Vol. 112, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 29, 1983 Pages 701-708

# ISOLATION AND STRUCTURE OF T-KININ

H. Okamoto and L.M. Greenbaum

Department of Pharmacology
Medical College of Georgia
Augusta, GA 30912

Received March 15, 1983

T-kinin, a previously undescribed peptide containing bradykinin, has been isolated following treatment of rat plasma with trypsin (1 mg/ml). The liberated T-kinin, which contracts the rat uterus, was isolated by procedures including CM-cellulose, Biogel P-4 and reverse-phase high-performance liquid chromatography. The final material had a single N-terminal isoleucine and was shown by amino acid analysis and sequence determination to have the structure of the undecapeptide Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (isoleucyl-seryl-bradykinin). The relationships of the protein from which T-kinin is cleaved (T-kininogen) to other known kininogens is discussed.

It is well known that bradykinin liberation by trypsin from mammalian plasmas reflects the levels of high (HMW) and low molecular weight (LMW) kininogen of the plasma (1, 2). Recent reports from our laboratory noted that rat plasma, in the presence of high concentrations of trypsin (1 mg/ml), released 7 fold more kinin than could be accounted for by reported kininogen levels (3,4). This discrepancy directed us toward the identification of the released kinin(s) both in terms of comparison with bradykinin and in terms of the kininogen from which it was released. Since the unknown kinin could be separated by CM-cellulose from bradykinin, Lys-bradykinin and Met-Lys-bradykinin we termed this material T-kinin (3, 4).

In this communication we report on the purification, amino acid composition and amino acid sequence of T-kinin. Our results are confirmation that T-kinin is a previously undescribed undecapeptide containing the bradykinin sequence.

## MATERIALS AND METHODS

Synthetic bradykinin, Lys-bradykinin and Met-Lys-bradykinin were purchased from Peninsula Laboratory, Inc. (California). TPCK-treated trypsin (bovine

0006-291X/83 $1.50

pancreas), aminopeptidase M (porcine kidney), soybean trypsin inhibitor, dansyl-chloride, dansyl-amino acids and fluorescamine were obtained from the Sigma Chemical Co. CM-cellulose (Cellex CM) and Biogel P-4 were from Bio-Rad Laboratories (California). 4-NN-dimethylaminoazobenzene 4'-isothiocyanate and phenylisothiocyanate were the products of Fluka Chemical Co. Polyamide sheets were obtained from Schleicher and Schüll (FRG). Octadecyl ($C_{18}$) disposable extraction column (3 ml) was obtained from J.T. Baker Chemical Co. The apparatus for high-performance liquid chromatography (HPLC) was a Beckman Model 334; the column was Ultrapore RPSC (4.6 x 75 mm) packed with spherical alkyl bonded silica (Beckman).

Bioassay - This was carried out on the estrus rat uterus (5).

Collection of rat plasma - Male Sprague-Dawley rats, weighing 400-450 g, were anesthetized with ether, and blood was collected from the abdominal aorta by a polyethylene syringe containing 1/10 volume of 3.8% sodium citrate. Plasma was separated from cells by centrifugation at 800 x g for 15 min. Sixty ml of citrated plasma was obtained from 9 animals.

Extraction procedure of kinin by n-butanol - The incubation mixture was acidified (pH 2.5) with 1 N HCl and saturated with solid NaCl. After centrifuging at 4,000 x g for 20 min at room temperature, the supernatant (730 ml) was stirred vigorously with 500 ml of n-butanol for 5 min. The butanol phase was collected after centrifugation at 1,000 x g for 5 min. The butanol extraction was repeated once again. The combined butanol phases (1 L) were stirred with 700 ml of ethyl ether and 200 ml of distilled water for 5 min. The aqueous phase was collected after centrifugation. This extraction, from butanol to distilled water, was repeated once again. The combined aqueous phases were lyophilized.

Modification of peptides with fluorescamine and HPLC separation - The methods previously described (6) were followed. Fifty µl of a kinin or peptide solution was diluted with an equal volume of 0.5 M sodium borate buffer, pH 8.5. Five µl of a freshly prepared fluorescamine solution in acetone (3 mg/ml) was then added and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 10 min before analyzing with reverse-phase HPLC as described (7).

