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PURIFICATION OF GUINEA-PIG PLASMA PREKALLIKREIN

ACTIVATION BY PREKALLIKREIN ACTIVATOR DERIVED FROM GUINEA-PIG SKIN

TETSURO YAMAMOTO [a], KIKUO KOZONO [a], TSUTOMU OKAMOTO [a], HISAO KATO [b],* and TAKESHI KAMBARA [a]

[a] *Department of Pathophysiology, Toxicology Institute, Kumamoto University Medical School, Kumamoto 860 and* [b] *Institute for Protein Research, Osaka University, Suita (Japan)*

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Summary

Prekallikrein was purified from guinea-pig plasma. The prekallikrein appeared homogeneous as a single-chain protein on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol. The apparent molecular weight was 82 000 by SDS-polyacrylamide gel electrophoresis, 99 000 by gel filtration on a Sephadex G-150 column and 84 500 (protein part) by amino acid analysis. The isoelectric point was approx. 9.0. The purification method yielded 3.8 mg (A_{280} 3.800) of prekallikrein from 500 ml of plasma.

Kallikrein was generated from the prekallikrein by limited proteolytic action of a prekallikrein activator which was derived from guinea-pig skin. From analysis using SDS-polyacrylamide gel electrophoresis, the kallikrein has two fragments with apparent molecular weights of 52 000 and 40 000 which are linked by disulfide bond(s). The 40 000 molecular weight fragment was shown to incorporate [^{3}H]diisopropylfluorophosphate. The kallikrein hydrolyzed the synthetic substrates containing the Phe-Arg sequence at the COOH-terminal, and it cleaved carbobenzyloxy-Phe-Arg-4-methylcoumaryl-7-amide more readily than Pro-Phe-Arg-methylcoumaryl-7-amide. The K_m for the kallikrein with carbobenzyloxy-Phe-Arg-methylcoumaryl amide was $2 \cdot 10^{-4}$ M. Also, the kallikrein showed negligible activities on peptide-methylcoumaryl amide-substrate for α-thrombin, Factor Xa or plasmin.

* Present address: Department of Biology, Faculty of Science, Kyushu University, Fukuoka, Japan.

Introduction

The presence of a protease-like permeability factor in the guinea-pig skin that existed in a latent form in its extract and pseudoglobulin fraction has been previously reported [1,2]. This latent permeability factor which was separated in the pseduoglobulin fraction has a molecular weight of 80 000, and was converted to an active form of molecular weight 35 000 by contact with kaolin at neutral pH [20]. This property resembles prekallikrein activators from human and guinea-pig plasma which were shown to be active forms of Hageman factor [3—5]. Moreover, in our recent experiments, a prekallikrein activation activity was observed in a fraction that included the skin permeability factor. It became essential to determine whether the permeability factor from skin directly activates plasma prekallikrein. Careful study of this question required purified plasma prekallikrein. In this manuscript, we report the purification and characterization of guinea-pig plasma prekallikrein, and its proteolytic activation by a prekallikrein activator derived from guinea-pig skin.

Recently, Iwanaga and his coworkers reported the development of fluorogenic substrates that have high specificity and sensitivity for some proteases including bovine and human kallikrein [6]. In our experiments, we measured the guinea-pig plasma kallikrein effectively using one of the fluorogenic substrates, carbobenzyloxyphenylalanylarginine 4-methylcoumaryl-7-amide, that was reported to be specific substrate for bovine plasma kallikrein.

Materials and Methods

Animals. Albino-Hartley strain guinea-pigs of both sexes (300—700 g body wt.) were used.

Plasma. Blood was collected from large (500—700 g) guinea-pigs of mixed breed by cardiac puncture, using 21-gauge needles and 10-ml polyethylene syringes, and mixing 9 parts blood to 1 part anticoagulant (Na_2EDTA (1.5%), glucose (5%) and polybrene (0.05%)). The plasma obtained by centrifugation at 3000 rev./min for 20 min was kept frozen at −80° C until use.

Substances. Diisopropylfluorophosphate, soybean trypsin inhibitor, ovalbumin, Blue Dextran 2000, myoglobin and cytochrome *c* were obtained from Sigma, St. Louis, U.S.A. Trasylol was a gift from Bayer, Osaka, Japan. Carbobenzyloxyphenylalanylarginine 4-methylcoumaryl-7-amide (z-Phe-Arg-methylcoumaryl amide), prolylphenylalanylarginine 4-methylcoumaryl-7-amide (Pro-Phe-Arg-methylcoumaryl amide), *t*-butyloxycarbonylglutamyllysyllysine 4-methylcoumaryl-7-amide (Boc-Glu-Lys-Lys-methylcoumaryl amide), *t*-butyloxycarbonylvalylleucyllysine 4-methylcoumaryl-7-amide (Boc-Val-Leu-Lys-methylcoumaryl amide), *t*-butyloxycarbonylisoleucylglutamylglycylarginie 4-methylcoumaryl-7-amide (Boc-Ile-Glu-Gly-Arg-methylcoumaryl amide), carbobenzyloxyleucylglycylarginine 4-methylcoumaryl-7-amide (z-Leu-Gly-Arg-methylcoumaryl amide) and leupeptine were purchased from Protein Research Foundation, Osaka, Japan. DEAE-Sephadex A-50, CM-Sephadex C-50, Sephadex G-75 and Sephadex G-150 were obtained from Pharmacia Co., Uppsala, Sweden. DEAE-cellulose (DE-52) was obtained from Whatman Bio-

chemicals, U.K. Carrier ampholites were products of LKB-Amikemi, Bromma, Sweden. All other chemicals were obtained from Wako Pure Chemicals, Osaka and from Nakarai Chemicals, Tokyo, Japan.

