Biochimica et Biophysica Acta, 438 (1976) 250—264

BBA 67826

HUMAN PLASMA KALLIKREINS AND THEIR INHIBITION BY AMIDINO COMPOUNDS

SYED S. ASGHAR, F.C.P.W. MEIJLINK, K.W. PONDMAN and R.H. CORMANE

Department of Dermatology, Binnengasthuis, University of Amsterdam and Laboratory of Immunochemistry, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam (The Netherlands)

(Received November 3rd, 1975)

Summary

Human plasma kallikreins (EC 3.4.21.8) were purified as three distinct enzyme entities which hydrolyzed arginine esters and were active in releasing kinin from heated human plasma as measured by guinea pig ileum contraction bio-assay. The three enzymatically active fractions were termed as 19 S, 7 S-I and 7 S-II kallikreins. They represented purifications of 262-, 2200- and 110-fold, respectively. These enzyme activities showed differences in physicochemical and biochemical properties as it appears from their elution profile on Sephadex G-200 and DEAE-cellulose columns, affinity for substrates and susceptibility of inhibition by various protease inhibitors such as trasylol and soya bean trypsin inhibitor. The data suggest that all these three enzyme preparations were most likely kallikreins.

All these three enzymes (19 S, 7 S-I and 7 S-II) were inhibited by a series of amidino compounds competitively. Diamidines consisting of two amidinophenyl residues linked in para position by molecular bridge were comparatively stronger inhibitors of all the three enzymes than those linked in meta position and those having single ring structure. The possibility that some of these amidino compounds might prove to be useful for treatment of disease states where the kallikrein-kinin system plays a role, is discussed.

Introduction

Kallikrein (EC 3.4.21.8) is the general designation for serine proteinases which effect the release of vasodepressor peptides called kinins from protein

Abbreviations: Bz-Arg-OEt, benzoyl-arginine ethyl ester; Ac-Tyr-OEt, N-acetyltyrosine ethyl ester; Tos-Arg-OMe, p-toluene sulphonyl-L-arginine methyl ester; Tos-Lys-OMe, tosyl-L-lysine methyl ester.

precursors by limited proteolysis [1]. Proteases with this property have been found in urine, pancreatic and other glandular secretions and plasma from various sources but appear to be different entities [2]. Human pancreatic, urinary and plasma kallikreins have been shown to be different in their susceptibility to proteolytic inhibitors, ability to hydrolyze synthetic substrates, electrophoretic mobility and immunochemical behaviour, in spite of the fact that they all liberate kinins [2—6].

Recently, serum kallikrein has received increasing attention after it was found that under pathological conditions an increase in activation of this enzyme from its precursor, kallikreinogen, occurs [7,8]. In clinical practice, an inhibitor from bovine organ, trasylol, is employed to inhibit this enzyme [9]. As a polypeptide, this inhibitor has the disadvantage of being effective only after parenteral application [9]. Moreover, since it is a foreign polypeptide for the patients, hypersensitive reactions might be expected following its repeated administration. It appeared worthwhile, therefore, to make a search for other non-toxic, low molecular weight inhibitors of human plasma kallikreins. From this point of view human plasma kallikreins were isolated, characterized and tested for their inhibition by various amidines. The results are the subject of this communication.

Materials and Methods

Chemicals. Benzoyl-arginine ethyl ester (Bz-Arg-OEt), ovomucoid trypsin inhibitor and lima bean trypsin inhibitor were purchased from Sigma, *N*-acetyltyrosine ethyl ester (Ac-Tyr-OEt) from Merck, *p*-toluene sulphonyl-L-arginine methyl ester (Tos-Arg-OMe) and tosyl-L-lysine methyl ester (Tos-Lys-OMe) from Calbiochem., soya bean trypsin inhibitor from BDH and trasylol from Bayer. Hexadimetherine bromide was obtained from Aldrich-Europe and heparin from Vitrum. *p*-Aminobenzamidine hydrochloride was purchased from Cyclochemical Division of Travenol Laboratories, *N*-amidinobenzamide from Eastman Organic Chemicals and the rest of the amidino compounds were gifts from May and Baker Ltd.

Antisera. Antiserum to human α_1-antitrypsin, inter-α-antitrypsin and α_1-antichymotrypsin were obtained from Behringwerke. Antisera to human fibrinogen, complement (β_1A, β_1E and α_2D), α_2-macroglobulin, Cl-esterase inhibitor, albumin, IgG and IgM were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. Antisera to human plasminogen, clotting factor II and VIII were obtained as gifts from Dr. E.A. van Royen, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

Preparation of C1-esterase inhibitor. C1-esterase inhibitor from human plasma was prepared by the method of Vogelaar et al. [10] after removal of prothrombin complex. The preparation was 150-fold purified in comparison to normal plasma and contained 1200 units/ml of reconstituted inhibitor preparation.

Purification of kallikreins. Cohn fraction IV was prepared by the method of Cohn and associates [11]. 4.3 mg paste was suspended in 10 ml phosphate-buffered saline, pH 7.4. The precipitate was removed by centrifugation and the

supernatant was applied on Sephadex G-200 column (3.4 × 90 cm), pre-equilibrated with phosphate-buffered saline, pH 7.4. The column was run at a speed of 0.1 ml/min and 2.0-ml fractions were collected. Two protein peaks were obtained. The first peak contained α_2-macroglobulin and the last peak contained albumin. They were therefore designated as 19 S and 4 S peaks. In between 19 S and 4 S peaks, several fractions contained IgG and this region without a protein peak was designated as 7 S region. Kallikrein was localized in 19 S peak and 7 S region. Some fractions in between 19 S peak and 7 S region contained factor XI which were rejected. The remaining fractions of 19 S and 7 S region were pooled separately.

