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A novel visceral excitatory neuropeptide from the brain tissue of cloudy dogfish (*Scyliorhinus torazame*)

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ABSTRACT

A visceral excitatory neuropeptide with a molecular weight of 1563 Da was isolated from the brain extracts of cloudy dogfish (*Scyliorhinus torazame*) discarded as a fishery by-product. Fish hindgut was employed to carry out physiological assay for its visceral excitation. During consecutive purification using C18 cartridge and high-performance liquid chromatography (HPLC), a neuropeptide (DF-2) was isolated from neuronal cell extracts of dogfish brain tissue and clearly exhibited potential to exert visceral excitatory effect on hindgut of dogfish. The primary structure of DF-2 established by ESI-Q-TOF tandem mass was LESLVYEQLWPWamide. The results of database search provided evidence for this peptide sequence to be novel. In visceral excitatory assay using myography, the threshold concentration of DF-2 required for the changes in spontaneous contraction of cloudy dogfish hindgut was found to be 10^{-6} M. Therefore, this study suggests that DF-2 isolated from brain of elasmobranches could be one of the sequences, which regulates spontaneous visceral contractions in dogfish digestive duct.

1. Introduction

Annually, over 100 million tonne of fish are harvested worldwide, and approximately 30% of the total catch is used for fishmeal and animal feed because of its poor functional properties. Moreover, more than 50% of the total catch is discarded as processing waste or by-product. Despite international attempts to decrease waste through various kinds of waste treatment systems, the quantity of the waste produced has been increasing annually (Jung, Mendis, et al., 2006). These by-products are important protein and mineral sources, and they can be converted to value-added products by enzymatic hydrolysis, which is widely applied to improve and upgrade the functional and nutritional properties of proteins. Although the nutritional value of these wastes is high, no substantial attempt is being made to utilise these potentially valuable resources. Recently, several studies have been reported on utilisation of fish by-products by enzymatic hydrolysis for the recovery of all valuable components (Bougatef et al., 2008; Cudennec, Ravallec-Ple, Courois, & Fouchereau-Peron, 2008; Jung, Lee, & Kim, 2006; Rajapakse, Jung, Mendis, Moon, & Kim, 2005). Bioactive peptides can be released by enzymatic proteolysis of food proteins and may act as potential physiological modulators of metabolism during the intestinal digestion of the diet. Bioactive peptides usually contain 3–40 amino acid residues and their activity is based on their amino acid composition and sequence. The possible regulatory effects of fishery peptides relate to anticoagulation, antioxidant and antihypertensive activities, amongst others (Jung, Mendis, et al., 2006; Jung et al., 2007; Rajapakse et al., 2005).

Neuropeptides that are synthesised and released by the nervous system often act as transmitters, hormones and modulators to control various biological functions at cellular level in the animal body. They interact with cell surface receptors, G-protein coupled receptors to trigger intracellular transduction pathways. The structurally diverse nature has led neuropeptides to exert different effects on living cells. Neuropeptides exhibit very small differences within biologically active sequences. However, these neuropeptides exert different functions and effects in different animal species (Jensen & Holmgren, 1989). During the last few decades, several fish neuropeptides from both teleosteans and elasmobranchs have been characterised structurally and their physiological roles have been partially identified. Most of these neuropeptides identified from teleosts and elasmobranchs belong mainly to tachykinin, neuropeptide Y, melanin-concentrating hormone, glucagon-family neuropeptide and FMRFamide peptide groups. The other neuropeptides also have been identified from fish that do not belong to these families (Jensen & Conlon, 1992).

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The cloudy dogfish, *Scyliorhinus torazame*, belongs to the family Scyliorhinidae, and this dogfish has mainly been found from Japan and Korea to Taiwan at depths down to 100 m. The dogfish has been consumed as s seafood; however, other parts such as backbone, head, intestine are usually discarded as by-products. In this study, to utilise dogfish by-products as a resource of bioactive substance, we isolated neuropeptide from neuronal cell extracts of its brain tissue using C18 cartridge and high-performance liquid chromatography (HPLC) and characterised visceral excitatory effects of isolated neuropeptide on hindgut using myographic system. This report can contribute to provide more knowledge about a novel visceral excitatory neuropeptides from brain of elasmobranches.