End-group analysis of peptide by dansylation - Purified peptide (0.8 µg bradykinin equivalent) was dansylated according to the method of Gray (8). The product was hydrolyzed with 50 µl of 6 N HCl at 110°C for 20 hr in a sealed tube. After the HCl was removed in vacuo over NaOH, the hydrolyzate was dissolved in 20 µl of ethanol. Five µl of the sample was spotted on a polyamide sheet (7.5 x 7.5 cm). The sheet was developed two dimensionally by ascending chromatography using two of the following solvent system: solvent I, 1.5% formic acid (w/v); solvent II, benzene-acetic acid (9:1, v/v). The spots of dansyl amino acids were examined under ultraviolet light.

Determination of amino acid composition and the sequence of purified peptide - 1.1 µg bradykinin equivalent (rat uterus) of purified peptide was hydrolyzed with 6 N HCl (2 ml) at 110°C for 24 hours in a sealed tube under $N_2$ gas. The hydrolyzate was evaporated and analyzed in a Beckman model 121 M amino acid analyzer.

The sequence of peptide was determined by the 4-NN-dimethylaminoazobenzene 4'-isothiocyanate-/phenylisothiocyanate-double coupling method (Edman degradation) reported by Chang et al. (9). The colored 4-NN-dimethylaminoazobenzene 4'-thiohydantoin-amino acids were identified by thin-layer chromatography on polyamide sheets (10). The amino acid content and sequence determinations were done through the courtesy of Dr. T.H.J. Huisman and Mr. H. Lam (Department of Cell and Molecular Biology, Medical College of Georgia).

## RESULTS

Generation of T-kinin from rat plasma by trypsin - Rat plasma (60 ml) was acidified to pH 2.0 by mixing with 9 volume of 0.03 N HCl and incubated at 37°C for 15 min to inactivate plasma kinases (2), and then neutralized with 10 N NaOH. 750 mg TPCK-treated trypsin in 150 ml of 0.2 M Tris-HCl buffer, pH 7.8 was added. After incubation at 37°C for 2 hours, the reaction was terminated by acidifying the mixture to pH 2.5 with 1 N HCl. For bioassay, 200 µl of the incubation mixture before acidification was treated with 1 mg soybean trypsin inhibitor and assayed against the estrus rat uterus. The incubation mixture was found to contain 0.95 µg bradykinin equivalents per ml (Table 1). Thus, 11.5 µg bradykinin equivalents of kinin was generated from 1 ml of rat plasma by trypsin.

Purification of T-kinin - Purification of T-kinin was performed by butanol extraction and chromatography on CM-cellulose, Biogel-P 4 and reverse-phase HPLC.

Extraction: Kinin was extracted from the incubation mixture by using n-butanol and aquous extraction as described in "Materials and Methods". About 85% of the kinin activity was recovered by this procedure (Table 1).

CM-cellulose column chromatography: The lyophilizate of the combined aqueous phases was dissolved in distilled water. The pH and the conductivity

Table 1

PURIFICATION OF T-KININ LIBERATED BY TRYPSIN FROM RAT PLASMA

| Purification Step | Volume (ml) | Kinin (µg BK equiv./ml) | Kinin (µg BK equiv.) | Recovery (%) |
|---|---|---|---|---|
| Incubation mixture | 750 | 0.95 | 690 | 100 |
| Butanol extraction | 20 | 29.3 | 585 | 84.8 |
| CM-cellulose (stepwise) | 20 | 21.4 | 427 | 61.9 |
| CM-cellulose (gradient) | | | | |
| Peak I | 6 | 44.8 | 268 | 38.9 |
| Peak II | 6 | 7.8 | 47 | 6.8 |
| Biogel P-4 | 4 | 59.7 | 239 | 34.6 |
| Reverse-phase HPLC | 3 | 41.3 | 124 | 17.9 |

Kinin was assayed by estrus rat uterus using bradykinin as a standard.

were adjusted to 5.0 and 1.0 mmho, respectively. The sample (480 ml) was applied to the column of CM-cellulose (2.5 x 5.0 cm) previously equilibrated with 0.01 M ammonium acetate (pH 5.0). After washing with 200 ml of the buffer, the elution was carried out with stepwise changes of ammonium acetate buffer concentration at a flow rate of 30 ml/hr; 100 ml each of 0.05 M (pH 5.0), 0.2 M (pH 7.0) and 0.5 M (pH 7.0), buffer was employed. Uterine contracting activity was eluted exclusively by the 0.2 M ammonium acetate buffer. The active fractions were combined and lyophilized.

