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Large-Scale Purification and Characterization of Human Urinary Kallikrein[1)]

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Kallikrein was highly purified from 1000 liters of fresh pooled urine collected from healthy men by the following procedure: silica gel adsorption, gel filtration on Sephadex G-75, DEAE-Sephadex chromatography, bentonite treatment, affinity chromatography on aprotinin-Sepharose 4B, and rapid gel filtration on a TSK Gel G-3000 SWG column. The final preparation, 17 mg of human urinary kallikrein (HUK), was purified 621.6-fold from the crude preparation obtained by silica gel adsorption. One mg of HUK showed activities of 5.58 PNA units toward H–D-Val–Leu–Arg–pNA, and about 1100 kallikrein units in the dog hypotensive assay.

HUK thus isolated was found to be pure by means of disc electrophoresis, immunoelectrophoresis, and gel filtration. The following enzymes and bioactive substances were not detected: kininase, urokinase, caseinolytic proteases, blood group substances, substances affecting plasma coagulation, and pyrogens.

On isoelectric focusing, HUK separated into microheterogeneous components. The p*I*'s of the dominant components were 3.5, 3.8, and 4.1, while the corresponding molecular weights were 5.4×10^4, 4.9×10^4, and 4.4×10^4, respectively.

An aqueous solution of HUK was stable in the pH range from 6 to 12, and was also stable at 60 °C for 2 h at neutral pH. Michaelis constants were from 4.0×10^{-5} to 4.0×10^{-4} M toward five kinds of synthetic substrates conventionally used for HUK assay. Optimal pH's for these substrates and human kininogen were in the alkaline pH range of 7.5 to 11.5.

Keywords——human urinary kallikrein; purification; characterization; H–D-Val–Leu–Arg–pNA; hypotensive assay; heterogeneity; anti-human urinary kallikrein rabbit serum; kininase; urokinase; caseinolytic protease

Kallikreins or kininogenases (EC 3.4.21.35) are endopeptidases that specifically and rapidly liberate vasodilative peptides from kininogen, a part of the α_2-globulin fraction of plasma. The enzymes have been classified into two groups, plasma and glandular kallikreins.[2)] Urinary kallikrein, which closely resembles the latter in several properties, is assumed to originate from the kidney[3)] and to participate in the regulation of renal functions.[4)]

Kallikrein extracted from hog pancreas has been used clinically, for example, to improve peripheral circulatory disturbances,[5)] essential hypertension,[6)] and male subfertility.[7)] Human kallikrein has been purified from saliva,[8)] pancreas,[9)] pancreatic juice,[10)] kidney,[11)] large intestine,[12)] and urine,[13)] but none of these has yet been used for medical purposes. Since human kallikrein has no antigenicity in patients, it can be administered without any adverse effect due to allergic reaction, even when given by repeated intravenous injection.

In this paper, we describe the purification of kallikrein from human urine on a large scale, and the characterization of its physico-chemical and enzymatic properties. A fairly large-scale preparation of HUK is essential in order to characterize the properties of HUK in detail, elucidate the origin and physiological role of HUK, and develop an immunological assay system such as radioimmunoassay, as well as to prepare medicines for clinical testing.

Materials and Methods

The following materials were obtained from commercial sources: silica gel, bentonite (Merck, Germany); Sephadex G-75, DEAE-Sephadex A-50, CNBr-activated Sepharose 4B, molecular weight determination kits for electrophoresis and gel filtration (Pharmacia Fine Chemicals, Sweden); H-D-Val-Leu-Arg-pNA (Kabi Diagnostica, Sweden); H-Pro-Phe-Arg-MCA, TAME, BAEE, CbzTyrONp, bradykinin (Protein Research Foundation, Osaka); SBTI (type I-S, Sigma Chem. Co., U.S.A.); aprotinin (Trasylol, Bayer, Germany); urokinase, Cohn's fraction I (Green Cross Co., Osaka); casein ("Hammarsten," Merck, Germany); Freund's complete adjuvant (Difco, U.S.A.); anti-human whole plasma rabbit serum (Behringwerke, Germany); anti-human urinary protein rabbit serum (Dako Immunochemicals, U.S.A.).

Aprotinin-Sepharose 4B (1.75×10^4 kallikrein inhibitor units/ml of swelled beads) was prepared according to the method of Oza *et al.*[14] Human kininogen was prepared by heating fresh plasma at 56 °C for 30 min followed by dialysis against phosphate-buffered saline (pH 7.0).[15] Neither kininase nor kallikrein activity was detected in the preparation. TSK Gel G-3000 SWG (2.15 × 60 cm) and TSK Gel G-3000 SW (0.75 × 60 cm) columns, which are products of Toyo Soda Manufacturing Co., Tokyo, were used for preparative and analytical studies, respectively, on a high-performance liquid chromatograph (Waters Limited, U.S.A.).