The kallikrein present in 19 S peak was precipitated with 45% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in phosphate-buffered saline and dialyzed against this buffer at 4°C until all $(NH_4)_2SO_4$ was removed. The dialyzed solution was designated as 19 S kallikrein.

The pooled fractions containing arginine esterase activity were dialyzed against 0.005 M phosphate buffer, pH 8.0. The dialyzed pool was applied on DEAE-cellulose column (1.6 × 24 cm) pre-equilibrated with 0.005 M phosphate buffer, pH 8.0. After washing the column with 120 ml equilibrating buffer, a gradient of NaCl was established using 300 ml of equilibrating buffer in mixing vessel and 300 ml of 0.005 M phosphate buffer, pH 8.0, containing 0.7 M NaCl in the other vessel. 11-ml fractions were collected at a speed of 0.5 ml/min. Two peaks of enzyme activity were obtained, one in the fractions containing unadsorbed proteins and the other in fractions containing 80—160 mequiv. NaCl per l.

The enzyme in unadsorbed fractions was dialyzed against phosphate-buffered saline, pH 7.4, and designated as 7 S-I enzyme.

The fractions containing 80—160 mequiv. NaCl per l were pooled and chromatographed on Sephadex G-200 to remove contaminating proteins belonging to the 4 S peak. The enzyme was dialyzed against phosphate-buffered saline and designated as 7 S-II enzyme.

Measurement of esterase activity. The esterolytic activity of kallikrein was measured using Bz-Arg-OEt as substrate. The hydrolysis of Bz-Arg-OEt was monitored continuously with the help of pH-stat (type TTT11b with titrigraph 3, Radiometer, Copenhagen, Denmark). In a total volume of 7.5 ml the titration vessel contained a suitable volume of enzyme preparation, 0.5 ml of 0.1 M Bz-Arg-OEt and phosphate-buffered saline. The pH was adjusted to 8.0 and the amount of 10 mM NaOH required to maintain the pH of the mixture to 8.0 was recorded by automatic recorder. Until mentioned otherwise, the reaction was carried out at 37°C. The enzyme activity was expressed in terms of μmol H^+ produced per h per ml enzyme and specific activity was expressed in terms of μmol H^+ produced per h per mg protein.

Other procedures. The clotting factor XI was estimated by the method of Horowitz et al. [12]. Caseinolytic and hemoglobin splitting activity was measured by the methods described by Geratz [13] and Press et al. [14] at pH 7.4 and 8.0. Esterolysis of various esters were measured by the method described above for measuring the hydrolysis of Bz-Arg-OEt.

The biological activity of kallikrein by kinin release was observed through the use of the guinea pig ileum [15] in Tyrode's solution at 36°C, measured

with a transducer and recorder. The substrate was freshly frozen human plasma heated at 60°C for 60 min. Bradykinin was used as standard reference.

Immunodiffusion experiments were performed according to the method described by Ouchterlony [16].

Protein was estimated by the method of Folin and Ciocalteu as described by Lowry et al. [17].

Results

Purification of plasma kallikrein

The easy availability of Cohn fraction IV isolated from human plasma and the fact that it was very rich in kallikrein activity as compared to kaolin-activated normal plasma made us use it as starting material for isolation of plasma kallikreins. The use of Cohn fraction IV as starting material also had an advantage over the normal plasma in that it was free of other major arginine esterases such as plasmin and thrombin. This prior separation was a fortunate feature of Cohn fractionation scheme in which these esterases appear in fractions other than fraction IV [18]. The arginine esterase activity present in Cohn fraction IV has been shown to be similar to that of kaolin-activated plasma and is also believed to be identical to plasma kallikrein which effects the release of kinin from plasma substrate [19,20].

The procedures used in the purification of plasma kallikreins from Cohn fraction IV are shown in the flow diagram (Fig. 1). The results obtained from these procedures are summarized in Table I. Since kaolin-activated individual

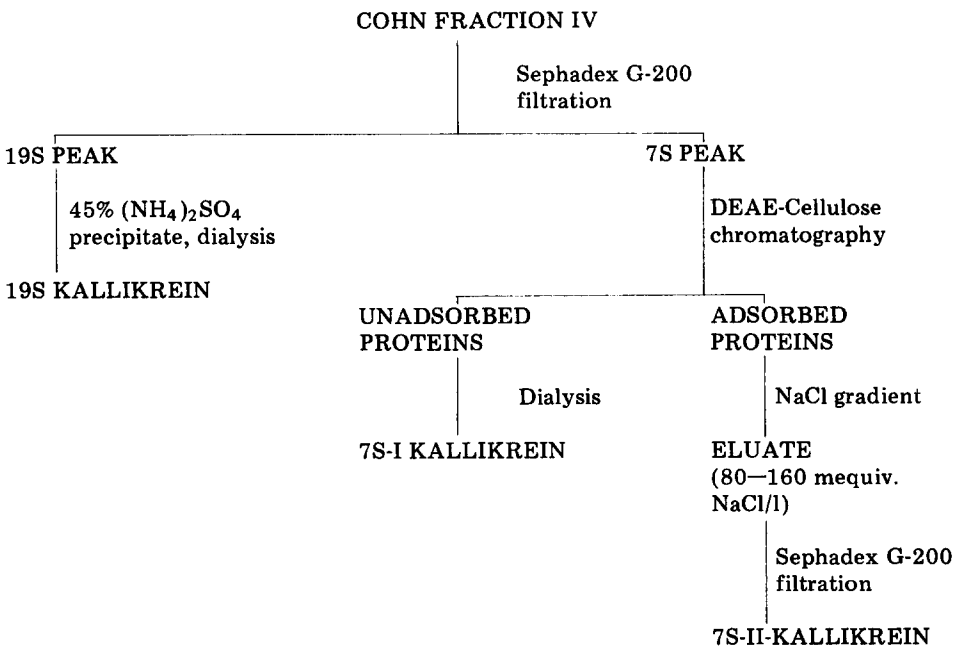

Fig. 1. Flow diagram showing the procedures used for the isolation of plasma kallikreins. Experimental details are described in the text.

