Biochemical Pharmacology, Vol. 38, No. 15, pp. 2421–2427, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

QUANTITATIVE DETERMINATION OF KININS RELEASED BY TRYPSIN USING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND IDENTIFICATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)*

KALYAN R. ANUMULA, RAYMOND SCHULZ and NATHAN BACK†
Department of Biochemical Pharmacology, State University of New York at Buffalo, Buffalo, NY 14260, U.S.A.

(*Received* 11 *May* 1988; *accepted* 16 *December* 1988)

Abstract—Rapid ELISA and HPLC procedures were developed for quantitation and identification of various natural kinins. Antibradykinin mouse monoclonal antibodies were used to determine kinin levels in the range of 20–200 ng. Bradykinin coupled to bovine serum albumin was used to coat the plates in a 3- to 4-hr ELISA. Synthetic kinin standards isoleucine-seryl-bradykinin (Ileu-Ser-BK), methionyl-lysyl-bradykinin (Met-Lys-BK), tyrosine-bradykinin (Tyr-BK) and bradykinin (BK) yielded almost identical curves with a mixture of A_5 and D_9 monoclonal antibodies. [Tyr^5]-BK, [Tyr^8]-BK and des-arginine^9 bradykinin (des-Arg^9-BK) showed negligible amounts of cross-reactivity. ELISA-compatible trypsin digestion developed for release of kinins from plasma of normal humans, rats and turpentine-treated rats gave values of 3.2, 6.9 and 70 μg/ml plasma, respectively. High performance liquid chromatography methods were developed for complete resolution of kinins on a C-18 reversed phase μ-Bondapak column before and after derivatization with phenyl isothiocyanate (PITC). The simple PITC derivatization procedure yielded good quantitation above 20 pmol. The ELISA and HPLC methods were used in a complementary fashion to assay and identify kinins in biological fluids as well as during the course of kininogen purification.

Kinins are part of the kininogen protein molecule and are released by proteolytic action of specific and general kininogenases in body tissues and fluids [1, 2]. The released kinin peptides mediate potent physiologic and pharmacologic actions presumably through kinin receptors [3, 4]. The recent discovery that kininogens indeed are identical to the cysteine proteinase inhibitors (for review see Refs 5 and 6) has increased further the need for a specific and sensitive method to estimate both kinin and kininogen levels. Traditionally kinins and kininogens (in terms of bradykinin [BK‡] equivalents) have been determined by bioassay in which the concentration of kinin is correlated to its smooth muscle contractile response [7]. The bioassay method also has been supplemented with or replaced by the more convenient and reproducible BK radioimmunoassay [8, 9]. In addition, kinins have been quantitated either directly by HPLC [10] or by radioimmunoassay after separation by HPLC [11]. Soluble enzyme immunoassay for BK determination using a β-galactosidase label has been reported recently [12]. In the present paper we report on a rapid and highly sensitive enzyme-linked immunosorbent assay (ELISA) for kinins in tryptic digests of both rat and human plasma and of purified kininogen. In addition, this report also describes a simple solvent system for the complete separation of native and phenylisothiocyanate (PITC)-derivatized kinins on an HPLC C-18 reversed phase column.

MATERIALS AND METHODS

Materials. Bradykinin (BK), Lys-BK, Met-Lys-BK, Ileu-Ser-BK, [Tyr^5]-BK, [Tyr^8]-BK and des-Arg^9-BK were purchased from either Chemical Dynamics (South Plainfield, NJ) or the Sigma Chemical Co. (St Louis, MO). Polystyrene ELISA plates were from Costar (Cambridge, MA). Bovine serum albumin (BSA) and γ-globulins (95–99% pure) were obtained from Sigma. TPCK-treated trypsin was from the Worthington Biochemical Corp. (Freehold, NJ). Citrated (0.38%) normal rat plasma was obtained either from Pel-Freeze (Rogers, AK) or collected from the abdominal aorta. Human plasma was prepared from freshly collected blood. Mouse monoclonal antibodies and BSA–BK conjugate were the same as reported from this laboratory [13]. Goat anti-mouse immunoglobulin horse radish peroxidase conjugate was purchased from Zymed Laboratories (South San Francisco, CA). Sepharose-4B bound papain for affinity chromatography was obtained from United States Biochemicals (Cleveland, OH).

* Presented in part at the Kinin 1987 Tokyo International Congress, November 29–December 3, 1987, Tokyo, Japan.

† Correspondence: Dr Nathan Back, Department of Biochemical Pharmacology, SUNY at Buffalo, School of Pharmacy, 313 Hochstetter Hall, Buffalo, NY 14260.

