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FOUR PLASMA KALLIKREINS GENERATED IN ACETONE-ACTIVATED HUMAN PLASMA

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Abstract—Four enzymatically active fractions were isolated from acetone-activated human plasma. These four esterases were termed KI, KII, KIII and KIV. KI, KII, KIII and KIV were found to represent free kallikrein, kallikrein–α_2-macroglobulin (α2M) complex, kallikrein–α2M-high molecular weight (HMW) kininogen complex and kallikrein–HMW kininogen complex, respectively. KI, KII, KIII and KIV showed K_m values 2.27×10^{-3}, 7.14×10^{-3}, 7.69×10^{-3} and 3.59×10^{-3} M for TAMe hydrolysis, respectively. Kininogenase activity in KII and KIII was much less than in KI and KIV. Soy bean trypsin inhibitor completely inhibited the esterase activity in KI and KIV but not in KII and KIII.

Several recent studies suggest that human plasma contains more than one kallikrein. In 1961, the possibility of the existence of two kallikreins was demonstrated by Webster and Pierce [1]. They reported that acetone activates not only plasma kallikrein but also other proteolytic enzyme(s) which attack arginine esters and are not inhibited by soy bean trypsin inhibitor (SBTI). An anionic kallikrein was described during the course of purifying the cationic kallikrein [2, 3]. Two serum kallikreins were separated by ion exchange chromatography by Eisen and Glanville [4]. Vogt postulated two kinin forming systems, on the basis of functional analysis of intrinsic plasma kinin formation [5], although this hypothesis was not confirmed by Nakahara [6]. Using Cohn fraction IV, three plasma kallikreins have been demonstrated by Colman *et al.* [7]. Their kallikreins, termed kallikrein I, II, and III, did not contain any α_2-macroglobulin (α2M). Asghar *et al.* also noticed three plasma kallikreins in Cohn fraction IV [8]. Many resemblances were noted between the results obtained by Colman *et al.* [7] and Asghar *et al.* [8]. Recently Vogt and Dugal have separated two kallikreins in acetone-activated human serum; one is free kallikrein and the other is a kallikrein–α2M complex which is not inhibited by SBTI [9]. In this paper the author demonstrates four enzymatically active kallikreins isolated from acetone-activated human plasma and some properties of the four kallikreins.

MATERIALS AND METHODS

Plasma. Human venous blood was collected in 0.1 volume of 3.1% sodium citrate using siliconized needles and plastic syringes, and was centrifuged at 3000 rev/min for 30 min at 4°. This freshly separated plasma was used for the experiment.

Acetone activation. Acetone activation was carried out by adding 16 or 25% (v/v) acetone to plasma, with stirring, in a siliconized glass vessel. The mixture was allowed to stand at room temperature for 3.5 hr. The acetone was immediately evaporated in a rotary evaporator after activation.

Column chromatography. The fresh plasma (15 ml) activated with acetone was diluted three-fold with deionized water and concentrated to the original volume using an Amicon Ultrafiltration Chamber with a UM-10 membrane. This plasma was subjected to column chromatography on a 3.5 × 49 cm polyethylene column (LKB) of diethylaminoethyl (DEAE) cellulose (DE 52) which was equilibrated with 0.05 M NaCl containing 0.0075 M phosphate buffer, pH 8.0. The column was eluted with a linear gradient of increasing concentrations of NaCl (0.05–0.70 M) using a mixing chamber. The u.v.-absorption at 280 nm, tosyl-arginine methyl ester (TAMe) hydrolysis, benzoyl-prolyl-phenylalanyl-arginine-*p*-nitroanilide (PPAN) cleavage, and kininogenase activity of the effluent fractions were measured.

Gel filtration. The acetone-activated plasma (15 ml) was also subjected to gel filtration on a 2.3 × 128 cm column of Sepharose 4 B which was equilibrated with 0.15 M NaCl containing 0.02 M Tris buffer, pH 7.4. The column was eluted with 0.15 M NaCl containing 0.02 M Tris buffer, pH 7.4. On the other hand, each of the four esterases eluted from the DEAE cellulose column was pooled and dialyzed overnight at 4° against 0.15 M NaCl containing 0.02 M Tris buffer, pH 7.4. The dialyzed esterase was concentrated to about 10 ml and applied to gel filtration on Sepharose 4 B.

