Thesis for the Degree of Master of Science

Isolation and Characterization of Visceral Excitatory Neuropeptides from Cloudy Dogfish (*Scyliorhinus torazame*) Brain

by

Young-Sook Cho

Department of Chemistry

The Graduate School

Pukyong National University

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두툽상어 (*Scyliorhinus torazame*) 뇌로부터 소화계 자극성 신경계 펩타이드의 분리 및 특성

Advisor: Prof. Se-Kwon Kim

by

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Approved as to style and content by:

Prof. Sung-Hoon Moon, Ph.D. (Chairman)

Prof. Byeng-Wha Son, Ph.D. (Member)

Prof. Se-Kwon Kim, Ph.D. (Member)

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Young- Sook Cho

Department of Chemistry, Graduate School,

Pukyong National University

Abstract

To investigate visceral excitatory neuropeptides of Chondrichthyes, peptides were purified from the brain of cloudy dogfish (*Scyliorhinus torazame*) using fish hindgut as the physiological assay system. Neuropeptides were purified from acetic acid brain extract of dogfish using reversed phase high performance liquid chromatography (HPLC) with sep-pak C18 cartridges. One of these visceral excitatory neuropeptides was determined by MALDI-TOF mass spectrometry and its amino acid sequence was determined by ESI-Q-TOF mass spectrometry. ESI-Q-TOF mass spectrometry showed that the peptide (Df-2) contained amino acid sequence of LESLVYEQLWPamide (1563Da). Threshold concentration of Df for the excitatory effect on the visceral segment of dogfish was 0.01㎍/㎖-1㎍/㎖ and minimum of 20min time was required for the complete recovery of intestine from the desensitization state brought by the Df-2 concentration of 1㎍/㎖.

Key words : neuropeptides, visceral excitatory, dogfish

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Introduction

Neuropeptide is a member of a class of protein-like molecules made in the brain. Neuropeptides consist of short chains of amino acids, with some functioning as neurotransmitters and some functioning as hormones. Neuropeptides are peptides involved in nervous system function (Grimmelikhuijzen, C., Leviev, I.K., Carstensen, K., 1996). They are synthesized in cells in large precursor proteins, and generally several biologically active peptides are contained in the same precursor molecule. Various biochemical processes control the quantities of neuropeptides, as well as the nature of their biological activity, through size, form and derivatization of the end product. In this way neuropeptides with different, opposite and often more selective properties are formed from the same precursor. The generation of neuropeptides is a cell/specific phenomenon (Fujimoto, M., Takishita et al, 1998). They are co-localized with classical neurotransmitters and released when the system is stimulated. Neuropeptides may act as neurotransmitters, but in most instances modulate neuronal activity in conjunction with the neurotransmitter, with which they are co-localized.v(Bhardwaj RS, Becher E, Mahnke K, et al, 1997). In recent years, receptors have been found for many of the neuropeptides in the brain. The effects on learning and memory processes by various neuropeptide families have been reviewed earlier.

The current period of studies on neuropeptides may be characterized by the appearance of a unifying neuropeptide concept (Grimmelikhuijzen et al, 1996) Various separate pieces of knowledge have been assembled and, finally, the idea of neuropeptides as a novel class of highly specific, physiologically active compounds possessing a number of specific properties have become widely recognized. Two major events in the mid and late 1970s gave powerful impetus to the studies of biologically active peptides: the discovery of the family of opioid peptides and the demonstration of the localization in the central nervous system of

the several peptides otherwise known to be produced in peripheral organs and tissues. These discoveries not only laid the groundwork for the development of new research areas, but also led to other important findings. The fact that substance P is present in both the brain and the viscera (gut and lungs) suggested that this type of distribution may be common for some other peptides as well. In 1974 it was established that somatostatin, known to be produced in the brain, is also produced in the pancreas. This finding was followed by a search for alternate localizations of other peptides, which resulted in the discovery of a number of biologically active peptides in the CNS. Studies on the physiological role of these peptides in the central nervous system revealed that they possessed neurotropic activities**.**

A number of new neuropeptides have been discovered since 1973. In 1974 Erspamer's group isolated a novel physaleimin-like peptide(Anastasi A, Erspamer et al. , 1975) uperolein. Theprimary structure of proctolin, an insect neuropeptide, was determined in 1975. Greenberg elucidated the primary structure of a mollusc cardioexcitatory neuropeptide, FMRF-amide. Another neuropeptide, head activator, was isolated from a coelenterate hydra and its structure was sequenced by H. C. Schaller and H. Bodenmuller in 1981; this undecapeptide was shown to be present in rat intestine and bovine and human hypothalamus. In 1977 G. A. Schoenenberger and M. Monnier elucidated the amino acid sequence of d sleep-inducing peptide (DSIP), discovered in 1964. Further studies of its effects, using a synthetic peptide and the antiserum developed against it, revealed that although DSIP is present in the CNS and the gut its role in the organism appeared to be other than sleep induction. This latter action was shown to be one of the effects of substance P and other neuropeptides. In 1979 a peptide was isolated from porcine stomach and intestines using the antiserum developed against bombesin by T. J. McDonald *et al.* from Mutt's laboratory. Determination of its primary structure revealed that it is not identical to bombesin but only structurally related to it. The peptide was named gastrinreleasing peptide, GRP, because of its ability to induce

gastrin secretion. Urotensin II, a peptide structurally related to somatostatin, was isolated from the fish caudal neurosecretory system in 1980. Traditionally, detection of a new biological activity preceded the isolation of its active principle, and during the isolation procedure bioassays based on this biological activity was used for screening fractions for the presence of the active principle. Tatemoto and Mutt proposed 258 MARIA M. KLAVDIEVA first to isolate new peptides from the tissues and then to study their biological effects. Taking into consideration the fact that C-terminal amide is present in the structure of many known peptides, the authors developed a technique for the isolation of C-amidated peptides. Using this method they discovered a number of C-amidated peptides in porcine intestine extracts. In 1982 Tatemoto and Mutt isolated from porcine brain extracts a neuropeptide Y (NPY) which consisted of 36 residues and resembled PYY and pancreatic polypeptide. NeuropeptideY, detected by such an unconventional technique, was found to be one of the most widespread of neuropeptides. In the CNS it was found to be localized in neurons ranging from the cerebral cortex to the spinal cord, while outside the CNS, NPY-containing neurons were found to constitute a distinct group in the enteric nervous system, and throughout the periphery NPY was found in adrenergic nerves innervating smooth muscle. It was shown that NPY behaves as a potent vasoconstrict or and it was subsequently established as an important neuromessenger in the regulation of anterior pituitary hormonesecretion. Galanin was discovered in the same laboratory in 1983. This 29-aminoacidpeptide originally isolated from porcine gut extracts was found to be widely distributed in the mammalian central nervous system, where it is colocalized with other neuropeptides. It exerts a wide spectrum of hormonal and neurotropic effects, including regulation of the release of pituitary hormones, growth promotion, and modulation of nociception. Galanin was shown to inhibit insulin release, hippocampal acetylcholine release, and firing of locus coeruleus cells and to stimulate feeding and the release of growth hormone. Subsequently galanin became implicated in the pathophysiology of Alzheimer's disease.

Neuroendocrine control of egg-laying in *Aplysia* was the object of extensive

studies for several years. By 1981, the work of D. H. Schlesinger, S. B. Babirak, and J. E. Blankenship had demonstrated a complicated and peculiar mechanism of regulation of egg-laying, which includes the release of several peptide neurohormones in a particular order. The structure of neurohormones was determined.

After several unsuccessful attempts by other groups, another releasing factor, GRF (growth hormone-releasing factor), was isolated, using HPLC. Its primary structure was determined in 1982 by two independent research groups.

