Agents and Actions
vol. 10/4 (1980)
Birkhäuser Verlag, Basel

Biochemistry and Functional Aspects of Human Glandular Kallikreins

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Abstract

Human urinary kallikrein was purified by gel filtration on Sephacryl S-200 and affinity chromatography on aprotinin-Sepharose, followed by ion exchange chromatography on DEAE-Sepharose. In dodecylsulfate gel electrophoresis two protein bands with molecular weights of 41,000 and 34,000 were separated. The amino acid composition and the carbohydrate content of the kallikrein preparation were determined; isoleucine was identified as the only aminoterminal amino acid. The bimolecular velocity constant for the inhibition by diisopropyl fluorophosphate was determined as 9 ±2 l mol^{-1} min^{-1}. The hydrolysis of a number of substrates was investigated and AcPheArgOEt was found to be the most sensitive substrate for human urinary kallikrein. Using this substrate an assay method for kallikrein in human urine was developed.

It was shown by radioimmunoassay that pig pancreatic kallikrein can be absorbed in the rat intestinal tract. Furthermore, in dogs the renal excretion of glandular kallikrein from blood was demonstrated by radioimmunological methods.

Introduction

In the mammalian organism, organ or tissue kallikreins are present in salivary glands, pancreas, kidney, urine, small and large intestine and in low concentrations also in blood [1]. Kallikreins found in these tissues are immunologically and biochemically very similar as demonstrated by FRITZ et al. for the pig [2]. Glandular kallikreins are assumed to be involved in various biological processes such as adsorption of water, electrolytes and hexoses in the intestine [3, 4], lowering of the blood sugar level, probably by glucose uptake into muscle cells [5], cell proliferation [6] and stimulation of other biological hormone systems [7, 8].

Of the palette of tissue kallikreins human urinary kallikrein was selected for investigations.

Materials and methods

CN-Br-activated Sepharose and Sephacryl S-200 were products from Pharmacia Fine Chemicals, Sweden. D-Val-Leu-ArgOEt, D-Val-Leu-ArgNHNp and other nitroanilides were kindly provided by KABI, Mölndal, Sweden; Z-TyrONp and BzArgOEt were purchased from SERVA, Heidelberg. AcPheArgOEt was synthesized according to FIEDLER et al. [9]. Trasylol was a gift from Bayer AG, Leverkusen. Human urinary kallikrein activity was assayed with peptide esters and nitroanilides as substrates as described [9, 10]. Dog blood pressure was measured according to FREY et al. [11]. Human urinary kallikrein content in urine samples was assayed by radioimmunoassay as described by MANN et al. [12]. Polyacrylamide gel electrophoresis were performed as described by MAURER [13]. For amino acid analysis samples were hydrolyzed in vacuo at 100°C with 5.7 *N* HCl for 20, 40 and 80 h and analyzed on a Durrum D 500. The bimolecular velocity constant for the inhibition of human urinary kallikrein by DFP was determined according to FIEDLER et al. [14]. Carbohydrate content of kallikreins were determined as described by KRYSTAL et al. [15]. The complex of human urinary kallikrein and Trasylol was obtained by incubating enzyme and inhibitor in a 1:2 ratio (w/w) at 37°C for 10 min before analysis.

Table 1
Isolation of human urinary kallikrein.

Steps	Purification[a]
Collection of human male urine	—
Dialysis	—
Lyophilization	—
Extraction of the crude urine powder	—
Sephacryl S-200	1
Trasylol Sepharose	89
DEAE-Sepharose	240

[a] Substrate: AcPheArgOEt.

Results and Discussion

Isolation and characterization

Isolation of human urinary kallikrein was achieved by the procedure summarized in Table 1, slightly different as reported earlier [16]. Human urinary kallikrein obtained after isolation was subjected to polyacrylamide gel electrophoresis at pH 7.5 as shown in Figure 1.

After reduction with dithioerythriol the following pattern was obtained (Fig. 2).

Amino acid analysis of human urinary kallikrein and the determination of carbohydrate content were done by common methods. The results are shown in Tables 2 and 3. According to our results, the amino acid composition of human urinary kallikrein and pig pancreatic β-kallikrein are very similar.

In Table 4 constants of the inhibition of kallikreins and other serine proteases by DFP are compiled. The constants obtained for tissue kallikreins are all of the same order of magnitude.

Isoleucine was identified by high pressure liquid chromatography after Edman degradation [17] as the *N*-terminal amino acid of human urinary kallikrein.

