รายงานวิจัยฉบับสมบูรณ์

โครงการ การโคลนและการศึกษาการแสดงออกของโปรตีนของตัวตอบรับ 5-HT ในกุ้งกุลาดำ

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สัญญาเลขที่ MRG4680199

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ซีโรโตนิน (5-HT) เป็นสารสื่อประสาทซึ่งมีบทบาทสำคัญทางสรีรวิทยาหลายอย่างทั้งในสัตว์ที่มีและไม่มีกระดูกสันหลัง ในสัตว์จำพวกครัสเตเชียน ซีโรโตนินมีบทบาทในการกระตุ้นการหลั่งฮอร์โมนที่กระตุ้นการเจริญเติบโตของอวัยวะสืบพันธุ์ จากปมประสาทส่วนสมองและส่วนอก และมีผลทำให้มีการพัฒนาการของรังไข่และเกิดการตกไข่ ซีโรโตนินสามารถออกฤทธิ์ทางสรีรวิทยาได้หลายอย่าง โดยการจับกับตัวตอบรับซีโรโตนินชนิดต่างๆ ซึ่งโดยส่วนใหญ่แล้ว จัดอยู่ในกลุ่มตัวตอบรับ G-protein ในการทดลองนี้ จึงมีวัตถุประสงค์เพื่อโคลน cDNA สายสมบูรณ์ที่ถอดรหัสได้ตัวตอบรับซีโรโตนินจากรังไข่ของแม่พันธุ์กุ้งกุลาดำ และสร้างโพลีโคลนอลแอนติบอดีต่อลูปที่สามด้านในของตัวตอบรับซีโรโตนิน เพื่อใช้ในการศึกษาการแสดงออกของโปรตีนและบทบาทหน้าที่ของตัวตอบรับเหล่านี้ในกุ้งกุลาดำ ในขั้นแรก RNA ที่ถูกสกัดจากรังไข่ของกุ้งแม่พันธุ์ที่ได้จากธรรมชาติซึ่งมีพัฒนาการอยู่ในระยะที่ 3 จะถูกนำมาสร้างสาย cDNA สายแรกด้วยกระบวนการ reverse transcription โดยใช้ Oligo (dT) primer และเอ็นไซม์ Impromll™ reverse transcriptase แล้วทำการสร้างและเพิ่มจำนวนชิ้น cDNA สายคู่ด้วยกระบวนการ PCR amplification โดยใช้ primer ที่ออกแบบอย่างจำเพาะต่อตัวตอบรับซีโรโตนินของกุ้งกุลาดำเพื่อโคลน cDNA สายสมบูรณ์ นอกจากนี้ดีเอนเอของลูปที่สามด้านในของตัวตอบรับ 5-HT (329 คู่เบส) ได้ถูกโคลนเข้าไปในเอ็กเพรสชันเวกเตอร์ (pGEX-5X1) เพื่อสร้างโปรตีนในเซลล์แบคทีเรียและสกัดให้บริสุทธิ์เพื่อนำไปใช้ในการสร้างโพลีโคลนอลแอนติบอดีในหนู จากการศึกษา พบว่า cDNA สายสมบูรณ์ ประกอบด้วย ลำดับนิวคลีโอไทด์ จำนวน 2291 ตัว ซึ่งมีรหัสซ้ำ GGC จำนวน 10 รหัสซ้ำในบริเวณ loop ที่ 3 ที่อยู่ภายในเซลล์ ลำดับนิวคลีโอไทด์นี้ ถอดรหัสโปรตีนตัวตอบรับ (5-HT_{1Pem}) ได้กรดอะมิโนจำนวน 591 ตัว ซึ่งเมื่อเปรียบเทียบในฐานข้อมูล GENBANK พบว่า ลำดับกรดอะมิโนของตัวตอบรับซีโรโตนินในกุ้งกุลาดำมีความคล้ายคลึงกับลำดับกรดอะมิโนของตัวตอบรับซีโรโตนินชนิดที่ 1 ของสัตว์ที่มีและไม่มีกระดูกสันหลังมากถึง 76% มีบริเวณ transmembrane 7 สาย ซึ่งเป็นลักษณะเด่นของตัวตอบรับในกลุ่ม G-protein มี loop ที่ 3 ที่อยู่ภายในเซลล์ที่มีขนาดใหญ่ และมีส่วนปลายคาร์บอกซีสั้น นอกจากนี้ ยังมีกรดอะมิโนอนุรักษ์ซึ่งพบเฉพาะในตัวตอบรับซีโรโตนินชนิดที่ 1 ผลของ western blot พบว่าแอนติบอดีตัวนี้สามารถจับกับโปรตีนที่มีขนาด 50 กิโลดาลตันของรังไข่ของกุ้งกุลาดำโดยที่ระดับการแสดงออกจะเพิ่มขึ้นเรื่อยๆ เมื่อมีพัฒนาการตั้งแต่ช่วงที่หนึ่งถึงช่วงที่สี่แล้วลดลงหลังจากกุ้งวางไข่ การศึกษาโดยใช้ immunohistochemistry ยังสามารถระบุตำแหน่งการแสดงออกของตัวตอบรับซีโรโตนินบนผนังเยื่อหุ้มเซลล์ของเซลล์ไข่ที่มีพัฒนาการเจริญเติบโตในช่วงที่สี่ และพบรอบๆเยื่อหุ้ม cortical rod จากข้อมูลดังกล่าว จึงเป็นไปได้ว่า ตัวตอบรับซีโรโตนินที่โคลนได้ในกุ้งกุลาดำ จัดอยู่ในกลุ่มตัวตอบรับซีโรโตนินชนิดที่ 1 และมีบทบาทสำคัญในการควบคุมพัฒนาการของรังไข่และการตกไข่ การศึกษานี้จึงมีความสำคัญในการทำให้

เกิดความรู้ความเข้าใจถึงกลไกทางสรีรวิทยาของสารสื่อประสาทซีโรโตนินและตัวตอบรับซีโรโตนินของกุ้งกุลาดำในช่วงที่มีพัฒนาการของรังไข่ ซึ่งจะเป็นประโยชน์ต่อการพัฒนาแม่พันธุ์กุ้งที่ให้ผลผลิตสูงต่อไป

คำหลัก: ตัวตอบรับ 5-HT, รังไข่, กุ้งกุลาดำ, RT-PCR

Abstract

Project Code: MRG4680199

Project Title: Molecular Cloning and Functional Expression of a 5-HT Receptor from *Penaeus monodon*

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Abstract—Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that plays an important role in many physiological functions in both vertebrates and invertebrates. In crustacean, serotonin has been proposed to stimulate the release of gonad stimulating hormone from brain and thoracic ganglion and resulting in an ovarian maturation and spawning. It mediates a widespread physiological function by interacting with multiple serotonin (5-HT) receptor subtypes which most of them are G-protein coupled receptors. Therefore, the purposes of this study are to clone the full-length cDNA of 5-HT receptor from black tiger shrimp *(Penaeus monodon)* and to produce polyclonal antibodies against the intracellular loop (i3) of 5-HT receptor. Total RNA was extracted from an ovary of a wild broodstock black tiger shrimp undergoing stage 3 of ovarian maturation. The first stranded cDNA was synthesized by reverse transcription using an oligo-dT primer and Improm-II™ reverse transcriptase. The full-length cDNA was obtained by using 5-HT receptor gene specific primers. In order to produce polyclonal antibodies against the intracellular loop (i3) of 5-HT receptor, the DNA fragment of i3-5-HT receptor (329bp) was cloned into an expression vector (pGEX-5X1). The i3-5-HT receptor protein was expressed in *E.coli* DH5α, purified, and injected into mice in order to raise polyclonal antibodies. The results showed that the full-length nucleotide sequences of 5-HT receptor contained 2291 nucleotides. A ten GGC tandem repeat was observed in the i3 loop. The sequences encoded a protein (5-HT_{1Pem} receptor) of 591 amino acids. Deduced amino acid sequences showed high amino acid sequence identities (up to 76%) with invertebrate and vertebrate 5-HT_1 receptors. This 5-HT_{1Pem} receptor contained seven transmembrane domains, a large i3 loop, a short carboxyl tail and several conserved amino acids characteristic of 5-HT_1 receptor family. The receptor expressed at relatively high levels in ovary and at variable levels in all tissues examined. Polyclonal antibody against the i3 loop of the 5-HT_{1Pem} receptor reacted specifically to an ovarian membrane protein of molecular weight approximately 50 kDa. The expression of 5-HT_{1Pem} receptor protein progressively increased in ovary undergoing stage 1, 2, 3 and 4 of ovarian maturation and was reduced during spent phase. Immunohistochemistry demonstrated the expression of 5-HT_{1Pem} receptor protein on the membrane of mature oocytes stage 4 and surrounding the cortical rod membrane supporting its role in regulation of ovarian maturation and spawning.

