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RAT LIVER KININASE, A SERINE PEPTIDASE*

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Abstract—A serine peptidase (RLK_1) was partially purified from rat liver homogenates. Its molecular weight was 80,000, and its optimum pH was 7.5. Bz-Tyr-O-Et was hydrolyzed by the enzyme, which was inhibited by $I_{p2}PF$, PMSF and by Tos-Phe-CH_2Cl. The bonds cleaved by the enzyme were Phe^5-Ser^6 and Phe^8-Arg^9, when bradykinin was used as substrate.

Kininases are proteolytic enzymes which are able to promote inactivation of bradykinin (Arg^1-Pro^2-Pro^3-Gly^4-Phe^5-Ser^6-Pro^7-Phe^8-Arg^9) and related kinins [1, 2]. The best studied plasma kininases are kininase I and II, the first being a carboxypeptidase with preference for basic amino acids, such as pancreatic carboxypeptidase B, but displaying some important differences [3], and the second enzyme being an angiotension-converting enzyme (peptidyl hydrolase, EC 3.4.15.1), which initially releases the dipeptide Phe^8-Arg^9 from bradykinin[4]. The rabbit brain also contains two kininases, A and B, both of which are SH-dependent enzymes that split Phe^5-Ser^6 and Pro^7-Phe^8 bonds respectively [5, 6]. Although lung has been considered the major site for inactivation of bradykinin [7], it was demonstrated recently that rat liver has a considerable capacity to inactivate bradykinin when perfused with this peptide [8]. The initial results suggested that the hydrolysis was promoted mainly by an angiotensin-converting enzyme, but later results indicated that the enzyme is a thiol-dependent endopeptidase similar to the rabbit brain kininase A and, thus, splits bradykinin at the Phe^5-Ser^6 bond [9]. Liver homogenates inactivate bradykinin at neutral pH more rapidly than does perfused liver [9], and this fact indicates that liver cells may contain kininases other than those described previously. The aim of this paper is to describe one of these neutral kininases.

MATERIALS AND METHODS

Bradykinin (BK), Lys-bradykinin (LBK), Met-Lys-bradykinin (MLBK), BK-fragments, PCA-Lys-Trp-Ala-Pro (BPP_5) and angiotensin were prepared by solid-phase synthesis and supplied by Dr. A. C. M. Paiva from the Department of Biophysics of the Escola Paulista de Medicina. Hippuryl-L-arginine, di-isopropyl-fluor phosphate ($I_{p2}PF$), D-tryptophan, lysyl-β-naphthylamide, leucyl-β-naphthylamide, tosylarginine methyl-ester (Tos-Arg-OMe) and benzoyl-Tyr-O-Et (BTEE) were from the Sigma Chemical Co. (St. Louis, MO). Sephadex G-25, G-100 and Sepharose 4B were Pharmacia (Piscotaway, NJ) products; Cellex-D and Aminex A6 were purchased from Bio-Rad (Richmond, CA). Tosyl-lysyl-chloromethylketone (Tos-Lys-CH_2Cl), tosyl-phenylalanyl-chloromethylketone (Tos-Phe-CH_2Cl) and phenyl-methane sulfonyl fluoride (PMSF) were from Cyclo (Los Angeles, CA). The amino acid standards and the reagents for the analyzer were from the Pierce Co. (Rockford, IL). Chymotrypsin, trypsin, aprotin and carboxypeptidase B were products from Worthington (Freehold, NJ). Human plasma kallikrein was prepared by a procedure described previously [10]. High molecular weight human kininogen was prepared by modification of the procedure of Kerbiriou and Griffin [11]. D-Tryptophan methyl ester-Sepharose-4B was prepared by following the procedure used by Cuatrecasas [12].

Kininase was prepared as follows: in a typical preparation, five Nembutal-anesthesized Wistar rats, weighing 200–300 g, had their livers perfused *in situ* with cold 0.14 M NaCl, until there was apparent removal of blood. The organs (about 10 g wet weight each) were then rapidly withdrawn from the abdominal cavities, finely chopped, and homogenized in 0.025 M Tris–HCl (pH 7.4) containing 0.25 M sucrose, with a Virtis homogenizer, in a cold room. The homogenates were centrifuged at 40,000 *g*, 5°, and the supernatant fraction was subjected to ammonium sulfate fractionation between 2.09 and 2.95 M. The subsequent desalting was performed in a 2.7 × 60 cm Sephadex G-25 column, equilibrated with 0.025 M Tris–HCl (pH 7.4), 20°, and run at a flow rate of 15 ml/hr, and the desalted material was collected in the void volume of the column (127 ml). In the next step, 100 ml of this material was applied to a DEAE-cellulose column (1.8 × 10 cm) equilibrated with the same Tris buffer and developed at a flow rate of 60 ml/hr, at room temperature, under stepwise KCl concentrations, with the effluent collected in 6 ml fractions.

