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Two tachykinin-like peptides from skin secretions of *Danio rerio*

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Tachykinin perform multiple physiological functions such as smoothing muscle contraction, vasodilation, inflammation, the processing of nerve signal, neuroprotection and neurodegeneration. Two novel tachykinin-like peptides named tachykinin-DR1 and -DR2 were identified from skin secretions of *Danio rerio* in current work. Their amino acid sequences were determined as SKSQHFHGLM-NH_2 and NKGEIFVGLM-NH_2, respectively. They share a conserved FXGLM-NH_2 *C*-terminal consensus motif. By cDNA cloning, the precursor encoding both tachykinin-DR1 and -DR2 was screened from the skin cDNA library of *D. rerio*. Tachykinin-DR1 and -DR2 share the same precursor, which is composed of 108 amino acid (aa) residues. Regarding the biological activity, tachykinin-DRs could induce the contraction of isolated strips of guinea pig ileum just like other tackykinins. To our best knowledge, this is the first report of tachykinin from fish skin. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: tachykinin; fish; *Danio rerio*; precursor

Introduction

The tachykinin peptide family is one of the most prevalent peptide families described in animals. Over the past 40 years, more than 40 tachykinins have been identified from invertebrates and vertebrates [1]. They perform multiple physiological functions such as smoothing muscle contraction, vasodilation, inflammation, the processing of nerve signal, neuroprotection, and neurodegeneration and so on [1–3]. These physiological effects of the tachykinins may substantially vary depending on the activation of different receptor subtypes. Up to now, three main receptor subtypes for the tachykinins have been identified: NK1, NK2, and NK3 [1]. The numbers of tachykinin receptors are still increasing.

So far, more than 20 tachykinin-like peptides have been isolated from fishes [1,4–15]. Tachykinin precursors of mammalians, amphibian, octopus, and ascidian have been identified [3,16–18], while the precursor of fish tachykinin was only reported from the research about goldfish gamma-preprotachykinin mRNA encoding the neuropeptides substance P, carassin and neurokinin A [9]. And most of fish tachykinins were reportedly found in gut, stomach and brain. Different from fish, many amphibian tachykinins are found to be distributed in skins [1]. In the current work, we first reported the tachykinin-like peptides from zebrafish *Danio rerio*, a model organism being extensively studied. The purification, characterization and cDNA cloning of two tachykinin-like peptides from skin secretions of the zebrafish were described.

Materials and Methods

Collection of Skin Secretions

The skin secretions of zeabrafish (*D. rerio*) were collected by gently scratching the surface of the fish skin. The skin secretions were mixed with the same volume of 0.1 M phosphate buffer (PBS), pH 6.0, containing protease inhibitor cocktail (Sigma). The mixture was quickly centrifuged and the supernatants were lyophilized.

Peptide Purification

Lyophilized skin secretions of *D. rerio* (3 g, total absorbance of 770 at 280 nm) were dissolved in 10 ml 0.1 M PBS. The sample was applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) gel filtration column equilibrated with 0.1 M PBS. Elution was performed with the same buffer, with collecting fractions of 3.0 ml. The absorbance of the elution was monitored at 280 nm. The fractions were screened with smooth muscle contraction activity. The protein peak containing smooth muscle contraction activity was pooled (40 ml), lyophilized and re-suspended in 2 ml 0.1 M PBS, then further purified by C_{18} reverse phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C_{18}, 30 × 0.46 cm) column using acetontrile as elution solution as illustrated in Figure 1B.

Structural Analysis

Complete peptide sequencing was undertaken by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer,

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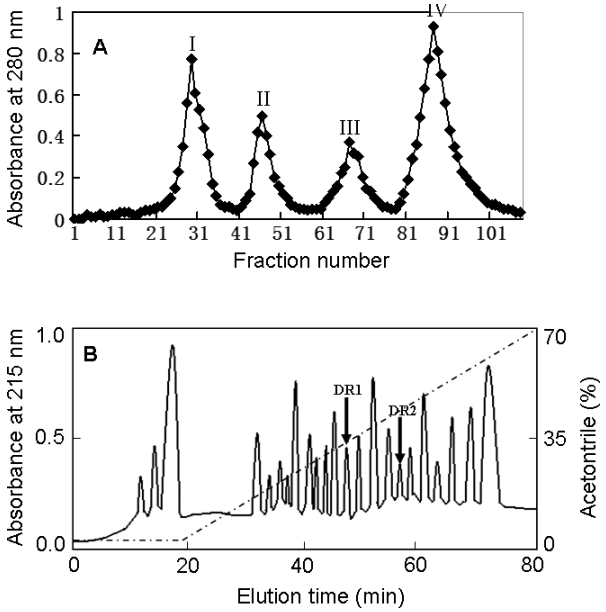