Keywords: 5-HT receptor, ovary, black tiger shrimp, RT-PCR

เนื้อหางานวิจัย

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a biogenic amine neurotransmitter found in both vertebrates and invertebrates that affect a wide variety of physiological and behavioral functions, including sleep, appetite, learning, pain perception, and circadian rhythm. Serotonin mediates these effects by interacting with various receptor subtypes. To date, fourteen 5-HT receptor subtypes have been cloned and identified (Gerhardt and van Heerikhuizen, 1997). These receptors can be classified into 7 families (5-HT_1 to 5-HT_7) according to their amino acid sequence homology, pharmacological binding properties, and coupling to second messengers. Most 5-HT receptors are G-protein coupled except for 5-HT_3 receptor which is a ligand-gated ion channel. 5-HT receptors couple to adenylate cyclase either negatively (5-HT_1 and 5-HT_5 receptor) or positively (5-HT_4, 5-HT_6, and 5-HT_7 receptor). In contrast, 5-HT_2 receptor primarily couples to phospholipase C.

In contrast, very little is known about the molecular properties and diversity of 5-HT receptors in invertebrates. Invertebrate 5-HT receptors have been cloned from *Drosophila melanogaster* (Colas et al., 1995; Saudou et al., 1992; Witz et al., 1990), *Caenorhabditis elegans* (Hamdan et al., 1999; Olde and McCombie, 1995), fresh water snail (*Lymnaea stagnalis)* (Gerhardt et al., 1996; Sugamori et al., 1993), sea snail (*Aplysia californica*) (Angers et al., 1998; Barbas et al., 2002; Li et al., 1995), *Aedes aegypti* (Pietrantonio et al., 2001), and the southern cattle tick (*Boophilas microplus*) (Chen et al., 2004). Recently, crustacean 5-HT_1 receptor has been cloned from crayfish, spiny lobster and fresh water prawn (Sosa et al., 2004). A number of these receptors share similar pharmacological and signaling properties to their mammalian counterparts. Most invertebrate 5-HT receptors are 5-HT_1, 5-HT_2, and 5-HT_7 -like receptors (Tierney, 2001). No cloned 5-HT receptor has been identified from *Penaeus* shrimp including *Penaeus monodon*. Indeed, it is not yet known if there is a 5-HT receptor in *Penaeus monodon*.

However, the effect of serotonin injection in inducing ovarian maturation and spawning in *Penaeus vannamei* (Vaca and Alfaro, 2000) suggested that a 5-HT receptor may exist in *Penaeus* shrimps including *Penaeus monodon*.

Serotonin has been proposed to induce ovarian maturation and spawning in penaeid shrimps similar to an eyestalk ablation technique (Vaca and Alfaro, 2000). Most shrimp farmers rely on wild broodstock for their supply of spawners and use an eyestalk ablation technique to induce ovarian maturation and to increase spawning activity. However, this approach leads to the problem of reduction of wild broodstocks and deterioration in spawning quantity and quality over time. Therefore, it is possible to use serotonin injection as an alternative approach to increase production of shrimps especially black tiger shrimps (*Penaeus monodon*) which are one of the most economically important animals in Thailand and many Southeast Asian countries. However, very little is known about biochemical and physiological functions of serotonin and its receptor in *Penaeus monodon*. In this study, we have cloned a full-length cDNA (2291 nucleotides) encoding a putative 5-HT receptor (591 amino acids) of *Penaeus monodon*. A tissue distribution study demonstrated that this receptor mRNA is expressed at relatively high levels in ovary and in all tissue examined. In addition, western blot analysis showed that the expression of 5-HT receptor protein is progressively increased during ovarian maturation suggesting that this receptor is involved in shrimp reproduction.

MATERIALS AND METHODS

Experimental animals

Live wild broodstock black tiger shrimps (100-120g) undergoing stage 1 to 4 of ovarian maturation and spent phase (an ovary stage after spawning) were obtained from commercial shrimp farms in the Southern part of Thailand. Stages of ovarian maturation were evaluated according to the external observation of ovarian size and color (Tan-Fermin and Pudadera, 1989). Animals were kept with continuously aerated in seawater.

Reverse transcription and PCR amplification

Total cellular RNA was isolated from a wild broodstock black tiger shrimp (undergoing stage 3 of ovarian maturation) by using TRIzol REAGENT® (Life Technologies, USA) according to manufacturer's protocol. Total RNA (5 μg) was used as a template to synthesize first strand cDNA in a 20μl reverse transcription reaction. Reverse transcriptions were performed according to the manufacturer's protocol by using PRT-oligo-dT primer (500ng) (5'CCGGAATTCAAGCTTCTAGAGGATCCT$_{12}$ 3') and ImProm-II™ reverse transcriptase (Promega, USA). The reaction was incubated at 42°C for 1 hour. The resulting cDNAs were subjected to amplification by PCR using 5-HT gene specific primers. Partial parts of the 5-HT receptor cDNA of *Penaeus monodon* was previously cloned by using degenerate primers corresponding to conserved amino acid sequences of the third (TM III) and the seventh (TM VII) transmembrane regions of the available invertebrate 5-HT receptor genes. Rapid amplification of cDNA ends (RACE) was employed to amplify the 3' and 5' regions of the ovarian 5-HT receptor gene.

In order to amplify the full-length cDNA of the putative 5-HT receptor, two gene specific primers, (5-HTF: 5'-GGTATTGGCTTGGATTTCGGAC-3' and 5-HTR: 5'-CTACCAAAGGTCATCGCGTGACG-3') corresponding to the 5' and 3' end were used

to perform PCR according to the following conditions: denaturation at 94°C for 2 min followed by addition of 1 unit of *Pfu* DNA polymerase (Strategene, USA), then 30 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec. and extension at 68°C for 5 min. followed by a final extension at 74°C for 7 min.

Sequence analysis of 5-HT$_{1Pem}$ cDNA

The full-length cDNA clones of the 5-HT$_{1Pem}$ receptor were sequenced from both ends using ABI Prism model 377 automated DNA sequencer. The nucleotide sequences were analyzed with the GenBank database and translated into the deduced amino acid sequences by Bioedit Sequence Alignment Editor program. Sequence comparison to other invertebrate 5-HT receptors was performed by ClustalX program. In addition, prediction of the transmembrane domains of the cloned receptor was performed by using TMPred program. The putative phosphorylation sites were predicted by the program in http:// cbs.dtu.dk.

Tissue distribution study

The expression of the 5-HT$_{1Pem}$ receptor was examined in various tissues of the wild broodstock shrimp undergoing stage 3 of ovarian maturation. Total RNAs were isolated from ovary, brain and thoracic ganglion, heart, hepatopancreas, muscle, and gill and used as a template for RT-PCR to amplify 5-HT$_{1Pem}$ receptor. Two gene specific primers corresponding to the most variable regions of 5-HT$_{1Pem}$ receptor (5HT1F1: 5'-ACTTCCTGGTGCGGGTCAAC-3'and 5HT1R: 5'-GGTTCCTTGGGCAGCATCTC-3') were used to perform RT-PCR in the presence and absence of reverse transcriptase. Actin was used as an internal control in this study.

Construction of the recombinant expression plasmid (pGEX-i3-5-HT)

The third intercellular loop (i3) of G-protein coupled receptors generally is the most variable region. Nucleotide sequence of 317 base pairs of the i3 loop was amplified by

using a 5' primer linking with *Eco*RI site (5' cggaattcGCACGGAAGAGGATCCACAAG 3') and a 3' primer linking with *Sal*I site (5' gcgtcgacGCTTCCAGGGTCTCCTTCTTG 3'). In order to construct the expression plasmid, the PCR product was cloned into *Eco*RI and *Sal*I sites of pGEX-5X-1 (Amersham Biosciences, USA) containing glutathione-S-transferase (GST) that facilitated fusion protein purification. The recombinant plasmids were sequenced in order to confirm for the corrected reading frame.

Expression of i3-5-HT receptor protein for antibody production

E.coli JM 109 transformed with recombinant plasmid (pGEX-i3-5-HT) was grown overnight at 30 °C in terrific broth (TB) media containing 100 μg/ml ampicillin. The expression of GST-i3-5-HT fusion protein was induced with 0.1 mM IPTG for 8 hours at 30 °C. The 5-HT fusion protein was purified by using glutathione-affinity agarose bead (Sigma, USA). The eluted protein product was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In order to separate receptor protein, i3-5-HT from GST, the bacterial protein lysate was allowed to bind glutathione agarose bead after the induction step. The cleavage of i3-5-HT fusion protein was performed by incubating the bead containing fusion protein with factor Xa (Amersham Biosciences, USA) (10 unit per 1 mg fusion protein). The reaction was incubated at 22 °C for 16 hours and then centrifuged at 8,000 g for 5 min. After centrifugation, the supernatant was collected and verified by SDS-PAGE using a 13% gel. The purified cleavage product of i3-5-HT protein was used as an antigen to generate antibody in mice.

