Action of the Kallikreins on Synthetic Ester Substrates. (26575)

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The kallikreins are hypotensive proteinases of animal origin which cause a marked fall in blood pressure and stimulate certain smooth muscles(1). They exert their hypotensive effect by enzymatic action on kallidinogen, an *α-2* globulin found in plasma, to release the polypeptide kallidin. Although it is likely that the kallikreins derived from various sources such as the urine, saliva, plasma and pancreas, may attack the same substrate in plasma to release the same polypeptide, they are not identical proteolytic enzymes since they vary in susceptibility to the action of various proteolytic inhibitors(1,2,3). Recently, Contzen, *et al.*(4), Habermann(5) and Werle and Kaufmann-Boetsch(6) have presented evidence that one or more of the kallikreins are capable of attacking various arginine esters. This report describes the action of 4 of the kallikreins derived from hog pancreas, human pancreas, human urine and human plasma on a number of synthetic ester substrates.

Materials and methods. Highly purified *hog pancreatic kallikrein* (240 Frey units mg) was kindly supplied by Dr. H. Moriya (7). Crude *human pancreatic kallikrein* (4 Frey units/mg) was prepared by adsorption on diethylaminoethyl cellulose (DEAE-C) as previously described(3). *Human urinary kallikrein* from 150 liters of human urine was adsorbed on Amberlite XE-64 (IRC-50) at pH 4.0(3) and eluted with 10 liters of 0.1 M sodium phosphate buffer, pH 6.0. The eluate was diluted to 32 liters with water and treated with 22.5 g of 0.36 meg/g DEAE-C prepared from Whatman Ashless Cellulose Powder, Standard Grade, by the method of Ellis and Simpson(8). The pH was adjusted to 6.7 with hydrochloric acid and the mixture was stirred for 2 hours at 15-20° and allowed to settle overnight. The adsorbent was removed by filtration, washed on the filter with distilled water, and eluted with about 500 ml of 0.2 M sodium phosphate buffer, pH 7.0, containing 0.5 M sodium chloride. The dialyzed and freeze-dried eluate represented 80-100% yield of the kallikrein at 6 Frey units/mg. *Human plasma kallikrein* was activated by addition of acetone as previously described(3). The solution was dialyzed to remove salts and acetone, and the precipitate which appeared on dialysis was removed by filtration. The clear supernatant was diluted with distilled water to 5 volumes (based on the original plasma volume of 1.4 liters); 73.5 g 0.84 meg/g DEAE-C were added; the pH adjusted to 8.0 and the mixture was stirred for 1 hour at 12°. The inactive adsorbate was removed by filtration and the clear supernatant stirred with an additional 71 g DEAE-C except that pH was adjusted to 6.5. The second inactive adsorbate was removed by filtration and the plasma kallikrein was adsorbed from the supernatant by addition of 70.5 g of 0.36 meg/g carboxymethyl cellulose (CM-C). The pH was adjusted to 6.0 with sodium hydroxide and the mixture was stirred for 1 hour at 12°. The adsorbent was recovered by filtration, washed with about 1 liter of distilled water and eluted with 500 ml of 0.3 M potassium phosphate buffer, pH 7.0. The eluate was adjusted to pH 6.9, dialyzed against running tap water for 16 hours at 15-17° and dried from the frozen state. A 15-25% yield of kallikrein with a potency of 0.3 Frey units/mg was obtained by these procedures. *Esterase* activity of the kallikrein preparations was studied using Robert's modification of the Hestrin method(9). To tubes containing 1.0 ml of .06 M ester and 1.0 ml of 0.75 M tris (hydroxymethyl) amino methane (Tris) buffer was added 1.0 ml of enzyme solution. After incubation at 37° the amount of residual ester was determined colorimetrically as previously described(9), except that the precipitate following addition of the trichloroacetic acid was removed by filtration. The highly purified hog pancreatic kallikrein and the crude human urinary kallikrein did not precipitate at this

TABLE I. Ability of Kallikreins to Digest Synthetic Esters.†

Substrate .02 M, pH 7.5	Kallikreins derived from Urine 6 FU‡	Pancreas 6 FU	Plasma 1.7 FU
Benzoyl-L-arginine methyl ester	5.4	14.9	11.2
Benzoyl-L-arginine ethyl ester	5.3	16.2	10.6
p-Toluenesulfonyl-L-arginine methyl ester	8.8	7.5	10.5
Benzoyl-dl-alanine methyl ester*	<1	<1	<1
Benzoyl-dl-alanine ethyl ester*	<1	<1	<1
L-leucine ethyl ester	<1	<1	<1
L-lysine methyl "	<1	<1	<1
L-tyrosine " "	<1	1.5	<1
Acetyl-L-tyrosine ethyl ester*	<1	11.2	<1
Acetyl-L-tryptophan ethyl ester*	<1	1.2	<1

* Esters dissolved in 25, 30, 15 and 50% methyl cellosolve, respectively. Determinations of these proteinases with TAMe in presence of 50% methyl cellosolve caused some inhibition of the enzymes as only 2.1, 3.2 and 9.4 μmoles were digested, respectively.