2. Materials and methods

2.1. Animals

Cloudy dogfish (*S. torazame*) specimens were obtained from a commercial outlet of the Jagalchi live fish market (Busan, South Korea). Prior to dissection, all animal were anesthetized using 0.1% of MS-222 (2-aminobenzoic acid ethyl ester methanesulphonate salt). Following decapitation, whole brain was removed and immediately was placed in ice-cold acidified (with 1 mM HCl) acetone (final concentration, 70%).

2.2. Preparation of neuronal cell extracts

Frozen brains were boiled in 1 l of distilled water for 20 min. Boiled brain tissues were pulverised by using a Waring commercial blender (Model 7005, Waring products Inc., Torrington, CT, USA) following acidification with 5% acetic acid. It was then homogenised by using a Polytron homogenizer (Polytron PT3000, Brinkman Instruments Co., Lucerne, Switzerland). After centrifugation (Hanil Science Industrial Co., Ltd., Incheon, South Korea) at 10 000 *g* for 20 min at room temperature to remove tissue debris the pellet was re-extracted twice and supernatants were collected. The two supernatants were combined and most of the methanol was removed using a rotary evaporator at 35 °C under vacuum (EYELA rotary evaporator-N1000SW, Rikakikai Co., Ltd., Tokyo, Japan). Next, ethanol was added into the concentrated solution. After that the sample was recentrifuged at 10 000 *g* for 30 min at 4 °C and the supernatant was concentrated. Concentrated solution was reacidified by adding 1 M HCl up to 1/10 of its volume. This solution was then centrifuged at 12 000 *g* for 30 min at 4 °C and collected supernatant was concentrated by using the vacuum evaporator and lyophilised.

2.3. Purification of visceral excitatory neuropeptide

Lyophilized matter was then dissolved in distilled water and passed through disposable sep-pak vac 20 cc (5.0 g) C18 cartridges. First these cartridges were activated with 100% methanol in 0.1% trifluoroacetic acid (TFA) and washed with distilled water containing 0.1% TFA. Unbound compounds were collected by the cartridges with 0.1% TFA. Retained materials in the cartridges were subsequently eluted with different concentrations of methanol (10%, 60% and 100%) in 0.1% TFA. Each fraction was vacuum concentrated and lyophilized. Then lyophilized samples were tested for their excitatory effect on hindgut segment removed from *S. torazame.*

HPLC separations were carried out by two reverse-phase C18 columns, CAPCELL Pak C18 (20 × 250 mm, Shiseido Co., Ltd., Tokyo, Japan) with 0–20% acetonitrile (ACN) and SynChropak RP C18 (4.6 × 250 mm, SynChrom Inc., Lafayette, IN, USA) with 0–10% ACN. The sample that had the highest excitatory effect on hindgut tissue was first subjected to HPLC and fractionation was done using a liner gradient of 0–20% ACN over 40 min at flow rate of 1.5 ml/min. Fractions were collected at every 2 min interval and each fraction was tested for its tension change in hindgut of *S. torazame* employing the physiological assay. Following identification of active fractions, they were rechromatographed and resulting fractions were further tested using the same physiological assay system to identify potent fractions.

2.4. Structure confirmation

The amino acid sequence of the isolated peptide was determined using electrospray ionisation hybrid quadrupole time of flight tandem mass spectrometry (ESI-Q-TOF MSMS) on a Q-TOF instrument (Micromass Q-TOF, Altrincham, UK). For that, the sample was dissolved in 70% CH_3CN with 0.1% formic acid and 1 μl of sample solution was loaded into a Long NanoES spray capillary for Micromass Q-TOF. During tandem mass spectrometry, fragment ions were generated from a selected precursor ion by collision-induced dissociation.

2.5. Assay for visceral excitation using myography

Fractions obtained from HPLC purification were checked for capability to produce excitatory effects on the hindgut isolated from *S. torazame* by using refined myography system (TIS8105R, Kent Scientific Corporation, Torrington, CT, USA). Hindgut tissues (10 mm long) were dissected from intestines of sacrificed well-fed fish after opening the abdomen ventrally. Isolated hindguts were mounted in a vertical Perspex chamber (3.5 ml) by connecting the posterior end with cotton thread to a hook in the bottom of the chamber and the anterior end to a force transducer (2.0 g). The transducer was connected with the chart recorder (REC101, Kent Scientific Cooperation, Torrinton, CT, USA) and the chamber was perfused with saline at room temperature (20 °C). Then the tissue was dipped and kept in the aerated tissue bath of bioassay setup for more than 1 h to equilibrate. Alternatively, solutions were continuously applied with a peristaltic pump at a flow rate of 2 ml/min to avoid the artificial stretch responses that were sometimes caused by withdrawal of the medium.

