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Expression of GFSKLYFamide-like neuropeptide in the digestive system of the sea cucumber *Holothuria scabra* (Echinodermata)

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Neuropeptides are key mediators of physiological processes in animals and a considerable amount of information has been accumulated on their diversity and functions across phyla. However, progress in echinoderm neurobiology has been much slower than others. The sea cucumber *Holothuria scabra* is an economically important tropical echinoderm species in which a neuropeptide is yet to be identified. This immunohistochemical study utilized antibody raised against GFSKLYFamide neuropeptide together with confocal laser scanning microscopy. GFSKLYFamide-like immunoreactivity was shown to be localized in the stomach and small intestines of *H. scabra*. This is the first report that provides evidence for the presence of GFSKLYFamide neuropeptide in the digestive tract of this species.

Key words: GFSKLYFamide, SALMFamides, immunohistochemistry, sea cucumber, *Holothuria scabra*.

INTRODUCTION

The sea cucumber *Holothuria scabra*, also called sandfish, is an echinoderm species in the class Holothuroidea, order Aspidochirotida, family Holothuriidae and genus *H. Metriatyla scabra* (Jaeger, 1833). Other classes in the echinoderm phylum are starfish (Astroidea), sea urchin (Echinoidea), sea lilies (Crinoidea) and brittle stars (Ophruroidea) (Hyman, 1955). *H. scabra* is a premium sea cucumber species that is widely consumed due to its medicinal value but there is a threat of extinction on the natural population due to overexploitation in some parts of Asia (Bussarawit and Thongtham, 1999). Consequently, efforts have been made to culture *H. scabra*, however, there is little knowledge about its biology, particularly the neurohormonal pathway (Cobb, 1988). Neuropeptides are key mediators of physiological processes in animals and a considerable amount of information has been accumulated on their diversity and functions across phyla (Strand, 1999). Echinoderms are of phylogenetic importance to chordates in that they are deuterostomes. They exhibit some remarkable biological phenomena

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such as evisceration and regeneration, the mediation of which may involve neuropeptides. Although, research in echinoderm neurobiology has advanced at a much slower pace compared with that of other phyla, the discovery of SALMFamide peptides, which are related to the FMRFamide-related peptides family, in echinoderm species has been very encouraging (Cobb, 1988; Elphick et al., 1991; Walker et al., 2009).

The tetrapeptide FMRFamide neuropeptide was isolated from the ganglia of a mollusc, the clam *Macrocallista nimbosa* by virtue of its cardioexcitatory effect and became the first known member of the family of FMRFamide-related peptides (Price and Greenberg, 1977). FMRFamide-related peptides are a large collection of neuropeptides found in vertebrates and invertebrates (Price and Greenberg, 1989; Walker et al., 2009). They are identified by the possession of a C-terminal -RFamide (-Arg-Phe-NH_2) amino acid sequence and are often coded for by multiple genes. In the *Caenorhabditis elegans* for example, at least 22 genes are known to encode FMRFamide-related peptides (Li et al., 1999). They have been implicated in roles that include cardiovascular function, modulation of muscle contraction, control of locomotor activity, water balance, neuroendocrine and neuromodulatory activities (Dockray, 2004). The effect of FMRFamide neuropeptide itself could be excitatory or inhibitory on cardiac rhythm and contractility in molluscs, depending on the species under investigation. It activates non-cardiac muscles in molluscs (Greenberg and Price, 1979). In rats, FMRF-amide excites brain stem neurons (Gayton, 1982) and induces the elevation of blood pressure (Mues et al., 1982). An FMRFamide-gated sodium channel, which was the first peptide-gated ion channel was reported in snails (Cottrell et al., 1990; Cottrell, 1997; Lingueglia et al., 1995), and it was shown in rats that brain FMRFamide-activated sodium channels may be involved in the mechanism of salt-sensitive hypertension through the regulation of the brain renin-angiotensin system (Nishimura et al., 2000).

