Isolation and Determination of Human Urinary Kallikrein

R. Geiger, E. Fink, U. Stuckstedte and B. Förg-Brey

Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik der Universität München, Nussbaumstr.20, D-8000 München 2, FRG

ABSTRACT

Human urinary kallikrein (EC 3.4.21.8) was purified to electrophoretic homogeneity by gel filtration on Sephacryl S-200, followed by affinity chromatography on Trasylol-Sepharose and chromatography on DEAE-Sepharose. Kallikrein activity in human urine samples was determined using Ac-Phe-ArgOEt. The hydrolysis of Ac-Phe-ArgOEt was compared with a radioimmunoassay, a biological assay and the cleavage of D-Val-Leu-ArgNHp. Clear correlations were found. These arguments are strongly in favour of the presumption that in the assay applied only the kallikrein content of urine is determined.

KEY WORDS

Kallikrein assay, urinary kallikrein, peptide esters, peptide p-nitroanilides, proteases, Trasylol-Sepharose, affinity chromatography,

INTRODUCTION

The possible role of urinary kallikrein in the regulation of kidney function or blood pressure has been debated for a long time (Pisano, 1976). We even think that the question whether urinary kallikrein is synthesized in the kidneys or whether it represents only filtered enzyme of pancreatic or salivary gland origin is not finally settled. When we started investigations in the hope of contributing to the clarification of these problems, we realized that the methods presently available for the determination of glandular kallikreins in human urine and other body fluids were rather cumbersome (Fiedler, 1978).

MATERIALS AND METHODS

D-Val-Leu-ArgOEt, D-Val-Leu-ArgNHNp and other nitroanilides were kindly provided by KABI, Mölndal, Sweden. Trasylol was a gift from Bayer AG, Leverkusen. Z-Ser-Pro-Phe-Arg-MNA was purchased from Enzyme Systems Products, Indianapolis, USA, and Fast Blue B Salt from SERVA, Heidelberg. Ac-Phe-ArgOEt was synthesized according to Fiedler (1978).

Human urinary kallikrein activity was assayed with peptide esters and nitroanilides as substrates as described (Fiedler, 1978). Dog blood

pressure was measured according to Dittmann (1978). Human urinary kallikrein content in urine samples was assayed by radioimmunoassay as described by Mann (1977).

RESULTS AND DISCUSSION

As the first step for the development of a radioimmunoassay and other assay methods for human urinary kallikrein we began with an isolation of this enzyme (see Table 1), somewhat modified as communicated in Mainz at the International Symposium on Kininogenases (Mann, 1977).

TABLE 1 Isolation of human urinary kallikrein (Geiger, 1977)

Steps	Purification
Collection of human male urine	-
Dialysis	-
Lyophilisation	-
Extraction of the crude urine powder	-
Sephacryl S-200	1
Trasylol Sepharose	89
DEAE-Sepharose	240

Different preparations of human urinary kallikrein obtained from different batches of starting material showed a startling behaviour on acrylamide gel electrophoresis at pH 6 as well as pH 8. Some displayed only one protein band as stated previously, but other preparations were resolved into three bands as described also by Moriya and coworkers (Matsuda, 1976). All these bands could be revealed also by active enzyme staining using Z-Ser-Pro-Phe-Arg-MNA and coupling with Fast Blue B Salt, a method used by Smith (1975) for the histochemical localization of proteolytical enzymes. After incubation with Trasylol, however, the bands merged into only one of slower electrophoretic mobility.

It has been observed in this laboratory that Ac-Phe-ArgOMe is a much better substrate than are simple α-N-acetylated arginine esters for porcine pancreatic (Fiedler, 1976) as well as submandibular and urinary (Fritz, 1977) kallikreins. Furthermore the corresponding ethyl ester, Ac-Phe-ArgOEt, was the most rapidly hydrolysed substrate for human urinary kallikrein described as yet (Fiedler, 1978). The reaction was followed in analogy to the test with BzArgOEt developed by Trautschold and Werle (1961, see Table 2).

TABLE 2 Scheme of kallikrein determination using Ac-Phe-ArgOEt as substrate

$$\text{Ac-Phe-ArgOEt} \xrightarrow{\text{KALLIKREIN}} \text{Ac-Phe-Arg} + C_2H_5OH$$

$$C_2H_5OH + NAD \xrightarrow[(\longleftarrow)]{\text{ALCOHOLDEHYDROGENASE}} CH_3-C\overset{O}{\underset{H}{}} + NADH_2$$

Ethanol release by kallikrein was determined by the alcohol dehydrogenase reaction in a buffer of pH 8.7 containing semicarbazide. The NADH formed was continuously monitored for 10 min at 366 nm. Results on the sensitivities obtained with this and other assay procedures for human urinary kallikrein are compiled in table 3.

