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Kinetic analysis of the interaction of human tissue kallikrein with single-chain human high and low molecular weight kininogens

(bradykinin/lysyl-bradykinin/human urinary kallikrein/human pancreatic kallikrein)

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ABSTRACT **Human low molecular weight kininogen (LMWK) and high molecular weight kininogen (HMWK) have been purified to apparent homogeneity as intact, single-chain molecules. When they interacted with homologous urinary kallikrein, 0.9 mol of kinin per mol of substrate was released from LMWK and 0.7 mol of kinin per mol of substrate was released from HMWK. These functionally and structurally intact substrates have been used to obtain the kinetic constants for kinin release by purified human tissue kallikreins. With human urinary kallikrein, apparent second-order rate constants (k_{cat}/K_m) of 1.46×10^5, 8.6×10^4, and 5.08×10^4 $M^{-1} \cdot s^{-1}$ were obtained with LMWK, HMWK, and α-*N*-*p*-tosyl-L-arginine methyl ester (TAMe), respectively; with human pancreatic kallikrein, values of 8.7×10^3 and 7.3×10^4 $M^{-1} \cdot s^{-1}$ were obtained with HMWK and TAMe. These values, which are comparable to those obtained for other enzyme–protein substrate interactions, indicate that LMWK is only slightly preferred to HMWK as the natural substrate for urinary kallikrein and that HMWK is a somewhat better substrate for urinary kallikrein than for pancreatic kallikrein. Although the data obtained have been shown by $NaDodSO_4$/polyacrylamide gel electrophoresis to reflect cleavage of the substrate at two points, the linear Lineweaver–Burk plots suggest that one cleavage is rate limiting. Because the plasma concentrations of both LMWK and HMWK are approximately 1/10th the K_m values obtained, substrate concentration may also play a role in determining the rate at which tissue kallikreins release kinins from kininogen substrates either in the circulation or extravascularly.**

Kininogens are plasma proteins that contain the peptide sequence of the vasoactive kinin polypeptides bradykinin (BK) and lysyl-BK. These peptides are released from their precursor molecules by limited proteolysis by enzymes known as kallikreins or kininogenases (EC 3.4.21.8). Human plasma contains two distinct forms of kininogen with M_r 120,000 (1–5) and 50,000–80,000 (6–9), designated high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK), respectively. HMWK, together with either plasma prekallikrein or factor XI, is an important component in contact activation of the Hageman factor-dependent pathways (10–13) and, in contrast to LMWK (6, 8), serves as a substrate for both tissue and plasma kallikreins.

The kinin moiety is located internally in bovine HMWK and LMWK, and its release from HMWK by bovine plasma kallikrein and from LMWK by snake venom kininogenase results in the formation of two-chain disulfide-linked "kinin-free" kininogens (14). The NH_2-terminal chains (termed "heavy chains") of the cleaved bovine HMWK and LMWK appear, by size, amino acid composition, and antigenic analysis, to have a high degree of homology, whereas their COOH-terminal chains ("light" chains) are clearly different by the same criteria. Cleavage of human HMWK by human plasma kallikrein or tissue kallikreins from saliva and urine also results in the formation of a two-chain, disulfide-linked kinin-free HMWK (3, 5, 15–17). Antibody specific for the human HMWK NH_2-terminal chain recognizes both HMWK and LMWK, whereas antibody specific for the HMWK COOH-terminal chain recognizes only HMWK (18). Furthermore, the COOH-terminal chains of both bovine and human HMWK exhibit all the procoagulant activity of the intact molecule (2, 19, 20) whereas human and bovine LMWK lack this activity (10, 20), suggesting that the features that distinguish bovine HMWK and LMWK are also characteristics of human HMWK and LMWK.

Glandular (tissue) kallikreins, which are found in kidney, pancreas, and salivary glands and in their secretions, are structurally, functionally, and antigenically different from plasma kallikrein but are related to each other (21–25). Glandular kallikrein has also been identified antigenically in human (26) and rat (27, 28) plasma, and the glandular kallikrein extracted from human plasma by a procedure that included immunoaffinity chromatography cleaved both synthetic substrates and HMWK (29). A single report (30) of the interaction of a glandular kallikrein—human salivary kallikrein—with human kininogen described the time-dependent cleavage of HMWK but did not present a detailed kinetic analysis or describe cleavage of LMWK.