Western blot analysis

Membrane proteins of the wild broodstock *P. monodon* from ovary undergoing various stages of ovarian maturation were prepared by homogenizing and lysing the tissues with 0.2 % Triton X-100 in buffer M (100 mM NaCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM PMSF, pH 7.4). The tissue debris was removed by

centrifugation at 600g and then at 6,000g. After centrifugation at 20,000g for 30 min at 4 °C, the membrane pellet was dissolved in 0.2% Triton X-100 in buffer M. Protein concentration was determined by using Coomassie blue dye protein assay (Pierce Biotechnology, USA). The membrane protein (100 µg) and purified GST-i3-5-HT fusion protein product (9 µg) were loaded on a 10% SDS-PAGE gel and subsequently transferred to nitrocellulose membrane by electroblotting. To block non-specific binding the membrane was incubated with blocking solution (0.5% skim milk in 1X PBS) for 1 hour at room temperature or overnight at 4 °C. The membrane was rinsed 3 times with PBST (0.2% tween 20 in 1X PBS) and then incubated for 1 hour at room temperature with antiserum (anti-i3-5-HT antibody, 1:500 in blocking solution). After the membrane was rinsed with PBST 3 times, it was incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-mouse IgG (1:4,000 in blocking solution). After the membrane was rinsed with PBST, the film was exposed to the blot for 1 hour. The signal was detected by using ECL plus kits (Amersham Biosciences, USA).

Immunohistochemistry

Live mature female shrimps (P. monodon broodstock) at stage 4 of ovarian development were fixed by injection of Davidson's fixative into the vicinity of the ovary. Then, the ovaries were dissected free from other tissues, cut into small pieces, and further immersed in the same fixative at room temperature for 24 hrs. Tissues were processed by standard paraffin technique and sectioned at 5 µm thickness. These sections were deparafinized with 2 changes of xylene for 10 min each and then hydrated with a sequentially decreasing concentration of ethanol: 100, 95, 80 and 70 % ethanol. The hydrated sections were immersed in 70% ethanol containing 1% saturated lithium carbonate for 15 min. The sections were subsequently incubated in 1% H_2O_2 in absolute methanol for 15 min to abolish endogenous peroxidase activity and then rinsed once with

0.1 M PBS, pH 7.4. The sections were covered with 4% bovine serum albumin (BSA) in 0.1 M PBS, pH 7.4 containing 0.1% glycine for 1 hr to block non-specific binding and free aldehyde groups. After the blocking step, the sections were incubated with 0.5% Triton X-100 for 15 min to permeabilize the membrane, which aided antibody penetration, then rinsed once with 0.1 M PBS, pH 7.4. Subsequently, the sections were incubated with primary antibody (polyclonal antibody against i3-5-HT_{1Pem} receptor) diluted 1:200 in 4% BSA in 0.1 M PBS, pH 7.4 containing 0.25% Triton X-100 overnight at room temperature. After the sections were washed 3 times with 0.1 M PBS, pH 7.4 containing 0.1% tween-20 for 5 min each, they were then incubated with secondary antibody (HRP-conjugated anti-mouse IgG). After the sections were washed 3 times with 0.1 M PBS, pH 7.4 containing 0.1% tween-20 for 5 min each, the staining was performed by incubating the sections with AEC reagent (3-Amino-9-ethylcarbazole) to develop a red color. The reaction was stopped in H_2O and the sections were dipped in haematoxylin solution for nuclei counter staining. The sections were mounted in 90% buffered glycerol solution and observed under a Nikon digital light microscope.

RESULTS

Cloning of the full-length ovarian 5-HT receptor

Previous studies obtained the nucleotides sequences of the middle, 5' and 3' end regions of the 5-HT receptor cDNA. The full-length cDNA of the 5-HT receptor was performed using gene specific primers corresponding to the 5' and 3' end of this gene. The resulting PCR product of 2.3 Kb was obtained. Three recombinant clones harbouring the full-length cDNA of 5-HT receptor were sequenced from both directions. According to the nomenclature rules proposed by Tierney, et al 2001 (Tierney, 2001), the putative ovarian 5-HT receptor has high sequence homology to vertebrate 5-HT receptor subtype 1. Therefore, the receptor was named 5-HT_{1Pem} receptor.

Sequence analysis of the full-length cDNA of the 5-HT_{1Pem} receptor

The complete nucleotide and deduced amino acid sequences of the full-length 5-HT_{1Pem} receptor is shown in Fig. 1. The full-length cDNA of the 5-HT_{1Pem} receptor consists of 2291 nucleotides with an open reading frame of 1773 nucleotides encoding a protein of 591 amino acids. A ten GGC tandem repeat (encoding for glycine) was observed in the third intracellular loop. The 5' and 3' untranslated regions (UTR) contain 348 and 170 nucleotides, respectively. The nucleotide sequence, AAGATGG between the position 346 to 352 resembles the Kozak consensus sequence, A/GXXATGG and gives one long open reading frame. However, no putative poly A signal was found in the 3'UTR. The deduced amino acid sequences of 5-HT_{1Pem} receptor was analyzed by blastp and showed high sequence homology to all known invertebrate and vertebrate 5-HT receptors especially 5-HT_1 receptor.

Hydrophobicity analysis of the deduced amino acid sequences of 5-HT_{1Pem} receptor suggests the presence of the seven hydrophobic regions corresponding to the seven transmembrane domains that are the major characteristic of all G-protein coupled receptors. Sequence comparison of the 5-HT_{1Pem} with other invertebrate 5-HT receptors

shows that the receptor shares high sequence identity especially within the conserved seven transmembrane regions (Fig. 2). This receptor displayed the highest sequence identity to 5-HT_{1Pan} (94%), followed by 5-HT_{1ADro} (75%), 5-HT_{Hel} (75%), and 5-HT_{1Bdro} (70%). Dendrogram analysis revealed that the 5-HT_{1Pem} receptor was grouped and clustered primarily with 5-HT_1-like receptors cloned from insect species such as 5-HT_{1ADro}, 5-HT_{1BDro}, 5-HT_{Hel}, and 5-HT_{Bom} (Fig. 3). The topology model of 5-HT_{1Pem} receptor showed the large third intracellular loop of 120 amino acids, a short carboxyl tail of 26 amino acids, and some conserved amino acids similar to the vertebrate 5-HT_1 receptor family (Fig. 4). Sequence analysis revealed 8 potential N-linked glycosylation sites in the extracellular N-terminal domain. One potential protein kinase A phosphorylation site was found in the second intracellular loop. Two protein kinase C phosphorylation consensus sequences were found within the third intracellular loop. No palmitoylation site was observed in the C-terminal end. In addition, amino acids that are conserved in all G-protein coupled receptors were found in this receptor as shown in Fig. 1.

Tissue distribution analysis

Tissue distribution of the 5-HT_{1Pem} receptor of wild broodstock shrimp was analyzed by semiquantitative RT-PCR using two gene specific primers corresponding to the sequences in the second extracellular loop and the third intracellular loop, respectively. The result demonstrated that 5-HT_{1Pem} receptor mRNA was expressed at different levels in all tissues examined. This receptor is expressed at relatively high levels in ovary, moderate levels in brain and thoracic ganglion, hepatopancreas, abdominal muscle, and gill, and slightly lower levels in heart (Fig. 5). No PCR product was observed in the control reactions performed in the absence of reverse transcriptase suggesting that no genomic DNA was present.

Expression of 5-HT$_{1Pem}$ receptor protein and mRNA

Polyclonal antibody against of the third intracellular loop (i3-5-HT) of 5-HT$_{1Pem}$ receptor was generated. The i3-5-HT was cloned into an expression vector (pGEX 5X-1) containing glutathione S-transferase (GST). The i3-5-HT/GST fusion protein was applied to a SDS-PAGE and demonstrated a major band with the molecular weight of 38 kDa corresponding to the calculated molecular weight for the fusion protein (12 kDa for the i3-5-HT and 26 kDa for GST). In addition, 2 minor bands were possibly degradation forms of the fusion protein. After cleavage with factor Xa, the i3-5-HT receptor protein (12 kDa) was obtained and used for immunization to raise anti i3-5-HT antibody. The antibody specifically reacted with i3-5-HT receptor protein (Fig. 6A and 6B).

Membrane proteins extracted from ovary of a wild broodstock shrimp were prepared and used for western blot analysis to detect the expression of 5-HT$_{1Pem}$ receptor protein. Western blot analysis using polyclonal antibody against i3-5-HT receptor showed that this antibody bound specifically to a protein of molecular weight approximately 50 kDa isolated from the ovarian tissues of a wild broodstock shrimp. The 5-HT$_{1Pem}$ receptor protein was expressed at relatively high levels in ovary. The observed molecular weight of this receptor is lower than the expected molecular weight (63 kDa) predicted from the cDNA sequences.