Amidolytic Activity——Amidolytic activity of HUK toward H-D-Val-Leu-Arg-pNA was determined by a slight modification of the method of Amundsen *et al.*[16] The mixture of 0.1 ml of sample solution, 1.95 ml of 0.2 M Tris-HCl buffer, pH 8.0, 0.05 ml of 0.25% (w/v) SBTI, and 0.2 ml of 1.5 mM substrate was incubated at 37 °C for 15 min. Thereafter, the reaction was terminated by addition of 0.2 ml of 50% (v/v) acetic acid. The absorbance of the mixture was spectrophotometrically measured at 405 nm (the molar extinction of *p*-nitroaniline was taken to be $10600\ M^{-1}\ cm^{-1}$). A blank reaction was carried out for each sample with aprotinin solution (4000 kallikrein inhibitor units/ml) in place of SBTI. The HUK concentration was adjusted so that ΔA_{405} did not exceed 0.35 in the 15 min reaction period. When untreated urine was used as a sample, the incubation time was increased to 45 min.

The amidolytic activity of HUK was expressed in terms of the PNA unit. One PNA unit was defined as the amount of enzyme that could hydrolyze 1 μmol of the substrate per minute under the conditions described above.

Hypotensive Assay[17]——A dog, weighing 8—12 kg, was anesthetized with sodium pentobarbital (25 mg/kg, *i.p.*). The blood pressure was measured at the femoral artery and recorded on a polygraph. Samples were dissolved in, or dialyzed against physiological saline, and injected through the femoral vein. Decrease of diastolic pressure caused by administration of sample solution was compared with that caused by a reference preparation, 14.4±0.4 kallikrein units (KU)/ampoule of hog pancreatic kallikrein.

Kininase, Urokinase, and Caseinolytic Activities——Kininase activity was determined by the method of Trautschold.[18] After incubation of the sample solution with 100 ng of synthetic bradykinin at pH 7.4 for 30 min, the remaining kinin was assayed with isolated rat uterus.

Urokinase activity was measured by a slight modification of the method of Astrup and Müllertz.[19] A solution consisting of clottable protein (Cohn's fraction I) and thrombin in 0.1 M Sörensen's buffer, pH 7.2, was transferred into a plastic plate (24 × 12 cm) and allowed to stand for gelation. An aliquot of 10 μl of sample solution or urokinase (as a reference, 0.125—10 IU) was placed on the gel surface and the plate was incubated at 37 °C for 16 h. Urokinase activity was indicated by the size of the clear circle produced by fibrinolysis.

Caseinolytic activity was determined according to Kunitz[20] with bovine pancreatic trypsin (1.25—10 μg) as a reference. One ml of sample solution was incubated at 35 °C for 20 min with the same volume of 1% (w/v) casein dissolved in 0.1 M Sörensen's buffer, pH 7.6. Then 3 ml of 5% (w/v) trichloro acetic acid (TCA) was added, and the mixture was centrifuged. The absorbance at 280 nm of the supernatant was measured.

Blood Group Substances——A slight modification of the ABO blood grouping test was applied. A mixture of 0.1 ml of sample solution and the same volume of anti-A determinant serum was allowed to stand at room temperature for 15 min, followed by addition of 0.2 ml of A-type red cells suspended in physiological saline. After centrifugation, the sediment was examined for agglutination, which occurred when the sample solution was lacking in blood group substance A. Blood group-specific substance A and saline were employed as positive and negative controls, respectively. The grouping test for B substance was carried out similarly.

Substances Affecting Plasma Coagulation——Plasma recalcification time was measured according to Alkjaersig *et al.*[21] A mixture of 0.1 ml of sample solution or 0.05 M Veronal buffer, pH 7.4, 0.1 ml of 0.3 M epsilon-aminocaproic acid, 0.1 ml of human plasma containing 0.4 mg of potassium oxalate, and an appropriate volume of 0.025 M calcium chloride was incubated at 37 °C. Fibrin clot-forming time was recorded.

Pyrogens——The pyrogen test was performed with rabbits weighing 1.5 to 2.7 kg. Sample solution was administered through an ear vein and the body temperature of the rabbit was determined by means of a thermometer inserted into the rectum. Assessment was done according to the Japanese Pharmacopoeia X.[22]

Protein Determination——The absorbance at 280 nm in a 1.0-cm cuvet was routinely used to estimate protein concentration of sample solutions. The final preparation of HUK was weighed after desalting and lyophilization.

Disc Electrophoresis——According to Davis,[23] disc electrophoresis was performed in 12% polyacrylamide gel (5 × 80 mm) at pH 8.9 at a constant current of 3 mA per gel column. The protein was stained with Coomassie Brilliant

Blue G-250 by the method of Diezel *et al.*[24] An unstained gel was cut into 2-mm slices. Each slice was extracted with 0.5 ml of 0.2 M Tris-HCl buffer, pH 8.0, and assayed for amidolytic activity with H-D-Val-Leu-Arg-pNA.

