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# Purification of a vasoactive peptide related to lysyl-bradykinin from trout plasma

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Incubation of plasma from the steelhead trout, *Oncorhynchus mykiss* with porcine pancreatic glandular kallikrein generated bradykinin-like immunoreactivity. The primary structure of the immunoreactive peptide was established as: Lys-Arg-Pro-Pro-Gly-Trp-Ser-Pro-Leu-Arg. This sequence shows two amino acid substitutions (Phe$^6$→Trp and Phe$^9$→Leu) compared with mammalian lysyl-bradykinin (kallidin). Bolus intra-arterial injection of the purified peptide produced a strong and sustained vasopressor response in the unanaesthetized trout. The data demonstrate that the kallikrein–kinin system predates the appearance of tetrapods and suggest a role for this system in cardiovascular regulation in fish.

Bradykinin; Kallidin; Kininogen; Kallikrein; Blood pressure; Trout

## 1. INTRODUCTION

The existence of a kallikrein–kinin system in birds and reptiles has been established unequivocally. In the chicken, treatment of plasma kininogen with bovine plasma kallikrein generates [Thr$^6$,Leu$^8$]bradykinin (ornitho–kinin) [1]. Activation of endogenous kallikrein by incubation of plasma from the turtle [2] and alligator [3] with glass beads generates [Thr$^6$]bradykinin. Synthetic replicates of the avian and reptilian kinins show potent vasodilator activity in their species of origin. The existence of a kallikrein–kinin system in other classes of lower vertebrates is less clear. Attempts to generate kinins in frog and fish plasma that were active on the isolated rat uterus have produced conflicting results [4,5]. More recently, however, it has been shown that trout tissues contain kallikrein-like esterolytic activity, determined with a range of synthetic substrates and specific kallikrein inhibitors, and kininogen, demonstrated by generation of bradykinin-like immunoreactivity after incubation with trypsin or porcine glandular kallikrein [6,7]. The substance(s) generated in trout plasma by kallikrein treatment, termed T60K, produced a dose-dependent pressor response in both trout and rats and T60K-contracted isolated trout and rabbit arteries [8]. In this study, we have used an antisera raised against mammalian bradykinin to facilitate the purification of a bradykinin-related vasoactive peptide from kallikrein-treated trout plasma that is probably identical to T60K.

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## 2. MATERIALS AND METHODS

### 2.1. *Generation of the kinin*

Plasma (60 ml) from 5 adult steelhead trout (*Oncorhynchus mykiss*, skamania strain) was incubated with porcine pancreatic glandular kallikrein (5 U/ml plasma) for 1 h at 37°C as previously described [8]. The reaction mixture was diluted with an equal volume of 1% (v/v) trifluoroacetic acid/water and peptide material was isolated using Sep-Pak C18 cartridges as described [2].

### 2.2. *Radioimmunoassay*

Bradykinin-like immunoreactivity was determined by radioimmunoassay using antiserum BT4, raised against mammalian bradykinin, using a procedure previously described [9].

### 2.3. *Purification of the peptide*

The plasma extract was redissolved in 1% (v/v) trifluoroacetic acid/water (4 ml) and chromatographed on a (90 × 1.6 cm) column of Sephadex G25 (Pharmacia-LKB) equilibrated with 1 M acetic acid at a flow rate of 24 ml/h. Fractions (2 ml), were collected and the presence of bradykinin-like immunoreactivity was determined by radioimmunoassay at appropriate dilutions. Immunoreactive fractions were pooled (total volume = 14 ml) and pumped at a flow rate of 2 ml/min onto a (250 × 10 mm) Vydac 218TP510 C18 column (Separations Group) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The concentration of acetonitrile in the eluting solvent was raised to 14% (v/v) over 10 min and to 35% (v/v) over 50 min using linear gradients. Absorbance was measured at 214 and 280 nm and 2 ml fractions were collected. The fraction denoted by the bar (Fig. 1A) was re-chromatographed on a (250 × 4.6 mm) Vydac 214TP54 C4 column equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 28% (v/v) over 40 min using a linear gradient. Trout lysyl-bradykinin was purified to apparent homogeneity by successive chromatographies on a (250 × 4.6 mm) Vydac 218TP54 C18 column and then on a (250 × 4.6 mm) Supelcosil LC-18-DB C18 column (Supelco Inc.) under the same conditions used with the C4 column.

### 2.4. *Structural characterization*

The primary structure of the peptide was determined by automated Edman degradation. Its amino acid composition was determined in

duplicate by pre-column derivatization with phenylisothiocyanate. Tryptophan and cysteine residues were not determined. The methods and instrumentation used have been described previously [2,3].