TABLE I

SUMMARY OF PROCEDURES OF PURIFICATION OF HUMAN PLASMA KALLIKREINS FROM COHN FRACTION IV

Experimental conditions are described in the text.

Procedures	Specific activity (μmol H^+ produced h/mg per protein)	Total esterase activity (%)	Purification factor (activated plasma = 1)
19 S Kallikrein			
Cohn fraction IV	2.7	100	57
Sephadex G-200 19 S peak	5.1	26	108
45% $(NH_4)_2SO_4$ precipitate —19 S Kallikrein	12.4	13	262
7 S-I Kallikrein			
Sephadex G-200 7 S peak obtained by passing Cohn fraction IV	4.5	10	95
DEAE-cellulose peak I (eluted with equilibrating buffer) —7 S-I Kallikrein	105	20	2200
7 S-II Kallikrein			
DEAE-cellulose peak II (eluted with 80—160 mequiv. NaCl/l) obtained by passing 7 S peak of Sephadex G-200	5.0	11	105
Rechromatography on Sephadex G-200, 7-S peak —7 S-II Kallikrein	5.2	8	110

* See also flow diagram (Fig. 1).

normal plasma samples as well as pooled normal serum showed similar and reproducible specific activity, the degree of purification has been expressed in terms of purification achieved over kaolin-activated plasma. Cohn fraction IV obtained from pooled normal plasma had a specific activity 57 times higher than kaolin-activated normal human plasma. When it was passed on Sephadex G-200 and the fractions tested for the presence of kallikrein, 19 S and 7 S regions were found to contain arginine esterase activity whereas 4 S peak was free of it (Fig. 2). 19 S, 7 S and 4 S regions were identified by detecting α_2-macroglobulin (19.6 S), IgG (7 S) and albumin (4 S) in the respective fractions immunochemically.

The enzyme in 19 S peak was further purified by precipitation at 45% $(NH_4)_2SO_4$ saturation. A 260-fold purification was achieved as compared to kaolin-activated plasma. For the sake of convenience in discussion, this enzyme will be referred to as 19 S kallikrein.

$(NH_4)_2SO_4$ treatment to 7 S peak resulted in a drastic loss of yield and therefore this step was avoided. DEAE-cellulose chromatography was used for further purification of enzyme present in 7 S peak. This separated the kallikrein peaks, one in fractions representing unadsorbed proteins and the other in fractions with 80—160 mequiv./l NaCl concentration (Fig. 2). The enzyme in the first peak representing unadsorbed proteins was 2200-fold purified with respect to kaolin-activated plasma. The estimated degree of purification was very high, perhaps partly due to actual purification and partly due to activation of the en-

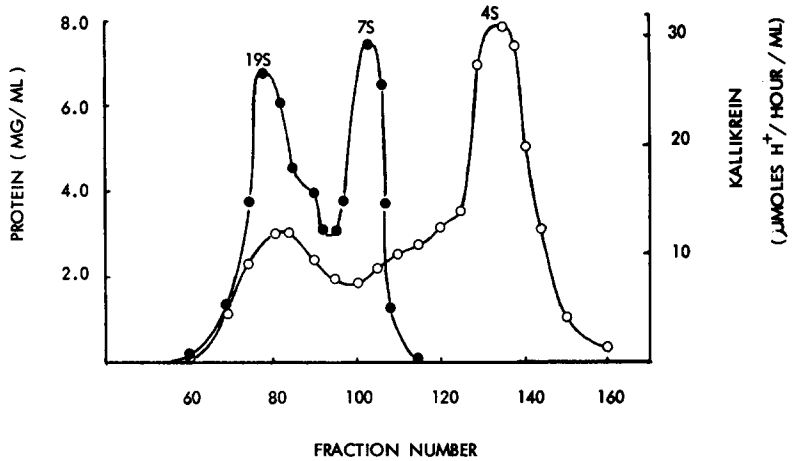

Fig. 2. A representative elution profile of kallikreins from a Sephadex G-200 column after application of Cohn fraction IV. ○———○, protein; ●———●, kallikrein.

zyme as evidenced by the increase in yield. This enzyme was designated as 7 S-I kallikrein.

The enzyme activity localized in fractions containing 80—160 mequiv./l NaCl concentration was contaminated with various proteins including some plasma protease inhibitors. These included α_1-antitrypsin, inter-α-trypsin inhibitor, α_1-antichymotrypsin, IgG and protein(s) reacting with anti-total complement (β_1A, β_1E and α_2D). However, all of these proteins except IgG were removed when these fractions were chromatographed again on Sephadex G-200. The enzyme appeared in the fractions which also contained IgG whereas the rest of the above-mentioned proteins appeared in later fractions. The enzyme preparation was 110-fold purified with respect to kaolin-activated plasma and was designated as 7 S-II kallikrein.

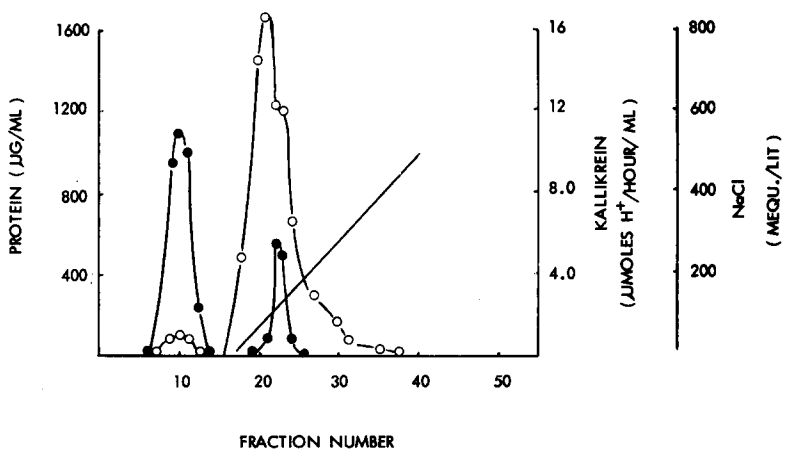

Fig. 3. A representative elution profile of kallikreins on DEAE-cellulose column after application of 7 S peak proteins obtained from Sephadex G-200 chromatography. ○———○, protein; ●———●, kallikrein, ———, NaCl.

