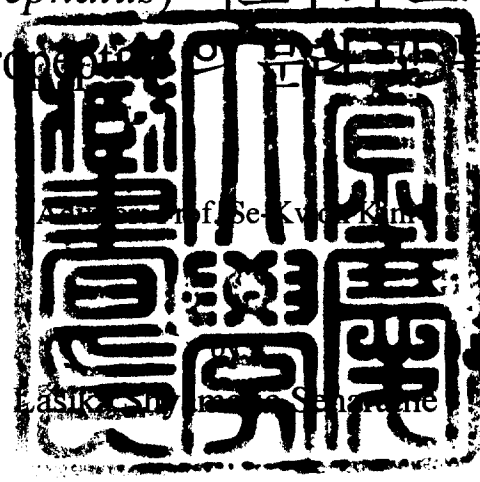
Isolation and Characterization of Visceral Excitatory Neuropeptides from Striped Mullet (*Mugil cephalus*) Brain

숭어 (*Mugil cephalus*) 뇌로부터 소화계 자극성 Neuropeptide의 분리 및 특성



A thesis submitted in partial fulfillment of the requirements
for the degree of
Master of Science
in Department of Chemistry, The Graduate School,
Pukyong National University

February 2006

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A dissertation

by

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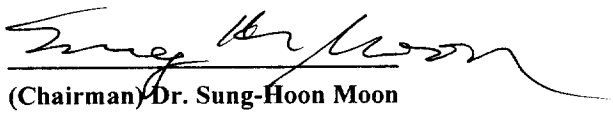

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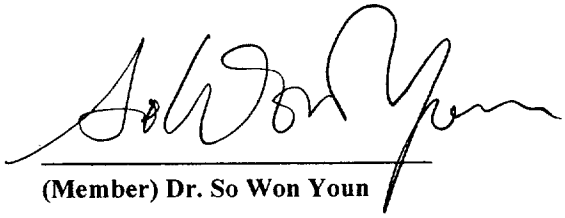

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Abstract

To investigate visceral excitatory neuropeptides of catadromous fish, peptides were purified from the brain of striped mullet (*Mugil cephalus*) using fish hindgut as the physiological assay system. Neuropeptides were purified from the acetic acid brain extract of mullet by using reversed phase chromatography with sep-pak C18 cartridges and high performance liquid chromatography (HPLC). Two visceral excitatory HPLC peaks (Mc-1 and Mc-2) were identified. Molecular weights of visceral excitatory neuropeptides were determined by MALDI-TOF mass spectrometry and their sequences were determined by ESI-Q-TOF mass spectrometry. ESI-Q-TOF mass spectrometry showed that Mc-1 contained two peptides with amino acid sequences of SGPAGVLamide (598.7 Da) and KLGELGNamide (728.8 Da). Threshold concentration of Mc-1 for the excitatory effect on the visceral segment of mullet was between 0.005 –0.05 μg/ml and 30 min or more was required for complete recovery of the intestine from the desensitization state brought by 0.5 μg/ml of Mc-1. Mc-1 was also found to have excitatory activities on other smooth muscle segments (oviduct and esophagus) of mullet. Mc-2 with the molecular weight of 1622.7 Da was determined as a neuropeptide belonged to tachykinin family. Therefore, the present study directly demonstrated the presence of visceral excitatory neuropeptides in striped mullet fish brain.

Key words: neuropeptides, visceral excitatory, isolation, striped mullet

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Introduction

Scientists from different disciplines, including chemists and biologists have been focusing their research interests towards peptides for a long time. As more biologically active peptides were isolated, it became apparent that a large number of biological functions in various life forms were controlled by different peptides. It is now widely accepted that some peptides released from endocrine as well as some released from nerve endings act as neurotransmitters. During last 30 years, the constant increase of identified neuropeptides illustrates that the chemical language of nerve cell communication is far more complicated than originally anticipated.

Since neuropeptides are involved in nervous system function and occur in nerves, they can be defined as peptides that are released from neurons as signaling molecules. These molecules exert effects as transmitters or modulators on other excitable cells or sometimes on their own cells. Therefore, they are known as biochemicals that regulate almost all life processes on the cellular level and thereby they link all body systems.

Neuropeptides transmit chemical messages from the brain to the receptor sites on cell membranes through out the body. Therefore, they are also called as messenger molecules. With respect to the clarification of biochemists, neuropeptides are known as nerve proteins that are produced and released by neurons in both central and peripheral nervous systems as signaling molecules in all animal groups possessing a nervous system (Holmgren and Jensen 1994; Thorndyke and Goldsworthy, 1988). Neurons release neuropeptides into the circular systems (blood or lymph or cerebrospinal fluid) of the animal body in order to send the chemical messages from the brain to receptor sites of target cells through out the body. Generally, neuropeptides are acting as "lock" and "key"

mechanism where the neuropeptide is the "key" that opens the "lock" on the cell membrane to cause complex and fundamental changes in the target cells they lock onto.

When considering the molecular structure of neuropeptides, they are composed of a few amino acid residues, generally between 4 to 40 residues. Therefore, neuropeptides are called as low molecular weight peptides or biochemicals. Furthermore, all neuropeptides studied so far have simple chain (linear) structures, but some like melanin-concentrating hormones (MCH) possess cyclic molecular conformations.

Neuropeptides differ in their way of biosynthesis and molecular structure from those of classical neurotransmitters. It is well known fact that, neuropeptides secreting neurons structurally differ from neurons secreting classical neurotransmitters. All the classical neurotransmitters identified until now are amino acids or monoamines formed from dietary sources by one of few intercellular enzymatic steps (Cooper et al., 1986). The product of these enzymatic actions is the active transmitter molecule, which is stored in small secretary vesicles (SSVs) until release. After release, classical neurotransmitters are reabsorbed into axon terminals by energy-dependent active mechanism and degenerated by monoamine oxidases into small molecules that can be reutilized to form neurotransmitters at the axon terminals. The release of classical transmitters from SSVs occurs at high Ca^{+2} concentration that is closer to the site of Ca^{+2} entry on the presynaptic membrane of the neuron. Although neuropeptides are usually in lower concentrations than classical neurotransmitters in their vesicles, they interact with their G-protein coupled receptors with higher affinity (Mains et al., 1998).

Although, neuropeptides are synthesized at the cell soma (cell body) of neurons in all types of nerve fibers, some neuropeptides have been localized to

different types of glial cell, which are non-neuronal cells in the nervous system. Therefore, neuropeptides can be released from both neurons and glial cells (Sandberg and Weber, 2003). Neuropeptides always coexist with either a classical neurotransmitter or one or several neuropeptides. There are number of significant differences between neuropeptides and classical neurotransmitters. Those differences are prominent at the stage of their synthesis in the neurons.

Neuropeptides are synthesized in the same way as all other peptides and proteins synthesis. DNA in the nucleus encodes mRNA (primary), which is released by the nucleus. The primary mRNA is spliced by the process called alternative mRNA splicing in order to produce prepropeptide mRNA (alternative spliced mRNA), which acts as a template for the polypeptide synthesis on the ribosomes of rough Endoplasmic Reticulum (ER). The precursor polypeptide, called as prepropeptide has three components: a signal sequence (16-30 amino acids) at the N-terminal, one or more neuropeptide sequence and one or more several spacer parts (few amino acid to 10 times the final active product) (figure 1A). The spacer parts help to lengthen enough the neuropeptide precursor molecule to enter the ER through its membrane (Dores et al., 1996).

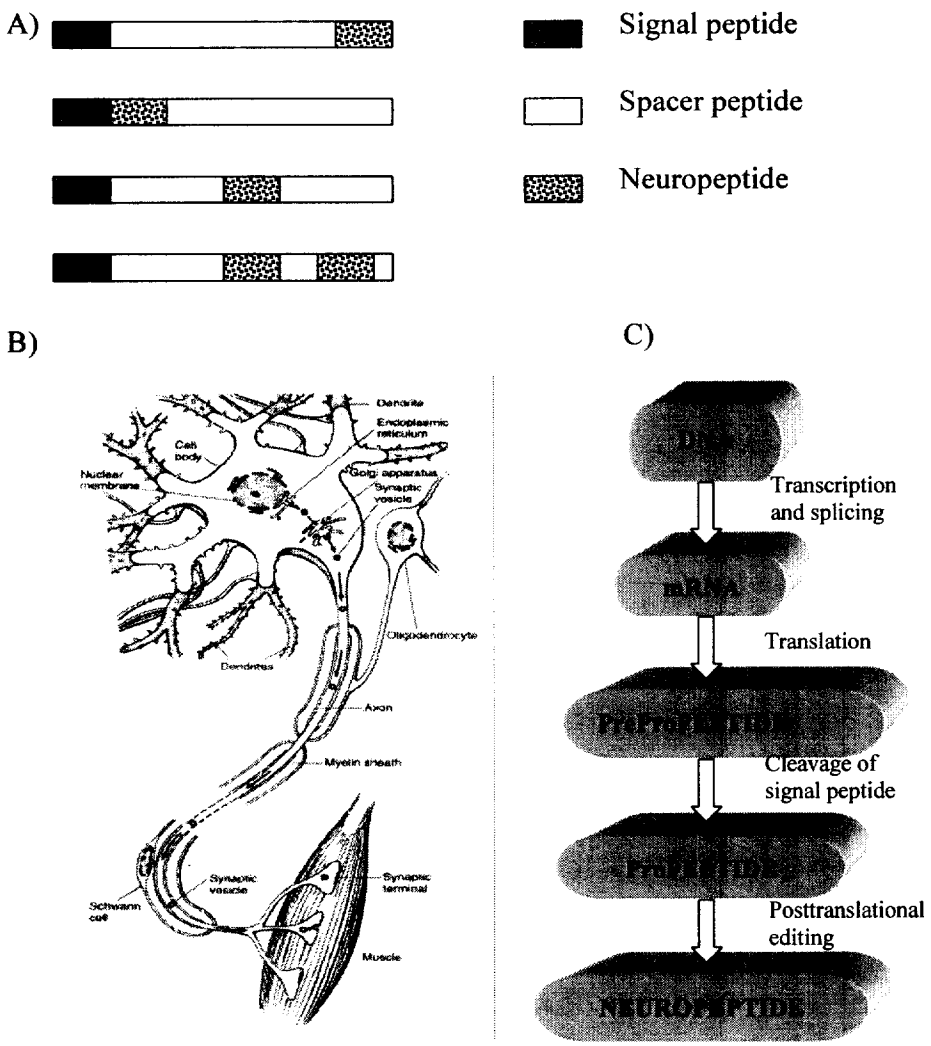

Figure 1. (A) Schematic diagram of the arrangement of neuropeptide precursor, prepropeptide, (B) simplified illustration of neuropeptide biosynthesis in a neuron, and (C) cascade of main steps involves in the neuropeptide synthesis.

Within ER, a part of the prepropeptide (signal peptide) is cleaved off to form propeptide by signal peptidases. Then the part of the propeptide is split off to form the active peptide called as neuropeptide in the Golgi apparatus. Then they are packed in large dense core vesicles (LDCVs) and transported along the axon to the nerve terminals until they are released at lower Ca^{+2} concentration or away from the site of calcium entry on the presynaptic membrane of the nerve endings (figure 1B and C).

Although the complete physiological function of these neuropeptides has not been understood, the common function identified is that they involve in the modulation of metabolism, sensitivity and transcription of their cellular targets via G-protein coupled receptors (Lombardin et al., 2002; Ferry et al., 2002; Zhou et al., 2002). Nevertheless, an exception has been discovered in the neuropeptide FMRFamide, which induces a fast excitatory depolarizing response through direct activation of an amiloride-sensitive sodium channel. Therefore, FMRFamide is the first instance of neuropeptides acting via ionotropic receptors.

