Isolation and Characterization of Bovine Plasma Prekallikrein (Fletcher Factor)†

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ABSTRACT: Prekallikrein (Fletcher factor) has been purified from bovine plasma approximately 25 000-fold with an overall yield of 14%. Purification steps included ammonium sulfate fractionation and column chromatography on heparin–agarose, DEAE-Sephadex, CM-Sephadex, benzamidine–agarose, and arginine methyl ester–agarose. The purified protein was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and amino-terminal sequence analysis. Bovine plasma prekallikrein is a glycoprotein with a molecular weight of 82 000 as determined by sedimentation equilibrium centrifugation. It contains 12.9% carbohydrate, including 6.2% hexose, 4.5% *N*-acetylglucosamine, and 2.2% *N*-acetylneuraminic acid. Prekallikrein is a single polypeptide chain with an amino-terminal sequence of Gly-Cys-Leu-Thr-Gln-Leu-Tyr-His-Asn-Ile-Phe-Phe-Arg-Gly-Gly. This sequence is homologous to the amino-terminal sequence of human factor XI (plasma thromboplastin antecedent). Both prekallikrein and kallikrein require kaolin to correct Fletcher factor deficient plasma. Kallikrein, however, has a specific activity 3.5 times greater than prekallikrein. Prekallikrein does not correct plasma deficient in factor XII (Hageman factor), factor XI, or high molecular weight kininogen (Fitzgerald factor).

Plasma prekallikrein (Fletcher factor) is the inactive precursor or zymogen form of kallikrein, a serine protease. It is involved in several physiological processes, including the generation of kinin (Werle, 1955; Margolis, 1958; Wuepper, 1973), fibrinolysis (Ogston et al., 1969; Laake & Vennerod, 1974; Mandle & Kaplan, 1977), and the intrinsic pathway of blood coagulation (Wuepper, 1973; Saito et al., 1974; Weiss et al., 1974). In 1965, Hathaway et al. (1965) described a coagulation defect which they designated as Fletcher factor deficiency. The disorder was characterized by a prolonged partial thromboplastin time which was shown to be a defect in the early stages of the intrinsic pathway of blood coagulation. It was subsequently shown by Wuepper (1973) that the Fletcher trait was corrected by a highly purified preparation of plasma prekallikrein. Furthermore, surface-mediated fibrinolysis and kinin formation were also shown to be defective in Fletcher trait plasma (Wuepper, 1973; Saito et al., 1974; Weiss et al., 1974).

Extensive purification of the human, bovine, and rabbit prekallikrein from plasma has been achieved in several laboratories. The chemical characterization of this protein, however, has not been made due to its low concentration in plasma and the difficulty of its purification. McConnell & Mason (1970) partially purified human prekallikrein by DEAE-cellulose chromatography in the presence of heparin, an inhibitor of the activation of prekallikrein (McConnell & Becker, 1966). Residual kallikrein activity was destroyed by treatment with diisopropyl phosphorofluoridate (iPr_2PF).[1] This preparation, however, was substantially contaminated with other proteins, including immunoglobulin IgG, and was not further characterized.

Takahashi et al. (1972) purified bovine prekallikrein about 1300-fold by procedures that included ammonium sulfate fractionation, DEAE-Sephadex chromatography, CM-Sephadex chromatogrpahy, affinity chromatography on arginine–Sepharose, and gel filtration on Sephadex G-150. The purified preparation was homogeneous on ultracentrifugation and isoelectric focusing. It also gave a single band on polyacrylamide disc gel electrophoresis. The molecular weight of bovine prekallikrein was found to be 90 000 by sedimentation equilibrium and sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Prekallikrein from human and rabbit plasma was extensively purified by Wuepper & Cochrane (1972) in the presence of polybrene. Their procedure for the rabbit preparation included ammonium sulfate fractionation, DEAE-Sephadex and CM-Sephadex chromatography, and zone electrophoresis. The overall purification was 6200-fold with a 6% yield. This preparation yielded a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis when stained for protein or carbohydrate and had a molecular weight of 100 000. Human prekallikrein, purified by essentially the same procedure, was contaminated with β_2-glycoprotein in the final step. This contaminant, however, was removed by sucrose density gradient centrifugation. The molecular weight of the human prekallikrein was 107 000, as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

More recently, Mandle & Kaplan (1977) isolated human prekallikrein in the presence of iPr_2PF by desalting plasma on Sephadex G-25, followed by chromatography on QAE-Sephadex, CM-Sepharose CL 6B, Sephadex G-150, and an immunoadsorbent prepared against human IgG and β_2-glycoprotein. Two molecular forms of prekallikrein were copurified with apparent molecular weights of 88 000 and 85 000, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

In order to study the various chemical properties of plasma prekallikrein and its mechanism of activation in detail, we have developed a method for the isolation of milligram quantities of this protein from bovine plasma. The highly purified preparation was then characterized as to its size, amino-terminal sequence, and chemical composition.

Experimental Section

Materials. Benzamidine hydrochloride, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate,

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[1] Abbreviations used: iPr_2PF, diisopropyl phosphorofluoridate; Tos-PheCH_2Cl, *N*-tosyl-L-phenylalanyl chloromethyl ketone.