TABLE 3 Absorbance changes in assays 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	1 100	46
D-Val-Leu-ArgOEt	0.6	430	20
B_z-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- "		3	
Tos-Gly-Pro-Arg-p- "		1	
Bz-Phe-Val-Arg-p- "		0.3	
Glu-Gly-Arg-p- "		0.3	

Sensitivity of the assay using Ac-Phe-ArgOEt allowed convenient measurement of the esterase activity of human urine. Urine samples of 20-100 µl provoked linear increase of absorbance from 0.04 to 0.2 absorbance units per 10 min. The coefficient of variation of the test was determined as ± 4%. Human urinary kallikrein added in known amounts to urine samples raised the esterase activity to the expected extent. There was found only one annoying disturbance. Ethanol, if contained in the urine sample, had to be removed by dialysis. Evidently, intake of ethanol has to be prevented during the collection of urine samples to be analyzed by the present assay. Samples not containing ethanol showed unchanged esterase activity after dialysis. A number of experiments were undertaken in order to verify that the measured esterase activity could be equated to the kallikrein content of urine. Trasylol completly inhibited the esterase activity. Urine samples of 10 different persons were assayed after dialysis, also on the blood pressure of the dog. The results of this test are shown in Fig.1.
Though the correlation coefficient of the results of the two different assays is rather close to 1, there is found a notable scatter in the data. This observation is not too surprising, as the coefficient of variation of the blood pressure assay for kallikrein amounts to ± 20%, according to Arens and Haberland (1973).

The results have also been checked by means of a radioimmunoassay (Fig.2) developed with antibodies to human urinary kallikrein produced in rabbits. Details of this assay have been communicated last year in Mainz (Mann, 1977).

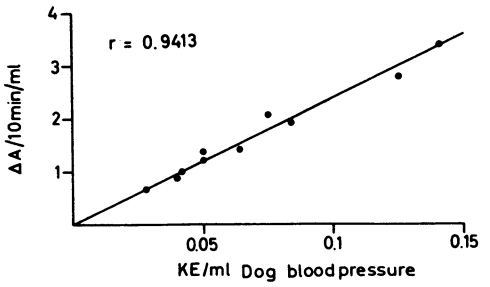

Fig. 1. Comparison of kallikrein activity in 10 human urine samples, as determined by bioassay and by hydrolysis of Ac-Phe-ArgOEt

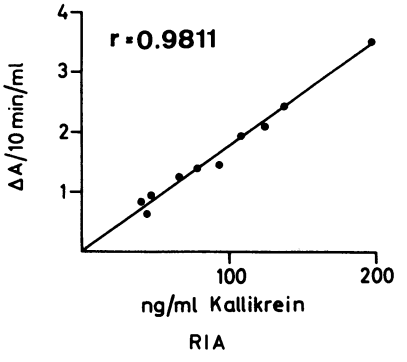

Fig. 2. Amount of kallikrein in human urine determined by radioimmunoassay compared with kallikrein activity, as determined by hydrolysis of Ac-Phe-ArgOEt

For further confirmation, parallel determinations were also conducted with D-Val-Leu-ArgNHNp. D-Val-Leu-Arg-p-nitroanilide has been suggested quite recently by KABI (Amundsen, 1978) as a substrate for the determination of human urinary kallikrein. Hydrolysis of p-nitroanilides can be monitored at 405 nm. Negligible spontaneous hydrolysis of these compounds allows working at 37 degrees centigrade. To obtain absorbance change of sufficient magnitude, an incubation time of 30 min was necessary with urine samples from 50 to 500 µl. This long incubation time precluded continuous monitoring of the reaction. The results had to be corrected for the inherent absorbance of the urine samples that was determined in parallel. Both assays for human urinary kallikrein then showed a satisfactory correlation, as demonstrated in Fig. 3. Such a correlation would only be expected from a mixture of different enzymes if these were excreted always in identical proportions. This

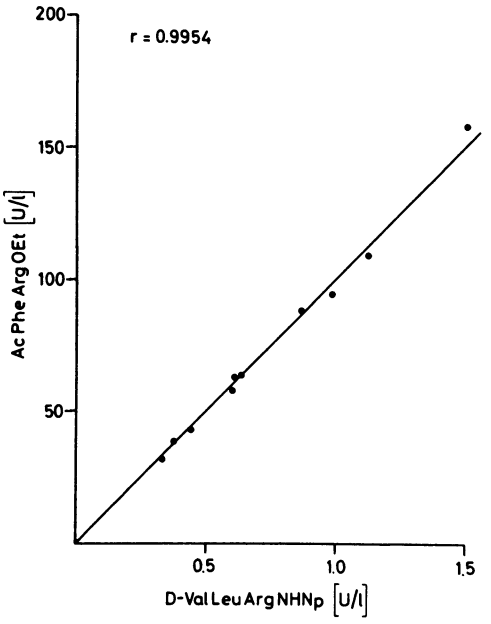

Fig. 3. Comparison of kallikrein activity in human urine, as determined by hydrolysis of Ac-Phe-ArgOEt and D-Val-Leu-ArgNHNp

argument is strongly in favour of the presumption that in both assays only the kallikrein content of urine is determined.