GRF characterized by Guillemin's group was a polypeptide which consisted of 44 amino acid residues and possessed high structural homology with the peptides of the ''secretin-glucagon'' family, especially PHI. It was shown that the (1–29)-fragment retains full intrinsic growth hormone-releasing activity *in vitro.* The same year Vale and associates succeeded in the isolation of another peptide, the existence of which had been demonstrated many years before—the famous, elusive CRF (''constant research frustration'' as Guillemin once called it). CRF was purified from the old fractions of ovine hypothalamic extracts remaining after the isolation of LH-RH. It was found to be a 41-amino-acid residue peptide with a structure highly homologous to that of sauvagine, a frog skin peptide isolated by Erspamer's group. Like sauvagine, CRF stimulated ACTH and b-endorphin elease. It was shown that CRF also releases MSH. The fact that many peptides were found to be structurally homologous with other peptides allowed the development of yet another approach to the search for new neuropeptides, based this time on the possible affinity of unknown peptides to antisera developed against the known ones. Thus G. J. Dockray andassociates isolated a novel hypertensive pentapeptide from chicken brain and identified it using antibodies to FMRF-amide. Simultaneously, R. E.Carraway and C. F. Ferris isolated a hypotensive neurotensin-related hexapeptide, Lys8-Asn9-neurotensin-(8–13) (LANT-6), from chicken intestine.

In 1982 Takagi's group isolated a new analgesic pentapeptide from bovine brain, neokyotorphin, and showed that it was an N-terminally extended kyotorphi .

Exogenous (food) opioid peptides were discovered in 1979. In 1981 Erspamer's group made a surprising discovery: they isolated from the skin of Phyllomedusa sauvagei a unique class of opioid peptides, dermorphins, containing a D-alanine residue in the 2 position. The heptapeptide dermorphin was much more potent than Met- and Leu-enkephalins, b-endorphin, and morphine in guinea pig ileum and mouse vas deferens assays and induced analgesia in mice injected intravenously and in rats injected intracerebroventricularly. Higher doses of intracerebroventricularly injected heptapeptide caused catalepsy. It was soon shown that dermorphin is also present in rat brain. A group of biochemists from Merck together with E. Costa of NIMH, Washingon, isolated a big polypeptide, 11 kDa, with chemical and physiological affinity to benzodiazepine receptors, the existence of which had been demonstrated in 1976.

In 1982 another neuropeptide was discovered when an approach originating in molecular biology was followed. Studies of alternate tissue-specific RNA processing in calcitonin gene expression revealed the generation of mRNAs encoding different polypeptide products (Tatemoto K,1982). Neuropeptide Y Calcitonin-encoding mRNA is formed predominantly in the thyroid parafollicular cells, while in the nervous system, the main product of calcitonin gene expression is an mRNA coding for a novel neuropeptide named calcitonin gene-related peptide, CGRP. CGRP has potent cardiovascular actions in humans and in animals. It acts as a powerful vasodilator and has a direct positive inotropic effect on the heart. It was demonstrated later that CGRP has specific effects on the human heart. These findings suggested an important physiological and therapeutic role for CGRP in cardiovascular diseases. Immunoreactive CGRP was found in the spinal cord, where it was colocalized with substance P and galanin, and it was shown to possess neurotropic actions in the CNS. In 1984 S. Nakanishi's group, using alternative RNA splicing, demonstrated a similar tissue-specific generation of the

two different preprotachykinin mRNAs from one gene.

ANF receptors have been identified in all putative target tissues (vascular smooth muscle, kidney, adrenal zona glomerulosa cells, posterior pituitary), as well as in the brain. In 1985, evidence for the presence of ANF in rat brain was isolated another peptide, the 26-amino-acid brain natriuretic peptide, BNP, from rat brain. BNP has marked structural homology to ANF. This peptide, as well as its bigger forms, was subsequently found in porcine, human, and rat heart.

Endothelin (ET), a vasoconstrictor 21-amino-acid peptide, was found during the search for the long-postulated endothelial vascular smooth muscle activator.
Endothelin was isolated in 1988 by M. Yanagisawa and associates at the University of Tsukuba from the supernatant of porcine aortic endothelial cells. It is one of the most potent and long-lasting vasoconstrictor agent known to date. Subsequently, it was shown that the endothelin is synthetized as a preprohormone which is cleaved by unusual proteolytic processing. The different isoforms of endothelin (endothelin-1, -2, and -3), originally predicted from the finding of three separate genes, appeared to differ in their localization and biological activity. Distribution patterns and pharmacological functions of endothelin isoforms have now been defined. Vascular endothelial cells were shown to produce only endothelin-1. Endothelin-1 and endothelin-2 are mainly distributed in the CNS, including spinal cord and peripheral tissues, while endothelin-3, although present in other tissues, is mainly concentrated in the pituitary. It was found that endothelins exert a number of different effects, such as positive inotropic and chronotropic actions on the heart, release of other regulatory agents and pituitary hormones, regulation of regional cerebral blood flow, and renal and mitogenic actions. Endothelins have been implicated in the pathophysiology of a number of cardiovascular, renal, and respiratory diseases (Ohtani et al.,1997)

Neuropeptides are ubiquitous in the nervous system at all levels of organization from hydrozoans to man and they are by far the most diverse signaling substances,

both structurally and functionally. A plethora of peptides have been characterized in the brain by immunohistochemical and molecular biological techniques. The development of retrograde tracing techniques, combined with immunohistochemistry, reveals that these peptides are localized in nerve fibers or cell bodies of the hypothalamus and medulla, which are important sites for autonomic nervous outflow to the gastrointestinal tract. Based on these studies, Taché et al.first reported the effect of central neuropeptides in the regulation of gastric function.

Since then, more than 40 peptides have been examined, and it is well established that many neuropeptides, such as thyrotropin-releasing hormone (TRH), corticotropin-releasing factor (CRF), neuropeptide Y (NPY), bombesin, and somatostatin, mediate the central nervous system-induced stimulation or inhibition of gastrointestinal function.(Brownstein MJ, 1974) The liver is also richly innervated, and a retrograde tracing technique has revealed hepatic innervation through the vagus originating in the medulla, where abundant neuropeptides exist.

Table 1. The classification of neuropeptides

Hypothalamic releasing peptides Thyrotropin releasing hormone Gonadotropin releasing hormone Somatostatin releasing hormone Corticotrpin releasing hormone Growth releasing hormone	**Gastrointestinal peptides** Vasoactive intestinal peptide Cholecytokinin Gastrin Neurokine A Insulin Glucagon Motilin
Neurohypophysial peptides Vasopressin Oxytocine	**Growth factors** Nerve growth factor Epidermal growth factor Fibroblast growth factor Endothelial growth factor
Adenopypophysial peptides Adenocorticotropic hormone α –Melanocyte stimulating hormone Prolactine Luteinizing hormone Tyrotropin	**Others** Angiotensin II Bombesin Calcitonin Camosine Neuropeptide Y Neurotensin Thymosin