The difficulty in purifying LMWK as a fully active molecule free of albumin (31), some α-globulins of similar size and charge (32), and plasminogen (33, 34) has precluded adequate kinetic studies of human LMWK–enzyme interactions. Hence, the previous studies with human LMWK have used partially purified kininogen isolated under denaturing conditions (33, 34). Purification of human LMWK to apparent homogeneity by a reproducible six-step procedure (9) and the availability of apparently homogeneous human HMWK (5) and tissue kallikreins (35–37) now permit the comparative kinetic analyses of kininogen cleavage by these enzymes.

MATERIALS AND METHODS

Reagents. Sources were as follows: human albumin (purest), Behringwerke AG (Marburg, Federal Republic of Germany);

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Abbreviations: HMWK, high molecular weight kininogen; LMWK, low molecular weight kininogen; TAMe, α-*N*-*p*-tosyl-L-arginine methyl ester; LBTI, lima bean trypsin inhibitor; iPr_2P-F, diisopropylfluorophosphate; BK, bradykinin; HUK, human urinary kallikrein; HPK, human pancreatic kallikrein.

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Polybrene (hexadimethrine bromide), Aldrich; diisopropylfluorophosphate (iPr_2P-F), Calbiochem–Behring; α-*N*-*p*-tosyl-L-arginine methylester (TAMe) and benzamidine, Sigma; BK and lysyl BK, Bachem Fine Chemicals (Torrance, CA).

Assays. Kinin generation was measured on the guinea pig terminal ileum suspended in 5 ml of Tyrode's solution at 37°C (38). BK and lysyl-BK standards were quantified by amino acid analysis. Twenty-five-microliter samples (1 mg/ml) were hydrolyzed in 6 M HCl for 24 or 48 hr at 110°C, 2% of the starting material was assayed in a Beckman 121MB amino acid analyzer, and the BK and lysyl-BK concentrations were calculated from the amino acid values obtained after extrapolation to zero hydrolysis time. In the bioassay, the dose–response curves for BK and lysyl-BK were parallel and lysyl-BK had 83–85% of the activity of equimolar concentrations of BK. In kinetic studies of kinin generation with purified kininogens and purified kallikreins, the reactants were incubated separately for 5 min at 37°C, mixed, and incubated for another 5 min at 37°C (unless otherwise stated), and the generated kinin was assayed immediately. The response of the guinea pig ileum was standardized after every fifth sample with at least four doses of synthetic BK. A smooth muscle preparation was used only if it responded to 2–5 ng of BK added to the organ bath and gave a linear dose–response curve up to 50 ng of BK. Kallikrein esterolytic activity was determined by direct spectrophotometric analysis of TAMe cleavage (39). Protein was measured by the method of Lowry *et al.* (40) with human serum albumin, calibrated on the basis of its extinction coefficient (41), as the standard.

Enzyme Purification. Human urinary kallikrein (HUK) was purified from pooled fresh urine by affinity chromatography on aprotinin-CH-Sepharose and gel filtration on Sepadex G-100 (35). The final product revealed a single band with M_r 48,000 on NaDodSO$_4$/polyacrylamide gel electrophoresis with and without prior reduction; a single stained protein band on alkaline gel electrophoresis with the same mobility as kallikrein function and antigen identified in eluates from a sliced gel run in parallel; and the same amino acid composition and single NH_2-terminal residue sequence as reported by others for HUK (42–44). The kinetic constants with TAMe as substrate (39) at 25°C and pH 8.0, shown in Table 1, agree with those reported by others (45). The purified enzyme released 925 μg of BK equivalents per min per mg of enzyme protein from an excess of heat-inactivated human plasma (46).