Protein concentration. Protein concentration of the prekallikrein fractions was determined from the absorbance at 280 nm (A_{280}) assumed an $E^{1\%}_{280}$ of 10. The measurement of A_{280} was made by subtracting the absorbance of benzamidine when it was needed.

Assay of prekallikrein. Prekallikrein activity was determined after activation with the prekallikrein activator prepared from guinea-pig skin as described below. Unless stated otherwise, 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and bovine serum albumin (0.1 mg/ml) was used for assay buffer. 5 μl of proenzyme source were mixed into 470 μl of the assay buffer and incubated with 10 μl of the prekallikrein activator (Sephadex G-75 fraction, A_{280} 0.030) for 30 min at 37°C. Prekallikrein in the mixture (from 0.1 to 20 munits) was maximally activated within 20 min and the maximum activity was retained for at least 60 min. The prekallikrein activity thus induced was measured by its amidolytic activity on plasma kallikrein substrate, z-Phe-Arg-methylcoumaryl amide, as previously described [6]. The final concentration of the peptide-methylcoumaryl amide-substrate used was usually 100 μM. It was adjusted so that 100 μM of substrate had an A_{320} value of 1.600, using a deuterium lamp. For estimating the elution position of prekallikrein from the several column chromatographies during purification, the end-point assay at 37°C was used. At 5 min after adding 10-μl of the kallikrein substrate, the reaction was stopped by addition of 5-μl of 5% acetic acid. The amount of 7-amino-4-methylcoumarin released by the amidolytic activity was fluorimetrically measured using a fluorescence spectrophotometer with excitation at 380 nm and emission at 440 nm. In other experiments, kallikrein activity was measured by an initial velocity assay at 37°C. The kallikrein concentration used in this assay method gave linear amidolytic rate for at least 10 min. 1 unit of z-Phe-Arg-methylcoumaryl amide hydrolytic activity was defined as the amount of enzyme which released 1 μmol of aminomethylcoumarin per min at 37°C, pH 8.0, at a substrate concentration of 100 μM. Specific activity was expressed as z-Phe-Arg-methylcoumaryl amide amidolytic units per mg of protein (A_{280} of 1.0). A linear relationship between amidolytic activity and prekallikrein concentration was observed from 0.1 to 2.5 munits.

For the amidolytic assay method, small amounts of bovine serum albumin were required and buffers containing bovine serum albumin from 0.1 to 1 mg/ml were used for the experiments.

Preparation of prekallikrein activator. Prekallikrein activator was purified from guinea-pig skin using the same methods for the protease-like permeability factor purification. Details are given in Ref. 20. The prekallikrein activator was precipitated with pseudoglobulin fraction (30—50% ammonium sulfate saturation) from skin extract, and was adsorbed on and released from kaolin particles. Further purification was performed using DEAE-cellulose and Sephadex G-75 column chromatography in this order. A pool of prekallikrein activator-rich fractions from the Sephadex column was usually used for kallikrein generation. For SDS-polyacrylamide gel electrophoretic analysis of kallikrein generation, prekallikrein activator was further purified using isoelectric

focusing and Sephadex G-50.

Purification of plasma prekallikrein. All procedures were performed at 0—4° C employing plastic or siliconized glass containers.

Step 1: DEAE-Sephadex chromatography. 500 ml of guinea-pig plasma were dialyzed against 20 mM Tris-HCl buffer containing 40 mM NaCl 3 mM EDTA, 50 mg/ml polybrene and 3.3 mM benzamidine (pH 8.0) for 15 h. The dialyzed sample was applied to a DEAE-Sephadex A-50 column (5 × 25 cm, bed volume 500 ml) equilibrated with the same buffer. Prekallikrein was eluted entirely in the precursor form in the break-through fraction.

Step 2: DEAE-Sephadex rechromatography. The prekallikrein-rich fractions were pooled and diisopropylfluorophosphate was added to achieve a final concentration of 1 mM. After 5 h, it was passed through the second DEAE-Sephadex A-50 colum (4 × 21 cm, bed volume 270 ml) with the same conditions as the first chromatography. Diisopropylfluorophosphate was added to the pooled prekallikrein fraction to a final concentration of 1 mM.

Step 3: CM-Sephadex chromatography. The prekallikrein-rich fraction of the second DEAE-Sephadex column was dialyzed against 50 mM acetate buffer containing 80 mM NaCl, 50 mg/ml polybrene and 3.3 mM benzamidine (pH 6.0) for 15 h. The sample was then applied to a CM-Sephadex C-50 column (4 × 5 cm, bed volume 60 ml) equilibrated with the acetate buffer containing 100 mM NaCl. The elution was accomplished by a linear NaCl gradient (500 ml of equilibration buffer and 500 ml of the acetate buffer containing 700 mM NaCl). Elution speed was approx. 200 ml/h. The prekallikrein-rich fractions appeared in the latter half of the gradient elution (Fig. 1) and were pooled. Diisopropylfluorophosphate was immediately added to achieve a final concentration of 1 mM. After stirring for 12 h, this was concentrated 20-fold with a Diaflo membrane (UM-10, Amicon) to 25 ml.