‡ Abbreviations: BK, bradykinin; ELISA, enzyme-linked immunosorbent assay; PITC, phenylisothiocyanate; PTC, phenylthiocarbamyl; TFA, trifluoroacetic acid; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; L-kininogen, low molecular weight kininogen; H-kininogen, high molecular weight kininogen; BSA, bovine serum albumin; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Preparation of kininogens. Agarose-bound papain was poured into a column and activated by washing with 20 vol. of 0.05 M sodium phosphate, pH 6.8, 2 mM EDTA and 10 mM 2-mercaptoethanol. The activated papain then was S-carboxymethylated on the column at ambient temperature by washing with 20 vol. of the above buffer without 2-mercaptoethanol but containing 10 mM iodoacetic acid. The column finally was equilibrated with 0.05 M sodium phosphate, pH 6.8, 0.5 M NaCl, 3 mM benzamidine HCl, 3 mM EDTA, 1 mM NEM and 0.01% polybrene. The binding capacity of the cm-papain column was determined by using an excess of plasma (10 ml/2 ml bed volume) after diluting with 1 vol. of the above buffer 2× concentrated. The column then was washed with 5 bed volumes of the above buffer and 5 bed volumes of pH 8.5 buffer. Bound kininogens (cysteine proteinase inhibitors) were eluted with 0.05 M sodium phosphate, pH 11.0, and fractions were neutralized with acetic acid. The kininogens thus isolated were purified further on a DEAE-Trisacryl column.

Tryptic digestion of plasma. An optimized standard digestion procedure was developed utilizing 50 μl of plasma. The plasma samples were placed in a 1.5-ml microcentrifuge tube and mixed thoroughly with 50 μl of 1.0 M NaCl followed by 0.9 ml of 0.5% HCl in 2-propanol. Samples were boiled for 10 min and spun in a microcentrifuge for 2 min. The protein precipitate pellets then were suspended in 0.25 ml of 0.05 M sodium phosphate containing 0.15 M NaCl, 5 mM EDTA and 2 mM 1,10-phenanthroline, pH 11.0. The final pH was 8.0. TPCK-treated trypsin, 50 μl of 10 mg/ml in the above buffer at pH 8.0, was added and incubated at 37° for 1 hr. The trypsin was inactivated by boiling the incubation digest for 10 min. After the digest was cooled to room temperature, 50 μl of a 7× concentration of the ELISA inhibitor–protein mixture was added consisting of 21 mM benzamidine–HCl, 14 mM EDTA, 14 mM 1,10-phenanthroline, 7 mM NEM, 1.4% Tween-20, 0.7% BSA and 0.7% bovine γ-globulin, pH 7.4. Samples then were compatible with the ELISA incubation buffer. Digests were spun in a microcentrifuge at maximum speed. Kinins present in the supernatant fraction were determined by ELISA. Kininogen preparations were digested with trypsin as described above after boiling the kininogen solution for 10 min without the HCl/2-propanol mixture.

ELISA of kinins. ELISA manipulations were carried out according to Voller and Bidwell [14]. Optimal concentrations of reagents were determined following the recommendations of Engvall [15]. The kininase inhibitor, 1,10-phenanthroline, 2 mM (0.2 M in a methanol stock), was included in all of the ELISA solutions. Plates were coated with BSA–BK conjugate, 0.5 μg/ml BK equivalent, at 4° overnight in 1.59 g Na_2CO_3:2.93 g $NaHCO_3$ per liter (pH 9.6) containing 0.01% Thimersol. Plates were rinsed with phosphate-buffered saline (PBS)–Tween-20 consisting of 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 2 ml Tween-20 per liter and 0.01% Thimersol, pH 7.4. Non-specific binding surfaces were blocked with 2% BSA in the above buffer for 2 hr at 37°. After the plates were rinsed with the buffer, it was possible to store them at −20° without any noticeable change for at least 4 months. First and second antibody-enzyme conjugate and serial dilutions were carried out in PBS–Tween-20 also containing proteinase inhibitors (3 mM benzamidine–HCl, 2 mM EDTA, 1 mM NEM) and proteins (BSA 0.1% and bovine γ-globulins 0.1%), pH 7.4. The first well contained either 1.0 μg of BK or 100 μl of the tryptic digest and was diluted serially (1:2) using 50-μl aliquots. Monoclonal antibodies, 50 μl of a 1:500 dilution, were added and the final volume (0.15 ml) incubated at 37° for 1 hr. Plates were rinsed three times, and 0.15 ml of a 1:1000 dilution of goat anti-mouse immunoglobulin-peroxidase conjugate was added. The plates were rinsed five times after 1 hr at 37°, and 0.15 ml of *o*-phenylene diamine, 0.2% in 0.1 M sodium citrate (pH 4.2) containing 0.03% H_2O_2, was added. Color development was complete in 30 min at room temperature and was stopped by the addition of 50 μl of 1:4 dilution of concentrated H_2SO_4. Color intensity was measured at 490 nm after dilution of 0.15 ml into 0.8 ml of water.