Assay of kallikrein. Kallikrein activity was measured by release of kinin from kininogen and cleavage of synthetic substrates. The kininogenase activity was estimated using kininogen by the method previously described [6]. The hydrolysis of TAMe was estimated according to Colman *et al.* [7]. The hydrolysis of benzoyl-arginine-ethyl ester (BAEe) was measured by the spectrophotometric method of Schwert and Tanaka [10]. The assay of PPAN for kallikrein was performed by the method of Claeson *et al.* [11]. The amount of enzyme giving hydrolysis of 1 μmole TAMe per min at 37° was taken as 1 unit.

Assay of prekallikrein. Prekallikrein was activated with trypsin by the method of Wuepper and Cochrane [12]. An aliquot of fraction eluted from Sepharose 4 B and trypsin 10 μg were incubated at 37° for 15 min. At the end of incubation, lima bean trypsin inhibitor was added to inhibit the trypsin activity. The activated prekallikrein was measured by TAMe hydrolysis.

Measurement of α2*M.* α2M was measured by radial immunodiffusion on M-Partigen plates.

Immunodiffusion. Immunodiffusion analysis of HMW kininogen was carried out on an Ouchterlony agarose plate. Anti-human HMW kininogen serum was kindly supplied by Dr. Nagasawa, Faculty of Pharmaceutical Sciences, Hokkaido University, Japan.

Chemicals. TAMe, BAEe and bradykinin were obtained from the Protein Research Foundation, Osaka, Japan; PPAN from A B KABI, Sweden; bovine pancreas trypsin (2 × crystalline) from NBC, U.S.A.: SBTI and lima bean trypsin inhibitor from Sigma Chemical Co., U.S.A.: agarose and M-Partigen from Behring Institut, Germany; *trans*-aminomethyl-cyclohexene-carboxylic acid (*t*-AMCHA) from Daiichi Seiyaku, Co., Ltd., Japan.

RESULTS

Chromatography on DEAE cellulose of acetone-activated plasma

A typical eluation pattern from column of DEAE cellulose of acetone-activated plasma revealed six distinct protein peaks as shown in Fig. 1a. Four esterase fractions (KI–KIV) containing the activity of TAMe hydrolysis and kininogenase were observed. The kininogenase activity was much higher in KI and KIV than in KII and KIII in relation to esterolytic potency, as shown in Fig. 1b. These esterases also cleaved *p*-nitroaniline from PPAN as shown in Table 1.

Each of these four esterase fractions was rechromatographed on DEAE cellulose. The esterase in KI and KII was not separated and the esterase in KI was not absorbed on DEAE cellulose. The esterase in KIII was separated into two esterase fractions; one which corresponds to about 80 per cent of the amount of rechromatographed sample remained in the KIII region and the other appeared in the KII region. The esterase in KIV was also separated into two esterase fractions; one which corresponds to about 70 per cent of the amount of rechromato-

