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Isolation of high-molecular-weight C-type natriuretic peptide from the heart of a cartilaginous fish (European dogfish, *Scyliorhinus canicula*)

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A high-molecular-weight form of C-type natriuretic peptide (CNP) was isolated from both cardiac atria and ventricles of European dogfish, *Scyliorhinus canicula*, and its primary structure was determined. The peptide consists of 115 amino acid residues, in which the C-terminal 22 residues show high homology to CNPs identified to date. This is the first direct evidence for the presence of natriuretic peptide in the cartilaginous fish, and for the presence of CNP in an organ other than the brain.

Natriuretic peptide; Cartilaginous fish; Amino acid sequence; Sequence homology; Heart; *Scyliorhinus canicula*

1. INTRODUCTION

It is well accepted that A-type natriuretic peptide (ANP) is a factor implicated in water and electrolyte homeostatis in mammals. In contrast to most other fishes, seawater cartilaginous fish maintain their plasma slightly hyperosmotic to environmental seawater by the retention of urea and trimethylamine oxide. They use a special organ called the rectal gland as a principal route for elimination of excess monovalent ions. The action of ANP in cartilaginous fish has received little attention apart from the in vitro study reporting that mammalian ANP stimulates fluid and solute excretion from the dogfish rectal gland [1,2]. Histochemical studies showed the presence of immunoreactive ANP in atria and ventricles of dogfish using an antiserum against human ANP [3]. In this study, we attempted to isolate ANP from the dogfish heart for future studies on osmoregulation in cartilaginous fish. This study results in the isolation of a high-molecular-weight form of C-type natriuretic peptide (CNP), which has so far been reported to exist only in the brain.

Abbreviations: ANP, A-type natriuretic peptide; Asp-N, endoprotease Asp-N; BNP, B-type natriuretic peptide; CM, carboxymethylated; CNP, C-type natriuretic peptide; HPLC, high-performance liquid chromatography; LE, lysyl endopeptidase; NP, natriuretic peptide; TFA, trifluoroacetic acid

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2. MATERIALS AND METHODS

2.1. *Extraction and purification*

The atria (7 g) and ventricles (42 g) of European dogfish, *Scyliorhinus canicula*, were isolated immediately after decapitation, frozen on dry ice, and stored at −40°C until use. The isolation was performed separately on atria and ventricles. The extraction was carried out as reported previously [4]. The extract was applied to a Sephadex G-25 fine column. Each fraction was assayed for the relaxant activity in the chick rectum [5]. Bioactive fractions were further subjected to cation-exchange high-performance liquid chromatography (HPLC) and reverse-phase HPLC, until the bioactive principle was purified to homogeneity.

2.2. *Sequence analysis*

A peptide purified from dogfish atria and ventricles, later named dogfish CNP-115 (CNP-(1–115)), was digested as follows: (i) lysyl endopeptidase (LE; Wako, E/S = 1/30, by weight) in 0.1 M $NaHCO_3$, pH 8.3, containing 4 M urea, at 30°C for 4 h; (ii) endoprotease Asp-N (Asp-N; Boehringer-Mannheim, E/S = 1/40, by weight) in 50 mM NaH_2PO_4-Na_2HPO_4, pH 8.0, containing 1 M urea and 20 mM $CH_3NH_2 \cdot HCl$, at 37°C for 4 h and (iii) *Staphylococcus aureus* V8 protease (Boehringer-Mannheim, E/S = 1/30, by weight) in 0.1 M NH_4HCO_3, pH 7.8, containing 1 M urea, at 37°C for 3 h. A reduced and S-carboxymethylated (CM) dogfish CNP-115 was also digested with LE. The resulting fragments were separated by reverse-phase HPLC on a ODS-120T column. Amino acid sequence of the intact molecule and fragment peptides were analysed on a gas-phase automatic protein sequencer (Model 470A/120A; Applied Biosystems). Amino acid composition was determined by an amino acid analyzer (PICO TAG Work Station; Waters) after hydrolysis of the peptides.

3. RESULTS

3.1. *Purification*

The elution pattern of an extract of dogfish ventricles

on Sephadex G-25 is shown in Fig. 1A. The peak marked by an arrow was subjected to cation-exchange HPLC (Fig. 1B). Each of the bioactive fractions was subjected to reverse-phase HPLC. The CNP-115 was isolated from fractions 12 and 13. The elution pattern of fraction 13 is shown in Fig. 1C. The fraction that exhibited the greatest bioactivity was re-chromatographed (Fig. 1D) and 8.3 nmol of CNP-115 was finally recovered.

