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Isolation and characterization of visceral excitatory neuropeptides from striped mullet (*Mugil cephalus* L.) brain

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Abstract

From a brain extract of the catadromous fish, striped mullet (*Mugil cephalus*), two visceral excitatory neuropeptides (Mvp-1 and Mvp-2) were isolated by means of reversed phase chromatography together with bioassay on fish hindgut. The primary structure of Mvp-1 was elucidated to be SGPAGVLamide by ESI-Q-TOF mass spectrometry. The threshold concentration of Mvp-1 that changes spontaneous contraction of mullet hindgut was between 10^{-9} and 10^{-8} M. In addition, Mvp-1 was found to exert excitatory activities on some other smooth muscle segments (oviduct and esophagus) of mullet but it did not show any effect on body wall muscle strips. Therefore, the present study suggests that Mvp-1 and Mvp-2 peptides act as factors that control visceral contractions of mullet gastrointestinal tract.

Keywords: Neuropeptides; Visceral excitation; *Mugil cephalus*; Brain

Introduction

Neuropeptides that are synthesized and released by the nervous system often act as transmitters, hormones and modulators to control various biological functions at cellular level in the animal body. They interact with cell surface receptors, G-protein coupled receptors to trigger intracellular transduction pathways. The structurally diverse nature has led neuropeptides to exert different effects on living cells. Neuropeptides are encoded from the same gene and exhibit very small differences within biologically active sequences. However, these neuropeptides exert different functions/effects in different animal species (Holmgren and Jensen, 2001; Kagstrom et al., 1996). Those are due to the differences in the gene splicing and the posttranslational processing of neuropeptide precursors in different species (Danielson and Dores, 1999).

During the last few decades, different neuropeptides from a wide range of animal phyla have been identified and characterized. These neuropeptides belonging to different families vary from the phylum cnidaria, which has the most primitive nerve system, to the phylum chordata (Morishita et al., 2003; Sithigorngul et al., 2003; Ohtani et al., 1997). Several fish neuropeptides from both teleosteans and elasmobranchs have been characterized structurally and their physiological roles have been partially identified. Most of these neuropeptides identified from teleosts and elasmobranchs belong mainly to tachykinin, neuropeptide Y, melanin-concentrating hormone, glucagon-family neuropeptide and FMRFamide peptide groups. However, other neuropeptides also have been identified from fish that do not belong to these families. Recently developed versatile proteomic techniques, including combined online nanoscale liquid chromatography (nanoLC) and electrospray-ionization quadrupole time-of-flight (ESI-Q-TOF) have revealed the presence of many undiscovered neuropeptides in the animal body (Skold et al., 2002; Baggerman et al., 2002). Identification of novel biologically active neuropeptides and neuropeptide families indicates the complicated nature of the chemical language of nerve cell communication.

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Although most neuropeptides from different vertebrates have been identified by means of chromatography and/or radioimmunoassay techniques, it is not possible to identify their actual biological functions in the body. However, it is important to characterize neuropeptides with respect to their biological functions. Therefore, bioassays can be employed successfully to identify the biological actions of similar neuropeptides from different species.

Although many experiments have been performed to isolate different neuropeptides from teleosts and elasmobranchs, only a few studies have been carried out to study neuropeptides from catadromous fish. Mullet (*Mugil cephalus*) living throughout coastal tropical-to-warm temperate waters are a good example of a catadromous fish. Since it lives mainly in fresh water or estuarine areas and migrate to sea for breeding, mullet possesses several characteristics such as tolerance to extreme salinity, temperature and low oxygen conditions required for acting as an estuarine sentinel species.

Therefore, the aim of the present study was to increase our understanding about visceral tissue excitatory neuropeptides occurring in the brain of a catadromous fish, *Mugil cephalus*, using a physiological assay technique.

Materials and methods

Animals

Adult striped mullet fishes were purchased from a local live fish market (Jagalchi, Pusan, South Korea). Fishes anesthetized with 0.1% of MS-222 (3-aminobenzoic acid ethylester methanesulfonate salt) were decapitated and heads were dissected to remove the whole brain (medulla oblongata, optic lobes, olfactory lobes, cerebrum and cerebellum). Following removal, the brains were immediately frozen in ice-cold acidified (with 1 mM HCl) acetone (final concentration of acetone, 70%) and transported to the laboratory on dry ice containing insulated boxes.