Neuropeptide receptors or G-protein coupled receptors are defined as cell surface receptors that bind specific neuropeptides with a high affinity and trigger intercellular changes influencing the behavior of target cell. The existence of these neuropeptide receptors was elusive until 1970s. Since neuropeptides coexisted with classical neurotransmitters, experts in the field of immunology insisted that the binding sites for neuropeptides might be located on the receptor molecules of classical transmitters. Therefore, the first structure of neuropeptide receptors for members of the tachykinin family neuropeptides was discovered by a group of Japanese scientists (Yakota et al., 1989; Yakota et al., 1992; Sasai et al., 1989). In 1987, due to further investigation on G-protein coupled receptors, the cDNA clone for a bovine substance K receptor was isolated from a stomach cDNA library and that was considered as the first cloned neuropeptide receptor in this

scenario (Musa et al., 1987). After that, many other receptors of different neuropeptides were cloned. Those receptors also belonged to the same G-protein coupled receptor family.

The corresponding gene in the genetic material of the organism determines amino acid sequence of the neuropeptide. During evolution, DNA sequence of the relevant neuropeptide in an organism changed due to mutation and that may cause amino acid substitution in the peptide sequence of neuropeptides in different species. Therefore, more distance related animal species have more substituted amino acid sequences in a particular type of neuropeptides compared to that of in closely related animal species (Holmgren and Jensen, 2001). The relationship between two peptides from different species is expressed as the percent of homology (identity). If the evolution time is longer, there may be more mutations in the neuropeptide gene and subsequently more changes may occur in the amino acid sequence. Some of those mutations can be signal base pair mutations and thereby causes no changes or conservative changes in the amino acid sequence. Other mutations cause severe changes in their neuropeptide properties. Therefore, they may no longer attach to the relevant receptors to initiate the cellular effects. Therefore, latter type of mutations in the genome is lethal (Holmgren and Jensen, 2001).

Generally, certain parts of the sequence of the neuropeptide molecule of a particular neuropeptide family are more conserved than the rest of the molecule in different species. Those conserved sequence parts are located either at C-terminal or N-terminal depending on the types of neuropeptide family. Generally, conserved part of the peptide sequence of tachykinin and neuropeptide Y families is located at the C-terminal of peptide in different species. The conserved peptide sequence of vasoactive intestinal polypeptides (VIP), pituitary adenylate cyclase activating peptides (PACAP) and growth hormone releasing factor (GRF) family

neuropeptides, is located at the N-terminal of the peptide in different species (Holmgren and Jensen, 2001). Usually that conserved part of sequence of a particular neuropeptide family determines the specific biological function. Meanwhile, there are much more variation in sequence at the spacer part of the neuropeptide precursor and non-transcribed sections between introns and exons of DNA in different species (Linder et al., 1987; Nishizawa et al., 1985).

Early days, neuropeptides had been primarily categorized with respect to their amino acid sequences. Therefore, neuropeptides with similar sequences belonged to the same family and families with similar sequences belonged to the same superfamily. Now-a-days neuropeptide categorization is done not only considering amino acid sequences but also considering the genes and their precursors (Conlon, 1989; Dores et al., 1996; Hoyle, 1998). It is believed that superfamilies and families of neuropeptides have originated from the same ancestral gene due to gene duplication, exon duplication and exon losses (Hoyle, 1998; Danielson et al., 1999). Although each cell has the potential to express all genes in the genome, the gene regulating system in the cell decides peptides that are finally produced in a particular cell. Therefore, posttranslational processing of neuropeptide synthesis is different in different cells. That may produce different types of neuropeptides in different cells.

Even though neuropeptides encoded from the same gene and showing very little difference in the biologically active parts may exert different functions/effects in different animals (Holmgren et al., 1994; Kagstrom et al., 1996a; Kagstrom et al., 1996b). Those are also due the differences in the gene splicing and the posttranslational processing of neuropeptide synthesis in different species (Danielson and Dores, 1999).

Since last few decades, scientists have been discovering different neuropeptides from wide range of animal phyla through out the animal kingdom.

As a result, up to now many neuropeptides belong to different families have been isolated and characterized from the phylum cnidaria, which has the most primitive nerve system, to the phylum chordata (Morishita et al., 2003a; Sithigorngul et al., 2003; Ohtani et al., 1997; Cowden and Stretton, 1994; Kuroda et al., 2003; Morishita et al., 2003b; Iwakoshi, et al., 2000; Sithigorngul et al., 2002; Liebrich et al., 1995; Hayashi and Camargo, 2005; Jensen and Conlon, 1992). Recently, neuropeptides produced in nerves and/or gastrointestinal tissues have been isolated from lower classes of vertebrates. Neuropeptides from fish, both teleosteans and elasmobranchs have been characterized structurally and their physiological roles were partially identified. Most of neuropeptides identified from teleosts and elasmobranchs mainly belong to tachykinin, neuropeptide Y, melanin-concentrating hormone, glucagon-family neuropeptide and FMRFamide peptide categories.

The amidated tetrapeptide FMRFaminde (Phe-Met-Arg-Phe-NH_2) are originally isolated from the central ganglia of the clam, *Macrocallista nimbosa*, based on its cardioexcitatory activity (Price and Greenberg, 1977). Later FMRFamindes and FMRFamide-like peptides were identified in the nervous system of invertebrates and vertebrates (Boer et al., 1980; Chen et al., 1989; Chronwall et al, 1984; Dockray et al., 1983; Kobierski et al., 1987; O'Donohue et al., 1984; Watson and Groome, 1984; Weber et al., 1981; Vallarino et al., 1991; Fujimoto et al., 1998; Sithigorngul et al, 1998). The tetrapeptide exerts different biological actions, including excitatory effects on brainstem neurons leading to alterations of blood pressure (Gayton, 1982; Wong and Greenberg, 1985), modifications of the electrical activity of cultured spinal neurons (Guzman et al., 1989), modulation of activity of morphine (Raffa, 1988; Tang et al., 1984 and Zhu and Raffa, 1986) and function as neuromodulators and neurotransmitters in vertebrate and invertebrate species. Many FMRFamide neuropeptides have been

isolated from organisms using immunoreactivity techniques. For the first time, FMRFamide-like neuropeptide (Carassius RFamide – C-RFa) was isolated from the brain of japanese carp based on its visceral excitatory effect. C-Rfa has also shown similar tension changes on other vertebrate visceral tissues (Fujimoto et al, 1998).

Neuropeptide Y (Neuropeptide tyrosine, NPY) family neuropeptides having 36 amino acid residues occur in the central and peripheral nervous systems. They exert control of vascular tone, satiety and sexual behavior. The neuropeptide Y family including NPY peptides, peptide YY (PYY), pancreatic polypeptide (PP or PPY) and polypeptide Y (polypeptide tyrosine, PY) can be seen in vertebrates while NPY-like peptides has been isolated from invertebrates. NPY and PPY are found in all vertebrate classes from agnatha to mammalia, but PP is only found in pancreas of tetrapodes (Jensen and Conlon, 1992; Conlon et al., 1991). PY has been identified only in four fish species belong to acanthopterigii (*Myoxocephalus scorpius* and *Tilapia niloticus*), paracanthopterigii (*Lophius americanus*) and elasmobranch (*Raja rhina*) and not found neither in angilliforms nor protacantheropterygian. Since PYY or PP has not been found in acanthopterigii and paracanthopterigii fish, it reveals that PY may be a mutant of PYY. Having similar degree of sequence homology with NPY and PYY, PY provides better evidence that acanthopterigii and paracanthopterigii fish have evolved from teleosts of euteleost lineage. Therefore, the fish, which contain PYY, are considered as phylogenetically older. The only fish expressing PYY is a euteleost coming under protocanthopterygii (*Oncorhynchus kisutch*).

Tachykinins comprise a large family of peptides, having a common C-terminal amidated amino acid sequence, -Phe-Xaa-Gly-Leu-Met-NH_2 (Xaa = Phe, Tyr, Val or Ile). This conserved amino acid sequence at the C-terminal is considered to be the most important part, which exerts biological roles in

myotropic actions on vascular, gastrointestinal and other smooth muscle preparations (Maggio et al., 1994; Schoofs et al., 1990 and Pernow, 1983). The first tachykinins isolated from fish tissues were scyliorhinin I and scyliorhinin II. They were purified from the intestine of an elasmobrach fish *scyliorhinus canicula* (Conlon et al., 1986) and *Toepedo marmorata* (Conlon and Trim, 1988). Subsequently, tachykinins, which have a similar primary structure of mammalian tachykinins, were purified from brain tissues of teleost fish (Jensen and Conlon, 1992).

Although many experiments have been performed to isolate different neuropeptides from teleosts and elasmobranches, few studies have been carried out regarding the presence of neuropeptides in the central nervous system of teleost fish, which have catadromous behaviors. Catadromous behavior can be defined as the migration of fresh water or estuarine living fish to seawater for breeding. Mullet (*Mugil cephalus*) living through out coastal tropical to warm temperate waters is a good example for a catadromous fish. Since they live mainly in fresh water or estuarine area and migrate to sea for breeding, mullet possesses several characteristics required for acting as an estuarine sentinel species such as the extreme salinity tolerance.

Since many 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. Therefore, it is important to discover neuropeptides with respect to their biological functions. Subsequently, this biological action can be employed as the bioassay for subsequent steps of the discovery process of the neuropeptides.

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

Experimental Procedures

1. Materials

Adult striped mullets (*Mugil cephalus*; 45 – 50 cm, n = 100) were purchased from a local live fish market (Jagalchi, South Korea). Disposable sep-pak vac 20 cc (5 g) C18 cartridges were bought from Waters Associates (Milford, MA, USA). All other chemical reagents used were of the highest analytical grade.

2. Methods

2-1. Collection of specimens

Anesthetized fish using 0.1% of MS-222 (3-aminobenzoic acid ethylester methanesulfonate salt) were decapitated. Whole brains (medulla oblongata, optic lobes, olfactory lobes, cerebrum and cerebellum) were removed from dissected heads, immediately frozen in ice-cold acidified (with 1mM HCL) acetone (final concentration of acetone-70%) in dry ice and quickly transported to the laboratory.

2-2. Extraction of neuropeptides

Frozen brains were boiled in 500 ml of distilled water for 15 min. Then boiled brain tissues were pulverized by using the Waring commercial blender (Model 7005, Waring Products Inc., Connecticut, USA). After acidifying pulverized mixture with acetic acid to the final concentration of 3%, it was homogenized by using the polytron homogenizer (Polytron® PT3000, Brinkmann Instruments Co., Lucerne, Switzerland). Then homogenate was centrifuged (Hanil Science

Industrial Co., Ltd, Incheon, South Korea) at 9 000 × g for 30 min at 4°C and supernatant was collected. The pellet was re-homogenized in 300 ml of 3% acetic acid and centrifuged at previous conditions. Both supernatants were combined and concentrated by using the vacuum evaporator (EYELA rotary evaporator-N1000S W, Rikakikai Co., Ltd., Tokyo, Japan). Concentrated solution was acidified by adding 1 M HCl up to 1/10 of its volume. Acidified solution was centrifuged at 9 000 × g for 30 min at 4°C to precipitate proteinous matter in the peptide solution. The supernatant was concentrated by using the vacuum evaporator and lyophilized in the freeze dryer (SFDSM 12, Samwon Freezing Engineering Co., Pusan, South Korea).