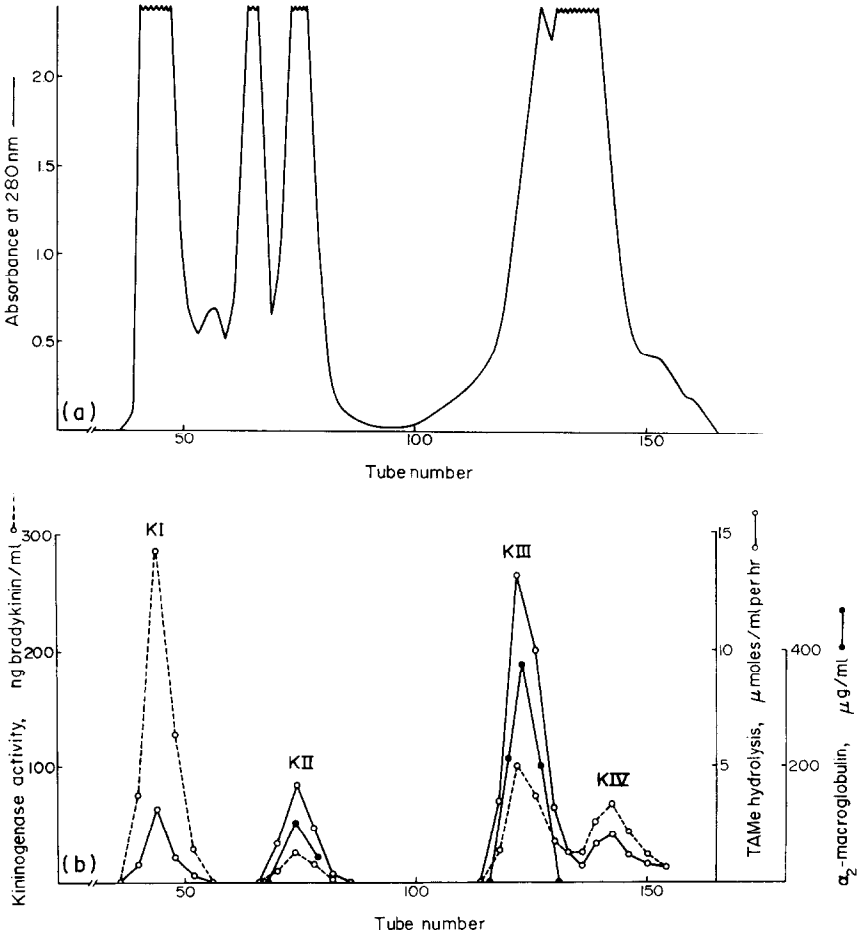

Fig. 1. DEAE cellulose chromatography of acetone-activated plasma. Fifteen ml of plasma were activated with 16% (v/v) acetone. Five ml fractions were collected at flow rate of 25 ml/hr. a: absorbance at 280 nm, b: TAMe hydrolysis, kininogenase activity and α_2-macroglobulin.

Table 1. TAMe hydrolysis and PPAN cleavage of four esterases in acetone-activated plasma

	KI	KII	KIII	KIV
PPAN cleavage / TAMe hydrolysis ratio	1.80	0.75	0.74	0.91
TAMe hydrolysis*	0.040	0.056	0.140	0.024
PPAN cleavage*	0.072	0.042	0.104	0.022

* TAMe hydrolysis and PPAN cleavage were expressed in μmoles/ml/min.

graphed sample remained in KIV region and the other appeared in KI region. When plasma was activated with 25% (v/v) acetone, most of the esterase activity appeared in KI and relatively small esterase activity was revealed in KII and KIII as shown in Fig. 2.

Gel filtration on Sepharose 4 B of acetone-activated plasma

The acetone-activated plasma was also subjected to a column of Sepharose 4 B as shown in Fig. 3. Fractionation revealed one major esterase peak and two minor esterase peaks. Occasionally these minor esterase peaks were negligible, but significant kininogenase activity was always detectable at the corresponding regions. Most prekallikrein coincided with the distribution of the major esterase peak. A trace of another prekallikrein was observed in the late descending limb of the major protein peak. These results suggest that the first prekallikrein represents the prekallikrein–kininogen complex and the second prekallikrein represents free prekallikrein. Mandle *et al.* have described that prekallikrein and high mol. wt. (HMW) kininogen circulate in plasma as a noncovalently linked complex [13].

When each of the four esterases obtained by DEAE cellulose chromatography was applied to a Sepharose 4 B column, KIII was first eluted, KII

