Articles

Purification and Characterization of [Ala²]-Neuromedin N from the Visceral Tissue of the African Lungfish, *Protopterus dolloi*

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A new biologically active peptide with structural similarity to neuromedin N (NMN) has been isolated from extracts of visceral tissue of the African lungfish, *Protopterus dolloi*, using the rectum of the quail as the bioassay system. The primary structure of NMN-related peptide was established as Lys-Ala-Pro-Tyr-Ile-Leu-OH ([Ala²]-NMN) and contained one substitution (Ala² → Ile) compared with the porcine NMN. [Ala²]-NMN was found to have an excitatory effect on rectal muscle tissues of quail (*Coturnix japonica*), newt (*Cynops pyrrhogaster*) and black bass (*Micropterus sulmoides*). The threshold concentration of [Ala²]-NMN for contraction of *C. japonica* muscle was found to be approximately 10^{-11} M. [Ala²]-NMN showed contractile activities in the following order: *C. japonica* > *C. pyrrhogaster* > *M. sulmoides*. The identification of [Ala²]-NMN provides evidence that NMN family, hitherto confined to mammals, has a widespread occurrence in lungfish.

Key Words : African lungfish, Purification, [Ala²]-Neuromedin N

Introduction

Neuromedin N (NMN) was recently isolated and purified from porcine spinal cord using a bioassay for a stimulant effect on guinea pig ileum.[1] The primary structure of NMN is Lys-Ile-Pro-Tyr-Ile-Leu-OH, which is close sequence homology with the C-terminal 8-13 sequence of neurotensin. Neurotensin[2] and NMN[1] are synthesized by a common precursor in the mammalian brain and intestine.[3,4]

Immunohistochemical investigations have shown that NMN-related peptides are also present in the brain, retina, and gut of several species.[5-12] NMN-related peptides which are structurally similar to NMN have been isolated from chicken[13] and frog.[14] NMN-related peptides share a common sequence Lys-Xaa-Pro-Tyr-Ile-Leu-OH, where Xaa can be a hydrophilic (Asn or Lys) or hydrophobic (Ile) amino acid. These peptides play a role in many different physiological actions, such as smooth muscle contraction, hypotensive response, hypothermic effect, histamine secretion, stimulation of phagocytic function and cyclic GMP stimulatory effects on the pancreas and splanchnic circulation.[2,15-19]

Abbreviations used: NMN, neuromedin N; ACN, acetonitrile; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high performance liquid chromatography; LANT-6, a chicken brain pentapeptide; MS-222, (3-aminobenzoic acid ethylester methanesulfonate salt; TFA, trifluoroacetic acid.
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Protopterus dolloi is an African lungfish and a member of class Sarcopterygii, which is more closely related to the early amphibians than the modern Neopterygians. Therefore, it seems interesting to identify new bioactive peptides in *P. dolloi* and to compare their structures and actions with those of ordinary fishes and amphibians, which are considered to have evolved from a lungfish-type ancestral animal.

In this study, we purified NMN-related peptide from the visceral tissue of the African lungfish, *P. dolloi*; we labeled these peptide as [Ala²]-NMN. Additionally, we report biological activities of this peptide.

Methods and Materials

Animals. Adult male and female red-bellied newts (*Cynops pyrrhogaster*) were purchased from Hiroshima Experimental Animals (Hiroshima, Japan). Animals were kept in a tank and individual cages, and were fed with commercially-available foods until bioassay. Adult male Japanese quails (*Coturnix japonica*), aged 3-4 months, were purchased from the Tokai Yuki Company (Toyohashi, Japan). Quails were housed in a temperature-controlled room (25 ± 2 °C) with a daily 16-h light and 8-h dark cycle. Specimens of the African lungfish, *P. dolloi*, of both sexes were used in the present study. The fish were deeply anaesthetized by MS-222, killed by decapitation.