PITC derivatization of kinins. Kinins, 0.1 to 10 μg in 10 μl of water, were incubated in a 1.5-ml microcentrifuge tube with 90 μl of fresh methanol/triethylamine/phenylisothiocyanate (7:1:0.5) mixture at 37° for 30 min with occasional mixing in a Vortex. In the case of the tryptic digests, an aliquot was removed before the addition of the inhibitor–protein mixture and then dried. The derivatization reaction was stopped by the addition of 500 μl of 2% phosphoric acid in 50% methanol. The PTC-kinins then were separated from the PITC reagent adducts by extraction of the reaction mixture with 0.8 ml of chloroform. After vigorous mixing the phases were separated by brief centrifugation, and the PTC-kinins remaining in the upper aqueous methanol phase were extracted once again with 0.8 ml chloroform in a separate tube. An aliquot representing 50–250 pmol of PTC-kinins was used for the HPLC analysis.

HPLC of kinins. Native and PTC-kinins were separated on a C-18 reversed phase μ-Bondapak (Waters) 3.9 mm × 30 cm column and detected at 214 and 254 nm, respectively. The mobile phase for the native kinins consisted of A: 0.2% trifluoroacetic acid (TFA) in non-organic water and B: 0.15% TFA in acetonitrile. Separation was achieved with TFA-acetonitrile gradient of 17% to 35% in 40 min at a flow rate of 1 ml/min. PTC-kinins were separated using solvents A: 0.1% TFA in water, B: 0.05% TFA in acetonitrile and a TFA–acetonitrile gradient of 25% to 60% in 40 min at a flow rate of 1 ml/min. For determination of immunologic activity, fractions of 0.5 ml were collected, and acetonitrile was allowed to evaporate overnight in the hood. Volumes were reconstituted equal with ELISA buffer, and 50-μl aliquots were used for kinin determination.

The HPLC system consisted of Waters M-45 pumps, a model 441 UV detector, and a solvent mixer installed between a U6K injector and the mixed solvent outlet from the pumps. The gradient programs, data acquisition and analyses were carried out with an IBM compatible Chromatochart-PC system from Interactive Microware (State College, PA)

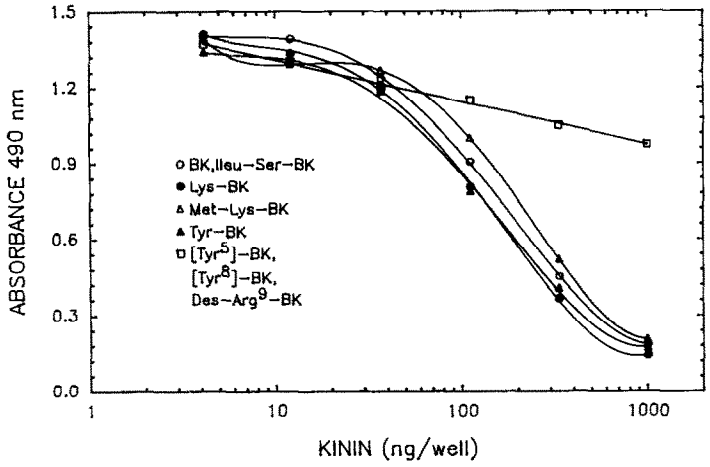

Fig. 1. Typical standard curves obtained with synthetic bradykinin and its analogues in the ELISA with a mixture of monoclonal antibodies D6A5, B6C9 and A3D9.

on a Zenith-148 computer with an Epson EX-1000 printer/Hewlett–Packard 7475A plotter. HPLC reagents were obtained from Pierce (Rockford, IL) and solvents from either Fisher Scientific (Pittsburgh, PA) or J. T. Baker (Phillipsburg, NJ). Non-organic water was prepared by passage of double glass distilled water through a Norganic cartridge (Waters, Milford, MA).

RESULTS

Kinin determination in tryptic digests by ELISA. The properties of the anti-BK mouse monoclonal antibodies prepared in our laboratory, clones D6A5, B6C9 and A3D9, have been described previously [13]. Incubation of various kinin analogues with a mixture of monoclonal antibodies in the ELISA system yielded essentially similar curves with BK, Lys-BK, Met-Lys-BK, Ileu-Ser-BK and Tyr-BK (Fig. 1). [Tyr^5]-BK, [Tyr^8]-BK and des-Arg^9-BK, on the other hand, showed relatively poor interaction. The data indicate that this ELISA test system quantitated intact natural kinins in the range of 10–200 ng under optimized conditions.

The release of kinin from tryptic digests of plasma and purified kininogen was determined by ELISA, as depicted in Fig. 2. Approximately 75% of the kinins were released by 30 min. No further increase in kinin levels occurred after 2 hr. The plasma kinin levels determined as BK for normal rats, rats treated with turpentine and normal humans were 6.9, 70 and 3.2 μg/ml, respectively. In the instance of the purified kininogen, the kinin content was 18.5 μg/mg of kininogen (68 kD). Only amounts less than 0.2 μg/ml were found in the non-trypsin control samples.