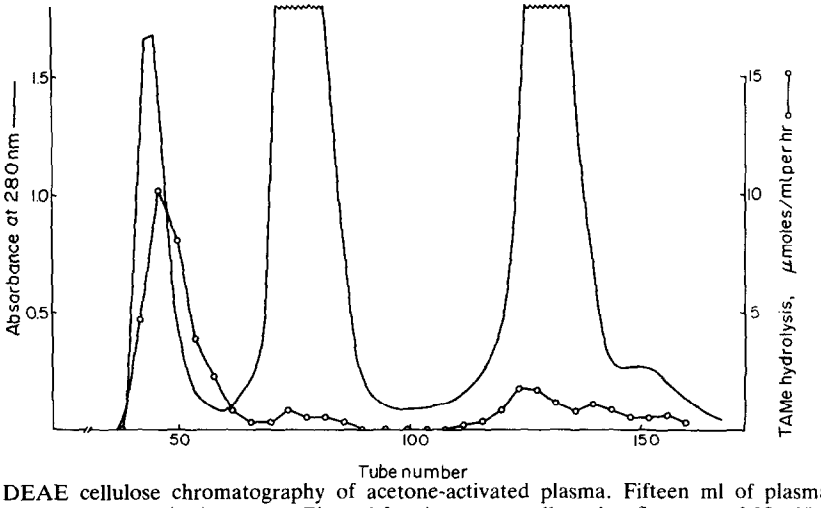

Fig. 2. DEAE cellulose chromatography of acetone-activated plasma. Fifteen ml of plasma were activated with 25% (v/v) acetone. Five ml fractions were collected at flow rate of 25 ml/hr.

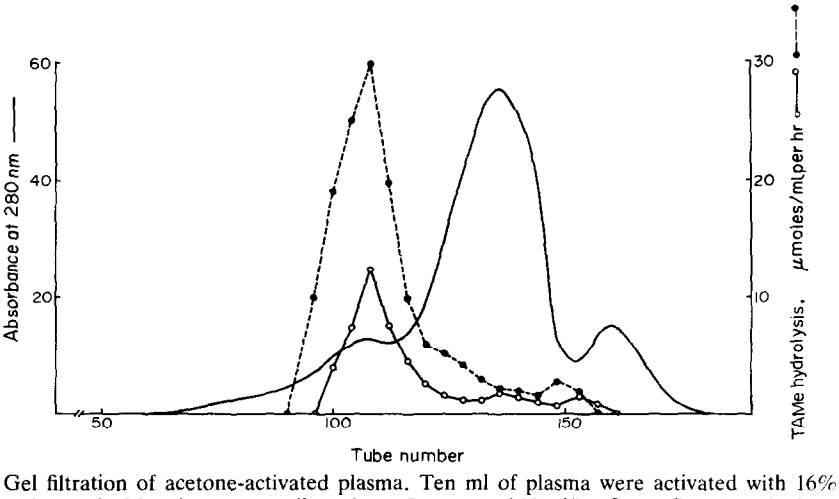

Fig. 3. Gel filtration of acetone-activated plasma. Ten ml of plasma were activated with 16% (v/v) acetone. Three ml of fractions were collected at a flow rate of 12 ml/hr. ○——○: TAMe hydrolysis by esterase generated after acetone-activation of plasma; ●----●: TAMe hydrolysis by esterase generated after trypsin treatment of effluent (prekallikrein). Esterase activity of activated prekallikrein was obtained by substracting the acetone-activated esterase activity from the activity obtained after incubation with trypsin.

came second, next order were KIV and KI without any separation of the esterase activity. From the behaviour of the four esterases on gel filtration, it is reasonably conceivable that the major esterase peak, the first minor esterase peak, and the second minor esterase peak represent KII + KIII, KIV and KI, respectively.

Properties

The four esterases from acetone-activated plasma are considered kallikreins from the findings that these esterases have the abilities such as cleavage of PPAN, hydrolysis of TAMe and kinin generation from kininogen. The ratio of PPAN cleavage to TAMe hydrolysis in Table 1 indicates that PPAN is the most cleavable substrate for the esterase in KI. The mol. wt. of the esterase in KI was estimated to be about 100,000 by gel filtration on a Sephadex G-200 column. The mol. wt. is agreement with various other plasma kallikrein described in previous reports [7, 14, 15]. The esterase in KII and KIII coincided with the distribution of α2M as shown in Fig. 1b. Ammonium sulphate [16] failed to separate α2M from the esterase activity in KII and KIII. In immunodiffusion of human HMW kininogen, KIII and KIV formed precipitation lines with human HMW kininogen antibody but not in KI and KIV as shown in Fig. 4. The alternations induced by protease inhibitors on TAMe hydrolysis were shown in Table 2. Table 2 indicates differences in behaviour between α2M-free kallikreins and kallikreins linked with α2M. K_m values of the four kallikreins were determined by the Lineweaver–Burk plot. The K_m values of KI, KII, KIII and KIV were 2.27×10^{-3}, 7.14×10^{-3}, 7.69×10^{-3} and 3.59×10^{-3} M for TAMe hydrolysis, respectively and 0.22×10^{-3}, 1.93×10^{-3}, 2.21×10^{-3} and 0.29×10^{-3} M for BAEe hydrolysis, respectively. Kinin generation by the kininogenase activity in KI progressively increased with incubation time, reaching a plateau in 10 min. A similar pattern of kinin generation was seen with the kininogenase activity in KIV but the maximum levels were less as shown in Fig. 5.