† μmole ester digested per 60 min., 37°.

‡ FU (Frey Units) of biological activity.

concentration of trichloroacetic acid. Inhibitors were dissolved in the Tris buffer and incubated with enzyme at 37°. The enzymatic reaction was initiated by addition of substrate. The *kallikrein* content of the various preparations was determined by measuring the increase in blood flow following injection of .01-.04 Frey units in the dog(3). Because the response to plasma kallikrein as compared to pancreatic kallikrein varied from one dog to another, a separate plasma kallikrein standard based on the average response furnished by 3 dogs was utilized for this proteinase. Crystalline soy bean trypsin *inhibitor* (SBTI) was purchased from Worthington Biochemical Corp. Pancreatic trypsin inhibitor (PTI) was a product of Nutritional Biochemicals Corp. Diisopropylfluorophosphate was purchased from K & K Laboratories, Inc.

Results. The esterase action of purified hog pancreatic kallikrein, crude human urinary kallikrein and human plasma kallikrein is shown in Table I. All 3 proteolytic solutions were capable of digesting arginine esters. Highly purified hog pancreatic kallikrein also gave some digestion of tyrosine and tryptophan esters, and acetyl-L-tyrosine ethyl esters appeared to be as good a substrate for this proteinase as the arginine esters. Urinary and plasma kallikrein, on the other hand, did not attack the aromatic substrates. Other esters, *i.e.*, alanine, leucine, lysine, were not attacked by the kallikrein solutions.

It is well established that certain inhibitors compete with the substrate for the active site of the enzyme. If the reversible plasma inhibitor of urinary and pancreatic kallikreins (10) is a competitive inhibitor, the presence of those esters which are substrates for the kallikreins should prevent the inhibition of the biological activity by the inhibitor. These studies should provide, despite the crudeness of the kallikrein preparations, more direct evidence that the kallikreins were actually capable of digesting arginine esters. In the absence of esters greater than 50% inhibition of crude human urinary and pancreatic kallikreins by the plasma inhibitor was obtained (Table II). However, in presence of the arginine esters the inhibition was competitively blocked. Leucine and lysine esters, on the other hand, failed to prevent the inhibition of the kallikreins. Benzoyl-L-arginine amide also competes with the inhibitor and may be a substrate for the kallikreins. Similar measurements were also made with human plasma kallikrein using diisopropylfluorophosphate as the inhibitor. Highly purified hog pancreatic kallikrein gave results in no way different from those reported for crude human pancreatic kallikrein. However, the crude human pancreatic kallikrein, at equivalent biological activity, was much more potent in its action on synthetic esters *in vitro* and is probably contaminated by other pancreatic proteinases. These data are compatible with the kallikreins as proteolytic enzymes capable of digesting arginine esters. The direct competitive inhibition of the kallikreins by these synthetic amino acid esters cannot be satisfactorily tested *in vivo*. Attempts to measure the plasma inhibitor to human urinary kallikrein or hog pancreatic kallikrein *in vitro* at pH 7.5 have shown that addition of 6 Frey units to a

TABLE II. Ability of Esters to Inhibit Plasma Inhibition of Human Urinary and Pancreatic Kallikrein and DFP Inhibition of Plasma Kallikrein.

Esters, .02 M	% inhibition by plasma of kallikreins derived from Urine	Pancreas	% inhibition by DFP of plasma kallikrein
None	>50	>50	>50
Benzoyl-L-arginine methyl ester	12	2	50
" ethyl "	20	0	8
p-Toluenesulfonyl-L-arginine methyl ester	8	29	20
L-leucine ethyl ester	>50	>50	>50
L-lysine methyl "	>50	>50	>50

To out-dated human ACD (acid citrate dextrose) plasma was added 3 volumes of 0.75 M Tris buffer, pH 7.5, and 2 volumes of enzyme at 1.5 Frey units/ml. Solutions were incubated for 2 hr at 37° in presence or absence of esters. To 1.0 ml of human plasma kallikrein, 0.6 Frey units in 0.1 M Tris buffer, pH 7.5, was added 1/10 volume of 1×10^{-3} M DFP (diisopropylfluorophosphate) in anhydrous isopropanol and solutions incubated for 1½ hr at 37° in presence or absence of esters. In presence of benzoyl-L-arginine amide 49, 22 and 60% inhibition of the 3 kallikreins was obtained, respectively.