Blood plasma composition of *S. torazame* was analysed in order to prepare a suitable physiological saline for bioassay. For this purpose blood was withdrawn from the caudal vein of fish anesthetized with 0.1% MS-222 by using heparinized syringes (1½ inch needle, 6 ml syringe) and centrifuged at 2000*g* for 10 min at 4 °C. Blood plasma pH was measured using a 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. The soluble sugar content in the blood plasma was quantified according to the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.6. Pharmacological Effects of DF-2 Peptide

To measure the threshold concentration of purified DF-2 on hindgut of *S. torazame,* different concentration of DF-2 ranging from 10^{-8} M to 10^{-6} M were prepared by mixing with physiological saline according to dilution series. One hundred microlitres from each concentrate were added to the tissue bath and tissue contractions were examined following same procedure mentioned in the physiological assay using myography method. To confirm the tachyphylaxis of the hindgut in the presence of DF-2, excitatory effects recorded following administration of active substance were compared. For this purpose DF-2 at a concentration of 10^{-6} M was applied onto different tissue preparation of dogfish hindgut at

Table 1
Composition of blood plasma from *Scyliorhinus torazame* and physiological saline used in the bioassay for in vitro tissue preparation.

Components	mM
Composition of blood plasma from Scyliorhinus torazame	
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
Physiological saline used in the bioassay for in vitro tissue preparation	
NaCl	170
KCl	19
$CaCl_2$	4
$MgSO_4$	1.28
$NaHCO_3$	6.5
NaH_2PO_4	0.89
D-glucose	5.3

different time intervals (10 and 20 min). Then the excitatory effects were discovered using the bioassay system set at previously mentioned conditions.

3. Results

3.1. Physiological saline for *S. torazame* tissues

The composition of plasma sample obtained from *S. torazame* is shown in Table 1A. Taking each elemental composition in plasma of dogfish into consideration, physiological saline solution was prepared by using laboratory-graded chemicals and is depicted in Table 1A.

3.2. Extraction and purification of visceral excitatory neuropeptides

All fractions (flow-through, methanol eluates) obtained following separation using Sep-pak Vac 20 cc C_{18} cartridges were tested for their excitatory effects on the hindgut by employing a physiological assay. Amongst all fractions, the fraction eluted with 10% methanol exhibited an effect on impulsive contractions of the hindgut of dogfish (Fig. 1). Based on tension changes, 10% methanol eluted fraction was selected for further purification using HPLC.

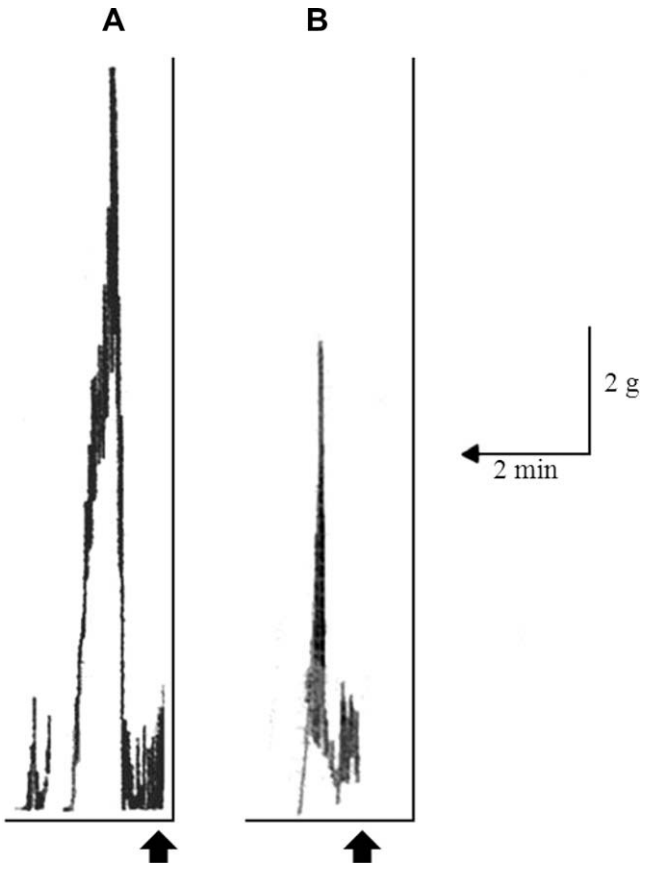

Fig. 1. Tension change of dogfish after application of purified fractions from sep-pak C18 cartridges (A) 10% methanol fraction (B) Flow-through fraction at 10^{-4} M. Arrow indicates the point of sample treatment.

