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Purification and Some Properties of Kallikreins from Human Urine and Pancreas[1)]

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Human urinary and pancreatic kallikreins were purified by immunoadsorbent column chromatography. The obtained enzymes each showed a single protein band on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

The properties of the two kallikreins were compared. By both gel filtration and SDS polyacrylamide gel electrophoresis, the molecular weights of urinary and pancreatic kallikreins were estimated to be 42000 ± 3000 and 55000 ± 3000, respectively. The isoelectric point, pH stability and optimum pH of the two enzymes were very similar, and both enzymes were inhibited by serine protease inhibitors such as diisopropyl fluorophosphate, phenylmethyl sulfonylfluoride, aprotinin and α_1-proteinase inhibitor. Both kallikreins were stable up to 56°C for 80 min at pH 8.0. Urinary and pancreatic kallikreins gave a fused precipitin line against anti-human urinary kallikrein antibody or anti-human pancreatic kallikrein antibody.

The comparisons of enzymic and immunological properties thus suggested that human glandular kallikreins, at least urinary and pancreatic kallikreins, are very similar.

Keywords——human kallikrein; urinary kallikrein; pancreatic kallikrein; purification of glandular kallikrein; immunoadsorbent column; enzymic and immunological properties of kallikrein

Kallikreins (EC 3.4.21.8) are serine proteases which produce kinin from kininogen by limited proteolysis.[2)] Plasma, pancreatic, urinary and salivary kallikreins are well known.[3–5)] However, their physiological functions are not yet clear[6–8)] and it has not been established whether or not urinary and pancreatic kallikreins are identical. At present, human kallikreins can be classified into two different types by means of immunological techniques, and in particular, human urinary and pancreatic kallikreins are known to have common determinants,[9)] though their enzymic properties have not yet been elucidated.

So far, porcine pancreatic kallikrein has been the subject of extensive study and there are at least two sub-components.[10–14)] Furthermore, these two sub-components have been proved to be immunologically identical. Such sub-components were also found in rat pancreatic,[15)] rat urinary,[16)] bovine serum,[17)] human serum,[18)] and human urinary kallikreins.[19)]

Physiological and pathological studies may be important in investigating the functions of human kallikreins. However, the functions for the glandular kallikreins may be complex, and it is difficult to obtain sufficient amounts of human kallikreins. In the present paper, we describe the purification of human urinary and pancreatic kallikreins by immunoadsorbent column chromatography, and compare their enzymic properties.

Materials and Methods

Materials——The following reagents were obtained commercially: Sephadex G-100 and CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals Co., Ltd.), diethylaminoethyl (DEAE)-cellulose (Brown Co., Ltd.), bovine serum albumin (BSA, Wako Pure Chemical Co., Ltd.) and L-prolyl-L-phenylalanyl-L-arginyl-4-methyl

coumaryl-7-amide (Pro–Phe–Arg–MCA, Protein Research Foundation, Osaka). α_1-Proteinase inhibitor was purified according to the method of Sugiura *et al.*[20] This specimen was confirmed to be homogeneous by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Other reagents were of analytical reagent grade.

Assay of Kallikrein Activity——The amidolytic activity of kallikrein towards Pro–Phe–Arg–MCA was determined according to the method of Kato *et al.*[21] with a minor modification.

Five μl of enzyme solution was added to 0.2 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl, 0.05% soybean trypsin inhibitor and 0.1% BSA and the mixture was preincubated at 37 °C for 5 min, then 10 μl of 10 mM Pro–Phe–Arg–MCA dissolved in dimethyl sulfoxide was added. The mixture was incubated at 37 °C for 60 min and the enzyme reaction was terminated by the addition of 2.5 ml of 0.1 M acetate buffer (pH 4.5). The amount of liberated 7-amide-4-methylcoumarin (AMC) was measured by fluorometry (excitation at 380 nm and emission at 440 nm). Under the above conditions, 1 unit of kallikrein activity was defined in terms of 1 μmol of AMC liberated per min, and this assay was used in the studies on enzyme properties.