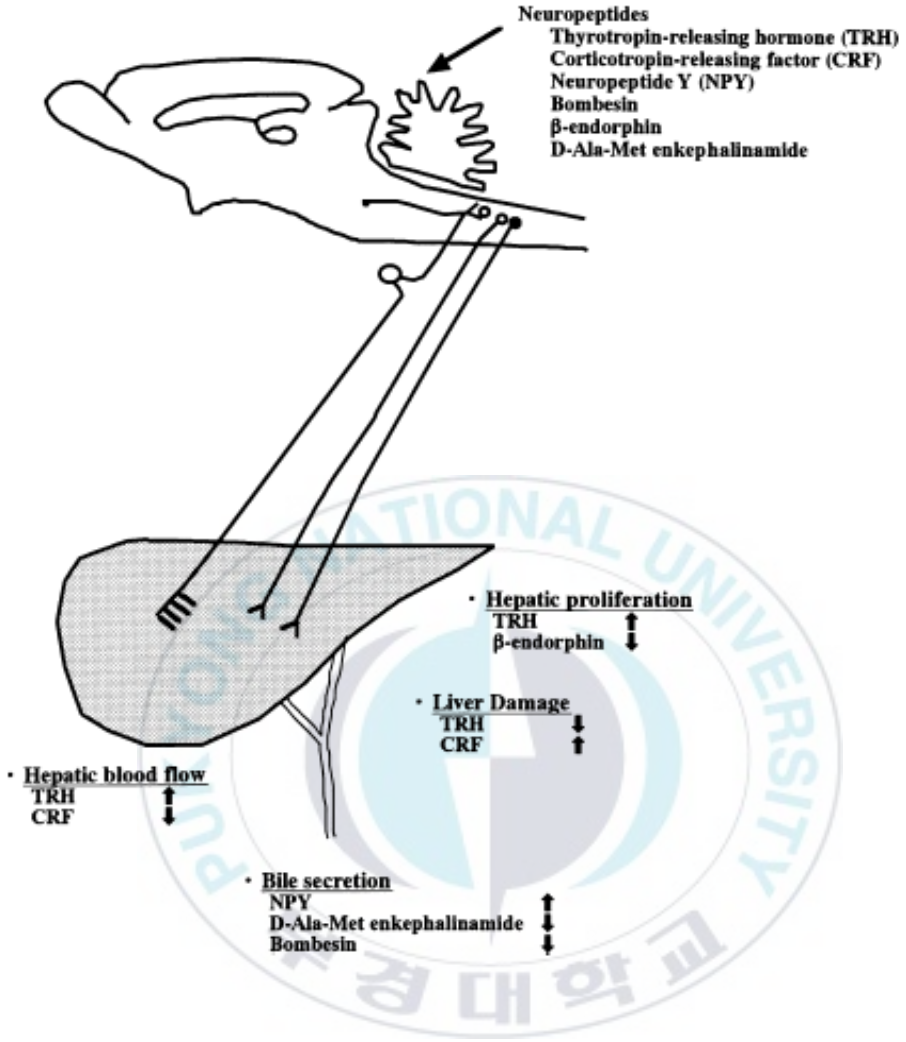


Fig. 1. Schematic illustration of interaction between neuropeptides in the central nervous system and liver

Neuropeptides as well as other mediators released by cells of the nervous system have been suspected of playing a role in the modulation of immune and inflammatory reactions. Accordingly, it has been demonstrated that neuropeptides are crucially involved in both conditioning and fine-tuning the host response to antigenic challenge.

The principal effects appear to be inflammatory and immunoregulatory, mainly in concert with other mediators, rather than potent direct effects on immune function (Chronwall, 1984). Among others, calcitonin gene related peptide (CGRP), vaso-intestinal peptide (VIP), substance P, prolactin (PRL) and proopiomelanocortin (P0MC)-derived hormones, such as melanocyte stimulating hormone ((rMSH) have been found to function as potent mediators of immunity and inflammation. In addition to neuronal tissue, a number of cell types have been shown to be able to produce these neuropeptides, including immunocompetent cells and epithelial cells such as keratinocytes. In particular, most leukocytic cell types are able to produce many of these factors, providing a possible link between the nervous and the immune system and a potential rationale for neurogenic modulation of immune functions. The significance of this finding is further supported by the observation that receptors for neuropeptides are expressed on immunocompetent cells.

Biosynthesis

Neuropeptides are generated from larger precursor proteins, prepropeptides, encoded in genes. Cell-specific gene transcription leads to the expression of neuropeptides in a limited and specific set of neurons in the nervous system.(Weber et al., 1981) After translation the peptide precursor enters the secretory pathway for further processing and maturation, as well as occasional

sorting. Often the peptide precursors contain multiple neuropeptides each surrounded by dibasic cleavage sites (or sometimes other cleavage sites) that direct enzymatic cleavage and liberation of the peptides (Gillis RA et al., 1989, ; Taghert, 1999; Veenstra, 2000). After cleavage the peptides can undergo posttranslational modifications such as carboxy terminus amidation, sulfation of tyrosyl groups, pyroglutamate formation in the amino terminus, disulphide bridge formation (between cysteines) and so on (Zupanc, 1996; Strand, 1999). While maturing, the peptides are transported to the release sites enclosed in vesicles. Commonly neuropeptides in neurons are found within so called large dense cored vesicles.

Cloudy dogfish is a kind of sharks which is Chondrichthyes. They live deep of the sea move on through bottom of the sea. *Scyliorhinus torazame* have special ability. Their brain has special receptors which perceive electricity. So many researchers study the correlation between their brain and their movement, controls of diversity physical reactions. This research is concentration on excitatory effects of visceral tissue.

Table 2. Amino acid sequence of neuropeptide from several molluscs

Animals (sources)	Peptides name	Amino acid sequence	Reference
Mytilus (pedal ganglia)	FMRFa	Phe-Met-Arg-Phe-amide	Kobayashi and Muneoka. 1990
Mytilus (muscle)	Opioid	Try-Gly-Gly-Phe-Met-Arg-Phe-amide	Muneoka and Matsuura, 1985
Mytilus (ABRM)	SCPs	Ala-Pro-Asn-Phe-Leu-Ala-Tyr-Pro-Arg-Leu-amide	Fujisawa et al., 1993
Snail (ganglia)	WW-1	Trp-Lys-Glu-Met-Ser-Val-Trp-amide	Minakata et al., 1993
	WW-2	Trp-Arg-Glu-Met-Ser-Val-Trp-amide	Minakata et al., 1993
	WW-3	Trp-lys-Glu-Met-Ser-Val-Trp-amide	Minakata et al., 1993
Leech (whole body)	LEP	Ala-Lys-Cys-Glu-Gly-Glu-Trp-Ala-Ile-His-Ser-Cyf-Leu-Gly-Gly Asn-amide	Minakata et al., 1997
Earthworm (gut)	EEP-1	Ala-Pro-Lys-Cys-Ser-Gly-Arg-Trp-Ala-Ile- His-Ser-Cyc-Gly- Gly-Gly-Asn-Gly	Oumi et al., 1995

Experimental procedures

1. Materials

Adult striped cloudy dogfish (*Scyliorhinus torazame*) were purchased from a local live fish market (Namchen , South Korea) . Disposable sep-pak vac12cc (2g) C18 cartridge were bought from waters Association (Milford , MA , USA).
All other chemical reagents were of the highest analytical grade.

2. Methods

2-1 Collection of specimens

Anesthetized fish using 0.1% of MS-222(2-aminobenzoic acid ethylester methanesulfonate salt) were decapitated. Whole brain were removed from heads and washed with distilled water. The samples were kept in a freezing compartment.

