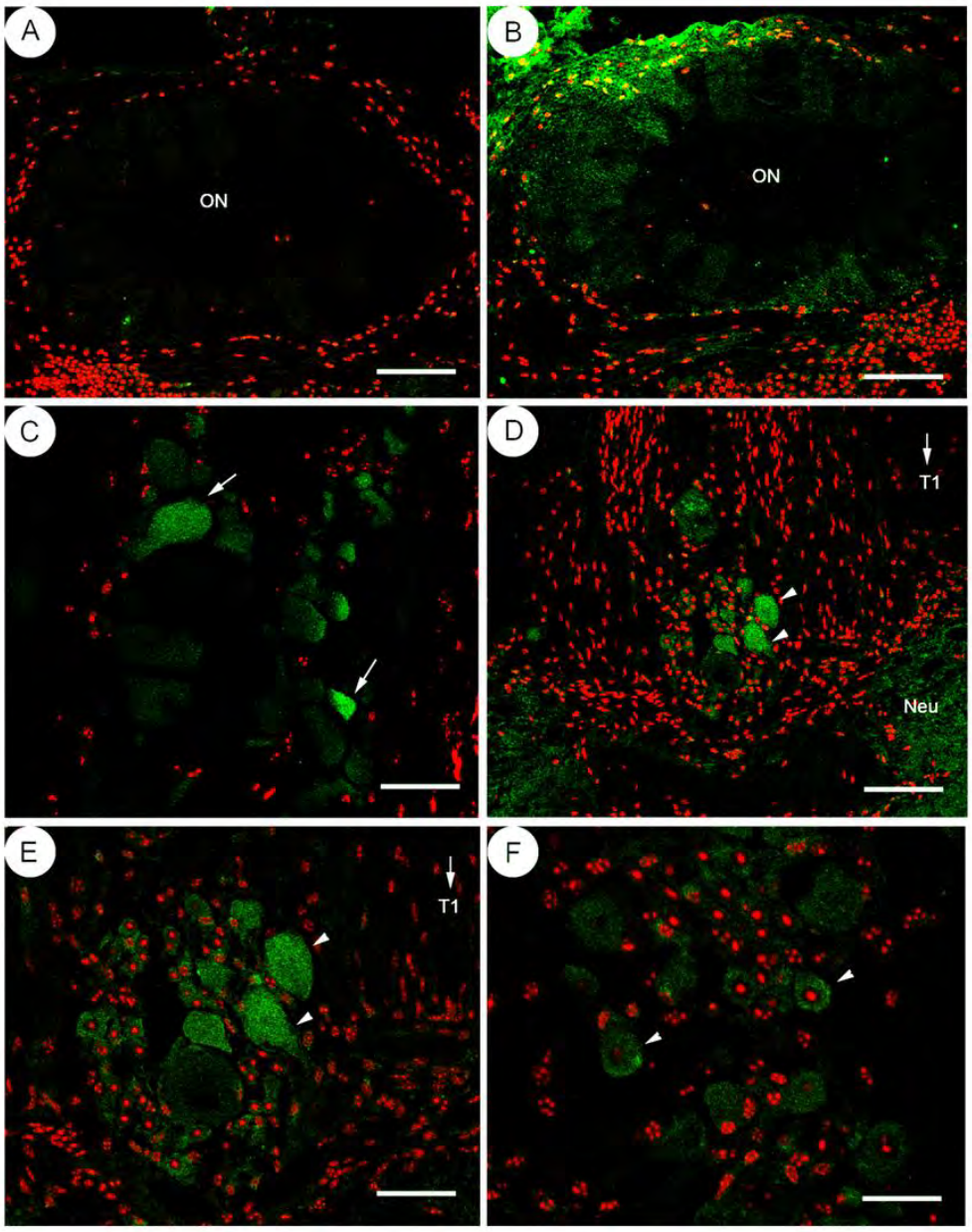

Fig. 9 Immunofluorescence detection of leptin-like immunoreactivity (green) in the central nervous system of *M. rosenbergii* with nuclei counterstained with ToPro-3 (red). (A) No immunofluorescence staining was observed in control section of the brain. (B) The leptin-like immunoreactivity was detected in the olfactory neuropil (ON). Magnification: 20x, Bar = 40 µm. (C) The leptin-like immunoreactivity was found in the adipocytes (arrows) surrounding thoracic ganglion. Magnification: 40x, Bar = 20 µm. (D) The high intensity of immunofluorescence staining of leptin-like peptide was observed in the medium-sized neurons of T1. Magnification: 20x, Bar = 40 µm. (E and F) The high micrographs showing