The lyophilizate was dissolved in distilled water, and the conductivity was adjusted to 1.0 mmho by adding 0.2 M ammonium acetate. The pH was adjusted to 5.0 by 0.2 M acetic acid. A column of CM-cellulose (0.9 x 15 cm) was equilibrated with 0.01 M ammonium acetate, pH 5.0, and the sample (22 ml) applied. After washing with 50 ml of the buffer, elution of the kinin was carried out with a linear concentration gradient from 0.01 M ammonium acetate, pH 5.0, to 0.5 M ammonium acetate, pH 7.5. The buffer mixing chamber had a volume of 100 ml. Flow rate was 20 ml/hr and 1 ml fractions were collected. Uterine contracting activity was eluted in the two peaks appearing between 10 mmhos and 20 mmhos (Fig. 1). Of the two peaks of activity, the second or smaller peak (peak II) corresponds to bradykinin (3), the eluates making up the first peak of activity (peak I) we termed "T-kinin", and were combined and lyophilized.

Biogel P-4 column chromatography: The T-kinin lyophilizate was dissolved in 0.5 ml of 0.2 M ammonium acetate, pH 6.0, and applied to a column of Biogel P-4 (1.5 x 80 cm) previously equilibrated with 0.2M ammonium acetate, pH 6.0. The flow rate was 5.5 ml/hr, the void volume was 43 ml. The active fractions appeared as a single peak and were combined and lyophilized.

Reverse-phase HPLC: The lyophilizate was dissolved in 0.5 ml of distilled water. Five to 15 µl of sample were injected and the column was isocratically eluted with 12% acetonitrile in 0.04 M triethylammonium formate (pH 4.4) at a flow rate of 1.0 ml/min (11). As seen in Fig. 2B, two peaks of absorbance at 210 nm was obtained. Only the peak with the retention time of

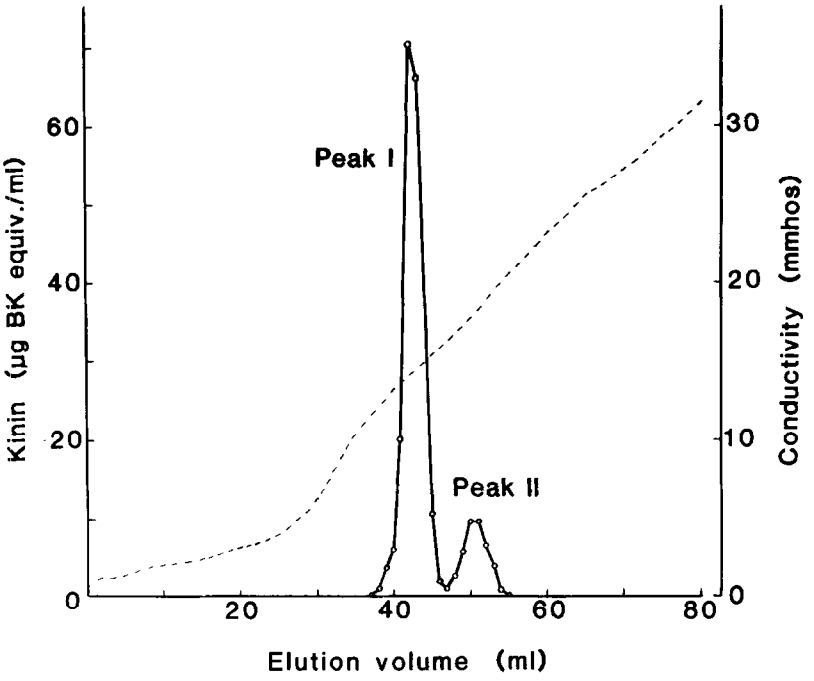


Figure 1

CM-cellulose column chromatography of trypsin-released kinin. Sample (427 µg bradykinin equivalents) was obtained from the stepwise elution of a CM-cellulose column (Table 1) and applied to a second column of CM-cellulose (0.9 x 15 cm). After washing the column with 0.01 M ammonium acetate, pH 5.0, the elution was performed by a linear gradient of the buffer concentration from 0.01 M to 0.5 M using a 100 ml of mixing chamber. o—o, kinin activity in each fraction (1 ml); ----, conductivity. Peak I is T-kinin; peak II is bradykinin.