Characterization of isolated kallikrein preparations

Since a large number of plasma proteolytic enzymes are capable of hydrolyzing basic amino acid esters, which include thrombin, plasmin, clotting factor XI, C1-esterase, C1r (one of the three subunits of the first component of complement possessing proteolytic and esterolytic activities) and PF/Dil (an enzyme found in 1 : 100 or more diluted plasma which increases capillary permeability, activates kallikrein and possesses esterase activity), a systematic study was carried out to ascertain whether or not the isolated preparations were kallikreins.

All the three enzyme preparations (19 S, 7 S-I and 7 S-II) effected the release of kinin from heated plasma which contracted guinea pig ileum when added directly to the tissue bath. These kallikrein preparations did not generate kinin activity in the absence of plasma. Such a dependence on plasma substrate is the property of kallikrein [21] which is also shared by PF/Dil. PF/Dil, however, does not generate kinin from plasma heated at 60° C, since it forms kinin by activation of endogenous plasma prekallikrein which is destroyed at 60° C. Thus, it appears that the three preparations functioned as kallikrein rather than PF/Dil.

When kallikrein is added to the tissue bath containing heated normal plasma and Tyrode's solution, a rapid kinin liberation, as evidenced by guinea pig ileum contraction, was noted which remained linear for 4—5 min in case of 7 S-I and 7 S-II and for 7—8 min in case of 19 S kallikrein. Thereafter, there was a plateau in the time curves of all the three preparations. From these curves it was possible to calculate the kinin-liberating activity arbitrarily. A comparison of the biological activity with esterolytic activity demonstrated that these activities were perhaps directly related. The ratios of biological and esterolytic activity of the three kallikreins were of the same order of magnitude (Table II). Similar results were obtained by Colman et al. [19] with the three forms of kallikreins they had isolated.

The well-known inhibitor of kinin generation by kallikrein, trasylol, completely abolished the contraction caused by all the three enzyme preparations. 2-Hydroxystilbamidine at a concentration necessary for complete inhibition of esterolytic activity also inhibited biological activity of all these preparations completely. This also suggests that esterolytic and contractile activities are closely related.

All the three preparations of kallikrein were unable to hydrolyse Ac-Tyr-OEt, lysine esters, casein and hemoglobin. These observations suggest that the isolated enzyme preparations were free of C1-esterase, those enzymes whose best substrates are lysine esters such as plasmin and thrombin, and non-specific proteolytic enzymes. Heparin (500 units/ml), ovomucoid trypsin inhibitor (1 mg/ml), lima bean trypsin inhibitor (1 mg/ml) and hexadimetherine bromide (500 μg/ml) did not inhibit any of the three preparations, thus excluding PF/Dil, trypsin and Hageman factor as entities responsible for arginine esterase activities. Trypsin is not expected to be present in the Cohn fraction even otherwise.

There were, however, some differences in the properties of 19 S, 7 S-I and 7 S-II preparations. Soya bean trypsin inhibitor was very effective in inhibiting esterolysis by 7 S-I preparation, less effective in inhibiting 7 S-II preparation and ineffective in inhibiting 19 S enzyme. Trasylol also inhibited the esterolytic

TABLE II

BIOLOGICAL AND ESTEROLYTIC ACTIVITIES OF PLASMA KALLIKREINS

Experimental conditions are described in the text.

Preparation	Biological activity * (mm contraction/min per mg protein)	Esterolytic activity (μmol H^+/h per mg protein)	$\frac{\text{Biological}}{\text{Esterolytic}}$ ratio
19 S	16	12.2	1.3
7 S-I	98.4	105	0.93
7S-II	4.1	5.2	0.8

* 0.3 ml heated plasma was used in these experiments.

activity of 7 S-I and 7 S-II but not of 19 S preparations. The arginine esterase activities of 19 S and 7 S-I enzymes were not inhibited by C1-esterase inhibitor. The 7 S-II kallikrein was gradually inactivated by a very high concentration of C1-esterase inhibitor. The manner of inactivation and the high concentrations required for complete inactivation suggest that 7 S-II preparation, like others, was also distinct from C1r, plasmin and C1-esterase. 7 S-I enzyme was slightly activated by heparin and appreciably activated by C1-esterase inhibitor. The possible explanation for such an activation may be that these molecules may interact with the enzyme bringing about some steric changes and thereby activating it. Some of the results described here are presented in Table III.

TABLE III

INHIBITION OF HUMAN PLASMA KALLIKREINS BY SOME PROTEASE INHIBITORS

Experimental conditions are described in the text.

Inhibitor	Concentration	Esterase * activity (%)	
		7 S-I **	7 S-II
No inhibitor	—	100	100
Trasylol	50 units/ml	72	48
	75 units/ml	55	15
	125 units/ml	22	0
Soya bean trypsin inhibitor	5 μg/ml	55	100
	10 μg/ml	<5	90
	66 μg/ml	0	63
	266 μg/ml	—	60
Heparin	50 units/ml	114	100
Cl-esterase inhibitor	16 units/ml	155	66 ***

* 19 S kallikrein was not inhibited by any of the inhibitors mentioned above. Moreover, none of the three preparations were inhibited by ovomucoid trypsin inhibitor, lima bean trypsin inhibitor and hexadimetherine bromide.