The esterolytic activity was measured by the colorimetric method[22] with chromotropic acid using *N*-α-tosyl-L-arginine methyl ester (TAME) as a substrate at 30 °C, pH 8.0. One unit of kallikrein activity was defined in terms of 1 μmol of methyl alcohol liberated per min.

The vasodilator activity was determined by the method of Moriya *et al.*[23] and the unit of kallikrein was defined according to the report of Moriya *et al.*[23]

Determination of Protein——Protein was determined either by measuring the absorbance at 280 nm or by he method of Lowry *et al.*[24] with BSA as a standard protein.

Disc Electrophoresis——Disc electrophoresis was performed on 7.5% polyacrylamide gel. The electrophoresis was carried out by the procedure of Davis.[25] The protein was stained with Coomassie Brilliant Blue R-250.

SDS Polyacrylamide Gel Electrophoresis——SDS polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn,[26] using 10% polyacrylamide gel containing 0.1% SDS. The protein was stained with Coomassie Brilliant Blue R-250.

Isoelectric Focusing——A dialyzed sample was applied to an ampholine column (110 ml) as described by Vesterberg and Svensson.[27] The pH range of carrier ampholite was 3.5—5.0 and electrophoresis was carried out for 44 h at constant voltage (700 V) at 4 °C.

Immunotitration——Immunotitration studies were done by incubating 20 μl of purified enzyme (15 μg/ml) in a 0.14 ml system in 10 mM potassium phosphate buffer (pH 7.0) with the antibody overnight at 4 °C. Then 20 μl of goat anti-rabbit immunoglobulin G serum was added and the mixture was further incubated overnight at 4 °C. Subsequently the reaction mixture was centrifuged at 3000 rpm for 20 min and the enzyme activity in the supernatant was measured with Pro–Phe–Arg–MCA.

Preparation of Anti-human Kallikrein Antibody——Human urinary kallikrein (10.2 U/mg) was purified according to the method of Matsuda *et al.*[28] Purified human pancreatic kallikrein (10.5 U/mg) was prepared according to the method of Amouric and Figarella.[9] The homogeneity of human urinary and pancreatic kallikreins were confirmed by disc and SDS polyacrylamide gel electrophoresis.

One mg of purified enzyme (1 mg/ml) was emulsified with an equal volume of complete Freund's adjuvant and injected into multiple intracutaneous sites in two rabbits. Injection of the antigen (200 μg/ml) was repeated three times at two-week intervals. One week after the last injection, the rabbits were bled. The antibody was purified according to the method of Peterson and Sober,[29] using DEAE–cellulose column chromatography.

In immunotitration, 10 μl of anti-human urinary kallikrein antibody precipitated 96.8% of human urinary kallikrein and 10 μl of this antibody also precipitated 95.8% of human pancreatic kallikrein. One hundred μl of anti-human pancreatic kallikrein antibody preincubated 94.4% of human pancreatic kallikrein and 93.6% of human urinary kallikrein.

Immunological Techniques——Double immunodiffusion in a mixed gel of 0.5% agar and 0.5% agarose was performed by the method of Ouchterlony.[30] Immunoelectrophoresis on a glass plate (10 × 10 cm) was employed as described by Graber and Williams,[31] and two-dimensional immunoelectrophoresis was carried out according to the method of Weeke.[32]

Preparation f Immunoadsorbent Column——Five g of CNBr-activated Sepharose 4B was allowed to swell and was washed on a glass filter (G-3) with 1 l of 1 mM HCl solution. The antibody (50 mg) was dissolved in 25 ml of 0.1 M bicarbonate buffer (pH 8.3) containing 0.5 M NaCl (coupling buffer). CNBr-activated gel was immediately washed with coupling buffer and subsequently mixed with the above antibody solution and gently stirred for 2 h at room temperature. Unbound antibody was washed away with the coupling buffer on the glass filter and remaining active groups on activated Sepharose 4B were blocked with 1 M ethanolamine (pH 8.0) for 2 h at room temperature.