Step 4: Isoelectric focusing. Half of the concentrated prekallikrein fraction

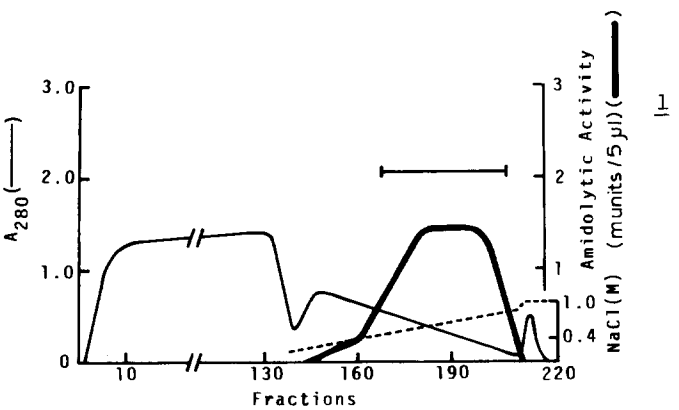

Fig. 1. CM-Sephadex C-50 column chromatography of the prekallikrein pool from the second DEAE-Sephadex column chromatography. A linear NaCl gradient elution from 100 to 700 mM (with 500 ml in each reservoir) was used. The fine line (———) denotes the protein concentrations (A_{280}). The broken line (- - - - -) denotes the NaCl concentration. The thick line (═══) denotes the z-Phe-Arg-methylcoumaryl amide amidolytic activity of 5 µl of each fraction (fraction volume 10.5 ml). Horizontal bar represents pooled fraction.

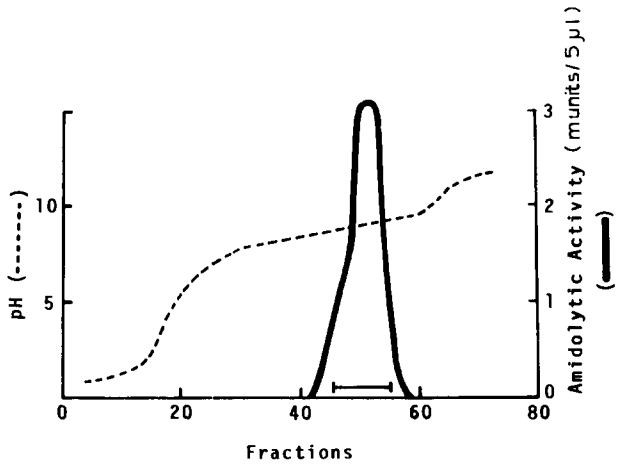

Fig. 2. Isoelectric focusing column of prekallikrein pool from CM-Sephadex column chromatography. The broken line (- - - - - -) denotes the pH gradient. The thick line (———) denotes the potential amidolytic activity on z-Phe-Arg-methylcoumaryl amide of 5 μl of each fraction (fraction volume 1.5 ml). Horizontal bar represents pooled fraction (from pH 8.5 to 9.5).

was dialyzed against 20 mM phosphate buffer containing 50 mM NaCl and 3.3 mM benzamidine (pH 7.4) for 6 h, and was applied into an isoelectric focusing column (110 ml volume, Kato-shoten, Osaka, Japan). Focusing was performed using carrier ampholite (final concentration 1%, mixture of pH 3.5—10 (1 part) and pH 9—11 (4 parts)) and a sucrose gradient. After focusing for 40 h at 500 V, 1.5-ml serial fractions were collected from the bottom of the column. The pH of each fraction was measured at 4° C. As shown in Fig. 2, the prekallikrein was concentrated in the fractions from pH 8.5 to 9.5. The prekallikrein-rich fraction were pooled, centrifuged to discard a small amount of insoluble material and diisopropylfluorophosphate was added to a final concentration of 1 mM. It was passed through a Sephadex G-50 column (4 × 16 cm, bed volume 200 ml) equilibrated with 20 mM Tris-HCl buffer containing 1 M NaCl (pH 7.0) to remove the ampholines. The prekallikrein-containing fractions were pooled, benzamidine added to 3.3 mM and concentrated with Diaflo to 7 ml. The remaining half of the prekallikrein fraction from step 3 was also treated in the same way.

Step 5: Sephadex G-150 column chromatography. 14-ml of the concentrated prekallikrein solution from step 4 were put on a Sephadex G-150 column (5 × 130 cm, bed volume 2500 ml). The equilibration and elution buffer was 20 mM Tris-HCl buffer containing 1 M NaCl (pH 7.0). As shown in Fig. 3A, the prekallikrein was eluted in the included volume and corresponded with the third protein peak. The prekallikrein-rich fractions were pooled, diisopropylfluorophosphate and benzamidine added (final concentration 1 and 3.3 mM, respectively) and concentrated with the Diaflo. This fraction was usually used for assay of prekallikrein activator from skin.

Step 6: Sephadex G-150 rechromatography. 14-ml of the prekallikrein solution thus obtained were subjected to gel filtration, again through the same column of Sephadex G-150 as used above. As shown in Fig. 3B, a small amount of contaminating protein was removed, and prekallikrein activity (measured as