Immunoelectrophoresis——Immunoelectrophoresis was carried out according to Scheidegger.[25] The antigens were electrophoresed in a 1.2% agarose gel plate (50 × 75 mm) in 0.05 M Veronal buffer, pH 8.0 (μ=0.03), at a constant current of 2 mA/cm at 4 °C for about 2 h. The antisera were then added to each trough and diffusion was allowed to occur over a period of 16 h at room temperature.

Antiserum against HUK was raised in rabbits. One-half ml of purified HUK solution (300 μg/ml) was emulsified with an equal volume of Freund's complete adjuvant and injected intracutaneously into multiple sites at the back of the rabbit. The injection was repeated five times at three-week intervals and antiserum was collected 10 d after the last injection.

Molecular Weight Estimation——Sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-gel electrophoresis)[26] was carried out to estimate the molecular weight of HUK. The following proteins were used as references: α-lactalbumin (MW: 14400), soybean trypsin inhibitor (MW: 20100), carbonic anhydrase (MW: 30000), ovalbumin (MW: 43000), bovine serum albumin (MW: 67000), and phosphorylase B (MW: 94000). The sample and reference solutions were each incubated in 1% (w/v) SDS at room temperature for 16 h then run in the gel at 4 mA/gel for about 4 h.

The molecular weight was also estimated by rapid gel filtration through TSK Gel G-3000 SW column with chymotrypsinogen A (MW: 25000), ovalbumin, bovine serum albumin and aldolase (MW: 158000) as markers.

Isoelectric Focusing——Isoelectric point was measured by the method of Vesterberg and Svesson,[27] with an LKB 110-ml column (LKB Produkter AB, Sweden) and ampholine (pH 3.5—5.0). The electrophoresis was carried out at 2 °C and 450 V for 70 h. The pH of the fractions collected from the column was measured at 2 °C with a PHM 84 pH meter (Radiometer, Denmark).

Heat-Stability and pH-Stability——The pH range studied was from 2.0 to 12.0. Aliquots of 1 ml of HUK solution in 0.04 M Britton–Robinson's wide-range buffer were allowed to stand at 25 °C for up to 24 h. The pH of the solution was then adjusted to 8.0 and the residual amidolytic activity toward H-D-Val-Leu-Arg-pNA was determined.

Thermostability of HUK was checked at 37, 60, and 90 °C in the Britton–Robinson's buffer. The HUK solution was allowed to stand for 30 min or 2 h, then the amidolytic activity was measured.

Kinetic Studies——The pH profiles of HUK activity toward human kininogen and synthetic substrates were estimated in the Britton–Robinson's buffer. Assays were carried out by the initial-rate method except for kininogenase assay. Michaelis constants with these substrates were obtained from Lineweaver–Burk plots under the following conditions: 0.1 M sodium phosphate buffer, pH 8.0, at 25 °C for BAEE[28] and TAME;[29] 0.05 M Tris-HCl buffer, pH 8.0, containing 12% (v/v) methanol, at 25 °C for CbzTyrONp;[13b,30] 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl and 0.01 M $CaCl_2$, at 37 °C for H-Pro-Phe-Arg-MCA;[31] and 0.2 M Tris-HCl buffer, pH 8.0, at 37 °C for H-D-Val-Leu-Arg-pNA.

Results

Purification of HUK

The whole purification process of HUK was carried out at about 4 °C unless otherwise noted. HUK activity was determined during the purification with H-D-Val-Leu-Arg-pNA as a substrate, as described under Materials and Methods. Fresh urine (1000 l) collected from healthy men was adjusted to pH 4.0 with 5 N HCl and treated with 10 kg of silica gel for 2 h under mild stirring. The gel was packed in a column (50 cm i.d.) and washed with 0.1 M sodium phosphate buffer, pH 7.0. HUK was eluted from the silica gel with 50 l of 0.3 M NH_4Cl, pH 8.0. Then 30 kg of solid ammonium sulfate was added to the resulting HUK solution. The precipitate formed was recovered by centrifugation and dissolved in 450 ml of 0.18 M sodium phosphate buffer, pH 8.0.

The solution was applied to a Sephadex G-75 column (7 × 90 cm) equilibrated with the same buffer. The HUK-containing fractions were combined and adjusted to pH 6.0. The solution was applied to a DEAE-Sephadex A-50 column (7 × 20 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 6.0. The column was washed with the starting buffer and the enzyme was eluted with the same buffer containing 0.2 M NaCl. The active fractions were pooled, adjusted to pH 5.0, and treated with 4 g of bentonite for 1 h. The mixture was centrifuged, and solid sodium bicarbonate and sodium chloride were added to the supernatant to make final concentrations of 0.1 and 0.5 M, respectively. The pH of the solution was