Purification of Human Urinary Kallikrein——Forty-five l of male urine was mixed with 450 g of the activated silica gel, the pH was adjusted to 4.0 with 5 N HCl and the mixture was allowed to stand for 15 min. The silica gel was collected on a Buchner funnel, and transferred into 300 ml of 0.2 M ammonium chloride adjusted to pH 8.0 with ammonium hydroxide, then allowed to stand for 15 min. The resulting supernatant was dialyzed against distilled water at 4 °C for 2 d. The dialyzed enzyme was applied to a column (2.8 × 30 cm) of DEAE–cellulose equilibrated with 50 mM phosphate buffer (pH 6.7) containing 50 mM NaCl, then the column was washed with 2 l of the above buffer.

The adsorbed enzyme was eluted with a linear gradient from 0.05 to 0.5 M phosphate buffer (pH 6.7) containing 50 mM NaCl.

The active fractions were concentrated to 8 ml with a membrane filter (Amicon UM-10) and applied to a column (2.8 × 80 cm) of Sephadex G-100 equilibrated with 50 mM phosphate buffer (pH 8.0) containing 50 mM NaCl at a flow rate of 15 ml/h.

Active fractions were pooled and dialyzed against 50 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl. The enzyme (2 ml) was applied to the immunoadsorbent column (1 × 2 cm) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl. The immunoadsorbent column was washed with the same buffer until the absorbance at 280 nm was less than 0.01, and then eluted with 0.2 M Na_2CO_3 containing 0.5 M NaCl. The active fractions were collected, the pH was adjusted to 8.0 with 1 N HCl, and the solution was concentrated with a membrane filter (Amicon UM-10) to 3 ml. The concentrated enzyme was applied to a Sephadex G-100 column (1 × 90 cm) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 50 mM NaCl. Active fractions were collected and used as purified human urinary kallikrein (880 μg). The purified enzyme showed a single protein band on disc and SDS polyacrylamide gel electrophoresis, and the specific activity was 10.4 U/mg for Pro–Phe–Arg–MCA, 26.1 U/mg for TAME and 1744 KU/mg for vasodilator activity.

Purification of Human Pancreatic Kallikrein——Human pancreas (370 g), obtained from autopsy cases and frozen, was minced, and homogenized with a Polytoron homogenizer in 300 ml of 50 mM phosphate buffer (pH 8.0) containing 50 mM NaCl, then autolyzed at 25 °C for 20 h. The homogenate was mixed with 5 volumes of acetone at 4 °C, and stirred vigorously. The precipitate was filtered off by means of a Buchner funnel, and the residue was washed with about 500 ml of acetone, then with 500 ml of ethyl ether.

The acetone powder was dissolved in 50 ml of 0.1 M phosphate buffer (pH 6.7) containing 50 mM NaCl, and then solution was centrifuged (10000 × *g*) for 15 min, then dialyzed against the same buffer. The dialyzed enzyme was applied to a DEAE–cellulose column (3 × 30 cm) equilibrated with 50 mM phosphate buffer (pH 6.7) containing 50 mM NaCl. The column was washed with 2 l of the above buffer, and the enzyme was eluted with a linear gradient from 0.05 to 0.5 M phosphate buffer (pH 6.7) containing 50 mM NaCl. The active fractions were concentrated to 8 ml with a membrane filter (Amicon UM-10), and the concentrated enzyme was applied to a Sephadex G-100 column (2.8 × 90 cm) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 50 mM NaCl at a flow rate of 15 m/h. Active fractions were collected, and dialyzed against 50 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl, and further purification of pancreatic kallikrein was performed according to the procedure described for urinary kallikrein, with an immunoadsorbent column. Active fractions were collected and used as purified enzyme (910 μg). The purified human pancreatic kallikrein showed a single protein band on disc and SDS polyacrylamide gel electrophoresis and the specific activity was 10.6 U/mg for Pro–Phe–Arg–MCA, 28.6 U/mg for TAME and 1778 KU/mg for vasodilator activity.

Molecular Weight——The molecular weights of human urinary and pancreatic kallikreins were determined by gel filtration at 4 °C on a Sephadex G-100 column and by SDS polyacrylamide gel electrophoresis. SDS polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gel containing 0.1% SDS in 0.1 M sodium phosphate buffer (pH 7.2), and protein bands were stained with Coomassie Brilliant Blue R-250.