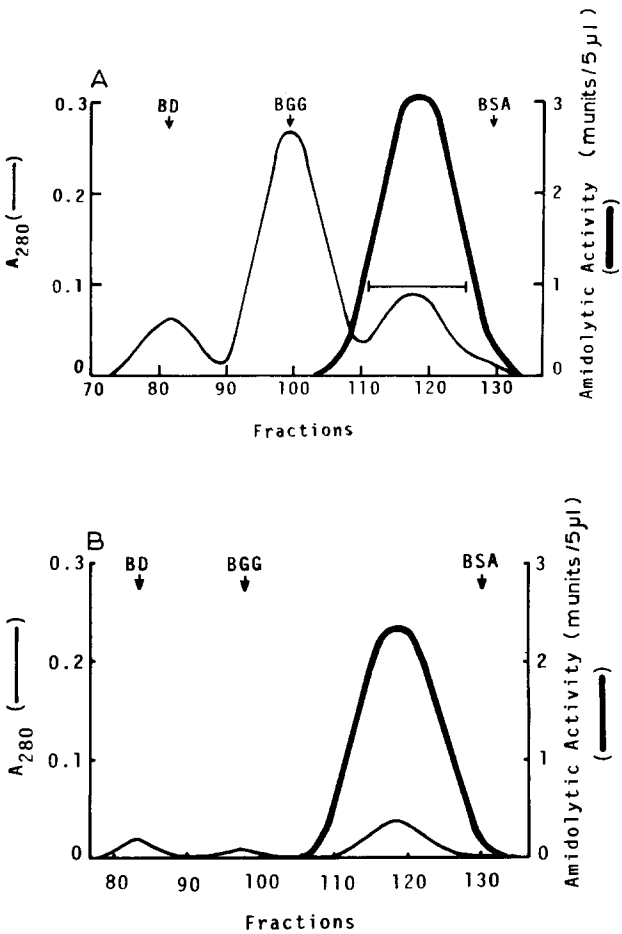

Fig. 3. Purification of prekallikrein using Sephadex G-150 column chromatography. The column size was 5 × 130 cm (bed volume 2500 ml) and fraction volume was 9.5 ml. 20 mM Tris-HCl buffer (pH 7.0) containing 1 M NaCl was used as elution buffer. The fine line (——) denotes the protein concentration (A_{280}). The thick line (⟶) denotes the potential amidolytic activity on z-Phe-Arg-methylcoumaryl amide of 5 µl of each fraction. (A) First Sephadex G-150 column chromatography of prekallikrein pool from the isoelectric focusing. Horizontal bar represents pooled fraction. (B) Second Sephadex G-150 column chromatography of prekallikrein pool from the first Sephadex G-150 column chromatography. For the reference to estimate the molecular weight of prekallikrein, Blue Dextran 2000 (BD) M_r 2 000 000 bovine gamma globulin (BGG) M_r 150 000, bovine serum albumin (BSA) M_r 67 000, ovalbumin M_r 45 000 and cytochrome *c* M_r 12 800 were used.

potential z-Phe-Arg-methylcoumaryl amide hydrolytic activity) completely paralleled the elution profile of the major peak of protein.

Polyacrylamide disc gel electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed by using the method of Weber et al. [7] using an 8% gel. Each sample was mixed with 20 µl of 1% SDS and 8 M urea and was heated for 5 min at 80° C with or without β-mercaptoethanol. For analysis of prekallikrein, the pooled and concentrated fraction from the second Sephadex G-150 column was used (A_{280} 0.440). After a brief dialysis against 20 mM Tris-HCl buffer (pH 7.0), 30 µl of the sample were applied to

each SDS gel. Analysis of kallikrein generated by prekallikrein activator from skin was performed as followed. The prekallikrein was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) for 5 h. 200 μl of the sample or 200 μl of the buffer (for negative control) were mixed with 20 μl of prekallikrein activator from skin (Sephadex G-50 fraction after isoelectric focusing, A_{280} 0.050) and with 20 μl of bovine serum albumin in the same buffer (3 mg/ml). The mixtures were incubated for 60 min at 37° C. Small amounts of DEAE-cellulose paste previously equilibrated in the same buffer were added to the incubation mixtures to remove the bovine serum albumin and skin prekallikrein activator. After centrifugation, 50 μl of each the supernatants were used as the sample for SDS-disc gel electrophoresis. For experiments using [^{3}H]diisopropylfluorophosphate, 200 μl of the sample prepared as described above were treated for 30 min at 37° C with 10-μl of [^{3}H]diisopropylfluorophosphate (1 mCi/200 μl, The Radiochemical Centre, Amersham, U.K.). Unreacted [^{3}H]diisopropylfluorophosphate was removed by exhaustive dialysis against 20 mM sodium phosphate buffer (pH 6.0) at 4° C, and then against 20 mM sodium phosphate buffer containing 1% SDS and 8% urea (pH 6.0) at room temperature. The SDS gels were sliced at a thickness of 1 mm and then each slice was incubated with 100-μl of 30% H_2O_2 at room temperature for 16 h. The radioactivity of 50 μl of each sample was counted in 10 ml of toluene scintillation fluid by a Packard Tri-Carb scintillation spectrometer (Model 3385).

Amino acid analysis of prekallikrein. 50-μg samples were hydrolyzed in vacuo with 5.7 N HCl at 110° C for 24, 48 and 72 h, and analyzed on a Hitachi KLA-5A amino acid analyzer. The values of threonine and serine were determined by extrapolation to zero hydrolysis time. Isoleucine and valine values were calculated from the 72-h hydrolysis time. Tryptophan and tyrosine were estimated by using the method of Matsubara and Sakaki [8], and calculated according to the method of Holmquist and Vallee [9]. Half-cysteine was determined as cysteic acid by using the method of Holmquist and Vallee [9].

Kinin-releasing activity of the plasma kallikrein. Kininogen was partially purified from guinea-pig plasma using DEAE-Sephadex followed by lysine-Sepharose column chromatography. Kinin liberated from the kininogen was detected by bioassay for its vascular permeability-enhancing activity. The prekallikrein, prekallikrein activator and kininogen were mixed together in 20 mM Tris-HCl buffer (pH 8.0), and incubated for 30 or 60 min at 37° C. Next, soybean trypsin inhibitor was added to the incubation mixture to a final concentration of $2 \cdot 10^{-4}$ M. This incubation was continued briefly to block completely the permeability-increasing activity attributed to the proteases in the solution. Then the samples were assayed for the vascular permeability-enhancement activity by using the method described in our previous paper [1].

