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SUBUNITS OF HUMAN PLASMA KININASE II GENERATED BY PLASMA KALLIKREIN

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Abstract—Partially purified human plasma kininase II was incubated with proteolytic enzymes such as plasma kallikrein, prekallikrein activator, plasmin, thrombin and trypsin. Digestion of plasma kininase II by plasma kallikrein generated 180,000 (I) and 95,000 (II) mol. wt subunits while plasmin, thrombin and trypsin inactivated plasma kininase II. These subunits have two activities just as plasma kininase has: they hydrolyze bradykinin, and they convert angiotensin I into angiotensin II. K_m values of the two subunits for bradykinin were 0.17×10^{-6} M, indicating that this value was identical with plasma kininase II. V_{max} values of the two subunits for bradykinin were higher than that of plasma kininase II. The bradykininase activities of the two subunits were significantly inhibited by inhibitors such as $HgCl_2$, ethylenediamine tetraacetic acid, *p*-chloromercuriphenyl sulfonic acid, SQ 20881(BPF9a), soy bean trypsin inhibitor and Trasylol. The behaviour of the two subunits towards these above inhibitors was also identical with plasma kininase II. A 180,000 mol. wt kininase was partially purified from human plasma. The kininase had bradykinin-inactivating and angiotensin I-converting activities. Its K_m value and the behaviour towards the inhibitors described above were the same as the subunit I.

In 1962 an enzyme that removes the C-terminal arginine of bradykinin and cleaves hippuryl-L-lysine (HLL) was first demonstrated in human plasma by Erdös and his colleagues [1–6], and was named carboxypeptidase *N* or kininase I [7]. Subsequently, they found a second kininase which cleaves the C-terminal phenylalanyl-arginine dipeptide of bradykinin but not HLL in human plasma [8–10], and named the newly found enzyme kininase II [7]. The microsomal fraction of the swine kidney cortex was found to be a rich source of kininase II [11]. The angiotensin I-converting enzyme that liberates the histyl-leucine dipeptide from angiotensin I, yielding angiotensin II, was described in horse plasma by Skegges *et al.* [12]. Erdös and his colleagues [13] have demonstrated that kininase II is also an angiotensin I-converting enzyme because the angiotensin I-converting activity could not be separated from kininase. Erdös *et al.* [6] have reported that during storage, plasma kininase I dissociates to 45,000 and 90,000 mol. wt subunits which inactivates bradykinin and C3a anaphylatoxin. Recently, Nishimura *et al.* [14] have demonstrated that after trypsin treatment angiotensin I-converting enzyme in rabbit lung dissociates to two active components which have the identical K_m value. The author found that two subunits of a mol. wt of 95,000 and 180,000 are generated from human plasma kininase II by incubation with human plasma kallikrein, and these two subunits have bradykinin-inactivating and angiotensin I-converting activities. The author also partially purified a 180,000 mol. wt kininase from human plasma. In this paper, the author describes the effect of plasma kallikrein and other proteolytic enzymes on human plasma kininase II, and some properties of the two subunits and a 180,000 mol. wt kininase from human plasma.

MATERIALS AND METHODS

All experiments were carried out in siliconized glassware and polyethylene equipment.

Plasma. Human blood from normal individuals was collected in 0.1 volume of 3.1% sodium citrate in plastic syringes and centrifuged at 3000 rev/min for 30 min at 4°C. This freshly separated plasma was used for the experiment.

Purification of plasma kininase. The fresh plasma (14 ml) was subjected to gel filtration on a 3.5×90 cm polyethylene column (LKB) of Sephacryl S-200 equilibrated with 0.05 M phosphate buffer, pH 7.0 containing 0.5 M NaCl. The active protein eluted from the column was dialyzed overnight at 4° against 0.05 M phosphate buffer, pH 8.0 containing 0.1 M NaCl. The dialyzed protein was concentrated to about 10 ml using an Amicon Ultrafiltration Chamber with a UM-10 membrane. The solution concentrated was chromatographed on a 2.8×45 cm column of DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer, pH 8.0 containing 0.1 M NaCl. The column was eluted with a linear gradient of increasing concentrations of NaCl (0.1–0.5–0.9 M) using a mixing chamber with three containers. Three active peaks which hydrolyzed bradykinin appeared to the eluent. The peak containing plasma kininase II was further purified by ascending gel filtration on a 3.5×46 cm column of Sephadex G-200 equilibrated with 0.05 M phosphate buffer, pH 7.4 containing 0.1 M NaCl. The purified enzyme was again subjected to gel filtration on a Sephadex G-200 column. The peak which was not identified as plasma kininase I or II was subjected to chromatography on a 1.0×40 cm column of Sepharose 4B-arginine equilibrated with 0.05 M phosphate buffer, pH 7.4 after concentration. The adsorbed protein was eluted with a linear gradient of increasing

concentrations of KCl (0–1.0 M) using a mixing chamber and the active peak was pooled.