Evidently, both Ac-Phe-ArgOEt and D-Val-Leu-ArgNHNp are useful substances for kallikrein determination in human urine. The advantage of the Ac-Phe-ArgOEt assay is the possibility of continuous monitoring during the test, a drawback is the alcohol sensitivity of the reaction. This is not found in the assay with D-Val-Leu-ArgNHNp but this method suffers from the disadvantages of a two point assay, due to its limited sensitivity.

ABBREVIATIONS

Bz-ArgOEt: N^{α}-benzoyl-arginine ethyl ester, Z-TyrONp: Carbobenzoxy-tyrosine nitrophenyl ester, Ac-Phe-ArgOMe: Acetyl-phenylalanyl-arginine methyl ester, Ac-Phe-ArgOEt: Acetyl-phenylalanyl-arginine ethyl ester, Z-Ser-Pro-Phe-Arg-MNA: Carbobenzoxy-seryl-prolyl-phenylalanyl-arginine-4-methoxy-ß-naphtylamine, D-Val-Leu-ArgNHNp: D-valyl-leucyl-arginine-p-nitroanilide, D-Val-Leu-ArgOEt: D-valyl-leucyl-arginine ethyl ester,

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REFERENCES

Amundsen, E., J. Püttner, P. Friberger, M. Knös, M. Larsbråten, and G. Claeson (1978). Methods for the Determination of Glandular Kallikreins by Means of a Chromogenic Tripeptide Substrate. In press.

Arens, A., and G.L. Haberland (1973). Determination of Kallikrein Activity in Animal Tissue Using Biochemical Methods. In G.L. Haberland, and J.W. Rohen (Eds.), Kininogenases - Kallikrein 1, F.K. Schattauer Verlag, Stuttgart, pp.43-53.

Dittmann, B. (1978). Personal communication.

Fiedler, F. (1976). Pig Pancreatic Kallikrein: Structure and Catalytic Properties. In J.J. Pisano, and K.F. Austen (Eds.), Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease, DHEW Publication No. (NIH) 76-791, pp. 93-95.

Fiedler, F., R. Geiger, C. Hirschauer, and G. Leysath (1978). Peptides esters and nitroanilides as substrates for the assay of human urinary kallikrein. Hoppe-Seylers Z. Physiol. Chem., in press.

Fritz, H., F. Fiedler, T. Dietl, M. Warwas, E. Truscheit, H.J. Kolb, G. Mair, and H. Tschesche (1977). On the Relationship between Porcine Pancreatic, Submandibular, and Urinary Kallikreins. In G.L. Haberland, J.W. Rohen, and T. Suzuki (Eds.) Kininogenases - Kallikrein 4, F.K. Schattauer Verlag, Stuttgart, pp.15-28.

Geiger, R., K. Mann, and T. Bettels (1977). Isolation of Human Urinary Kallikrein by Affinity Chromatography. J. Clin. Chem. Clin. Biochem. 15, 479-483.

Mann, K., and R. Geiger (1977). Radioimmunoassay of Human Urinary Kallikrein. In G.L. Haberland, J.W. Rohen, and T. Suzuki (Eds.) Kininogenases - Kallikrein 4, F.K. Schattauer Verlag, Stuttgart, pp.55-61.

Matsuda, Y., K. Miyazaki, H. Moriya, Y. Fujimoto, Y. Hojima, and C. Moriwaki (1976). Studies on Urinary Kallikreins, I. Purification and Characterization of Human Urinary Kallikrein. J. Biochem. 80, 671-679.

Pisano, J.J., and K.F. Austen (1976). Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease, DHEW Publication No. (NIH) 76-791.

Smith, R.E., and R.M. Van Frank (1975). The use of amino acid derivatives of 4-methoxy-ß-naphtylamine for the assay and subcellular localization of tissue proteinases. In A. Neuberger and E.L. Tatum (Eds.) Frontiers of Biology 43, North-Holland Publishing Co., Incl., New York, 193-249.

Trautschold, I., and E. Werle (1961). Spektrometrische Bestimmung des Kallikreins und seiner Inaktivatoren. Hoppe-Seylers Z. Physiol. Chem. 325, 48-59.