Human pancreatic kallikrein (HPK) was purified from pancreas obtained at autopsy within 6 hr of death. Homogenization, freeze-thawing, and isoelectric precipitation were performed as described (36). The supernatant from the isoelectric precipitation step, which contained all the detectable glandular kallikrein antigen as assessed by Ouchterlony diffusion against anti-urokallikrein (47), was filtered on lima bean trypsin inhibitor-CH-Sepharose to remove remaining trypsin and chymotrypsin and then subjected to chromatography on hydroxylapatite (37). The active fractions were pooled, concentrated, and subjected to aprotinin-CH-Sepharose affinity chromatography and Sephadex G-100 gel filtration as described (36). HPK eluted as a single, superimposable peak of kinin-generating and TAMe-esterase activities with an apparent M_r of 50,000. Alkaline polyacrylamide gel electrophoresis of 35 μg of the purified HPK revealed a single protein band stained with Coomassie blue, with the same mobility as the kinin-generating and [^{3}H]TAMe-esterase (48) activities eluted from slices of a duplicate gel. NaDodSO$_4$ gel electrophoresis of 30 μg of the HPK with or without prior reduction yielded a single stained protein band with M_r = 52,000. The kinetic constants are shown in Table 1. The purified enzyme released 740 μg BK equivalents per min per mg of enzyme protein from an excess of heat-inactivated plasma.

Kininogen Purification. HMWK was isolated as described (5) from citrated human plasma which was made 0.02 M in benzamidine, 0.01 M in Na_2EDTA, 0.01 M in iPr_2P-F and contained Polybrene at 50 mg/liter. It was used either fresh or after storage at −70°C for no more than 3 weeks. Briefly, 3 liters of plasma was subjected to stepwise chromatography on QAE-Sephadex A-50 at room temperature and pH 8.0, and then to linear salt gradient elution from SP-Sephadex C-50 at pH 5.3 at 4°C and from QAE-Sephadex A-50 at pH 8.0 at 4°C. Both kinin generation and procoagulant activities were followed at each step (5). The purified HMWK had a specific clotting activity of 18.1 units/mg of protein and contained 5.2 μg of kinin per mg of HMWK (0.63 mol/mol) as assessed with purified HUK after 5 min of incubation and 0.7 mol/mol after 30 min. Twenty-five micrograms migrated on NaDodSO$_4$/polyacrylamide gel electrophoresis as a single major protein band of apparent M_r 120,000 with or without prior reduction. No plasma prekallikrein, Hageman factor, or factor XI was detected by clotting assay with the appropriate deficient plasmas; no $\overline{C1}$INH, α_2-macroglobulin, α_1-antitrypsin, antithrombin III, or inter$_\alpha$-trypsin inhibitor was detected by Ouchterlony analysis of 20 μg of HMWK protein (5).

The initial stepwise QAE-Sephadex A-50 fractionation of inhibitor-treated plasma also yielded a pool of LMWK as defined by the capacity to release kinin in the presence of HUK and to be immunoprecipitated by anti-kininogen antiserum (31) and by the failure to correct the coagulation defect in HMWK-deficient plasma (9). In the six-step LMWK isolation procedure, which was carried out entirely between pH 7 and 8, the QAE-Sephadex chromatography was followed by reverse ammonium sulfate gradient solubilization, hydrophobic chromatography on phenyl-Sepharose, gel filtration through Sephadex G-200, and removal of the remaining contaminants by binding them to Affi-Gel blue and to zinc (9). The specific functional activity of the purified human LMWK was 0.8 mol of kinin per mol of substrate after a 5-min incubation with HUK and 0.9 mol/mol after a 30-min incubation. On alkaline polyacrylamide gel electrophoresis of 18 μg of the purified human LMWK, the position of the single stained protein band corresponded to the only region of the replicate gels from which functional substrate could be eluted. Twenty-two micrograms of unreduced LMWK appeared as a single stained band of M_r 65,000 on NaDodSO$_4$/polyacrylamide gel electrophoresis. With reduction, the mobility of the protein reflected a M_r of 68,000, further indicating that the LMWK was purified as the intact single polypeptide chain.

RESULTS

The rate of kinin release from HMWK and LMWK by glandular kallikreins was determined by mixing prewarmed reagents and incubating them at 37°C and pH 8.0 (0.1 M Tris·HCl) for various times. With either substrate, a linear relationship between kinin release and incubation time was obtained beyond the 5-min incubation period usually used for further experiments. To determine the pH dependence, replicate samples of HMWK (25 μl) and LMWK (50 μl) in 3 mM sodium phosphate, pH 7.4/0.15 M NaCl were adjusted to varying pH values by the addition of 20 or 40 μl of 0.1 M H_3PO_4/K_3PO_4 solutions with pH values between 2.0 and 12.0 adjusted to a conductivity of 40 mS with KCl. After preincubation at 37°C for 5 min, 2–10 μl of prewarmed enzyme in 0.05 M Tris/0.15 M NaCl, pH 8.0, was added, incubation was continued for another 5 min, and the released kinin was assayed immediately.