** Calculated from 15 min reading because of gradual inactivation of enzyme with time.

*** There was progressive increase in inactivation with time in presence of inhibitor. The activity here is calculated from 30 min readings.

Since clotting factor XI is a known arginine esterase, it was important to exclude this entity as responsible for arginine esterase activity. During the purification on Sephadex G-200, factor XI peak appeared in between 19 S and 7 S peaks. In one of the several chromatographic runs in which the two peaks were quite distant there were several fractions in which Bz-Arg-OEt-splitting activity was at its lowest ebb, almost negligible, but factor XI was showing a peak value in these fractions. This suggests that factor XI, if present in the final preparation, should not contribute to significant arginine esterase activity. During purification procedure, however, after Sephadex G-200 chromatography, fractions containing factor XI were removed and the final preparations were found not to be contaminated with it. The impression that even if factor XI is present as contaminant it will not contribute to significant arginine esterase activity under the assay conditions was supported by an earlier report [22] that plasma severely deficient in factor XI showed a kaolin-activated arginine esterase activity comparable in amount to normal plasma.

As tested by immunodiffusion method, 7 S-I and 7 S-II preparations were free of plasminogen, fibrinogen, complement components (β_1A, β_1E and α_2D), clotting factors II and VIII, C1-esterase inhibitor, α_2-macroglobulin, α_1-antitrypsin, inter-α-antitrypsin and α_1-antichymotrypsin. The 19 S preparation, was also free of these proteins except α_2-macroglobulin.

The enzyme activity was gradually lost from the preparation after incubation at room temperature with solidified anti-α_2-macroglobulin prepared according to Avrameas and Ternynck [23]. Perhaps it was adsorbed on solidified antisera.

When the effect of substrate concentration on the activities of the isolated enzymes was studied and K_m values calculated from Lineweaver-Burke plots, 19 S, 7 S-I and 7 S-II kallikreins showed K_m values $5.4 \cdot 10^{-2}$, $8.3 \cdot 10^{-3}$ and $4.76 \cdot 10^{-3}$ M respectively for Tos-Arg-OMe hydrolysis. Colman and coworkers [19] have reported a K_m value $7.4 \cdot 10^{-3}$ M with their kallikrein I preparation and Hial and coworkers [24] obtained $1.14 \cdot 10^{-3}$ M for urinary kallikrein for the same substrate. Table IV shows the K_m values obtained with our three preparations using Bz-Arg-OMe and Tos-Arg-OMe as substrates. Unlike 19 S and 7 S-II preparations, 7 S-I kallikrein showed inhibition at high substrate concentration (Fig. 4). Colman et al. [19] also observed substrate inhibition at high Tos-Arg-OMe concentrations with their kallikrein III preparation. Esterolysis by the three enzyme entities was not inhibited by benzoyl arginine.

The above-mentioned results showed that the activities of the isolated preparations could not be ascribed to other known arginine esterases and some properties of these kallikreins resemble those described before for plasma kallikreins. Although 19 S kallikrein was contaminated with α_2-macroglobulin, its activity was still retained and therefore could be used for further experiments. However, 7 S-I and 7 S-II enzymes were considered free of possible interfering protease inhibitors.

Inhibition of isolated plasma kallikrein by amidino compounds

A series of amidino compounds were examined for their inhibitory effect on the hydrolysis of Bz-Arg-OEt by the isolated kallikreins. The inhibition was measured at two substrate and at varying inhibitor concentrations. The initial rate of reaction was calculated and the results were plotted according to the

TABLE IV

K_m VALUES OBTAINED WITH HUMAN PLASMA KALLIKREINS

Experimental conditions are described in the text.

Enzyme	K_m (M)	
	Bz-Arg-OEt	Tos-Arg-OEt
19 S	$1.2 \cdot 10^{-2}$	$5.4 \cdot 10^{-2}$
7 S-I	$3.3 \cdot 10^{-2}$	$8.3 \cdot 10^{-3}$
7 S-II	$1.6 \cdot 10^{-2}$	$4.76 \cdot 10^{-3}$

method of Dixon [25]. Since 7 S-I enzyme showed progressive rapid inactivation during its assay at 37°C, its activity was measured at 30°C at which temperature the inactivation was comparatively less. The kinetic parameters with this enzyme were calculated from 15 min readings.

Table IV shows the K_i values for inhibition of the three enzymes by amidino compounds as calculated from Dixon plots. The inhibition caused by almost all these compounds was competitive in nature, as evidenced by the intersection of the lines above x-axis. Because of the irregular kinetic behaviour of all the enzyme preparations in presence of M and B 4596, K_i values could not be calculated, although this compound was a strong inhibitor of all these preparations. ϵ-Aminocaproic acid was also included in this study for comparison pur-

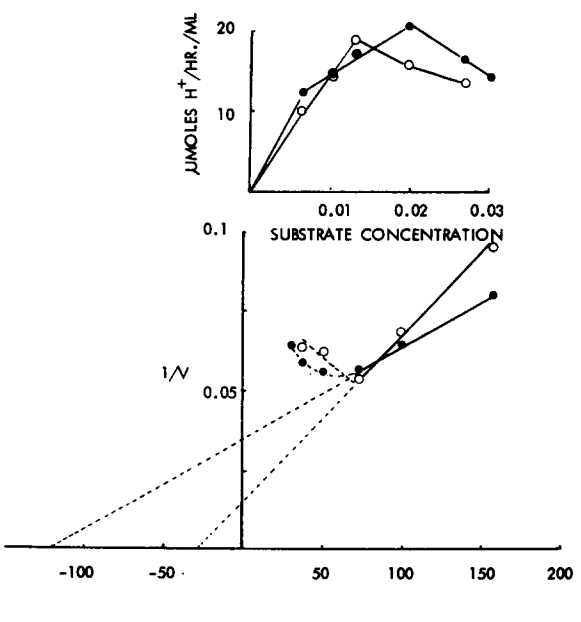

Fig. 4. Inhibition of 7 S-I kallikrein by excess of substrate. Double reciprocal plot of initial velocities of ester hydrolysis at different substrate concentrations. Experimental conditions are described in the text. ○———○, Bz-Arg-OEt; ●———●, Tos-Arg-OMe. $1/V$ is expressed as reciprocal of initial velocity calculated from 15 min readings and 1/S as reciprocal of ester concentration.