the strong intensity of leptin-like ir in the medium sized neurons (arrowheads) in the thoracic ganglion (T1). Neu: Neuropils. Magnification: 60x, Bars = 20 µm.

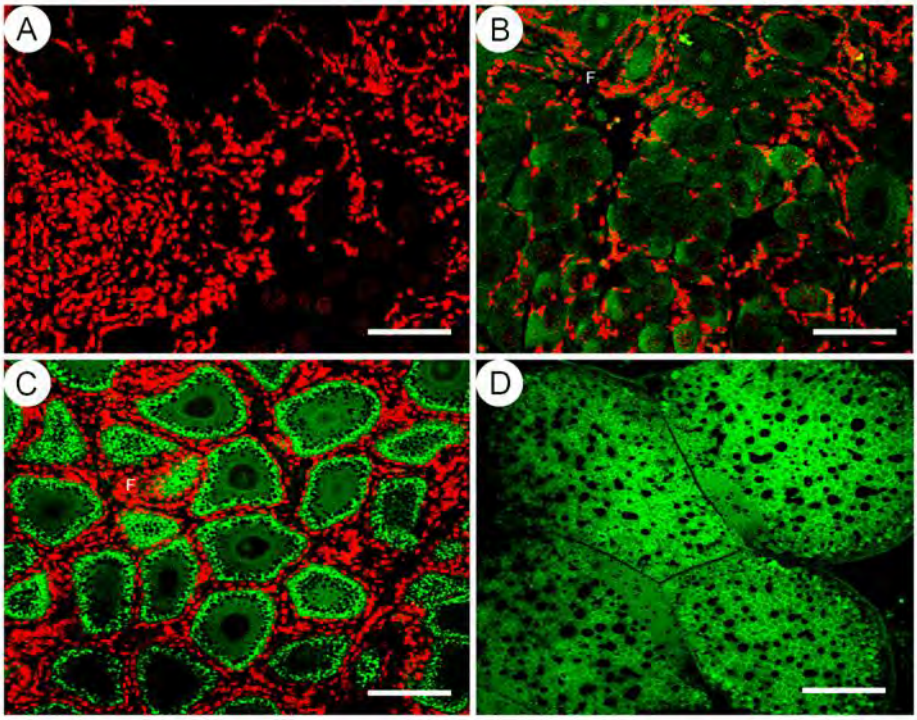

Fig. 10 Immunofluorescence detection of leptin-like immunoreactivity (green) in the ovary of *M. rosenbergii* with nuclei counterstained with ToPro-3 (red). (A) No immunofluorescence staining was observed in control section of ovary stage 1. (B) The leptin-like staining was detected in the oocyte stage 1. (C) The strong leptin-like immunoreactivity was detected in the cytoplasm of oocytes stage 3. (D) The leptin-like immunoreactivity was detected in the cytoplasm of oocyte stage 4. F: follicular cells, Magnification: 20x, Bars = 40 μm.