2-2 Extraction of neuropeptides

Frozen brains were boiled in 1L 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 5% , it was homogenized by using the polytron homogenizer (Polytron PT3000, Brinkman Instruments Co., Lucerne, Switzerland). Then homogenate was centrifuged (Hanil Science Industrial Co., Ltd, Incheon, South Korea) at 10000 x g for 20min at room temperature and supernatant was collected . the pellet was re-homogenized 500ml of 5% acetic acid and centrifuged at previous condition. Both supernatant were combined and concentrated by using the vacuum evaporator (EYELA rotary evaporator-N1000SW, Rikakikai CO., Ltd., Tokyo, Japan). Then added at concentrated solution ethanol, which is 4 times of volume more than sample. And then the samples were kept for 12 hours at -4 ℃. After that the sample was centrifuged at

10000 x g for 30min at 4ºC and the supernatant was concentrated. Concentrated solution was acidified by adding 1M HCl up to 1/10 of its volume. Acidified solution was centrifuged at 12000 x g for 30min at 4ºC to precipitated proteinous matter in peptide solution. The supernatant was concentrated by using the vacuum evaporator and lyophiled in the freeze dryer (SFDAM12, Samwon Freezing Engineering Co., Busan, 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. It was activated with 100% methanol in 0.1% trifluoroacetic acid (TFA) and washed distilled water in 0.1% TFA. Unbound compound were collected by the cartridges with 0.1% TFA. Retained materials in the cartridges were subsequently eluted with different concentration of methanol (10%, 60%, and 100%) in 0.1% TFA. Each eluted fraction was vacuum concentrated and lyophilized. Then lyophilized sample were tested for their excitatory effect on hindgut segment removed from Scyliorhinus torazame . The fraction 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 10% methanol contained substances that induced the highest hindgut contraction. Therefore, it was subject to HPLC using a C18 reversed-phase column (CAPCELL Pak C18 20mm x 250mm, Shiseido Co., Ltd, Tokyo, Japan) . Fractionation was done using a liner gradient of 0-10% acetonitrile (ACN) over 40 min at flow rate of 1.5ml/min. Fraction were collect for every 2 min . A portion of each fraction (1/300) was tested for tension change on the hindgut of *Scyliorhinus torazame*. The fractions contained tension changing substances were further purified by HPLC using another reverse-phase C18

analytical column (CAPCELL Pak C18,4mm x 250 mm, Watchers Co., Ltd,) and column was eluted with a linear gradient of 0-10 % ACN over 10min at a flow rate of 0.7ml/min. Each peak was collected and tested for excitatory effect on isolated hindgut of *Scyliorhinus torazame* as described in the physiological assay.

2-5 Physiological assay

Fraction 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 *Scyliorhinus torazame* by using refined myography system (TIS8105R, Kent Scientific Cooperation, Torrinton, USA). A portion hindgut (10mm) was removed from the intestine 10mm 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 *Scyliorhinus torazam* . Both end of tissues were tightened with cotton threads. Then the tissues was dipped in the aerated tissue bath containing physiological saline (4ml) after connecting other end of threads to the pre-calibrated transducer head (2 g) and 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 water-jacked tissue bath was set at 25ºC. The tissue was kept in the tissue bath of bioassay setup for more than 1 hour for the equilibration and the saline was changed rapidly in 10min intervals. Purified fraction from sep-pak C18 cartridge and HPLC were lyophilized, dissolved in physiological saline (100µl) and directly applied to the tissue bath to test the tension changes of the hindgut segment of *Scyliorhinus torazame.* Mechanical responses of the tissue due to sample application were recorded. In between successive sample application, the

tissue was washed thoroughly with physiological saline for 20min to avoid previous sample effect.

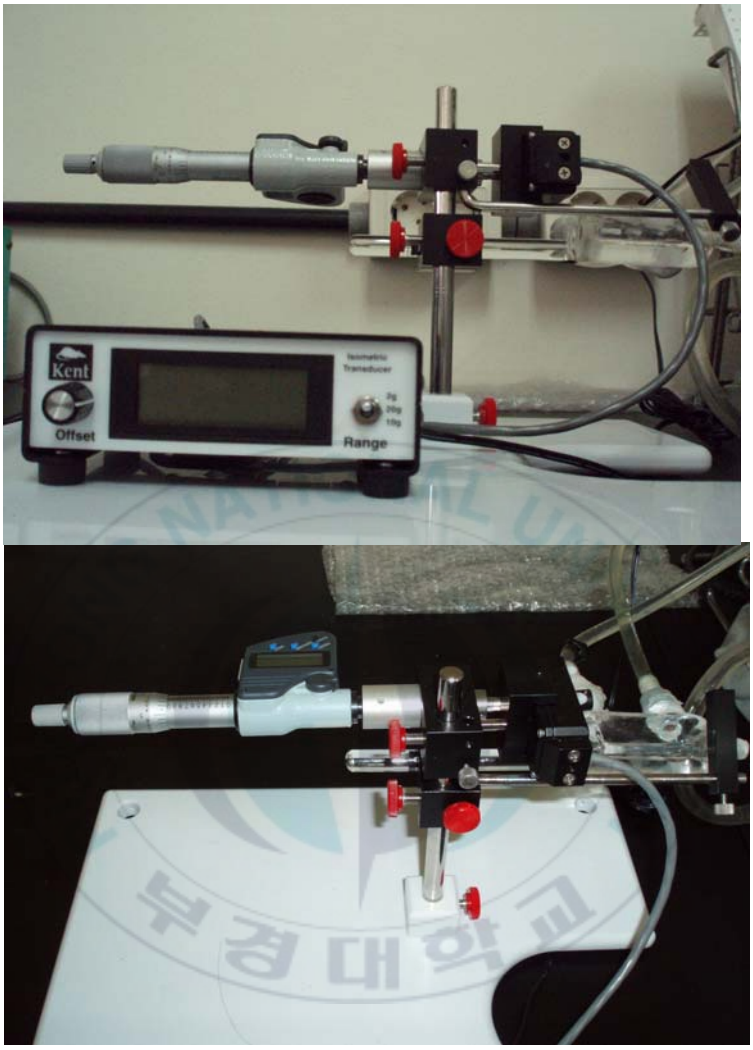


Fig. 2. Instrument of myography

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-hydroynnamic acid was prepared at a concentration of 20mg/ml in 50% CAN with 10% TFA(v/v) and 10mg/ml of peptide solution was prepared with 50% CAN 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 3000kWcm^{-2}.

Determination of amino acid sequence

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 approach reveals more about neuropeptide precursors than the neuropeptides express ultimately. Although it ca predict what peptides processed from a precursor considering their putatin be 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 development or tissues specific regulation of neuropeptide precursor processing. A single gene can be encode several neuropeptides and processing of individuals 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 modification, which are often essential for the activity of neuropeptides.The purification 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 dog fish

Blood plasma composition of dogfish was analyzed in order to prepare a suitable physiological saline used in the bioassay for the dogfish tissues. Fish were anesthetized using 5ml of 0.1% (V/V) MS-222 (99.5% Pure Tricaine Methamesulfonate) . Blood was withdrawn from the caudal vein of anesthetized fish by using a heparinised syringe. The syringe was inverted 5 to 6 times in order to proper mixing of collected blood with heparine. The blood sample were transferred into heparinised caps screw tubes and centrifuged at 2000 x g for 10 min. then the plasma-containd supernatant was collected. Blood plasma was stored at -80ºC for further analysis. Electrolytes (Na^+, Ca^{2+} ,Mg^{2+}, K^+ , PO_4^{3-}, NO_3^- , F^- , Cl^- and SO_4^{2-}) were determined using ICP-MS (Inductively coupled Plasma Mass Spectrometery – Perkin Elmer optima 3300 XL , Perkin Elmer INC., New York , USA) and ionic chromatography (Model 650 , Alltech Associates Inc,. USA). Soluble sugar in blood plasma was quantified according to the colorimetric method, Phenol-sulfuric acid analysis (Dubois et al,. 1955)

2-8 Pharmacological effects on Df peptides

Dose dependent response on dogfish hindgut tissue

To measure the threshold concentration of purified Df (after 2nd step of HPLC purification) on hindgut of *Scyliorhinus torazame,* different concentration of Df from 0.01㎍/㎖ - 1㎍/㎖ were prepared mixing with physiological saline according to dilution series. Hundred micro liter 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 dogfish hindgut tissue

To confirm the tachyphylaxis of the muscle on Df, i.e the desensitization of a tissue to the increase of active substance administration, the influence of interval time between applications was compared. Df at a concentration of 1㎍/㎖ was applied onto different tissue preparation of dogfish hindgut at different time intervals (10, 20min). Then the excitatory effects were detected using the bioassay system set at previously mentioned conditions.

Results and Discussion

1. Preparation of physiological saline for *Scyliorhinus torazame* tissues

Since neuropeptides from brain extract of *Scyliorhinus torazame* were identified with respect the physiological assay using in vitro tissue preparation, a suitable medium for the maintenance of in vitro tissue preparation of *Scyliorhinus torazame* needed.