Kininase activity was measured by the remaining

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biological activity of bradykinin or its homologs, or isolated guinea pig ileum [13]. Routinely, BK (1 μg/ml) was incubated with the enzyme preparation in 0.025 M Tris–HCl buffer (pH 7.4), 37°, and the biological activity was compared at 1, 4, 7 and 10 min with the BK control. One unit of the kininase was defined as the amount of enzyme which hydrolyzed 1 nmole BK/min under the conditions described. In the experiments performed in the presence of compounds that interfered with the bioassay, appropriate controls were run. Kininase activity was also measured using an excess of kinin (30–50 μg/ml), essentially as described previously [13]. Angiotensinase activity was tested in the same way as BK. Esterolytic activity and carboxypeptidase and arylamidase activities were measured by procedures described previously [10, 14]. Caseinolytic activity was determined by incubation of 1% casein in 0.1 M phosphate buffer (pH 8.0) at 30°, in a final volume of 1 ml for 1 hr, followed by measurement of trichloroacetic acid (TCA) soluble products at 280 nm. Proteins were measured by absorbance at 280 nm. After incubation of 150 nmoles of BK with 6.0 μl of 0.025 M Tris–HCl (pH 7.4), 30°, for 3 hr, analysis of BK fragments was performed in an automatic amino acid analyzer at 80°, with a special buffer system for bradykinin fragments: 160 ml of 0.65 N citrate buffer (pH 4.20) and 70 ml of 0.39 N citrate buffer (pH 7.25) at a flow rate of 1 ml/min in a short column (0.9 × 17 cm) of Aminex A6 [5].

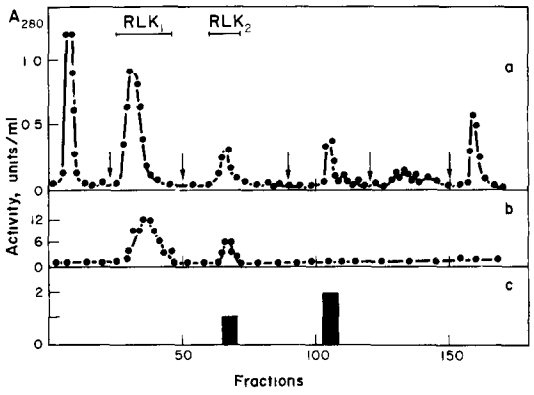

Fig. 1. DEAE-cellulose chromatography of rat liver kininase (column, 1.8 × 10 cm) in 0.025 M Tris–HCl (pH 7.4), 20°; sample (100 ml) from Sephadex G-25 (see Table 1). Key: (a) protein profile; (b) kininase activity expressed in nmoles of BK inactivated per min per ml of enzyme solution (units/ml); and (c) relative arylamidase activity upon Lys-naphthylamide, expressed in arbitary units per ml. The arrows indicate increasing amounts of KCl (0.1, 0.2, 0.3, 0.4 and 0.5 M KCl). The bars indicate the pools of the kininase activity (RLK_1 and RLK_2 respectively).

RESULTS

The kininase recovered from the 40,000 *g* supernatant fraction represented about 75% of the total activity in the homogenates. From 15 to 20% of the activity could be extracted from that precipitated by Triton X-100, and it was verified that angiotensin-converting enzyme accounts for most of this kinin-destroying activity, either by direct conversion of angiotensin I to angiotensin II or by the formation of Phe-Arg during the hydrolysis of bradykinin. Table 1 summarizes a process for the rapid preparation of the enzyme by a procedure involving primarily ion-exchange chromatography. The kinin-destroying activity eluted in two peaks in DEAE-cellulose (Fig. 1), the first one in an ionic strength of 0.1 M KCl (RLK_1) and the second one in 0.2 M KCl (RLK_2). The RLK_1 was devoid of aminopeptidase activity and no angiotensin-converting activity was detected under the conditions in which BK was hydrolyzed. The RLK_2 was associated with an arylamidase which acted on neutral and basic amino acids. Further attempts at purification of RLK_1 were made using gel filtration or other conditions of ion-exchange, including gradient, but the recovery of the activity after these steps was quite low due to the instability of the enzyme. Affinity chromatography using D-tryptophan methyl ester bound to Sepharose-4B was not effective.