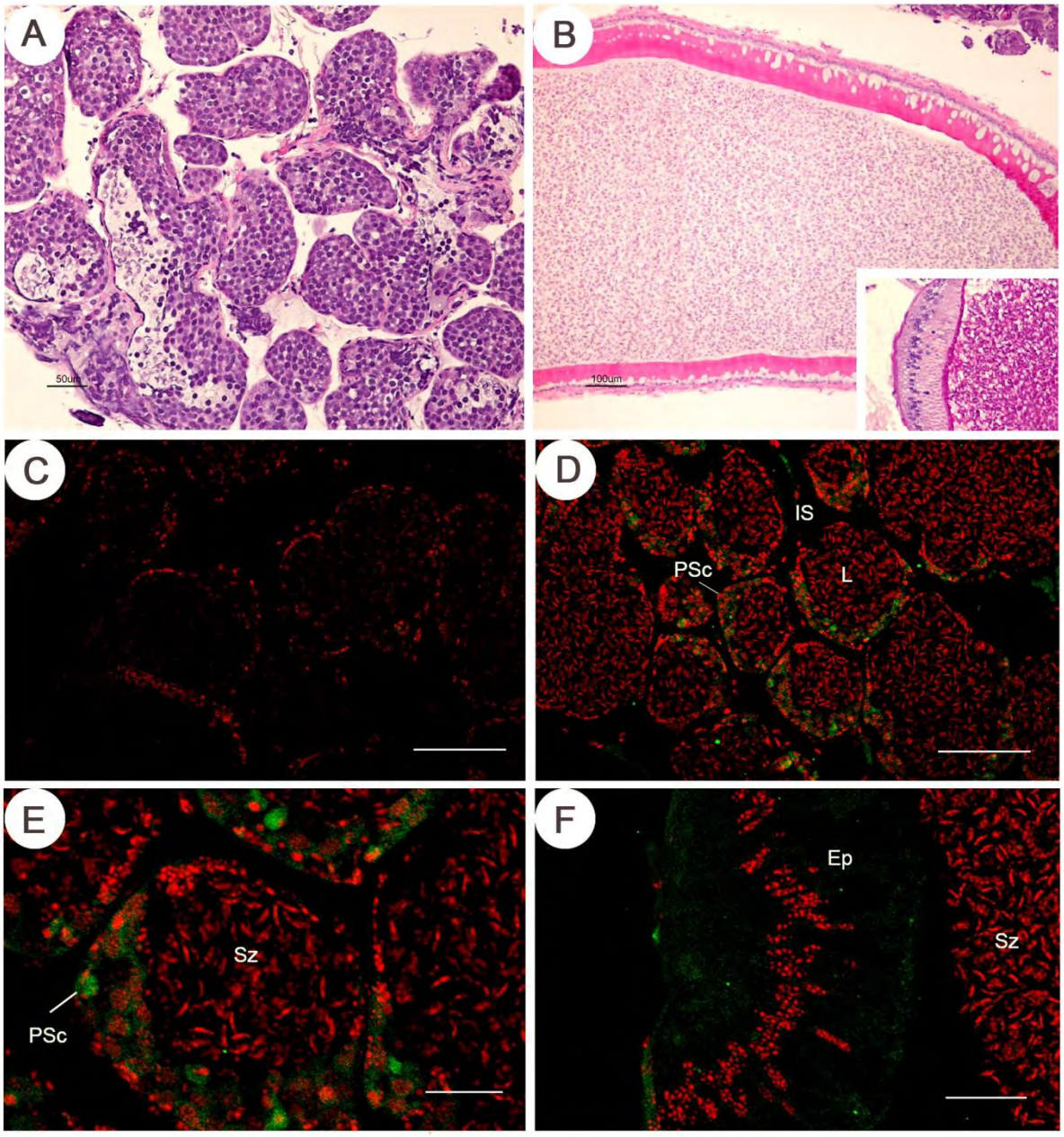

Fig. 11 (A) Histological structure of testis of *M. rosenbergii*. (B) Histological structure of spermatic duct. High epithelium of spermatic duct was shown in the inset. (C-F) Immunofluorescence detection of leptin-like immunoreactivity (ir; green) in the testis of *M. rosenbergii* with nuclei counterstained with ToPro-3 (red). (C) No immunofluorescence staining was observed in control section of testis. Magnification: 20x, Bar = 40 µm. (D) The leptin-like ir was found in the primary spermatocytes (PSc). L: lumen; IS: Interstitial space. Magnification: 20x, Bar = 40 µm. (E) Higher magnification of seminiferous tubules showing the leptin-like ir was detected in primary spermatocytes (PSc). No immunofluorescence staining was observed in the spermatozoa (Sz) and also in the lumen of the tubules (L). Magnification: 40x, Bar = 20 µm. (F) There was no positive staining of leptin-like peptide in

the lumen and the high epithelium of the spermatic duct (Ep). Magnification: 20x, Bar = 40 μm.