Figure 1. Fractionation of *D. rerio* skin homogenates. Figure 1A shows Sephadex G-50 gel filtration of *D. rerio* skin secretions. *D. rerio* skin secretions were applied on a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 × 100 cm) column equilibrated with 0.1 M PBS, pH 6.0. Elution was performed with the same buffer, collecting fractions of 3.0 ml (Figure 1A). Peak III with tachykinin-like activity from Sephadex G-50 was further purified on a Hypersil BDS C_{18} RP-HPLC column (30 × 0.46 cm) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The elution was performed with the indicated gradient of acetonitrile at a flow rate of 0.7 ml/min, and peaks were tested for tachykinin-like activity. The purified tachykinin-like peptides is indicated by arrows (DR1 and DR2) (Figure 1B).

model 491. Mass fingerprints (MFPs) were obtained using a Matrix-assisted Laser Desorption Ionization Time-Of-Flight mass spectrometer (MALDI-TOF-MS) AXIMA CFR (Kratos Analytical) in positive ion and liner mode. The specific parameters were as follows: the ion acceleration voltage was 20 kV, the accumulating time of single scanning was 50 s, polypeptide mass standard (Kratos Analytical) serving as external standard. The accuracy of mass determinations was within 0.1%. α-Cyano-4-hydroxycinnamic acid (CHCA) was used as matrix.

cDNA Synthesis

Total RNA was extracted using TRIzol (Life Technologies, Ltd.) from the skin of single sample of *D. rerio*. cDNA was synthesized by SMART™ techniques by using a SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA). The first strand was synthesized by using cDNA 3′ SMART CDS Primer II A, 5′-AAGCAGTGGTATCAACGCAGAGTACT (30) N-1N-3′ (N = A, C, G or T; N − 1 = A, G or C), and SMART II oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′. The second strand was amplified using Advantage polymerase by 5′ PCR primer II A, 5′-AAGCAGTGGTATCAACGCAGAGT-3′. Finally, the PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI).

Screening of cDNA Encoding Tachykinin

The skin cDNA library of *D. rerio* was used as template for PCR to screen the cDNAs encoding tachykinin peptide. Two oligonucleotide primers, DR1 (5′-(a/t/c/g)cccat(a/t/c/g)cc(a/c)acaaa(a/t/c/g)at(a/t/c/g)tc-3′), in the antisense direction, a primer designed according to the tachykinin peptide sequence reported in this paper and primer II A as mentioned in *'cDNA synthesis'* in the sense direction were used in PCR reactions. The DNA polymerase was Advantage polymerase from Clontech (Palo Alto, CA) The PCR conditions were: 2 min at 94 °C followed by 30 cycles of 10 s at 92 °C, 30 s at 50 °C, 40 s at 72 °C. DNA sequencing was performed on an Applied Biosystems DNA sequencer, model ABI PRISM 377.

Bioassay

Tachykinin activity was tested by assaying the contractile activity on an isolated guinea pig ileum as described earlier [19]. About 10 cm of the distal ileum of guinea pig of either sex (150–250 g body weight) was removed immediately after death and washed with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 5.04 mM D-glucose). 2 cm of cut segments of isolated ileum were mounted isotonically, under 1-g load, in 5 ml muscle bath containing Tyrode solution maintained at 37 °C and bubbled with air. PcLab software package was used for the collection and analysis of biological signal (Beijing Microsignalstar Technology Development Co. Ltd.). All the peaks from Sephadex G-50 gel filtration and RP-HPLC were dissolved in Tyrode solution and added into the muscle bath with different concentration.

Peptides Synthesis

Tachykinin-DR1 and -DR2 were synthesized by AC SCIENTIFIC (Xi An) INC. (Xi An, China) and analyzed by HPLC and MALD–TOF mass spectrometry to confirm purity higher than 95%.