2-3. Purification using sep-pak C18 cartridges

Lyophilized matter was dissolved in distilled water and passed through disposable sep-pak vac 20 cc (5 g) C18 cartridges conditioned with 100% methanol in 0.1% trifluoroacetic acid (TFA) and with 0.1% TFA, respectively. Unbound compounds were collected by washing the cartridges with 0.1% TFA. Retained materials in the cartridges were subsequently eluted with different concentrations of methanol (20%, 60%, 80% and 100%) in 0.1% TFA. Each eluted fraction was vacuum concentrated and lyophilized. Then lyophilized samples were tested for their excitatory effects on the hindgut segment removed from *M. cephalus*. Fractions showing the highest excitatory effects on hindgut tissues were further purified by using high performance liquid chromatography (HPLC).

2-4. High performance liquid chromatography

The fraction eluted with 60% methanol contained substances that induced the highest hindgut contractions. Therefore, it was subjected to HPLC using a C18 reversed-phase column (CAPCELL Pak C18, 20 mm × 250 mm, Shiseido Co., Ltd, Tokyo, Japan). Fractionation was done using a linear gradient of 0-60% acetonitrile (ACN) in 0.1%TFA over 70 min at a flow rate of 3.5 ml/min. Fractions were collected for every 3 min. A portion of each fraction (1/300) was tested for the tension changes on the hindgut of *Mugil cephalus*. Fractions contained tension changing substances were further purified by HPLC using another reverse-phase C18 column (CAPCELL Pak C18, 10 mm × 250 mm, Shiseido Co., Ltd, Tokyo, Japan) and column was eluted with a linear gradient of 0-40% ACN in 0.1% TFA over 50 min at a flow rate of 2 ml/min. Each peak eluted was collected and tested for excitatory effects on the isolated hindgut of *M. cephalus* as described in the physiological assay. Then bioactive substances were applied on to a reverse phase analytical column (SynChropak RP C18, 4.6 mm × 250 mm, SynChrom Inc., Indiana, USA) which was then eluted with a linear gradient of 0-40% ACN in 0.1% TFA over 25 min at a flow rate of 0.3 ml/min. Then peaks were collected and tested for bioassay using a portion of *Mugil cephalus* hindgut. Two peaks (Mc-1and Mc-2) that caused excitations on the hindgut were further purified isocratically by injecting to the same column with relevant ACN concentrations (26.2% and 27.3%) in 0.1% TFA.

2-5. Physiological assay

Fractions collected after solid phase extraction by using C18 cartridges and HPLC purification were checked for their capabilities to produce excitatory

effects on the hindgut isolated from *Mugil cephalus* by using refined myograph system (TIS8105R, Kent Scientific Cooperation, Torrinton, USA). A portion of hindgut (15 mm) was removed from the intestine 10 mm away from the anus of a scarified well-fed fish after opening the abdomen ventrally. Then the tissue segment was cleaned after trimming off all the adhered fat/connective tissues and ingesta was removed with a flush of physiological saline. The physiological saline was prepared with respect to the plasma composition of *M. cephalus*. Both ends of the tissue were tightened with cotton threads. Then the tissue was dipped in the aerated tissue bath containing physiological saline (3 ml) after connecting other ends of threads to the pre-calibrated transducer head (2 g) and to the supporting bar, respectively. The transducer was connected with the chart recorder (REC101, Kent Scientific Cooperation, Torrinton, USA) (chart speed- 0.5cm/min, sensitivity – 2 volts) via an amplifier (TRN005-220, Kent Scientific Cooperation, Torrinton, USA). The temperature of the water-jacketed tissue bath was set at 25°C. The tissue was kept in the tissue bath of the bioassay setup for more than 1 h for the equilibration and the saline was changed rapidly in 10 min intervals. Purified fractions from sep-pak C18 cartridges and HPLC were lyophilized, dissolved in physiological saline (100 µl) and directly applied to the tissue bath to test the tension changes of the hindgut segment of *M. cephalus*. Mechanical responses of the tissue due to sample applications were recorded. In between successive sample applications, the tissue was washed thoroughly with physiological saline for 20 min to avoid previous sample effect.

2-6. Structure analysis

Determination of molecular weight

Mass spectra was acquired by matrix-assisted laser desorption ionization time-of-flight on a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK). The matrix, α-cyano-4-hydroxycinnamic acid was prepared at a concentration of 20 mg/ml in 50% ACN in 10% TFA (v/v) and 10 mg/ml of peptide solution was prepared with 50% ACN with 10% TFA (v/v). One microliter of sample mixture was added to the MALDI sample plate followed by 1 μl of matrix and dried at room temperature. Then the spectrometric spectrum was obtained at a laser power of 3000 $kWcm^{-2}$.

Determination of amino acid sequence

The purified materials 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).

2-7. Determination of physiological saline for mullet tissues

Blood plasma composition of mullet was analyzed in order to prepare a suitable physiological saline used in the bioassay for the mullet tissues. Fish were anesthetized using 3 ml of MS-222 (99.5% Pure Tricaine Methanesulfonate). Blood was withdrawn from the caudal vein of anesthetized fish by using a heparinised syringe (1½ inch needle, 6 ml syringe). The syringe was inverted 5 to 6 times in order to proper mixing of collected blood with heparin. The blood

samples were transferred into heparinised caps screw tubes and centrifuged at 2000 × g for 10 min at 4°C. Then the plasma-contained supernatant was separated. Blood plasma was stored at –80°C for further analysis. pH of the blood plasma was measured using the potable pH meter at 25°C. Electrolytes (Na^+, Ca^{2+}, Mg^{2+}, K^+, PO_4^{3-}, HCO_3^- and SO_4^{2-}) were determined using ICP-MS (Inductively coupled Plasma Mass Spectrometry – Perkin Elmer optima 3300 XL, Perkin Elmer INC., New York, USA) and ionic chromatography (Model 650, Alltech Associates Inc., USA) respectively. Soluble sugar in the blood plasma was quantified according to the colorimetric method, phenol-sulfuric acid analysis (Dubois et al., 1955).

2-8. Pharmacological effects of Mc-1 peptides

Dose dependent response on mullet hindgut tissue

To measure the threshold concentration of purified Mc-1 (after 3rd step of HPLC purification) on hindgut of *Mugil cephalus,* different concentrations of Mc-1 from 500 µg/ml to 0.005 µg/ml were prepared mixing with physiological saline according to a dilution series. Hundred micro liters (100 µl) of each concentration were added to the tissue bath and tissue contractions were examined following same procedure mentioned in the physiological assay.

Time dependent response on mullet hindgut tissue

To confirm the tachyphylaxis of the muscle on Mc-1, i.e the desensitization of a tissue to the increment of active substance administration, the influence of interval time between applications was compared.

Mc-1 at a concentration of 0.5 μg/ml was applied onto different tissue preparations of mullet hindgut at different time intervals (5, 15, 30 min). Then the excitatory effects were detected using the bioassay system set at previously mentioned conditions.

Effects on different tissues of mullet

Effects of purified Mc-1 on different tissues of mullet were tested using segments of oviduct, esophagus and body wall muscle. Each tissue was mounted in the tissue bath of the bioassay setup and all the conditions for the bioassay were set as described above. Tension changes after adding Mc-1 at a concentration of 0.5 μg/ml onto different tissues were compared.

Results and Discussion

1. Preparation of physiological saline for *Mugil cephalus* tissues

Since neuropeptides from brain extract of *M. cephalus* were identified with respect to the physiological assay using in vitro tissue preparations, a suitable medium for the maintenance of in vitro tissue preparations of *M. cephalus* was needed.

The maintenance of tissue viability in terms of biochemical and physiological function is critical to demonstrate any experimentally induced effect using in vitro tissue preparations. The degree of viability of isolated tissue preparations mainly depends on the artificial serum or saline solutions used (Burton, 1975 and Rees, 1989). Therefore, the prerequisite for in vitro maintenance of isolated gut preparation from *M. cephalus* fish was to establish a physiological medium that would be more reflective and supportive of the in situ biochemical and physiological processes of those tissues. The method used to prepare commercially available Ringer's solutions, which are used commonly in vitro physiological studies is the preparation of a medium by substituting the blood plasma composition of the relevant animal (Ringer, 1882, 1883, 1885; Clement and Rees, 1998). Therefore, ionic composition and pH of collected blood plasma from anesthetized *M. cephalus* fish were analyzed.

The observed ionic and biochemical profile of plasma sample of *M. cephalus* has been shown in Table 1. Therefore, a physiological saline solution with a composition similar to the plasma composition of mullet was formulated by using laboratory-graded chemicals as mentioned in the Table 2. In general, physiological saline solutions used for marine invertebrates, marine teleosts and

elasmobranches contain higher Na^+ concentrations than that of in saline solutions used for tissues of fresh water teleosts (Young, 1933; Burton, 1988).

Mullet blood plasma contained a lower Na^+ concentration (129 mM) than that of in marine fish. The reason for low content of Na^+ in mullet blood plasma was due to their catadromous behaviour. In general, mullet fish lives in fresh water or in estuarine areas where the Na^+ concentration is lower than that of in seawater and they migrate to sea for breeding. Therefore, adult mullets can tolerate a wide rage of salinity from 0 ppt to 75 ppt. The ability of these fish to adapt to changes in salinity is mediated by hormones, especially prolactin (fresh water) and cortisol (more saline water). All these hormonal adaptations of the body allow catadromous fish to maintain the ionic balance at salinity fluctuations. Therefore, the ionic composition of their blood plasma is similar to that of fresh water teleosts.

However, in 1953 Keynes and his fellow scientists have prepared a physiological saline with a higher Na^+ concentration for the catadromous fish, electric eel (*Electrophorus electrics*). Therefore, that proves catadromous fish tissues can perform well both in high and low ionic conditions. In this study, we prepared the physiological saline according to the composition mentioned in the Table 2 for each step of bioassay used to isolate visceral excitatory neuropeptides from *M. cephalus* brain.

Table 1. Composition of blood plasma from *M. cephalus*. Results are in millimolar concentration per liter. The pH of blood plasma was 7.2.

Component	**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

Table 2. Composition of physiological saline used in the bioassay for in vitro tissue preparation of *M. cephalus*. Results are in millimolar concentration per liter. The pH was 7.2.

Component	**mM**
NaCl	120
KCl	10
$CaCl_2$	1.3
$MgSO_4$	3.87
NaHCO3	7.0
NaH_2PO_4	2
D-glucose	5.7

2. Purification of visceral excitatory peptides using sep-pak C18 cartridges

Since neuropeptides are synthesized in the nervous system, we decided to collect whole brain of mullet fish as the sample material. Brains were dissected from live fish after decapitation, frozen immediately in dry ice and boiled for 15 min to prevent enzymatic degradation of neuropeptides by peptidases and proteases. Brain peptides were extracted in 3% acetic acid.

Five fractions were obtained from acetic acid brain extract of *M. cephalus* by using sep-pak C18 cartridges. They were named as flow-through fraction, 20% methanol fraction, 60% methanol fraction, 80% methanol fraction and 100% methanol fraction. Since sep-pak C18 cartridges were made of more hydrophobic stationary phase (silicate with C18), more hydrophilic peptides were eluted first. Therefore, the flow-through fraction contained more hydrophilic peptides than that of in other four methanol eluted peptide fractions. Due to lack of sample materials, 20% methanol eluted and 100% methanol eluted fractions were not tested for tension changes on hindgut segments of *M. cephalus*. Therefore, flow-through, 60% methanol and 80% methanol fractions were examined for the excitatory effects on the hindgut using the myograph system. The results indicated that flow-through fraction and 60% methanol fraction contained substances that caused tension changes on the hindgut segment (Figure 2). We could not see any tension change on the hindgut after adding 80% methanol fraction. After computing percentage of tension change using the equation mentioned below, it showed that compounds in flow-through fraction and in 60% methanol fraction caused 38% and 80% tension changes on the isolated hindgut, respectively (Figure 3).

$$\text{Tension change } \% = \left(\frac{\text{Indused tension - Intrinsic tension}}{\text{Transducer sensitivity}} \right) \times 100$$

According to the results of tension change, 60% methanol eluted fraction, which caused the highest tension change on hindgut, was selected for further purification using HPLC.