ELISA also was used for the detection, as well as quantitation, of kinins during the course of purification of kininogens from plasmas (Fig. 3). Rat plasma proteins were bound to a papain-affinity column and eluted with a pH 11.0 buffer [16] in the presence of protease inhibitors as described in Materials and Methods. These proteins were applied onto a DEAE-Trisacryl column in 0.01 M Tris–HCl, pH 8.0, and developed with a 0 to 0.5 M NaCl gradient in the same buffer. The major protein peak was confirmed by SDS–PAGE to be a 68 kD kininogen showing kinin immunologic activity in ELISA, and papain inhibitory activity [6] (data not shown).

Resolution of kinins by HPLC. Natural kinins as well as nearly all of their synthetic analogues were separated completely within 20 min on a μ-Bondapak C-18 reversed phase column. Trifluoroacetic acid concentrations of 0.15% or more in both water and acetonitrile were required for complete separation. The retention times (min) for the following peptides were: [Tyr^5]-BK, 13.7; [Tyr^8]-BK, 15.0; Lys-Bk, 19.3; BK, 21.3; Met-Lys-BK, 22.4; Ileu-Ser-BK, 23.6; and des-Arg^9-BK, 25.0 (Fig. 4). Synthetic Tyr-BK separated poorly from Ileu-Ser-BK, and des-Arg^1-BK had an identical retention time as that of Met-Lys-BK. ELISA of fractions following HPLC separation of kinins showed an identical profile as obtained by 214 nm monitoring of native kinins (data not shown). A model peptide was included as an internal standard for both retention times as well as peak area reference for computer analysis (data not shown).

PTC-kinins also were separated on a μ-Bondapak column with only water, acetonitrile and TFA mixtures (Fig. 5). In these separations, TFA concentrations in excess of 0.05% were not critical. While natural kinins were separated completely, the [Tyr^5]-BK/[Tyr^8]-BK and Ileu-Ser-BK/Tyr-BK peptide pairs would not separate under the conditions studied. Lys-BK and Met-Lys-BK eluted much later than Ileu-Ser-BK, consistent with their structures, reacting with 2 moles of PITC/mole of kinin and showing twice the peak area of kinins with no lysine. PTC-kinins were retarded more than native kinins. Good quantitation was observed with concentrations of 30 pmol or more of PTC-kinin. The PTC-kinins were relatively stable and underwent 15–20% degradation overnight at room temperature. Derivatization of peptides from tryptic digests with PITC and HPLC analysis revealed large numbers of pep-

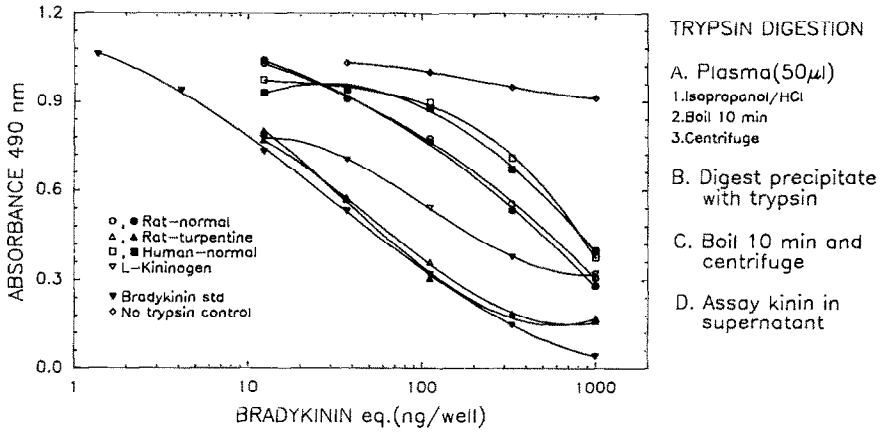

Fig. 2. Determination of kinin in various plasmas and in a purified kininogen preparation by ELISA following trypsin digestion. Plasma from turpentine-treated (0.5 ml/100 g) rats was collected after 48 hr. Kinin content was measured as bradykinin using synthetic standard. Open and closed symbols represent 30-min and 2-hr digestion times, respectively.

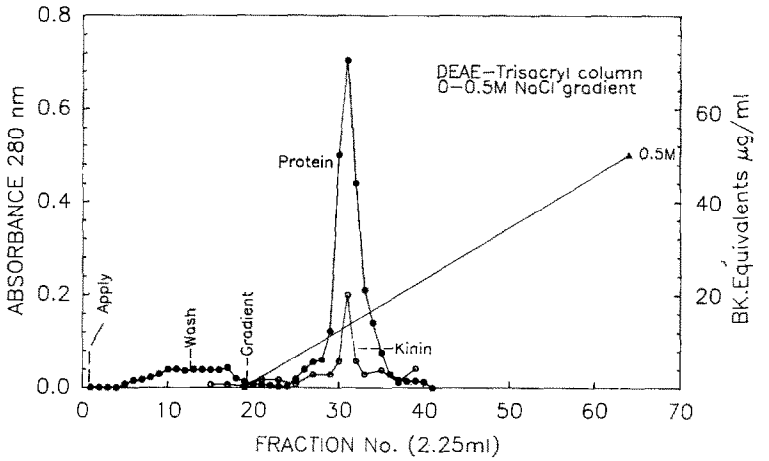

Fig. 3. Determination of kinin immunological activity in column fractions by ELISA. An aliquot (50 μl) was denatured by boiling and digested with trypsin as described above. Kinin was estimated using bradykinin standard. A DEAE-Trisacryl column (1.1 × 17 cm) was equilibrated with 0.01 M Tris-HCl, pH 8.0, and developed with a linear gradient consisting of 50 ml each of buffer and buffer with 0.5 M NaCl.

