J. Clin. Chem. Clin. Biochem.
Vol. 15, 1977, pp. 479–483

Isolation of Human Urinary Kallikrein by Affinity Chromatography

Determination of human urinary kallikrein, I.

By R. Geiger,

Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik der Universität München

K. Mann

Klinikum Großhadern, Medizinische Klinik II, and

T. Bettels

Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik der Universität München

(Received February 3/April 1, 1977)

Summary: Human urinary kallikrein (EC 3.4.21.8) was purified to electrophoretically homogeneity by gel filtration on Sephadex G-100, followed by affinity chromatography on aprotinin-Sepharose. The molecular weight, estimated by gel filtration, is 64,000 Daltons, by dodecyl sulfate electrophoresis 45,000 Daltons and 29,000 Daltons, and it is microheterogeneous on electrofocusing, yielding isoelectric point values of 3.8, 3.9 and 4.05. The kinetic constants for hydrolysis of BzArgOEt, TosArgOMe, CbzTyrONp, and human HMW-kininogen were determined. Benzamidine and aprotinin are competitive inhibitors of human urinary kallikrein.

Isolierung von menschlichem Harnkallikrein durch Affinitätschromatographie.

Bestimmung von menschlichem Harnkallikrein, I. Mitteilung

Zusammenfassung: Menschliches Harnkallikrein (EC 3.4.21.8) wurde erstmals mittels Affinitätschromatographie an Aprotinin-Sepharose isoliert. Das Molekulargewicht wurde mit Hilfe der Gelfiltration zu 64 000 Dalton, und mit der SDS-Elektrophorese zu 29 000 Dalton und 45 000 Dalton bestimmt. Bei der Isoelektrischen Fokussierung wurden drei Isoenzyme mit isoelektrischen Punkten von 3,8, 3,9 und 4,05 gefunden. Die kinetischen Konstanten für BzArgOEt, TosArgOMe, CbzTyrONp und menschliches HMW-Kininogen wurden bestimmt; Benzamidin und Aprotinin hemmten menschliches Harnkallikrein kompetitiv.

Introduction

Kallikreins (EC 3.4.21.8)¹⁾ are endopeptidases, which are found in the blood, tissues, and urine of several species (1–13). They liberate kinins (biologically active polypeptides) from kininogen, which is a part of the α_2 -globulin fraction of the plasma.

Since the discovery of kallikrein in 1926 (14), many investigators have shown that the kidney kallikrein plays an important role in the physiological processes of the kidney. In essential hypertension, Elliot & Nuzum (15) and Werle & Korsten (16) found lower than normal levels of kallikrein in urine. Work by Margolius et al. (17–19) has also shown a decreased excretion of urinary kallikrein in vascular hypertension. In contrast, animals with deoxycorticosterone-induced hypertension

(20) and men with primary hyperaldosteronism (21) have higher urinary kallikrein levels. A role of kallikrein in the regulation of sodium excretion is suggested (22–24). In patients showing reaction to kidney transplant, urinary kallikrein levels are lower (25) and vasodilators such as prostaglandins, acetylcholine, bradykinin and isoproterenol increase urinary kallikrein excretion (26–31). Recently, some investigators have shown that urinary kallikrein levels are decreased in glomerular basement membrane nephritis (32).

Urinary kallikrein is normally measured by the cleavage of synthetic acyl-arginine esters. This is not a very specific method, since there are other proteases in urine with the same esterolytic activity. In this and the following paper we report the isolation of human urinary kallikrein by affinity chromatography, the preparation of antibodies against it, and the specific determination by a sensitive radioimmunoassay. The radioimmunoassay for human urinary kallikrein can be used for the evalua-

¹⁾ Enzymes
Kallikrein (EC 3.4.21.8); Alcohol dehydrogenase
(EC 1.1.1.1). KIE = Kallikrein-Inhibitor-Einheiten.

tion of the importance of the kallikrein-kinin-system in physiological and pathophysiological processes in the kidney.

Materials and Methods

Human male urine was collected in the Ambulance of the Chirurgische Universitätsklinik. Kininogen was partially purified according to Mann & Geiger (33).

Synthetic bradykinin was a gift from Sandoz AG, Basle. Aprotinin (Trasylo^l, ampoule: 100 000 KIE/5 ml) was a gift from Bayer AG, Leverkusen. BzArgOEt²⁾ was purchased from Merck, Darmstadt. TosArgOMe²⁾ and CbzTyrONp²⁾ were obtained from Serva, Heidelberg. Benzamidine-HCl was purchased from Aldrich-Europe, Beerse, Belgium. Alcohol dehydrogenase¹⁾ from Boehringer, Mannheim. Sepharose 4B, Sephacryl and Sephadex from Pharmacia, Uppsala, Sweden, and Ampholine from LKB, Bromma, Sweden.