TABLE V

INHIBITION OF 19 S, 7 S-I AND 7 S-II KALLIKREINS BY AMIDINO COMPOUNDS

Experimental conditions are described in the text.

Compound	K_i (M)		
	19 S	7 S-I *	7 S-II
ε-Aminocaproic acid	no inhibition	no inhibition	no inhibition
N-Amidinobenzamide	no inhibition	no inhibition	no inhibition
p-Aminobenzamide	—	$2 \cdot 10^{-4}$	$1 \cdot 10^{-4}$
Amicarbalide	$1.1 \cdot 10^{-4}$	$1 \cdot 10^{-4}$	$2.4 \cdot 10^{-4}$
Propamidine	$1.75 \cdot 10^{-5}$	$9 \cdot 10^{-5}$	$5 \cdot 10^{-5}$
Stilbamidine	$4.5 \cdot 10^{-5}$	$7 \cdot 10^{-5}$	$4 \cdot 10^{-5}$
2-Hydroxystilbamidine	$3.6 \cdot 10^{-5}$	$7 \cdot 10^{-5}$	$8 \cdot 10^{-5}$
Pentamidine	$1.5 \cdot 10^{-5}$	$7 \cdot 10^{-5}$	$4 \cdot 10^{-5}$
Dibromopropamidine	$1.4 \cdot 10^{-5}$	$8 \cdot 10^{-5}$	$4 \cdot 10^{-5}$
M & B 4596	**	**	**

* Calculated from 15 min readings because of gradual inactivation of enzyme.

** K_i values were not calculated because of irregular kinetic behaviour.

poses which was found to have no effect on any of these preparations at as high a concentration as $1.2 \cdot 10^{-2}$ M.

As is evident from Table V, amidino compounds having a two-ring skelton linked in para position by a molecular bridge were the strongest inhibitors of kallikreins than those with single ring structure or two ring structure linked in meta position. Propamidine, stilbamidine, pentamidine, 2-hydroxybamidine and dibromopropamidine were stronger inhibitors of all the free forms of kallikrein than amicarbalide, *N*-amidinobenzamide and *p*-aminobenzamide.

In spite of the fact that we have studied structurally different amidines, it will not be inappropriate to say that our results are consistent with those of Sampaio et al. [26] who have shown inhibition of human plasma kallikreins by benzamidine. The esterolytic activity of human urinary kallikrein has also been shown to be inhibited by benzamidine but not its biological activity [24]. Davies and Lowe [27] have shown that amidines inhibit guinea pig plasma kallikrein. Geratz and Webster [28] have demonstrated the inhibition of procine pancreatic kallikrein by various amidines.

Discussion

The purification of human plasma kallikreins has been a challenging problem because of the close resemblance of kallikrein to other plasma proteases. Harpel [29] was unable to separate the kallikrein from factor XI by isoelectric focussing, acrylamide gel electrophoresis, or Sephadex G-200. Schrieber et al. [30] found that the isoelectric points of kallikrein, plasminogen activator and factor XI were very close to each other. Similar problems have been encountered by Bagdasarian and coworkers [31]. In our experiments many such problems were overcome by the use of Cohn fraction IV as starting material, since it was free

of many arginine esterases. Factor XI was removed by simply rejecting the fractions between the 19-S and 7-S regions which contained it. The purification procedure finally resulted in separation of three different kallikrein entities one of which was highly pure. The comparison of the degree of purification obtained in the present work with that obtained by others is particularly difficult because of the enzyme activation and its separation into different entities during the purification procedure. However, the purification achieved with 7 S-I kallikrein is comparable to that recently obtained by other procedures [19,26, 31]. The method described is relatively simple and the enzyme preparations obtained appear to be pure enough for enzymic studies involving the evaluation of inhibitors which was our initial goal.

The arginine esterase activities obtained after purification were most probably identical with plasma kallikreins. Three lines of evidence have been obtained to support this conclusion. (1) The isolated preparations released kinins from heated plasma that contracted the guinea pig ileum. (2) The activities could not be ascribed to other known esterases. (3) The properties of the isolated kallikreins resemble those described before the human plasma kallikreins. It is recognized that this evidence is indirect and that anyone of the abobe-mentioned criteria by itself is indufficient to establish the identity of the isolated kallikreins. However, the ability to meet all these criteria allow one to conclude that there is a high probability that the enzyme preparations were kallikreins.

The isolated preparations (19 S, 7 S-I and 7 S-II) released substance(s) from heated plasma that contracted the guinea pig ileum. The quickly generated contraction-causing substance(s) was most likely kinin since the well-known inhibitor of kinin generation by kallikrein, trasylol, completely abolished the contraction caused by all the three enzyme preparations. Furthermore, 2-hydroxystilbamidine strongly inhibited the esterolytic as well as the biological activities of all these preparations. This suggested that the esterolytic and biological activities were closely related. The ratios of biological and esterolytic activities of the three preparations were of the same order of magnitude, suggesting that these activities were directly related. It was therefore concluded from these observations that the isolated preparations were kallikreins possessing esterolytic as well as proteolytic (kinin-forming) activities. The enzyme preparations did not hydrolyze casein, hemoglobin and lysine esters, thus distinguishing them from various non-specific proteases, thrombin and plasmin. The clotting factor XI was removed during purification as already described, and was not present in the final enzyme preparations. The unability to catalyze the hydrolysis of Ac-Tyr-OEt, the unability of C1-esterase inhibitor to inhibit 19 S and 7 S-I enzyme, the manner and the quantitty required for appreciable inhibition of 7 S-II enzyme suggest that the enzyme preparations were free of C1s and C1r. None of the three enzymes was inhibited by heparin and hexadimetherine bromide, suggesting that their esterase activities can not be attributed to PF/Dil and clotting factor XII. Failure to detect clotting factor II, VIII and XI in the final enzyme preparations suggest that these factors are also not responsible for esterase activities of the three enzymes.