Visceral tissue extraction. The visceral tissue was removed from 40 male and female lungfish specimens. The

visceral organs, including the spleen, liver, bile, and intestine, were frozen in liquid nitrogen, and stored in a deep freezer (−80 °C). Each frozen sample was boiled in 1.5 L of water for 10 min, and 45 mL of acetic acid was then added to the boiled material. The material was homogenized with a Polytron homogenizer (Kinematica, Luzerne, Switzerland) and centrifuged at 18,000 × g for 40 min at 4 °C. The supernatant was pooled and concentrated to 100 mL using a rotary evaporator at 40 °C. To this solution, 10 mL of 1 N HCl was added, and centrifuged at 20,000 × g for 40 min at 4 °C. The resulting supernatant was forced through Sep-Pak C-18 cartridges (Waters, Milford, MA, USA). The retained material was eluted in 100% methanol (RM 100). RM 100 was concentrated using vacuum centrifugation. We used the peptide-induced contraction of the rectums of *C. japonica* as a bioassay at each purification step.

Purification of [Ala²]-NMN. In the first step of [Ala²]-NMN purifications, RM 100 was applied to a reversed-phase HPLC column (CAPCELL PAK C18 SG-120, Shiseido, 10 mm × 250 mm) and eluted with a linear gradient of 0-60% ACN in 0.1% TFA for 120 min at a flow rate of 1.0 mL/min. An aliquot of each fraction was examined by bioassay. Bioactive fractions were concentrated and subjected to cation-exchange HPLC (SP-5PW, Tosoh, 7.5 mm × 75 mm) with a linear gradient of 0-0.4 M NaCl in 10 mM phosphate buffer (pH 7.2) for 40 min at a flow rate of 0.5 mL/min. The bioactive fractions that had been eluted at approximately 0.06-0.1 M NaCl were again subjected to C-18 reversed-phase HPLC (ODS-80TM, Tosoh, 4.6 mm × 150 mm) with a linear gradient of 20-30% ACN in 0.1% TFA for 50 min at a flow rate of 0.5 mL/min. Bioactive fractions were subsequently subjected to the next HPLC (ODS-80TM) with an isocratic elution of 22% ACN at a flow rate of 0.3 mL/min; the active fraction was finally purified as a single peak on the same column and conditions (Fig. 1).

Structure determination. Isolated peptide was subjected to amino acid sequence analysis by automated Edman degradation with a gas-phase sequencer (PSQ-1, Shimadzu, Kyoto, Japan). Molecular weight was determined using fast atom bombardment mass spectrometry (FAB-MS; JEOL JMS-HX 100/110A, Peabody, MA). [Ala²]-NMN was synthesized by standard solid-phase method (PSSM-8, Shimadzu, Japan), followed by TFA-anisol cleavage and HPLC purification, as described previously.[20] The structure of the synthetic peptide was confirmed by amino acid sequence and FAB-MS analysis. The purified peptide was compared to synthetic [Ala²]-NMN by reversed-phase (ODS-80TS) and cation-exchange (SP-5PW) HPLC. The FAB-MS data of the synthetic peptide was as follows: [Ala²]-NMN; base peak, 703.4, calcd. for, $C_{35}H_{57}O_8N_7$.

Bioassay and pharmacology. The activities of both the lungfish-derived, HPLC-fractionated peptides and the synthetic peptide were examined by monitoring the effects of these samples on the spontaneous contraction of the quail rectums. Methods for dissection and recording the tension of the muscle have been described previously.[21] To assess the activity during the purification process, quail rectums were
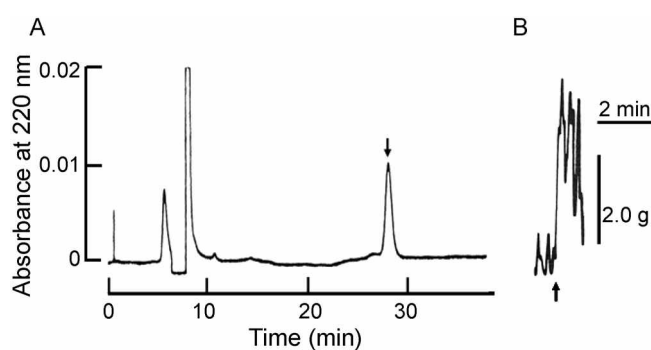