The SALMFamide peptides are the first neuropeptides to be fully characterized and sequenced from any echinoderm and they share some similarities with FMRFamide in that they were initially discovered based on radioimmunoassay using antibodies against FMRFamide (Díaz-Miranda et al., 1992; Elphick et al., 1991), and the presence of a Famide (-Phe-NH_2) at their carboxyl terminal (Walker, 1992). Though it was noted that the FMRFamide immunoreactivity earlier observed in echinoderms could have been due to other peptide families (Díaz-Miranda et al., 1992), the proposed criteria by Price and Greenberg (Price and Greenberg, 1989) for identifying any FMRFamide-related peptide seemed to have been met, at least in part, by SALMFamides. Interestingly, none of the newly characterized peptides possess the penultimate Arginine (-RFamide) motif that is signature of FMRFamide-related peptides hence, the notion that SALMFamides represent a new family of neuropeptides in Echinoderms, and the name 'SALMFamide' was first proposed by Elphick et al. (Elphick et al., 1991). The first fully sequenced echinoderm SALMFamide neuropeptides were identified about two decades ago (Elphick et al., 1991). The two peptides, GFNSALMFamide (S1) and SGPYSFNSGLTFamide (S2), were isolated from the starfish *Asterias rubens* and *Asterias forbesi* both in the class Asteroidea. They were designated SALMFamides because of the conserved "SALMF" at the C-terminal of their amino acid sequence since they lack the penultimate –R motif that characterizes FMRFamide-related peptides. A number of SALMFamide peptides were also identified in other echinoderm species (Elphick and Thorndyke, 2005). These include GFNSALMFamide (S1), SGPYSMTSGLTFamide (MagS2), AYHSALPFamide (MagS3), and AYQTGLPFamide (MagS4) which were isolated from the starfish, *Marthasterias glacialis* (Yun et al., 2007). Interestingly, S1 was discovered in two species of starfish. GFSKLYFamide and SGYSVLYFamide were isolated from the digestive tract of the sea cucumber, *Holothuria glaberrima* and they constitute the first neuropeptides to be isolated from any sea cucumber species (Díaz-Miranda et al., 1992). However, it is yet to be determined if the isolated peptides from the sea cucumber *H. glaberrima* are also expressed in other sea cucumber species as is the case with the expression of S1 in starfish.

Our study of the sea cucumber *H. scabra* is premised on it being a tropical species with high economic value. Moreover, adequate knowledge of the neurohormonal pathway of this species is important for its aquaculture and conservation. Of particular interest is the digestive system, which is critical for effective nutrition and growth of *H. scabra* under culture conditions. Unfortunately, a neuropeptide or peptide hormone is yet to be identified in this species. Hence, this project aims to determine the presence and distribution of GFSKLYFamide neuropeptide in the stomach and small intestine of *H. scabra*.

MATERIALS AND METHODS

Animals

Adult sea cucumber *H. scabra* were sourced from the Andaman Sea at Koh Jum Island in Krabi province of Thailand. Six adult *H. scabra* used in this study have an average weight of 123 g (62 to 175 g) and were collected at different times between May, 2009 and April, 2010. They were maintained in filtered natural sea water within a temperature range of 28 to 31°C and salinity of about 32 ppt at the Shrimp Genetic Improvement Centre, Chiya, Suratthani before being transferred to the laboratory in Bangkok in oxygenated sealed plastic bags.

Antibody

Polyclonal antibody against GFSKLYFamide was generously provided by Professor García-Arrarás (University of Puerto Rico, USA). The antibody was raised (as described by Díaz-Miranda et al., 1995), using 63 g of synthetic GFSKLYFamide coupled to 15 mg BSA with 0.3% glutaraldehyde. The reaction was stopped by the addition of 1 M Glycine, and the mixture dialyzed. Aliquot of the dialysate BSA-GFSKLYFamide conjugate was emulsified with complete Freund's adjuvant and injected into two rabbits with half of the emulsion each, subcutaneously and intraperitoneally. Two boosters of the aliquot mixed with incomplete Freund's adjuvant were given after the initial injection and sera was collected 7 and 14 days after each injection, preabsorbed with BSA and assayed by immunohistochemical reactivity on sections of sea cucumber intestine and by dot blot.