Results

Purification of Kallikreins

Elution profiles from the immunoadsorbent column are presented in Fig. 1. The enzymes were each eluted as a single peak, and the maximum capacities of the column were calculated to be about 300 and 35 μg for urinary and pancreatic kallikreins, respectively. The purification procedures for urinary and pancreatic kallikreins are summarized in Table I. The homogeneity of each purified enzyme was confirmed by disc and SDS polyacrylamide gel electrophoresis; as shown in Fig. 2, each purified enzyme showed a single protein band.

Molecular Weight

By the gel filtration and SDS polyacrylamide gel electrophoresis, the molecular weights of huamn urinary and pancreatic kallikreins were estimated to be 42000 ± 3000 and 55000 ± 3000, respectively.

Isoelectric Points of Urinary and Pancreatic Kallikreins

The isoelectric points of the purified enzymes were investigated by the isoelectrofocusing technique. The urinary kallikrein showed two main peaks of activity at pH 4.0 and 4.2, and pancreatic kallikrein showed two main peaks of activity at pH 4.2 and 4.3.

TABLE I. Purifications of Human Urinary and Pancreatic Kallikreins

Step of purification	Human urinary kallikrein				Human pancreatic kallikrein			
	T.A.[a] (U)	T.P.[b] (mg)	S.A.[c] (U/mg)	Purification factor	T.A.[a] (U)	T.P.[b] (mg)	S.A.[c] (U/mg)	Purification factor
Silica gel adsorption	34.9	1050	0.033	1				
Acetone powder					120	5300	0.023	1
DEAE-cellulose column chromatography	12.2	44.9	0.272	8.2	56	138	0.406	17.7
Sephadex G-100 gel filtration	9.6	21.6	0.444	13.5	20.6	7.5	2.73	119
Immunoadsorbent column chromatography	9.3	0.94	9.9	300	9.8	1.07	9.16	398
Sephadex G-100 gel filtration	9.1	0.88	10.4	315	9.6	0.91	10.6	460

a) T.A., total activity. *b*) T.P., total protein. *c*) S.A., specific activity.
Forty-five liters of human urine and 370 g of human pancreas were used as starting materials, respectively.
Protein was determined by the method of Lowry *et al.*[24] with BSA as a standard protein.

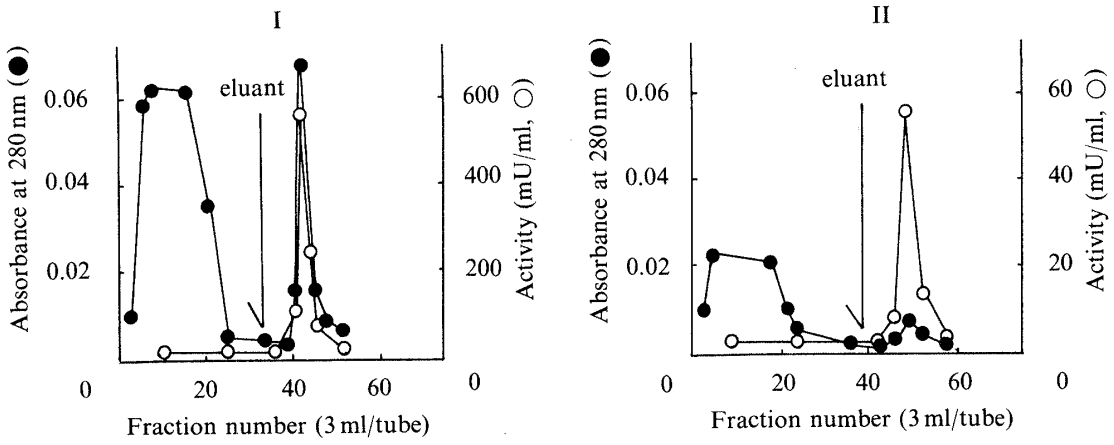

Fig. 1. Immunoadsorbent Column Chromatographies of Kallikreins from Human Urine and Pancreas

The immunoadsorbent column was equilibrated with 50 mM phosphate buffer (pH 8.0) containing 0.5 M NaCl. The eluant was 0.2 M Na_2CO_3 (pH 11.4) containing 0.5 M NaCl. The flow rate was 50 ml/h.
I, urinary kallikrein; II, pancreatic kallikrein.