In the first step of HPLC, the fraction eluted with 6–7% ACN at 12–14 min greatly affected the tension change of the visceral tissues (Fig. 2). The same fraction obtained following several runs was pooled according to excitatory effects and subjected to further purification. From the purification step conducted using an analytical column, three peaks were obtained (Fig. 3A). One peak (DF-2)

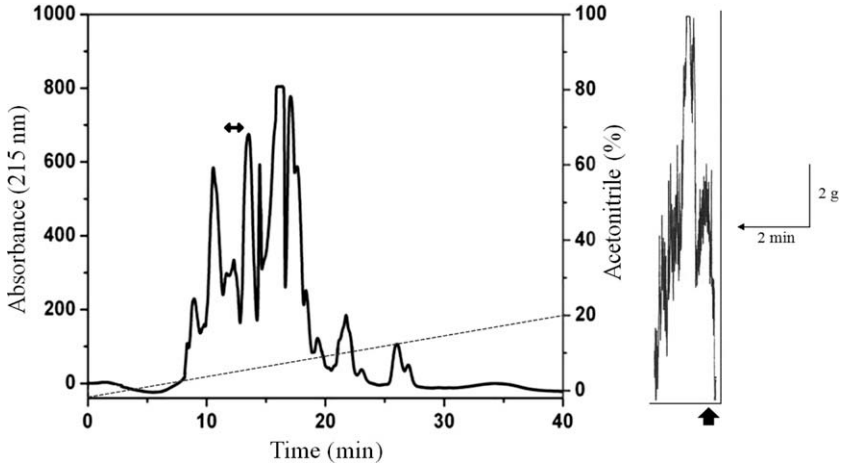

Fig. 2. Chromatogram obtained from the first HPLC purification of visceral excitatory peptides performed using C18CAPCELL Pak C18 semi-preparative HPLC column. Fraction were collected at every 2 min. Elution conditions: a liner gradient of ACN (0–20%) for 40 min at flow rate of 1.5 ml/min. Fraction showing effective excitatory effects are indicated using an arrowhead. Arrow indicates the point of sample treatment.