3.2. *Amino acid sequence*

Digestion of an intact molecule with LE resulted in 4 fragments (Fig. 2A). Complete sequences of LE10, 13, and 17 were determined in the sequencer. Two half-cystines (residues 99 and 115) were identified as CM-Cys using CM-LE17 (data not shown). LE13 consisted of CNP-(93-102) and (103-115) which were linked by the disulfide bond (Fig. 3). LE24 was derived from the N-terminal region of the intact molecule, since the sequence of the first 38 residues were identical (Fig. 3). The complete sequence of LE24 was determined by overlapping the fragments of intact molecule digested with Asp-N (Fig. 2C) and those of LE24 (Fig. 2D). Digestion with Asp-N of intact molecule and LE24 gave similar elution patterns of the fragments in HPLC except for the one eluted at 59 min. DN-A16 from the intact molecule contained CNP-(79-104) and (105-115) linked by a disulfide bond, in addition to DN-B14 from LE24. Sequence analysis revealed that DN-A27 consisted of CNP-(5-16) and (47-78). The results of sequencing peptides obtained with V8 protease (Fig. 2B) demonstrate CNP-115 is arranged as LE24-LE10-LE17.

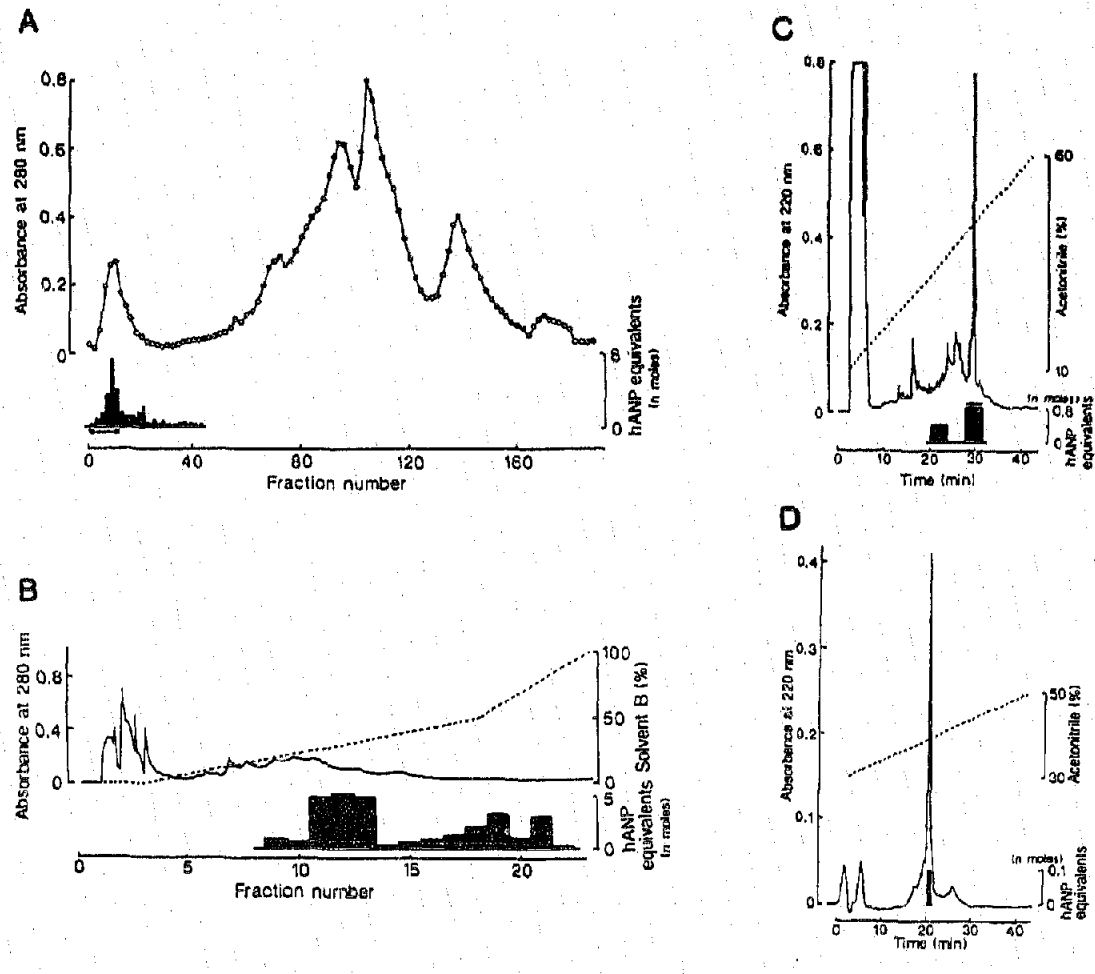

Fig. 1. Purification of dogfish CNP-115. (A) Gel filtration chromatography of on a Sephadex G-25 fine column (5 × 82 cm). Sample, acid extract of dogfish ventricle; flow rate, 85 ml/h; eluent, 1 M AcOH; fraction size, 7 ml. Black bars represent activities equivalent to human ANP in the chick-rectum relaxant assay. (B) Cation-exchange HPLC on a Biofine IEC-CM column (7.5 × 75 mm). Sample, bioactive fractions marked in (A). Linear-gradient elution was performed from the Solution A (10 mM NH_4OAc, pH 6.8/CH_3CN, 9:1) to B (1 M NH_4OAc, pH 6.8/CH_3CN, 9:1) for 80 min. Flow rate, 1 ml/min; fraction size, 4 ml/tube. Dogfish CNP-115 was recovered from fractions 12 and 13. (C) Reverse-phase HPLC on a Tosoh ODS-120T column (4.6 × 250 mm). Dotted line represents a gradient of CH_3CN in 0.1% TFA. Flow rate, 1 ml/min. Sample, fraction 13 of cation-exchange HPLC. (D) Rechromatography of the bioactive fraction marked in panel (C) by an arrow. The peak between 2 nicks was processed to amino acid sequencing.