Effect of pH on Activity and Stability

The optimum pH values for activity of human urinary and pancreatic kallikreins towards Pro–Phe–Arg–MCA were investigated with various pH buffers. The optimum pH of both enzymes was found to be 11.0. In order to investigate the pH stability, the enzymes were incubated with buffers of various pH values at 37 °C for 80 min. As shown in Fig. 3, both kallikreins were found to be stable in the pH range of 6.0—11.0.

Thermal Stability of Human Urinary and Pancreatic Kallikreins

One-tenth ml of kallikrein (0.1 U/ml) was mixed with 2 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.15 M NaCl and the mixture was kept for 80 min at various temperatures ranging from 0 to 80 °C. The samples were then cooled and the remaining activities were determined. Both enzymes were stable below 56 °C.

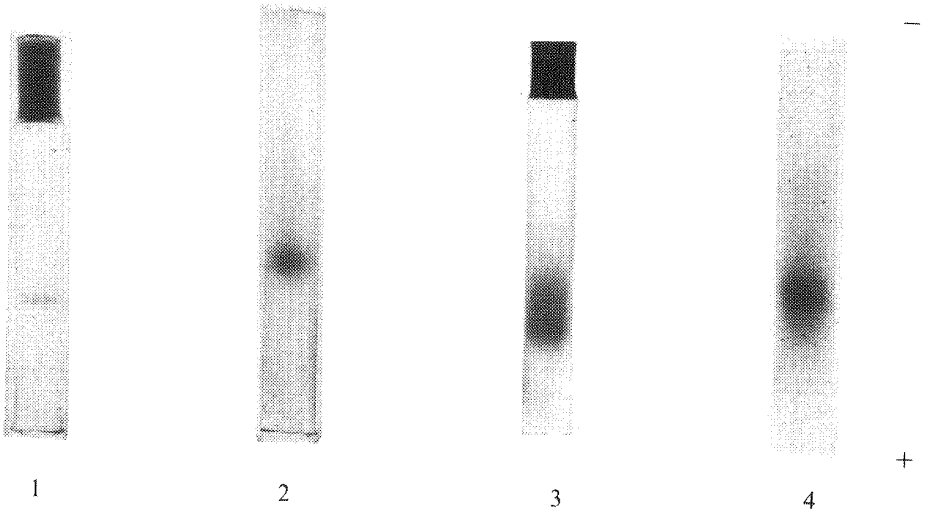

Fig. 2. Disc and SDS Polyacrylamide Gel Electrophoresis of Kallikreins from Human Urine and Pancreas

Disc electrophoresis 1, 3 was carried out with 7.5% polyacrylamide gel and 0.04 M Tris-glycine buffer (pH 9.4) at 4 °C, and 40 μg of sample was applied per gel. SDS polyacrylamide gel electrophoresis 2, 4 was carried out on 10% polyacrylamide gel at room temperature, and 50 μg of sample was applied per gel.

Gels 1 and 2, purified urinary kallikrein; gels 3 and 4, purified pancreatic kallikrein.

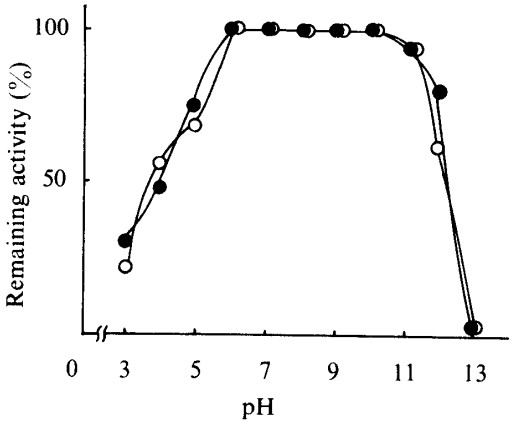

Fig. 3. pH Stability Human Urinary and Pancreatic Kallikreins

The enzymes (25 mU/ml) were incubated at 37 °C for 80 min and then diluted 50-fold with 0.1 M phosphate buffer (pH 8.0). The activity was determined with Pro–Phe–Arg–MCA as a substrate.

pH 3—6, McIlvain buffer; pH 7—8, 0.1 M phosphate buffer; pH 9—13: 0.1 M glycine–NaOH buffer.