In order to investigate the involvement of 5-HT$_{1Pem}$ receptor protein during ovarian maturation of wild broodstock shrimps, membrane proteins were extracted from ovaries undergoing developmental stage 1, 2, 3, and 4 and during spent phase. The anti-i3-5-HT receptor antibody was specifically interacted with an ovarian membrane protein of molecular weight approximately 50 kDa. The signal increased progressively in ovary undergoing stage 1 to 4 and decreased in the ovary during spent phase. The expression of receptor protein corresponded to their mRNA levels (Fig. 7).

Immunohistochemistry

The expression of 5-HT_{1Pcm} receptor can be localized at the cellular level by immunohistochemistry using primary antibody against i3-5-HT_{1Pcm} receptor in 1:200 dilution and secondary antibodies (HRP-conjugated anti-mouse IgG). The positive signal was developed by using AEC reagent (3-Amino-9-ethylcarbazole) resulting in a red color development. The results showed that the positive signal (dark red color) of the 5-HT_{1Pcm} receptor was observed at the membrane of mature oocyte stage 4 and at the cortical rods membrane (Fig. 8). These results suggest that 5-HT_{1Pcm} receptor may be involved in ovarian maturation and spawning.

DISCUSSION

In this study, we have cloned and sequenced a putative 5-HT_1 receptor that belongs to the G-protein coupled receptor family from penaeus shrimp. The complete nucleotide and deduced amino acid sequences are shown in Fig. 1. The longest open reading frame consists of 1773 nucleotides encoding a protein (5-HT_{1Pem} receptor) of 591 amino acids. A ten GGC tandem repeat was found in the i3 loop. Sequence analysis of the 5-HT_{1Pem} receptor revealed the major characteristics of the seven transmembrane domains of G-protein coupled receptors (Fig. 2). The 5-HT_{1Pem} receptor has high sequence similarity to invertebrate and vertebrate 5-HT_1 receptors (Fig. 3 and 4). This receptor contained no intron, has a large third intracellular loop, a short carboxyl tail and several conserved amino acids of the G-protein coupled receptors. Based on the analogous structure of β-adrenergic receptor, the aspartate amino acid in TM III corresponding to Asp-287 of the 5-HT_{1Pem} receptor is predicted to be the counter ion for the amine group of 5-HT (Bockaert and Pin, 1999; Shih et al., 1991). From the sequence analysis, it is possible that 5-HT_{1Pem} receptor is a 5-HT_1 -like receptor of penaeus shrimp.

Invertebrate 5-HT_1-like receptors have been identified from *Drosophila melanogaster* : 5-HT_{1A}dro and 5-HT_{1B}dro (Saudou et al., 1992). *Caenorhabditis elegans* : 5-HT_{Ce} (Olde and McCombie, 1995), sea snail (*Aplysia californa*) : 5-HT_{Ap1} and 5-HT_{Ap2} (Angers et al., 1998; Barbas et al., 2002), and fresh water snail (*Lymnaea stagnalis)* : 5-HT_{1Lym} (Sugamori et al., 1993). Most of the receptors encode 445 to 834 amino acids. Most 5-HT_1- like receptors couple to Gαi protein and result in a reduction of cAMP levels. Some receptors also couple to stimulate phospholipase C leading to an increase in inositol triphosphate. However, the pharmacological binding properties of the invertebrate 5-HT_1-like receptors do not follow the mammalian counterparts (Tierney, 2001).

Recently, a 5-HT_1 receptor has been cloned from brain and thoracic ganglion of a crustacean, the 5-HT_{1Pan} of spiny lobster (Sosa et al., 2004). Comparison of the deduced amino acid sequences of 5-HT_1 receptor from lobster and penaeus shrimp demonstrated 94% sequence identity in the transmembrane domains (Fig. 2). The most variable region existed in the N-terminal region and the beginning of the i3 loop (data not shown). In contrast, 5-HT_{1Pan} receptor has a shorter extracellular amino terminal than that is observed in the invertebrate 5-HT_1–like receptor of black tiger shrimp and *Drosophila*. The functional significant of the long extracellular amino terminal is not known. Both receptors have not been functionally characterized in their signal transduction pathway and pharmacological binding property. Comparison of the partial amino acid sequences between TM3 and TM7 of 5-HT_1 receptor among crustacean species such as spiny lobster, crayfish, fresh water prawn and black tiger shrimp demonstrated high sequence identity (Data not shown). The difference was observed only in the beginning of the third intracellular loop. However, a glycine tandem repeat in the i3 loop of 5-HT_{1Pem} receptor was not observed in other crustaceans or invertebrate 5-HT_1 receptors. The functional significant of the glycine repeat is not known. In *Drosophila melanogaster*, a ten glycine-serine repeat was found in the first putative extracellular domain of the 5-HT_7-like receptor and proposed to be putative attachment sites for glycosaminoglycans (Witz et al., 1990). In addition, a polyglutamine motif was found in the i3 loop of 5-HT_{1ADro} (or previously named 5-HT_{dro2A}) receptor (Saudou et al., 1992).

Tissue distribution analysis demonstrated that 5-HT_{1Pem} receptor mRNA was expressed at variable levels in many tissues examined (Fig. 5), suggesting that this receptor is involved in many physiological functions such as reproduction, muscle contraction, escape behavior, etc. It has been proposed that 5-HT stimulates the release of gonad stimulating hormone from brain and thoracic ganglion and results in ovarian

maturation (Sarojini et al., 1995). The presence of 5-HT_{1Pem} receptor in brain and thoracic ganglion of the wild broodstock shrimp suggests that 5-HT may mediate the effect through an interaction with 5-HT_{1Pem} receptor. In addition, 5-HT has been shown to increase heart rate and force of cardiac contraction in crustacean (Saver and Wilkens, 1998)

In this study, the i3 loop of 5-HT_{1Pem} receptor which contained the lowest sequence homology between G-protein coupled receptor was chosen to generate an i3-5-HT-GST-fusion protein. The fusion protein was cleaved by factor Xa to give 12 kDa of i3-5-HT protein to use as an antigen for raising anti-5-HT_{1Pem} receptor antibody in mice (Fig. 6). This antibody was further used in western blot analysis on various membrane proteins and for subcellular localization of this receptor in shrimp. The antibody reacted specifically with an ovarian membrane protein of molecular weight 50-55 kDa. Interestingly, the expression of the 5-HT_{1Pem} receptor protein was progressively increased from ovary stage 1 to 4 and decreased during spent phase (Fig. 7). The expression of 5-HT_{1Pem} receptor especially during stage 4 of ovarian maturation was observed on the oocyte membrane and cortical rod membrane (Fig. 8). During the final stages of oocyte maturation in penaeid shrimp, the cortical rods appear as a rod-like structure arranged radially around the periphery of the oocyte plasma membrane. The cortical rods are expelled upon spawning allowing direct contact of the spawned egg with sea water. Therefore, the expression of 5-HT_{1Pem} receptor may be tightly regulated during shrimp ovarian development and spawning. The reduction of the expression of 5-HT_{1Pem} receptor in the ovary after spawning during spent phase suggests that this receptor is not only expressed in ovarian membrane but also expressed in the spawning oocytes. Previous studies showed that an injection of 5-HT in *Penaeus vannamei* induced ovarian maturation and spawning comparable to the effect induced by an eyestalk ablation technique (Vaca and

Alfaro, 2000). In addition, an injection of 5-HT in *M. rosenbergii* stimulated ovarian maturation, increased the number of mature oocytes and shortened the period from mating to spawning (Chen et al., 2003). Therefore, it is possible that 5-HT caused an induction of ovarian maturation and spawning by interaction with a 5-HT_1–like receptor. The functional characterization of the cloned 5-HT receptor of *Penaeus monodon* will be essential for understanding the signaling mechanisms of 5-HT receptors and to define a specific agonist that can be potentially used to inject in black tiger shrimp to induce ovarian maturation and spawning without an eyestalk ablation.

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FIGURE LEGEND

Fig 1. Nucleotide and deduced amino acid sequences of the full-length 5-HT_{1Pem} receptor. The deduced amino acid sequence of the 5-HT_{1Pem} is shown in a single letter under the respective codon. The asterisk represents the terminator codon. Seven putative transmembrane domains are boxed and numbered (I-VII). Amino acid letters circled represent residues that are conserved in all G-protein coupled receptors. Triangles represent potential N-linked glycosylation sites. Solid circles represent putative protein kinase C phosphorylation sites. A solid square represents a putative protein kinase A phosphorylation site. The nucleotide sequence is available in the GenBank database under accession number AY 6661549.