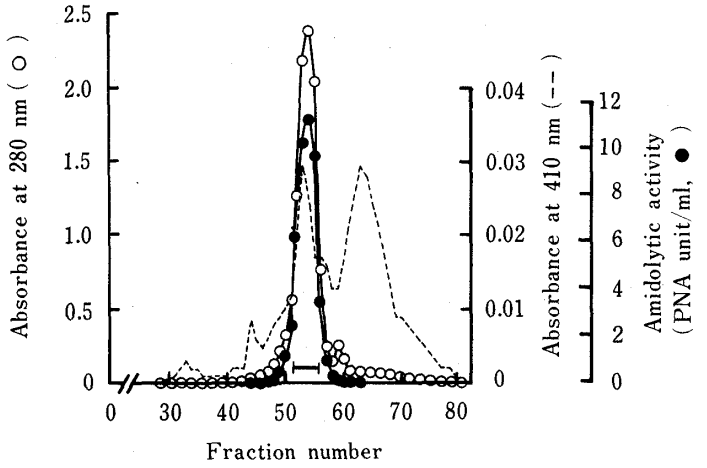

Fig. 1. Rapid Gel Filtration of Human Urinary Kallikrein

The enzyme obtained from affinity chromatography (131 PNA units) was filtered through a TSK Gel G-3000 SWG column (2.15 × 60 cm). The eluant was 0.1 M Na Phosphate buffer, pH 6.8, 0.3 M in NaCl. Three-ml fractions were collected at a flow rate of 3.0 ml/min. Yellowish-brown pigment was measured spectrophotometrically at 410 nm. The bar at the bottom represents the pooled fractions.

TABLE I. Purification of Kallikrein from 10001 of Human Urine

Step	Total activity (PNA unit)	Recovery (%)	Specific activity (mPNA unit/A_{280})	Purification (fold)
Human urine	534.5	100	0.0127	—
Silica gel	467.2	87.4	7.18	1
Sephadex G-75	438.8	82.1	19.39	2.7
DEAE-Sephadex A-50	319.6	59.8	76.21	10.6
Bentonite	251.7	47.1	168.4	23.5
Aprotinin-sepharose	131.0	24.5	3770	525.1
TSK Gel G-3000 SWG	120.5	22.5	4463	621.6

adjusted to 9.5 with 6 N NaOH.

Aprotinin-Sepharose 4B (450 ml of settled beads) was suspended in the HUK solution and gently stirred for 20 h. The gel was then packed in a column and washed with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.6 M NaCl. HUK was eluted from the gel with 0.5 M glycine–HCl buffer, pH 3.5, containing 0.6 M NaCl. The active fractions were combined, dialyzed against distilled water, and lyophilized.

The resulting powder was dissolved in 2 ml of 0.1 M sodium phosphate buffer, pH 6.8, containing 0.3 M NaCl, and subjected to rapid gel filtration on a TSK Gel G-3000 SWG column. The filtration was performed at room temperature. Fractions of 3 ml were collected at a flow rate of 3 ml/min (Fig. 1). The active fractions were combined, desalted by dialysis, and lyophilized.

Table I summarizes the purification procedure of HUK. Seventeen mg (dry weight) of purified HUK was obtained with 622-fold purification from the crude HUK preparation after silica gel treatment. Overall recovery was 23%.

The specific activity of the purified HUK was 4.463 PNA units/A_{280}. The extinction coefficient, $E_{280}^{1\%}$, of HUK in 0.1 M sodium phosphate buffer, pH 7.1, was determined as 12.5.

The preparation of purified HUK thus obtained will simply be referred to as HUK below.

Purity Analysis

Polyacrylamide Gel Electrophoresis——In 12% polyacrylamide gel disc electrophoresis at pH 8.9, HUK gave, after staining with Coomassie Brilliant Blue, a major and a minor protein band which coincided well with the activity toward H-D-Val-Leu-Arg-pNA (Fig. 2). These proteins, microheterogeneous components, of HUK, moved much faster than human serum albumin.

In standard 7% polyacrylamide gel at pH 8.9, HUK moved with the dye, Bromophenol Blue, to the gel front (not shown).

Immunoelectrophoresis——As shown in Fig. 3, a clear precipitin line between HUK and anti-HUK rabbit serum appeared at a more anodic position than that of albumin. There was no precipitation between HUK and anti-human urine or anti-human plasma rabbit serum.

Other Enzymes and Bioactive Substances——The following enzymes and bioactive substances were not detectable in 0.1 PNA unit of HUK: kininase, urokinase, caseinolytic proteases, blood group substances, substances affecting plasma coagulation, and pyrogens. Urokinase and caseinolytic proteases were completely removed from HUK in the purification procedure by the bentonite treatment, whereas other bioactive substances were mainly eliminated from HUK by the affinity chromatography on aprotinin-Sepharose 4B.

Properties

Molecular Weight——In SDS-gel electrophoresis, HUK gave a single band corresponding to a molecular weight of 5.0×10^4 (Fig. 4).