Figure 1. HPLC profile of the final purification procedure of [Ala²]-NMN (A) and bioactivity of the purified peptide (B). The C18 column (TSKgel ODS-80TM) was eluted with 22% acetonitrile in 0.1% TFA (pH 2.2). Fraction of the absorbance peak (indicated by the downward arrow) showed contractile activity on rectum of the quail, *Coturnix japonica*.

excised and cut transversely to a 10-20 mm length. Both ends of each excised rectum were tied with two cotton threads. The preparation was then mounted vertically in a recording chamber, with one end connected to the bottom of the chamber and the other end attached to a force-transducer (Type 45196A, NEC-Sanei Instrument Ltd., Tokyo, Japan). The chamber was filled with physiological saline and aerated. The saline was of the following composition (mM): NaCl 187.0, KCl 5.6, $CaCl_2$ 2.2, Glucose 11.0, HEPES 10.0, pH adjusted to 7.4 using NaOH. Output from the force-transducer was monitored by pen recorder (EPR-221A, TOA Electronics Ltd., Tokyo, Japan) *via* an amplifier (AS1302, NEC-Sanei, Tokyo, Japan) which recorded the mechanical responses of the device. An aliquot of each fraction was evaporated, redissolved in 50 μL saline, and added to the chamber. To examine the contraction effects of the synthetic [Ala²]-NMN, the rectums of black bass (*M. sulmoides*) and newt (*C. pyrrhogaster*) were also used along with quail. To record intestinal responses, we utilized a similar bioassay as that described above for the assessment of purification. The composition of the physiological saline used for black bass was the same as that of the saline of the newt. The saline used for the newt was of the following composition (mM): NaCl 110.0, KCl 2.0, $CaCl_2$ 1.0, Glucose 10.0, TRIS 5.0, pH adjusted to 7.4 using NaOH.

All bioassay and pharmacological experiments on the muscle tissues of the fish and newt were performed at room temperature, between 22 °C and 27 °C. The experiment on the muscle tissues of the quail was performed at 37 °C.

Results and Discussion

As shown in Figure 1, an active substance was finally purified on the C-18 reversed-phase column from the visceral tissue of *P. dolloi*. Aliquots (1/200) of the purified peptide potentiated spontaneous contractions of the isolated rectal preparations of the quail. The determined sequence and detected amounts (picomoles) of each PTH-amino acid in the amino acid sequence analysis were as follows: Lys₅₃-
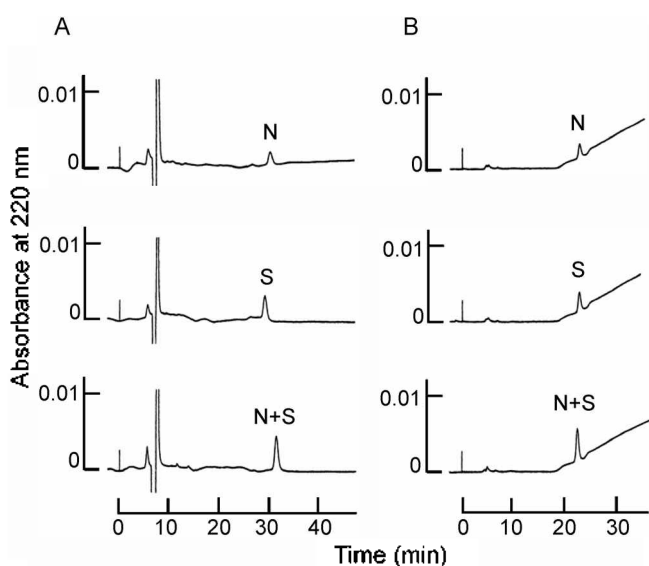
A   B

Figure 2. Comparison between HPLC profiles of the native (N) and synthetic (S) [Ala²]-NMN. A: [Ala²]-NMN was injected into a C18 reversed-phase column (TSKgel ODS-80TM) and eluted with 22% acetonitrile in 0.1% TFA (pH 2.2). B: [Ala²]-NMN was injected into a cation-exchange column (TSKgel SP-5PW) and eluted with a linear gradient of 0–0.3 M NaCl in 10 mM phosphate buffer (pH 6.2) for 30 min. N+S, represents a mixture of native and synthetic peptides.