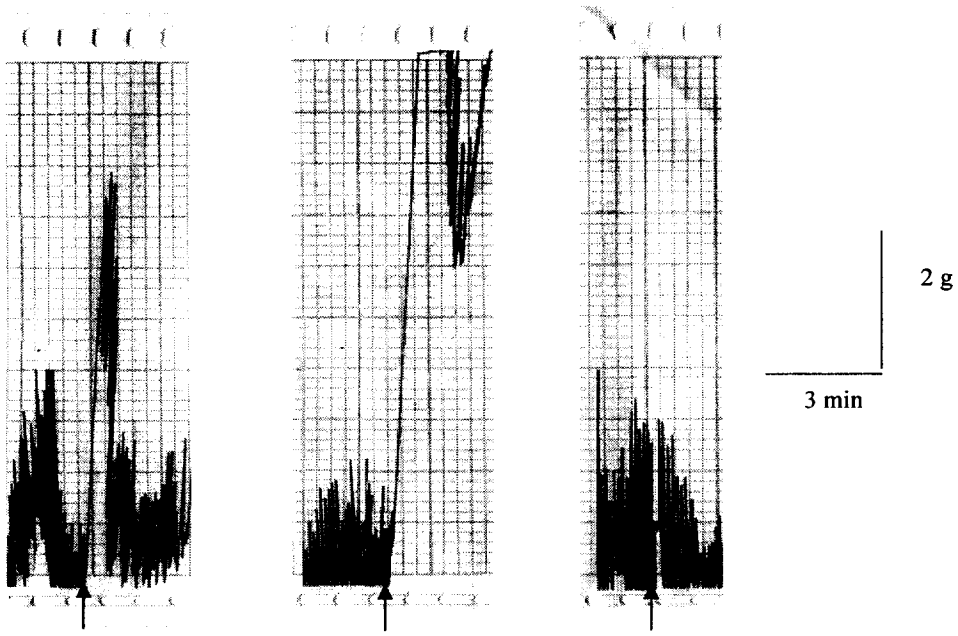

Figure 2. Tension changes of mullet hindgut after application of purified fractions from sep-pak C18 cartridges. (A) Flow-through fraction, (B) 60% methanol fraction and (C) 80% methanol fraction. Time points of sample application are indicated by the arrow.

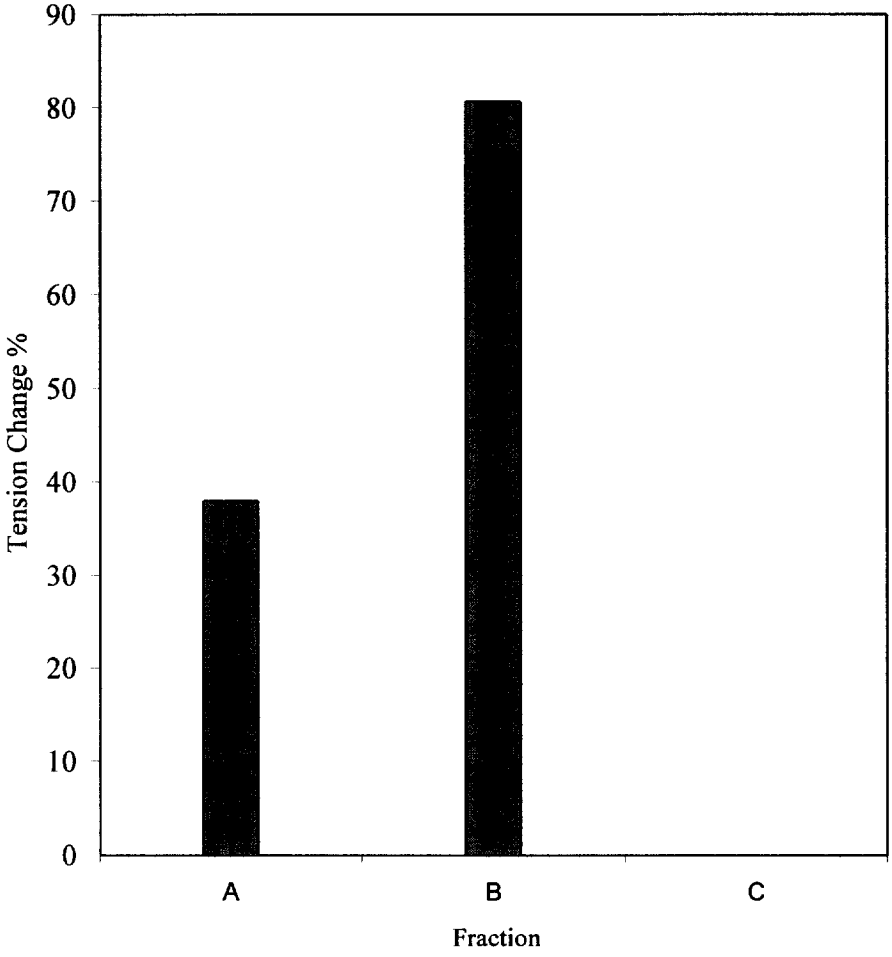

Figure 3. Percentage of tension changes of hindgut after adding purified fractions from sep-pak C18 cartridges. (A) Flow through fraction, (B) 60% methanol fraction and (C) 80% methanol fraction.

3. Purification of visceral excitatory peptides using HPLC

Peptides can be separated using reversed phase liquid chromatography, which is based on their hydrophobicity. Therefore, 60% methanol fraction was further purified by HPLC with different reversed phase C18 columns successively. As a practical matter for C18 stationary phases in peptide purification, eluents should be acidic. Therefore, we used 0.1% TFA to give acidic pH (2.2 pH) to ACN which was used as the eluent through out the HPLC purification.

In the first step of HPLC purification, lyophilized 60% methanol fraction was applied onto the CEPCELL PAK C18 semi preparative column and fractions were collected at every 3 min. After applying lyophilized fractions of first step of HPLC onto the hindgut tissue preparation of mullet, only the fraction eluted with 28-31% ACN in 0.1% TFA at 33-36 min contained compounds that affected the tension change of the visceral tissues (Figure 4).

Then the lyophilized bioactive fraction was further purified using another reversed phase C18 column at the second step of HPLC purification. Each eluted peak was collected and lyophilized. Only the 6^{th} peak eluted at 28% ACN concentration in 0.1% TFA showed significantly higher visceral contractions on the hindgut tissue preparation (Figure 5).

After freeze-drying, the bioactive matter was further purified using a reversed phase C18 analytical column. Only two peaks, designated as Mc-1 and Mc-2 were obtained (Figure 6). Both showed excitatory effects on hindgut tissue preparation of mullet. Finally, Mc-1 and Mc-2 were applied on to the same reversed phase C18 analytical column and they were purified isocratically with 26.2% and 27.3% ACN concentrations in 0.1% TFA, respectively (Figure 7and 8).

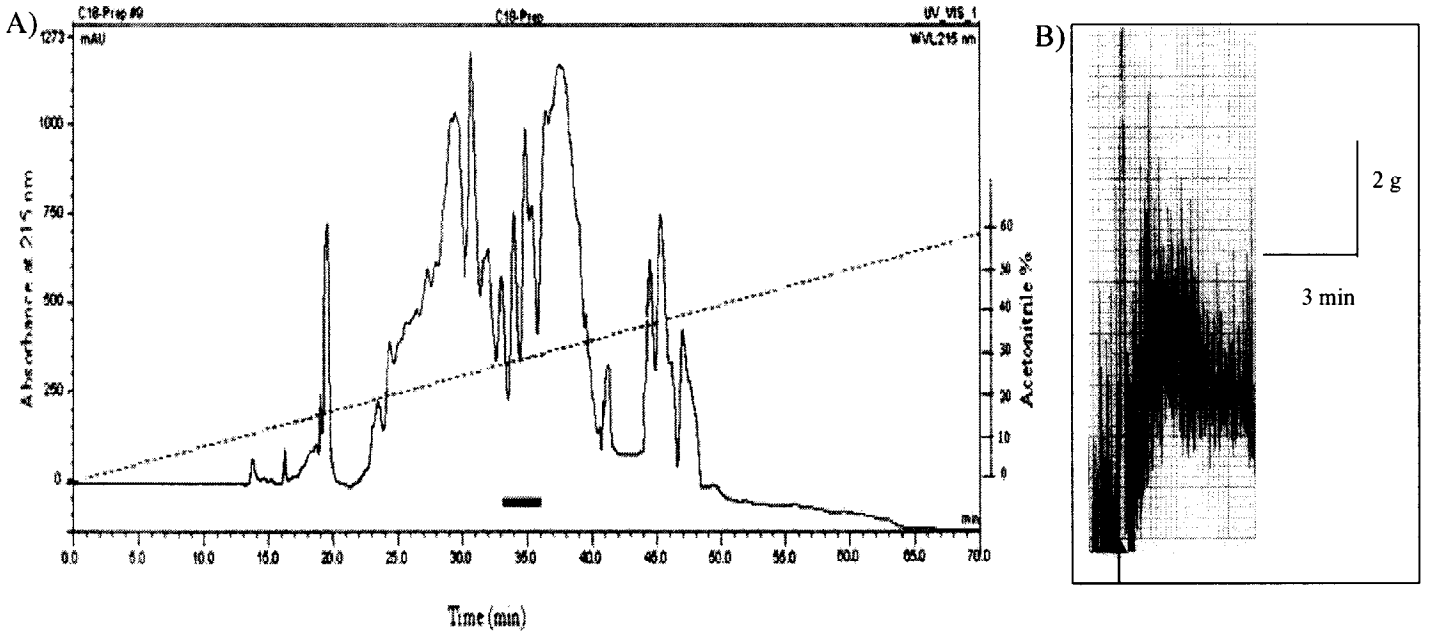

Figure 4. A) Chromatogram of the first step of HPLC purification of visceral excitatory peptides. Fractions were collected for every 3 minutes. Elution conditions: a linear gradient of ACN (0-60% v/v) in 0.1% TFA for 70 min at a flow rate of 3.5 ml/min. Excitatory effective fraction is indicated by the dark horizontal bar.

B) Visceral excitatory effect of the fraction indicated by the dark horizontal bar in the chromatogram. Arrow indicates the point of application of bioactive substance.