Immunoblot detection

From the result of SDS-PAGE, the protein band was found at about 75 kDa in the extract from all tissue (Fig. 12A). The leptin (Ob) peptide was present at 75 kDa (Fig. 12B). In the nervous tissue, including brain, thoracic ganglion and abdominal ganglion, the protein band was also mainly found at 75 kD (Fig. 12B, lane 3, 4 and 5). As well, the same pattern of protein band was found in the hepatopancreas, foregut, midgut and hindgut (Fig. 12A, lane 6, 7, 8 and 9). In the ovary, the protein bands were mainly found about 75 kD (Fig. 12A, lane 10-13). The 75 kD of protein band were also present in testis, spermatic duct and muscle (Fig. 12A, lane 14-16). Moreover, the protein bands were found about 18, 20, and 37 kD in the muscle (Fig. 12A, lane 16). After transferring protein into the nitrocellulose membrane, the TMB membrane detection kit was used as a visualizer of antigen-antibody complex on the membrane. The leptin-like positive protein bands were shown in Figure 12B. It was found that the intense leptin-like immunoreactivity was observed in the brain, thoracic ganglia and abdominal ganglia at 48 and 70 kD (Fig. 12B, lane 3-5). In the gastrointestinal tract, the leptin-like immunoreactivity was found in the foregut, midgut and hindgut at molecular weight 50 and 75 kD (Fig. 12B, lane 7-9). However, there was no leptin positive protein band in the hepatopancreas (Fig. 12B, lane 6). Moreover, the leptin-like immunoreactivity was found at molecular weight about 40 kD in the hind gut (Fig. 12B, lane 9). In ovary, two positive bands were mainly found at molecular weight about 50 and 70 kD (Fig. 12B, lane 10-13). As well, in testis and spermatic duct, the leptin-like positive protein bands were detected about 50 and 70 kD (Fig, 12B, lane 14-15). In addition, the leptin-like immunoreactivity was found in the muscle at molecular weight at 40 and 50 kD (Fig. 12B, lane 16). There was no any protein band in the negative controls (Fig. 12C).