25 min 30 s had uterine contracting activity. The retention time differed from Lys-bradykinin (7 min 10 s), Met-Lys-bradykinin and bradykinin (12 min 25 s) (Fig. 2A). In order to obtain a large sample of T-kinin, the lyophilized material eluted from the Biogel P-4 column was repeatedly subjected to HPLC and the fraction collected between the retention times of 22 min and 30 min. The combined fractions were then lyophilized and dissolved in 3 ml of distilled water. It contained a total of 124 µg bradykinin equivalents kinin (Table 1). The purified T-kinin was examined for homogeneity by using reverse-phase HPLC after fluorescamine treatment (7). A single peak was eluted.

Determination of end-group of T-kinin - The N-terminal amino acid of the purified peptide was analyzed by dansylation and thin-layer chromatography of the dansylated end-group. As shown in Fig. 3, one spot of dansylated amino

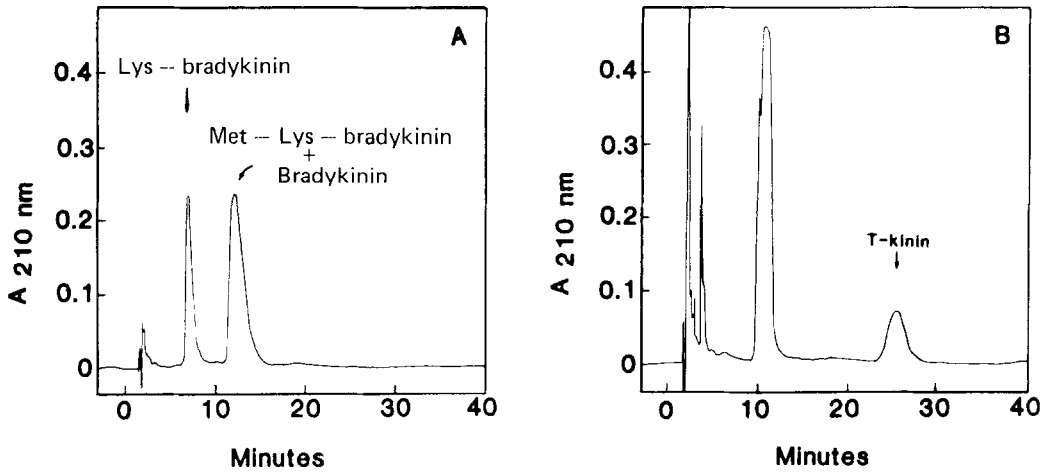


Figure 2

Reverse-phase HPLC of bradykinin and its analogs (A). Reverse-phase HPLC of T-kinin (B). Ten nmol of synthetic bradykinin and its analogs and 6 μg bradykinin equivalents of T-kinin fraction were injected at 0 min.

acid was detected on the two-dimensionally developed thin-layer plate, except for those of dansyl-OH and dansyl-$NH_2$. This spot had the same Rf as the dansyl-isoleucine standard.

Amino acid composition and the sequence of T-kinin - As seen in Table 2, purified T-kinin was composed of 11 amino acids. When compared with the amino acid composition of bradykinin, the peptide contained additional single

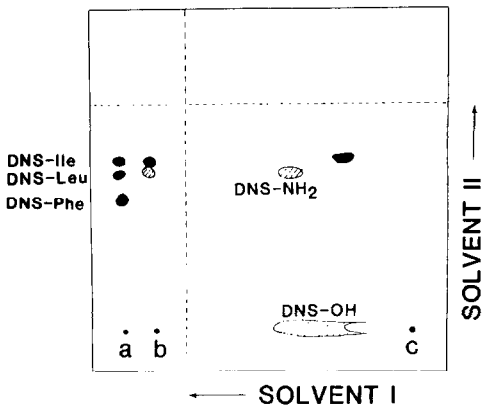


Figure 3

Two-dimensional thin-layer chromatography of the dansylated end-group of T-kinin. Origin a, mixture of standard dansyl-amino acids (dansyl-Ile, dansyl-Leu, dansyl-Phe; each 0.2 nmol); origin b and c, hydrolysate of dansylated T-kinin. Origin a and b were spotted after first dimension and developed in solvent II. Origin c was developed two-dimensionally.