Studies of enzymatic properties. The first Sephadex G-150 fraction was used a prekallikrein source. The Sephadex G-75 fraction was used a prekallikrein activator source. Studies of substrate specificities were performed using seven kinds of synthetic peptide-methylcoumaryl amide-substrates. Kallikrein was generated by prekallikrein activator as described above. Each peptide-methylcoumaryl amide-substrate was added to the kallikrein solution which still contained a small amount of prekallikrein activator. Solutions containing the same amount of the prekallikrein activator were also treated. The final concen-

tration of each substrate was 100 μM, and the initial velocities of aminomethylcoumarin release were measured. Linearity of these reactions was observed for at least 5 min. Amidolytic activities attributed to the kallikrein were calculated by subtracting the activities of kallikrein activator from the total. To study the pH dependency of kallikrein activity, a higher concentration of kallikrein was prepared in 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and bovine serum albumin (0.1 mg/ml). 60 μl of the kallikrein solution and 10 μl of z-Phe-Arg-methylcoumaryl amide were mixed with 430 μl of assay buffer of various pH values (200 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 6), 200 mM Tris-HCl buffer (pH 7, 8 and 9), $NaHCO_3$-NaOH buffer (pH 10), all containing 0.3 mg/ml bovine serum albumin). The pH of the reaction mixture was measured again at 37° C.

For kinetic studies on z-Phe-Arg-methylcoumaryl amide, kallikrein was generated in the usual manner. Initial velocities for various substrate concentrations from 17 to 150 μM final concentration were measured at pH 8.0, 37° C. The kallikrein concentration in the reaction mixtures was 0.5 munits/500 μl. Data were plotted by using the method of Lineweaver and Burk [10] and K_m was determined.

Studies of kallikrein susceptibility to various trypsin inhibitors were performed as followed. Kallikrein was generated in the usual buffer (pH 8) containing 0.3 mg/ml bovine serum albumin. z-Phe-Arg-methylcoumaryl amide was used as its substrate. The amidolytic activity on the substrate attributed to prekallikrein activator in the kallikrein solution was 3% of the total activity. The trypsin inhibitors were dissolved in the same buffer without bovine serum albumin. The mixture of enzyme and inhibitor was preincubated for 20 min at 37° C before the initial velocities of their amidolytic activities were measured.

Heat stability of the prekallikrein. The thermal stability of the purified prekallikrein (second Sephadex G-150 fraction) was examined by heating in 200 mM Tris-HCl buffer containing 0.1 mg/ml bovine serum albumin (pH 8.0), for 10 min at various temperatures. After this treatment, the remaining potential amidolytic activity of the prekallikrein on z-Phe-Arg-methylcoumaryl amide was measured.

Results

Preparation of guinea-pig plasma prekallikrein and some properties of the purified material

Purification of prekallikrein. The purification steps and the yield for a typical preparation of guinea-pig plasma prekallikrein are shown in Table I. Detailed descriptions of each step are given in Materials and Methods. The first DEAE-Sephadex column was used to separate almost all of the prekallikrein in the gamma globulin fraction from the bulk of plasma protein. Trace amounts of prekallikrein activator still present in the gamma globulin fraction in some preparations would be almost completely removed by the second DEAE-Sephadex column chromatography. Elution of prekallikrein from the CM-Sephadex column was usually complete within 5 h. In the isoelectric focusing step, the prekallikrein was concentrated in the fractions at about pH 9.0. In some preparations, during the isoelectric focusing step approx. 1/30 of the

TABLE I

PURIFICATION OF GUINEA-PIG PLASMA PREKALLIKREIN

	Volume (ml)	Total A_{280}	Recovery of A_{280} (%)	Total units amidolysis (units)	Specific activity units/ A_{280}	Recovery of activity (%)
1st DEAE-Sephadex	840	1599.36	100	296.5	0.19	100
2nd DEAE-Sephadex	855	1460.34	91.3	290.5	0.20	98.0
CM-Sephadex	625	52.82	3.3	252.0	4.77	85.0
Isoelectric focusing	140	45.50	2.8	154.0	3.39	52.0
1st Sephadex G-150	162	10.34	0.65	143.5	13.88	48.5
2nd Sephadex G-150	143	3.84	0.24	54.0	14.06	18.1

amount of prekallikrein was activated to kallikrein. Because the active kallikrein was concentrated in the fractions at approx. pH 8.5, this showed that the kallikrein molecule was separated from its precursor form. As shown in Table I, the specific activity of prekallikrein was not increased by the isoelectric

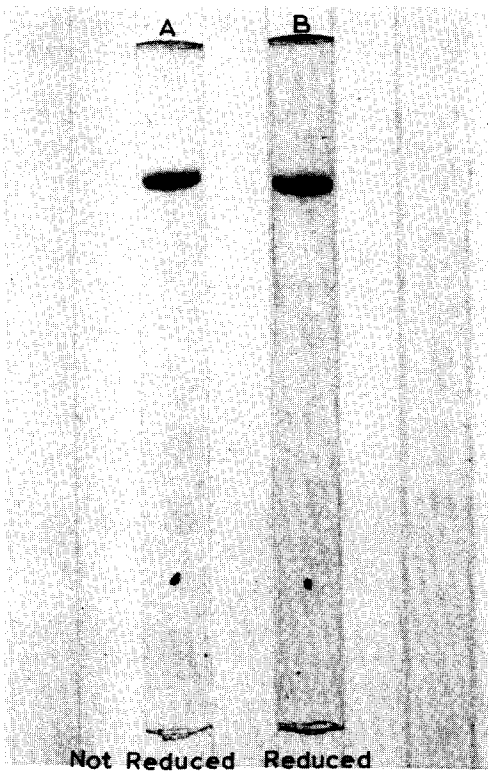

Fig. 4. Guinea-pig prekallikrein on 8% SDS-polyacrylamide gels. Gel A was run in the absence of reducing agent and gel B in its presence (final 10% β-mercaptoethanol). Gels contained 10 μg/30 μl of prekallikrein from the second Sephadex G-150 column chromatography and 20 μl of 1% SDS and 8 M urea. Protein staining was carried out with Coomassie brilliant blue R-250.

focusing step; however, this step was necessary for the purification procedure, because if this step was omitted, one other protein, which behaved very similarly in SDS-polyacrylamide gel electrophoresis, would contaminate the final prekallikrein preparation.