tides masking the PTC-kinin regions despite the fact that a significant amount of large molecular weight peptides and insoluble peptides in 2% phosphoric acid/50% methanol was removed during the chloroform extraction process (data not shown).

DISCUSSION

The determination of kinins in biologic fluids and tissue most often is carried out by bioassay, a technique that is inconvenient, time consuming, unpredictable and requiring a certain manipulative expertise. The use of radioimmunoassay (RIA) and HPLC as alternative methods has been reported [8, 9, 11]. Homogenous soluble enzyme immunoassay (EIA) using β-galactosidase-labeled BK for differential activation of high and low molecular weight kininogens was found unsuitable for measuring Ileu-Ser-BK in the rat [12]. Other reported ELISA methods were lengthy (about 48 hr) and limited only to a description of standard curves [17].

The tryptic digestion procedure developed for release of kinins from plasmas and the ELISA of kinins reported in this study permit rapid, specific and sensitive estimation of kinins in biologic fluids and tissue as well as in purified kininogen preparations. Furthermore, unlike the BK determination by EIA [18], the present method does not involve initial separation of kinin fractions from the digests but rather estimates kinins directly in the supernatant fraction of the plasma tryptic digest. Treatment of plasma samples with 0.5% HCl in 2-propanol followed by boiling for 10 min assured highly reproducible kinin estimates. The extraction of plasma

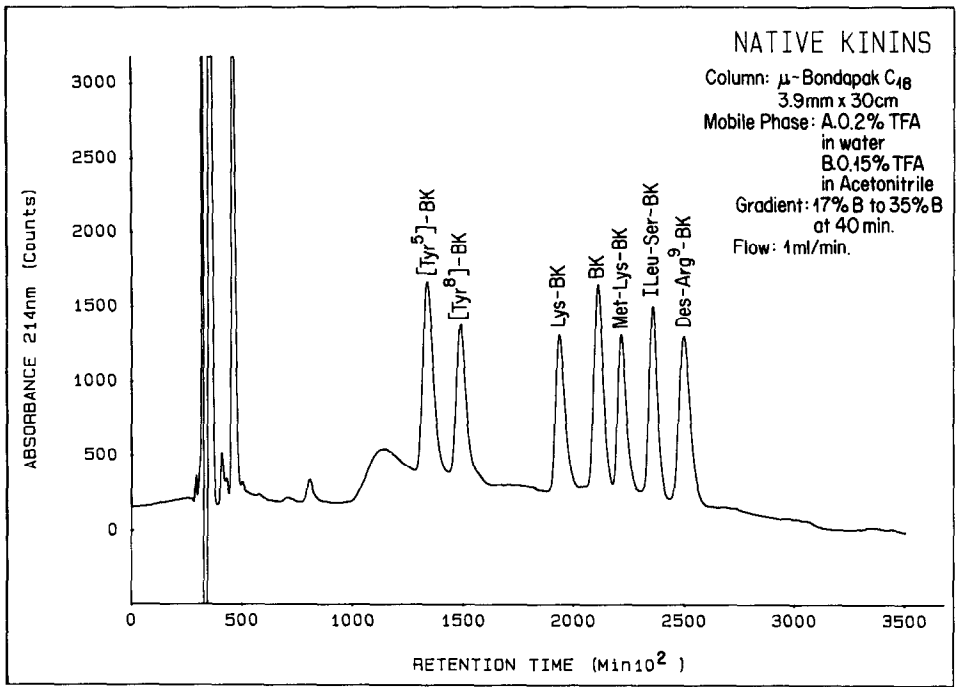

Fig. 4. HPLC separation of native kinin mixture containing BK, Ileu-Ser-BK, Lys-BK, Met-Lys-BK, [Tyr^5]-BK, [Tyr^8]-BK and des-Arg^9-BK. Separation was carried out on a μ-Bondapak C-18 reversed phase column. Native kinins, 1.0 μg each, and BK, 1.5 μg, were injected, and absorbance was recorded at 214 nm at 0.1 AUFS.