Table 2

AMINO ACID COMPOSITION OF T-KININ

| Amino acid | nmol | mol/mol of isoleucine | Residue/molecule |
|---|---|---|---|
| Arginine | 3.14 | 2.05 | 2 |
| Glycine | 2.09 | 1.37 | 1 |
| Isoleucine | 1.53 | 1.00 | 1 |
| Phenylalanine | 3.00 | 1.96 | 2 |
| Proline | 4.35 | 2.90 | 3 |
| Serine | 3.16 | 2.07 | 2 |

Purified peptide (1.1 μg bradykinin equivalent on rat uterus) was subjected to amino acid analysis (see text).

residues of isoleucine and serine. The sequence determination indicates that the structure for T-kinin is as shown:

Ile-Ser-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Mr = 1202.3)

## DISCUSSION

The isolation of T-kinin (Ile-Ser-bradykinin) indicates that rat plasma contains a kininogen with a unique sequence that differs from other known kininogens. Kato et al. has identified the sequence of Leu-Met-Lys-bradykinin in both HMW and LMW kininogen of bovine plasma (12). A human kininogen is reported to have the sequence of Met-Lys-Bradykinin (13). However, not all kininogens contain this sequence. Horse HMW kininogen was shown to contain the sequence Met-Leu-Lys-bradykinin (14). The sequence of T-kinin differs from the other kininogen sequences in that kallidin (Lys-bradykinin) is not a component of the molecule.

At this time, it is not known whether T-kinin is contained in the HMW or LMW kininogen found in rat plasma (15). The sequence of these kininogens is unknown. It is possible that T-kinin is generated from T-kininogen, a third kininogen of rat plasma.

The generation of T-kinin from rat plasma by trypsin is of importance in terms of its potential as a circulating kinin in rat plasma. It is not clear

whether or not trypsin is directly cleaving T-kinin from T-kininogen or is activating a plasma enzyme which in turn generates T-kinin. If the latter is the case, T-kinin may represent a circulating kinin in the rat under conditions in which trypsin-like enzymes are released into the plasma. Studies are now proceeding to determine whether glandular and plasma kallikreins can also generate T-kinin from rat plasma.

It will be of interest to determine whether T-kininogen is present in the plasma of other species and whether there are endogenous proteases which can generate T-kinin from this substrate.

## ACKNOWLEDGEMENTS

The authors wish to gratefully thank Professor T.H.J. Huisman and Mr. H. Lam of the Cell and Molecular Biology Department for the amino acid analysis and the sequence determinations.

## REFERENCES

1. Diniz, C.R. and Carvalho, I.F. (1963). Ann. N.Y. Acad. Sci. 104, 77-89.
2. Uchida, Y. and Katori, M. (1978). Biochem. Pharmacol. 27, 1463-1470.
3. Okamoto, H. and Greenbaum, L.M. (1983). Life Sci., In press.
4. Okamoto, H. and Greenbaum, L.M. (1983). Fed. Proceed. 42, 1020.
5. Freer, R., Chang, J. and Greenbaum, L.M. (1972). Biochem. Pharmacol. 21, 3107-3110.
6. Prakash, A., Roffman, S. and Greenbaum, L.M. (1978). Anal. Biochem. 89, 257-263.
7. Narayanan, T.K. and Greenbaum, L.M. (1982). Fed. Proceed. 41, 1138.
8. Gray, W.R. (1972). Methods Enzymol. 25, 121-138.
9. Chang, J.Y., Brauer, D. and Wittmann-Liebold, B. (1978). FEBS Lett. 93, 205-214.
10. Chang, J.Y. (1977). Biochem. J. 163, 517-520.
11. Powers, C.A. and Nasjletti, A. (1982). J. Biol. Chem. 257, 5594-5600.
12. Kato, H., Han, Y.N., Iwanaga, S., Suzuki, T. and Komiya, M. (1976). J. Biochem. (Tokyo) 80, 1299-1311.
13. Hial, V., Keiser, H.R. and Pisano, J. (1976). Biochem. Pharmacol. 25, 2499-2503.
14. Sugo, T., Kato, H., Iwanaga, S. and Fujii, S. (1979). Biochim. Biophys. Acta 579, 474-478.
15. Jacobsen, S. (1966). Br. J. Pharmacol. 28, 64-72.