In the following step, Sephadex G-150 column chromatography was very effective for the removal of IgG. The elution buffer, 20 mM Tris-HCl buffer containing 1 M NaCl (pH 7.0) was also suitable for storage buffer. The yield of purified protein was about 3.8 mg (A_{280} 3.800) from 500 ml of guinea-pig plasma, and specific amidolytic activity of 14 units per mg (A_{280} 1.000) was observed.

Polyacrylamide gel electrophoresis of purified prekallikrein. As shown in Fig. 4, a single protein band was observed in the SDS-polyacrylamide gels in the absence or in the presence of reducing agent. Thus, guinea-pig prekallikrein appeared to be a single polypeptide chain.

Determination of the molecular weight. On the basis of its elution position from the first Sephadex G-150 column, the molecular weight of prekallikrein was estimated to be 99 000. It was measured at 92 000 by SDS-polyacrylamide gel electrophoresis without a reducing agent or 82 000 with a reducing agent. From amino acid analysis, the molecular weight attributed to protein was estimated to be 84 539.

Amino acid composition of prekallikrein. The amino acid composition of the guinea-pig plasma prekallikrein is shown in Table II. Total amino acids were estimated to be 753.

Limited proteolysis as the mechanism of prekallikrein activation by a prekallikrein activator from skin. The generation of kallikrein by a prekallikrein

TABLE II

AMINO ACID COMPOSITION OF GUINEA-PIG PREKALLIKREIN

Amino acids	Percent	Nearest integral *
Trp	0.290	2
Lys	6.766	51
His	3.837	29
Arg	4.551	34
Asx	8.574	65
Thr	8.224	62
Ser	8.612	65
Glx	11.218	84
Pro	2.483	19
Gly	8.648	65
Ala	5.209	39
Cys	2.242	17
Val	6.653	50
Met	1.760	13
Ile	4.825	36
Leu	6.990	53
Tyr	3.403	26
Phe	5.717	43
Total residues	100.002	753
Protein M_r	84 539	

* Nearest integral number of residues per molecule (a molecular weight of 82 000 is assumed by SDS-polyacrylamide gel electrophoresis).

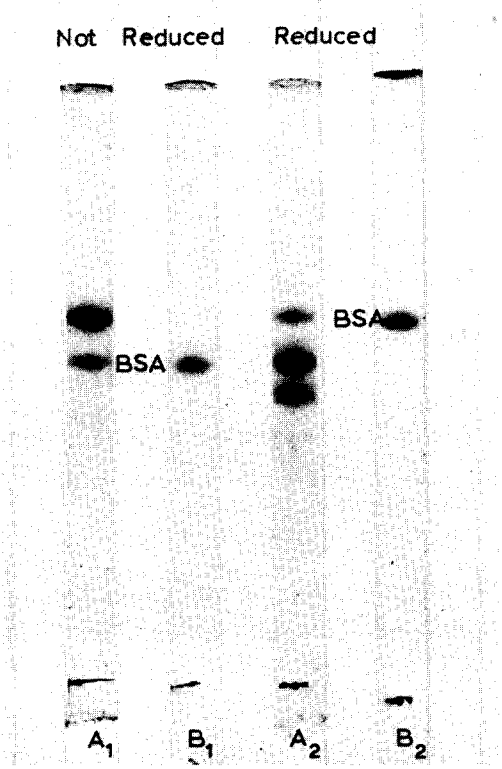

Fig. 5. Kallikrein on 8% SDS-polyacrylamide gels. Prekallikrein activation using prekallikrein activator from skin was performed with bovine serum albumin. After activation most of the prekallikrein activator and bovine serum albumin were removed by DEAE-cellulose (see text for experimental details). As control, a sample containing the prekallikrein activator and bovine serum albumin (without prekallikrein) was prepared in the same way and run in gels. A_1, non-reduced kallikrein; B_1, non-reduced control; A_2, reduced kallikrein; B_2, reduced control.

activator derived from skin was performed in Tris-HCl buffer (pH 8.0) containing bovine serum albumin for 60 min at 37°C, under these conditions the complete activation of the prekallikrein was observed functionally. Analysis on SDS-polyacrylamide gels in the presence of reducing agent showed that the prekallikrein band was cleaved into two new bands with apparent molecular weights of 52 000 and 40 000. In the absence of reducing agent, the activated kallikrein gave one band with an apparent molecular weight identical to that of the prekallikrein protein band. This indicates that the prekallikrein is proteolytically cleaved during activation by the prekallikrein activator and that the resultant polypeptide fragments of kallikrein are linked by a disulfide bond (Fig. 5). The kallikrein was found to incorporate [^{3}H]diisopropylfluorophosphate, and after reduction the [^{3}H]diisopropylfluorophosphate was localized within the 40 000 molecular weight polypeptide chain of kallikrein (Fig. 6).