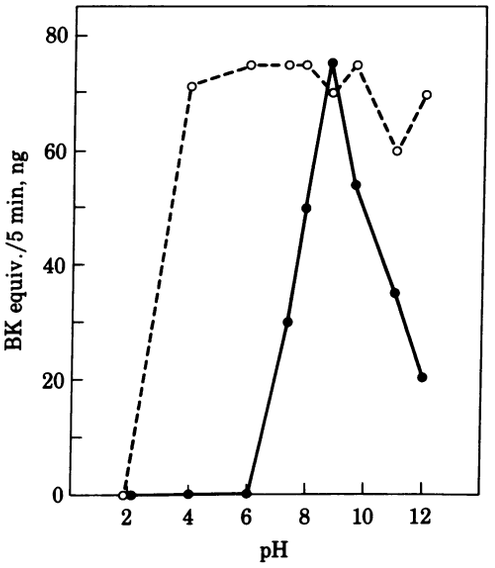

FIG. 1. Effect of pH on the release of kinin from HMWK by HUK. Enzyme and substrate were incubated together at the indicated pH (●) or the enzyme was preincubated at the indicated pH and allowed to react with substrate at pH 8.8 (○).

When HUK was used as the enzyme, there was a sharp optimum for generation of kinin at pH 8.8 with either HMWK (Fig. 1) or LMWK (Fig. 2) as the substrate. When HPK and HMWK were incubated together at various pH values, there was a broad peak of kinin release between pH 7.9 and 9.6 (Fig. 3). Preincubation of HUK at the different pH values followed by interaction with substrate at pH 8.8 yielded maximal activity between pH 4.0 and 12.0 (Fig. 1). In contrast, preincubation of HPK before interaction with HMWK at pH 8.8 showed a marked loss of activity below pH 6.5 (Fig. 3).

With the optimal pH conditions for kinin release by tissue kallikreins established, K_m, V_{max}, and k_{cat} were determined for HMWK and LMWK. Prewarmed samples (ranging from 8 to 90 μl) of 10 μM HMWK were incubated with 10 μl of HUK (54 ng) or 10 μl of HPK (330 ng) in 0.1 M Tris·HCl (pH 8.8) in a final volume of 100 μl for 5 min at 37°C, and the generated kinin was immediately determined by bioassay. A Lineweaver–Burk plot of the data obtained with HUK is shown in Fig. 4 *Upper* and with HPK in Fig. 4 *Lower*. Velocity is given as mol of BK equiv. generated per min per mg of enzyme. From these calculated plots the kinetic constants were determined (Table 1).

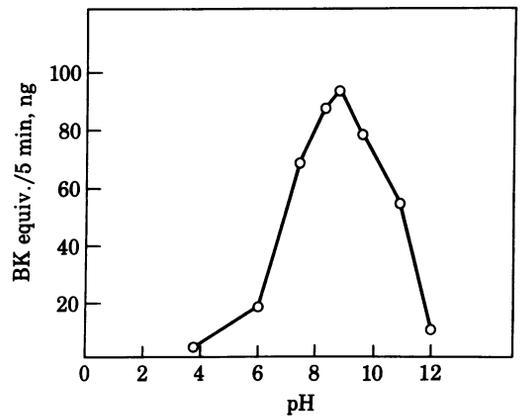

FIG. 2. Effect of pH on the release of kinin from LMWK by HUK. Enzyme and substrate were incubated together at the indicated pH.