Homogeneity of the enzyme preparation is strongly indicated by the presence of only one amino terminal amino acid and by the results of the electrophoretic investigations (cf. Fig. 1). No residual protein remains at the position of the single kallikrein band (gel A) after complex formation with Trasylol (gel B), i.e. the kallikrein

Table 2
Amino acid composition of kallikreins from human urine and porcine pancreas (residues per mole). Tryptophan was not determined, cystein after performic acid oxidation.

	Human urinary kallikrein	Porcine pancreatic β-kallikrein [18]
Asp	23	28
Thr	14	15
Ser	14	14
Glu	28	23
Pro	14	16
Gly	22	22
Ala	14	13
Cys	10	10
Val	18	10
Met	4	4
Ile	8	12
Leu	20	20
Tyr	6	7
Phe	10	10
Lys	8	10
His	9	8
Arg	6	3
Trp	?	7

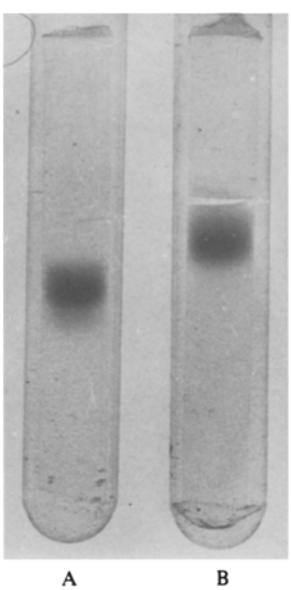

Figure 1
Acrylamide gel electrophoresis (7.5% gel, pH 7.5) of human urinary kallikrein. A, purified enzyme; B, purified enzyme samples with Trasylol.

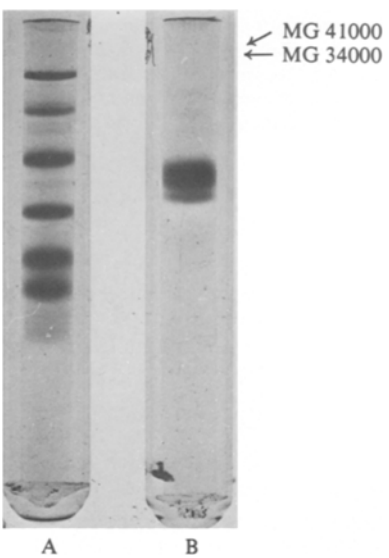

Figure 2
SDS-polyacrylamide gel electrophoresis (7.5% gel, pH 7.2; the enzyme was incubated with 10% dithioerythriol, 6 *N* urea and 10% SDS at 45°C for 60 min) of human urinary kallikrein (B). Molecular weight was estimated by SDS electrophoresis using a protein standard of Bio-Rad (A).

Table 3
Carbohydrate content of human urinary kallikrein and porcine pancreatic kallikrein.

Kallikrein from	Carbohydrate content g/100 g protein
Porcine pancreas [18]	
form A	5.6
form B	11.5
Human urine	10.5–15.6

Table 4
Kinetic constants for the inhibition of different serin proteases by DFP (pH 7.2, 25°C), R. Geiger and U. Stuckstedte, unpublished.

Enzyme	k (l mol^{-1} min^{-1})
Human urinary kallikrein	9 ± 2
Human large intestine kallikrein [19]	4
Porcine pancreatic kallikrein [14]	8 ± 1
Human plasma kallikrein	320 ± 20
Trypsin [20]	300
Chymotrypsin [20]	2,700
Acetylcholinesterase [20]	13,000

Table 5
Assay of human urinary kallikrein using the substrate Ac-Phe-ArgOEt.

2.00 ml	0.15 *M* sodium diphosphate buffer, pH 8.7, containing 0.15 *M* semicarbazidium chloride and 0.0375 *M* glycine
0.10 ml	0.03 *M* NAD
0.10 ml	0.015 *M* AcPheArgOEt acetate
0.02 ml	alcohol dehydrogenase (100 mg/3.4 ml)
(0.28 + x) ml	water
	5 min preincubation at 25°C
(0.5 − x) ml	enzyme solution
Final volume:	3 ml

The change in absorbance is monitored for 10 min at 366 nm.

preparation contains exclusively active enzyme molecules.

Assay methods

The methods for the determination of tissue kallikreins in urine and other body fluids are either rather cumbersome or insensitive. With preparations of human urinary kallikrein, AcPhe-ArgOEt was found to be the most rapidly hydrolyzed substrate described as yet [9].