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iPr_2PF, cyclohexanone, and polybrene were obtained from Aldrich Chemical Co., Milwaukee, WI. Acrylamide, *N,N,N',N'*-tetramethylethylenediamine and Bio-Gel A-15m were products of Bio-Rad Laboratories, Richmond, CA. Barium sulfate and 4-vinylpyridine monomer (practical grade) purified further by vacuum distillation were products of J. T. Baker Chemical Co., Phillipsburg, NJ, and acid-washed kaolin was obtained from Fisher Scientific Co., Pittsburgh, PA. *N,N'*-Methylenebis(acrylamide) and 2-mercaptoethanol were purchased from Eastman Kodak Co., Rochester, NY. Cyanogen bromide was a product of Pierce Chemical Co., Rockford, IL. Sephadex G-50, CM-Sephadex C-50, and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Ovomucoid trypsin inhibitor (type II-O), soybean trypsin inhibitor (types I-S and II-S), rabbit brain cephalin, dithiothreitol, L-arginine methyl ester, lithium heparin, 2-(*N*-morpholino)ethanesulfonic acid, thiobarbituric acid, ϵ-aminocaproic acid, *p*-aminobenzamidine, bovine serum albumin, carbonic anhydrase, ovalbumin, myoglobin, Coomassie brilliant blue R, and imidazole (grade I) were purchased from Sigma Chemical Co., St. Louis, MO. Tos-PheCH_2Cl-treated trypsin (278 units/mg) was a product of Worthington Biochemical Co., Freehold, NJ. Guanidine hydrochloride (extreme purity) was obtained from Heico, Inc., Delaware Water Gap, PA. *N*-Benzoyl-L-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide hydrochloride was obtained from Pentapharm, Ltd., Basel, Switzerland, and sodium dodecyl sulfate was purchased from British Drug House, Poole, England. Human Fitzgerald factor deficient plasma and prekallikrein deficient plasma were obtained from George King Biomedical, Inc., Salem, NH. Human factor XII[2] deficient plasma from a Hageman trait patient in the Seattle area was kindly provided by the Puget Sound Blood Center, Seattle, WA, and bovine factor XI deficient plasma was a generous gift of Dr. G. Kociba of The Ohio State University. Phosphorylase *b* was kindly provided by Dr. E. Fischer of this department. Dialysis tubing was prepared according to McPhie (1971) prior to use. All other chemicals were commercial preparations of the highest quality available.

Methods. Prekallikrein concentrations were determined from the absorbance at 280 nm by using $E_{280}^{1\%} = 10.9$. This value was determined in the analytical ultracentrifuge by employing the methods of Babul & Stellwagen (1969). For carbohydrate analyses, protein concentration was determined by amino acid analysis after the sample was hydrolyzed in 6 N HCl for 24 h at 110 °C in evacuated tubes.

Amino acid analyses and the preparation of samples were carried out by the methods of Moore & Stein (1963) and Spackman et al. (1958) employing a Durrum Model D500 amino acid analyzer. Samples were hydrolyzed in 6 N HCl at 110 °C for 24, 48, 72, and 96 h in evacuated tubes. The values for serine and threonine were determined by extrapolation to zero-time hydrolysis, and isoleucine and valine values were calculated from the 96-h hydrolysis time. Half-cystine was determined as cysteic acid by the method of Hirs (1967). Tryptophan was determined by the spectrophotometric procedure of Edelhoch (1967).

Hexose was determined according to the method of Carter & Hakomori (1979). The sample (200 μg) was hydrolyzed in 90% glacial acetic acid containing 0.5 N H_2SO_4 for 8 h at 80 °C. Inositol was employed as an internal standard. Sugars were identified and quantitated by employing a Hewlett-Packard Model 402 gas chromatograph and a Finnigan Model 3300 gas chromatograph–mass spectrometer (Lindberg, 1972; Laine et al., 1974). Neuraminic acid was determined by the thiobarbituric acid method of Warren (1959), using *N*-acetylneuraminic acid as a standard. Hexosamine was determined with the amino acid analyzer on samples previously hydrolyzed in 2 N HCl for 22 h at 110 °C.

Amino-terminal sequence analysis was performed with a Beckman Model 890C sequencer, using 3 mg of the *S*-pyridylethyl derivative of prekallikrein. The operation of this instrument and the methods employed are adaptations (Hermodson et al., 1972) of the original technique of Edman & Begg (1967). The specific program employed was the dimethylbenzylamine system of Hermodson et al. (1972). Phenylthiohydantoin amino acids were identified by gas chromatography after silylation or directly by high-pressure liquid chromatography (Bridgen et al., 1976). Protein concentration for the sequence analysis was determined by amino acid analysis.

The *S*-pyridylethyl derivative of plasma prekallikrein was made according to Friedman et al. (1970). The protein was separated from salt and excess reagents by gel filtration on Sephadex G-50 (1.8 × 50 cm) in 9% formic acid.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the method of Weber & Osborn (1969) using 7.0% gels. Samples (5–10 μg) were incubated for at least 30 min at 65 °C in the presence or absence of 10 μL of 2-mercaptoethanol in 0.1 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing 2% sodium dodecyl sulfate and 6 M urea. Electrophoresis was performed at 8 mA/gel for 4 to 5 h. Gels were stained with 0.25% Coomassie brilliant blue R and destained electrophoretically.

Ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control. Sedimentation equilibrium experiments were performed according to Yphantis (1964) by employing a six-channel Kel-F centerpiece as described by Kisiel & Davie (1975). Sedimentation equilibrium studies were carried out in 0.05 M sodium acetate (pH 5.5), containing 6 M guanidine hydrochloride (extreme purity). Prekallikrein was dialyzed at room temperature against this solvent for 72 h and then diluted with the diffusate to obtain the appropriate protein concentration. Analyses were performed at three different protein concentrations (1.4, 0.7, and 0.35 mg/mL) at 20 °C at a rotor speed of 20000 rpm. A partial specific volume of 0.704 was calculated from the amino acid composition and corrected for 12.9% carbohydrate content according to Gibbons (1966) and Lee & Timasheff (1974). The solvent density at 20 °C was obtained from its index of refraction at 23.5 °C as measured by an Abbe refractometer (Kielley & Harrington, 1960). Point-by-point molecular weight averages were calculated from the data by utilizing a computer program developed by Teller (1973).