Kinin-releasing ability of kallikrein from guinea-pig plasma. The kinin released from guinea-pig kininogen by purified and activated kallikrein was estimated by the enhancement of local vascular permeability as described above. As shown in Table III, the enhancement of permeability unsusceptible

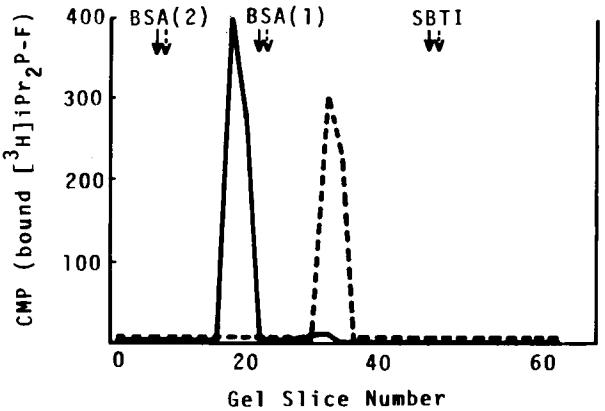

Fig. 6. SDS-polyacrylamide gel electrophoresis of [^{3}H]diisopropylfluorophosphate ([^{3}H]iPr$_2$P-F) labelled kallikrein. The thick line (———) denotes non-reduced kallikrein. The broken line (- - - - - -) denotes reduced kallikrein. BSA(1), BSA(2), bovine serum albumin (monomer, M_r 67 000) and (dimer, M_r 134 000), respectively; SBTI, soybean trypsin inhibitor (M_r 21 000).

to soybean trypsin inhibitor was significantly increased in the incubation time. Furthermore, the permeability-enhancing activity was completely lost by dialysis overnight at 4° C.

Enzymatic properties of kallikrein

(1) Substrate specificities on various synthetic peptide-methylcoumaryl amide-substrates. As shown in Table IV, the guinea-pig plasma kallikrein hydrolyzed the substrates containing the Phe-Arg sequence at the COOH-terminal, and it cleaved z-Phe-Arg-methylcoumaryl amide more readily than Pro-Phe-Arg-methylcoumaryl amide. Only negligible activities were observed on substrates for α-thrombin (Boc-Val-Pro-Arg-methylcoumaryl amide), Factor Xa (Boc-Ile-Glu-Gly-Arg-methylcoumaryl amide) or plasmin (Boc-Val-Leu-Lys-methylcoumaryl amide, Boc-Glu-Lys-Lys-methylcoumaryl amide).

TABLE III

KININ-GENERATING ABILITY OF GUINEA-PIG PLASMA KALLIKREIN

0.1 ml of each mixture was injected intracutaneously on the clipped flank of guine-pig immediately after giving Evans blue intravenously. The extravascular dye of each blued lesion was extracted in hot formamide, estimated spectrophotometrically, and expressed in micrograms by means of a standard calibration curve. The calculation was made by subtracting buffer background. Procedures 2—4 were prepared as control. Prekallikrein, 10 μl of second Sephadex G-150 pool. PKA (prekallikrein activator from skin), 10 μl, A_{280} 0.05. Kininogen, 50 μl of lysine-Sepharose pool. Expt. 1, A_{280} 0.48; Expt. 2, A_{280} 0.99. SBTI (soybean trypsin inhibitor), 30 μl of $5 \cdot 10^{-3}$ M. Buffer, 20 mM Tris-HCl (pH 8.0). Total volume of mixture, 330 μl. Incubation (→) at 37°C: for Expt. 1, 60 min; Expt. 2, 30 min.

Procedure	Extravascular dye (μg)	
	Expt. 1	Expt. 2
(1) Prekallikrein + PKA + kininogen →+ SBTI	9.8	20.6
(2) Prekallikrein + PKA + kininogen + SBTI →	1.8	1.5
(3) PKA + kininogen →+ SBTI	2,2	14.2
(4) Kininogen →+ SBTI	0.4	3.1

TABLE IV

SUBSTRATE SPECIFICITY STUDIES OF PLASMA KALLIKREIN AND PREKALLIKREIN ACTIVATOR FROM SKIN USING VARIOUS PEPTIDE-METHYLCOUMARYL AMIDE-SUBSTRATES

The activities are expressed as velocity of aminomethylcoumarin release (nM/min). MCA, methylcoumaryl amide; AMC, aminomethylcoumarin. PKA, prekallikrein activator (1) Prekallikrein (first Sephadex G-150 pool) dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 0.1 mg/ml bovine serum albumin was activated with prekallikrein activator from skin for 30 min at 37°C. 10 μl of various peptide-methylcoumaryl amide-substrates (final concentration 100 μM) were added to 490 μl of the mixture containing kallikrein and prekallikrein activator and initial velocity was measured. (2) Prekallikrein activator containing pool alone was dissolved in the buffer and the experiment was carried out as described in 1. The amount of prekallikrein activator was 10 μl of A_{280} 0.03 per 500 μl of assay mixture. (3) Balance of activity (1—2) was referenced to that of plasma kallikrein. The kallikrein used in these experiments corresponded to 0.155 munits.

Substrate	Total activity (1)	PKA skin (2)	Kallikrein (3)
z-Phe-Arg-MCA (Plasma kallikrein)	316	6	310
Pro-Phe-Arg-MCA (Gland kallikrein)	169	61	108
Boc-Ile-Glu-Gly-Arg-MCA (Factor Xa)	20	10	10
Boc-Val-Pro-Arg-MCA (α-thrombin)	42	26	16
z-Leu-Gly-Arg-MCA (Horseshoe crab protease)	62	23	39
Boc-Val-Leu-Lys-MCA (Plasmin)	12	0	12
Boc-Glu-Lys-Lys-MCA (Plasmin)	24	2	22

TABLE V

SUSCEPTIBILITY OF GUINEA-PIG PLASMA KALLIKREIN TO VARIOUS TRYPSIN INHIBITORS

Prekallikrein (first Sephadex G-150 pool) dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and 0.3 mg/ml bovine serum albumin was activated with prekallikrein activator from skin for 30 min at 37°C. Then, aliquots (480-μl) of the kallikrein solution were mixed with 500 μl of inhibitor solution and were incubated for a further 20 min. Then, 20 μl of z-Phe-Arg-methylcoumaryl amide solution (final concentration 100 μM) were added and initial velocity was measured. 100% activity was 0.05 munits of amidolytic activity. 3% of the activity was attributed to prekallikrein activator-containing fraction.