Results

Two Tachykinin-like Peptides were found in the Skin Secretions of *D. rerio*

The supernatant of *D. rerio* skin secretions was fractionated into four peaks by Sephadex G-50 as illustrated in Figure 1A. The contractile activity on the isolated guinea pig ileum was detected in the third peak. Hence, the peak III was collected and purified further by an RP-HPLC. More than 20 peaks were obtained (Figure 1B). Two eluted peaks showed smooth muscle contractile activity (marked by DR 1 and DR 2, respectively). These two peaks were collected for further study.

Structural Characterization

The purified peptides were subject to the amino acid sequence analysis by automated Edman degradation. Treating with carboxypeptidase Y did not lead to the release of free amino acids under conditions that free amino acids were released from a peptide with free *C*-terminal -COOH group. The result indicated that the *C*-terminal ends of these two peptides were amidated, which were further confirmed by mass spectrometry analysis. Their amino acid sequences were determined as SKSQHFHGLM-NH_2 (tachykinin-DR1) and NKGEIFVGLM-NH_2 (tachykinin-DR2), respectively. They are both composed of 10 amino acid residues. Similar to other tachykinin-like peptides, they contain a conserved FXGLM-NH_2 *C*-terminal consensus motif. There are two serine residues in the *N*-terminus of tachykinin-DR1. MALDI–TOF mass spectrometry analysis gave tachykinin-DR1 and -DR2 an observed mass of 1170.5 and 1107.4, respectively, which match well with the calculated mass of 1170.3 and 1107.3.

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atggacatatttaaactttctgctttagcgttcatcctttacttgcagttgcacaatgct   60
 M  D  I  F  K  L  S  A  L  A  F  I  L  Y  L  Q  L  H  N  A    20
ggagccagtcccagtgaagaggggatatctggactgtggagaacttggaggagaagcca  120
 G  A  S  P  S  E  E  G  D  I  W  T  V  E  N  L  E  E  K  P    40
caggtgacagatgtattccttcgcattgctgatctgatgaaacgatccaaatctcagcat  180
 Q  V  T  D  V  F  L  R  I  A  D  L  M  K  R  S  K  S  Q  H    60
tttcatggattaatgggcagcagcgcaggcaatactcaacctttgcgacttggcaggaga  240
 F  H  G  L  M  G  S  S  A  G  N  T  Q  P  L  R  L  G  R  R    80
agaaacaagggtgaaatctttgttggacttatgggaagaagatcttcgagtgatttgcga  300
 R  N  K  G  E  I  F  V  G  L  M  G  R  R  S  S  S  D  L  R   100
gagcaactggagagacgtcaactataacttcaagcaagtgctcatgcaagccttctgttt  360
 E  Q  L  E  R  R  Q  L  *                                    108
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Figure 2. The nucleotide sequences encoding tachykinin-DRs and the deduced amino acid sequence of the precursor polypeptide. The sequence of mature tachykinin is indicated in box. * indicates stop codon.

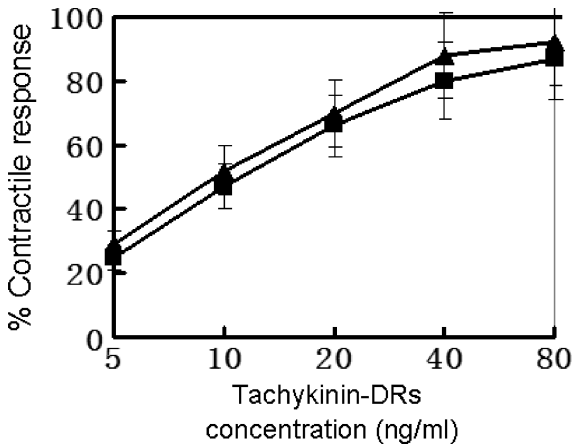

Figure 3. Concentration–response curve of tachykinin-DRs on isolated rat ileum. The ileum was stimulated with increasing concentrations of tachykinin previously incubated for 10 min, at 30 °C, with Tyrode solution. Each point represents the mean ± SEM of five different experiments.