The maintenance of tissue viability in terms of biochemical and physiological function is critical demonstrate any experimentally induced effect using in vitro tissue preparation. The grade of viability of isolated tissues preparations mainly depends on the artificial serum used (Burton, 1975 and Rees, 1989). Therefore, the prerequisite for in vitro maintenance of isolated gut prepare from ***Scyliorhinus torazame*** was to establish a physiological medium that would be more reflective and supportive of the situ biochemical and physiological processes of those tissue. The method used to prepare commercially available Ringer's solution, which are used commonly in vitro physiological studies is preparation of amedium by substituting the blood plasma composition the relevant animal. (Ringer, 1882, 1883, 1885 ; Clement and Rees, 1988).

Therefore, ionic composition and pH of collected blood plasma form anesthetized *Scyliorhinus torazame* fish were analyzed.

The observed ionic and biochemical profile of plasma sample of *Scyliorhinus torazame* gas been shown in Table 1. Terefore, a physiological saline solution with a composition similar to the plasma composition of dogfish 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).

Table 3. Composition of blood plasma from *Scyliorhinus torazame.*

Results are in miilmolar concentration per liter. The pH of blood plasma was 7.3

Components	mM
Na^+	170
Ca^{2+}	4.03
Mg^{2+}	1.28
K^+	19.58
PO_4^{3-}	21.45
NO_3^-	2.43
F^-	6.84
SO_4^{2-}	1.45
Cl^-	220
D-glucose	5.3

Table 4. Composition of physiological saline used in the bioaasy for in vitro tissue preparation of *Scyliorhinus torazame*.
Results are in milimolar concentration per liter. The pH was 7.3.

Components	mM
NaCl	170
KCl	19
$CaCl_2$	4
$MgSO_4$	1.28
$NaHCO_3$	6.5
NaH_2PO_4	0.89
D-glucose	5.3

Table 5. Analysis of total blood

Components	Amount
BUN	**13.0 mg/dL**
Glucose	**83 mg/dL**
Calcium	**9.3 mg/dL**
Phosphorus	**3.4 mg/dL**
Alk. Phos	**85 U/L**
Creatine Kinase	**139 U/L**
Total protein	**5.8 g/dL**

2. Purification of visceral excitatory peptide using Sep-Pak C18 cartridge

Because the neureopeptide synthesized in the nervous system, we decide to collect whole brain of dogfish as the sample materials. Brain selected from live fish after decapitation, frozen immediately in dry ice and boiled for 15min to prevent enzymatic degradation of neuropeptides by peptides and proteases. Brain peptides were extracted condition of 5% acetic acid.

Total four fractions were obtained from acetic acid brain extract *Scyliorhinus torazame* by using Sep-Pak C18 cartridges. They were named as flow-through fraction, 10% methanol fraction, 60% methanol fraction, 100% methanol fraction. Science 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 peptide than that of in other three fractions. Because samples were lacked during flowing time and large amount of samples were flowed the first and the second step , 60% methanol eluted , 100% methanol eluted fraction were not tested for tension changes on hindgut segment of *Scyliorhinus torazame* . Therefore flow-through and 10% methanol eluted fraction were examined for the excitatory effect on the hindgut using the myography system. The result indicated that 10% methanol fraction contained substances that caused tension changes on the hindgut segment. We could see a little tension change on the hindgut after adding 60% methanol fraction.

According to the results of tension change, 10% methanol eluted fraction, which caused the highest tension change on hindgut , was selected for further purification using HPLC.

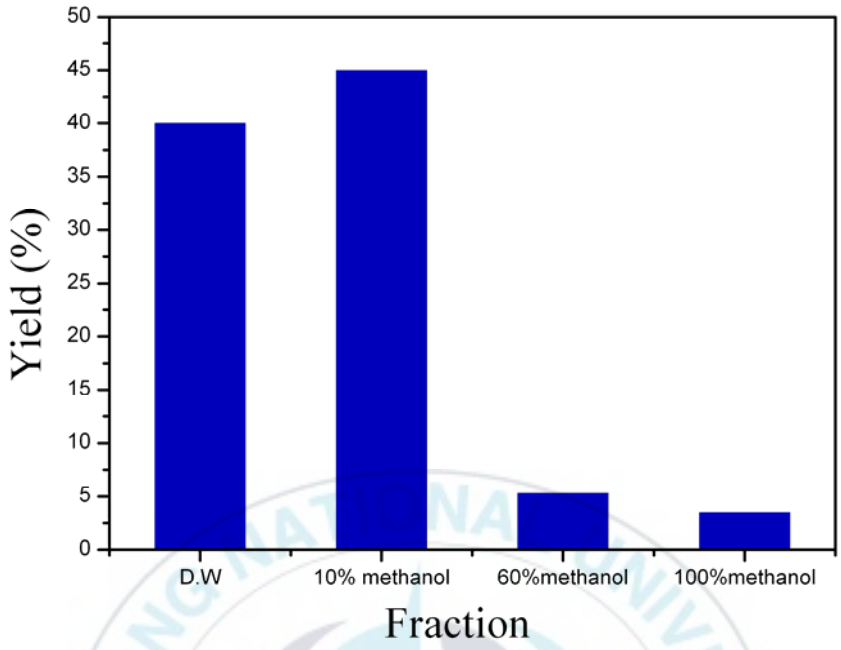


Fig. 3 Yield of Sep-Pak C18 cartridges. Yield of D.W fraction and methanol fractions of three different concentrations were shown.

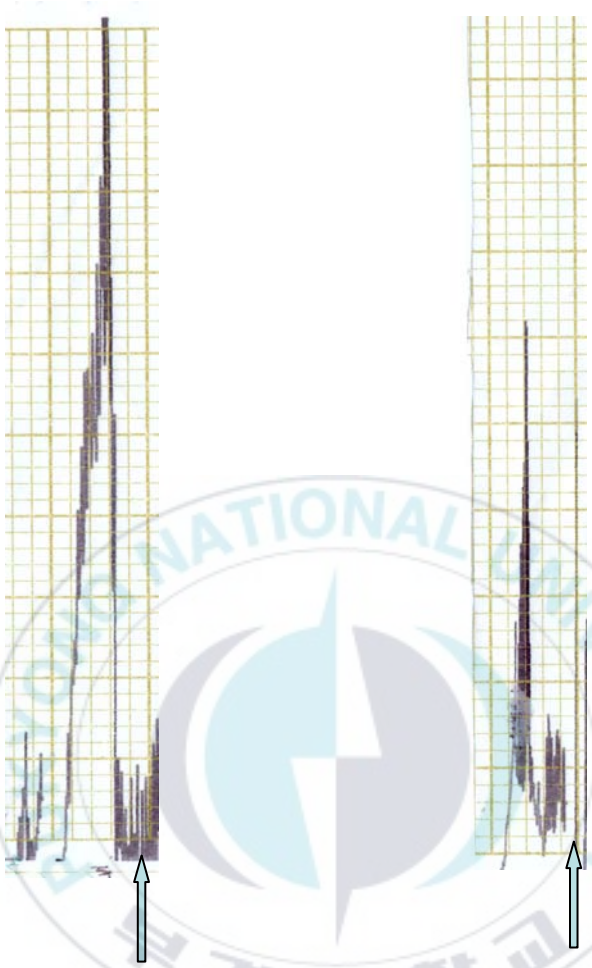


Fig. 4. Tension change of dogfish after application of purified fractions from sep-pak C18 cartridges (A) 10% methanol fraction (B) Flow-through fraction

3. Purification of visceral excitatory peptides using HPLC

Peptides can be separated using reversed phase liquid chromatography, which based on their hydrophobicity. Therefore, 10% methanol fraction was further purified by HPLC with different reversed phase C18 columns successively. (Fig.5) As a practical matter C18 stationary phase in peptide purification on acidic condition their resolution is more higher. Hence we used 0.1% TFA to adjusted acidic pH to ACN which was used as the eluent through out the HPLC.