The amidase activity of kallikrein was measured by using the chromogenic kallikrein substrate Bz-Pro-Phe-Arg-*p*-nitroanilide, as modified from Griffin & Cochrane (1976). The sample (0.01–0.1 mL) was diluted to 0.9 mL with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and incubated with 10 μL of trypsin (0.2 mg/mL in 1 mM HCl and 0.1 mM $CaCl_2$) at 37 °C. After 20 min, the trypsin was inactivated with 100 μg of ovomucoid trypsin inhibitor (10 mg/mL in 0.05 M Tris-HCl buffer, pH 8.0) and 0.1 mL of kallikrein substrate (1 mM) was added. The reaction mixture was incubated at 37 °C for 3 min and stopped with 10 μL of glacial acetic acid, and the absorbance at 405 nm was read in a Gilford spectrophotometer.

For the coagulant assay of plasma prekallikrein, the sample

[2] The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

was diluted 50–200-fold with Michaelis buffer (0.036 M sodium acetate, 0.036 M sodium barbital, and 0.145 M sodium chloride, pH 7.4) containing 0.1 mg/mL bovine serum albumin. The sample (0.05 mL) was then incubated at room temperature for 5 min with 0.05 mL of prekallikrein deficient plasma and 0.05 mL of kaolin suspension (50 mg of kaolin per 10 mL of saline). One-tenth milliliter of an equal mixture of the stock cephalin suspension (1 vial in 100 mL of 0.15 M NaCl) and 0.025 M $CaCl_2$ was then added, and the clotting time was determined by the tilting method. Activity was calculated from a calibration curve where the log of prekallikrein concentration was plotted against the log of the clotting time. One unit of activity was defined as the amount of activity present in 1.0 mL of pooled platelet-poor normal bovine plasma. The assay for high molecular weight kininogen was carried out in a similar manner with Fitzgerald factor deficient plasma. Factor XII was assayed as described by Fujikawa et al. (1977), and factor XI was assayed according to Koide et al. (1977).

Heparin–agarose and arginine methyl ester–agarose were prepared as previously described by Fujikawa et al. (1973). Fifty milliliters of freshly activated agarose was added to 1 g of heparin or 2.5 g of L-arginine methyl ester in 50 mL of 0.1 M sodium bicarbonate buffer (pH 8.3) and gently stirred overnight at 4 °C. Benzamidine–agarose was prepared according to the procedure of Schmer (1972) using ϵ-aminocaproic acid as a spacer between benzamidine and the agarose beads.

Trypsin was coupled to agarose by stirring 50 mL of freshly activated agarose with 100 mg of trypsin in 50 mL of 0.1 M sodium bicarbonate buffer (pH 8.5) containing 0.5 M NaCl. After 20 h at 4 °C, the agarose was washed with 0.05 M Tris-HCl buffer (pH 8.0) containing 1 M NaCl, followed by 0.05 M acetate buffer (pH 4.0) containing 1 M NaCl. Plasma kallikrein was prepared by activating bovine prekallikrein with trypsin–agarose. In these experiments, prekallikrein (0.16 mg) was incubated at 37 °C for 60 min with 50 μL of settled trypsin–agarose in 0.05 M Tris-HCl buffer (pH 8.0) and 0.05 M NaCl in a volume of 1.65 mL. The reaction was stirred every 2 min with a vortex mixer, and the trypsin–agarose was then removed by centrifugation. The activation of prekallikrein was monitored by following the generation of esterase activity.

Purification of Bovine Plasma Prekallikrein. Bovine blood was collected and rapidly mixed with 0.1 volume of anticoagulant solution [0.1 M sodium oxalate, heparin (100 mg/L), and crude soybean trypsin inhibitor (100 mg/L)]. The plasma was isolated at room temperature with a continuous flow separator (DeLaval Model BLE519). All subsequent steps were performed at 4 °C, employing plastic containers, columns, and tubes. The plasma (14 L) was stirred with barium sulfate (20 g/L) for 30 min, and the slurry was centrifuged for 8 min at 7800*g* in a Sorvall RC3 centrifuge. The supernatant was made 0.1 mM in ethylenediaminetetraacetic acid and then brought to 20% saturation by the slow addition of solid ammonium sulfate. After the mixture was stirred for 15 min, the precipitate was removed by centrifugation for 15 min at 7800*g*. The supernatant was then brought to 40% saturation with solid ammonium sulfate and stirred for 30 min, and the suspension was centrifuged for 40 min at 7800*g*. The precipitate was dissolved in 4.5 L of cold distilled water containing soybean trypsin inhibitor (20 mg/L), polybrene (50 mg/L), and iPr_2PF (0.2 mM). The solution was then dialyzed for 15 h against 60 L of distilled water, followed by dialysis against 80 L of 0.03 M Tris-HCl buffer (pH 7.5) and 0.05 M NaCl for 14 h.

After dialysis, the conductivity of the sample was adjusted with cold 0.03 M Tris-HCl buffer (pH 7.5) to 5.0 mΩ at 4 °C by using a radiometer conductivity meter, type CDM2, and soybean trypsin inhibitor, polybrene, and iPr_2PF were added at the same concentrations as noted above. The sample was divided in half, and each fraction was applied at a flow rate of 400 mL/h to a DEAE-Sephadex A-50 column (10 × 32.5 cm) previously equilibrated with the dialysis buffer. Each column was washed with 2 L of 0.03 M Tris-HCl (pH 7.5) containing 0.05 M NaCl and iPr_2PF (0.1 mM). The pass-through and wash fractions were then combined, and the pH was adjusted to 7.2 with 1 N HCl.