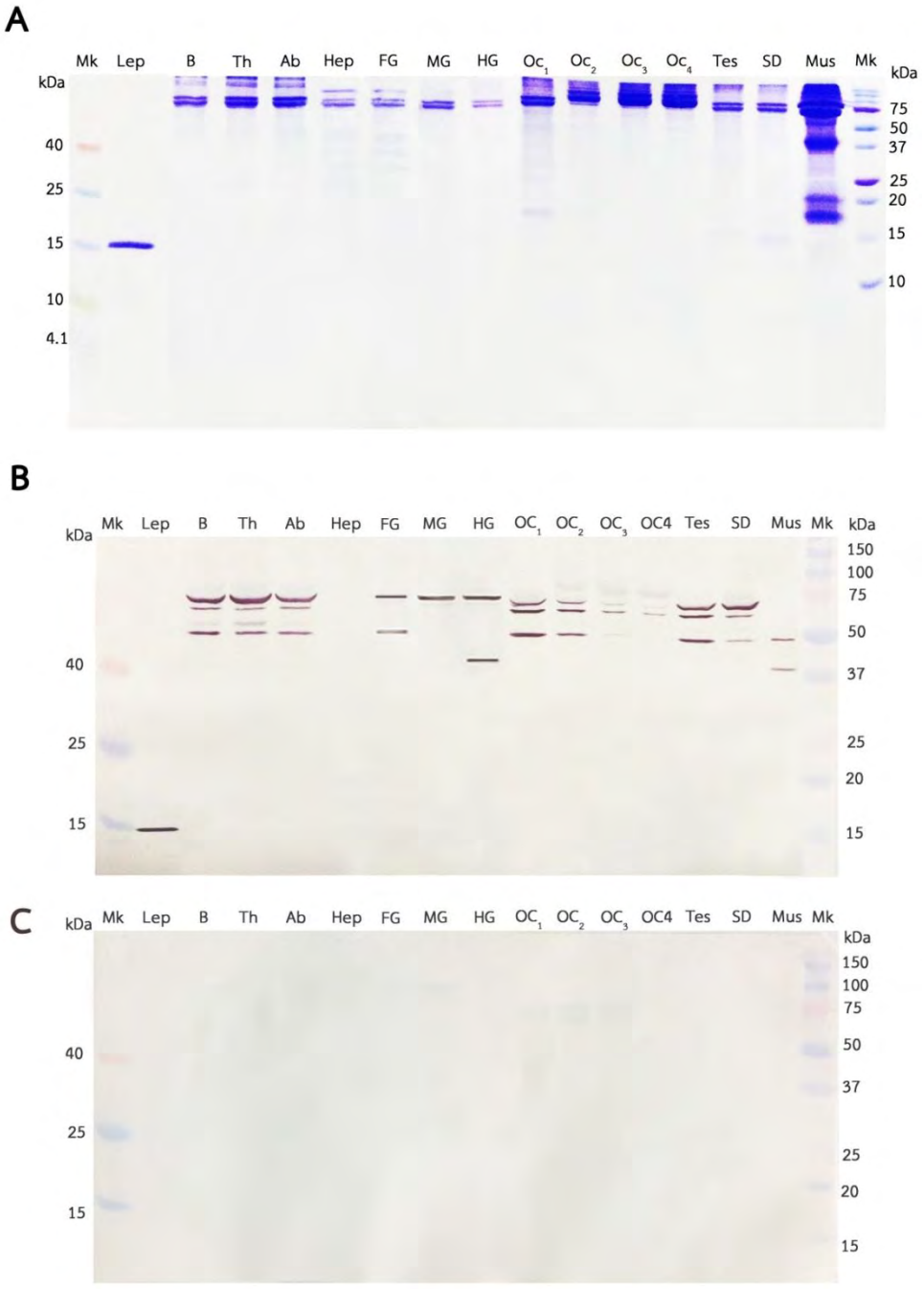

Fig. 12 The 15% SDS-PAGE of protein extract from various tissue of *M. rosenbergii*. (A) The 15% gel was stained with Coomassie blue solution showing the protein profile of CNS, gastrointestinal tract and gonad. (B) Immunoblot detection of leptin-like peptide in various organs of *M. rosenbergii*. (C) No leptin-like immunoreactivity present in the negative controls. Mk: standard protein marker, B: brain, Th: thoracic ganglia, Ab: abdominal ganglia, Tes: testis, SD: spermatic duct, Hep: hepatopancreas, Mus: muscle, FG: foregut, MG: midgut, HG: hindgut, OC_1: oocyte stage 1, OC_2: oocyte stage 2, OC_3: oocyte stage 3, OC_4: oocyte stage 4, Lep: leptin peptide.

Discussions

The results of this study represent the first evidence on the existence of leptin-like peptide in various tissue of *M. rosenbergii*. Immunohistological analysis showed leptin-liked immunoreactivity in central nervous system, gastrointestinal tract, adipose tissue and also in the gonad. The immunoreactivity of leptin was found in the medium sized neuron represented the role of this hormone for synthesis and regulation of hormonal release as in vertebrate, although the function of leptin in the neurons and nerve fiber are still unclear. Leptin was synthesized and secreted by adipose tissue before sending through the blood circulation and it can bind to their receptor in the brain for controlling food intake and reproduction (Tena-Sempere and Barreiro, 2002). Otherwise, leptin was produced locally in the brain, thoracic ganglion and also gonad, where it may directly effect to the neuron in the brain, thoracic ganglion or even in germ cell. In addition, it was suggested that leptin receptor was found in the central nervous system and peripheral organs of crustacean and vertebrate both in male and female (Ahima et al., 2000; Caprio et al., 2001; Halaas and Friedman, 1997; Jiang et al., 2010). Therefore, it was also possible that leptin was found in the brain as described in the previous study (Ahima et al., 2000; Caprio et al., 2001; Halaas and Friedman, 1997; Jiang et al., 2010; Czaja et al., 2007).