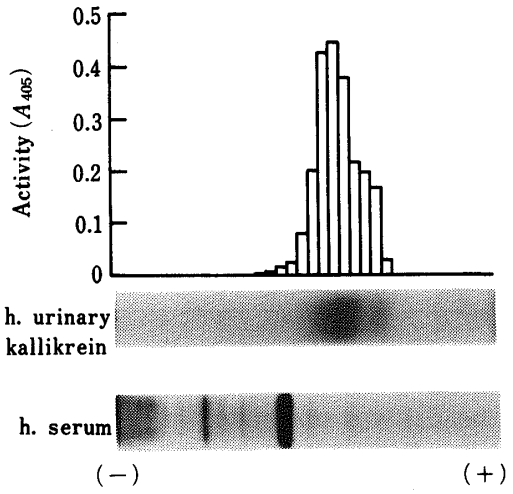

Fig. 2. Polyacrylamide Gel Electrophoresis of Human Urinary Kallikrein

Kallikrein (50 μg) was applied to a 12% polyacrylamide gel (pH 8.9) and developed at 3 mA/column. Migration was from left to right. Amidolytic activity toward H-D-Val-Leu-Arg-pNA coincided with the protein bands stained by Coomassie Brilliant Blue G-250. Human serum was employed as a reference.

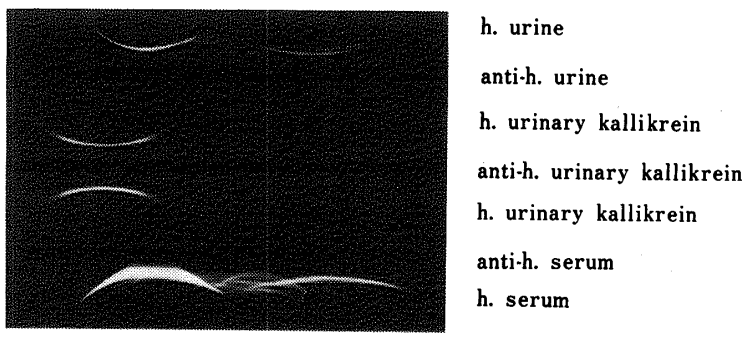

Fig. 3. Immunoelectrophoresis of Human Urinary Kallikrein

The antigens were subjected to electrophoresis in 1.2% agarose gel, pH 8.0, at 2 mA/cm for about 2 h and diffusion was allowed to occur for 16 h at room temperature. Antigens and antisera used are indicated in the figure. Migration was from right to left.

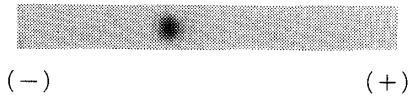

Fig. 4. SDS-Polyacrylamide Gel Electrophoresis of Human Urinary Kallikrein

The enzyme, 50 μg, was incubated in 1% SDS at room temperature for 16 h and run at a constant current of 4 mA/column for about 4 h.

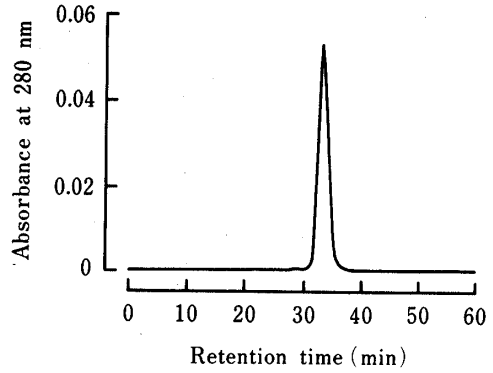

Fig. 5. Second Gel Filtration of Human Urinary Kallikrein

The enzyme (100 μg) was applied to TSK Gel G-3000 SW (0.75 × 60 cm, 2 columns). The flow rate was 1.0 ml/min. The eluant was 0.1 M Na phosphate buffer, pH 6.8, 0.3 M in NaCl. Absorbance at 280 nm was recorded.

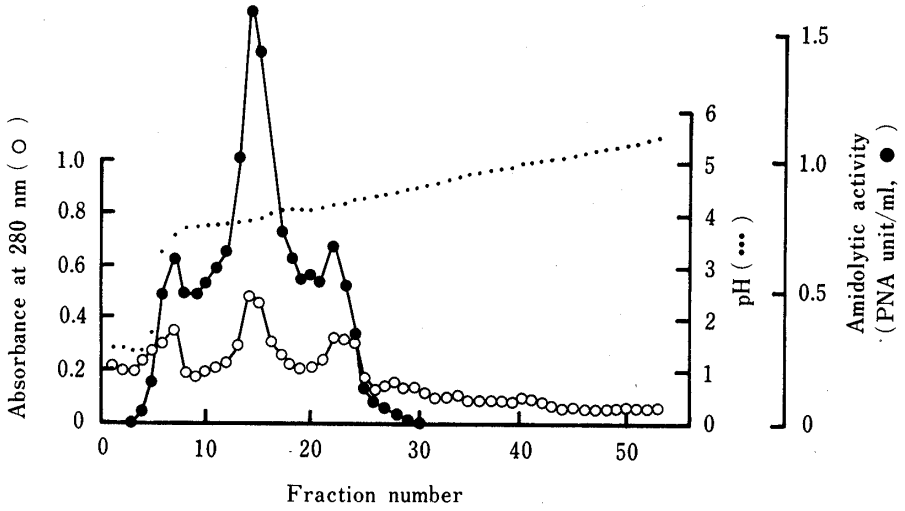

Fig. 6. Isoelectrofocusing of Human Urinary Kallikrein

Isoelectrofocusing was carried out with ampholine, pH 3.5—5.0, on an LKB 110-ml column. The conditions were 450 V, 70 h, and 2 °C. Fractions of 2 ml were collected.