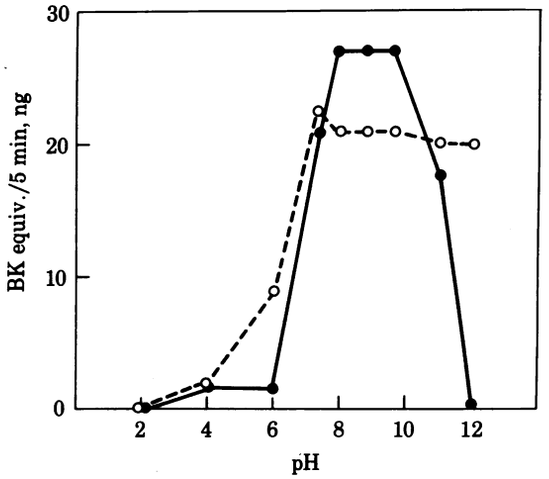

FIG. 3. Effect of pH on the release of kinin from purified HMWK by purified HPK. Enzyme and substrate were incubated together at the indicated pH (●), or the enzyme was preincubated at the indicated pH and incubated with substrate at pH 8.8 (○).

To determine K_m and V_{max} of kinin release from LMWK, prewarmed samples (12.5, 15, 20, 25, and 35 μl) of 30.6 μM LMWK were incubated with 5 μl of HUK (27 ng) in 0.1 M Tris·HCl (pH 8.8). Final reaction volume was maintained at 50 μl with the same buffer. A Lineweaver–Burk plot in which velocity is given as mol of BK equiv. generated per min per mg of enzyme is shown in Fig. 5 and the kinetic constants for HUK are given in Table 1.

DISCUSSION

The availability of HMWK and LMWK, purified to apparent homogeneity as single-chain proteins (5, 9) that were fully active with homologous tissue kallikreins (35–37), has permitted

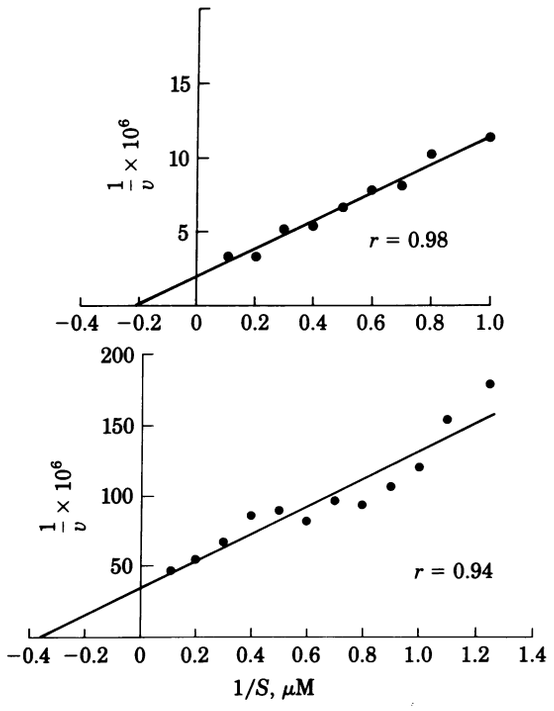

FIG. 4. Lineweaver–Burk analysis of the release of kinin from HMWK by HUK (*Upper*) and HPK (*Lower*). Each point represents the mean of duplicate analyses.

Table 1. Kinetic constants determined for the cleavage of HMWK, LMWK, and TAMe by purified human tissue kallikreins

	TAMe		HMWK		LMWK
	HUK	HPK	HUK	HPK	HUK
K_m, M × 10^5	10.0	14.2	0.5	0.285	1.25
V_{max}, mol/min/mg × 10^6	6.1	12.0	0.52	0.029	2.2
k_{cat}, s^{-1}	5.08	10.5	0.43	0.025	1.83
$\frac{k_{cat}}{K_m}$, $M^{-1}\cdot s^{-1}$ × 10^{-4}	5.08	7.3	8.6	0.87	14.6

kinetic analysis of these interactions as assessed by release of the active kinin moiety. The kinetic constants for the release of kinin by HUK from LMWK and HMWK and for the hydrolysis of TAMe by HUK are compared in Table 1. The turnover number for LMWK was >4 times that of HMWK but approximately 1/3rd that of the synthetic substrate, suggesting that under optimal conditions LMWK appears to be the preferred natural substrate for HUK. However, when k_{cat}/K_m was calculated to relate the reaction rate to the concentration of free rather than bound enzyme (49), as is the case for low substrate concentrations, the values for each of the two natural substrates and for TAMe varied by <3-fold, and LMWK appeared to be only slightly preferred as the natural substrate for HUK. Of the previous reports of human kininogen purification, only one included kinetic analyses of the substrates with kininogenase enzymes (33). However, the purification procedure (which included immunoaffinity elution with 8 M guanidine) yielded 15 different sizes and charge forms, the structural integrity of the two subforms selected for kinetic analysis was not documented by $NaDodSO_4$ analysis (33), and the LMWK subform was subsequently shown to be contaminated with plasminogen (34).