2.5. *Effects on arterial blood pressure*

The dorsal aorta of 300 g male rainbow trout (*O. mykiss*, unknown strain) was cannulated percutaneously as described [6] and the fish allowed to recover for 48 h. Approximately 500 pmol of purified trout lysyl-bradykinin in 0.25 ml 0.9% (w/v) NaCl was injected in a single bolus. Arterial blood pressure was measured as described [6]. A second injection of 250 pmol of peptide was made after an interval of 6 h. Vehicle controls were injected 2 h after each lysyl-bradykinin treatment.

## 3. RESULTS

### 3.1. *Generation of the kinin*

Incubation of trout plasma, in which proteolytic enzymes had been inactivated by heat treatment, with porcine glandular kallikrein generated bradykinin-like immunoreactivity equivalent to approximately 5 ng of bradykinin/ml of plasma. However, serial dilutions of the immunoreactivity did not diminish in parallel with the synthetic bradykinin standard in radioimmunoassay. Bradykinin-like immunoreactivity was not detected in trout plasma that had not been incubated with kallikrein.

### 3.2. *Purification of trout bradykinin*

Bradykinin-like immunoreactivity in the plasma extract was eluted from a Sephadex G-25 gel permeation column as a single major peak with $K_{av}$ between 0.80 and 0.95. The fractions containing maximum immunoreactivity were pooled and chromatographed on a semi-preparative C18 column (Fig. 1A). Bradykinin-like immunoreactivity was associated with the prominent peak denoted by the bar and with a minor peak that eluted later. Chromatography of the more abundant component on an analytical C4 column (Fig. 1B) showed that bradykinin-like immunoreactivity was associated with the well-resolved peak delineated by the arrows. Successive chromatographies of the fraction on an analytical Vydac C18 column (Fig. 1C) and on an analytical Supelcosil C18 column (Fig. 1D) led to the purification to apparent homogeneity of the trout bradykinin-related peptide. The final yield of pure material was approximately 2 nmol. Attempts to purify the minor peak with bradykinin-like immunoreactivity (Fig. 1A) were not successful.

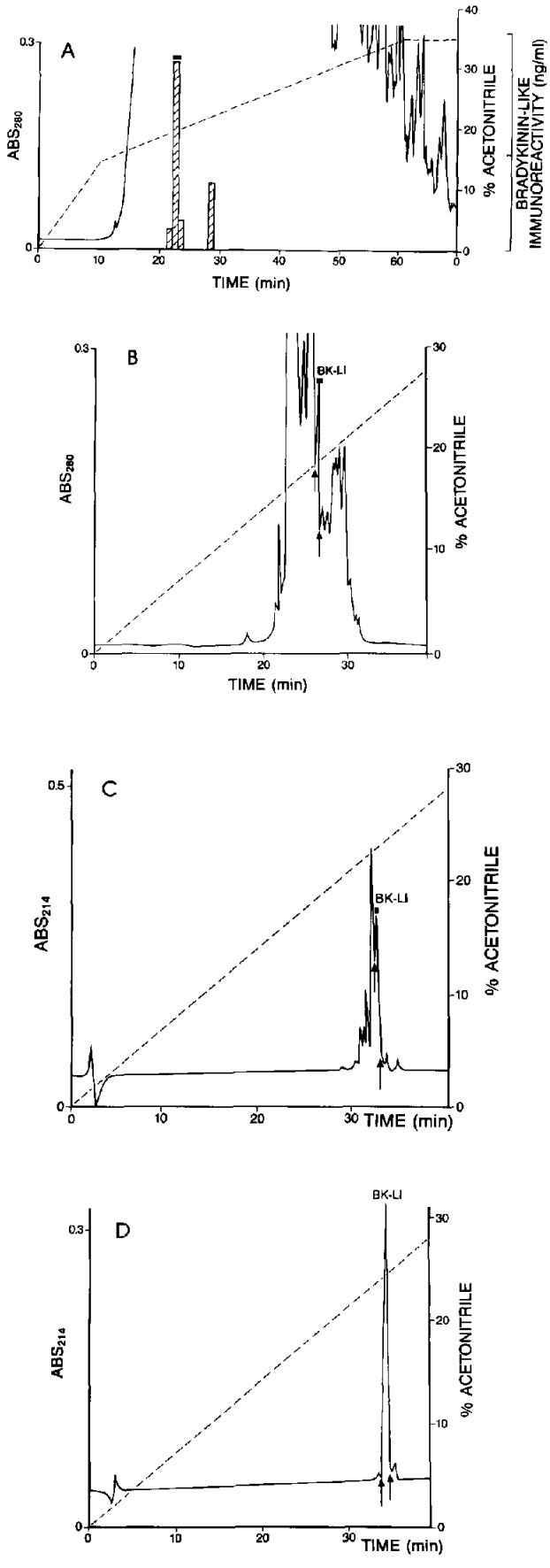


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Fig. 1. Purification by reverse-phase HPLC of trout bradykinin on (A) semipreparative Vydac C18, (B) analytical Vydac C4, (C) analytical Vydac C18, and (D) analytical Supelcosil C18 columns. BK-LI denotes the peaks containing bradykinin-like immunoreactivity. The arrows show where peak collection began and ended, and the dashed line indicates the concentration of acetonitrile in the eluting solvent.