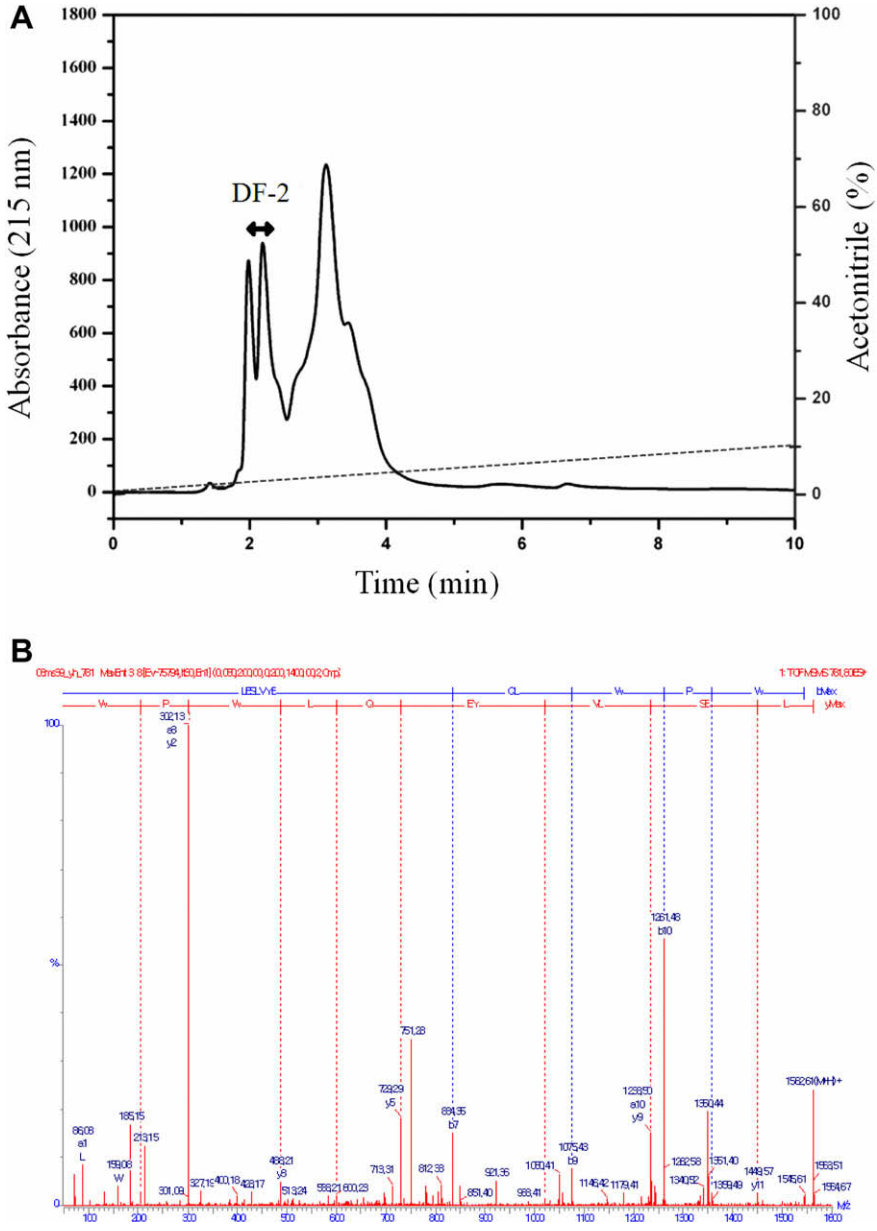

Fig. 3. (A) Chromatogram of HPLC purification of the visceral excitatory neuropeptides performed using SynChropak RP analytical HPLC column. Elution was performed using a liner gradient of ACN (0–10%) over 10 min at a flow rate of 0.7 ml/min. (B) Amino Acid sequence of visceral excitatory neuropeptide Df-2.

exhibited the strongest excitatory effect on hindgut tissue of dogfish. This peak was further purified with 2.5% ACN concentration.

3.3. Structure determination

Following isocratic elution of DF-2 in HPLC, it was lyophilized and sequenced using an electrospray-quadrupole-time of flight (ESI-Q-TOF) mass spectrometer. After amino acid sequence analysis, DF-2 fraction contained one visceral excitatory neuropeptide. Amino acid sequence of DF-2 was LESLVYEQLWPWamide and molecular weight of DF-2 was 1563 Da (Fig. 3B).

3.4. Threshold concentration of DF-2 for the excitatory effects on dogfish hindgut tissue

Dose dependent excitatory effects of DF-2 on hindgut of dogfish were checked to find out its threshold concentration using concentrations ranging from 10^{-8} M to 10^{-6} M. When the DF-2 concentration was 10^{-8} M, there was a little visible tension change on the hindgut. At 10^{-6} M concentration of DF-2, a small response of muscle tone on the hindgut could be observed. These data suggest that the threshold concentration of DF-2 for excitatory effects on the hindgut lies between 10^{-8} and 10^{-6} M (Fig. 4).

To decide the tachyphylaxis of the muscle in response to repeated application of DF-2 at 10^{-6} M concentration, contractions at 10 and 20 min intervals were recorded and compared. No effect could be seen at the second and third application of DF-2 in 10 min intervals (Fig. 5). However, when DF-2 was administered at 20 min intervals, at each application excitatory effects were clearly observed in the hindgut tissue.

4. Discussion

We reported here in the isolation of a novel neuropeptide sequence from *S. torazame* that exerts visceral excitatory effects on hindgut. Effects of this neuropeptide from brain extract of