Purification of plasma kallikrein. Plasma kallikrein partially purified through column chromatography on DEAE cellulose and DEA-Sephadex A-50 described in the previous paper [15] was subjected to affinity chromatography on a Trasylol-Sepharose 4B according to the method of Cuatrecasas *et al.* [16] and Oza *et al.* [17]. Its specific activity of Tosyl-Arginine methyl ester (TAMe) esterase was 34.7 μmoles/min/mg.

Purification of prekallikrein activator. Prekallikrein activator (PKA) in plasma was purified by the method described by Venneröd and Laake [18].

Measurement of kininase activity and angiotensin-converting activity. The inactivation of bradykinin was established by measuring contractions of an isolated guinea-pig ileum suspended in a muscle bath containing 10 ml of oxygenated Tyrode solution as described previously [15, 19]. The enzyme concentration was adjusted to a level that a 50 per cent hydrolysis was not exceeded during incubation time. Fifty to 100 ng of bradykinin dissolved in 0.5 ml of 0.05 M phosphate buffer was used for the assay. The same technique was used to assay the conversion of angiotensin I to angiotensin II on the isolated guinea-pig ileum. The amount of angiotensin II generated was assayed using angiotensin II as a standard. The sample was not preincubated with cobalt for the assay of kininase activity or angiotensin I-converting activity.

Measurement of kininogenase activity. Kininogenase activity of plasma kallikrein was measured by the method previously described [15, 20].

Other enzyme activities. The hydrolysis of hippuryl-histidyl-leucine (HHL) was measured according to Folk *et al.* [21] and Yang *et al.* [13]. The hydrolysis of HLL was measured as described by Folk *et al.* [21] and Erdös *et al.* [2]. TAMe esterase activity was measured by the method of Sherry *et al.* [22] and Colman *et al.* [23].

Protein. The protein content of the solutions was determined according to the method of Lowry *et al.* [24].

Chemicals. Bradykinin, angiotensin I, angiotensin II, TAMe, HHL and HLL were obtained from the Protein Research Foundation, Osaka, Japan; crystallized bovine serum albumin, human gamma-globulin, bovine thrombin (about 67 NIH units/mg), soy bean trypsin inhibitor (SBTI) (1 mg inhibits about 1 mg trypsin), and lima bean trypsin inhibitor (LBTI) from Sigma Chemical Co. U.S.A.; plasmin KABI (25 CU per vial) from AB KABI, Stockholm, Sweden; bovine pancreas trypsin (2 × crystalline) from NBC, USA; epsilon aminocaproic acid (EACA) and *trans*-aminomethyl-cyclohexene-carboxylic acid (t-AMCHA) from Daiichi Seiyaku, Co., Ltd., Tokyo, Japan. SQ 20881(BPT9a) [25] was kindly supplied by Professor Erdös, Department of Pharmacology and Internal Medicine, University of Texas Health Science Center, U.S.A; Trasylol® by Bayer, Germany.

RESULTS

Purification of plasma kininase II. The results of purification of plasma kininase II are summarized in Table 1. The progress of purification was followed by measuring the hydrolysis of Bradykinin. The enzyme eluted from a column of Sephacryl S-200 was subjected to a second column of DEAE-Sephadex A-50. As shown in Fig. 1, a peak showing HLL hydrolysis was found in tubes 89–120, indicating that this peak is responsible for plasma kininase I activity. Two peaks showing kininase and HHL hydrolysis were found in tubes 19–32 and 33–49, respectively. Yang *et al.* [13] prepared kininase II from plasma by precipitation with ammonium sulfate in the first step of purification and then the precipitate was used for further purification. The kininase corresponding to tubes 19–32 was mostly eliminated in the ammonium sulfate precipitate. Thus, this kininase did not clearly appear on DEAE-Sephadex A-50 column chromatography as shown in Fig. 1, when the ammonium sulfate precipitate was used for the enzyme source. For this reason the peak corresponding to tubes 33–49 was found to be plasma kininase II. Plasma kininase II, after the final stage of purification, was purified 971-fold and the yield of activity was 28 per cent.