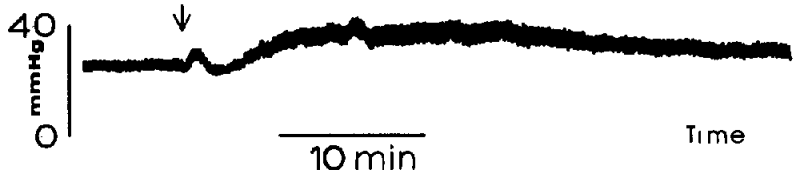


Fig. 2. Effect of an intra-arterial injection (arrow) of purified trout lysyl-bradykinin (approx. 500 pmol) on arterial blood pressure (mmHg) in an unanesthetized trout. The response in a single experiment is shown.

### 3.3. *Peptide characterization*

The primary structure of trout lysyl-bradykinin was determined by automated Edman degradation. The amino acid sequence of the peptide was established as: Lys (146)-Arg (240)-Pro (307)-Pro (319)-Gly (327)-Trp (90)-Ser (36)-Pro (169)-Leu (101)-Arg (78). The values in parentheses show the yields of phenylthiohydantoin amino acids in pmol. The amino acid composition of the peptide was determined as: Ser 1.2 (1), Gly 1.4 (1), Arg 2.5 (2), Pro 3.1 (3), Leu 1.0 (1), Lys 0.7 (1) residues/mol peptide. The values in parentheses show the values predicted from the proposed structure. The strong absorbance at 280 nm of the purified peptide was consistent with the presence of a tryptophan residue.

### 3.4. *Biological activity*

Intra-arterial injection of trout lysyl-bradykinin produced a bi-phasic pressor response (Fig. 2). An initial rapid but transient increase in arterial blood pressure was followed by a sustained hypertensive period exceeding 30 min. The response to both injections of the peptide was the same. Injection of vehicle alone had no effect on blood pressure.

## 4. DISCUSSION

This study has described the first isolation and structural characterization of a bradykinin-related peptide from a fish, and demonstrates that the appearance of the kallikrein–kinin system predates the appearance of tetrapods. As shown in Table I, trout lysyl-bradykinin shows two substitutions compared with human kallidin (Phe$^6$→Trp and Phe$^9$→Leu). The peptide shares with chicken bradykinin (ornitho–kinin) [1] the presence of a penultimate leucine residue but, unlike chicken and reptilian [2,3] bradykinins, contains a serine rather than a threonine residue. Although a peptide related to mammalian lysyl-bradykinin rather than to bradykinin was isolated in the present study, the peptide was generated under non-physiological conditions i.e. incubation with pig glandular kallikrein. Further studies are required to determine whether lysyl-[Trp$^5$,Leu$^8$]bradykinin or [Trp$^5$, Leu$^8$]bradykinin are generated in trout plasma when the animal's own kallikrein–kinin system is activated under physiological conditions. It may be significant, however, that the eel intestine contracts in response to kallidin whereas synthetic bradykinin is without effect [10].

Table I

A comparison of the amino acid sequences of peptides related to trout lysyl-bradykinin

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Trout lysyl-bradykinin | Lys | Arg | Pro | Pro | Gly | Trp | Ser | Pro | Leu | Arg |
| Human Lysyl-bradykinin | - | - | - | - | - | Phe | - | - | Phe | - |
| Chicken ornitho-kinin | | - | - | - | - | Phe | Thr | - | - | - |
| Alligator/turtle bradykinin | | - | - | - | - | Phe | Thr | - | Phe | - |

(–) denotes residue identity.

The vasopressor response of trout lysyl-[Trp$^6$,Leu$^9$]-bradykinin in the trout contrasts with hypotensive action of lysyl-bradykinin in mammalian species (reviewed in [11]) but is consistent with the known pressor effect of mammalian kinins in trout [12]. The vasodilator action of bradykinin (and kallidin) in mammals is mediated primarily through interaction with both $B_1$- and $B_2$-subtype receptors, and involves endothelial production of the potent vasodilators, prostacyclin [13] and nitric oxide [14]. Although prostanoid vasodilators can be released from trout endothelium by the calcium ionophore, A23187, this release is not stimulated by mammalian bradykinin, nor is nitric oxide released by trout endothelium [15]. Thus it appears that bradykinin originally evolved as a vasoconstrictor hormone and was more recently adapted to a vasodilator role by terrestrial vertebrates.

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