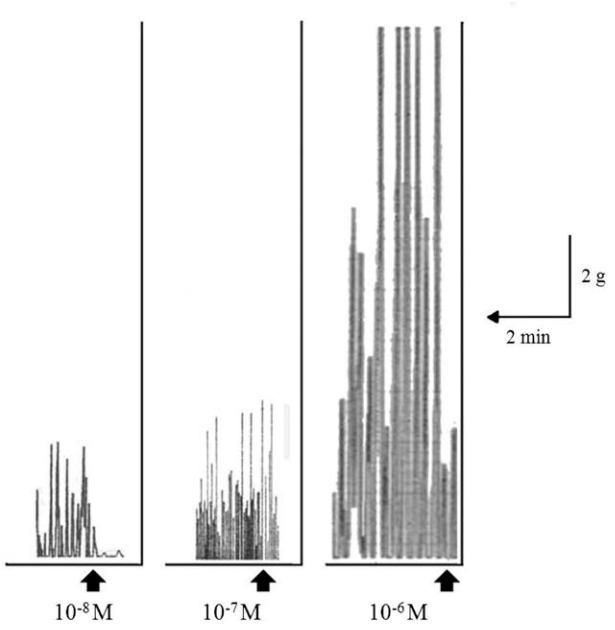

Fig. 4. Excitatory effects of different Df-2 concentrations on the isolated hindgut preparation on dogfish. Arrow indicates the point of sample treatment.

S. torazame was identified by employing the physiological assay using *in vitro* tissue preparation. The in vitro physiological assay has proven to be a particularly simple and efficient method for the detection of biological activities of neuropeptides mostly in extracts from the nervous system. Therefore, this assay has been employed in a similar research to test physiological effects of a number of neuropeptides, and a suitable medium for the maintenance of in vitro tissue preparations is a prerequisite because the grade of viability of isolated tissue preparations chiefly depends on the composition of serum used (Burton, 1988; Rees, 1989). Therefore, physiological solutions have been formulated based on biochemical analyses of plasma constituents of particular organism to maintain the functional viability of tissues over extended periods during assay. In order to prepare a physiological solution to maintain the gut isolated from *S. torazame*., blood plasma was collected from anesthetized *S. torazame* and after analysis of ionic composition pH physiological saline solution was prepared similar to the plasma composition of dogfish by using laboratory-graded chemicals as shown in Table 1. This physiological solution made the tissue more reflective and supportive of physiological processes.

Marine elasmobranchs have a blood plasma tonicity just slightly above that of seawater (approximately 1000 milliosmoticity; mOsm), and they regulate pressure principally by controlling plasma level of organic ions and compounds, especially urea and trimethylamine. In marine lampreys and teleosts the tonicity of blood plasma is approximately one third of seawater, being around 300 mOsm, and is due almost exclusively to dissolve inorganic ions. However, the diversity of vasotocin-like peptides in elasmobranch may reflect pressures associated with maintaining water and electronic balance through renal control of organic ions, such as magnesium and sulphate. So the concentration of magnesium and sulphate in elasmobranch blood plasma tend to be lower than marine lampreys and teleosts.

Knowledge of the structures of naturally occurring neuropeptides is required to determine their synthesis, mode of action and function. In the past, to determine primary structures of neuropeptides, Edman degradation method was frequently employed. More advanced spectrometric techniques such as neuroproteomic approach that combines online nanoscale liquid chromatography (nanoLC) and ESI-Q-TOF are currently employed to obtain better results (Skold et al., 2002). Therefore, to determine the structure of visceral excitatory peptide, ESI-Q-TOF was employed.

Neuropeptides were earlier grouped together in families according to sequence similarities. Neuropeptides discovered from teleosts have been classified into several neuropeptide families that include tachykinins, FMRFamides, neuropeptide Y, melanin-concentrating hormones and glucagons-family neuropeptides. Currently, the basis for grouping of the neuropeptides involves gene analyses and peptides are considered to belong to the same gene-family if they originate from the same ancestral gene. However, there is a possibility that multiple forms of peptides have different functional properties from the same gene transcript.

Regarding the novel neuropeptide sequence identified in this study, this peptide had virtually no effects on the tissue preparations from oviduct, heart, body wall muscle strip and oesophageal (data not shown) during the bioassay system. Moreover, based on the molecular size and its excitatory effect on smooth muscle (hind gut), we can predict that this peptide may represent the tachykinins family of neuropeptides. Several other related research have identified that the molecular weights of peptides from tachykinins family fall between 1000 and 2500 Da (Jensen & Conlon, 1992). The tachykinins are phylogenetically very ancient neuropeptides and have been isolated from a diverse array of species from invertebrates and vertebrates. These function as neurotransmitters in the central nervous system, primary afferent neurons, and enteric nervous system, and they exert various actions, such as smooth muscle contraction, vasodilatation, secretion, and neuronal excitation