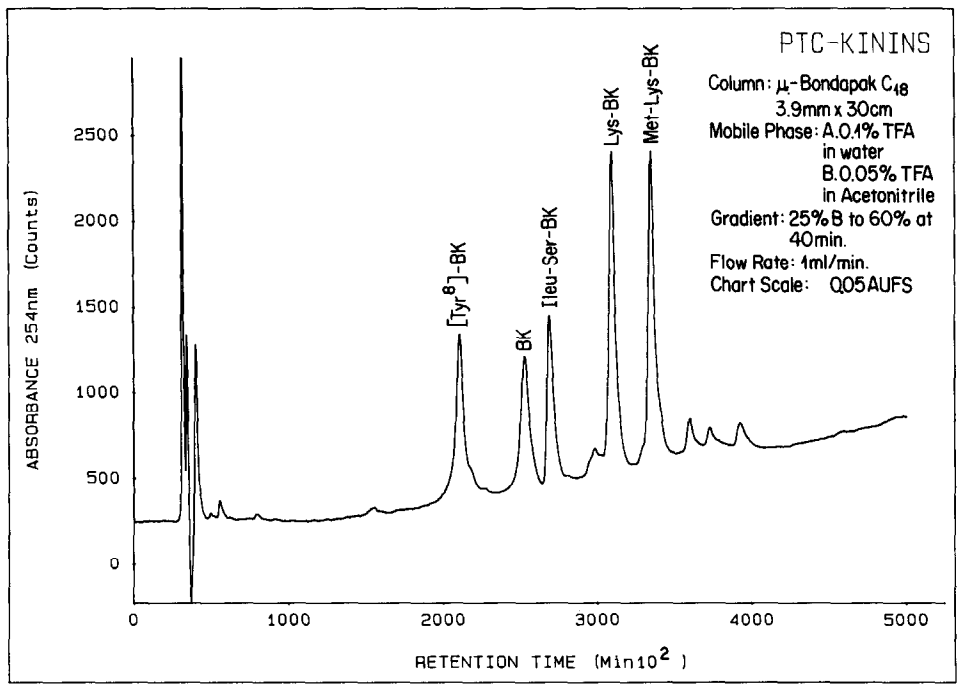

Fig. 5. HPLC separation of a mixture of PITC-derivatized kinins containing BK, Ileu-Ser-BK, Lys-BK, Met-Lys-BK and [Tyr^8]-BK. Separation was carried out on a μ-Bondapak C-18 reversed phase column. PITC-derivatized kinins, 0.25 μg each, were injected, and absorbance was recorded at 254 nm at 0.05 AUFS.

with hot HCl/2-propanol resulted in the complete inactivation of proteinases as well as initial clarification of the sample in preparation for derivatization of the peptides and analysis by HPLC. Relatively high concentrations of trypsin (10 mg/ml plasma) were necessary for the complete release of kinins from plasma collected 48 hr after treatment of rats with turpentine (0.5 ml/100 g body weight). Such turpentine treatment in the rat elevates, by as much as 10- to 20-fold [19], levels of a third low molecular weight kininogen synonymous with the major acute phase rat plasma protein, α_1-cysteine proteinase inhibitor [20], and with T-kininogen in rat adjuvant arthritis [21]. Additionally, a new vasopeptide kinin with the novel Ileu-Ser-amino terminus isolated by Bedi *et al.* [22] from a tumor acid protease-rat kininogen digest and later further characterized [23] also was isolated from rat plasma following treatment with non-catalytic concentrations of trypsin and named T-kinin [24].

The kinin levels from the respective plasmas and kininogen digests reported in this study are in good agreement with accepted kininogen/kinin levels [12, 16, 19, 25]. Turpentine treatment resulted in a 10-fold increase in the plasma kininogen levels. Digestion of normal rat plasma with hog pancreatic kallikrein (200 μg/ml plasma) yielded kinin levels equal to that obtained with trypsin. However, plasma from turpentine-treated rats yielded only 14 μg kinin/ml plasma when digested with the hog pancreatic kallikrein. These results indicate that small amounts ($\approx$ 10%) of the total available Ileu-Ser-BK were released from the "turpentine" plasma by the tissue kallikrein. Previous studies in our laboratory showed that a rat plasma low molecular weight kininogen, purified by ion exchange chromatography [26] and by a single-step passage through an immunoaffinity column prepared with monoclonal antibodies against this kininogen [27], is resistant to kallikrein from rat and human plasma, human urine and hog pancreas [26, 28]. Comparable amounts of kinin are released both by trypsin and an acid protease purified from the rodent Murphy–Sturm lymphosarcoma [29]. T-Kininogen forms subsequently purified from rat adjuvant arthritis plasma also were found resistant to rat submandibular gland kallikrein [30]. Thus, the results demonstrate that, by use of appropriate enzymes, the ELISA system could be used for the differential estimation of different kininogen species present in plasma. The results also show that high concentrations of trypsin do not affect the kinins released during the digestion process.