●, urinary kallikrein; ○, pancreatic kallikrein.

Effects of Various Reagents and Metal Ions on Activity of Kallikrein

The human urinary and pancreatic kallikrein activities were measured in the presence of various reagents. The results are shown in Table II. The enzymes were inhibited by serine protease inhibitors such as diisopropyl fluorophosphate (DFP) and phenylmethyl sulfonyl-fluoride (PMSF), and by proteinase inhibitors such as aprotinin and α_1-proteinase inhibitor. As shown in Fig. 4, the inhibition of pancreatic kallikrein by α_1-proteinase inhibitor was found to be somewhat stronger than that of urinary kallikrein. A similar results was obtained with both serum and plasma. None of the metal salts tested, such as 1 mM $CuCl_2$, $HgCl_2$, $ZnCl_2$, $CaCl_2$, $MgCl_2$, $CdCl_2$ and $BaCl_2$, affected the enzymes.

Immunological Study of Human Urinary and Pancreatic Kallikreins

In double immunodiffusion tests, anti-human urinary kallikrein antibody produces a single fused precipitin line against purified urinary and pancreatic kallikreins, and *vice versa*. Moreover, as shown in Fig. 5, on immunoelectrophoresis, anti-human urinary kallikrein antibody gave a single fused precipitin line against human urinary and pancreatic kallikreins, and *vice versa*. Furthermore, these antibodies gave a single protein precipitin line against

TABLE II. Effects of Various Reagents on the Activities of Human Urinary and Pancreatic Kallikreins

Reagent (10 mM)	Remaining activity (%)	
	Urinary kallikrein	Pancreatic kallikrein
None	100	100
o-Phenanthroline	97.3	95.2
EDTA	105	97.2
Monoiodoacetate	23.9	2.80
Cysteine	100	94.0
Dithiothreitol	97.6	93.6
DFP	79.6	65.6
PMSF	15.4	16.6
Aprotinin (1 mg/ml)[a]	2.10	3.80
SBTI (1 mg/ml)[a]	100	100
α_1-Proteinase inhibitor[a] (0.5 mg/ml)	22.3	25.7

The enzyme was incubated with each reagent in 0.1 M phosphate buffer (pH 8.0) at 37 °C for 30 min.
a) The enzyme was incubated with aprotinin, SBTI or α_1-proteinase inhibitor in the same buffer at 4 °C for 16 h.
DFP, diisopropyl fluorophosphate; PMSF, phenylmethyl sulfonylfluoride; SBTI, soybean trypsin inhibitor.

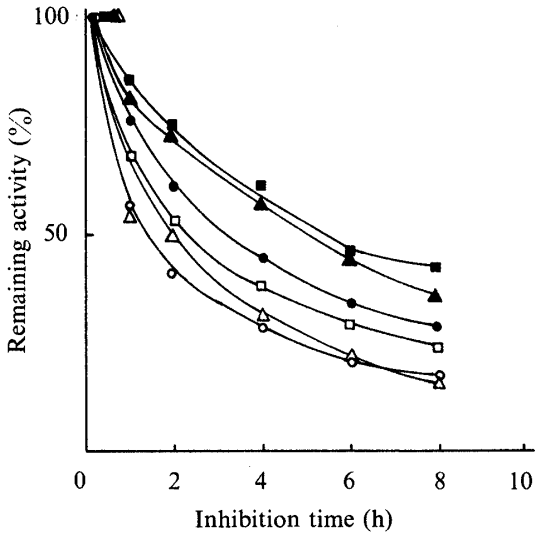

Fig. 4. Inhibition of Human Urinary and Pancreatic Kallikreins by Human α_1-Proteinase Inhibitor, Plasma and Serum