Fig. 2. Comparison amino acid sequence of the 5-HT_{1Pem} receptor with other 5-HT_1 receptors and putative invertebrate 5-HT receptors. The amino acid sequences of the seven transmembrane domains and the adjacent regions of the 5-HT_{1Pem} were aligned with 5-HT_1 receptors from *Drosophila melanogaster* (Dro), *C. elegans* (Ce), *Aplysia californica* (Ap), *Lymnaea stagnalis* (Lym), human *Homo sapiens* (Hum), and mouse *Mus musculus* (Mou) and with putative 5-HT receptors from *Heliothis virescens* (Hel), *Bombyx mori* (Bom), *Panulirus interruptus* (Pan), *Boophilus microplus* (Boo) by Clustal X. Predicted transmembrane domain I-VII are overlined. Numbers in parentheses correspond to the number of amino acids at the amino terminal end and the third intracellular loop that are not presented in the alignment.

Fig. 3. Dendrogram analysis of the 5-HT_{1Pem} receptor of *P. monodon* and other 5-HT receptors. The amino acid sequences of the seven transmembrane domains and the adjacent regions of 5-HT receptors from *Drosophila melanogaster* (Dro), *Heliothis virescens* (Hel), *Bombyx mori* (Bom), *C. elegans* (Ce), *Aplysia californica* (Ap), *Lymnaea stagnalis* (Lym), human *Homo sapiens* (Hum), mouse *Mus musculus* (Mou), *Penaeus monodon* (Pem), *Panulirus interruptus* (Pan), and *Boophilus microplus* (Boo) were aligned by Clustal X. The phylogenetic tree was drawn with the NJ plot program. The amino acid sequences were retrieved from the GenBank database and renamed as proposed by Tierney, 2001. Invertebrate 5-HT_2 and 5-HT_7 receptors were included in dendrogram analysis.

Fig. 4. Topology model of the 5-HT$_{1Pem}$ receptor of *P. monodon*. Amino acid residues that are conserved in all known G-protein coupled 5-HT receptors are indicated by solid circles. Consensus sites for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) and for N-linked glycosylation (Y) are indicated. Two conserved cysteine residues thought to form a disulfide bond are shown.

Fig. 5. Tissue distribution of the 5-HT$_{1Pem}$ mRNA in wild broodstock (stage 3) *P. monodon*. Upper panel shows semi-quantitative RT-PCR products of 5-HT$_{1Pem}$ from total RNA isolated from ovary (O), brain and thoracic ganglia (TG), heart (H), hepatopancreas (HP), muscle (Ms) and gill (G) of a wide broodstock undergoing stage 3 ovarian maturation. The PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. Lower panel shows RT-PCR products of a control gene (actin gene) size fractionated on a 2% agarose gel. Plus (+) and minus (-) under letters are reactions in the presence and absence of the reverse transcriptase, respectively. Positive control (+ve) was performed with plasmid harbouring 5-HT$_{1Pem}$ cDNA or actin cDNA. Negative control (-ve) was performed with distilled water.

Fig. 6. Anti-i3-5-HT antibody verification after immunization step by western blot analysis. A) Coomassie blue staining of the purified fusion protein (FP) containing i3-5HT receptor, purified GST alone of *Schistosoma japonicum* and the factor Xa cleavaged protein products containing i3-5-HT receptor protein were run on 13% SDS-PAGE. B) Western blot analysis using anti-i3-5-HT antibody (1:8,000 dilution) from mouse immunization. M represents a protein broad range marker.

Fig. 7. Expression of 5-HT$_{1Pem}$ receptor protein (A) and mRNA (B) from the ovary of *P. monodon* undergoing developmental stage 1 to 4 (OV1 to OV4) and during spent phase (SP). A) Western blot analysis using anti-i3-5-HT antibody (1:500 dilution). M represents a protein broad range marker. B) Semi-quantitative RT-PCR of the expression of the i3-5-HT$_{1Pem}$ receptor was analyzed. Actin was used as an internal control.

Fig. 8. Localization of 5-HT_{1Pem} receptor from the ovary of *P. monodon* undergoing developmental stage 4. A) Ovarian sections incubated without antibody was used as a negative control. B) The section was incubated with the polyclonal antibody against i3-5-HT_{1Pem} receptor (1: 200 dilution) and subsequently incubated with the secondary antibody, HRP conjugated anti-mouse IgG. The arrow represents the positive signal of 5-HT_{1Pem} receptor (dark red region) at the membrane around oocyte and around cortical rods membrane of oocyte stage 4.

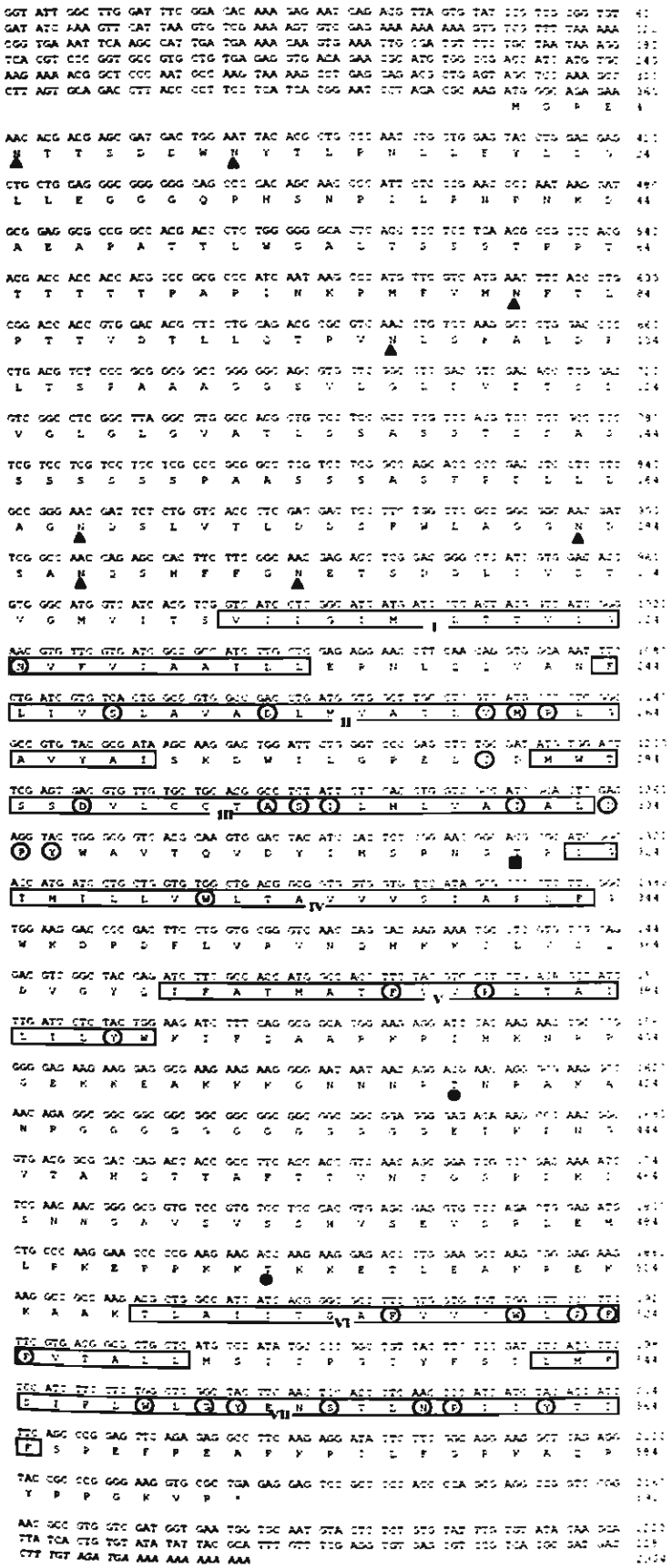
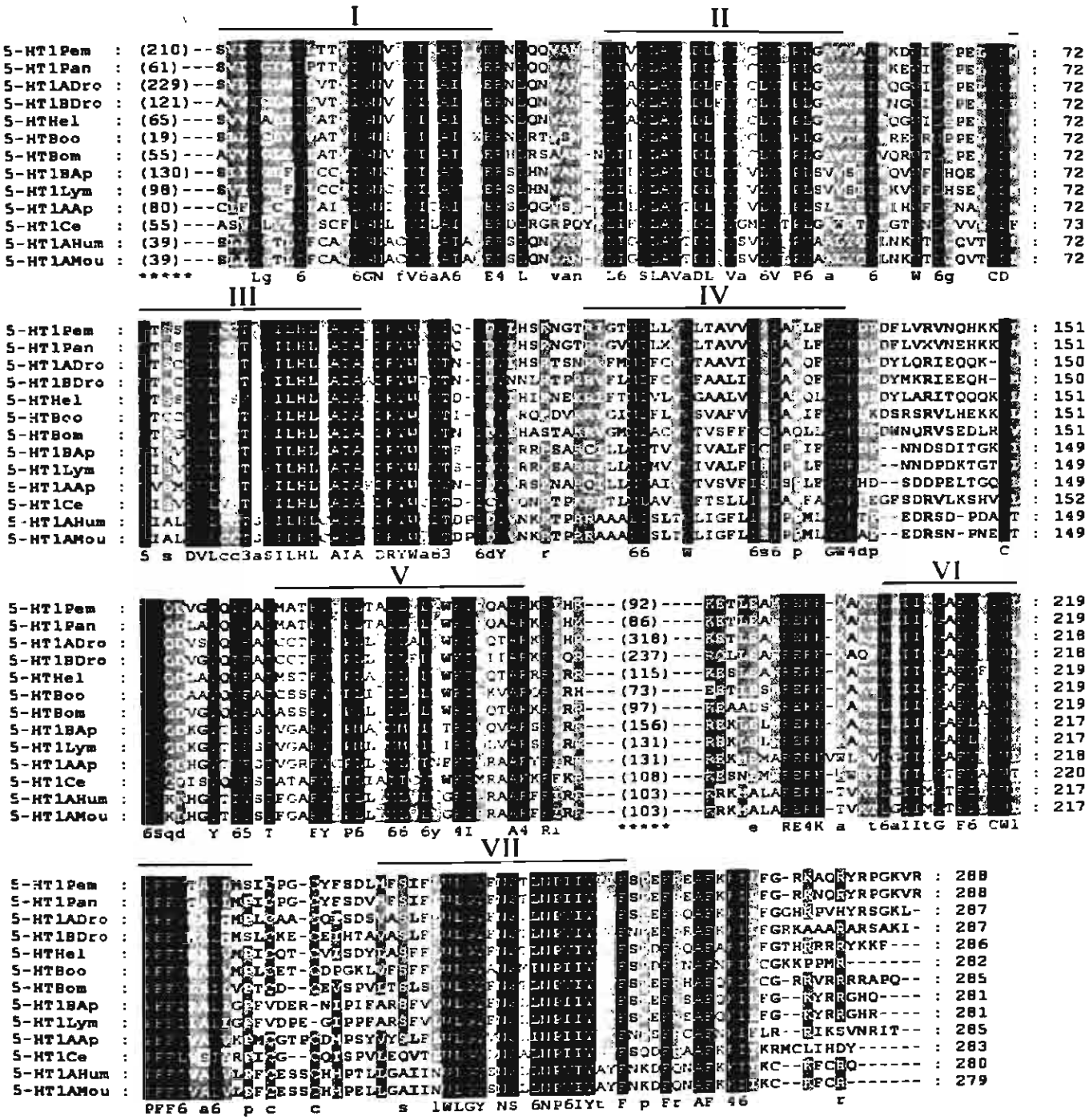