cDNA Cloning

Upon screening of a fish skin cDNA library, several clones containing inserts of around 310 base pairs were identified and isolated. Both strands of these clones were sequenced (Figure 2). One cDNA sequence encoding both tachykinin DR1 and -DR2 was obtained, and the deduced amino acid sequence is shown in Figure 2. The precursors of the tachykinin-like peptides were composed of 108 amino acid residues including an *N*-terminal signal peptide followed by an acidic spacer peptide and two mature tachykinin-like peptides. There are two dibasic sites of -RR- for possible enzymatic cleavage which flanked the mature tachykinin DR2, while there is only one dibasic site of -KR- at the *N*-terminus of mature tachykinin DR1. At the *C*-terminus of these mature tachykinin-like peptides, a glycine provides the amide for the *C*-terminal methioninamide (Figure 2). The deduced amino acid sequence from cloning is identical to the sequence determined by Edman degradation.

Myotropic Effects on Isolated Guinea Pig Ileum

The contractile activities of tachykinin DR1 and -DR2 on an isolated ileum smooth muscle of the guinea pig were evaluated in aerated Tyrode's solution at 37 °C. To a 5-ml organ bath, 5, 10, 20, 40, and 80 ng of these tachykinins were added for testing as indicated in Figure 3. These peptides were found to elicit contractile effects on the isolated guinea pig ileum in a concentration-dependent manner.

Table 1. Tachykinin-like peptides from fishes

Species	Organ	Sequences
Oncorhynchus mykiss		
	Brain	KPRPHQFFGLM-NH2
	Gut	HRINSFVGLM-NH2
Gadus morhua		
	Brain	KPRPQQFIGLM-NH2
Carassius auratus		
	Brain	HRINSFVGLM-NH2
		KPRPHQFIGLM-NH2
Amia calva		
	Stomach	SKSHQFYGLM-NH2
Scaphirhyncus platorhyncus		
	Gut	SKTHQFYGLM-NH2
Scyliorhinus canicula		
	Gut	AKFDKFYGLM-NH2
		SPSNSKCPDGPDCFVGLM-NH2
	Brain	KPRPGQFFGLM-NH2
Torpedo marmorata		
	Gut	SNSKCPDGPDCFVGLM-NH2
Sphyrna lewinini		
	Gut	AKFDKFYGLM-NH2
Raja rhina		
	Brain	AKHDKFYGLM-NH2
		HKLGSFVGLM-NH2
Lampetra fluviatilis		
	Brain	RKPHPKEFVGLM-NH2
		HFDEFVGLM-NH2
Petromyzon marinus		
	Brain	RKPHPKEHVGLM-NH2
Neoceratodus forsteri		
	Brain	KPRPDEFYGLM-NH2
Pallid sturgeon, Scaphirhynchus albus and *Polyodon spathula*		
	Brain	KPKPHQFFGLM-NH2
Danio rerio		
	Skin	SKSQHFHGLM-NH_2
		NKGEIFVGLM-NH_2

Discussion

The current work reported two novel tachykinin-like peptides (Tachykinin-DR1 and -DR2) from skin secretions of the model organism, zebrafish. Tachykinin-DR1 (SKSQHFHGLM-NH_2) shows high similarity to the tachykinin-like peptide (SKSHQFYGLM-NH_2) from the stomach of *Amia calva*. Tachykinin-DR2 (NKGEIFVGLM-NH_2) has a unique *N*-terminal end (NKGEIF). This is the first report ever about the tachykinin-like peptides found in fish skin. A large

amount of fish tachykinins were found in gut, stomach and brain but skin (Table 1).

Some neuroendocrine peptides distributed in fish gastrointestinal tract and central nervous system are also found in the skin, and these peptides are classified into skin-gut-brain triangle peptides, such as bombesins and caerulein [20]. The present data demonstrated that fish tachykinin could also be expressed in gastrointestinal tract, central nervous system and skin as other fish skin-gut-brain triangle peptides.

Similar to other tachykinin-like peptides, tachykinin-DRs possessed contractile activity on smooth muscle isolated from guinea pig ileum. However, the physiological roles of these tachykinin-like peptides from fish skins are still not clear. Tachykinins have algesic functions and are also involved in pain transmission at the spinal level [21]. Therefore, the fish skin tachykinin-like peptides reported in current work may perform defensive roles alone or synergetically with other biomolecules. These tachykinin-like peptides with algesic activity may make hosts escape injury and/or defend predators.

Acknowledgement

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