The pooled fraction was applied at a flow rate of 400 mL/h to a heparin–agarose column (8 × 20 cm) previously equilibrated with 0.02 M Tris-HCl buffer (pH 7.2) containing 0.05 M NaCl. The column was washed with 5.0 L of 0.02 M Tris-HCl buffer (pH 7.2) containing 0.065 M NaCl and 0.1 mM iPr_2PF, and the protein was eluted by a linear salt gradient formed by 3 L of 0.065 M NaCl in 0.02 M Tris-HCl buffer (pH 7.2) and 3 L of 0.55 M NaCl in 0.02 M Tris-HCl buffer (pH 7.2). Both solutions also contained polybrene (50 mg/L) and 0.1 mM iPr_2PF. Fractions (200 mL) were collected in plastic bottles by employing a Pharmacia preparative fraction collector. Polybrene (50 mg/L), purified soybean trypsin inhibitor (20 mg), and iPr_2PF (0.1 mM) were added to the pooled fractions containing prekallikrein from the heparin–agarose column, and the sample was dialyzed overnight against 40 L of 0.02 M sodium phosphate buffer (pH 8.0).

A precipitate which formed during dialysis was removed by centrifugation (15 min at 7800*g*), and the sample was applied to a second DEAE-Sephadex A-50 column (6 × 35 cm) at a flow rate of 150 mL/h. The DEAE-Sephadex column was previously equilibrated with 0.02 M sodium phosphate buffer (pH 8.0). After application of the sample, the column was washed with 4 L of equilibration buffer and the protein was eluted from the column by a linear salt gradient formed by 1.5 L of 0.02 M sodium phosphate buffer (pH 8.0) and 1.5 L of 0.02 M sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl. Each solution also contained 0.1 mM iPr_2PF.

The prekallikrein sample from the second DEAE-Sephadex column was dialyzed overnight, after addition of polybrene (100 mg/L) and soybean trypsin inhibitor (5 mg), against 40 L of 0.05 M sodium acetate buffer (pH 5.6) containing 0.02 M NaCl, and the sample was applied to a CM-Sephadex C-50 column (5 × 25 cm) equilibrated with the same buffer at a flow rate of 150 mL/h. The column was then washed with 2 L of 0.05 M sodium acetate buffer (pH 5.6) containing 0.1 M NaCl and polybrene (100 mg/L), and prekallikrein was eluted from the column with a linear gradient composed of 1.2 L of 0.05 M sodium acetate buffer (pH 5.6) containing 0.1 M NaCl and polybrene (100 mg/L) and 1.2 L of 0.05 M sodium acetate buffer (pH 5.6) containing 0.35 M NaCl and polybrene (100 mg/L).

Fractions containing prekallikrein from the CM-Sephadex column were combined and dialyzed overnight against 18 L of 0.05 M imidazole hydrochloride (pH 6.0) containing 0.025 M NaCl. The dialyzed solution was applied at a flow rate of 75 mL/h to a benzamidine–agarose column (2.6 × 25 cm) which had been previously equilibrated with 0.05 M imidazole hydrochloride buffer (pH 6.0) containing 0.025 M NaCl. The column was washed with 250 mL of 0.05 M imidazole hydrochloride buffer (pH 6.0) containing 0.025 M guanidine hydrochloride and 0.025 M NaCl and was eluted by a linear gradient consisting of 250 mL of 0.05 M imidazole hydrochloride buffer (pH 6.0) containing 0.025 M guanidine hydrochloride and 0.025 M NaCl and 250 mL of 0.05 M imidazole hydrochloride buffer (pH 6.0) containing 0.75 M

Table I: Purification of Bovine Plasma Prekallikrein

purification step	vol (mL)	total protein (mg)[a]	total act. (units)[b]	sp act. (units/mg)	recovery (%)	purification (x-fold)
plasma	14000	9.8×10^5	2300	0.0023	100	1
NH_4SO_4 fractionation	6500	4.4×10^5	2070	0.005	90	2.2
first DEAE-Sephadex	10500	1.3×10^5	1920	0.015	84	6.5
heparin-agarose	1880	6.1×10^3	1250	0.21	55	90
second DEAE-Sephadex	1050	820	850	1.0	37	450
CM-Sephadex	905	120	790	6.6	34	2900
benzamidine-agarose	227	27.6	490	18	21	7700
arginine methyl ester-agarose	181	5.5	320	58	14	25000

[a] Protein concentration was determined by absorption employing $E_{280}^{1\%} = 10.0$ for plasma and subsequent steps up to the arginine methyl ester-agarose column, for which $E_{280}^{1\%} = 10.9$ was employed. [b] Activity of prekallikrein was assayed with kaolin as described under Methods. Before assaying, the first three samples (1.0 mL) were dialyzed for 3 h against 0.03 M Tris-HCl buffer (pH 7.5) and 0.05 M NaCl and were passed through a DEAE-Sephadex column (0.7 × 4 cm) which was then washed with 4.0 mL of the same buffer.

guanidine hydrochloride and 0.025 M NaCl.

iPr_2PF (0.1 mM) was added to the pooled fractions of prekallikrein from benzamidine–agarose, and the sample was dialyzed against 4 L of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 M NaCl. The sample was applied to an arginine methyl ester–agarose column (1.6 × 30 cm) previously equilibrated with the dialysis buffer at a flow rate of 40 mL/h. The column was washed with 150 mL of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.1 mM iPr_2PF and was eluted with a linear gradient consisting of 250 mL of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 0.1 mM iPr_2PF and 250 mL of 0.05 M Tris-HCl buffer (pH 8.0) containing 1.0 M NaCl and 0.1 mM iPr_2PF. Fractions containing plasma prekallikrein were pooled, and iPr_2PF was added to a final concentration of 0.1 mM. The solution was then concentrated to ~15 mL and stored at −70 °C. The column was washed with 300 mL of 0.1 M Tris-HCl buffer (pH 7.5) containing 2 M NaCl and 1 M guanidine hydrochloride and was reusable for at least 12 preparations.