Although comparative studies of the human glandular kallikreins have been conducted in terms of the kinetic constants with synthetic substrates and relative susceptibility to various inhibitors (reviewed in ref. 50), comparable studies with purified native substrate have not been reported. HPK purified to apparent homogeneity (36) exhibited a V_{max} and k_{cat} with TAMe that were somewhat higher than for HUK. In contrast, with HMWK (Table 1) the K_m of HPK was approximately half that of the HUK, the V_{max} was 1/18th, and the k_{cat} was 1/20th that of HUK (Fig. 4). The k_{cat}/K_m ratios, which relate the rate of HMWK cleavage to the concentration of free enzyme, were 8.6 × 10^4 $M^{-1}\cdot s^{-1}$ and 8.7 × 10^3 $M^{-1}\cdot s^{-1}$ for HUK and HPK, respectively. Although these values reflect cleavage at two sites in HMWK, the k_{cat}/K_m for HUK cleavage of this substrate is similar to the results obtained for TAMe hydrolysis by HUK (5.08 × 10^4 $M^{-1}\cdot s^{-1}$) or HPK (7.3 × 10^4 $M^{-1}\cdot s^{-1}$). Thus, all the kinetic data obtained suggest that HMWK is a somewhat better substrate for HUK than for HPK. The two enzymes also differed in that HPK was rapidly inactivated at acidic pH but HUK was not (Figs. 1 and 3).

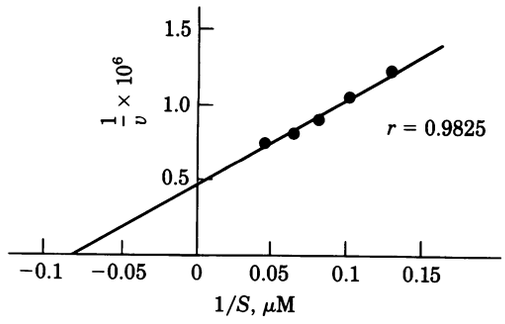

FIG. 5. Lineweaver–Burk analysis of the release of kinin from LMWK by HUK. Each point represents the mean of duplicate analyses.

The apparent second-order rate constants (k_{cat}/K_m) for the interaction of a number of enzymes with substrates such as acetylcholine or H_2O_2 range from 10^7 to 10^8 $M^{-1}\cdot s^{-1}$ (reviewed in ref. 49). Recent kinetic analyses of the interaction of enzymes with higher molecular weight protein substrates, such as urokinase with human or canine plasminogens (51, 52), cobra venom factor-dependent C3 convertase with the human third component of complement (C3) (53), and factor XI_a with human coagulation factor IX (54), have yielded k_{cat}/K_m values ranging from 2.2 × 10^3 to 4.6 × 10^5 $M^{-1}\cdot s^{-1}$, similar to the range of values obtained with tissue kallikreins and LMWK and HMWK. Of particular note is the fact that the release of an activation peptide from factor IX by factor XI_a, which requires cleavage at two sites within the substrate (54), yielded values similar to those obtained in the present study for the release of kinin from kininogens. Although the kinetic data obtained (Figs. 4 and 5) reflect the overall reaction leading to the release of biologically active kinin, the linear plots observed suggest that one cleavage reaction is rate limiting. Because "nicked" kininogens have undergone a single cleavage on the COOH-terminal side of the kinin moiety (30; reviewed in ref. 33), it is likely that the rate-limiting cleavage occurs at the NH_2-terminal side of the kinin moiety. The plasma concentrations of HMWK and LMWK, approximately 0.6 μM (15) and 2 μM (55), respectively, are 1 order of magnitude less than the K_m values for the interactions of tissue kallikreins with these substrates (Table 1), indicating that substrate limitation may also play a role *in vivo* in determining the rate at which kinins are liberated both in the circulation and extravascularly.

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