RLK_1 was shown to be extremely labile also during storage, and the best conditions found to preserve its activity were 0.025 M Tris–HCl (pH 7.4) at 0° without freezing. Under these conditions the activity was constant for about 2 weeks. No procedure was found to restore lost activity once inactivation had occurred.

The relative molecular weight of RLK_1 was estimated using a calibrated Sephadex G-150 column, to be 80,000 (Fig. 2). The second peak from DEAE-cellulose (RLK_2) eluted right after the void volume of the column, indicating a molecular weight of over 150,000. The recovery of RLK_1 by chromatography was less than 15%, and the specific activity had decreased.

The homogenates showed two pH optima for the hydrolysis of BK (Fig. 3), one between 5 and 6 and

Table 1. Purification of rat liver kininase

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity* (units/mg protein)	Yield (%)	Purification
Supernatant fraction (40,000 *g*)	140	4.144	5.800	1.4	(100)	(1)
$(NH_4)_2SO_4$ fractionation	26	780	3.360	4.3	58	3.1
Sephadex G-25	100	480	2.600	5.4	45	3.9
DEAE-cellulose (RLK_1)	210	50.4	1.620	32.0	28	22.9
DEAE-cellulose (RLK_1)	115	18.4	232	12.6	4	9.0

* One unit is one nmole of BK (1 μg/ml) hydrolyzed per minute in 0.025 M Tris–HCl (pH 7.4), 30°.

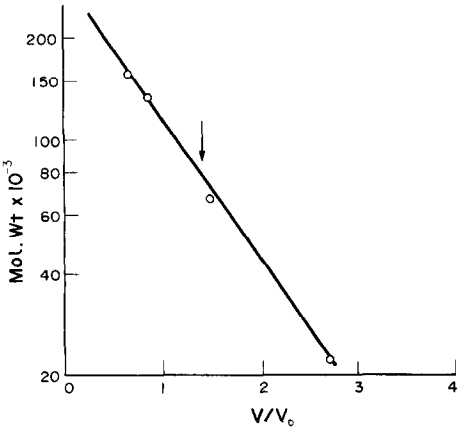

Fig. 2. Determination of the relative molecular weight of RLK_1. Gel filtration was in a Sephadex G-150 column (2.2 × 100 cm) equilibrated with 0.025 M Tris–HCl buffer (pH 7.4). The flow rate was 35 ml/hr; the void volume was 99 ml. Standards: γ-globulin (170,000), bovine serum albumin (dimer: 134,000, and manomer: 67,000), and soybean-trypsin inhibitor (Kunitz: 22,000); 10 mg of RLK_1 was applied in a final volume of 2.0 ml.

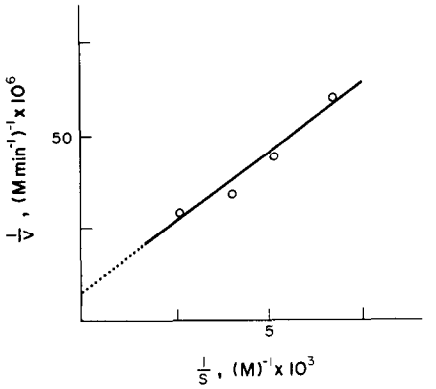

Fig. 4. Determination of kinetics of RLK_1 activity toward Bz-Tyr-O-Et. The substrate concentration was between 1 and 4 mM, 0.025 M Tris–HCl (pH 7.4), 30° in a final volume of 1 ml. The enzyme concentration was 25 μg/ml.

the other around 7.5; the pH profile for RLK_1 indicated that this partially purified preparation had no activity under pH 6.0, showing that cathepsins—largely present in the homogenates—were already eliminated at this stage of purification.