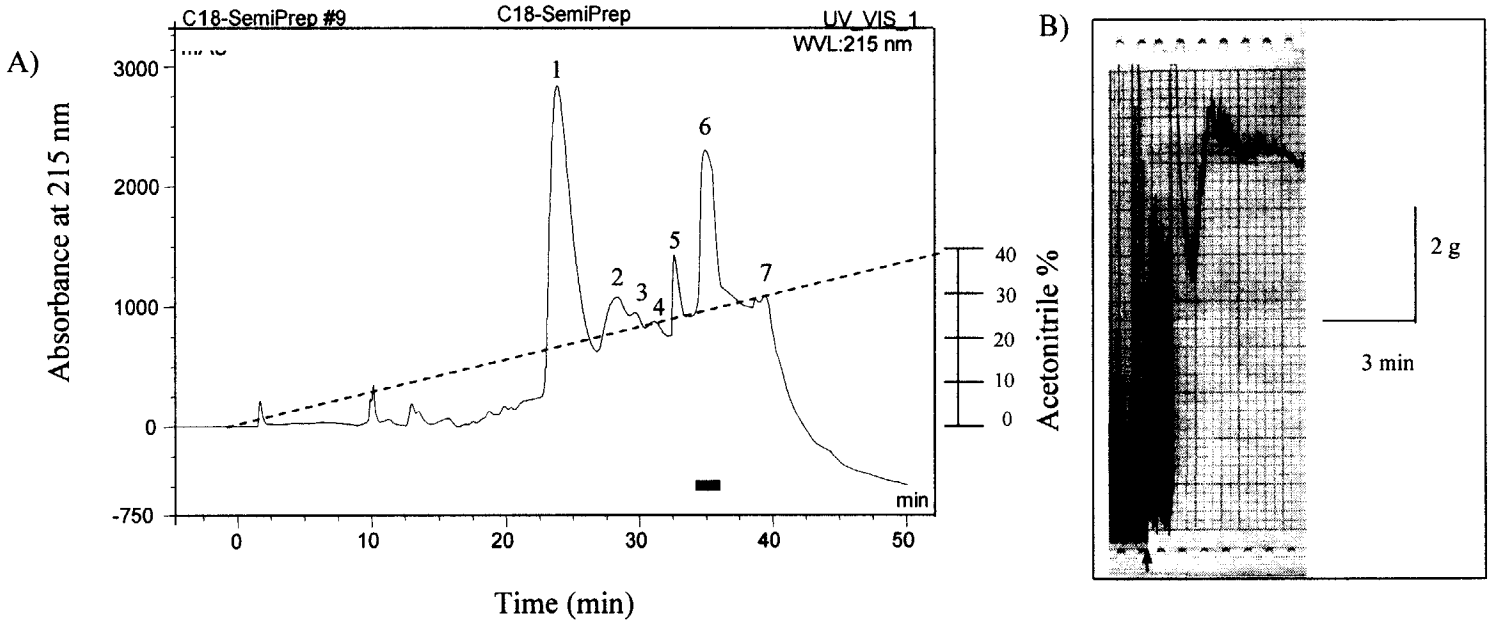

Figure 5. A) Chromatogram of the second step of HPLC purification of the bioactive substances. Each peak was collected. Elution conditions: a linear gradient of ACN (0-40% v/v) in 0.1% TFA over 50 min at a flow rate of 2 ml/min. Excitatory effective peak is indicated by the dark horizontal bar.

B) Excitatory effect of the fraction indicated by the dark horizontal bar in the chromatogram. Arrow indicates the point of application of bioactive substances.

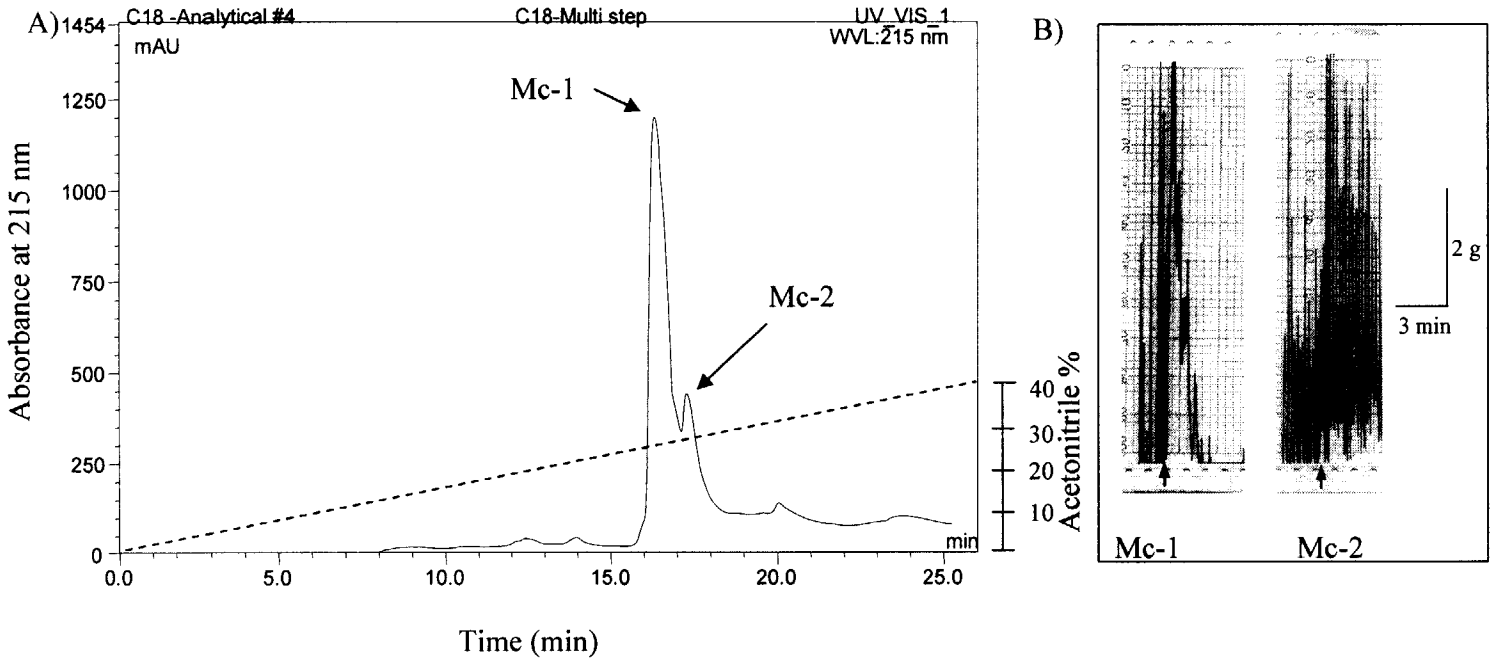

Figure 6. A) Chromatogram of the third step of HPLC purification of the visceral excitatory neuropeptides. Elution conditions: a linear gradient of ACN (0-40% v/v) in 0.1% TFA over 25 min at a flow rate of 0.3 ml/min.
B) Visceral excitatory effects of Mc-1 and Mc-2. Arrows indicate the points of application bioactive substances.

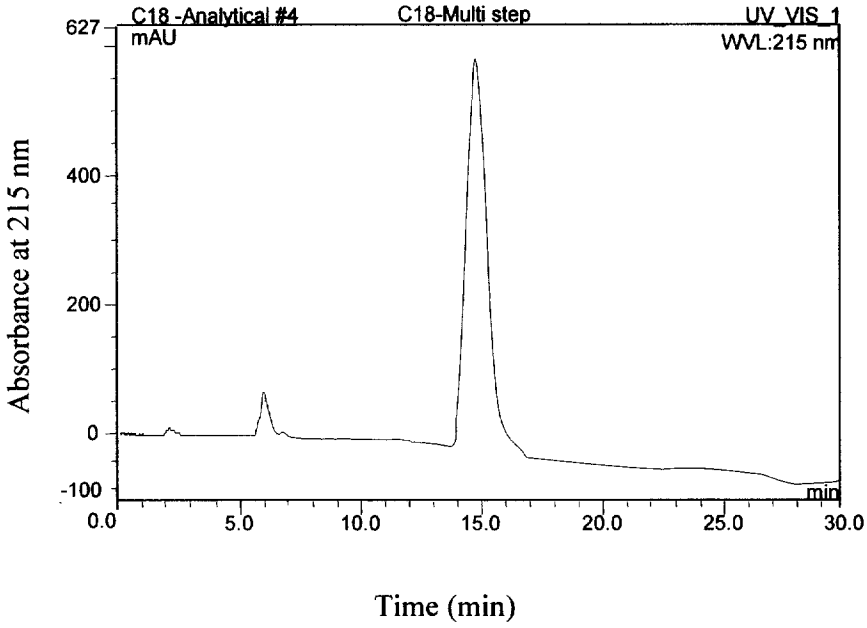

Figure 7. Chromatogram of the isocratic elution of Mc-1. Elution conditions: 26.2% ACN in 0.1%TFA at a flow rate of 0.3 ml/min.

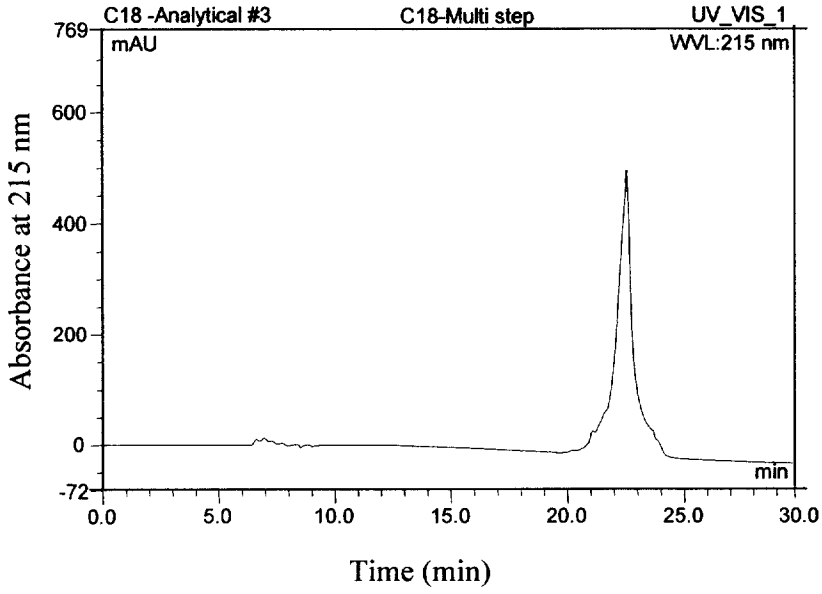

Figure 8. Chromatogram of the isocratic elution of Mc-2. Elution conditions: 27.3% ACN in 0.1%TFA at a flow rate of 0.3 ml/min..

4. Structure determination

Knowledge of the structure of naturally occurring neuropeptides is required to decipher their synthesis, to delineate mode of action and to identify their functions. The amino acid sequence of neuropeptides can be deduced from the relevant nucleotide sequence in the genome, but this approach reveals more about neuropeptide precursors than the neuropeptides expressed ultimately. Although it can predict what peptides processed from a precursor considering their putative proteolytic cleavage sites, not all proteolytic cleavage sites are used and processing can occur at unconventional sites. Moreover, nucleotide sequence does not provide information on the development or tissue specific regulation of neuropeptide precursor processing. A single gene can encode several neuropeptides and processing of individual peptides from a common precursor can differ during development or between tissues. In addition, predicting peptides from nucleotide sequence provides the information about putative primary sequence and does not decipher posttranslational modifications, which are often essential for the activity of neuropeptides.

Earlier, the primary structure of a neuropeptide was determined by Edman degradation after it was purified to homogeneity, but purification is often a time consuming procedure. Low abundance of naturally occurring neuropeptides also makes it challenging to isolate enough of a particular neuropeptide to determine its structure using Edman degradation. Therefore, in this study novel approach ESI-Q-TOF was used to determine the structures of visceral excitatory peptides (Skold et al, 2002).

After isocratic elution of Mc-1 and Mc-2 in HPLC, both were lyophilized and sent for peptide sequencing using ESI-Q-TOF.