DISCUSSION

The four esterase preparations isolated in this paper are considered to be identical with plasma kallikreins. The following lines of evidence have been obtained to support this conclusion. (1) The preparations have kininogenase activity. (2) PPAN is cleaved by these preparations. The kallikrein in KI is not absorbed by DEAE cellulose. These findings indicate that the kallikrein in KI represents free kallikrein. The coincidence of α2M with the distribution of esterase activity on chromatography and gel filtration, the potent esterase activity with slight kininogenase activity, and the ineffectiveness in inhibiting esterase activity by SBTI indicate that the kallikrein in KII and KIII represents a kallikrein–α2M complex. SBTI is ineffective in inhibiting kallikrein esterase activity when kallikrein binds with α2M [16, 17]. The HMW kininogen in KIII and KIV coincided with the distribution of the esterase activity on gel filtration on Sepharose 4 B. But it was partially separated on chromatography on DEAE cellulose. These properties resemble the prekallikrein–

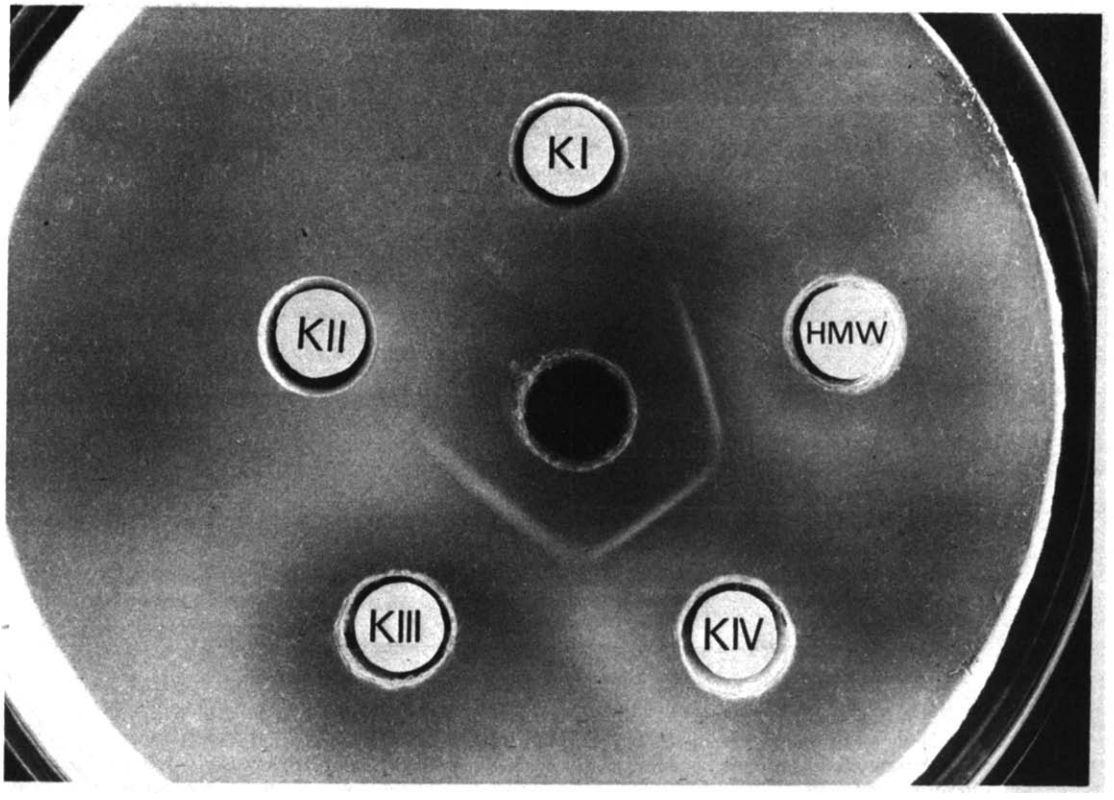

Fig. 4. Double immunodiffusion analysis of KI, KII, KIII, KIV and human high molecular weight (HMW) kininogen. The center well contains rabbit anti-human HMW kininogen serum.

Table 2. Inhibition of TAMe hydrolysis by various kallikrein preparations

Inhibitor	Concentration	Kallikrein	Inhibition (%) Kallikrein–α2M complex	Kallikrein–α2M–HMW kininogen complex	Kallikrein–HMW kininogen complex
SBTI	5 μg	84			50
	50 μg	96	0	0	70
	125 μg	100	0	0	75
Trasylol	5 KIU	46			74
	50 KIU	71	9	16	91
	125 KIU	77	18	23	95
DFP	1×10^{-4} M	100	77	75	100
AMCHA	1×10^{-3} M	0	0	0	0

The enzymes eluted from DEAE cellulose column were separately applied to Sepharose 4 B column, and used for the experiments.

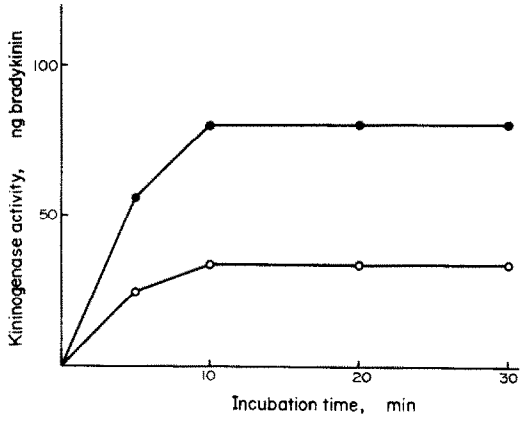