In the first step of HPLC purification, lyophilized 10% methanol fraction was applied onto the CEPCELL PAK 18 semi preparative column and fraction were collected at every 2 min. After applying lyophilized fraction of the first step of HPLC onto hindgut tissue preparation of dog fish, only the fraction eluted with 6-7% ACN at 12-14min contained compounds that affected the tension change of the visceral tissues (Fig. 6.)

After freeze-drying, the bioactive matter was further purified using a reversed phase C18 analytical column. Four peaks, designated as Df-1, Df-2 and Df-3 were obtained. Only two peaks showed excitatory effects on hindgut tissue preparation of dogfish. They were purified isocratically with 2.5% and 3.4% ACN concentration respectively.

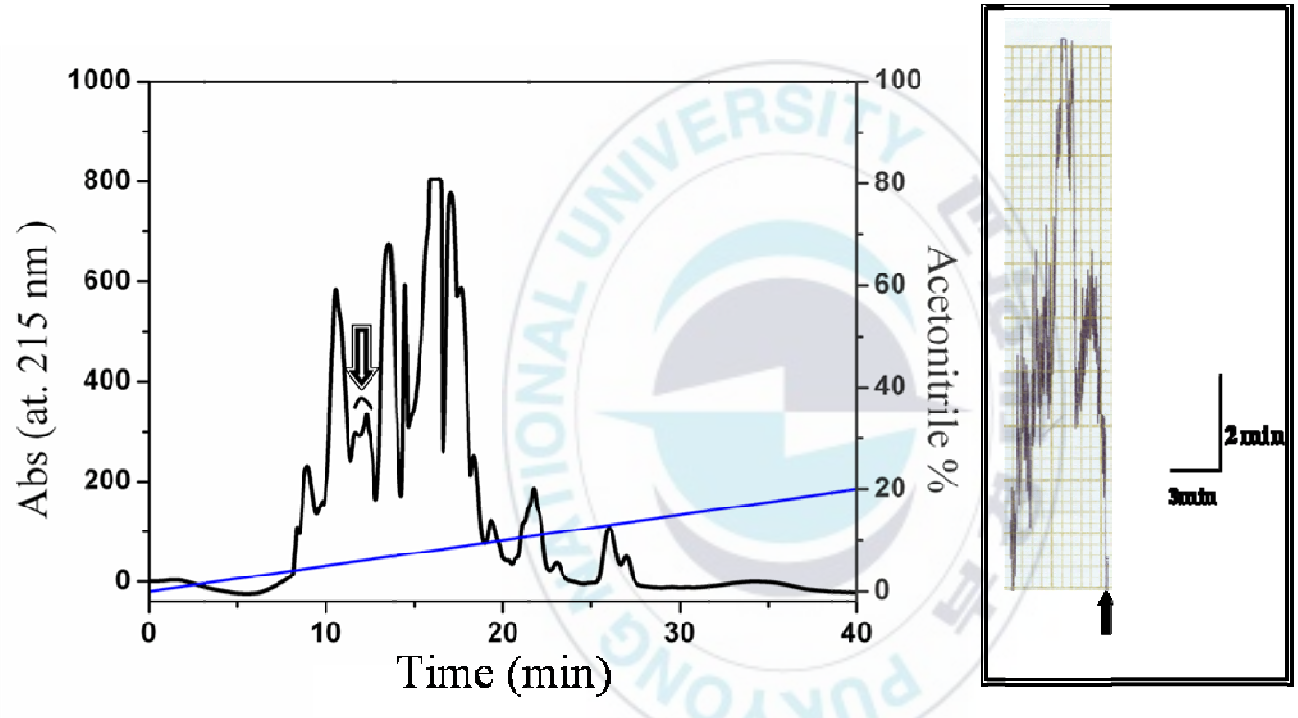


Fig.5 (A) Chromatogram of the first step of HPLC purification of visceral excitatory peptides. Fractions were collected for every 2min. Elution Conditions: a linear gradient of ACN (0-20%) for 40min at flow rate of 1.5 ml/min. Excitatory effective fraction in indicated by an arrow .(B) Visceral excitatory effect of the fraction indicated by an arrow.

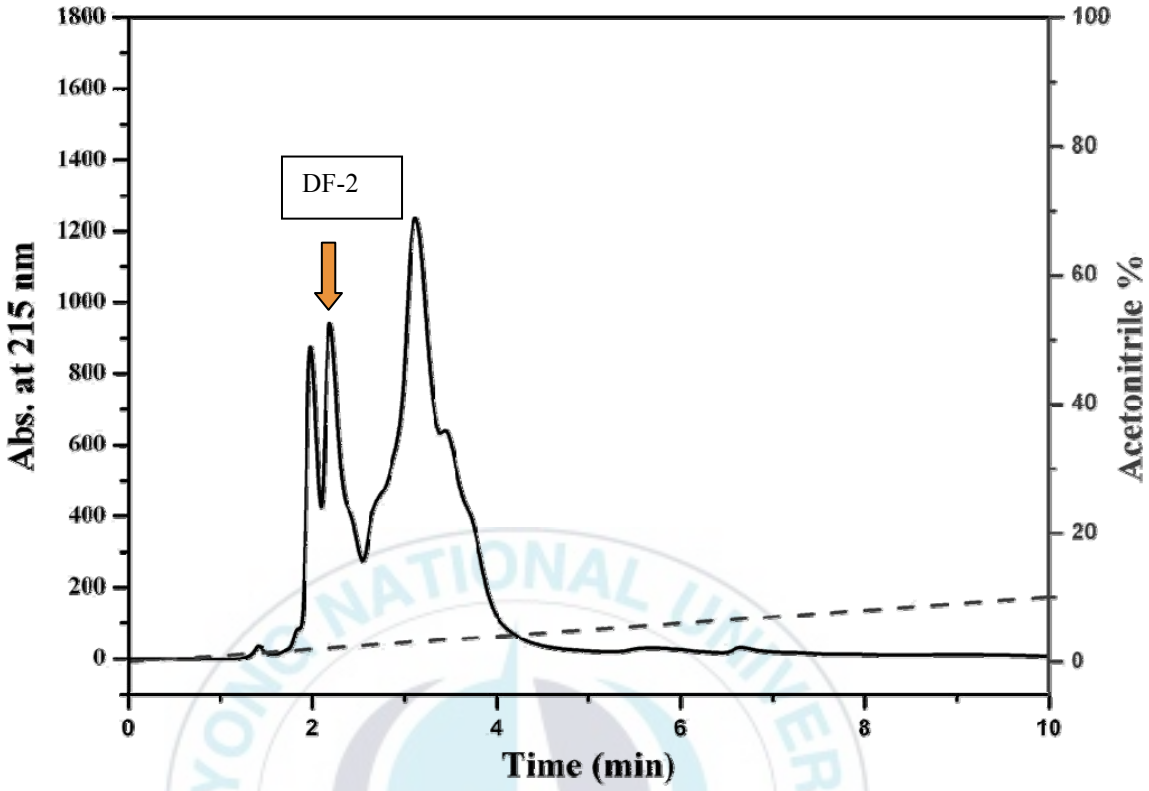


Fig. 6. Chromatogram of HPLC purification of the visceral excitatory neuropeptide. Elution conditions: a linear gradient of ACN (0-10%) over 10min at a flow rate of 0.7ml/min. Arrow indicates the visceral excitatory neuropeptides

3. Structure determination

Earlier, the primary structure of 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 neuropeptide also make it challenging to isolate enough of particular neuropeptide to determine its structure using Edman degradation. Therefore, in this study novel approach ESI-Q-TOF was used to determine the structure of visceral excitatory peptides. (Fig.7) After isocratic elution of 3 fractions in HLC, they were lyophilized and sent for peptide sequencing using ESI-Q-TOF.