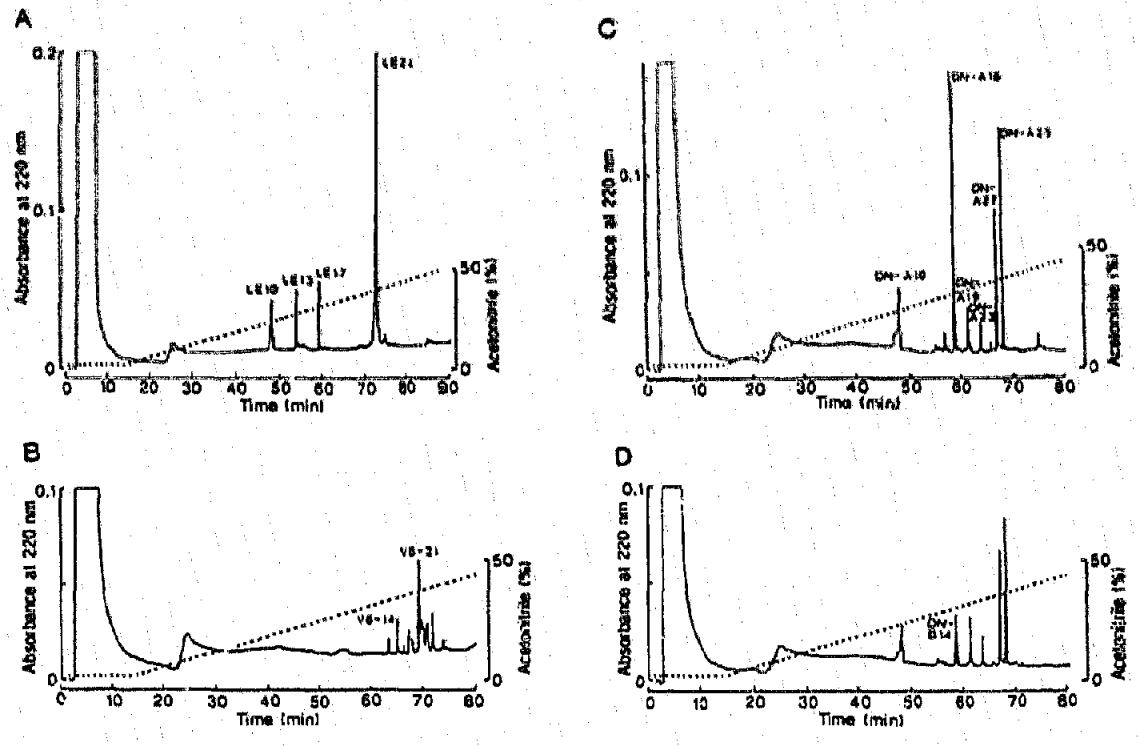

Fig. 2. Reverse-phase HPLC of enzymatic digests of dogfish CNP-115 on a Tosoh ODS-120T column. Dotted line represents a gradient of CH_3CN in 0.1% TFA. (A) Digest of CNP-115 with lysyl endopeptidase. (B) Digest of CNP-115 with *Staphylococcus aureus* V8 protease. (C) Digest of CNP-115 with endoprotease Asp-N. (D) Digest of LE 24 with endoprotease Asp-N.

Thus, the complete amino acid sequence was determined (Fig. 3). The result is consistent with the result of amino acid analysis of CM-CNP-115: Asp, 12.1(13); Thr, 1.1(1); Ser, 10.7(12); Glu, 16.5(15); Pro, 7,7(8); Gly, 11.1(10); Ala, 6.9(7). CM-Cys, 1.5(2); Val, 1.6(2); Met, 1.6(2); Ile, 1.9(2); Leu, 15.5(17); Tyr, 1.4(2); Phe, 3.0(3); Trp, not determined(1); His, 0.4(1); Lys, 3.6(4); Arg, 11.3(13). The numbers in parentheses were deduced from the sequence.

The same CNP-115 (0.9 nmol) was isolated from the dogfish atria. The elution pattern by HPLC of LE digests of CNP-115 from atria was identical to that from ventricles (data not shown).

4. DISCUSSION

According to Takei et al. [6], the natriuretic peptides (NPs) identified to date are classified into 4 groups based on structural similarities and sites of production, that is, ANP, BNP, CNP and ventricular NP. It is well accepted that their mature peptides are C-terminal fragments cleaved from prohormones of approximately 100 amino acid residues by specific proteolysis. The peptide isolated in this study consists of 115 amino acids, and its C-terminal segment has a ring-structure of 17 amino acids with a disulfide bond, which is common to NPs. The C-terminal 22 amino acid residues show the highest homology to CNP compared to the other NPs; sequence identity is 77–82% to porcine, rat, eel and killifish CNP-22 [7–10]. It lacks the sequence that follows the second half-cystine, which is a common characteristic of CNPs. CNP-115 is thought to be a prohormone, since a dibasic sequence, such as Lys-Lys, which is a cleavage site of proCNP to generate the mature form in the mammalian brain, is present at positions 92–93. However, such mature peptide was not found in the heart in the present study.

Since CNP has so far been identified only in the brain, the peptide is thought to be intrinsic to brain [6]. In the present study, however, CNP-115 was purified from the dogfish heart. Thus, there seems to be a species difference in the organ that produces a specific NP. However, a possibility remains that the dogfish heart produces other NPs which could not be detected by a rectum-relaxant assay.

Since the sequence homology of the C-terminal 22 amino acids of proCNP (CNP-22) between dogfish and mammals is as high as 82% (Fig. 4), CNP-22 could be a principal hormone among NP families. On the other hand, the sequence homology of the N-terminal segment is very low between dogfish and mammals, although the 2 mammalian species have a similar sequence (Fig. 4). These data suggest that CNP-22 has an important biological role among vertebrates.