Immunohistochemistry

Indirect immunofluorescence method was used to analyze the occurrence of GFSKLYFamide-like immunoreactivity in the digestive tract of *H. scabra* using frozen sections. Stomach and intestinal tissues of *H. scabr*a were processed for immunohistochemical analysis according to a previously described method (Ajayi and Withyachumnarnkul, 2013). Briefly, animals were anaesthetized in ice for about 30 min before dissection. Dissected tissues were fixed immediately in 4% paraformaldehyde for 5 to 24 h at 4°C, washed three times in PBS for 10 min each and cyoprotected in 30% sucrose overnight. Sections of 7 µm thickness were cut with a cryostat (LIECA CM 1850), mounted on poly-L-lysine-coated slides and permeabilized in PBS-Triton X-100 (0.1%) for 5 min before blocking with normal goat serum (1:50 in PBS) for 1 h. Sections were incubated overnight at room temperature with primary antibody (1:1000 in PBS) followed by three washes in PBS-Tween 20 (0.05%) for 10 min each. This was followed by 1 to 2 h incubation in Alexa 488-conjugated goat anti-rabbit IgG (1:500 in PBS) at room temperature. Sections were then washed 3 times, 10 min each, in PBS-Tween 20. Slides were incubated in TOPO-3 (1:500 in PBS) at room temperature for 1 h then rinsed in PBS-Tween 20 (0.05%) and mounted in buffered glycerol (pH 8.6). Tissues were examined and photographs taken with an Olympus Confocal laser scanning microscope (FV 1000). Images were processed using OLYMPUS FLOVIEW 1.7b viewer and Adobe Photoshop CS3. Preabsorption control was done by substituting the primary antibody with PBS or preabsorbed antibody. Working dilutions of GFSKLYFamide antibody (1: 1000 in PBS) were preabsorbed overnight at 4°C with 100, 50 and 10 ng/µl of synthetic GFSKLYFamide peptide.

RESULTS

In this study, portions of the stomach and descending intestine were examined for immunoreactivity to GFSKLYFamide antiserum. Histological features of the stomach and small intestine of *H. scabra* are similar to those of other sea cucumber species. Histologically, the mucosa of the digestive tract of echinoderms consists of pseudostratified luminal epithelium, adjacent to which there is a layer of internal connective tissue. The connective tissue layer is followed by a muscle layer and a layer of visceral coelomic epithelium. A basal lamina separates the internal connective tissue layer from the luminal epithelium and from the muscle layer. The muscle layer is usually subdivided into an inner circular muscle layer and an outer longitudinal layer. External connective tissue layer is associated with the coelomic epithelial layer (Garcia-Arraras et al., 2001; Hyman, 1955). Results show the presence of GFSKLYFamide-like immune-reactivity in the stomach and small intestines of the sea cucumber *H. scabra*. In the stomach, GFSKLYFamide-like immunoreactivity was localized within the submucosal and muscular layers. Immunoreactive cell bodies could also be seen in the submucosal layer (Figure 1). The small intestine expresses intensely stained immunoreactive fibers in mucosa as well as the coelomic epithelium, adjacent to the muscle tissue (Figure 2).

DISCUSSION

The digestive system of *H. scabra* consists of mouth, esophagus, stomach, ascending intestine, descending small intestine, large intestine, rectum, cloaca and anus, supported by a mesentery (Hyman, 1955; Bai, 1971). In this study, only the stomach and small intestines of *H. scabra* were examined for immunoreactivity against GFSKLYFamide neuropeptide. Results indicate wide-spread distribution of GFSKLYFamide-like immune-reactivity in the stomach and small intestines of the sea cucumber *H. scabra* suggesting that GFSKLYFamide neuropeptide might be present in the digestive tract of *H. scabra*. The expression of GFSKLYFamide-like immunoreactivity in the digestive tract of this species, as shown in this study, also provides evidence in support of the potential role of GFSKLYFamide neuropeptide in the digestive functions *H. scabra*. Although, this is the first report of the occurrence of GFSKLYFamide-like immunoreactivity in *H. scabra*, the neuropeptide has previously been reported in the sea cucumber *H. glaberrima*. In earlier report, GFSKLYFamide-like immunoreactivity was observed to be present in the radial nerve cords as well as in the digestive, haemal, respiratory and reproductive systems; in the tentacles; and in tube feet of *H. glaberrima* (Díaz-Miranda et al., 1995). Although, the present study only examined the digestive system, there are some similarities in the expression of GFSKLYFamide immunoreactivity in both species. It was observed that in the intestine of *H. glaberrima*, GFSKLYFamide immunoreactive nerve fibres were found mainly within a dense nerve plexus overlying and in close contact with smooth muscle cells of the intestine. In the stomach, GFSKLYFamide immunoreactivity was observed within the internal connective tissue as well as in the muscular and external connective tissue. These observations are consistent with the pattern of expression of GFSKLYFamide-like