The same molecular weight of HUK, 5.0×10^4, was estimated from the retention time in gel filtration on TSK Gel G-3000 SW column (Fig. 5).

Isoelectric Point——HUK was subjected to isoelectric focusing in ampholine (pH 3.5 to 5.0), and gave several components active toward H–D-Val–Leu–Arg–pNA. The pI values of the dominant components were 3.5, 3.8 and 4.1, as shown in Fig. 6. The molecular weights of these components were estimated as 5.4×10^4, 4.9×10^4, and 4.4×10^4, respectively, by SDS-gel electrophoresis.

Heat-Stability and pH-Stability——Figure 7 shows that HUK was stable at neutral and alkaline range and unstable at pH below 6.

The thermostability of HUK depended upon the pH of the solution. HUK activity decreased rapidly at pH 3.5 on heating. At pH 7.0, on the other hand, about 90% of HUK activity remained after 120 min at 60 °C, and 44% after 30 min at 90 °C (Fig. 8).

The thermostability of HUK was increased by addition of albumin. When HUK was dissolved in 0.1 M sodium phosphate buffer, pH 7.0, in the presence of 0.1% (w/v) human

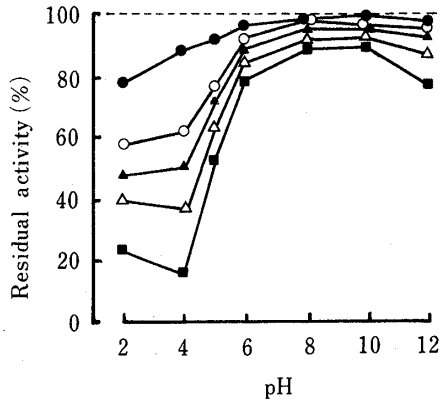

Fig. 7. Influence of pH on the Stability of Human Urinary Kallikrein

Kallikrein solutions in 0.04 M Britton–Robinson's wide-range buffer were allowed to stand at 25 °C and the residual activity toward H-D-Val-Leu-Arg-pNA was measured. ●, 30 min; ○, 2 h; ▲, 4 h; △, 6 h; ■, 24 h.

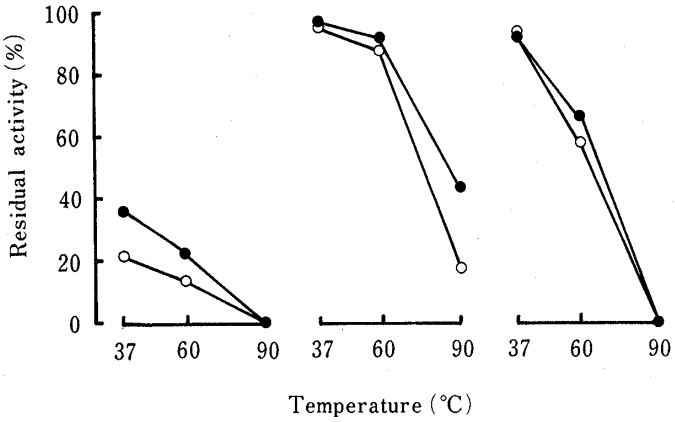

Fig. 8. Influence of pH on the Thermostability of Human Urinary Kallikrein

Kallikrein solutions in 0.04 M Britton–Robinson's wide-range buffer were heated at 37, 60, or 90 °C for 30 min or 2 h. The residual activity toward H-D-Val-Leu-Arg-pNA was measured. left, pH 3.5; middle, pH 7.0; right, pH 10.5. ●, 30 min; ○, 2 h.

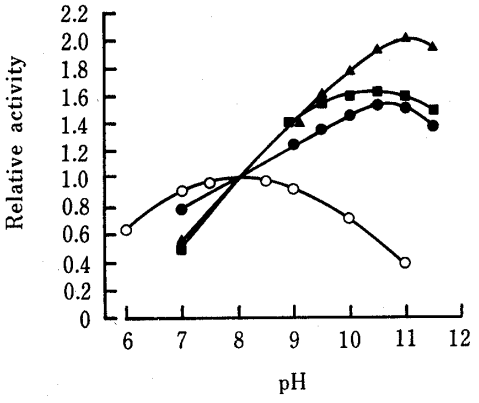

Fig. 9. Optimal pH of Human Urinary Kallikrein

Kallikrein was dissolved in 0.04 M Britton–Robinson's wide-range buffer and assayed for enzymatic activity. Hydrolytic activities toward synthetic substrates were measured by the initial-rate method, and kininogenase activity was assayed by the end-point method. The activities of the enzyme at pH 8.0 were taken as 1.0. ○, human kininogen; ●, H-D-Val-Leu-Arg-pNA; ▲, H-Pro-Phe-Arg-MCA; ■, BAEE.