The above-mentioned facts suggest that the isolated enzyme activities could not be ascribed to other known arginine esterases. As regards the resemblance of isolated kallikreins with human plasma kallikreins described before, many of

our results resemble, mostly with those of Colman et al. [19,20], Dyce et al. [32] and Webster and Innerfield [33]. Because of many resemblances and despite some differences in the results obtained, the overall conclusion remains that the isolated preparations were most likely kallikreins particularly because similar differences could be noted between the results of previous authors. Colman et al. [19,20] obtained three forms of kallikreins where as Webster and Innerfield [33] concluded that two forms of kallikreins exist in human plasma. We have, however, found three forms of kallikreins (19 S, 7 S-I and 7 S-II). Our 19-S kallikrein resembles in properties the kallikrein III of Colman et al. [19, 20] and the kallikrein of Dyce et al. [32] in that its esterolytic activity is not inhibited by plasma inhibitors or other natural protease inhibitors such as trasylol and soya bean trypsin inhibitor etc. However, it differs from kallikrein III of Colman et al. [19,20] in that it appears in 19 S fraction of Sephadex G-200 whereas kallikrein III does not. These differences may perhaps be accounted for by the binding of kallikreins to a carrier protein. Dyce et al. [32] reported his enzyme to be associated with α_2-macroglobulin of serum. Colman et al. [19, 20] were unable to detect any α_2-macroglobulin in kallikrein III preparation. Our 19-S preparation was always associated with α_2-macroglobulin. There are striking resemblances between 7 S-I and 7 S-II kallikreins on one hand and kallikrein I and kallikrein II of Colman and coworkers [19,20] on the other, particularly with regard to their inhibition by protease such as trasylol and SBTI etc. In inhibition by high substrate concentration our 7 S-I resembles kallikrein III of Colman and associates [19,20]. From these observations it appears that the isolated preparations are most probably kallikreins.

The differences in the properties of the three types of kallikreins as regards their inhibition by natural protease inhibitors could perhaps be explained on the basis of possible dimer formation by kallikrein molecules, as suggested by Colman and coworkers [19] or on the basis of binding of kallikrein with certain proteins such as α_2-macroglobulin or C1-esterase as demonstrated by Bagdasarian and coworkers [31]. The binding with protein carrier may perhaps bring about conformational change in kallikrein so that while active site may still be free to catalyze enzyme reaction, a comparatively larger inhibitor may not bind to it. Such an explanation can perhaps account for the differences in properties of 19 S, 7 S-I and 7 S-II kallikreins. The low molecular weight inhibitors (Table V) may perhaps still reach the active site and inhibit all the three types of kallikreins in spite of possible binding with other proteins. It might not be inappropriate to mention here the observations by Harpel [34] that enzymes which interact with α_2-macroglobulin such as thrombin, kallikrein and trypsin form a complex in which the enzymes retain their ability to degrade low molecular weight substrate while their proteolytic activity is lost.

The esterolytic activity of all isolated preparations was found to be competitively inhibited by amidines. Diamidines consisting of two amidinophenyl residues linked in para position were comparatively stronger inhibitors of all the three enzyme preparations than those linked in meta position and those having single ring structure. The inhibition of all the three forms by amidino compounds suggests similarities in the structure of their sites. There seem to be some similarities in the active sites of these kallikreins and with those of C1s, C1r, plasmin and trypsin. Possibly all these enzymes may have a hydrophobic

region at their active sites, through which aromatic skeleton of these inhibitors may form hydrophobic bonds. They may also have an anionic binding site to which the amidino group can bind through its positive charge. No attempt was made to draw conclusions about the conformational differences in the active sites of the three kallikreins on the basis of K_i values (or ΔF values calculated from it) obtained with different amidino compounds, since possible binding with other component such as α_2-macroglobulin might have brought about significant steric changes in enzymes molecule.

It will not be inappropriate here to mention that in vitro effectiveness of amidino compounds suggests their possible in vivo use in diseased conditions where the kallikrein system is thought to play a role. Such pathological conditions may include various types of inflammatory edema, shock, arthritis and carcinoid syndrome [35—38]. Davies and Lowe [27] have shown the beneficial effects of some of their inhibitors of kallikrein on artificially induced edema. However, thorough clinical trial is needed in various conditions. It has been suggested that they might also be useful to prevent the attack of edema in patients with hereditary angioneurotic edema [39].

Most of these amidino compounds have been in medical or veterinary use for years [40—45]. Their use as medicine is associated with some side-effects. Their main side-effect is their tendency to lower the blood pressure. It is suggested that this may be due to their property of releasing histamine [44]. Another explanation is the possible adrenogenic blocking effect [44]. However, the reversal of hypotensive effects by $CaCl_2$ has been described [43,28].

The fundamental skeleton of the most effective of these inhibitors is two benzamidines; linked in para position by a bridge of varying length. Further modifications in structure may increase their activity towards one enzyme and decrease it towards others. Furthermore, studies with agents that can counteract their side-effects, such as $CaCl_2$ and antihistamines, may lead us to believe that their toxicity could be neutralized. Further research in these directions may perhaps lead to their wide therapeutic use.

Acknowledgements

The authors are thankful for the skilful technical assistance of Miss Maria Voss and acknowledge the assistance of Mrs. E. Jansen-van den Bosch and Mr. Barend Vrees in preparing the manuscript for publication. As always, thanks are due to Mrs. M.C.F. Theuns-Jongen for her secretarial assistance.