Results

Preparation of Bovine Plasma Prekallikrein. A summary of the purification of prekallikrein from bovine plasma is shown in Table I. The purification was approximately 25 000-fold with an overall yield of 14%. The isolation procedure involves barium sulfate adsorption of contaminants, ammonium sulfate fractionation, and chromatography on DEAE-Sephadex A-50, heparin–agarose, CM-Sephadex C-50, benzamidine–agarose, and arginine methyl ester–agarose. The final preparation had a specific activity of nearly 60 units/mg of protein in the coagulant assay. The number of units of activity was determined from a standard reference curve based on the ability of pooled normal bovine plasma to correct human Fletcher factor deficient plasma. Factor XII, however, in addition to prekallikrein, also corrects Fletcher factor deficiency (Saito et al., 1975; Fujikawa et al., 1977). Therefore, in the quantitation of prekallikrein levels in plasma and after ammonium sulfate fractionation, it was necessary to pass the sample through a DEAE-Sephadex column, similar to that described below, to remove factor XII. When factor XII was removed from the plasma by passing it through a DEAE-Sephadex column and the treated plasma was used as a standard, the specific activity of the highly purified preparation was 320 units/mg.

Protease inhibitors, including soybean trypsin inhibitor and iPr_2PF, were added during the purification procedure to protect prekallikrein from proteolytic degradation. Polybrene, which prevents the surface contact activation of factor XII (Eisen, 1964), was also added to various buffers throughout the procedure.

Barium sulfate was employed to remove prothrombin,

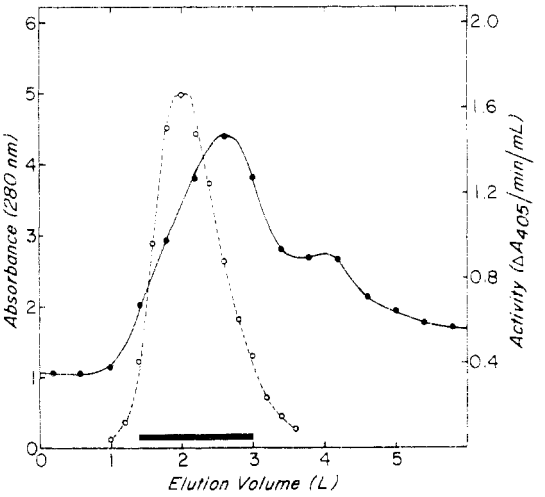

FIGURE 1: Elution pattern for heparin–agarose column chromatography. Prekallikrein was eluted from the column (8 × 20 cm) with a linear gradient formed by 3 L of 0.02 M Tris-HCl buffer (pH 7.2) containing 0.065 M NaCl and 3 L of 0.02 M Tris-HCl buffer (pH 7.2) containing 0.55 M NaCl. Both solutions also contained polybrene (50 mg/L) and 0.1 mM iPr_2PF. The flow rate was 400 mL/h. Prekallikrein activity was measured after activation by trypsin as described under Methods. (●) Absorbance; (○) amidase activity. Fractions shown by the solid bar were pooled.

protein C, protein S, factor X, factor IX, and factor VII, and a DEAE-Sephadex column was employed to remove factor XII following ammonium sulfate fractionation. Activated factor XII (Komiya et al., 1972; Wuepper & Cochrane, 1972; Mandle & Kaplan, 1977) and factor XII exposed to negatively charged surfaces (Cochrane & Wuepper, 1971) were previously shown to activate prekallikrein. Accordingly, conditions were selected which permitted prekallikrein to pass directly through the DEAE-Sephadex column while factor XII remained adsorbed to the resin. After this step, the clotting activity in factor XII deficient plasma was reduced from 1.9 units/mL to less than 0.05 unit/mL.

The pass-through fraction from the DEAE-Sephadex column was then applied to a heparin–agarose column. The elution profile of prekallikrein from the heparin–agarose column is shown in Figure 1. More than 90% of the proteins passed through the column before the gradient was started. Prekallikrein, assayed with a kallikrein chromogenic substrate after activation with trypsin, then appeared in the leading edge of the main protein peak.

The upper panel in Figure 2 shows the chromatography profile of prekallikrein from the second DEAE-Sephadex column. After the gradient was started, prekallikrein appeared in the descending portion of the first protein peak. The chromatography profile for prekallikrein on CM-Sephadex is

Table II: Effect of Prekallikrein and Kallikrein on Various Deficient Plasmas[a]

plasma	clotting time (s)					
	+kaolin			−kaolin		
	prekallikrein	kallikrein	control	prekallikrein	kallikrein	control
prekallikrein deficient	122	90	273	453	413	>600
HMW[b] kininogen deficient	237	159	239	>600	>600	>600
factor XII deficient	432	362	553	>600	>600	>600
factor XI deficient	355	330	366	>600	>600	>600

[a] Assay conditions were as described under Methods with either 50 μL of kaolin (50 mg/10 mL in 0.15 M NaCl) or 0.15 M NaCl added where indicated. Each assay contained 45 ng of prekallikrein or kallikrein. Kallikrein was prepared by incubating trypsin–agarose with prekallikrein as described under Methods. The clotting times shown are an average of four different experiments. [b] HMW, high molecular weight.