Due to poor ionization problem occurred in ESI-Q-TOF, Mc-2 could not be sequenced. Therefore, it was subjected to MALDI-TOF mass spectrometry to determine its molecular weight. Sufficient amount of materials of Mc-2 was not available for further testing like its pharmacological functions. MALDI-TOF mass spectrum of Mc-2 showed that it contained a peptide with a molecular weight of 1622.7 Da at higher intensity (Figure 9). According to molecular size of the peptide and its excitatory effect on smooth muscle (hindgut), we can predict that it may be a tachykinin family peptide or neurokinin-related peptide. There are many tachykinin family, smooth muscle contractive peptides were isolated from brain of different teleosts. For example trout-substance P (Jensen and Conlon, 1992), Cod-substance P (Jensen and Conlon, 1992), trout - neurokinin A (Jensen and Conlon, 1992), Cod–neurokinin A (Jensen and Conlon, 1992) and goldfish-carassin (Conlon et al., 1991). Tachykinins compose a large family of peptides, having a common amino acid sequence of Phe-Xaa-Gly-Leu-Met-amide at their C-terminus (Schoofs et al., 1990). Tachykinins are widely distributed in nerves and endocrine cells of vertebrates and several studies have suggested that these peptides may play in the regulation of cardiovascular and gastrointestinal functions both in mammals (Pernow, 1983) and lower vertebrates (Jensen, 1989). Several tachykinnins have been isolated from mammalian tissues, including substance P, neurokinin A, neurokinin K and neuropeptide γ (Nakanishi, 1987)

After amino acid sequence analysis, Mc-1 contained two visceral excitatory neuropeptides, which were denoted as Mc-1a and Mc-1b. Both were c-terminal amidated peptides. Amino acid sequence of Mc-1a was SGPAGVLamide and its observed molecular weight was 598.7 Da. Mc-1b was a peptide, which had an amino acid sequence of GLGELGNamide and its observed molecular weight was 728.8 Da.

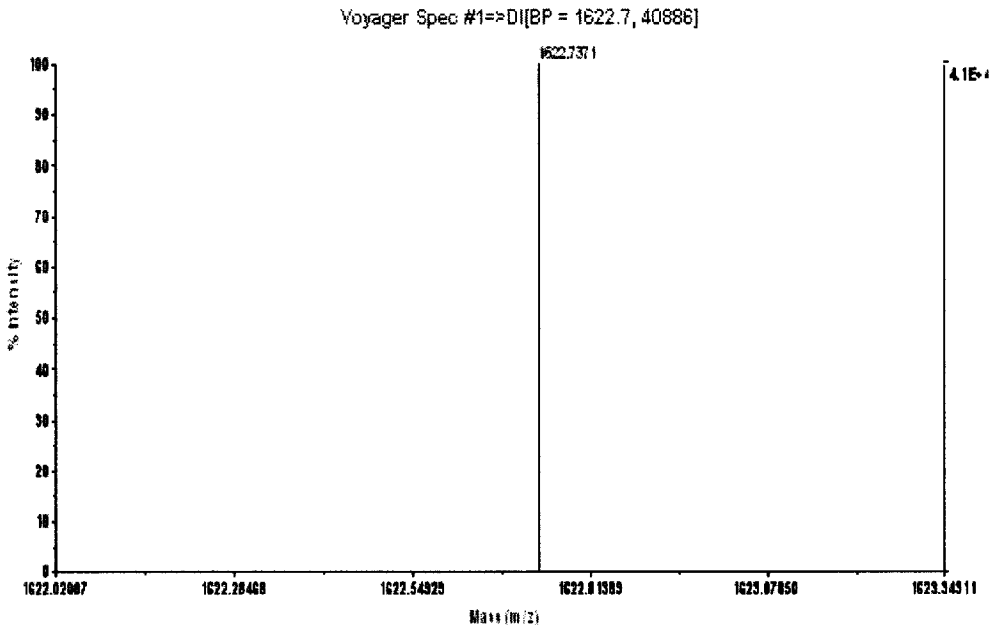

Figure 9. MALDI-TOF mass spectrum of the MP-2.

5. Pharmacological effects of Mc-1

Dose dependent response on mullet hindgut tissue

The dose dependent response of Mc-1 was tested to find out its threshold concentration for the excitatory effects on hindgut using different concentrations of Mc-1 from 0.005 -5 μg/ml.

When the Mc-1 concentration was at 0.005 μg/ml, there was no any visible tension change on the hindgut. At 0.05 μg/ml concentration of Mc-1, a slight increase of muscle tone on the hindgut could be seen. Therefore, the threshold concentration of Mc-1 for the excitatory effects on the hindgut was found to be between 0.005 μg/ml and 0.05 μg/ml (Figure 10). A significant excitatory effect can be seen at 0.5 μg/ml of Mc-1 concentration. Moreover, Mc-1 at higher concentrations than 0.05 μg/ml, exhibited the excitatory actions on hindgut segment in a dose-depended manner. Since bioactive peptides or neuropeptides are highly effective and react with their receptors on target tissues with high affinity, they are needed in minute concentrations to initiate an effect (Mains et al., 1998). It is conceivable that the Mc-1 peptides are involved in the regulation of the hindgut movement of the mullet acting as neurotransmitters or as neuropeptides. Threshold levels of many isolated brain neuropeptides from different animal phyla to have excitatory effects on visceral tissues have been reported to be between 10^{-9} and 10^{-7} M (Takahashi et al., 1995; Fujimoto et al., 1998; Ohtani et al., 1997).

Mc-1 at a concentration of 0.5 μg/ml was used for testing other pharmacological actions in this study.

Time dependent response on mullet hindgut tissue

To determine the tachyphylaxis of the muscle on Mc-1, the effects of interval times between successive applications were compared. The excitatory effects made by the application of 0.5 μg/ml of Mc-1 in 5 min, 15 min and 30 min intervals were compared. No effect could be seen at the second and third application of Mc-1 in 5 and 15 min intervals (Figure 11A and B). However, application of same dose of Mc-1 in 30 min intervals exhibited excitatory actions at each time (Figure 11C). From these results, it revealed that within 30 min, the intestine completely recovered from its desensitization state brought by the application of 0.5 μg/ml Mc-1.

Effects on different tissues of mullet

Effects of Mc-1 at a concentration of 0.5 μg/ml on different tissues (oviduct segment, body wall muscle strip and esophageal segment) of mullet were compared using the bioassay system.

Mc-1 raised the tone of the esophagus and the oviduct (Figure 12 A and C) but it did not affect the relaxation or contraction of the mullet body wall muscle strip (Figure 12 B). Therefore, Mc-1 peptides also affected the contraction of smooth muscles located in the viscera and the oviduct of *M. cephalus*. In 1998, Fujimoto and his colleagues have found a smooth muscle excitatory peptide (C-RFa) from Japanese crucian carp and it was found to have excitatory effects on visceral tissues of other non-fish vertebrates as well. Ohtani and his colleagues (1997) have also found several peptides, which caused gastrointestinal tract contractions in *Helix aspera*. Takahashi and his fellow scientists (1995) have found four different smooth muscle excitatory peptides from *Perinereis vancaurica* and all

those did not appear to be members of any other previously identified neuropeptide families.

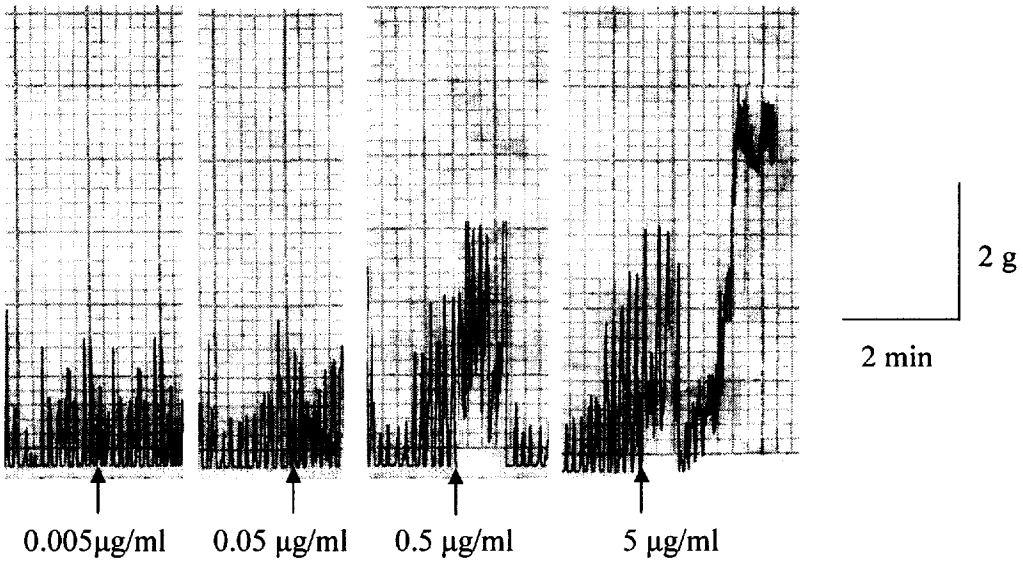

Figure 10. Excitatory effects of different Mc-1 concentrations on the isolated hindgut preparation of *M. cephalus*.

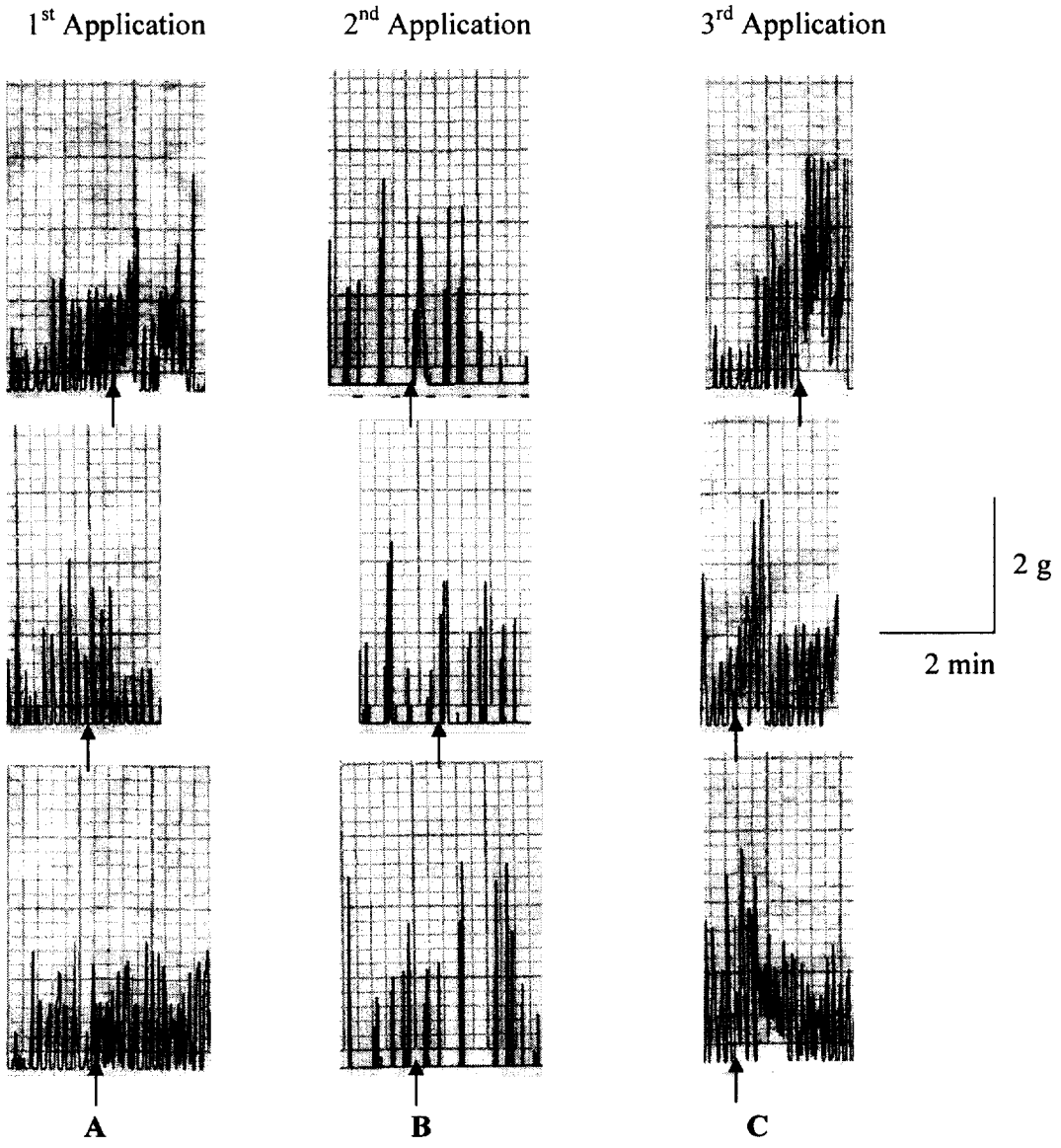

Figure 11. The excitatory effect of Mc-1 applied at a concentration of 0.5 µg/ml on the hindgut segment of *M. cephalus.* (A) At 5 min intervals, (B) at 15 min intervals and (C) at 30 min intervals. Observations were made from 3 different tissue preparations.