1:2 dilution of plasma in Tris buffer caused little or no inhibition of the p-toluene sulfonyl-L-arginine methyl ester (TAMe) activity after incubation for 2 hours at 37°. These results are in agreement with the biological data, and suggest that TAMe effectively competes with the reversible plasma inhibitor.

Because TAMe appeared to give the same esterolytic activity at equivalent biological activity with both urinary and pancreatic kallikreins (Table I), this substrate was chosen for more detailed investigation. When the μmoles of TAMe digested in 60 minutes are plotted against pH (Fig. 1), the pH optima of the 3 proteinases in Tris buffer was found to be 8.5. TAMe itself undergoes a small digestion at the higher pH's and the figures reported have been corrected for the decrease in the blank. Comparison of the μmoles of TAMe digested in 60 minutes with the biological activity in terms of Frey units (Fig. 2) showed that the hog pancreatic and human urinary kallikreins digested identical quantities of TAMe at pH 8.5 except in the higher regions around 9 Frey units/ml. Plasma kallikrein, on the other hand, appeared to be a much more potent protease against this synthetic ester, requiring about ⅕ of the Frey units to liberate the same μmoles of TAMe.

In an attempt to ascertain how much of

TABLE III. Comparison of % Inhibition of Esterase and Biological Activities of the Kallikreins by Trypsin Inhibitors.

Inhibitors, μg/ml		% inhibition at pH 8.5 of kallikreins derived from Human urine Esterase	Human urine Biological	Hog pancreas Esterase	Hog pancreas Biological	Human plasma Esterase	Human plasma Biological
SBTI	.25					24	
	.5					47	
	1					73	18
	2						45
	4						>50
	500	0	0	0	0		
PTI	.5	23	20		20		
	1	46	44	17	35		
	2	73	>50	31	>50		32
	4			50			56
	8						>50
	10					29	
	20					45	
	40					60	

Inhibitors were incubated with the kallikreins for 15 min. at 37°. Urinary and pancreatic kallikreins were inhibited at 3 Frey units/ml and plasma kallikrein at 0.6 Frey unit/ml. Soy bean trypsin inhibitor (SBTI). Pancreatic trypsin inhibitor (PTI).