Esterolytic activity of RLK_1 was detected toward benzoyl-L-tyrosine ethyl ester; the kinetics were followed spectrophotometrically. The K_m for the substrate was determined to be 1.1×10^{-3} M (Fig. 4) and the rate of hydrolysis was at least 600-fold less than for commercial α-chymotrypsin; on the other hand, the rate of hydrolysis of an excess of BK by a partially purified preparation was around 35 nmoles · min^{-1} · mg^{-1} which is the same order of magnitude as that of chymotrypsin (44 nmoles · min^{-1} · mg^{-1}). The pH profile for this esterase activity followed the kininase activity (Fig. 3); Tos-Arg-O-Me (20 mM) was not hydrolyzed using up to 10-fold the amount used to cleave Bz-Tyr-O-Et.

Carboxypeptidase B and aminopeptidase activities also were not found, using hippuryl-L-arginine, leucyl and lysyl-β-naphthylamides as substrates. BK homologs were inactivated by the enzyme faster than BK itself; Table 2 shows that the ratios of kinin-destroying activities followed the same pattern that was found previously for chymotrypsin [13], with the longer kinins being more susceptible than the shorter ones, whereas the opposite pattern was observed for angiotensin-converting enzyme [15]. Unspecific hydrolysis of casein was not detected using as much as 50 μg of RLK_1 under conditions in which chymotrypsin was easily quantified. The native purified human high molecular kininogen was not inactivated by previous incubation with RLK_1 (Fig. 5, left panel), because human plasma kallikrein released the same amount of kinin in both treated and untreated kininogen. The heat-denatured kininogen was also not cleaved by the RLK_1 (Fig. 5, right panel). The same amount of kinin was released by trypsin in this case. This observation indicates that, although the kininogen contained BK, the high molecular weight polypeptide chain was not cleaved, at least, at the bradykinin peptide bonds. Angiotensin II was not cleaved under conditions in which BK was totally inactivated by RLK_1.

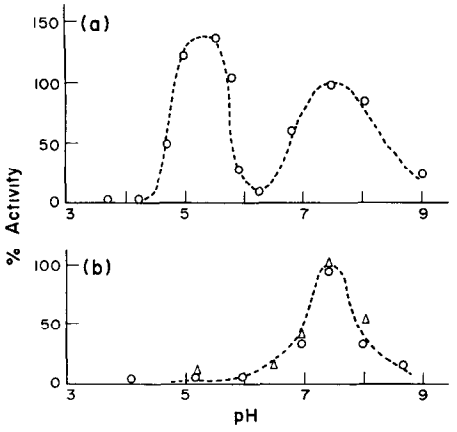

Fig. 3. Kininase activity and pH dependence in liver homogenate and in RLK_1. Hydrolysis: 1 μg/ml of BK; 0.05 M acetate buffer from pH 3 to 6; 0.05 M Tris–HCl from pH 7 to 9. Key: (a) activity of liver homogenates (200 μg/ml final concentration) and (b) RLK_1 (20 μg/ml); kininase activity (○) and esterase activity (△).

Table 2. Inactivation of BK, LBK and MLBK by RLK_1 and α-chymotrypsin

Kinin	Rate of inactivation by RLK_1*	Relative inactivation rate RLK_1	Relative inactivation rate Chymotrypsin†
BK	10.6	1	1
LBK	21.5	2.03	1.27
MLBK	28.5	2.65	1.59

* Rates are expressed as nmoles of BK hydrolyzed per min per ml of enzyme preparation (0.3 mg/ml).

† Sampaio *et al.* [13].