ELISA conditions were optimized to give about 1.0 absorbance unit in 30 min of color development in non-competing wells. The entire ELISA procedure can be completed within 3–4 hr. Proteinase inhibitors and bovine γ-globulins were included in the ELISA buffer system to prevent kinin degradation during the 37° incubation period and also to prevent background color respectively. With some preparations of goat second antibody-enzyme conjugates, 0.01% of goat γ-globulins was added to the incubation buffers to reduce the non-specific background color. The sensitivity of the ELISA was in the 10–200 ng kinin range, a range amply suitable for kinin measurement in normal plasma (Fig. 2). Furthermore, the kinin ELISA system can be used to advantage to monitor immunologically the kinin activity in fraction samples obtained either during the purification of kininogens or during the separation of kinins on an HPLC column.

Methods for the HPLC resolution of kinins have employed complex mixtures of solvents with the objective of achieving complete separation [10, 31, 32]. In contrast to the studies here reported, the kinins in the previously reported studies were not resolved to baseline. The HPLC method reported for the separation of kinins derivatized with fluorescamine [10] also was unable to resolve these peptides to baseline.

The solvent systems developed in this study are simple, employing only TFA as an additive. TFA concentrations of 0.15% or greater were found necessary for complete separation and base line resolution of those basic peptides presumably due to effective ion pairing/ion exchange with the C-18 matrix at those concentrations.

Detection of peptides at 214 nm is quite non-specific and also subject to various interferences such as accumulated solvent impurities eluting from the column with a gradient and subsequent changes in refractive indices of the solvent in the flow cell. Specific detection of kinins is improved when monitored at 254 nm following PITC derivatization. Presumed interaction between PTC groups and the C-18 column matrix retarded the PTC-kinins much more than the native kinins. Procedures were designed for extraction of basic peptide derivatives into the upper aqueous methanol phase. des-Arg^9-BK could not be extracted into the upper phase, and further extraction with chloroform resulted in higher losses of Lys-BK and Met-Lys-BK than with other kinins. The HPLC profiles of PTC-peptides obtained from plasma tryptic digests were complex and respective kinin regions seriously overlapped. Efforts continue towards direct determination of kinins in the tryptic digests by HPLC rather than by identification and quantitation in the eluted fractions by ELISA or RIA.

The procedures herein described for the tryptic digestion of plasma or protein samples for the ELISA of kinins and HPLC separation of kinins before and after derivatization with PITC should prove of value for the study of the complexities of the kallikrein–kininogen–kinin system.

Acknowledgements—We wish to express our thanks to Dr G. S. Bedi for the preparation of the monoclonal antibody immunoreagents. We wish to express our appreciation to Ms Patricia Poczkalski for her skillful and dedicated typing and transcription of this manuscript. This research was supported in part by USPH Service Grant 5R01CA38270-02.

REFERENCES

1. Schachter M, Kallikreins (kininogenases)—A group of serine proteases with bioregulatory actions. *Pharmacol Rev* **31**: 1–17, 1979.
2. Prado JL, Proteolytic enzymes as kininogenases. In: *Handbook of Experimental Pharmacology* (Ed. Erdos EG), pp. 156–192. Springer, New York, 1970.
3. Miller DH and Margolius HS, Kallikrein–kininogen–