Effect of proteolytic enzymes on plasma kininase II. Plasma kallikrein, PKA, plasmin, trypsin and thrombin were used for this study and the results are shown in Fig. 2. When a mixture of plasma kininase II and proteolytic enzyme was incubated, kininase activity in the mixture changed with increasing concentration of proteolytic enzymes. Plasmin, 0.25 CU, 10 μg of trypsin and 10 U of thrombin were able to cause complete inactivation of kininase activity. PKA did not affect kininase activity up to 0.3 U. Plasma kallikrein alone, showing no effect on the amplitude of guinea-pig ileum contraction, increased kininase activity in the mixture and a 27 per cent enhancement was obtained at a dose of 60 μg. During these experiments, careful

Table 1. Purification of plasma kininase II

Step	Treatment	Protein (mg)	Specific activity (ng/min/mg)	Degree of purification	Yield (%)
1	Plasma	980	0.32	1	100
2	Sephacryl S-200	232	1.1	3.4	74
3	DEAE-Sephadex A-50	15.7	20.8	65.0	101
4	Sephadex G-200 (I)	1.7	109.4	340.6	58
5	Sephadex G-200 (II)	0.29	310.8	971.3	28

The progress of purification was followed by measuring the hydrolysis of bradykinin.

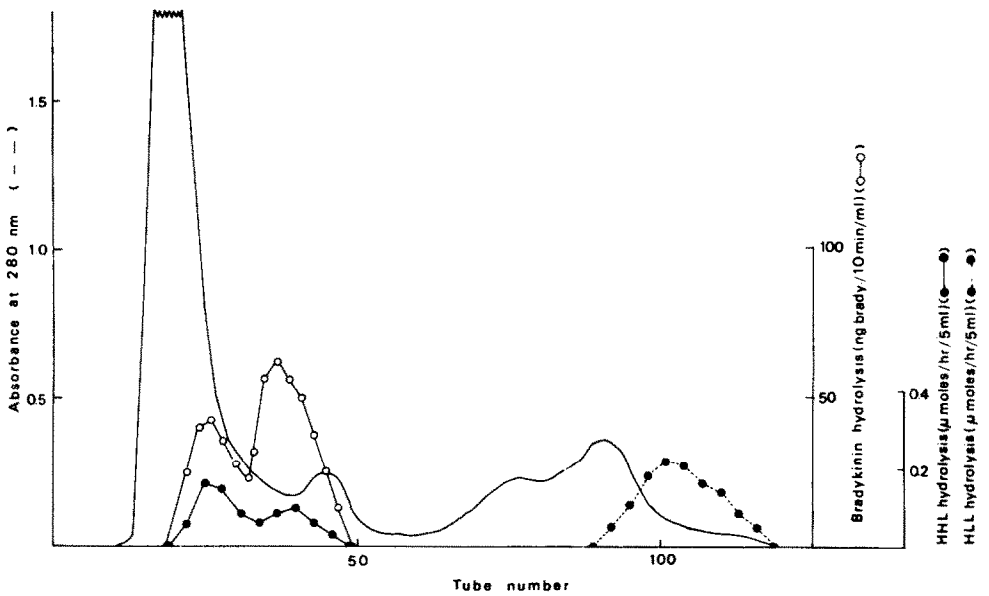

Fig. 1. DEAE-Sephadex A-50 chromatography of plasma kininase, 252 mg of the enzyme obtained by Sephacryl S-200 gel filtration was subjected to a DEAE-Sephadex A-50 column chromatography. 5 ml fractions were collected at a flow rate of 25 ml/hr. Each fraction was preincubated with 10^{-4} M $CoCl_2$ for HHL or HLL hydrolysis assay. For lower activity of HHL hydrolysis, each fraction was used after concentration.

attention was given to sensitization of guinea pig ileum by proteolytic enzymes.

In order to examine the extent of the influence of incubation time on enhancement of kininase activity, a mixture of plasma kallikrein and plasma kininase II was incubated much longer as shown in Fig. 3. Kininase activity in the mixture enhanced with increasing incubation time, reaching a 70 per cent enhancement at 60-min incubation. These findings suggest that a change occurred in plasma kininase II during the period of incubation. To discern this suggestive phenomenon, the following experiments were carried out.