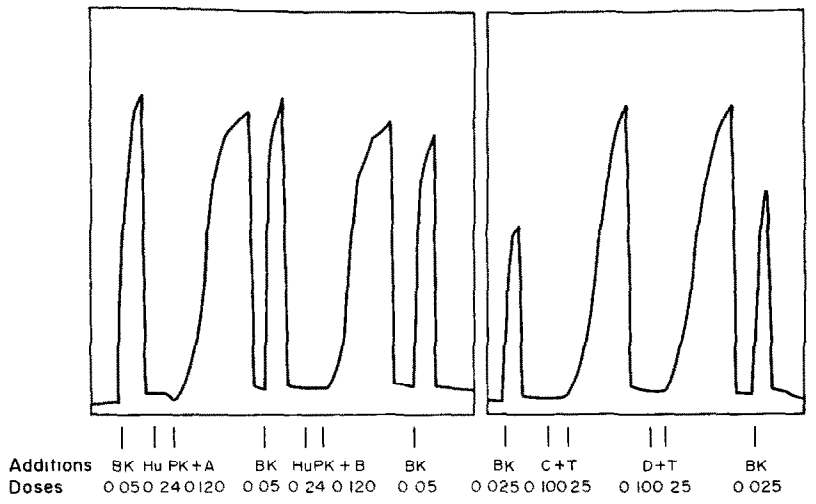

Fig. 5. Left panel: Native high molecular human kininogen preincubated with RLK_1-kinin released by human plasma kallikrien. Key: (A) 3 ml of preincubation mixture containing 5.8 mg kininogen (sp. act. 0.21 units/mg protein), 500 μl RLK_1 (0.145 mg, sp. act. 31.8 units/mg protein) in pH 7.4, 0.2 M phosphate buffer, 37°, 30 min; (B) Control incubation mixture without RLK_1. (HuPK) 7 Tos-Arg-O-Me units/ml (sp. act. 3.5 units/mg protein). Following BK additions, recordings took 60 sec. Following HuPK additions, recordings took 60 sec and then the preincubation mixtures were added for the direct assay of the bradykinin released. Right panel: Denatured high molecular weight kininogen preincubated with RLK_1-kinin released by trypsin. Key: (C) 2.2 ml of preincubation mixture containing 2.5 mg heat-treated kininogen (5 min in boiling water bath), 200 μl RLK_1 (0.116 μg; sp. act. 31.8 units/mg protein) in pH 7.4, 0.2 M phosphate buffer, 37°, 30 min; (D) Control incubation mixture without RLK_1; and (T) Trypsin, 1 mg/ml in 10^{-3} M HCl. The additions were made in the same way as described for the left panel.

Several well-known inhibitors of RLK_1 were tested (Table 3). The pentapeptide (BPP_5) was a powerful inhibitor, and chelating agents such as *o*-phenanthroline and EDTA also caused inactivation. No metal was found to restore this lost activity, indicating that the removal of the metal caused an irreversible alteration of the molecule; this may explain the instability of the enzyme in procedures such as gel filtration. SH-blocking agents did not cause inactivation under conditions in which other SH-proteases are inhibited and reducing agents were not effective in increasing the activity. On the contrary, dithiothreitol caused inactivation of the enzyme; these properties demonstrate marked differences between RLK_1 and the rabbit brain kininases that are very well characterized thiol-proteinases [5].

Table 3. Inactivation of RLK_1 by various inhibitors*

Inhibitor	Concn (M)	Inhibition (%)
BPP_5	0.82×10^{-6}	100
o-Phenanthroline	10^{-3}	100
EDTA	10^{-3}	50
Dithiothreitol	10^{-4}	65
Aprotinine	10^{-6}	0
β-Mercaptoethanol	10^{-4}	0
p-Hydroxy-mercuribenzoate	10^{-6}	0
Sodium tetrathionate	10^{-4}	0

* RLK_1 (0.26 mg/ml) was preincubated with the inhibitors in 0.05 M Tris–HCl (pH 7.4), 30°, for 30 min and then assayed for the kininase activity with BK. Controls for each inhibitor were carried out in order to avoid non-specific effects on the biological preparation.

The preincubation of RLK_1 with serine-proteinase inhibitors caused total inhibition of the enzyme (Table 4). The time course of the inactivation was followed for PMSF (10^{-3} M); the time for reaching 50% of inactivation was less than 15 min. The inactivation caused by $I_{p2}PF$ and PMSF was not reversed by dithiothreitol (10^{-4} M) which would be expected in the case of an unspecific inactivation of a thiol-proteinase [16]. The inactivation of RLK_1 by Tos-Phe-CH_2Cl was expected since the enzyme has a specificity which resembles chymotrypsin, such as in the hydrolysis of Bz-Tyr-O-Et; the enzyme was not sensitive to Tos-Lys-CH_2Cl as it does not cleave *N*-substituted basic aminoacyl esters. The esterase activity toward Bz-Tyr-O-Et was inhibited by both PMSF and Tos-Phe-CH_2Cl under the same conditions as that inhibited the kininase activity. Inhibition