References

1 Webster, M.E. (1970) Handbuch der experimentellen Pharmakologie (Erdös., E.G., ed.) Vol. 25, pp. 659—665, Springer-Verlag, New York
2 Werle, E. (1970) Handbuch der experimentellen Pharmakologie (Erdös. E.G., ed.) Vol. 25, pp. 1—6, Springer-Verlag, New York
3 Webster, M.E., Emmart, E.W., Turner, W.A., Moriya, H. and Pierce, J.V. (1963) Biochem. Pharmacol. 12, 511—519
4 Webster, M.E. and Pierce, J.V. (1963) Ann. N.Y. Acad. Sci. 104, 91—107
5 Moriya, H., Pierce, J.V. and Webster, M.E. (1963) Ann. N.Y. Acad. Sci. 104, 172—185
6 Webster, M.E. and Pierce, J.V. (1961) Proc. Soc. Biol. Med. 107, 186—191
7 Saldeen, T. (1973) New Aspects of Trasylol Therapy (Haberland, G.L. and Lewis, D.H., eds.) pp. 9—20, F.K. Schattauer Verlag, Stuttgart, New York

8 Webster, M.E. (1968) Fed. Proc. 27, 84—89
9 Haberland, G.L. (1973) New Aspects of Trasylol Therapy (Haberland, G.L. and Lewis, D.H. eds), pp. 1—7, F.J. Schattauer Verlag, Stuttgart, New York
10 Vogelaar, E.F., van der Meer, J., Brummelhuis, H.G.J. and Krijnen, H.W. (1972) Protides Biol. Fluids 20, 447—450
11 Cohn, E.J., Strong, L.E., Hughes, W.L., Mulford, D.J., Ashworth, J.N., Melin, M. and Taylor, H.J. (1946) J. Am. Chem. Soc. 68, 459—475
12 Harowitz, H.J., Wilcox, W.P. and Fujimoto, M.M. (1963) Blood 22, 35—43
13 Geratz, J.D. (1970) Thromb. Diath. Haemorrh. 23, 486—499
14 Press, E.M., Porter, R.R., Cebra, J. (1960) Biochem. J. 74, 501—514
15 Webster, M.E. and Prado, E.S. (1970) Methods in Enzymology (Perlmann, G.E. and Lorand, L., eds.), Vol. 19, pp. 681—699, Academic Press, New York
16 Ouchterlony, O. (1953) Acta Pathol. Microbiol. Scand. 32, 231—240
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Schultze, H.E. and Heremans, J.F. (1966) Molecular Biology of human proteins, Vol. I, pp. 250—252, Elsevier, New York
19 Colman, R.W., Mettler, L. and Sherry, S. (1969) J. Clin. Invest. 48, 11—12
20 Colman, R.W., Mettler, L. and Sherry, S. (1969) J. Clin. Invest. 48, 23—32
21 Webster, M.E. and Gilmore, J.P. (1965) Biochem. Pharmacol. 14, 1161—1163
22 Sherry, S., Alkjaersig, N.K., Fletcher, A.P. (1966) Throm. Diath. Haemorrh. 16 (Suppl. 20), 243—251
23 Avrameas, T. and Ternynck, T. (1969) Immunochemistry 6, 53—66
24 Hial, V., Diniz, C.R. and Mares-Guia, M. (1974) Biochemistry 13, 4311—4318
25 Dixon, M. (1953) Biochem. J. 55, 170—171
26 Sampaio, C., Wong, S. and Shaw, E. (1974) Arch. Biochem. Biophys. 165, 133—139
27 Davies, G.E. and Lowe, J.S. (1970) Adv. Exp. Med. Biol. 8, 453—460
28 Geratz, J.D. and Webster, W.P. (1971) Arch. Int. Pharmacodyn. 194, 359—370
29 Harpel, P.C. (1971) J. Clin. Invest. 50, 2084—2090
30 Schrieber, A.D., Kaplan, A.P. and Austen, K.F. (1973) J. Clin. Invest. 52, 1394—1401
31 Bagdasarian, A., Lahiri, B., Talmo, R.C., Wong, P. and Colman, R.W. (1974) J. Clin. Invest. 54, 1444—1454
32 Dyce, P., Wong, T., Adham, N., Mehl, J. and Haverback (1967) Clin. Res. 15, 101
33 Webster, M.E. and Innerfield, I. (1965) Enzymol. Biol. Clin. 5, 129—148
34 Harpel, P.C. (1973) J. Exp. Med. 138, 508—521
35 Back, N., Wilkens, H. and Streger, R. (1968) Ann. N.Y. Acad. Sci. 146, 491—509
36 Webster, M.E. and Maling, H.M. (1970) Adv. Exp. Biol. Med. 8, 493—499
37 Webster, M.E. (1968) Fed. Proc. 27, 84—89
38 Kellermeyer, R.W. and Graham, R.C. (1968) New Eng. J. Med. 279, 859—866
39 Asghar, S.S., Pondman, K.W. and Cormane, R.H. (1973) Biochim. Biophys. Acta 317, 539—548
40 Schoenback, E.B. and Greenspan, E.M. (1948) Medicine 27, 327—377
41 Ivady, G., Paldy, L., Koltay, M., Toth, G. and Covacs, Z. (1967) Lancet I, 616—617
42 Ashley, J.N., Berg, S.S. and Lucas, J.M.S. (1960) Nature 185, 461
43 Ellis, H.V., Johnson, A.R. and Moran, N.C. (1970) J. Pharmacol. Exp. Ther. 175, 627—631
44 Goodman, L.S. and Gilman, A. (1970) The Pharmacological Basis of Therapeutics, 4th edn. pp. 1144—1153, The Mac Millan Co., New York
45 Wien, R. (1943) Ann. Trop. Med. Parasit. 37, 1—18