Treatment (final concentration)	Remaining activity (%)
Buffer	100
Diisopropylfluorophosphate	
$1 \cdot 10^{-3}$ M	2
$1 \cdot 10^{-4}$ M	10
Trasylol	
500 units	1
50 units	5
5 units	28
Leupeptine	
$1 \cdot 10^{-4}$ M	0
$1 \cdot 10^{-5}$ M	9
Soybean trypsin inhibitor	
$1 \cdot 10^{-4}$ M	0
$1 \cdot 10^{-5}$ M	4
$1 \cdot 10^{-6}$ M	10
Limabean trypsin inhibitor	
$1 \cdot 10^{-4}$ M	38
Ovomucoid trypsin inhibitor	
$1 \cdot 10^{-4}$ M	76
Benzamidine	
$1 \cdot 10^{-3}$ M	25
$1 \cdot 10^{-4}$ M	76

(2) Effect of pH. The optimum pH for the amidolytic action on z-Phe-Arg-methylcoumaryl amide was at about pH 8.

(3) Kinetics of activity on z-Phe-Arg-methylcoumaryl amide at pH 8. The K_m value for kallikrein with z-Phe-Arg-methylcoumaryl amide was determined to be $2 \cdot 10^{-4}$ M according to a Lineweaver-Burk plot. In these experiments the kallikrein concentration was 0.5 munits/500 µl. This gave 1 µM aminomethylcoumarin release per min in 100 µM substrate concentration, but was calculated to give 3.03 µM aminomethylcoumarin release per min at maximum velocity.

(4) Susceptibility to trypsin inhibitors. As shown in Table V, the guinea-pig plasma kallikrein was strongly inhibited by diisopropylfluorophosphate, Trasylol, leupeptin and soybean trypsin inhibitor. It was also susceptible to benzamidine and lima bean trypsin inhibitor but had less susceptibility to ovomucoid trypsin inhibitor.

Heat stability of the prekallikrein. The prekallikrein was stable at 50°C; however, loss of potency began above 60°C (30% of activity lost) and complete inactivation was observed in 10 min at 80°C and pH 8.0.

Discussion

The purification of bivine [11,12], human [13—17] and rabbit [18,19] plasma prekallikrein has been reported. The guinea-pig prekallikrein described in the present paper was homogeneous as a single chain protein in SDS-polyacrylamide gel electrophoresis (Fig. 4), as was observed in bovine [11] and rabbit prekallikrein [19]. Two different molecular forms of purified human prekallikrein were observed as two different bands on SDS gels (molecular weights 88 000 and 85 000) [15,17]. The apparent molecular weight of guinea-pig prekallikrein (82 000 by SDS-polyacrylamide gel electrophoresis) is slightly smaller than other prekallikreins (from 85 000 to 100 000 by SDS-polyacrylamide gel electrophoresis) [11—19]. The apparent isoelectric point of the guinea-pig prekallikrein was 9.0 (Fig. 2), which is very different from that of the bovine prekallikrein molecule (pH 6.98) [11] or the rabbit prekallikrein molecule (pH 5.9) [18]. The behavior of guinea-pig prekallikrein in DEAE-Sephadex column chromatography is also different from that of the bovine [11] or rabbit prekallikrein molecule [18] but similar to that of the human prekallikrein molecule [17]. However, amino acid analysis (Table II) of the guinea-pig prekallikrein was similar to that of rabbit [19] and human prekallikrein molecules [17].

Kallikrein was generated from the prekallikrein by limited proteolytic action of the prekallikrein activator from skin. It had a similar molecular weight to its zymogen form but was a two-chain peptide having chains of 52 000 and 40 000 daltons. The smaller fragment contained the catalytic center (Fig. 6). This generation pattern is very similar to activation of human or rabbit prekallikrein by active Hageman factor [15,17,19]. It will be important to determine the relationship between the prekallikrein activator from skin and plasma Hageman factor.

In the experiments described in this paper, the fluorogenic substrate, z-Phe-Arg-methylcoumaryl amide, was found to be useful for enzymatic studies of

guinea-pig plasma kallikrein as well as for purification of the prekallikrein. In this assay a linear time-dependent curve of kallikrein amidolytic activity on z-Phe-Arg-methylcoumaryl amide was observed as well as a linear relationship between prekallikrein content and the potential amidolytic activity. As the kinetic study shows, determination of kallikrein amidolytic units was performed at a substrate concentration of 100 μM, which is less than half of the K_m value ($2 \cdot 10^{-4}$ M), due to the poor solubility of thus substrate. Also, for the assay procedure, small amounts of bovine serum albumin were required, the role of which is still unknown. The susceptibility of the guinea-pig kallikrein amidolytic acitivty to various trypsin inhibitors (Table V) resembles that of rabbit kallikrein [18]. Both are very sensitive to soybean trypsin inhibitor, Trasylol, and diisopropylfluorophosphate, but resistant to ovomucoid trypsin inhibitor. However, the rabbit kallikrein seems more resistant to lima bean trypsin inhibitor than this guinea-pig enzyme.

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