Kallikrein activity

Kallikrein activity was measured with BzArgOEt (34), TosArgOMe (35), CbzTyrONp (36), and Kininogen (33) as described.

Kinin activity

Bradykinin or kinin activity was assayed on the rat uterus as described by Mann (33). Bradykinin served as a reference standard for kinin activity.

Protein concentration was determined as described by Richterich (37). Standard serum (E. Merck, Darmstadt) was used as a reference.

Electrophoresis

Polyacrylamide gel electrophoresis was performed in a DESAGA flat gel apparatus. To check the purity of the preparation, gel no. 1a (75 g/l acrylamide gel, pH 8.9) according to Maurer (38) was employed.

Molecular weight

Molecular weight estimation was performed after reduction of -S-S- bonds by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (39). Coomassie Blue R-250 (Serva, Heidelberg) was used for staining. The determination of the molecular weight by gel filtration was performed on a Sephadex G 100 column (140 × 1.8 cm).

Aprotinin-Sepharose 4B

The affinity absorbent was synthesised according to l. c. (40).

Electrofocusing

Human urinary kallikrein was submitted to electrofocusing in ampholine in a pH gradient from 3 to 5, according to (41).

Isolation of human urinary kallikrein

Isolation of human urinary kallikrein was achieved by the following procedure: 30 l urine of male subjects were dialysed against running water for 24 h and then lyophilized. The lyophilized urine was suspended under gentle stirring (20 min) in 70 ml dist. water at 0°C. The suspension was centrifuged (8000 g), the supernatant

(30 ml) was applied to a water cooled Sephadex G-100 column (60 × 4.3 cm) and eluted with dist. water. (flow rate 50 ml/h; fractions of 12 ml were collected).

Affinity chromatography

The kallikrein-containing fractions were combined, lyophilized, dissolved in 4 ml phosphate buffer (0.025 mol/l Na₂HPO₄, 0.025 mol/l KH₂PO₄, 0.75 mol/l NaCl) pH 7.0, and applied to a aprotinin-Sepharose 4B column (15 × 1 cm) equilibrated with phosphate buffer. The column was washed with phosphate buffer, and eluted with 50 ml of the same buffer containing 1 mol/l benzamidine hydrochloride. The eluted fractions were dialysed against 50 l dist. water for 48 h, lyophilized, dissolved in 0.05 mol/l ammonium acetate buffer pH 7.0, applied to a Sephadex G-25 column (70 × 1.5 cm), equilibrated with the same buffer and eluted with 0.05 mol/l ammonium acetate buffer pH 7.0 (flow rate 30 ml/h; fractions of 2.5 ml were collected).

The kallikrein-containing fractions were combined, lyophilized, dissolved in 5 ml 0.05 mol/l ammonium acetate buffer pH 7.4 and applied to a Sephacryl S-200 superfine column, equilibrated with the same buffer.

Results and Discussion

The protein was eluted with 0.05 mol/l ammonium acetate buffer pH 7.4 and the fractions appeared homogeneous on polyacrylamide gel electrophoresis. In this way human urinary kallikrein was purified as shown in table 1.

The molecular weight was estimated by gel filtration to be 64 000 (fig. 1.). By dodecylsulfate polyacrylamide gel electrophoresis values of 45 000 and 29 000 (fig. 2) were obtained. In figure 3, and figure 4 the pH-optimum and the heat stability of human urinary kallikrein are shown.

When human urinary kallikrein was submitted to electrofocusing in ampholine in a pH gradient from 3 to 5, three components active on CbzTyrONp were detected. The isoelectric point values are 3.8, 3.9, and 4.09 as shown in figure 5.

Human urinary kallikrein is competitively inhibited by benzamidine and aprotinin. The kinetic data are given in table 2.

A kallikrein from human urine was purified for the first time by Moriya in 1963 (1) using DEAE-Sephadex A-50 as an initial step. In the present work, the important purification step is the aprotinin-Sepharose 4B affinity chromatography. After separation of the bile pigments by gel filtration, the fractions containing kallikrein activity are very heterogeneous and it is possible to remove nearly all the proteins from the kallikrein by affinity chromatography. The gel filtration on Sephacryl

²⁾ Abbreviations

BzArgOEt: N^α-benzoyl-L-arginine ethyl ester

TosArgOMe: Tosyl-L-arginine methyl ester

CbzTyrONp: Carbobenzoxy-L-tyrosine-p-nitrophenyl ester

HMW-kininogen: High molecular weight kininogen

SDS: Sodium dodecylsulfate

Tab. 1. Summary of the purification of human urinary kallikrein.