Sephadex G-200 gel filtration of plasma kininase

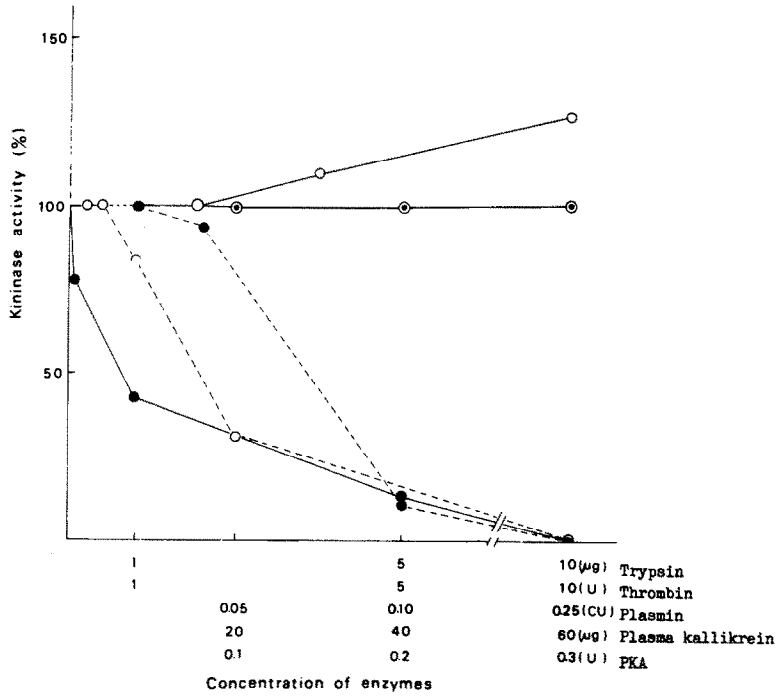

Fig. 2. Effect of the concentration of proteolytic enzymes on plasma kininase II activity. About 100 μg of plasma kininase II obtained from the Sephadex G-200 (II) gel filtration was incubated with varied concentrations of proteolytic enzymes at 37° for 10 min before assay of kininase. O——O: plasma kallikrein, O- - - - -O: plasmin, ●——●: trypsin, ●- - - - -●: thrombin, ⊙——⊙: PKA

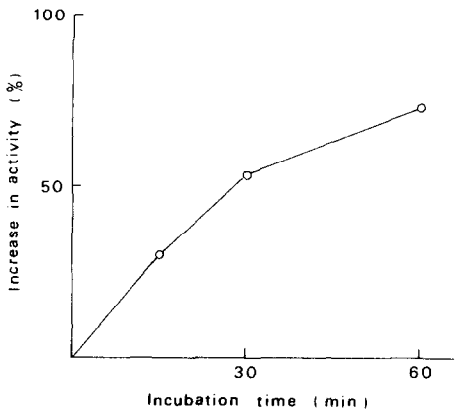

Fig. 3. Kininase activity in a mixture of plasma kininase II and plasma kallikrein during incubation. 96 μg of plasma kininase II obtained from the Sephadex G-200 (II) gel filtration (Table 1, step 5) was incubated with 63 μg of plasma kallikrein at 37°. After different intervals of incubation, aliquots of the mixture were taken and used for assay of kininase.

II, plasma kallikrein, and the mixture of plasma kininase II and plasma kallikrein. Plasma kininase II (720 μg) was eluted following Sephadex G-200 gel filtration and its kininase activity appeared at a position consistent with its mol. wt as shown in Fig. 4 (top panel). The total protein content of kininase activity was 223 μg. Plasma kallikrein was eluted from Sephadex G-200 and one peak for kininogenase activity was identified as shown in Fig. 4 (middle panel). After 60-min-incubation of the mixture of the same concentrations of plasma kininase II and plasma kallikrein as before, three peaks having kininase activity were eluted as shown in Fig. 4 (bottom panel), indicating in respect to a position eluted from gel filtration that the first peak apparently corresponds to plasma kininase II, and the second peak (subunit I) and the third peak (subunit II) are subunits. The total protein content of kininase II, the subunit I, and the subunit II was 122 μg, 40 μg and 131 μg, respectively. Unfortunately the subunit II was contaminated with plasma kallikrein as shown in Fig. 4 (bottom panel). Therefore, it was impossible to estimate its real concentration.