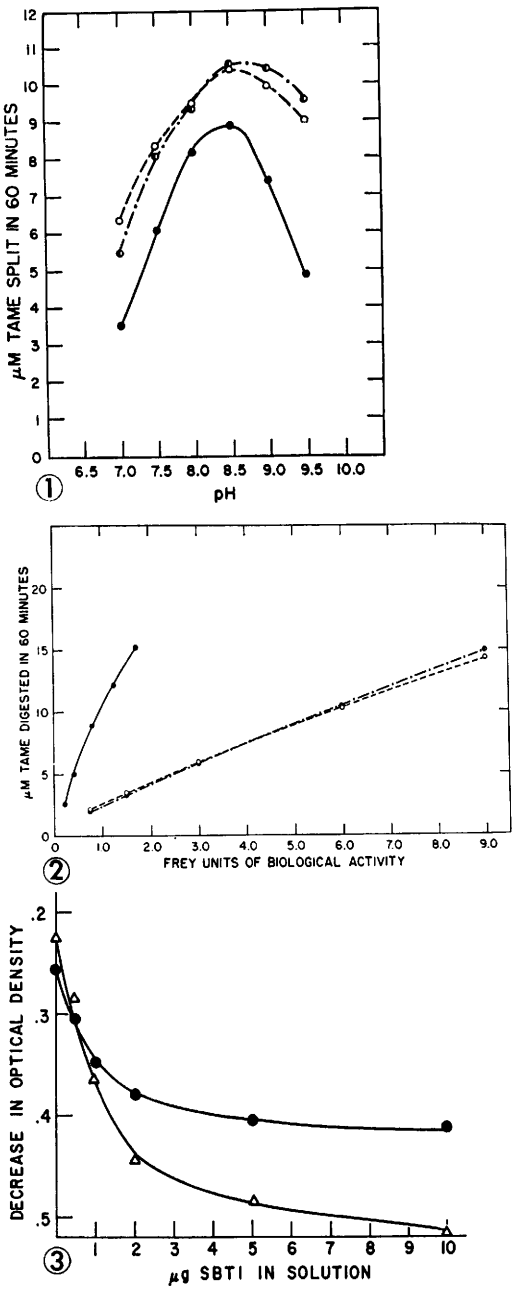

FIG. 1. Effect of pH on digestion of p-toluene sulfonyl-L-arginine methyl ester (TAMe) by the kallikreins; ◒—•—◒, hog pancreatic kallikrein; ○——○, human urinary kallikrein; ●——●, human plasma kallikrein.

FIG. 2. Comparison of biological and esterase activities of the kallikreins; ◒—•—◒, hog pancreatic kallikrein; ○——○, human urinary kallikrein; ●——●, human plasma kallikrein.

FIG. 3. Ability of soy bean trypsin inhibitor (SBTI) to inhibit 0.3 ml of acetone-activated and dialyzed plasma, ●——●, and partially purified plasma kallikrein, △——△, 4.2 mg. Addition of 20 and 100 μg SBTI gave little increase in inhibition.

TAMe activity was due to kallikreins and how much could be due to possible contamination with other proteases, the same concentration of kallikrein was compared by the esterase and biological methods for its ability to be inhibited by SBTI and PTI trypsin inhibitors. Crude human urinary kallikrein gave identical percentage of inhibition with both procedures (Table III). However, with the highly purified hog pancreatic kallikrein, an approximately 2-fold discrepancy was found with PTI inhibitor. This would suggest that even at this great purity other proteases from the pancreas were still present as contaminants. Neither biological nor esterase activity was inhibited with SBTI, which would indicate that trypsin was not one of the contaminating enzymes. Crude human plasma kallikrein was most grossly contaminated by other esterolytic activity as determined by this procedure. The esterolytic activity required about 4 times as much SBTI as did the biological activity and was much more sensitive to PTI. The ratio of inhibition of plasma kallikrein by SBTI and PTI was found in these studies to be 1:3 as compared to the 1:10 found with an earlier preparation(3). It is possible, therefore, that each lot of crystalline PTI will vary in its ability to inhibit the kallikreins. Investigation of the esterases liberated by acetone activation of plasma showed that, unlike the partially purified plasma kallikrein, not all TAMe activity could be inhibited by SBTI (Fig. 3). These data were a clear indication that at least 2 esterases were activated by acetone. During the purification of the acetone-activated and dialyzed plasma, it was found that a portion of the esterase activity, which was not inhibited by SBTI, could be adsorbed at pH 8.0 on DEAE-C and that the remainder of this activity could be removed by adsorption at pH 6.5 (Table IV). The esterase activity which was inhibitable with SBTI, on the other hand, failed to adsorb on DEAE-C at either pH and could be adsorbed from solution by CM-C at pH 6.0. The kallikrein activity paralleled the SBTI-inhibited esterase activity. Although it appears, from the com-

TABLE IV. Esterolytic and Kallikrein Activity during Purification of Human Plasma Kallikrein.

Enzyme source	Esterase activity in EU* Total	Inhibited by SBTI	Kallikrein activity in FU*	Protein, mg/ml†
Acetone-activated and dialyzed plasma	4.1	2.7	5.3	53
DEAE-C adsorption, pH 8.0				
Filtrate	2.3	1.5	1.2	13.8
Eluate	.98	.18	.3	27.0
DEAE-C adsorption, pH 6.5				
Eluate	.50	.11	.2	5.3
CM-C adsorption, pH 6.0				
Filtrate	.06	.06	.1	5.8
Eluate	.9	.84	.84	2.8

* Arbitrary esterase units (EU) based on Frey units (FU) found in the purest plasma kallikrein preparation (eluate from CM-C adsorption at pH 6.0).

† mg non-dialyzable protein recovered.

All results calculated/ml original concentration of plasma. Esterase activity was inhibited by addition of 100 μg soy bean trypsin inhibitor (SBTI). Acetone-activated and dialyzed plasma was fractionated by adsorption on diethylaminoethyl cellulose (DEAE-C) at pH 8.0, filtrate was then adsorbed with DEAE-C at pH 6.5, and filtrate from this adsorption was further fractionated by adsorption on carboxymethyl cellulose (CM-C) at pH 6.0.

parison of the esterase and biological activities (Table III) of this crude preparation of kallikrein, that plasma kallikrein is not responsible for all of the esterase activity, attempts to separate the kallikrein and residual esterase activity by hydroxyapatite chromatography have not been successful. To date, a single proteolytic and biologically active peak has been obtained.