Fig. 5. Kininogenase activity of kallikrein and kallikrein–high molecular weight (HMW) kininogen complex. Fifty units of kallikrein or kallikrein–HMW kininogen complex, which was isolated by DEAE cellulose chromatography and Sepharose 4 B, was used for the experiment. ●——●: kallikrein, ○——○: kallikrein–HMW kininogen complex.

HMW kininogen complex described by Mandle *et al.* [13]. These facts indicate that the kallikrein in KIII and KIV is probably a kallikrein–HMW kininogen complex. The evidence that the kallikrein in KIV is linked with HMW–kininogen alone was supported by the behaviour of kallikrein–HMW kininogen complex preparation on Sephacryl S-200 or Sepharose 4 B (unpublished data). From the above conclusion, it is probably demonstrated that KII is kallikrein–α2M complex and KIII is kallikrein–α2M–HMW kininogen complex.

Two forms of kallikrein [1–5, 9] and three forms of kallikreins [7, 8] have been described in human plasma. Free kallikrein, aggregation or dimer formation of kallikrein, and kallikrein–α2M complex have been included in these reports. Prekallikrein is complexed with HMW kininogen in plasma [13]. Therefore, it is likely to produce kallikrein–HMW kininogen complex by the activation of plasma. But no report has been demonstrated on the kallikrein–HMW kininogen complex such as the kallikrein complex in KIII and KIV.

The predominant production of kallikrein–α2M–HMW kininogen complex may reflect that the generated kallikrein–HMW kininogen complex reacts with naturally occurring plasma kallikrein inhibitors. HMW kininogen might gradually dissociate from the kallikrein–HMW kininogen complex and kallikrein–α2M–HMW kininogen complex in plasma resulting in the production of kallikrein and kallikrein–α2M complex, although investigation is required for the process of HMW kininogen dissociation.

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