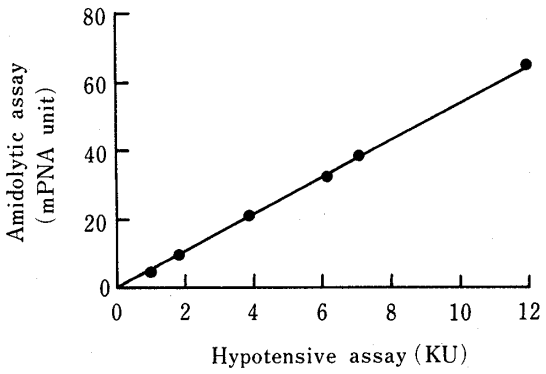

Fig. 10. Correlation between Amidolytic and Hypotensive Activities of Human Urinary Kallikrein

Samples were taken at each purification step. Amidolytic activity was measured at pH 8.0, 37 °C with H-D-Val-Leu-Arg-pNA as the substrate. Hypotensive activity was assayed by the dog blood pressure method with a reference preparation of hog pancreatic kallikrein.

TABLE II. Optimal pH's and Michaelis Constants of Human Urinary Kallikrein

Substrate	Optimal pH	Michaelis constant (M)
Human kininogen	8.0	—
Peptidyl-PNA	10.5—11.0	4.0×10^{-5}
Peptidyl-MCA	11.0—11.5	4.0×10^{-5}
TAME	7.5— 8.5	5.7×10^{-4}
BAEE	10.0—11.0	5.4×10^{-4}
CbzTyrONp	8.5— 9.0	4.0×10^{-4}

Assays were performed by the initial-rate method except for kininogenase assay. Michaelis constants were obtained Lineweaver–Burk plots under the following conditions: 0.1 M Na phosphate buffer, pH 8.0, at 25 °C for BAEE and TAME; 0.05 M Tris-HCl buffer, pH 8.0, 12% (v/v) methanol, at 25 °C for CbzTyrONp; 0.05 M Tris-HCl buffer, pH 8.0, 0.1 M in NaCl, 0.01 M in $CaCl_2$ for H–Pro–Phe–Arg–MCA; 0.2 M Tris-HCl buffer, pH 8.0, at 37 °C for H–D-Val–Leu–Arg–pNA.

serum albumin, its activity was unchanged after heating at 60 °C for 10 h, that is, conditions suitable for pasteurization against hepatitis virus.[32]

Optimal pH and Michaelis Constant——The effects of pH on the HUK activity toward human kininogen, H–D-Val–Leu–Arg–pNA, H–Pro–Phe–Arg–MCA, and BAEE are shown in Fig. 9.

Table II summarizes the optimal pH's and Michaelis constants of HUK toward these substrates. The optimal pH's of HUK were weakly alkaline toward human kininogen, TAME, and CbzTyrONp, and more alkaline toward H–D-Val–Leu–Arg–pNA, H–Pro–Phe–Arg–MCA, and BAEE. The *K*m values for the synthetic substrates studies were from 4.0×10^{-5} to 4.0×10^{-4} M.

Hypotensive Activity——Like hog pancreastic kallikrein, HUK lowered blood pressure in dogs. By comparison with the reference preparation of hog pancreatic kallikrein, HUK was found to possess hypotensive activity of approximately 1100 KU/mg dry weight.

A good relationship was found between dog hypotensive assay and amidolytic assay with H–D-Val–Leu–Arg–pNA (Fig. 10). One PNA unit of HUK corresponded to 201.6 KU.

Discussion

Although highly purified HUK has been obtained and characterized in several laboratories,[13] considerable differences in properties were found especially in molecular weight and enzymatic parameters. Therefore, Fiedlar[33] suggested that different enzymes had been isolated as kallikrein. Thus, we attempted to derermine HUK specifically and accurately with H–D-Val–Leu–Arg–pNA as a substrate, using protease inhibitors, aprotinin and SBTI. Besides kallikrein, proteases such as trypsin, plasmin, thrombin, and urokinase are known to be contained in human urine and are able to digest the substrate. HUK is inhibited completely by aprotinin, but is not inhibited by SBTI under the assay conditions used in this experiment. Trypsin and most similar enzymes are inhibited by both inhibitors. Accordingly, HUK activity toward the substrate is represented as the difference between activity in the presence of SBTI and that in the presence of aprotinin. The PNA activity obtained by this method correlated well with the hypotensive activity (Fig. 10).