Extraction of neuropeptides from fish

Frozen brains were boiled in distilled water for 15 min. After acidifying with 3% acetic acid, boiled brains were pulverized with a waring blender and homogenized by using a Polytron homogenizer (Polytron® PT3000, Brinkmann Instruments Co., Lucerne, Switzerland). The homogenate was centrifuged at 9000 ×*g* for 30 min at 4 °C and the supernatant was collected. Pellet was re-extracted into 3% acetic acid and the second supernatant was collected following centrifugation. Both supernatants were combined and concentrated. To precipitate proteinous matter, 1 M HCl was added up to 1/10 of its volume, centrifuged (9000 ×*g*, 30 min., 4 °C) and lyophilized. After dissolving lyophilized matter in 0.1% trifluoroacetic acid (TFA), it was passed through Sep-pak Vac 20 cc (5 g) C18 cartridges (Waters Associates, Milford, MA, USA) conditioned with 100% methanol in 0.1% TFA. Cartridges were washed with 0.1% TFA and subsequently with different methanol concentrations (60% and 80%) in 0.1% TFA. Each eluted fraction was vacuum-concentrated and lyophilized. These lyophilized fractions were then tested for their excitatory effects on hindgut tissues separated from *Mugil cephalus*.

Purification

The most potent peptide fraction showing the highest excitatory effect on hindgut tissues was subjected to HPLC using a C18 reversed phase column (CAPCELL Pak C18, 20 mm × 250 mm, Shiseido Co., Ltd, Tokyo, Japan). Separation was done using a linear gradient of 0–60% acetonitrile (ACN) in 0.1% TFA for 70 min at a flow rate of 3.5 ml/min and fractions were collected at 3-minute intervals. Tension changes in hindgut of *Mugil cephalus* in the presence of each fraction were tested. Fractions showing tension changes were further purified by HPLC using two reverse-phase C18 columns; RP-HPLC semi preparative column (CAPCELL Pak C18, 10 mm × 250 mm, Shiseido Co., Ltd, Tokyo, Japan; 0–40% ACN in 0.1% TFA for 50 min at a flow rate of 2 ml/min) and RP-HPLC analytical column (SynChropak RP C18, 4.6 mm × 250 mm, SynChrom Inc., Indiana, USA; 0–40% ACN in 0.1% TFA for 25 min at a flow rate of 0.3 ml/min). Finally, the peptide fractions exerted the highest tension changes on the hindgut of mullet were further purified isocratically using the same analytical column.

Structure analysis

The purified peptides were subjected to amino acid sequence analysis using electro spray ionization time-of-flight mass spectrometry (ESI-Q-TOF-MS) (Micromass Q-TOF, Altrincham, UK). Mass spectra of purified peptides were acquired by matrix-assisted laser desorption ionization time-of-flight on a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK).

Physiological assay

Peptide fractions collected after each purification stage were analyzed for their capacity to produce excitatory effects on the hindgut of *Mugil cephalus* by using a refined myograph system (TIS8105R, Kent Scientific Cooperation, Torrinton, USA). A small portion of hindgut (15 mm) was collected 10 mm away from the anus of a sacrificed well-fed fish after opening the abdomen ventrally. After cleaning the tissue segment, both ends were tightened with cotton threads. Then the tissue was mounted horizontally in the aerated tissue bath containing

Table 1
Composition of blood plasma from *M. cephalus*

Component	Concentration (mM)
Na^+	129 ± 1.23
K^+	10 ± 0.48
Ca^{2+}	1.3 ± 0.24
Mg^{2+}	3.87 ± 0.06
HCO_3^-	7.0 ± 0.02
PO_4^{3-}	2.0 ± 0.37
SO_4^{2-}	3.5 ± 0.63
D-glucose	5.7 ± 0.40

Results are mean mM ± SD (n = 3). The pH of blood plasma was 7.2 ± 0.08.