kinin systems. In: *Endocrinology* (Ed. DeGroot LJ), Vol. 3, 2nd Edn. pp. 2491–2503. W. B. Saunders, Philadelphia, 1989.
4. Regoli D and Barabe J, Pharmacology of bradykinin and related kinins. *Pharmacol Rev* **32**: 1–46, 1980.
5. Barrett AJ, Rawlings ND, Davies ME, Machleidt W, Salvesen G and Turk V, Cysteine proteinase inhibitors of the cystatin superfamily. In: *Proteinase Inhibitors* (Eds. Barrett AJ and Salvesen G), pp. 515–569. Elsevier, Amsterdam, 1986.
6. Müller-Esterl W, Iwanaga S and Nakanishi S, Kininogens revisited. *Trends Biochem. Sci.* **11**: 336–339, 1986.
7. Wilkens HJ and Steger R, Agents with kinin-like activity. In: *Screening Methods in Pharmacology* (Eds. Turner RA and Hebborn P), Vol. 2, pp. 61–73. Academic Press, New York, 1971.
8. Goodfriend TL and Odya CE, Bradykinin. In: *Methods of Hormone Radioimmunoassay* (Eds. Jaffe BM and Behrman HR), 2nd Edn, pp. 909–923. Academic Press, New York, 1979.
9. Mashford ML and Roberts ML, Determination of blood kinin levels by radioimmunoassay. *Biochem Pharmacol* **21**: 2727–2735, 1972.
10. Narayanan TK and Greenbaum LM, Detection and quantitation of fluorescamine-labeled bradykinin, its analogues and metabolites using high-performance liquid chromatography. *J Chromatogr* **306**: 109–116, 1984.
11. Fejes-To'th G, Naray-Fejes-To'th A and Frölich JF, Measurements of urinary kinins by HPLC-radioimmunoassay. *Clin Chim Acta* **140**: 21–29, 1984.
12. Uchida Y, Majima M and Katori M, A method of determination of human plasma HMW and LMW kininogen levels by bradykinin enzyme immunoassay. *Pharmacol Res Commun* **18**: 831–834, 1986.
13. Bedi GS and Back N, Monoclonal antibodies to bradykinin inhibit smooth muscle contractile action of bradykinin. *Biochim Biophys Acta* **842**: 90–99, 1985.
14. Voller A and Bidwell D, Enzyme-linked immunosorbent assay. In: *Manual of Clinical Laboratory Immunology* (Eds. Rose NR, Friedman H and Fahey JL), 3rd Edn, pp. 99–109. American Society for Microbiology, Washington, DC, 1986.
15. Engvall E, Enzyme immunoassay ELISA and EMIT. In: *Methods in Enzymology* (Eds. Vunakis HV and Langone JJ), Vol. 70A, pp. 419–439. Academic Press, New York, 1980.
16. Gounaris AD, Brown MA and Barrett AJ, Human plasma α-cysteine proteinase inhibitor. Purification by affinity chromatography, characterization and isolation of an active fragment. *Biochem J* **221**: 445–452, 1984.
17. Geiger R and Miska W, Determination of bradykinin by enzyme immunoassay. In: *Kinins—IV. Proceedings of the Fourth International Kinin Congress, October 21–25, 1984, Savannah, GA, USA. Adv Exp Med Biol* (Eds. Greenbaum LM and Margolius HS), Vol. 198B, pp. 531–536. Plenum Press, New York, 1986.
18. Ueno A, Oh-ishi S, Kitagawa T and Katori M, Enzyme immunoassay of bradykinin using β-D-galactosidase as a labeling enzyme. *Biochem Pharmacol* **30**: 1659–1664, 1981.
19. Esnard F and Gauthier F, Rat α_1-cysteine proteinase inhibitor. An acute phase reactant identical with α_1 acute phase globulin. *J Biol Chem.* **258**: 12443–12447, 1983.
20. Cole T, Inglis AS, Roxburgh CM, Howlett GJ and Schreiber G, Major acute phase α_1-protein of the rat is homologous to bovine kininogen and contains the sequence for bradykinin: Its synthesis is regulated at the mRNA level. *FEBS Lett* **182**: 57–61, 1985.
21. Barlas A, Okamato H and Greenbaum LM, T-Kininogen—The major plasma kininogen in rat adjuvant arthritis. *Biochem Biophys Res Commun* **129**: 280–286, 1985.
22. Bedi GS, Balwierczak J and Back N, Purification and characterization of an acid protease and vasopeptide kinins from Murphy–Sturm lymphosarcoma. In: *Kinins—III. Proceedings of the International Conference "Kinin 81", November 2–5, 1981, Munich, Federal Republic of Germany. Adv Exp Med Biol* (Eds. Fritz H, Back N, Dietze G and Haberland GL), Vol. 156B, pp. 705–726. Plenum Press, New York, 1983.
23. Bedi GS, Balwierczak J and Back N, A new vasopeptide formed by the action of a Murphy–Sturm lymphosarcoma acid protease on rat plasma kininogen. *Biochem Biophys Res Commun* **112**: 621–628, 1983.
24. Okamoto H and Greenbaum LM, Isolation and structure of T-kinin. *Biochem Biophys Res Commun* **112**: 701–708, 1983.
25. Damas J, The novel rat kininogen, T-kininogen. A pro-inflammatory factor or a thiostatin? *Arch Int Physiol Biochim* **95**: 67–73, 1987.
26. Bedi GS, Balwierczak J and Back N, Rodent kinin-forming enzyme systems—I. Purification and characterizations of plasma kininogen. *Biochem Pharmacol* **32**: 2061–2069, 1983.
27. Bedi GS and Back N, Production and characterization of monoclonal antibodies to rat plasma kininogen. *Hybridoma* **6**: 521–526, 1987.
28. Bedi GS and Back N, Single-step purification of "kallikrein-resistant" kininogen from rat plasma using monoclonal-antibody immunoaffinity chromatography. *Prep Biochem* **15**: 159–269, 1985.
29. Bedi GS, Balwierczak J and Back N, Rodent kinin-forming enzyme systems—II. Purification and characterization of an acid protease from Murphy–Sturm lymphosarcoma. *Biochem Pharmacol* **32**: 2071–2077, 1983.
30. Okamoto H and Greenbaum LM, Isolation and properties of two rat plasma T-kininogens. In: *Kinins—IV. Proceedings of the Fourth International Kinin Congress October 21–25, 1984 Savannah, GA, USA. Adv Exp Med Biol* (Eds. Greenbaum LM and Margolius HS), Vol. 198A, pp. 69–75. Plenum Press, New York, 1986.
31. Hayashi I, Kato H, Iwanaga S and Oh-ishi S, Rat plasma high-molecular-weight kininogen. A simple method for purification and its characterization. *J Biol Chem* **260**: 6115–6123, 1985.
32. Mindroiu T, Scicli G, Perini F, Carretero OA and Scicli AG, Identification of a new kinin in human urine. *J Biol Chem* **261**: 7407–7411, 1986.