Figure 1. Ongvarrasopone, et al.



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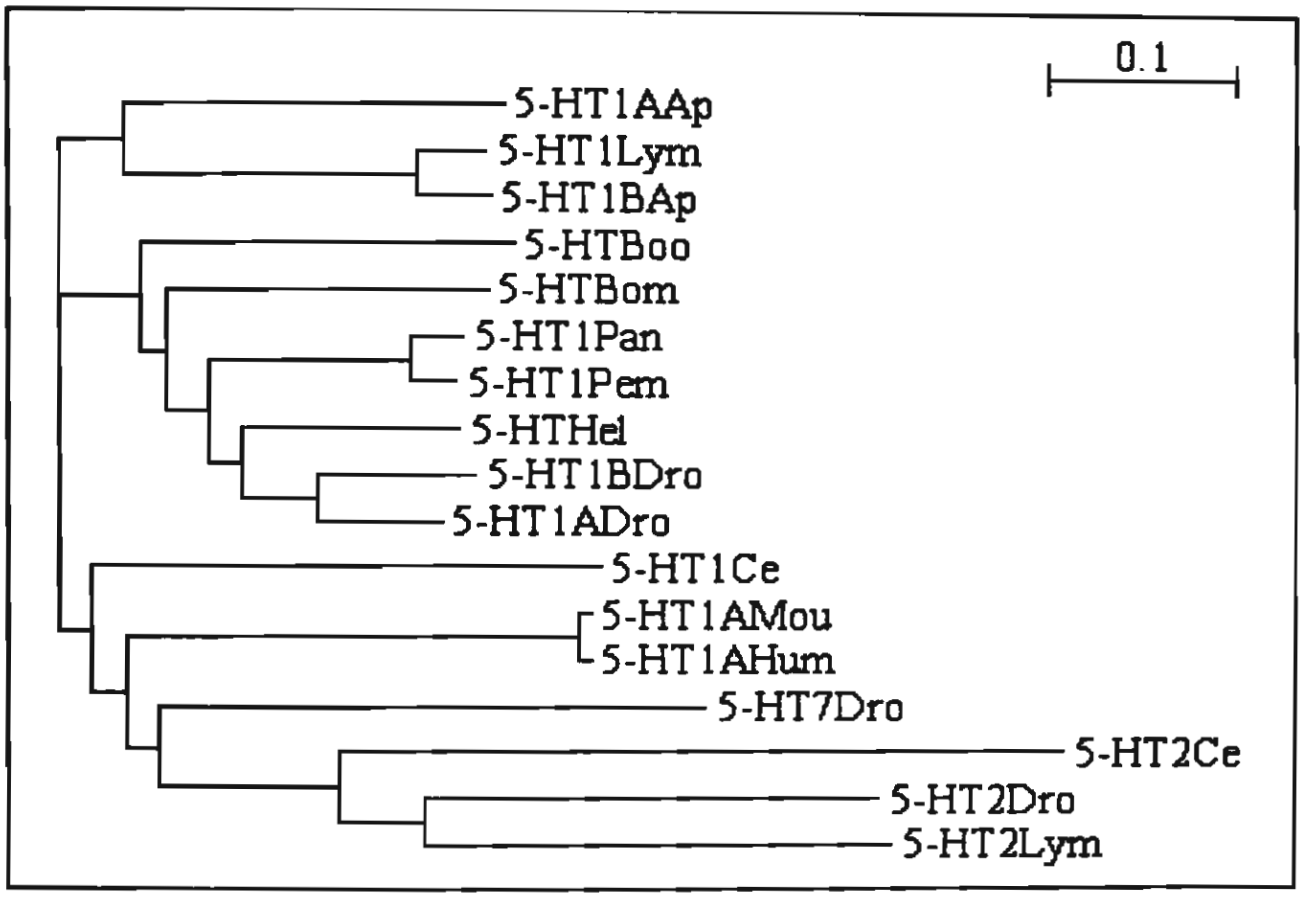

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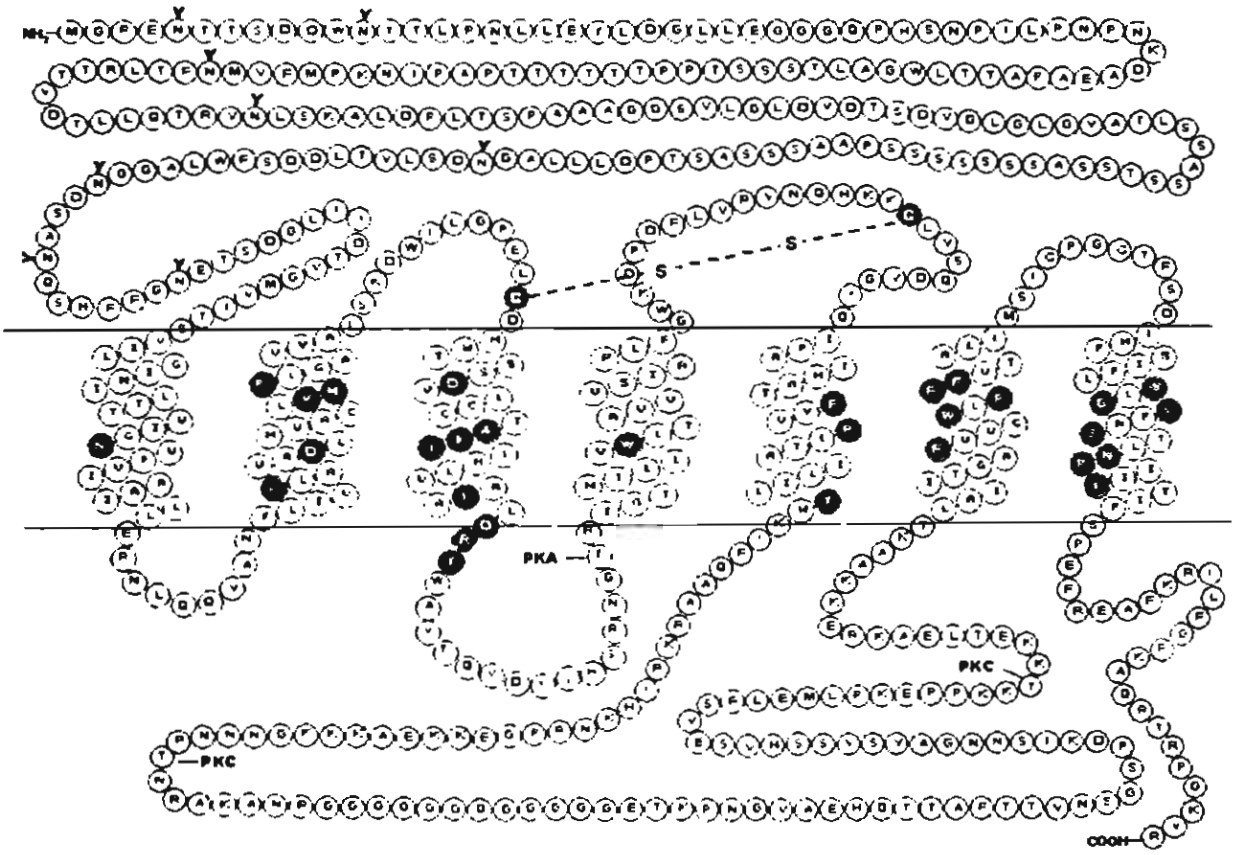