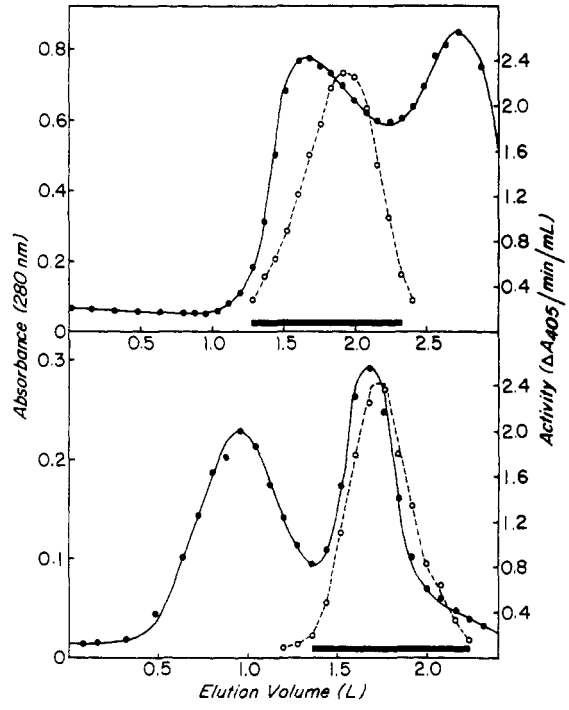

FIGURE 2: Elution pattern for DEAE-Sephadex and CM-Sephadex column chromatography. The top panel shows the elution pattern for the second DEAE-Sephadex column. Protein was eluted from the column (6 × 35 cm) with a linear gradient formed by 1.5 L of 0.02 M sodium phosphate buffer (pH 8.0) and 1.5 L of 0.02 M sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl. Both solutions also contained 0.1 mM iPr_2PF. The flow rate was 150 mL/h. The bottom panel shows the elution pattern for the CM-Sephadex column. The fractions from the second DEAE-Sephadex column were dialyzed and applied to the CM-Sephadex column (5 × 25 cm) previously equilibrated with 0.05 M sodium acetate buffer (pH 5.6) containing 0.02 M NaCl. Protein was eluted from the column with a linear gradient formed with 1.2 L of 0.05 M sodium acetate buffer (pH 5.6) containing 0.1 M NaCl and 100 mg/L polybrene and 1.2 L of 0.05 M sodium acetate buffer (pH 5.6) containing 0.35 M NaCl and 100 mg/L polybrene. The flow rate was 150 mL/h. Prekallikrein was measured after conversion to kallikrein by trypsin as described under Methods. (●) Absorbance; (○) amidase activity. Fractions shown by the solid bar were pooled.

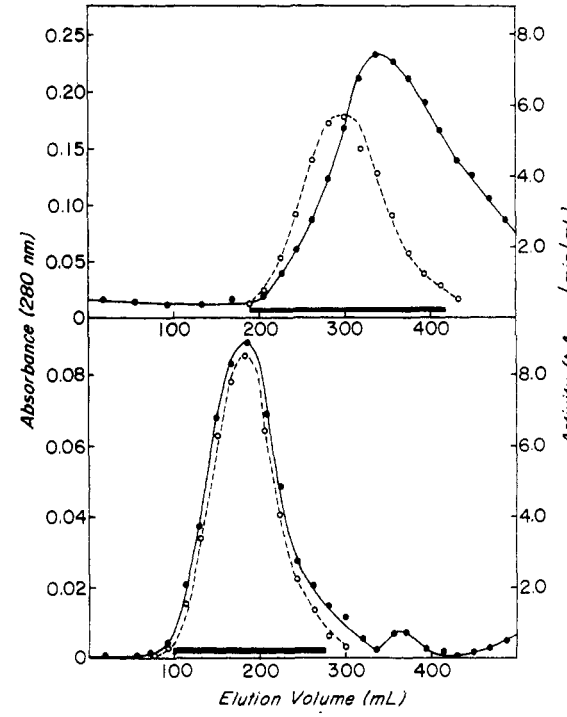

FIGURE 3: Elution pattern from benzamidine–agarose and arginine–agarose column chromatography. The top panel shows the elution pattern from the benzamidine–agarose column for prekallikrein activity pooled from the CM-Sephadex column. Protein was eluted from the column (2.6 × 25 cm) with a linear salt gradient formed by 250 mL of 0.05 M imidazole hydrochloride buffer (pH 6.0) containing 0.025 M NaCl and 0.025 M guanidine hydrochloride and 250 mL of 0.05 M imidazole hydrochloride buffer (pH 6.0) containing 0.025 M NaCl and 0.75 M guanidine hydrochloride. The flow rate was 75 mL/h. The bottom panel shows the elution pattern for the arginine methyl ester–agarose column. The fractions from the benzamidine–agarose column were dialyzed and applied to an arginine methyl ester–agarose column (1.6 × 30 cm) previously equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.05 M NaCl. Protein was eluted from the column with a linear gradient formed with 250 mL of 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 250 mL of 0.05 M Tris-HCl buffer (pH 8.0) containing 1 M NaCl. Both solutions also contained 0.1 mM iPr_2PF. The flow rate was 40 mL/h. Prekallikrein activity was measured after conversion to kallikrein by trypsin as described under Methods. (●) Absorbance; (○) amidase activity. Fractions shown by the solid bar were pooled.

shown in the lower panel of Figure 2. On this column, prekallikrein eluted in the second protein peak. Approximately 50% of the protein (not shown) passed through the column before the gradient was started.

A small purification of prekallikrein was obtained by chromatography on benzamidine–agarose (Figure 3, upper panel). Prekallikrein eluted with the ascending edge of the protein peak. When kallikrein contaminated the preparation, it appeared as a second peak in the descending portion of the protein peak. The final purification of prekallikrein was performed by chromatography on arginine methyl ester–agarose. As shown in the lower panel of Figure 3, prekallikrein eluted as a single peak in which the esterase activity paralleled that of the protein.