It was thought possible that some of the esterases activated by acetone might be capable of digesting other synthetic ester substrates. However, when the acetone-activated and dialyzed plasma and the esterase activity prepared by adsorption on DEAE-C at pH 6.5 were tested on the various synthetic esters at a level capable of digesting 10-12 μmoles of the arginine esters, they, like the product prepared by CM-C adsorption, failed to digest the other esters.

Discussion. These data would suggest that the kallikreins are capable of digesting arginine ester. Perhaps the strongest evidence that these synthetic amino acid esters are substrates for the kallikreins has been derived from inhibition studies. It was readily demonstrated that the arginine esters acted to prement inactivation of the biological activity by their inhibitors. However, comparison of the inhibition of esterase and biological activities with the trypsin inhbtors revealed that neither the pancreatic nor the plasma kallikreins gave parallel inhibition. The data for urinary kallikrein are most promising and suggest that urinary kallikrein may be the main TAMe esterase in this preparation.

Highly purified hog pancreatic kallikrein, which has been shown to be 40% pure(7), hydrolyzes acetyl-L-tyrosine ethyl ester as well as arginine esters. Unfortunately the ability of this ester to prevent inhibition of the biological activity by the plasma inhibitor could not be tested because of its limited solubility. It is also evident from the inhibition studies with PTI that the apparent correlation of esterolytic and bioolgical activities of human urinary kallikrein and hog pancreatic kallikrein is fortuitous. Homogeneous hog pancreatic kallikrein will probably not be as potent a TAMe esterase as human urinary kallikrein.

The fact that all of the kallikreins appear to be capable of attacking arginine esters is in good agreement with the studies of Elliot, Lewis and Horton(11) who have shown that bradykinin, prepared by the action of trypsin on bovine globulin, is a linear nonapeptide containing both N-terminal and C-terminal arginine residues. If it is assumed that kallidin and bradykinin are similar or identical,

the kallikreins are probably splitting kallidinogen at a specific peptide link of which arginine is one component.

The esterolytic activity of acetone-activated plasma kallikrein is of particular interest because of the many esterases which have already been tentatively identified in human plasma. Of those esterases known to be inhibited by SBTI, *e.g.*, plasmin, thrombin-E, thrombokinase and the permeability factor, only plasmin has been shown not to be activated by acetone(3). The observation that intravenous infusion of thrombin-E(12) did not alter the blood pressure in dogs clearly differentiates this proteinase from plasma kallikrein. In addition, although definitive data are lacking, it would appear unlikely that thrombokinase(14) and plasma kallikrein were identical. The premeability factor(13), on the other hand, cannot be definitely distinguished from plasma kallikrein. There are, therefore, at least 5 proteinases present in human plasma-plasmin, thrombin-E, thrombokinase, C'_1 component of complement and plasma kallikrein. To these can be added the acetone-activated proteinase(s) which are not inhibitable with SBTI since they differ from C'_1 in not attacking tyrosine esters. Austen (15) has also noted the presence in chloroform-activated human serum of a TAMe esterase which is not inhibited by SBTI.

Summary. Evidence is presented which suggests that the kallikreins derived from hog pancreas, human pancreas, human urine and human plasma are capable of digesting those synthetic esters which contain arginine as the specific amino acid residue. Highly purified hog pancreatic kallikrein also digests acetyl-L-tyrosine ethyl ester. Arginine esters and benzoyl-L-arginine amide prevented both inhibition of human urinary and pancreatic kallikreins by human plasma and inhibition of human plasma kallikrein by diisopropylfluorophosphate. Acetone activates not only plasma kallikrein but another proteolytic enzyme(s) which attack arginine esters and are not inhibited by SBTI.

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Received February 23, 1961. P.S.E.B.M., 1961, v107.

Tumors Induced in Hamsters by Injection of Rhesus Monkey Kidney Cell Extracts. (26576)

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Previous experiments have shown that hamsters <1 to 3 days of age are susceptible to tumor induction by the SE polyoma virus, (1) a virus isolated from the mouse. Since a large number of simian viruses have been recovered from monkey kidney tissue cultures (2-7) the present experiments were undertaken to find out if rhesus monkey kidney