Table 4. Inhibition of RLK_1 by agents specific for serine proteinases

Inhibitor	Concn (M)	Inhibition (%)
DFP	10^{-2}	100
PMSF	2×10^{-3}	88*
	10^{-4}	85*
Tos-Phe-CH_2Cl	5×10^{-5}	47
Tos-Lys-CH_2Cl	10^{-3}	0

* The same inhibition was observed for both kininase and esterolytic activity. RLK_1 (0.26 mg/ml) was preincubated with the inhibitors in 0.025 M Tris–HCl (pH 7.4), 37°, for 30 min. The kininase activity was tested with BK on the guinea pig ileum. The inhibition, in the case of PMSF, was followed by peptide analysis.

Table 5. Hydrolysis of BK by RLK_1*

Peptide	μmoles/ml
Arg	0.014
Arg^1-Phe^5	0.039
Ser^6-Arg^9	0.034
Arg^1-Phe^8	0.012

* Hydrolysis of 150 nmoles of BK by 6 μg of RLK_1 (sp. act. 31.8 nmoles BK hydrolyzed/min/mg protein) in 0.2 ml, 0.025 M Tris–HCl buffer (pH 7.4), 30°, for 3 hr. The reaction was interrupted by heating in a boiling bath, and the incubates were mixed with equal volumes of 0.2 M citrate buffer (pH 2.2), 15% polyethylene glycol (400); 0.3 ml was applied to the analyzer column.

Table 6. Hydrolysis of BK by RLK_2*

Peptide	μmoles/ml
Arg	0.205
Arg^1-Pro^7	0.214
Arg^1-Phe^5	0.011
Ser^6-Arg^9	0.033
Phe^8-Arg^9	0.013
Arg^1-Gly^4	Traces
Phe^5-Arg^9	Traces

* Hydrolysis of 500 nmoles of BK by 15 μg of RLK_2 (sp. act. 12.6) in 0.5 ml, 0.02 M phosphate buffer (pH 7.4), 30°, for 4 hr. The reaction was interrupted by heating in a boiling bath, and the incubates were mixed with equal volumes of 0.2 M citrate buffer (pH 2.2), 15% polyethylene glycol (400); 0.8 ml was applied to the short column of the analyzer as described in the legend of Fig. 6.

of enzyme was observed also when bradykinin was incubated in the presence of 10^{-3} M $CuSO_4$, $CoCl_2$ or $ZnCl_2$. No activation was found using cations such as Li^+, Ca^{2+}, Mg^{2+} or Fe^{2+}. The dependence of RLK_1 on chloride anions was tested by extensive dialysis against 0.025 M phosphate buffer (pH 7.4) at 0°. The activity of the enzyme remained constant and was not increased by adding from 10^{-3} to 10^{-1} M NaCl; this is an important difference between RLK_1 and the kininase II (angiotensin-converting enzyme) that shows an increased activity in the presence of chloride anions [15].

The site of cleavage of the enzyme was found to be in the Phe residues of the BK molecule, and the major bond to be cleaved was Phe^5-Ser^6 (Table 5) although the Phe^8-Arg^9 was also cleaved; these findings illustrate an important difference between RLK^1 and chymotrypsin because this latter enzyme cleaves the Phe^8-Arg^9 bond preferentially as was demonstrated earlier [13], and was confirmed by the analysis of the products in the amino acid analyzer. The peak, corresponding to the product Ser-Pro-Phe-Arg (Fig. 6), was collected from the Aminex A6 column and hydrolyzed in 6 N HCl for 24 hr; the free amino acids found were, as expected, Arg (6.2 nmoles), Ser (5.1 nmoles), Pro (4.7 nmoles) and Phe