The final preparation of bovine plasma prekallikrein readily corrected human Fletcher factor deficient plasma (Table II). In the absence of prekallikrein, the clotting time was 273 s. Correction of Fletcher factor deficient plasma shows an absolute requirement for kaolin. Prekallikrein was free of other coagulation factors such as factor XII, factor XI, and high molecular weight kininogen when measured in a clotting assay. No detectable kallikrein was observed in the preparation as

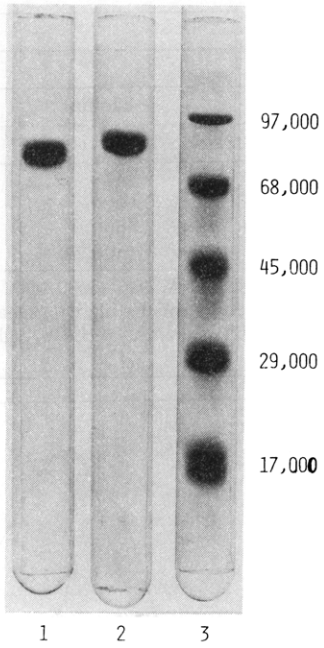

FIGURE 4: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of bovine plasma prekallikrein. Electrophoresis was carried out in 7% polyacrylamide gels and stained with Coomassie brilliant blue as described under Methods. Gel 1 was 10 μg of prekallikrein before reduction; gel 2 was 10 μg of prekallikrein after reduction; gel 3 included the following standards: phosphorylase (M_r 97 000), bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (29 000), myoglobin (17 000). The anode was at the bottom of the gel.

measured by amidase activity.

When prekallikrein was incubated with trypsin–agarose (see Methods), there was a generation of amidase activity which reached a maximum at 40 min. The resulting specific activity toward the kallikrein substrate *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide was 9.2 μmol of substrate hydrolyzed per min per mg of enzyme at 37 °C. Kallikrein also readily corrected Fletcher factor deficient plasma (Table II). Furthermore, the specific activity of kallikrein was 3.5 times greater than that of prekallikrein when assayed in the presence of kaolin. Kallikrein also has some clotting activity in human high molecular weight kininogen deficient plasma but little or no activity in factor XI deficient or factor XII deficient plasma. In the absence of kaolin, kallikrein has essentially no detectable coagulant activity. These data indicate that prekallikrein, as well as kallikrein, requires kaolin for activity in the coagulant assay.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis of Bovine Plasma Prekallikrein. Analysis of purified prekallikrein by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate is shown in Figure 4. A single protein band was observed before or after reduction with 2-mercaptoethanol (samples 1 and 2). The apparent molecular weight estimated by this technique was 88 000 for the reduced protein. When the amount of the reduced sample applied to the gel was decreased, the stained protein band was resolved into a closely spaced doublet with apparent molecular weights of 89 000 and 86 000.

Sedimentation Equilibrium Studies on Bovine Plasma Prekallikrein. Sedimentation equilibrium experiments on prekallikrein in 6 M guanidine hydrochloride indicated that the glycoprotein was essentially homogeneous. When the reciprocal of the apparent average molecular weights was plotted against concentration (Roark & Yphantis, 1969), a linear plot was obtained with a positive slope except for some minor deviation near the meniscus. Furthermore, the plot of $2M_n - M_w$ against concentration was nearly horizontal, suggesting that the minor variation is due to nonideality. The smallest molecular weight species calculated according to the method of Teller (1973) was 82 000 ± 200. The number-average molecular weight, the weight-average molecular weight, and the z-average molecular weight were 81 300 ± 200, 80 600 ± 400, and 79 000 ± 600, respectively.

Table III: Amino Acid and Carbohydrate Compositions of Bovine Plasma Prekallikrein

component	residues/82 000 g of glycoprotein
amino acid	
lysine	38.3
histidine	19.7
arginine	30.1
aspartic acid	54.0
threonine	52.6
serine	59.4
glutamic acid	66.9
proline	26.9
glycine	55.7
alanine	33.3
half-cystine	38.8[a]
valine	29.8
methionine	4.7
isoleucine	40.3
leucine	46.3
tyrosine	16.5
phenylalanine	26.1
tryptophan	9.2[b]
carbohydrate	
hexose	28.3 (6.2%)
N-acetylglucosamine	16.7 (4.5%)
N-acetylneuraminic acid	5.8 (2.2%)
protein (%)	87.1
carbohydrate (%)	12.9

[a] Determined as cysteic acid according to Hirs (1967). [b] Determined by the spectrophotometric assay of Edelhoch (1967).

Amino Acid and Carbohydrate Compositions of Bovine Plasma Prekallikrein. The amino acid and carbohydrate compositions of prekallikrein are shown in Table III. Bovine plasma prekallikrein is composed of 87.1% protein and 12.9% carbohydrate. This corresponds to 71 400 g of protein and 10 600 g of carbohydrate per 82 000 g of glycoprotein. The carbohydrate includes 6.2% hexose, 4.5% *N*-acetylglucosamine, and 2.2% *N*-acetylneuraminic acid.

Amino-Terminal Sequence of Bovine Plasma Prekallikrein. The amino-terminal sequence for the first 15 residues of prekallikrein is shown in Figure 5 along with the amino-terminal sequences for human factor XI (Kurachi & Davie, 1977) and bovine factor XII (Fujikawa et al., 1977). Glycine (~0.4 equiv/82 000 molecular weight) was identified as the amino-terminal residue of bovine plasma prekallikrein, and no other amino acids were detected. The repetitive yields for the degradations were 98% based upon the yield of glycine at positions 1, 14, and 15. The yields for residues 1–15 were as follows: 0.4, not determined, 0.6, not determined, 0.5, 0.6, 0.5, not determined, 0.4, 0.6, 0.7, 0.7, not determined, 0.3, and 0.3 equiv, respectively.