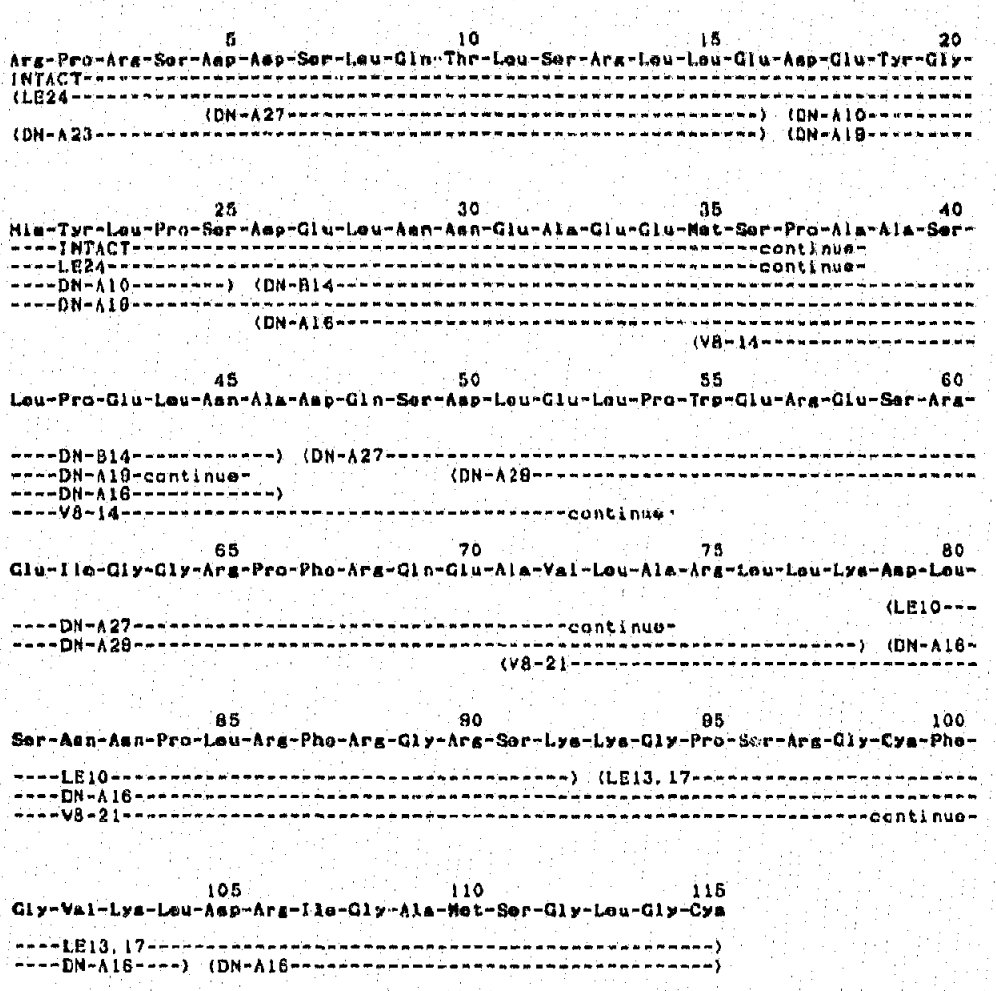

Fig. 3. Complete proposed amino acid sequence of dogfish CNP-115.

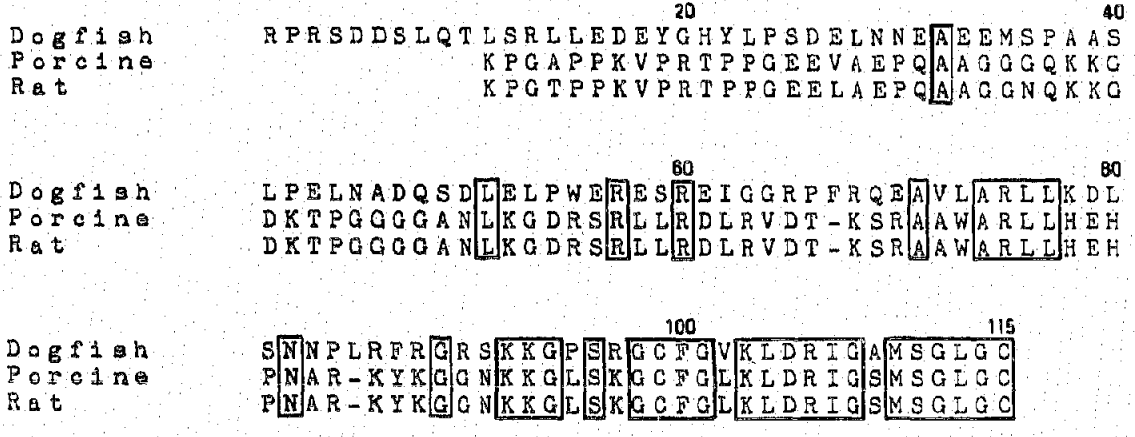

Fig. 4. Comparison of the structure of dogfish CNP-115 with mammalian proCNP. Identical residues between dogfish and mammals are boxed. There are only 3 replacements in 103 amino acid residues between porcine and rat proCNP [7,8].

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