Steps	Vol- ume (ml)	Total protein (mg)	Total content (mU) ¹⁾	Total content (mU) ²⁾	Total content (mU) ²⁾	Specific activity (U/mg protein)	Specific activity (U/mg protein)	Specific activity (U/mg protein)	a c	b c	x ³⁾
			BzArg- OEt	CbzTyr- ONp	Kinino- gen	BzArg- OEt (a)	CbzTyr- ONp (b)	Kinino- gen (c)			
Crude urine extract	30	3120	29 400	25 200	19.9	0.0094	0.008	0.006	1.492	1.28	—
G-100 gel filtration	565	876	158 200	943 550	423.8	0.181	1.077	0.483	0.377	2.23	1
Affinity chromatography	40	Fractions contain benzamidine. Values for activity cannot be given.									
G-25 gel filtration	25	5.7	27 120	506 240	244.1	4.76	88.8	42.8	0.111	2.075	88.6
S-200 Gel filtration	10	2	12 347	448 269	215	6.17	224	107.5	0.057	2.08	212

¹⁾ Alcohol dehydrogenase-linked assay for kallikrein, substrate: BzArgOEt (34)

²⁾ μ mol substrate per minute

³⁾ purification

Fig. 1. Molecular weight determination by gel filtration. For experimental details, see text. The molecular weight values of the marker proteins are given in parentheses.

- 1 = cytochrome c (13 400),
- 2 = carbonic anhydrase (30 000),
- 3 = hemoglobin (68 000),
- 4 = phosphorylase (98 000),
- 5 = bovine serum albumin (66 000),
- 6 = bovine serum albumin, dimer (132 000),
- 7 = bovine γ -globulin (169 000),
- 8 = human urinary kallikrein (64 000).

Fig. 2. Molecular weight determination by sodium dodecyl-sulfate electrophoresis. For experimental details, see text.

- 1 = cytochrome c (13 400),
- 2 = carbonic anhydrase (30 000),
- 3 = ovalbumin (46 000),
- 4 = phosphorylase (98 000),
- 5 = bovine serum albumin (66 000),
- 6 = bovine serum albumin, dimer (132 000),
- 7 and 8 = human urinary kallikrein (45 000, 29 000).

Fig. 3. pH effect on human urinary kallikrein. 0.3 ml human urinary kallikrein was tested at different pH values as described in 1. c. (33, 36).

- substrate CbzTyrONp,
●—● substrate HMW-kininogen.

Fig. 4. Effect of heat treatment. 0.3 ml human urinary kallikrein was heated for 15 min at the indicated temperature, and then measured as described in 1. c. (30, 36).

- substrate CbzTyrONp,
●—● substrate HMW-kininogen.

Tab. 2. Kinetic data for human urinary kallikrein and various substrates. K_m values were determined by *Lineweaver-Burk* plots, K_i values according to *Green & Work* (42).

	BzArgOEt ⁽³⁴⁾	TosArgOMe ⁽³⁵⁾	CbzTyrONp ⁽³⁶⁾	HMW-kininogen ⁽³³⁾
K_m (mol/l)	1.1×10^{-3}	0.67×10^{-3}	7.4×10^{-6}	0.133×10^{-6}
V (μ mol/min · mg protein)	7.14	1.9	1.4	2.5
K_i (mol/l) Benzamidine	—	—	0.2×10^{-3}	—
K_i (mol/l) Aprotinin	—	—	0.36×10^{-9} (M_r 45 000)	—
			0.102×10^{-9} (M_r 29 000)

Fig. 5. Isoelectric focusing of human urinary kallikrein. For experimental details, see text.

○—○ kallikrein, substrate CbzTyrONp,
..... Absorbance at 280 nm
----- pH.

S-200 yields a purified human urinary kallikrein, which is used for the preparation of antibodies.

The enzyme is considered pure by the following criteria: it yields a single peak on rechromatography in a Sephacryl S-200 column, with constant specific activity and

it shows a single band upon electrophoresis in polyacrylamide at 75 g/l.

After incubation of the polyacrylamide gel with CbzTyrONp at pH 8.0, the region of human urinary kallikrein shows esterolytic activity.

After gel filtration on Sephadex G-100, the kallikrein activity is increased. Two possible explanations of this phenomenon can be suggested. Either a kallikrein-inhibitor complex is dissociated during the elution, or a precursor is activated by the procedure.

It is interesting that the quotients of the specific activities are constant during the isolation steps for CbzTyrONp and kininogen, but not for BzArgOEt and kininogen. These characteristics indicate that there are other proteinases in urine with esterolytic activities on BzArgOEt.

Acknowledgement

This work was supported by Deutsche Forschungsgemeinschaft Sonderforschungsbereich 51. We are indebted to Miss *Ulrike Stuckstedte* and Miss *Karin Schuster* for their excellent technical assistance. We would also like to thank Prof. Dr. H. Fritz for his interest in this work and for many helpful discussions.

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Dr. Reinhard Geiger
Abteilung für Klinische Chemie und Klinische Biochemie
in der Chirurgischen Klinik der Universität München,
Nußbaumstraße 20,
D-8000 München 2