Molecular weight. The mol. wt of the two subunits was estimated by gel filtration on a Sephadex G-200 column using bovine serum albumin (mol. wt, 67,000), plasma kallikrein (mol. wt, 100,000), human gamma-globulin (mol. wt, 160,000), and human plasma kininase I (mol. wt, 280,000) [5] as protein marker. Kininase activity of the subunit I appeared between human γ-globulin and plasma kininase I. The subunit II was eluted from the gel immediately after plasma kallikrein. The mol. wt of the subunit I and II were estimated to be approximately 180,000 and 95,000, respectively.

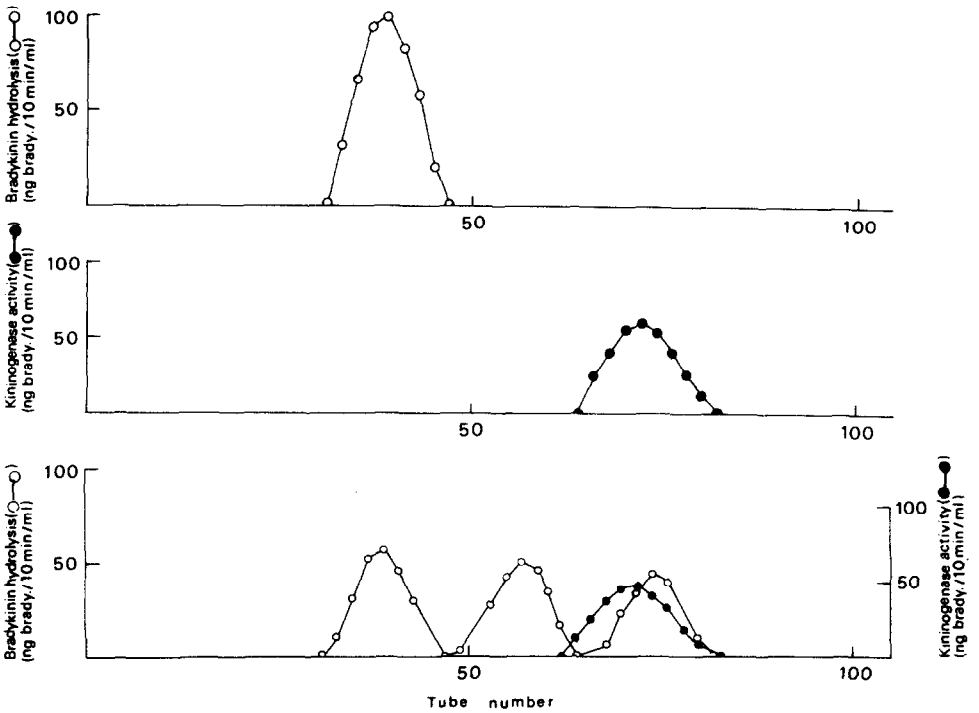

Fig. 4. Sephadex G-200 gel filtration of plasma kininase II, plasma kallikrein, and a mixture of plasma kininase II and plasma kallikrein. Plasma kininase II obtained from the Sephadex G-200 (II) gel filtration (Table 1, step 5) and plasma kallikrein were used 720 μg and 430 μg, respectively. Each sample of plasma kininase II, plasma kallikrein, and a mixture of plasma kininase II and plasma kallikrein was subjected to a 3.5 × 46 cm column of Sephadex G-200 equilibrated with 0.05 M phosphate buffer, pH 7.4 containing 0.1 M NaCl. Three ml fractions were collected at a flow rate of 10 ml/hr. The same amount of plasma kininase II as used in the top panel was incubated with the same amount of plasma kallikrein as used in the middle panel at 37° for 60 min and then subjected to the column as shown in the bottom panel. For protein determination each peak was pooled and concentrated because of lower content.

Table 2. Hydrolysis of bradykinin and angiotensin by plasma kininase II and subunits

	Protein (μg)	Hydrolysis of bradykinin (ng)	Generation of angiotensin II (ng)
Plasma kininase II	2.1	24 ± 2.4	23 ± 2.9
Subunit I	0.7	24 ± 3.3	22 ± 1.9
Subunit II	2.9	25 ± 3.7	25 ± 2.6

Kininases eluted from the Sephadex G-200 column in Fig. 4 were used as enzyme sources. For angiotensin I-converting enzyme assay, angiotensin I 200 ng dissolved in 0.5 ml 0.05 M phosphate buffer, pH 7.4 was incubated with 0.5 ml kininase sample at 37° for 30 min and the reaction was stopped in a boiling water bath.