Due to poor ionization problem occurred in ESI-Q-TOF, two fractions could not be sequenced. Sufficient amount of materials of two fractions were not available for further testing like its pharmacological function.

After amino acid sequence analysis, Df-2 contained one visceral excitatory neuropeptide, which were denoted as Df-2. Amino acid sequence of Df-2 was LESLVYEQLWPWamide and it observed molecular weight was 1563 Da(Fig. 7.)

DF-2_: LESLVYEQLWPW

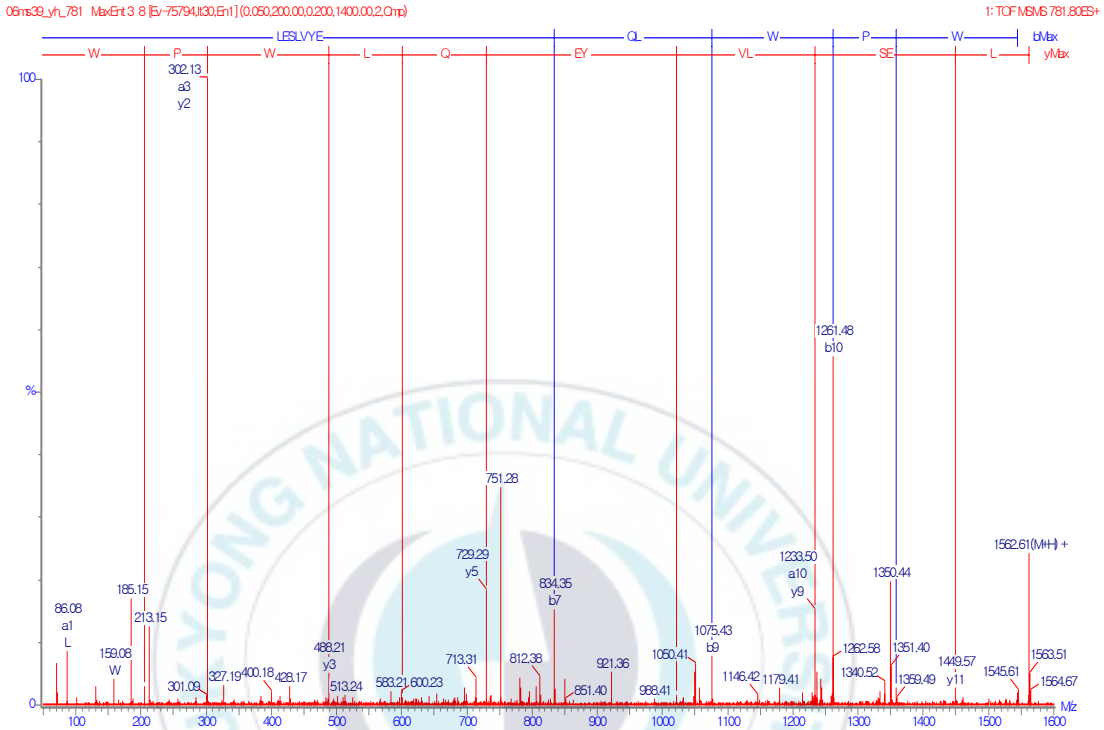


Fig. 7. Amino Acid sequence of a visceral excitatory neuropeptide Df-2

3. Pharmacological effect of Df-2

Dose dependent response on dogfish hindgut tissue

Acetylcholine is the neurotransmitter in all autonomic ganglia. It is an example of a totally untraceable poison. It was checked for excitatory effect on hindgut using different concentrations of Df-2 from 10^{-5}M - 10^{-7}M. (Fig. 8.)

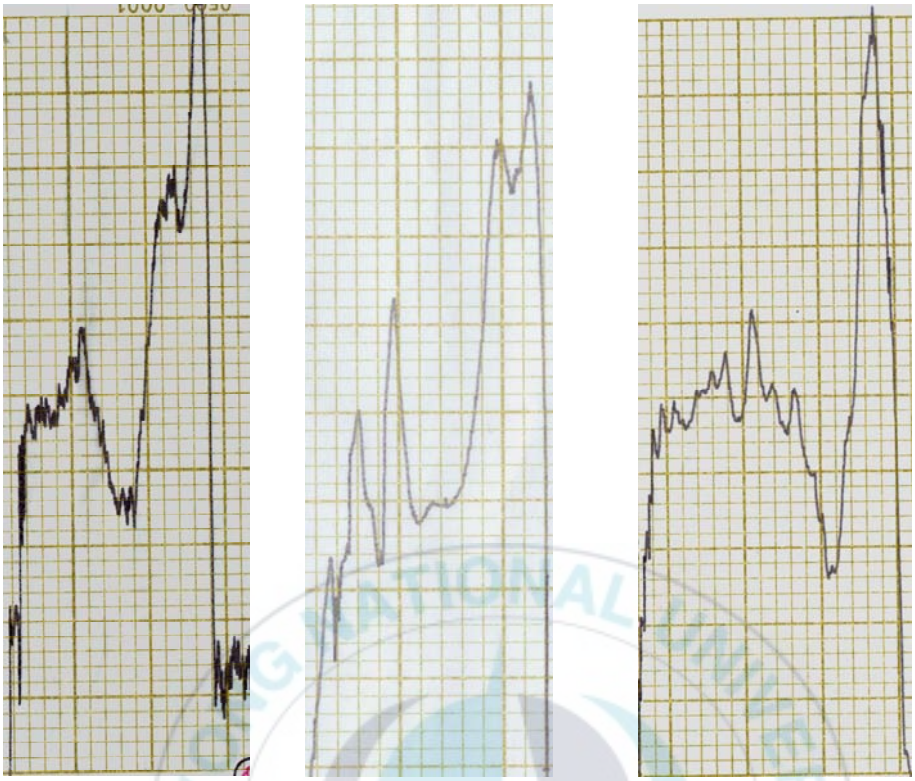
The dose dependent response of Df-2 was tested to find out its threshold concentration for excitatory effect on hindgut using different concentrations of Df-2 from 0.01㎍/㎖- 1㎍/㎖

When the Df-2 concentration was 0.01㎕/㎖, there was no any visible tension change on the hindgut. At 0.1㎕/㎖ concentration of Df-2, a slight increase of muscle tone on the hindgut could seen. Therefore, the threshold concentration of Df-2 for excitatory effects on the hindgut was found to be between 0.01㎍/㎖- 0.1 ㎍/㎖. (Fig. 9.) A significant excitatory effect can be seen at 1㎍/㎖, exhibited the excitatory actions on hindgut segment in dose-depended manner. Since bioactive peptides or neurepeptides are highly effective and react with their receptors on target tissues with high affinity, they are needed in minute concentration to initial an effect. It is conceivable that the Df-2 peptide involve in the regulation of the hindgut movement of dogfish acting as neurotrasmitters or as neuropeptides.

We used the acetylcholine as the positive controls. It was the first neurotransmitter to be identified. It is a chemical transmitter in both the peripheral nervous system (PNS) and central nervous system (CNS) in many organisms including humans.

Time- dependent respond on dogfish hindgut tissue

To determine tachyphylaxis of the muscle on Df-2, the effects of interval times between successive applications were compared. The excitatory effects made by the application of 0.5μg/ml of Df-2 in 10min, 20min intervals were compared. Df -2 effect could be seen at second and third application of Df-2 in 10min intervals. (Fig. 10.) However, application of same dose of Df-2 in 20min interval exhibited actions at each time (Fig. 10.). From these result, it revealed that whit in 20min, the intestine completely recovered from its desensitization state brought by the application of 0.5μg/ml Df-2.