Kallikrein (50 mU) was incubated with α_1-proteinase inhibitor (0.25 mg), plasma or serum at 37 °C in a 0.35 ml system in 0.2 M Tris-HCl buffer (pH 7.8). Five μl of sample was taken from the reaction tube and enzyme activity was determined using Pro–Phe–Arg–MCA as a substrate.
●, urinary kallikrein-α_1-proteinase inhibitor; ▲, urinary kallikrein-plasma; ■, urinary kallikrein-serum; ○, pancreatic kallikrein-α_1-proteinase inhibitor; △, pancreatic kallikrein-plasma; □, pancreatic kallikrein-serum.

crude kallikrein samples. These results suggest that the two enzymes have common antigenic determinants, and are immunologically indistinguishable.

Discussion

In the present works, human urinary and pancreatic kallikreins were purified by immunoadsorbent column chromatography. Several investigations have already been reported on the purification of human glandular kallikreins by means of conventional column chromatographies, affinity chromatographies and immunoadsorbent column chromatographies. In general, the use of biospecific reactions and immunological reactions has proved to be a very useful means of purification of proteins and sugars. In the purification of kallikreins,

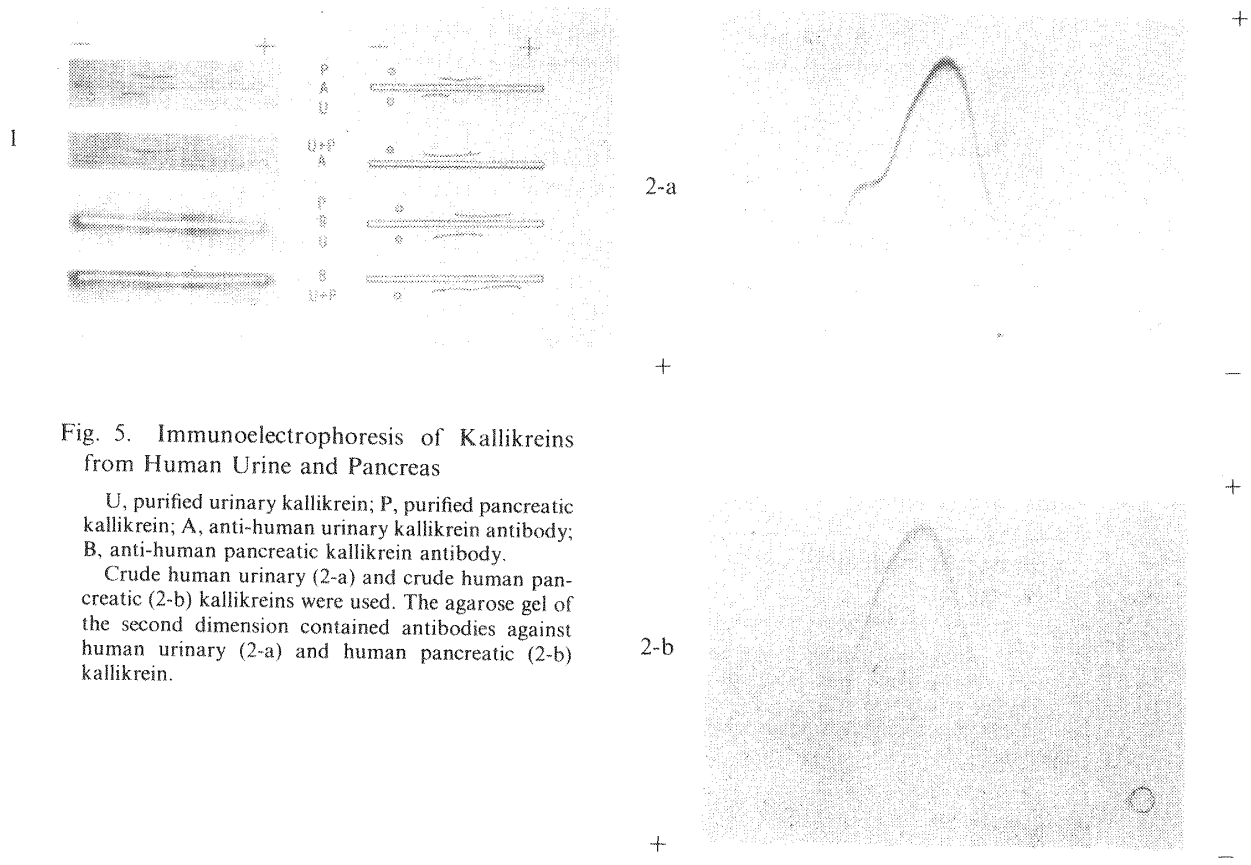

Fig. 5. Immunoelectrophoresis of Kallikreins from Human Urine and Pancreas

U, purified urinary kallikrein; P, purified pancreatic kallikrein; A, anti-human urinary kallikrein antibody; B, anti-human pancreatic kallikrein antibody.

Crude human urinary (2-a) and crude human pancreatic (2-b) kallikreins were used. The agarose gel of the second dimension contained antibodies against human urinary (2-a) and human pancreatic (2-b) kallikrein.

TABLE III. Effects of Various Reagents on the Recovery of Human Urinary Kallikrein from the Immunoadsorbent Column

Reagent	Recovery (%)
0.1 M Na_2CO_3 (pH 11.4)	87
0.2 M Na_2CO_3 (pH 11.4)	90
0.2 M Na_2CO_3 (pH 11.4) containing 0.5 M NaCl	98
0.1 M glycine–NaOH buffer (pH 11.0)	67
0.1 M glycine–NaOH buffer (pH 11.5)	83
0.1 M glycine–NaOH buffer (pH 12.0)	84
4 M guanidine–HCl (pH 8.0)	23
4 M guanidine–HCl (pH 8.0) containing 0.5% BSA	92
8 M urea (pH 8.0)	28

aprotinin affinity chromatography[33] and immunoadsorbent column chromatography[34–36] have been used, but there were problems regarding the recovery and the stability of the enzyme at the elution step.