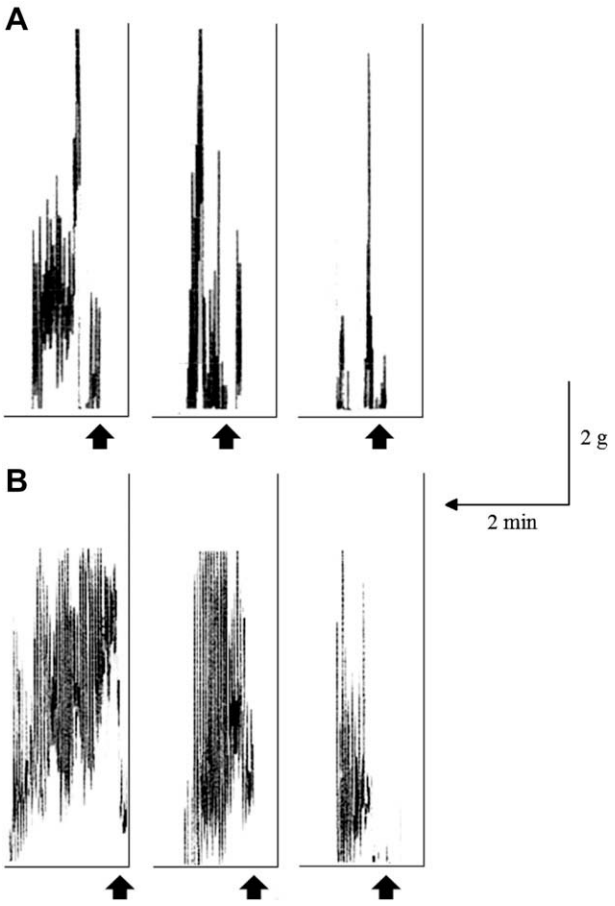

Fig. 5. The excitatory effect of Df-2 applied at a concentration of 10^{-6} M on the hindgut segment of dogfish. (A) At 10 min intervals (B) At 20 min intervals. Observations were made from three different tissue preparations. Arrow indicates the point of sample treatment.

(Otsuka & Yoshioka, 1993). In particular, this group of peptides has displayed excitatory and inhibitory actions on cardiovascular and gastrointestinal functions in vertebrates (Jensen & Holmgren, 1989). Within the gastrointestinal tract, tachykinins are important messenger molecules of enteric neurons that are intrinsic to the gut and that control various aspects of digestive activity. Many tachykinins family peptides were isolated from brain of different marine sources. Further, neuropeptides coming under tachykinins also have been isolated from gut of dogfish (*Scyliorhinus canicula*) (Conlon, Deacon, O'Toole, & Thim, 1986). However, neuropeptides coming under tachykinins exhibit the common amino acid sequence of Phe-Xaa-Gly-Leu-MetNH_2 in their sequences. Therefore, when considering its structural properties it is unlikely that this peptide fully represents the tachykinin group that has functional significance in physiological control of intestine. In this context the structure of DF-2 (LESLVYEQLWPWamid) cannot be exactly classified under any reported neuropeptide family belonging to marine chondrichthyes. Also there is a possibility that this peptide belongs to a family of neuropeptides that has not been identified to date. However, further experimentation is needed to resolve these issues.

Dose dependent excitatory effects in hindgut DF-2 was tested to find out its threshold concentration using concentrations ranging from 10^{-8} M to 10^{-6} M. As shown in Fig. 4, DF-2 demonstrated its capability to act on neuropeptide or neurotransmitter on gastrointestinal tract at 10^{-6} M. In other researches reported, threshold levels of many neuropeptides isolated from different animal species are in the range of 10^{-9}–10^{-5} M (Fujimoto et al., 1998; Takahashi et al., 1995).

Further, repeated application of DF-2 made the hindgut tissue desensitized to the substance or the stimulus. However, after 20 min, the intestine completely recovered from its desensitization state brought by the application of 10^{-6} M DF-2 and usual impulsive contraction movement in the hindgut was observed (Fig. 5).

In summary, we could purify and isolate a peptide from the brain of dogfish that has visceral contraction efficacy. Amino acid sequences of DF-2 revealed that it was a novel neuropeptide that has no structural similarity in the reported neuropeptide families. Therefore, this study suggests that DF-2 is a novel peptide sequence that plays an important role in the regulation of gastrointestinal activity in dogfish.

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