Properties. Plasma kininase II, the subunit I and the subunit II in Fig. 4 were used as enzyme sources. K_m values of plasma kininase II, the subunit I and the subunit II for bradykinin hydrolysis were determined by the Lineweaver–Burk plot carried out four to five times at each concentration. The K_m value of these enzymes was identical and its value was 0.17×10^{-6} M. This value is close to 0.94×10^{-7} M obtained from human plasma kininase II by radiometric assay using [^{14}C]bradykinin [26]. The corresponding V_{max} values of plasma kininase II, the subunit I and the subunits II was 5.0 nmoles/min/μg protein, 16.6 nmoles/min/μg protein, and 7.1 nmoles/min/μg protein, respectively.

The hydrolysis of bradykinin and angiotensin I by plasma kininase II, the subunit I and the subunit II is shown in Table 2. The findings indicating that plasma kininase II cleaved bradykinin and angiotensin I well agree with the results demonstrated by Erdös and his colleagues [13]. With great interest the subunit I and II also hydrolyzed bradykinin and angiotensin I.

The alternations induced by heavy metals and enzyme inhibitors on the hydrolysis of bradykinin of plasma kininase II, the subunit I, and the subunit II are shown in Table 3. Table 3 shows similarities in behaviour between the subunit I and the subunit II except for $CdSO_4$ and $ZnSO_4$; $HgCl_2$, EDTA, PCMS, 1,10-phenanthroline, SQ 20881, SBTI and Trasylol acted as potent inhibitor. Except for SBTI [1] and Trasylol [9], most of the inhibitors against plasma kininase II agree with the results reported previously by many investigators in spite of slight discrepancy which may be due to differences in experimental conditions [7].

Purification of 180,000 molecular weight plasma kininase. The peak corresponding to tubes 19–32 in Fig. 1 showing kininase activity and HHL hydrolysis was identified as neither plasma kininase I nor plasma kininase II for its behaviour towards ammonium sulfate and HLL hydrolysis. The pooled fraction of this peak was concentrated to 3 ml by ultrafiltration and applied to a Sepharose 4B-arginine column. Representative pattern of absorbence at 280 nm of the eluates are shown in Fig. 5. Most of the kininase activity appeared in the first protein peak which was weakly bound to Sepharose 4B-arginine. The kininase was purified 148-fold and the yield of activity was 27 per cent as shown in Table 4. The mol. wt of the kininase was estimated to be approximately 180,000 by the gel filtration. The mol. wt is identical with that of the subunit I. Under the same experimental condition in Table 2, the behaviour of the 180,000 mol. wt plasma kininase (45 μg) towards $HgCl_2$, $CdSO_4$, $ZnSO_4$, arginine, EACA, *t*-AMCHA, EDTA, PCMS, 1,10-phenanthroline,

Table 3. Inhibition of hydrolysis of bradykinin by plasma kininase II and subunits

Inhibitor	Concentration (M)	Plasma Kininase II	Subunit I	Subunit II
$HgCl_2$	1×10^{-4}	++	++	++
$CdSO_4$	1×10^{-4}	0	+	
$ZnSO_4$	1×10^{-4}	0	+	
Arginine	1×10^{-3}	+	+	+
EACA	1×10^{-4}	0	0	
t-AMCHA	1×10^{-4}	0	0	0
EDTA	1×10^{-4}	++	++	++
PCMS	1×10^{-4}	++	++	++
1,10-Phenanthroline	1×10^{-4}	++	++	++
SQ 20881	1×10^{-6}	++	++	++
SBTI	10 μg	++	++	++
LBTI	100 μg	0	0	0
Trasylol	10 U	++	++	++

++, more than 60 per cent inhibition; +, 20–59 per cent inhibition; 0, 0–19 per cent inhibition. For assay of inhibition, 0.5 ml of kininase sample was preincubated with 0.2 ml of inhibitor at 37° for 10 min.