Discussion

In the present procedure, bovine plasma prekallikrein was isolated in reasonable yield and high stability in the presence of various protease inhibitors. In addition, it was essential to remove factor XII from prekallikrein early in the isolation procedure and this was achieved by chromatography on the

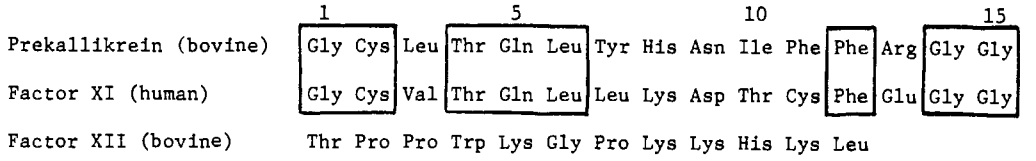

FIGURE 5: Amino-terminal sequence of bovine plasma prekallikrein. The amino-terminal sequences of human factor XI (Kurachi & Davie, 1977) and bovine factor XII (Fujikawa et al., 1977) are also shown. Amino acids that are identical with prekallikrein are shown in blocks.

first DEAE-Sephadex column. Fujikawa et al. (1977) used a similar DEAE-Sephadex column which adsorbed factor XII but allowed a factor XII activator to pass through. Chromatography of the sample on heparin–agarose made it possible to concentrate prekallikrein to a manageable volume for further purification. Two affinity columns were then employed for the purification of prekallikrein, including benzamidine–agarose and arginine methyl ester–agarose. The benzamidine–agarose was used earlier by Sampaio et al. (1974) in the purification of human plasma kallikrein and was useful in the separation of prekallikrein from kallikrein which was spontaneously generated during the isolation procedure. In the final purification step, chromatography on arginine methyl ester–agarose was employed. Takahashi et al. (1972) reported that bovine plasma prekallikrein binds to L-arginine–agarose but not to L-lysine–agarose. In the present studies, prekallikrein was found to have a higher affinity for the methyl ester derivative of L-arginine, and this derivative was found to be more effective in the final chromatography step.

The overall yield of prekallikrein was ~5.5 mg from 14 L of bovine plasma. This is equivalent to 40 mg of prekallikrein, assuming a 100% recovery of activity. These data suggest that prekallikrein occurs in bovine plasma at a concentration of ~3 μg/mL of plasma.

The present data indicate that bovine plasma prekallikrein is a single-chain glycoprotein with a molecular weight of 82 000, as determined by sedimentation equilibrium. This value is somewhat lower than the value of 88 000 estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This is probably due to decreased binding of sodium dodecyl sulfate to glycoproteins, as reported by Segrest & Jackson (1972). The present preparation of bovine plasma prekallikrein was further shown to migrate as a doublet of 89 000 and 86 000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The doublet was observed with each of the various bovine preparations of plasma prekallikrein. The ratio, however, between the two species varied. Since only a single glycine residue was observed in the first turn of the amino-terminal sequence analysis, it seems likely that the observed heterogeneity is due to the heterogeneity in carbohydrate content or a modification at the carboxyl-terminal region of the polypeptide chain. Since the activation of prekallikrein by factor XII_a yields a heavy chain and two light chains (Heimark et al., 1978), the latter case seems more probable. Similar results were observed by Mandle & Kaplan (1977) for human plasma prekallikrein which migrated with apparent molecular weights of 88 000 and 85 000.

The molecular weights reported in the present studies are slightly smaller than those reported for bovine, human, and rabbit prekallikrein by other investigators. Takahashi et al. (1972) purified bovine plasma prekallikrein and observed a single band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate with an apparent molecular weight of 90 000. A molecular weight of 90 000 was also determined by the Archibald method, but no correction of the partial specific volume was made for the presence of carbohydrate. Analysis of highly purified human and rabbit prekallikrein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis gave molecular weights of 107 000 and 100 000 (Wuepper & Cochrane, 1972). The rabbit preparation, furthermore, was stained with the periodic acid–Schiff method. Thus, the human, bovine, and rabbit preparations all appear quite similar in size.

Bovine plasma prekallikrein shows a great deal of homology in its amino-terminal region with human factor XI in that 8 of the first 15 residues are identical (Figure 5). There is no homology of the amino-terminal region of prekallikrein with factor XII, the other known proteins involved in the contact activation phase, or the various vitamin K dependent proteins of plasma (Davie & Hanahan, 1977). Both prekallikrein and factor XI are activated by factor XII_a or trypsin by limited proteolysis to yield a heavy chain and a light chain held together by a disulfide bond(s) (Bouma & Griffin, 1977; Kurachi & Davie, 1977; Mandle & Kaplan, 1977). The light chain in kallikrein and factor XI_a contains the active site. Factor XI_a, however, is composed of an identical pair of heavy chains and a pair of light chains, each with an active site. This suggests that a least two regions in prekallikrein and factor XI (the amino-terminal portion and the catalytic portion) have evolved from a common ancestor.

Acknowledgments

The authors thank Dr. Kazuo Fujikawa for his helpful advice and stimulating discussion and Dr. Walter Kisiel for performing the sedimentation experiments. Thanks are also due to Dr. Bill Carter and Mark Powell for assistance in the carbohydrate analyses. The authors are indebted to Karen Lebens and Richard Granberg for excellent technical help. The P. D. and J. Meats Co., Kent, WA, kindly provided the bovine blood.

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CORRECTIONS

Ligand Binding and Self-Association of Phosphorylase b_2, by R. F. Steiner,* L. Greer, and R. Bhat, Volume 18, Number 7, April 3, 1979, pages 1380–1385.

Page 1382. In Figure 1, the second sentence of the legend should read as follows: The ordinate is the number of moles of AMP bound per phosphorylase monomer.

Proteolytic Activity of Nerve Growth Factor: A Case of Autocatalytic Activation, by Michael Young, Volume 18, Number 14, July 10, 1979, pages 3050–3055.

Page 3053. In column 1, the third line is repetitious and should be deleted. Also in column 1, the last sentence should read as follows: The lag phase was completely absent. Consequently, the activation reaction initially induced by dilution of the protein is irreversible.