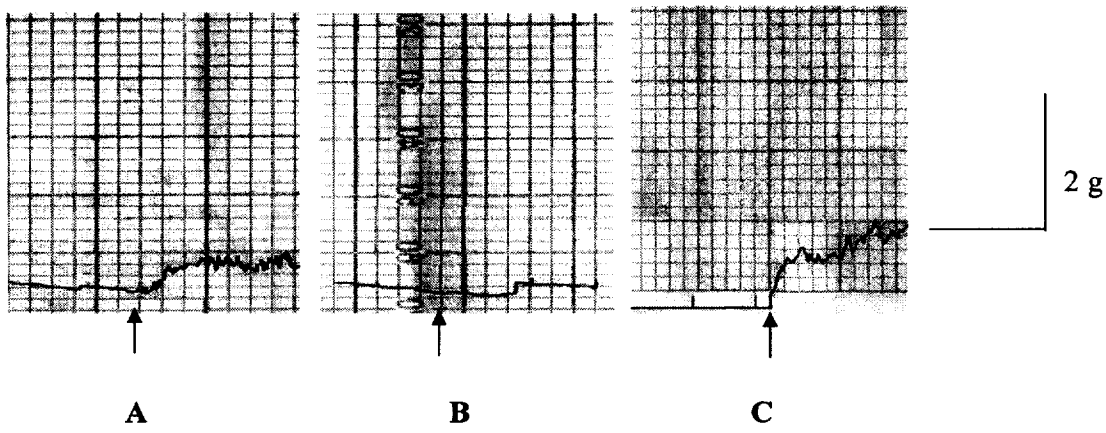

Figure 12. Excitatory effects of Mc-1 at a concentration of 0.5 μg/ml on different tissues of *M. cephalus*. (A) Oviduct segment, (B) body wall muscle strip and (C) esophageal segment.

Summary

In the present study, we identified visceral excitatory peptides from brain extract of a catadromous fish, stripped mullet (*Mugil cephalus*). Since neuropeptides from brain extract of *M. cephalus* were identified according to the physiological assay using in vitro tissue preparations, a suitable medium for the maintenance of in vitro tissue preparations of *M. cephalus* was prepared with respect to its blood plasma composition. The composition of the prepared physiological saline was NaCl 120 mM, KCl 10 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 3.87 mM, $NaHCO_3$ 7 mM, NaH_2PO_4 2 mM, D-glucose 5.7 mM and pH 7.2. 60% methanol fraction obtained from sep-pak C18 cartridges showed a significant tension change on the isolated hindgut of mullet in the bioassay. Therefore, visceral excitatory peaks (Mc-1 and Mc-2) were identified from the 60% methanol fraction using different reversed phase chromatographic techniques together with hindgut physiological assay. Due to lack of sample materials and poor ionization, Mc-2 was unable to be sequenced or its pharmacological properties to be studied. Molecular weight of Mc-2 was determined as 1622.7 Da by MALDI-TOF mass spectrometry. According to its molecular weight and smooth muscle excitatory effect, Mc-2 can be predicted as a neuropeptide belongs to the tachykinin family. Mc-1 contained two peptides, which raised the tone of hindgut in a dose dependent manner with a threshold concentration of 0.005-0.05 μg/ml and within 30 min the hindgut recovered completely from its desensitization state brought by 0.5 μg/ml of its concentration. Amino acid sequences of Mc-1 peptides were SGPAGVLamide and KLGELGNamide. Moreover, Mc-1 was found to have excitatory effects on esophageal and oviduct smooth muscle tissues. Therefore, Mc-1 (Mc-1a and Mc-1b) and Mc-2 are brain neuropeptides involved in regulation of visceral tissues of striped mullet.

References

Boer, H. H., Schot, L. P. C., Veenstra, J. A. and Reichelt, D. Immunocytochemical identification of neural elements in the central nervous systems of a sail, some insects, a fish, and a mammal with an antiserum to the molluscan cardio-excitatory tetrapeptide FMRFamide. *Cell Tissue Res.* 1980, 213; 21-27.

Burton, D. Further assessment of a pharmacological effect of Tris buffer. *Comp. Biochem. Physiol. C* 1988, 90 (1); 263-265.

Burton, R. F. Ringer solutions and physiological salines. *Bristol, Wright-Scientechnica, 1975.*

Chen, S. T., Tsai, M. S. and Shen, C. L. Distribution of FMRFamide-like immunoreactivity in the central nervous system of the Formosa monkey (*Macaca cyclopesis*). *Peptides* 1989, 10; 825-834.

Chronwall, B. M., Olschowka, J. A. and O'Donohue, T. L. Histochemical localization of FMRFamide-like immunoreactivity in the rat brain. *Peptides* 1984, 5; 569-584.

Clement, K. D. and Rees, D. Preservation of inherent contractivity in isolated gut segments from herbivorous and carnivorous marine fish. *J. Com. Physiol. B.* 1998, 168; 61-72.

Conlon, J. M., O'Harte, F., Peter, R. E. and Kah, O. Carassin: a tachykinin that is structurally related to neuropeptide-gamma from the brain of the goldfish. *J. Neurochem.* 1991, 56; 1432-1436.

Conlon, J. M., Bjenning, C., Moon, T. W., Youson, J. H. and Thim, L. Neuropeptide Y-related peptides from the pancrease of a teleostean (Eel), holostean (Bowfin) and elasmobranch (skate) fish. *Peptides* 1991,12; 221-226.

Conlon, J. M. Biosysthesis of regulatory peptides. Evolutionary aspects. In: Holmgren, S. eds. The comparative physiology of regulatory peptides. *London, Chapman and Hall*, 1989: 344-369.

Conlon, J. M. and Thim, L. Isolation of the thachykinin, des[Ser^{1}, Pro^{2}]scyliorhinin II from the intestine of the ray *Torpedo marmorata*. *Gen. Comp. Endocrinol.* 1988, 71; 383-388.

Conlon, J. M., Deacon, C. F., O'Toole, L. and Thim, L. Scyliorhinin I and II: two novel tachykinins from dogfish gut. *FEBS Let.* 1986, 200; 111-116.

Cooper, J. R., Bloom, R. E., Roth, R. H. Neuroactive peptides. In: The Biochemical Basis of Neuropharmacolog. 5th edition. *New York, Oxford University Press,* 1986: 352-393.

Cowden, C. and Stretton, A. O. W. Eight novel FMRFamide-like neuropeptides isolated from the nematode *Ascaris suum*. *Peptides* 1994, 16(3); 491-500.

Danielson, P. B. and Dores, R. M. Molecular evolution of the opioid/orphanin gene family. *Gen. Comp. Endocrinol.* 1999, 113; 169-186.

Dockray, G. J., Reeve, J. R., Shively, J., Gayton, R. J. and Barnard, C. S. A novel active pentapeptide from chicken brain identified by antibodies to FMRFamide. *Nature* 1983, 305; 328-330.

Dores, R.M., Rubin, D.A. and Quinn, T.W. Is it possible to construct phylogenetic trees using polypeptide hormone sequences?. *Gen. Com. Endocrinol.* 1996,103; 1-12.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* 1956, 28(3); 350-356.

Ferry, X., Brehin, S., Kamel, R. and Landry, Y. G protein-dependent activation of mast cell by peptides and basic secretagogues. *Peptides* 2002, 23(8); 1507-1515.

Fujimoto, M., Takishita, K.I., Wang, X., Takabataka, I., Fujisawa, Y., Taranishi, H., Ohtani, M., Muneoka, Y. and Ohta, S. Isolation and characterization of a novel bioactive peptide, Carassius Rfamide (C-Rfa), from the brain of the Japanese crussian carp. *Biochem. Biophys. Res. Commun.* 1998, 242; 436-440.

Gayton, R. J. Mammalian neuronal actions of FMRFamide and the structurally related opioid Met-enkephalin-Arg-Phe. *Nature* 1982, 298; 275-276.

Guzman, A., Legendre, P., Allard, M., Geoffre, S., Vincent, J. D. and Simmonet, G. Electrophysiological effects of FLFQPQRFamide, an endogenous brain morphine modulating peptide on cultured mouse spinal-cord neurons. *Neuropeptides* 1989, 14; 253-261.

Hayashi, M. A. F. and Camargo, A. C. M. The bradykinin-potentiating peptides from venom gland and brain of *Bothrops jararaca* contain highly site specific inhibitors of the somatic angiotensin-converting enzyme. *Toxicon* 2005, 45; 1163-1170.

Holmgren, S and Jensen, J. Evolution of vertebrate neuropeptides. *Brain Research Bulletin* 2001, 55; 723-735.

Holmgren, S. and Jensen, J. Comparative aspects on the biochemical identity of neurotransmitters of autonomic neuron. In: Burnstock, G. eds. Comparative physiology and evolution of the autonomic nervous system. *New York, Gordon and Breach Science Publishers*, 1994: 69-95.

Holmgren, S., Fritsche, R., Karila, P., Gibbins, I. G., Axelsson, M., Franklin, C., Grigg, G. and Nilsson, S. Neuropeptides in the Australian lungfish *Neoceratodus forsteri*: Effects in vivo and presence in autonomic nerves. *Am. J. Physiol.* 1994, 266; 1568-1577.

Hoyle, C. H. V. Neuropeptide families: Evolutionary perspectives. *Regulatory peptides* 1998, 73; 1-33.

Iwakoshi, E., Hisada, M. and Minakata, H. Cardioactive peptides isolated from the brain of a Japanese octopus, *Octopus minor*. *Peptides* 2000, 21; 623-630.

Jensen, J. and Conlon, J. M. Substance-P-related and neurokinin-A-related peptides from the brain of the cod and trout. *Eur. J. Biochem.* 1992, 206; 659-664.

Jensen, J. and Conlon, J. M. Characterization of peptides related to neuropeptide thyrosine and throsine-tyrosine from the brain and gastrointestinal tract of teleost fish. *Eur. J. Biochem.* 1992, 210; 405-410.

Jensen, J. and Conlon, J. M. Substance-P-related and neurokinin-A-related peptides from the brain of the cod and trout. *Eur. J. Biochem.* 1991, 206; 659-664.

Jensen, J. In: Holmgren, S., eds. The comparative physiology of regulatory peptides. *London, Chapman & Hall,* 1989: 130-149.

Kagstrom, J., Holmgren, S., Olson, K. R., Conlon, J. M. and Jensen, J. Vasoconstrictive effects of native tachykinins in the rainbow trout *Oncorhynchus mykiss*. *Peptides* 1996, 17; 39-45a.

Kagstrom, J., Axelsson, M., Jensen, J., Farrell, A. P. and Holmgren, S. Vasoactivity and immunoreactivity of fish tachykinins in the vascular system of the spiny dogfish. *Am. J. Physiol.* 1996, 270; 585-593b.

Keynes, R. D. and Ferreira, H. M. Membrane potentials in the electroplates of the electric eel. *J. Physiol. London* 1953, 119; 315-251.