In our preliminary studies, human urinary and pancreatic kallikreins were demonstrated to be stable in the range from pH 5 to 11, but unstable below pH 4.0. This finding led us to develop a new purification method for kallikreins. The elution solutions listed in Table III were investigated and the best result, from the viewpoints of recovery (98%) and avoidance of denaturation of kallikrein under acidic conditions, was obtained when 0.2 M Na_2CO_3 containing 0.5 M NaCl was used. At the immunoadsorption step, this new eluant permits the

effective purification of kallikreins.

When the immunoadsorbent column with anti-human urinary kallikrein antibody was used, no human pancreatic kallikrein activity was detected in the pass-through fractions and *vice versa*. This is in accordance with the immunological results, and suggests that human urinary and pancreatic kallikreins could be immunologically identical. These observations are also consistent with the report by Amouric and Figarella,[9] who found that kallikrein from human pancreatic juice had common antigenic determinants with urinary and submandibular kallikreins.

Urinary kallikreins, released from the kidney, show microheterogeneity[19] which may be due to the sugar moeity. However, it is not known whether the heterogeneity is due to tissue-specificity or arises at the release step from the tissue. Matsuda *et al.*[37] attempted to purify human renal kallikrein, but this question was not clarified. At least two kinds of kallikreins whose mobility on electrophoresis is different exist in porcine pancreas.[10-14] It is thought that heterogeneity may be due to their sugar content and composition, and similar conclusions were reached for kallikreins of other species.[38,39] It is interesting that glandular kallikreins from different tissues seem to have similar properties, and studies are needed to determine how their activities are controlled, and how modifications occur in specific tissues.

Human urinary and pancreatic kallikreins were strongly inhibited by serine protease inhibitors such as DFP and PMSF and also by aprotinin and α_1-proteinase inhibitor. Regarding the inhibition by α_1-proteinase inhibitor, pancreatic kallikrein released into the circulatory system is thought to be in an inactive form,[40] and kallikrein from the pancreas was more inhibited than that form urine. The kallikreins were also inhibited by monoiodoacetate, in accordance with Chao's report.[41] Serine and histidine residues may be involved in the catalytic and/or binding sites.

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