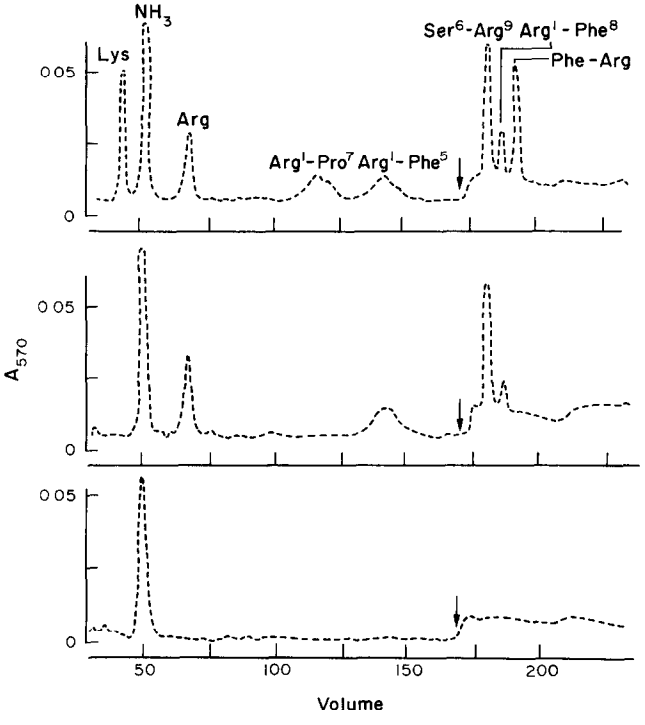

Fig. 6. Products of hydrolysis of BK incubated with RLK_1. Top panel: Standard amino acids and BK fragments (nmoles): Lys (10), Arg (10), Arg^1-Pro^7 (20), Arg^1-Phe^5 (60), Ser^6-Arg^9 (30), Phe^8-Arg^9 (17); center panel: products from BK (150 nmoles) incubated with RLK_1 (6.0 μg) in 200 μl of 0.025 M Tris–HCl (pH 7.4) for 3 hr at 30°; bottom panel: same conditions as for the center panel, in the presence of 2 mM PMSF. In all cases, samples were applied to the short column, and the arrows indicate change in the buffer (see text for details).

(5.3 nmoles). There was also traces of Glu (0.9 nmoles), and Gly (1.1 nmoles) and Ala (0.7 nmole). Both Phe^5-Ser^6 and Phe^8-Arg^9 cleavages were prevented by the pretreatment of the enzyme with PMSF (Fig. 6). The activity of RLK_2 (second peak from DEAE-cellulose) toward BK indicates that this was a mixture of proteases (Table 6) for the analysis of the products showed that, besides Phe^5-Ser^6, Pro^7-Phe^8 and Phe^8-Arg^9 were also split. Thus, at least an angiotensin-converting enzyme, a carboxypeptidase *N*-type, and a peptidase similar to the enzyme that splits the Phe^5-Ser^6 bond, described in the perfused rat liver [9], were present under this peak.

DISCUSSION

The properties of RLK_1 indicate that this kininase is a serine-proteinase; that is the major difference between these enzymes and the kininase A found in rabbit brain. Another difference is that the RLK_1 showed some splitting of the Phe^7-Arg^8 bond. This finding could be explained by a contaminating carboxypeptidase, but the simultaneous inhibition of cleavage at Phe^5-Ser^6 and Phe^8-Arg^9 favors the hypothesis that RLK_1 hydrolyzes both; hydrolysis by serine-proteinase of the C-terminal peptide bond was found, although not commonly, as demonstrated for chymotrypsin [16]. The fact that RLK_1 did not cleave native or denatured kininogen indicates that the enzyme has, at least with the BK sequence, a preference for smaller peptide chains.

Rabbit liver may contain a similar enzyme [17] but in this case the analysis of the crude homogenates showed a complex mixture of proteases able to hydrolyze several bonds in bradykinin, which makes the comparison rather difficult. RLK_1 differs from other serine proteinases described for liver such as lysosomal elastase (EC 3.4.21.11) and cathepsin G (EC 3.4.2.20), since these enzymes have lower molecular weights and are able to hydrolyze casein [18]. Red blood cell protease [19] and group specific proteases [20] are distinct from the kininase described in this paper, both in substrate specificity and molecular weight.

The kininase extracted from the liver by perfusion with detergent [9] is being purified, and the comparison of its properties with those of RLK_1 is under investigation.

RLK_1 resembles chymotrypsin in many ways since it is inhibited by Tos-Phe-CH_2Cl and shows esterolytic activity toward Bz-Tyr-O-Et. However, the hydrolysis of bonds in bradykinin which involve the carboxyl sides of the residues, and marked preference for Phe^5-Ser^6 when compared to Phe^8-Arg^9, makes RLK_1 distinct from chymotrypsin, which splits Phe^8-Arg^9 in the same substrate more efficiently than the internal Phe^5-Ser^6 bond [13].

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