In *M. rosenbergii*, the presence of leptin-like peptide in various part of gastrointestinal tract may indicated the role of this hormone for food intake, nutrient absorption, and energy homeostasis as described in the marine animals (Morton et al., 1998; Yarandi et al., 2011). The gastric part of the stomach may be the major source of the leptin production in most vertebrate (Oliver et al., 2002, Gambardella et al., 2010). In fish, leptin has been detected in the endocrine and mucous secreting cells in the gastric pit of the stomach showed the role of leptin for hydrochloric and pepsinogen secretion (Gambardella et al., 2010). In this study, the leptin-liked immunoreactivity was found in the brush border of the epithelial cell of the gastric and intestinal mucosa indicate the possible role of leptin for controlling the biological process of gastrointestinal tissues, including the nutrient absorption in this species as described in the marine animal and vertebrate (Cammisotto et al., 2006; Russo et al., 2012). Likewise, the leptin receptor has been found in the brush border suggest that leptin entering into the lumen of intestine to control the absorption of nutrient (Buyse et al., 2001; Ducroc et al., 2005). In

addition, the presence of leptin-like immunoreactivity in the lumen of foregut and midgut suggesting that leptin could be produced, stored and secreted into the lumen related to the food intake as described in the previous study (Oliver et al., 2002).

At present, there was no evidence about the presence of leptin peptide in the gonad of decapod crustacean. However, leptin receptor gene was first identified in the Chinese mitten crab, *Eriocheir sinensis* which may be involved in the regulation of metabolism and reproduction in Chinese mitten crabs (Jiang et al., 2010). In *M. rosenbergii*, there was leptin-like immunoreactivity in the spermatocyte and oocyte indicate that this peptide may be secreted by these cells or regulated by specific neuron in the brain for controlling germ cell differentiation and maturation. In mammal, leptin can bind to a membrane receptor (Ob-R) of arcuate neurons in the hypothalamus; possibly Kiss neurons, that control the release of GnRH and gonadotropins which controls the gonad maturation (Yu et al., 1997; Barb and Kraeling, 2004; Zieba et al., 2005; Smith et al., 2006). Likewise, leptin may be regulated the reproductive process in the same manner as described in vertebrate (Zieba et al., 2005; Smith et al., 2006; Jia et al., 2012). In addition, the presence of leptin receptor in the peripheral organs of the reproductive system suggests that leptin may have also a direct effect on the downstream endocrine targets of the reproductive axis, such as anterior pituitary, ovary, testis, uterus, placenta, and adrenal glands (Caprio et al., 2001; Moschos et al., 2002; Cassy et al., 2004). However, the existence of leptin receptor in this decapod crustacean could be investigated for the further study.

For immunoblot analysis, the leptin-like peptide was mainly detected in brain, thoracic ganglia, abdominal ganglia, gastrointestinal tract and also in the gonads at molecular weight 70 kD. Actually, the leptin peptide has 167 amino acid sequence and 16 kD. It was possible that the leptin-like peptide detected in these organs may be the preprohormone which undergo several stages to become an active peptide. Moreover, in mammals, the specific protein, called leptin binding proteins, is derived from leptin receptor gene or protein bind the circulating leptin (Sinha et al., 1996). In fish, this protein was identified in the plasma at molecular weight ranging from 70 to 100 kDa (Gong et al., 2013). It was possible that the leptin detected by western blotting in this study may be related to the expression of leptin binding protein in this specie. In addition, leptin binding protein may have an important role for modulating the leptin expression in different tissue (Gong et al., 2013). However, the information of leptin binding protein are still lacking in the decapod crustacean. It was also need to be further investigated.

Conclusion

In summary, it was suggested that leptin-like peptide was present in the prawn species. However, this is the first report for existence of this peptide in this prawn. It was also need to characterize the leptin encoding gene for the further study. The result of this study could improve the basic knowledge of hormonal regulation in the prawn reproduction in relation to nutrition and lipid status, and in helping to find the way to stimulate the gonadal maturation, increased fertilization rate, and the production of the prawn larvae for aquaculture.

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Publication

กำลังอยู่ในระหว่างการเตรียม manuscript

Benefit

The information from the result of this project could improve the basic knowledge of hormonal regulation in the decapods crustacean as well as yield practical applications, such as, the discoveries of neuropeptides that could stimulate the gonadal maturation, increased fertilization rate, and the production of the prawn broodstock in aquaculture.