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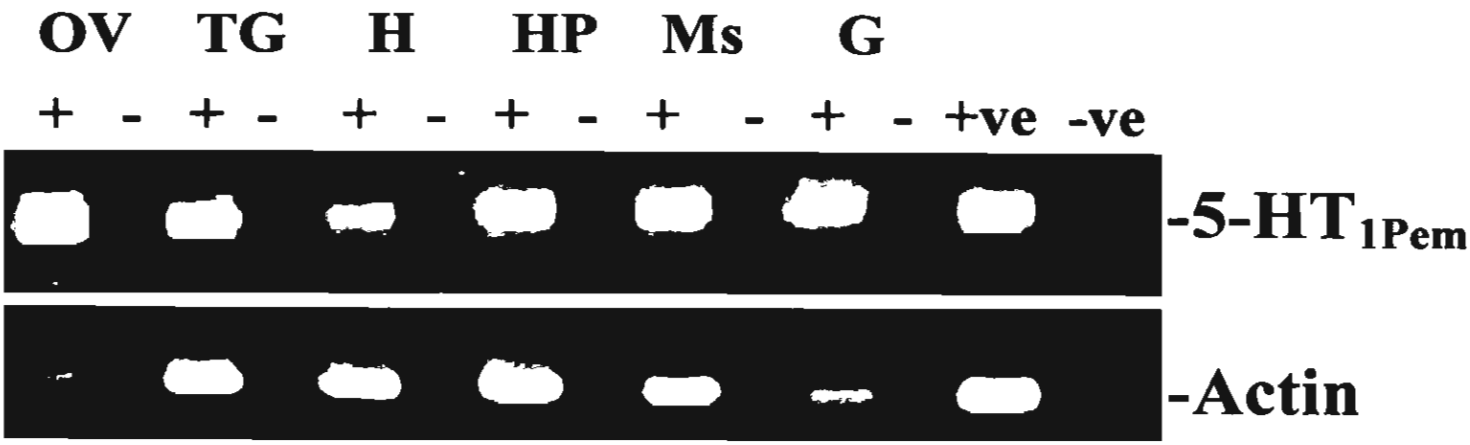

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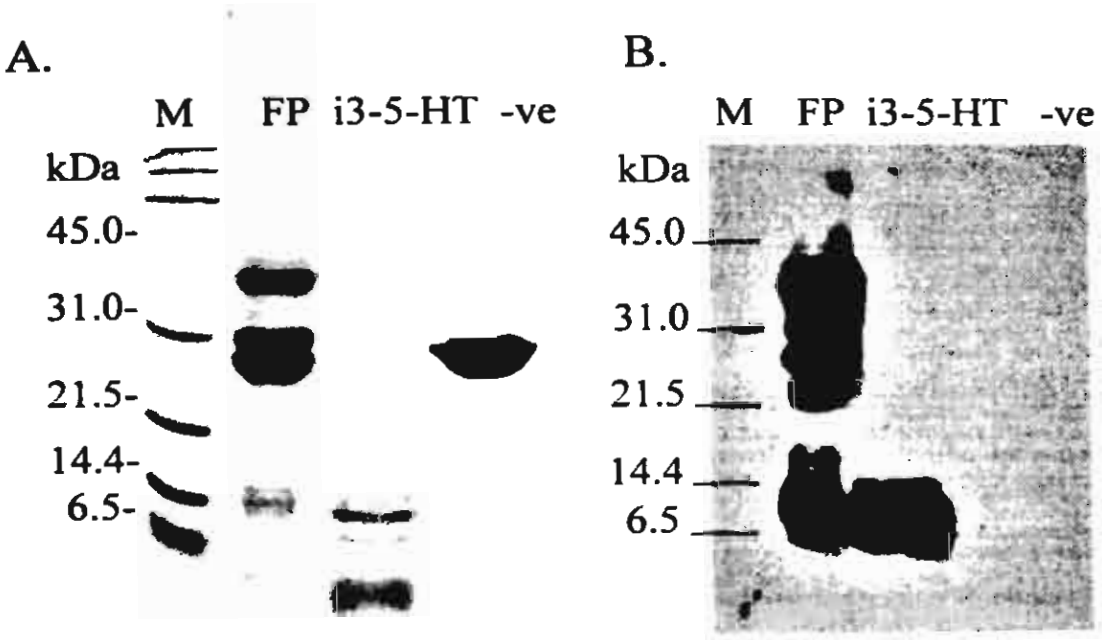

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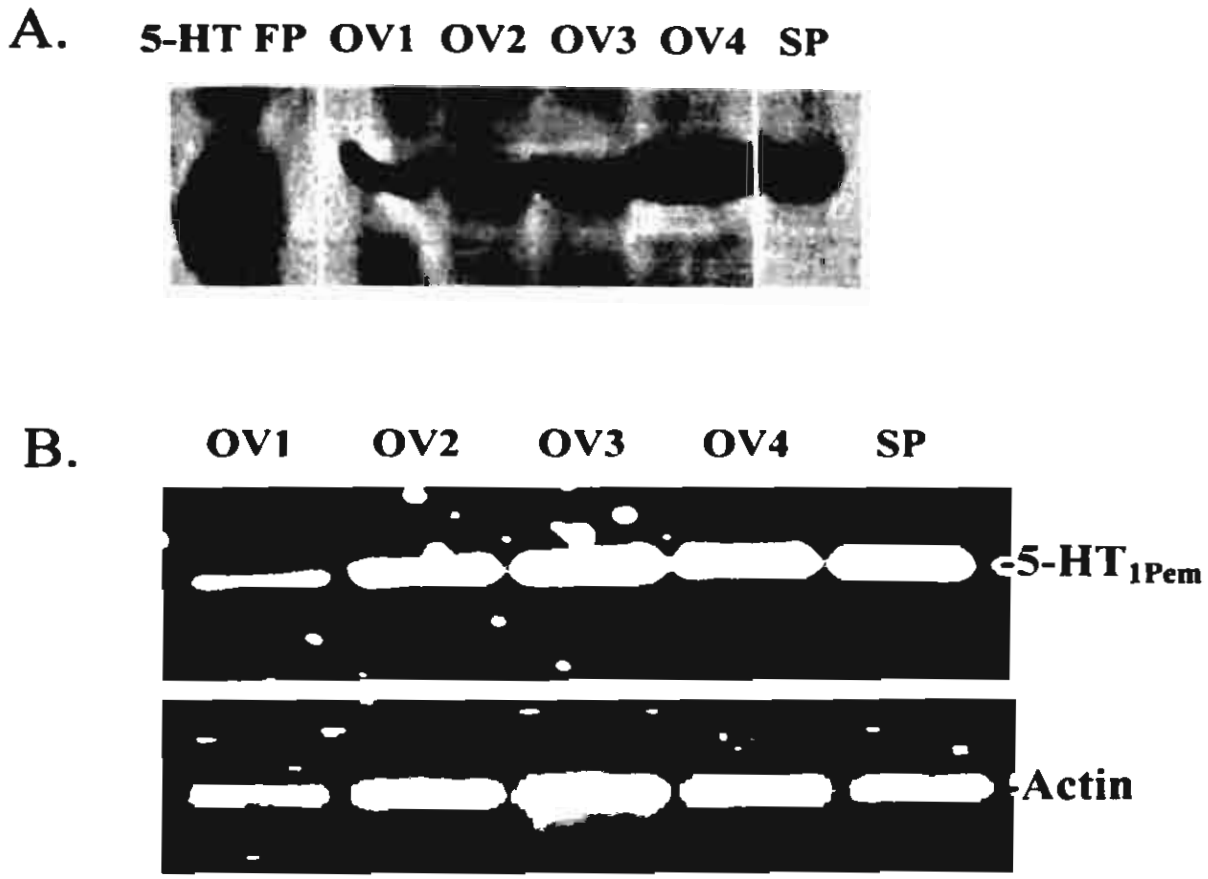