From 1000 l of human fresh urine, 17 mg of pure HUK was obtained with a 23% recovery of activity (Table I). This is the largest-scale purification of HUK so far reported. In this purification, bentonite treatment was effective to remove urokinase and caseinolytic

proteases from kallikrein. Affinity chromatography on aprotinin-Sepharose 4B gave an excellent result. In this step, specific activity of HUK was increased 22 times and bioactive substances such as blood group substances, plasma thrombopoietic substances, and pyrogens were removed. Rapid gel filtration on a TSK Gel G-3000 SWG column effectively excluded the yellowish-brown pigment which is difficult to remove by conventional procedures (Fig. 1). Thus, purified HUK should be safe for clinical use, even for intravenous administration.

It is generally agreed that glandular kallikreins consist of microheterogeneous components. In our laboratories, HUK has been resolved by isoelectric focusing into 3—7 components. In the present investigation, three main components having pI's of 3.5, 3.8, and 4.1 were separated (Fig. 6), and their molecular weights were found to be 4.4×10^4, 4.9×10^4, and 5.4×10^4, respectively, by SDS-gel electrophoresis. These molecular weights are close to that of human renal kallikrein,[11] $4.7—4.9 \times 10^4$, suggesting that urinary kallikrein is physiologically derived from the kidney. HUK was also separated into components in 12% polyacrylamide gel disc electrophoresis (Fig. 2). However, HUK behaved like a homogeneous protein in SDS-gel electrophoresis (Fig. 4) and in gel filtration on TSK Gel G-3000 SW column (Fig. 5). This is because the molecular weights of the heterogeneous components are similar, and, moreover, the medium-sized, 49000-dalton component is the major one. Further detailed investigation on the properties of these components is in progress. Pierce and Nustad[34] observed six components of HUK on isoelectric focusing, and one of the main fractions further gave three bands in SDS-gel electrophoresis corresponding to molecular weights of 23800, 28300, and 36400. They[35] also found that rat urinary kallikrein gave four fractions with pI's of 3.50, 3.68, 3.73, and 3.80, and molecular weights of 35300, 33600, 33100, and 32300, respectively.

Widely different values for the molecular weight of HUK have been reported: Moriya and co-workers[9,13d] gave 40500 and $2.7—2.9 \times 10^4$, Geiger *et al.*[13e,36] reported 64000, 45000, 29000, and 50000, ole-Moi Yoi *et al.*[13f,i] gave 25000—40000, 48700, 48000, and 48213, values of 37000 and 35400 were presented by Porcelli *et al.*,[13b] and Hial *et al.*[13a] and Oza and Ryan[13g] gave 43600 and 45000, respectively. It is likely that the variation of molecular weight of HUK is due to (1) different contents of sialic acid and carbohydrate,[37] and consequent conformational changes of the HUK molecule, (2) degradation and denaturation of HUK during the purification procedure, (3) incorrect identification of non-kallikrein protease as kallikrein, and, of course, (4) different methods for estimation of molecular weight. The first assumption seems more likely to explain the microheterogeneity of our HUK.

Hypotensive activity of our HUK appeared to be approximately 1100 KU/mg. It is of interest that this value is similar to those of several kinds of glandular kallikreins in spite of the differences of species and organs from which the enzymes were isolated: 1350 and 1400 KU/mg (Ikekita *et al.*)[38] and 1414 KU/mg (Kutzbach and Schmidt-Kastner)[39] for hog pancreatic kallikrein, 810—870 KU/mg for rate pancreatic kallikrein,[40] 1117 KU/mg (Fujimoto *et al.*)[41] and 930—1260 KU/mg (Fukuoka *et al.*)[42] for cat submaxillary kallikrein, and 1250 KU/mg for dog urinary kallikrein.[43] These values can be concidered as approximately equal since the reference materials necessary for biological assay are not uniform between laboratories, and the assays are subject to some variation.

HUK was very stable to heating at neutral pH, as was found for dog kallikreins from urine[43] and kidney,[44] and human urinary kallikrein[13d] previously described. The thermostability of HUK was enhanced by albumin. In the presence of human serum albumin, HUK was completely resistant to heating at 60 °C for 10 h, an indispensable process in the manufacture of medicines from human source materials.

Optimal hydrolytic activities of HUK toward H–D-Val–Leu–Arg–pNA, H–Pro–Phe–Arg–MCA, and BAEE were found at remarkably alkaline pH (Fig. 9) as observed with hog

pancreatic kallikrein and BAEE.[45] Such an optimal pH value is, as mentioned by Kato,[31] a characteristic of glandular kallikreins.

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References and Notes

1) Abbreviations: HUK, human urinary kallikrein; pNA, *p*-nitroanilide; MCA, 4-methylcoumarin amide; TAME, *N*-α-tosyl-L-arginine methyl ester; BAEE, *N*-α-benzoyl-L-arginine ethyl ester; CbzTyrONp, *N*-α-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester; SBTI, soybean trypsin inhibitor.
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