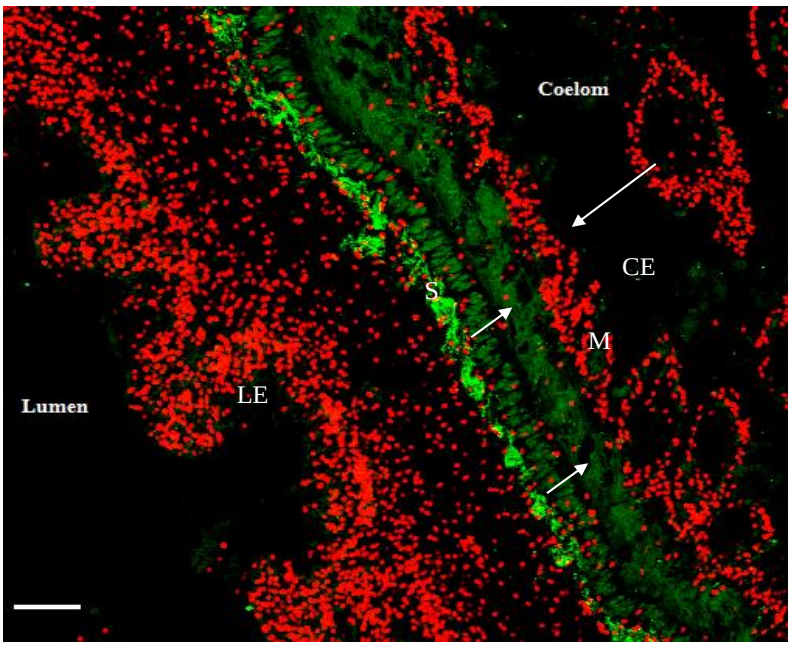

Figure 1. GFSKLYFamide-like immunoreactivity in the stomach of *H. scabra*. Immunoreactivity (green), as indicated by arrows, is present in the submucoca layer, muscular layer and in the coelomic lining of the serosa. Nuclei were counterstained with TOPO-3 (red). LE = Luminal epithelium of the mucosa; S = Submucosa; M = muscular layer; CE = coelomic epithelium of the serosa layer. Scale bar = 50 µm