Ala_{74}-Pro_{60}-Tyr_{61}-Ile_{30}-Leu_{17} ([Ala²]-NMN). Molecular ion peaks in the FAB-MS spectra of [Ala²]-NMN was displayed at 703.4 m/z $(M+H)^+$. Based on these results, the structures of [Ala²]-NMN was H-Lys-Ala-Pro-Tyr-Ile-Leu-OH. This peptide was thus synthesized, and chemical properties of the synthetic [Ala²]-NMN were compared to those of native peptide. Both the synthetic (S) and native (N) peptides indicated identical behaviors on reversed-phase and cation-exchange HPLC (Fig. 2). The elution profiles of both the native and synthetic [Ala²]-NMN showed the same retention times, and their mixture were eluted as single absorbance peaks.

Using the synthetic [Ala²]-NMN, we then performed the pharmacological experiments. [Ala²]-NMN demonstrated contractile activities on the isolated rectums of *C. pyrrhogaster*, *C. japonica* and *M. sulmoides* (Fig. 3). The threshold concentration of [Ala²]-NMN for contraction of *C. japonica*
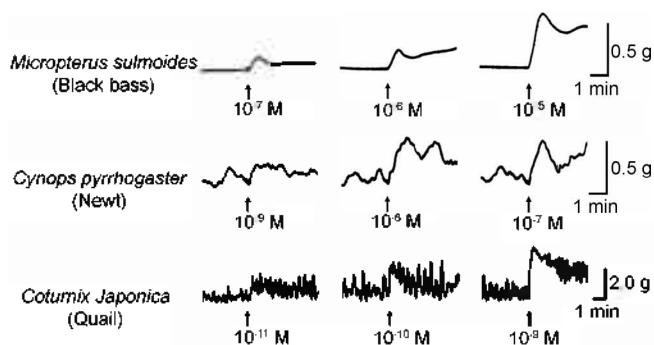
Figure 3. Typical tracings illustrating contractile responses to [Ala²]-NMN on the rectums of black bass, newt, and quail.

Table 1. A comparison of the primary structures of peptides related to neuromedin N from various species

Peptide	Sequence						Reference
Porcine	K	I	P	Y	I	L	1
Chicken	–	N	–		–	–	13
Frog	–	K	–		–	–	14
Lungfish	–	A	–		–	–	In this study

muscle was found to be approximately 10^{-11} M. [Ala²]-NMN showed contractile activities in the following order: *C. japonica* > *C. pyrrhogaster* > *M. sulmoides*. However, [Ala²]-NMN didn't show vasodilatory effect on the guinea-pig aorta (data not shown).

[Ala²]-NMN from the lungfish is the first member of NMN family to be isolated from a Sarcopterygii. [Ala²]-NMN has a novel primary structure. The amino acid sequence of [Ala²]-NMN is Lys-Ala-Pro-Tyr-Ile-Leu-OH (703.4 Da). Since the discovery of LANT-6 by Carraway and Ferris,[13] NMNs have been identified in a porcine[1] and a frog.[14] All the NMN-related peptides have conform to the sequence Lys-Xaa-Pro-Tyr-Ile-Leu-OH, Xaa being Asn, Ile, or Lys (Table 1). [Ala²]-NMN from the African lungfish contains one amino acid substitution (Ala^2 → Asn) compared with chicken NMN,[13] one substitution (Ala^2 → Ile) compared with the porcine NMN,[1] and one substitution (Ala^2 → Lys) compared with frog NMN.[14] This result indicates that the NMN-related peptides are well conserved in various vertebrates. The present biological studies suggest that [Ala²]-NMN plays important roles in the regulation of gastrointestinal and vascular smooth muscles in lungfish, similar to the roles seen in mammals. Furthermore, identification of [Ala²]-NMN provides evidence that NMN family, hitherto confined to mammals, have a widespread occurrence in lungfish.

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