Kobierski, L. A., Belz, B. S., Trimmer, A. and Kravitz, E. A. FMRFamide-like peptides of Hoarus americanus: Distribution, immunocytochemical mapping, and ultrastructural localization in terminal varicosities. *J. Comp. Neurol.* 1987, 266; 1-15.

Kuroda, K. T., Ukena, E. I., Kanda, A. and Minakata, H. Octopus, which owns the most advanced brain in inverebrates, has two members of vasopressin/oxytocin superfamily as in vertebrates. *Regulatory Peptides* 2003, 115; 139-149.

Liebrich, W., Kellner, R. and Gade, G. Isolation and primary structure of neuropeptides of the AKH/RPCH family from various termite species. *Peptides* 1995,16; 559-564.

Linder, S., Barkhem, T., Norberg, A., Persson, H., Schalling, M., Hokfelt, T., Magnusson, G. Structure and expression of the gene encoding the vasoactive intestinal peptide precursor. *Proc. Natl. Acad. Sci. USA* 1987, 84; 605-609.

Lombardin, M.S., Karelaars, A. and Hijnen, C. J. Role and Modulation of G-protein coupled receptor signaling in inflammatory processes. *Critical Review in Immunology* 2002, 22(2); 141-163.

Maggio, J. E. and Mantyh, P. W. History of tachykinin peptides. In: Buck, S. H. eds. Tachykinin receptors. *Totowa, NJ:Human Press.* 1994:1-21.

Mains, R. E. and Eipper, B. A. Peptides. In: Siegel, G. J., Agranoff, B. W., Albers, R. W. and Molinoff, P. B. eds. Basic neurochemistry. 6th edition. *New York, Raven Press* 1998: 363-382.

Morishita, F., Nitagai, Y., Furukawa, Y., Matsushima, O., Takahashi, T., Hatta, M., Fujisawa, T., Tunamoto, S. and Koizumi, O. Identification of a vasopressin-like immunoreactive substances in hydra. *Peptides* 2003, 24; 17-26a.

Morishita, F., Minakata, H., Sasaki, K., Tada, K., Furukawa, Y., Matsushima, O., Mukai, S. T. and Saleuddin, A. S. M. Distribution and function of an *Aplysia* cardioexitatory peptide, NdWFamide, in pulmonate snails. *Peptides* 2003, 24; 1533-1544b.

Musa, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M. and Nakanishi, S. cDNA eloping of bovine substance K receptor through oocyte expression system. *Nature* 1987, 329; 836-838.

Nakanishi, S. Substances P precursor and kininogen; their structure, gene organizations and regulation. *Physiol. Rev.* 1987, 67: 1117-1142.

Nishizawa, M., Hayakawa, Y., Yanaihara, N. and Okamoto, H. Nucleotide sequence divergence and functional constraints in VIP precursor mRNA distribution between man and rat. *FEBS Lett.* 1985, 183; 55-59.

O'Donohue, T. L., Bishop, J. F., Chronwall, B. M., Groome, J. and Watson, W. H. Characterisation and distribution of FMRFamide immunoreactivity in the rat central nervous system. *Peptides* 1984, 5; 563-568.

Ohtani, M., Muneoka, Y., Matsushima, O., Takao, T., Shimonishi, Y., White, A. R., Pedder, S., Sharma, R., Lennon, M. A., Katugampola, S. and Walker, R. J. Isolation of bioactive compounds from *Helix aspera* nerve tissue and the effect of pQPPLPRYamide on heart, esophagus and central neurons of *H. aspera* and Rectum of *Anodonta woodiana*. *Gen. Pharmac.* 1997, 29(1); 103-111.

Pernow, B. Substance P. *Pharmacol. Rev.* 1983, 35; 85-141.

Price, D. A. and Greenberg, M. J. Structure of a molluscan cardioexcitatory neuropeptide. *Science*1977, 197; 670-671.

Raffa, R. B. The action of FMRFamide (Phe-Met-Arg-Phe-NH_2) and related peptides on mammals, *Peptides* 1988, 9; 915-922.

Rees, D. Consideration of the inorganic and organic composition of mammalian perfusion solutions. In: Dőring, H. J., Dehnert, H. eds. Isolated perfused organ preparations. *Biomesstechnik,* 1989: 85-94.

Ringer, S. Regarding the influence of the organic constituents of the blood on the contractility of the ventricle. *J. Physiol. Lond.* 1885, 6; 361-381.

Ringer, S. A further contribution regarding the influence exerted of the different constituents of the blood on the contraction of the heart. *J. Physiol. Lond.* 1883, 4; 29-42.

Ringer, S. Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. *J. Physiol. Lond.* 1882, 3: 380-393.

Sandberg, M and Weber, S. G. Techniques for neuropeptide determination. *Trends in analytical chemistry* 2003, 22(9); 522-527.

Schoofs, L., Holman, G. M., Hayes, T. M., Nachman, R. J. and DeLoof, A. Locustatachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. *FEBS lett.* 1990, 261; 397-401.

Sasai, Y. and Nakanishi, S. Molecular characterization of rat substance K receptor and its mRNAs. *Biochem Biophys Res Communi.* 1989, 165; 695-702.

Sithigorngul, P., Nanda, J. C. and Stretton, A. O. W. A strategy for isolating rare peptides: isolation and sequencing of a large peptide present in a single neuron of the nematode *Ascaris suum*. *Peptides* 2003, 24; 1025-1033.

Sithigorngul, P., Pupuem, J., Krungkasem, C., Longyant, S., Panchan, N., Chaivisuthangkuru, P., Sithigorngul, W. and Petson, A. Four novel PYFs: members of the giant tiger prawn *Penaeus monodon*. *Peptides* 2002, 23; 1895-1906.

Sirhigorngul, P., Saraithongkum, W., Jaideechoey, S., Longyant, S. and Sithigorngul, W. Novel FMRFamide-like neuropeptide from the eyestalk of the giant freshwater prawn. *Com. Biochem. Physiology Part B* 1998, 120; 587-595.

Skold, K., Svensson, M., Kalpan A., Bjorkesten, L., Astrom, J. and Andren, P. E. Aneuroproteomic approach to targeting neuropeptides in the brain. *Proteomics* 2002, 2; 447-454.

Takahashi, T., Furukawa, Y., Muneoka, Y., Matsushima, O.,Ikeda, T., Fujita, T., Minakata, H. and Nomoto, K. Isolation and characterization of four novel bioactive peptides from a polychaete annelid, Perinereis vancaurica. *Com. Biochem. Physiol.* 1995, 110C; 297-304.

Tang, J., Yang, H. Y. T., Costa, E. Inhibition of spontaneous and opiate-modified nociception by an endogenous neuropeptide with Phe-Met-Arg-Phe-NH_2-like immunoreactivity. *Proc. Natl. Acad. Sci. USA* 1984, 81; 5002-5005.

Thorndyke, M.C. and Goldsworthy, G. J. Neurohormones in invertebrates. Cambridge *University Press* 1988: 336.

Vallarino, M., Salsotto-Cattaneo, M. T. Feuilloley, M. and Vaudry, H. Distribution of FMRFamide-like immunoreactivity in the brain of the elasmobranch fish *Scyliorhinus canicula. Peptides* 1991, 12; 1321-1328.

Watson, W. H. and Groome, J. R. Presence and distribution of immunoreactive and bioactive FMRFamide-like peptides in the nervous system of the horseshoe crab, limulus polyphemus. *Peptides* 1984, 5; 585-592.

Weber, E. and Evans, C. J. samuelsson, S. J., Barchas, J. D. Novel peptide neuronal system in rat brain and pituitary. *Science* 1981, 214; 1248-1251.

Wong, T. M., Greenberg, M. J. and Tse, S. Y. H. Cardiovascular effects of intraventricular injection of FMRFamide, met-enkephalin and their common analogues in the rat. *Comp. Biochem. Physiol.* 1985, 81C; 175-179.

Yakota, Y., Akazawa, C., Ohkubo, H. and Nakanishi, S. Delineation of structural domains involved in the subtype specificity of tachykinin receptor through chimeric formation of substance P/Substance K receptor. *EMBO J.* 1992, 11; 3585-3551.

Yakota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H. and Nakanishi, S. Molecular characterization of a functional cDNA for rat substance P receptor. *J Biol Chem.* 1989, 264; 17649-17652.

Young, J. Z. The preparation of isotonic solutions for use in experimentals with fish. *Publ. Staz. Zool. Nap.* 1933, 12; 425-431.

Zhu, X. Z., Raffa, R. B. Low affinity inhibition of opioid receptor binding by FMRFamide. *Neuropeptides* 1986, 8; 55-61.

Zhou, C. J., Shioda, S., Yada, T., Inagaki, N., Pleasure, S. J. and Kikuyama, S. PCACP and its receptors exert pleiotropic effect in the nervous system by activating multiple signaling pathways. *Current Peptide and Protein Science* 2002, 3 (2); 423-439.

Acknowledgements

First, I would like to extend my sincere gratitude with a deep appreciation to my supervisor, Prof. Kim Se-Kwon for kindly offering a valuable opportunity for me to study under his supervision. As a girl who lives far away from home in a foreign country, I like to thank my dear supervisor for his boundless caring, kindness and a lot of support over the last two years of my studies in South Korea.

I am deeply grateful to Mrs. Eresha Priyangika Mendis, for her guidance, enlightening discussions and her boundless tolerance to my unending questions during the completion of my thesis. I wish to thank Mr. Niranjan Prabhath Rajapakse who provided invaluable help and unselfish support throughout the preparation of this thesis. Again, I am owing to sister Eresha and brother Niranjan for their continue advices, encouragements when I was facing hardships and affectionate caring, to make my life comfortable during last couple of years of my studies in a foreign country. I am grateful to my colleague Mr. Sanjeewa Dilshan Ranatunga for his helpful comments to make my efforts fruition throughout my study period.

I must be sincerely grateful to Dr. Fumihiro Morishita, Graduate School, Hiroshima University, Japan for generously offering me the technical knowledge to carry out neuropeptide related experiments and I am sure my task would not be fruition without your advices.

I also wish to thank all of my lab members especially, Dr. Hee-Guk Byun, Dr. Moon- Moo Kim, Dr. Jae-Young Je, Dr. Won-Kyo Jung and Mr. Lee-Bae-Jin for creating a friendly working atmosphere and mutual encouragements through out my study period. I would also like to thank my lab colleagues Zhong-Ji Qian, Cho Hee-Yeon, Kim Soo-Young, Eom Tae-Kil, Cho Young-Sook, Tai Van Quang, Ngo Dai Nghiep, Ryu Bo-Mi, Song Yun-Hee and Kim Jung-Ae for their fullest

cooperation over the course of research. Sincere acknowledgement goes to staff members of Marine Bioprocess Research Center, Pukyong National University for their fullest cooperation in every aspect.

Grateful acknowledgments are made to Prof. Sung-Hoon Moon and Prof. So Won Youn for reviewing the thesis giving valuable advices and I like to acknowledge all other professors in the chemistry department, Pukyong National University for their guidance during my course of studies. I also express my sincere gratitude to officials of International Relations Bureau, Pukyong National University for their kind support in every aspect.

I sincerely thank Mrs. Seon-Min Park and other researchers at Research Center for Ocean Industrial Development in Pukyong National University for their technical assistants to complete the research successfully.

Sincere thanks to all my friends especially Komini Naoko and Zhang Xiao Li in the dormitory for creating a peaceful and enjoyable environment where I could always be certain to find at home. Thank you very much Park Sung Il and Yoon Yeon-Wook for your fullest cooperation and friendship every time to make my life easy.

Finally, I would like to express my eternal gratitude to my ever-loving parents for bringing sunshine into my life when all else failed. I extremely grateful to them and to my loving sister Inoka for their unconditional love, care, advice and emotional support throughout the period I stayed away from home.