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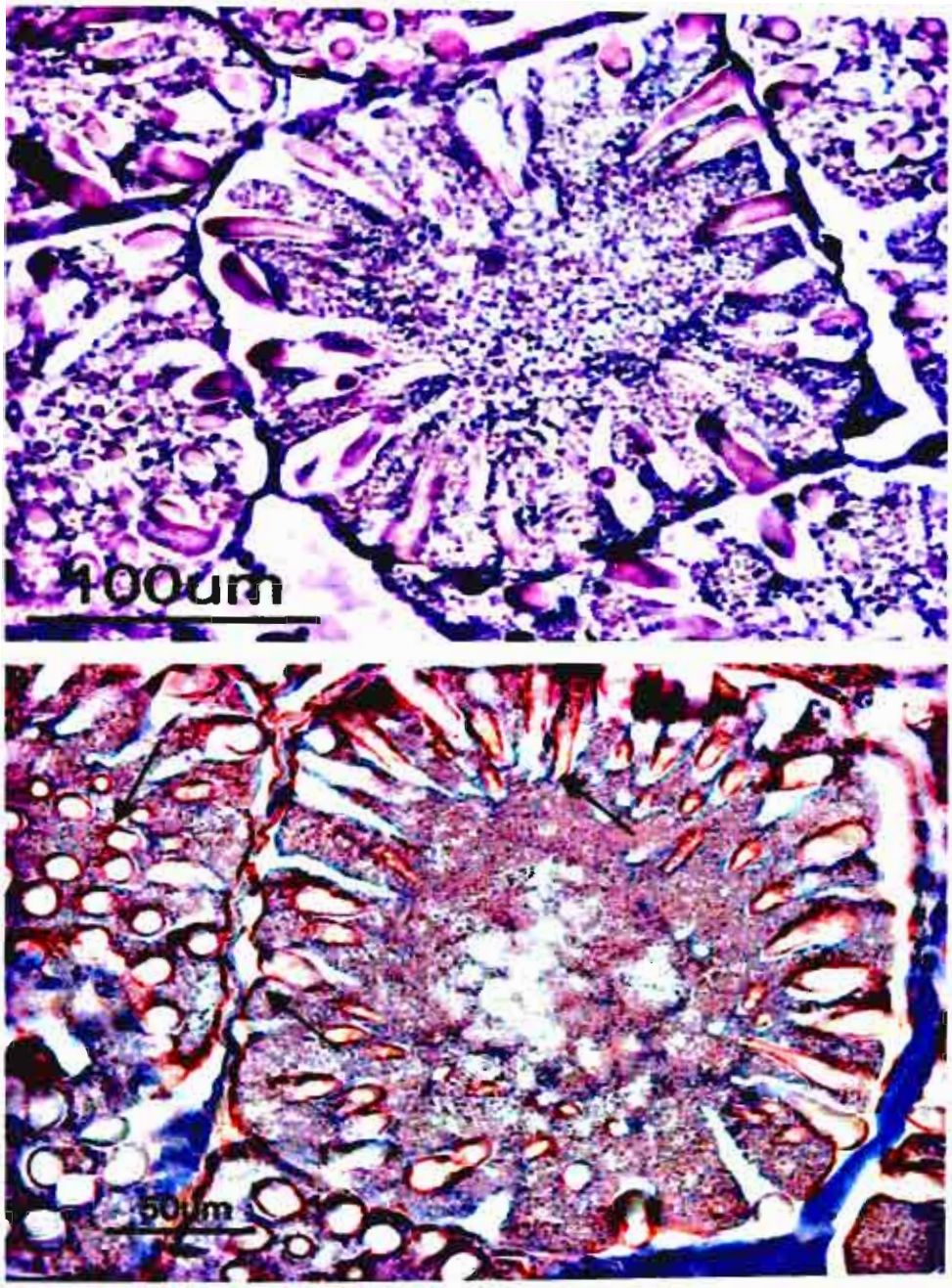

Figure 8. Ongvarrasopone, et al.

PROJECT OUTPUTS

1. Publication

Ongvarrasopone, C., Roshorm, Y., Somyong, S., Pothiratana, C. and Panyim. S. Molecular cloning and expression of an ovarian 5-HT receptor cDNA from *Penaeus monodon. (Submitted to Journal of Experimental Biology)*

2. Oral Presentation

Chalermporn Ongvarrasopone, Yaowaluck Roshorm, Suthasinee Somyong, and Sakol Panyim. Molecular cloning and expression of a 5-HT receptor from *Penaeus monodon* นำเสนอในการประชุมเพื่อเสนอผลงานวิจัยของนักวิจัยรุ่นใหม่ และพบกับเมธีวิจัยอาวุโส สกว. วันที่ 14-16 มกราคม 2548 ณ โรงแรมเฟลิกซ์ กาญจนบุรี

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Category Research Article

Title Molecular cloning and expression of an ovarian 5-HT receptor cDNA from Penaeus monodon

Abstract Serotonin 5-hydroxytryptamine, 5-HT mediates a widespread physiological function by interacting with multiple 5-HT receptor subtypes. Using strategies based on the amino acid sequence homology of invertebrate 5-HT1 receptors and Rapid Amplification of cDNA Ends, we have isolated the full-length cDNA encoding a putative 5-HT receptor from an ovary of black tiger shrimp Penaeus monodon. The full-length nucleotide sequences contained 2291 nucleotides. A ten GGC tandem repeat was observed in the third intracellular loop i3. The sequences encoded a protein 5-HT1Pem receptor of 591 amino acids. Deduced amino acid sequences showed high amino acid sequence identities up to 76 with invertebrate and vertebrate 5-HT1 receptors. This 5-

	HT1Pem receptor contained seven transmembrane domains, a large i3 loop, a short carboxyl tail and several conserved amino acids characteristic of 5-HT1 receptor family. The receptor expressed at relatively high levels in ovary and at variable levels in all tissues examined. Polyclonal antibody against the i3 loop of the 5-HT1Pem receptor reacted specifically to an ovarian membrane protein of molecular weight approximately 50 kDa. The expression of 5-HT1Pem receptor protein progressively increased in ovary undergoing stage 1, 2, 3 and 4 of ovarian maturation and was reduced during spent phase. Immunohistochemistry demonstrated the expression of 5-HT1Pem receptor protein on the membrane of mature oocytes stage 4 and surrounding the cortical rod membrane supporting its role in regulation of ovarian maturation and spawning.
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Molecular cloning and expression of a 5-HT receptor from *Penaeus monodon*

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Abstract—Serotonin (5-hydroxytryptamine, 5-HT) mediates a widespread physiological function by interacting with multiple 5-HT receptor subtypes. Using strategies based on the amino acid sequence homology of invertebrate 5-HT$_1$ receptors and Rapid Amplification of cDNA Ends, we have isolated the full-length cDNA encoding a putative 5-HT receptor from an ovary of black tiger shrimp *(Penaeus monodon)*. The full-length nucleotide sequences contained 2282-2291 nucleotides varying in the GGC tandem repeat (7-10 repeat) in the third intracellular loop (i3). It encoded a protein (5-HT$_{1Pem}$ receptor) of 588-591 amino acids. Deduced amino acid sequences showed high amino acid sequence identities (up to 76%) with invertebrates and vertebrates 5-HT$_1$ receptors. This 5-HT$_{1Pem}$ receptor contained seven transmembrane domains, a large i3 loop, a short carboxy tail and several conserved amino acids characteristics of 5-HT$_1$ receptor family. It expressed at relatively high levels in ovary and at variable levels in all tissues examined. Polyclonal antibody against i3 loop of 5-HT$_{1Pem}$ receptor reacted specifically to an ovarian membrane protein of molecular weight approximately 50-55 kDa. The expression of 5-HT$_{1Pem}$ receptor protein was progressively increased in ovary undergoing ovarian maturation from stage 1 to 4 and reduced during spent phase. Immunohistochemistry study showed that 5-HT$_{1Pem}$ receptor was expressed on the cortical rod membrane and surrounding the membrane of mature oocyte of an ovary undergoing stage 4 of ovarian maturation suggesting its role in regulation of ovarian maturation and spawning in *Penaeus monodon*.

Keywords—G-protein coupled receptor; cloning; black tiger shrimp; ovarian maturation; polyclonal antibody.

Output

1. Ongvarrasopone, C., et al., Manuscript in preparation, 2004.

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