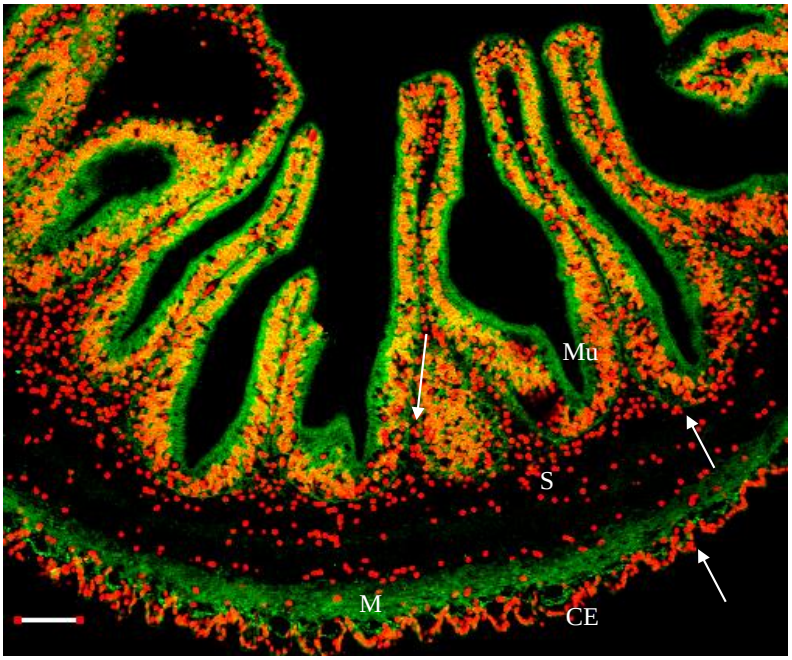

Figure 2. GFSKLYFamide-like immunoreactivity in the small intestine of *H. scabra*. Immunoreactivity (green) is indicated by arrows on the mucosa, submucosal, muscular and in the coelomic epithelium of the serosa lining. Nuclei were counterstained with TOPO-3 (red). Mu = Mucosa; S = submucosa; M = muscular layer; CE = coelomic epithelium of the serosa layer. Scale bar = 50 µm.

immunoreactivity in *H. scabra*. One important difference between the two species though, is the presence of a distinct stomach in *H. scabra* which was said to be absent in *H. glaberrima* (Díaz-Miranda et al., 1995). Since the isolation of the SALMFamide neuropeptide, GFSKLYFamide, alongside SGYSVLYFamide, from the sea cucumber *H. glaberrima* we have begin to gain some insight into the activity of SALMFamides and their global expression in the echinoderm phylum. In *H. glaberrima*, GFSKLYFamide neuropeptide has been observed to cause relaxation of intestinal muscles (Díaz-Miranda and García-Arrarás, 1995). SALMFamide peptides act as inhibitory neuromuscular transmitters in starfish (Elphick and Melarange, 2001). They may act as inhibitory transmitters not only in the starfish neuromuscular system but also more generally in other parts of their nervous systems because S1 inhibits secretion of the hormone gonad-stimulating substance from the radial nerves in the starfish *Asterina pectinifera* (Mita et al., 2004). Furthermore, SALMFamides have been observed to be involved in feeding behavior (Dockray, 2004; Melarange et al., 1999).

Investigations into the expression of SALMFamides in the sea cucumber *Stichopus japonicus* reveal the existence of two peptides, GYSPFMFamide and FKSPFMFamide (Ohtani et al., 1999). These peptides along with twenty others were shown to influence muscle activity in *S. japonicus* although GYSPFMFamide and FKSPFMFamide were the only peptides found to have a direct relaxing effect on muscle (Iwakoshi et al., 1995; Ohtani et al., 1999). Analysis of the genome of the sea urchin *Strongylocentrotus purpuratus* also reveals a SALMFamide gene. The gene encodes seven putative SALMFamide neuropeptides; PPVTTRSKFTFamide, DAYSAFSFamide, GMSAFSFamide, AQPSFAFamide, GLMPSFAFamide, PHGGSAFVFamide and GDLAFAFamide. These peptides were named SpurS1, SpurS2, SpurS3, SpurS4, SpurS5, SpurS6 and SpurS7, respectively. SpurS1, SpurS1 and SpurS3 have the C-terminal sequences -TFamide or -SFamide, which are identical or similar to the C-terminal region of the starfish SALMFamide S2 (Elphick and Thorndyke, 2005). The expression of GFSKLYFamide-like immunoreactivity in the digestive tract of *H. scabra* is remarkable as it underscores the potential physiological roles of the GFSKLYFamide-like peptide in the digestive system of *H. scabra*. Understanding for example, the potential effects of GFSKLYFamide neuropeptide on gastric motility and functions of digestive enzymes in *H. scabra* is vital, considering the economic importance of this species (Bai, 1971).

In conclusion, results from this study indicate that GFSKLYFamide-like immunoreactivity is localized within the stomach and small intestines of the sea cucumber *H. scabra*. This is to the best of our knowledge that, it is the first report providing evidence for the presence of GFSKLYFamide neuropeptide in the digestive tract of *H. scabra*. However, further studies on the structure and activity of the peptide(s) producing GFSKLYFamide-like immunoreactivity in *H. scabra* is warranted.

Conflict of interests

The authors did not declare any conflict of interest.

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