Therefore, a highly sensitive assay for human urinary kallikrein could be developed employing Ac-Phe-ArgOEt as substrate. The assay (Table 5) is analogous to that with Bz-ArgOEt developed by TRAUTSCHOLD and WERLE [21]. The reaction sequence is shown in Figure 3. The sensitivities of this and other assays for human urinary kallikrein are compiled in Table 6. The sensitivity of the assay allows the convenient measurement of the esterase activity of human urine. Urine samples of 20–100 µl cause a linear absorbance increase of 0.04 to 0.2 per 10 min. Known amounts of human urinary kallikrein added to urine samples raised the esterase activity to the expected extent. If the urine samples contain ethanol it has to be removed by dialysis. Therefore, ethanol intake should be avoided during the urine collection period.

A number of experiments were undertaken to verify that the esterase activity reflects the kallikrein content of urine. Trasylol completely inhibited the esterase activity. Dialysed urine samples of ten different persons were assayed. The results were compared with those obtained by the dog blood pressure assay, by a radioimmunoassay for human urinary kallikrein and by the assay with D-Val-Leu-ArgNHNp as substrate (Fig. 4). Though the correlation coefficient of the results of the Ac-Phe-ArgOEt assay and the dog blood pressure assay (Fig. 4a) is rather close to 1, a considerable scattering of the data is observed. This is not too surprising, since the coefficient of variation of the blood pressure assay for kallikrein amounts to 20%. The correlation between the Ac-Phe-ArgOEt

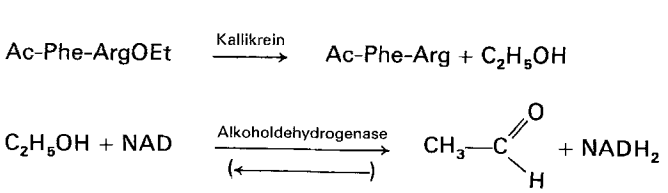

Figure 3
Reaction scheme of kallikrein assay using Ac-Phe-ArgOEt as substrate.

Table 6
Absorbance changes in assay of human urinary kallikrein with various substrates. (Reaction volume 3 ml, cuvette light path 1 cm.)

	Blank ($\Delta A \cdot 10^3 \times min^{-1}$)	Reaction	
		($\Delta A \cdot 10^3 \times min^{-1} \times U^{-1}$)	Relative sensitivities
Ac-Phe-ArgOEt	0.6	1100	46
D-Val-Leu-ArgOEt	0.6	430	20
Bz-ArgOEt	0.5	24	1
Z-TyrONp	15	460	19
D-Val-Leu-Arg-*p*-nitranilide	0.0	29	1.2
D-Pro-Phe-Arg-*p*-nitranilide		3	
Tos-Gly-Pro-Arg-*p*-nitranilide		1	
Bz-Phe-Val-Arg-*p*-nitranilide		0.3	
Glu-Gly-Arg-*p*-nitranilide		0.3	

assay and both the radioimmunoassay (Fig. 4b) and the D-Val-Leu-Arg-*p*-nitroanilide assay is even more satisfactory. The excellent correlation between the two enzymatic assays (Fig. 4c) strongly suggests that in both assays the same enzyme is determined. For a mixture of enzymes a similarly good correlation would only be expected if these enzymes were always excreted in identical ratios.

These arguments are strongly in favour of the presumption that in the assay using AcPhe-ArgOEt only the kallikrein content of urine is determined.

Functional aspects

During recent years we widely applied the radioimmunoassay methods in studies on the