(A) **(B)** **(C)**

Fig. 8. The excitatory effect of Ach applied at different concentrations of positive controls. (A) 10^{-4}M (B)10^{-5}M (C) 10^{-6}M

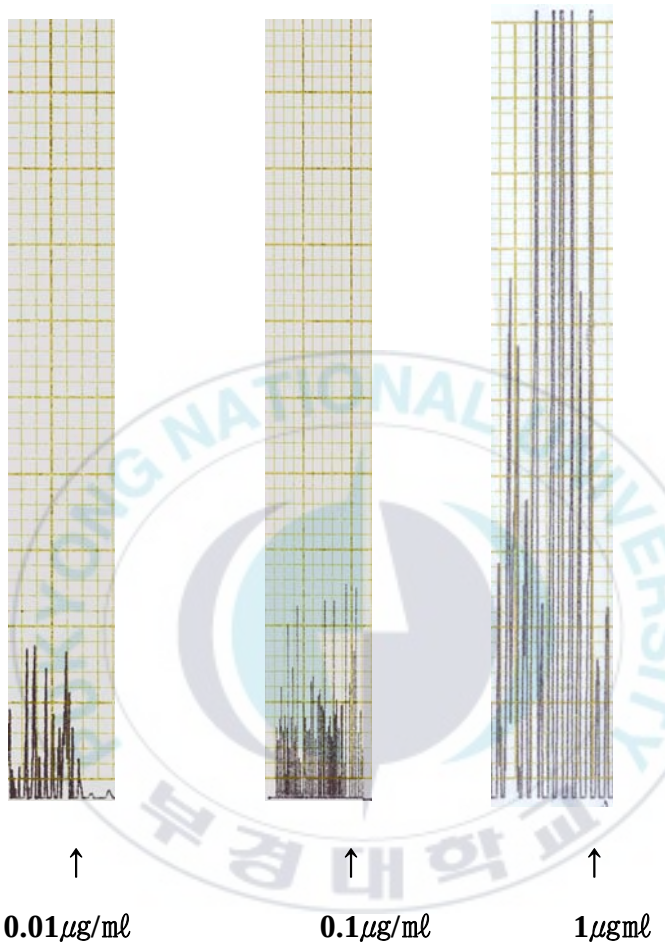


Fig. 9. Excitatory effects of different Df-2 concentrations on the isolated hindgut preparation from dogfish

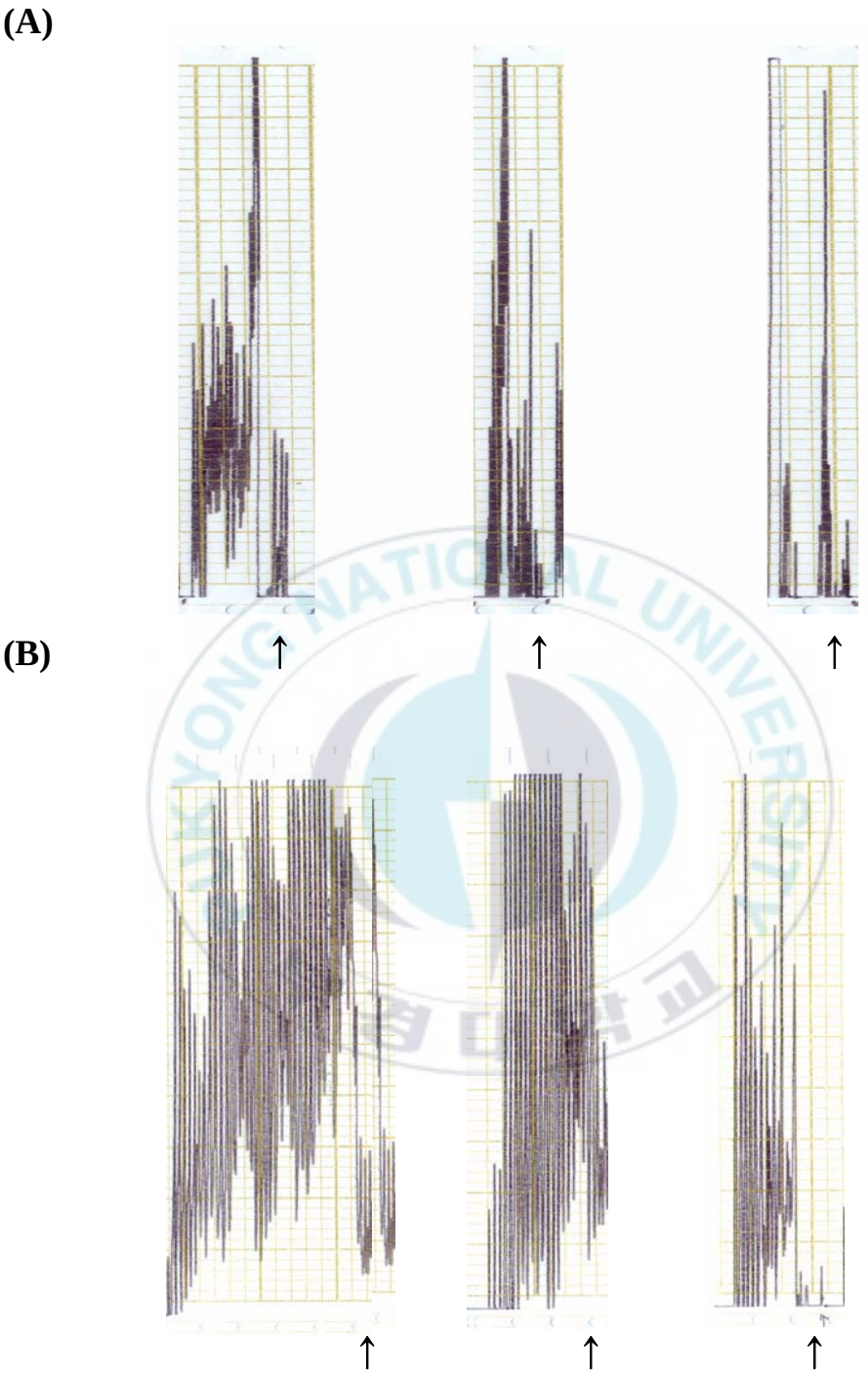


Fig. 10. The excitatory effect of Df-2 applied at a concentration of 1 μg/ml on the hindgut segment of dogfish. (A) At 10min intervals,(B) at 20min intervals. Observation were made from 3 different tissue preparations.

Summary

In the present study, we identified visceral excitatory peptides from brain extracts of a cloudy dogfish (*Scyliorhinus torazame).* Since neuropeptide from brain extract of *Scyliorhinus torazame* were identified according to the physiological assay using in vitro tissue preparations, a suitable medium for the maintenance of in vitro tissue preparation of *Scyliorhinus torazame* was prepared with respect to its blood plasma condition. The composition of the prepared physiological saline was NaCl 170mM, $CaCl_2$ 4mM, $MgSo_4$ 1.28mM, $NaHCO_3$ 6.5mM, NaH_2PO_4 0.89mM, D-glucose 5.3mM, KCl 19mM and pH 7.3. 10% methanol fraction obtained from Sep-Pak C18 cartridge showed a significant tension change on isolated hindgut of dogfish in bioassay. Therefore, visceral excitatory peaks were identified from the 10% methanol fraction using different reversed phase chromatographic techniques together with hindgut physiological assay. Due to lack of sample materials and poor ionization, other two fractions were unable to be sequenced or its pharmacological properties to be studied.

Df-2 contained one peptide, which raised the tone of hindgut in a dose-dependent manner with a threshold concentration of 0.01-1 μg/ml and within 20min the hindgut recovered completely from desensitization state brought by 1 μg/ml of its concentration. Amino acid sequence of Df-2 peptide was LESLVYEQLWPWamide.

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