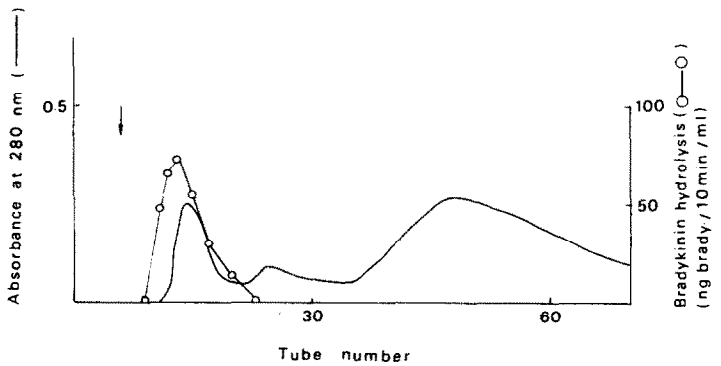

Fig. 5. Sepharose 4B-arginine chromatography of 180,000 mol. wt plasma kininase. The peak corresponding tubes 19–32 in Fig. 1 showing kininase activity was concentrated and chromatographed on a column of Sepharose 4B-arginine. Three ml fractions were collected at a flow rate of 7 ml/hr. Arrow indicates void volume.

Table 4. Purification of 180,000 molecular weight plasma kininase

Step	Treatment	Protein (mg)	Specific activity (ng/min/mg)	Degree of purification	Yield (%)
1	Plasma	980	0.32	1	100
2	Sephacryl S-200	232	1.1	3.4	74
3	DEAE-Sephadex A-50	56	3.7	11.6	64
4	Arginine-Sepharose4B	1.8	47.6	148.7	27

The progress of purification was followed by measuring the hydrolysis of bradykinin.

SQ 20881, SBTI, LBTI and Trasylol listed in Table 3 was identical with the subunit I. Its K_m value of 0.17×10^{-6} M for bradykinin hydrolysis was also in accordance with the subunit I.

DISCUSSION

The results in this paper demonstrated that two subunits of a mol. wt of 180,000 (subunit I) and 95,000 (subunit II) are generated from plasma kininase II by incubation with plasma kallikrein. The subunit I and II have a dual function in which it hydrolyzes bradykinin and converts angiotensin I into angiotensin II as well as plasma kininase II [13]. The V_{max} values of the subunit I and II for bradykinin are higher than that of plasma kininase II, while the K_m values of the two subunits for bradykinin are the same as plasma kininase II. Increasing kininase activity during enzymatic interaction of plasma kallikrein with plasma kininase II is confirmed by the findings in Fig. 4. (1) The total kininase activity in three peaks obtained from the mixture of plasma kallikrein (bottom panel) is greater than the kininase activity of plasma kininase II alone (top panel). (2) The kininase activity in the first peak corresponding to plasma kininase II (bottom panel) is smaller than that of plasma kininase II alone (top panel). Furthermore, the subunit I is very likely identical with the 180,000 mol. wt plasma kininase considering from the many similar properties in mol. wt, hydrolysis of substrates, K_m value, and inhibition by heavy metals and protease inhibitors. These findings suggest that the 180,000 mol. wt plasma kininase is generated by plasma kallikrein in plasma. Thus, plasma kallikrein may be responsible for enhancement of plasma kininase activity under pathological conditions in which prekallikrein is activated to kallikrein. In fact, the author has found that the 180,000 mol. wt kininase activity in synovial fluid was higher than that of plasma in patients with rheumatoid arthritis who showed increased kallikrein activity in synovial fluid (unpublished data).

Activated factor XII triggers the coagulation, fibrinolysis and kinin-forming pathway. Recently the more important relations with plasma proteolytic enzymes have been found in the kinin-forming system. The positive enzymatic feedback effect of kallikrein has been described in the kallikrein-kinin system [27–29]. Kallikrein has been shown to convert unactivated factor XII to activated factor XII [30]. High mol. wt kininogen is required for the activation of factor XII by kallikrein [31, 32]. Kallikrein activates plasminogen to plasmin [33, 34]. Digestion of activated factor XII by plasmin generates PKA [35]. The bradykinin level in plasma is the result of the balance between simultaneous processes of generation and inactivation. From the results of the enzymatic interaction of plasma kallikrein, PKA, plasmin, and thrombin with plasma kininase II, it is considered that plasma kallikrein may shift the balance of bradykinin level to reduction by generating the potent subunits from plasma kininase II while plasmin and thrombin may shift the balance to enhancement by inactivating of plasma kininase II.

Many controversial results, described in various conditions, may account for not only the differences in methods of determination but also enhanced activities of proteolytic enzymes such as plasma kallikrein, plasmin and thrombin [36]. The relationship

between plasma kininase activity and proteolytic enzyme activities is under investigation.

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