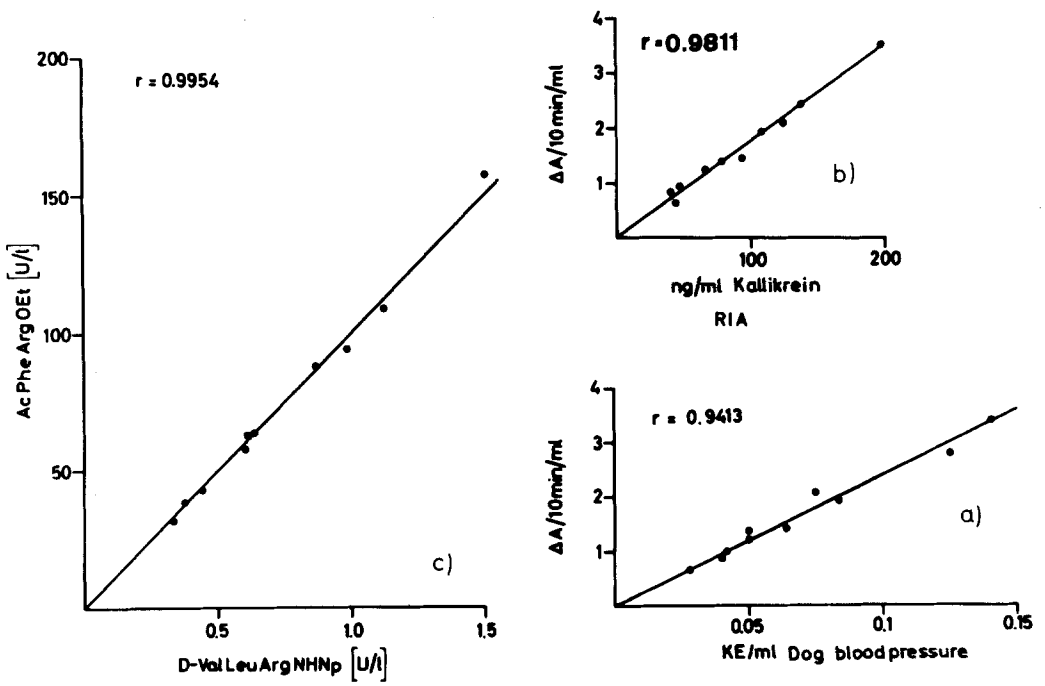

Figure 4
Comparison of the Ac-Phe-ArgOEt assay for human urinary kallikrein with the blood pressure assay (a), the radioimmunoassay (b) and the D-Val-Leu-ArgNHNp assay (c). Ten samples of human urine were measured.

Table 7
Biological effects of pig pancreatic kallikrein.

1. Stimulation of intestinal absorption of various substances like glucose, amino acids and vitamin B12
2. Stimulation of cell proliferation
3. Stimulation of number and motility of spermatozoa in patients with astenozoospermia and oligozoospermia
4. Enhancing effect on muscular glucose uptake in patients with maturity onset diabetes
5. Reduction of blood pressure in patients with essential hypertension

physiological role and pharmacological mechanism of action of glandular kallikreins.

Intestinal absorption. In certain pathological disorders an improvement is observed after oral administration or intramuscular injection of pig pancreatic kallikrein (3, 4 and 5 in Table 7). These findings suggest that glandular kallikrein can be absorbed intestinally and causes the observed effects after being transferred into the circulation.

In order to examine whether kallikrein can be absorbed by the intestine pig pancreatic kallikrein was injected into the lumen of the duodenum of anesthetized rats; the thoracic duct lymph was collected and blood samples were obtained from the tail vein. By applying the radioimmunoassay it was possible to show that pig pancreatic kallikrein appeared both in the thoracic duct lymph and in the blood demonstrating intestinal absorption of the kallikrein. These results provide a rational basis for the oral administration of pig pancreatic kallikrein: the kallikrein can be transferred from the lumen of the intestine into the circulation. The mechanism of action of the absorbed kallikrein which results in the improvement of pathologic disorders is not yet understood.

Renal excretion of glandular kallikrein. Recently it was possible to demonstrate the presence of a glandular kallikrein-like antigen in porcine and human blood by radioimmunoassays [1, 22, 23]. This finding led us to the deduction that glomerular filtration of the glandular kallikrein in the blood might partly be responsible for the occurrence of glandular kallikrein in urine. Our experiments in the renal excretion of pig pancreatic kallikrein after intravenous infusion in dogs corroborate this deduction [23, 24].

Pig pancreatic kallikrein was infused into the femoral vein of anesthetized dogs. Blood samples were drawn from the artery and urine was collected from the catheterized urinary bladder. The urine and blood samples were measured by radioimmunoassay. The total excreted kallikrein in five experiments was 1–3% of the administered dose. The relative clearance of porcine pancreatic kallikrein, related to inulin, was approximately 4%, being in the same range as the amylase clearance determined in man. Calculated from the clearance found for pig pancreatic kallikrein in the dog (approximately 2.5 ml/min) and the (preliminary) concentration of glandular kallikrein in blood (5–20 ng/ml) a daily excretion of about 50 μg kallikrein would result which is in the range of several 10% of the daily excretion of urinary kallikrein in man.

The results suggest that urinary kallikrein does not represent exclusively a kallikrein synthesized by the kidney and secreted directly into the urine. Glomerular filtration of glandular kallikrein from the blood may contribute significantly to the excretion of kallikrein in urine.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 51, Munich. We are indebted to Dr C. Kutzbach, Bayer AG, for providing preprocessed starting material for the isolation of human urinary kallikrein. We thank very much Prof. H. Fritz for his interest in this work and his stimulating discussions and comments.

Abbreviations

Bz-ArgOEt: N^{α}-Benzoyl-arginine ethyl ester; Z-TyrONp: Carbobenzoxytyrosine nitrophenyl ester; Ac-Phe-ArgOEt: Acetyl-phenylalanyl-arginine ethyl ester; D-Val-Leu-ArgOEt: D-valyl-leucyl-arginine ethyl ester; DFP: diisopropyl fluorophosphate.

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