Isolation and Characterization of Bovine Factor XII (Hageman Factor)†

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ABSTRACT: Factor XII was purified approximately 14 000-fold from bovine plasma by ammonium sulfate fractionation followed by heparin–agarose, DEAE-Sephadex, CM-cellulose, arginine–agarose, and benzamidine–agarose column chromatography. By this method, about 15 mg of protein was purified from 15 L of plasma with an overall yield of 18%. The purified protein was homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and amino-terminal analysis. Bovine factor XII is a glycoprotein with a mol wt of 74 000 as determined by sedimentation equilibrium centrifugation. It contains 13.5% carbohydrate including 3.4% hexose, 4.7% *N*-acetylhexosamine, and 5.4% *N*-acetylneuraminic acid. Factor XII is a single polypeptide chain with an NH_2-terminal sequence of Thr-Pro-Pro-Trp-Lys-Gly-Pro-?-Lys-His-. This sequence is homologous to the reactive-site regions of a number of protease inhibitors. The amino acid sequence of a carboxyl-terminal fragment prepared by cyanogen bromide digestion was found to be Leu-Cys-Ala-Gly-Phe-Leu-Glu-Gly-Gly-Thr-Asp-Ala-Cys-Gln-Gly-Asp-SER-Gly-Gly-Pro-Leu-Val-Cys-Glu-Asp-Glu-. This sequence is homologous with the active site of a number of plasma serine proteases including thrombin, factor IX_a, factor X_a, and plasmin. These data indicate that bovine factor XII is a precursor to a serine enzyme with an inhibitor sequence and a catalytic site located in the same single polypeptide chain.

Factor XII (Hageman factor)[1] is a plasma protein which participates in several important physiological processes including blood coagulation (Ratnoff and Colopy, 1955; Ratnoff and Rosenblum, 1958), fibrinolysis (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961; Ogston et al., 1969), and kinin formation (Margolis, 1959). When blood is placed in a glass container, a series of reactions occurs which leads to fibrin formation. These reactions are initiated by the surface activation of factor XII (Ratnoff and Rosenblum, 1958; Biggs et al., 1958; Nossel, 1964). In vivo, the activation of factor XII may occur by the binding of factor XII to collagen or vascular basement membranes (Niewiarowski et al., 1965; Cochrane et al., 1972) or platelets (Walsh, 1972). The enzymatic activation of human factor XII by plasmin, kallikrein, factor XI_a, and trypsin has also been reported by Kaplan and Austen (1971), Burrowes et al. (1971), Wuepper (1972), Cochrane et al. (1973), and Bagdasarian et al. (1973).

Factor XII has been extensively purified from human, bovine, and rabbit plasma during the past 15 years. Ratnoff et al. (1961) and Ratnoff and Davie (1962) reported a 3000- to 5000-fold purification of human factor XII by procedures that included DEAE-cellulose and CM-cellulose column chromatography. Their preparation apparently contained substantial amounts of factor XII_a, however, since it accelerated the clotting of normal or factor XII deficient plasma in siliconized tubes. Speer et al. (1965) extensively purified human factor XII by steps that included isoelectric precipitation, ammonium sulfate fractionation, and CM-Sephadex and phosphocellulose chromatography. This preparation had a molecular weight of 20 000 as determined by sedimentation equilibrium and contained an amino-terminal arginine.

Kaplan and Austen (1970) partially purified factor XII and factor XII_a from human plasma and serum. These preparations showed several bands on disc gel electrophoresis with molecular weights estimated at 30 000 and 40 000. Cochrane and coworkers (Cochrane and Wuepper, 1971; Cochrane et al., 1972; Revak et al., 1974) employed ammonium sulfate fractionation, DEAE-Sephadex chromatography, gel filtration, and CM-Sephadex chromatography for the isolation of human and rabbit factor XII. The purified protein from each source migrated as a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with a molecular weight of 80 000 to 90 000. The human preparation consisted of a single polypeptide chain, while the rabbit preparation appeared to contain three similar polypeptide chains held together by disulfide bonds. Movat and Ozge-Anwar (1974) purified human factor XII about 12 000-fold with an overall yield of about 1%. The molecular weight of this preparation was estimated to be 92 000. More recently, they estimated the molecular weight at about 78 000 (Chan and Movat, 1976).

Bovine factor XII was purified by Haanen et al. (1961) and Schoenmakers et al. (1963, 1965) by column chromatography on glass powder, ethanol fractionation, gel filtration, and CM-Sephadex and DEAE-Sephadex column chromatography. This preparation had substantial esterase activity toward benzoylarginyl ethyl ester and was primarily factor XII_a. More recently, Komiya et al. (1972) purified the bovine preparation by ammonium sulfate fractionation and CM-Sephadex and DEAE-Sephadex column chromatography. This preparation, which showed one main band and several faint bands on polyacrylamide disc gel electrophoresis, had considerable tosyl-L-arginine methylesterase activity and was readily inhibited by diisopropyl phosphorofluoridate (iPr_2FP)[2] and lima bean trypsin inhibitor suggesting that the final preparation was primarily factor XII_a.

In the present paper, we wish to report the isolation and

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

[2] Abbreviations used are: iPr_2FP, diisopropyl phosphorofluoridate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tos-PheCH_2Cl, *N*-tosyl-L-phenylalanyl chloromethyl ketone.

characterization of bovine factor XII. The final preparation was purified approximately 14 000-fold and was free of factor XII_a.

Materials

Soybean trypsin inhibitor (type II-S), rabbit brain cephalin, dithiothreitol, L-arginine, *N*-acetylneuraminic acid, galactosamine, mannose, galactose, thiobarbituric acid, *p*-dimethylaminobenzaldehyde, acetylacetone, ϵ-aminocaproic acid, *p*-aminobenzamidine, 2-(*N*-morpholino)ethanesulfonic acid (Mes), 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, N.J. 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (water-soluble carbodiimide), iPr_2FP, cyclohexanone, and polybrene were obtained from Aldrich Chemical Co., Milwaukee, Wis. Acrylamide and Bio-Gel A-15m (agarose), 100–200 mesh, were the products of Bio-Rad Laboratories, Richmond, Calif. Lithium heparin (158 units/mg) was purchased from Riker Laboratories, Inc., Northridge, Calif. Tosyl-L-arginine methyl ester HCl was obtained from Mann Research Laboratories, New York, N.Y. 4-Vinylpyridine monomer (practical grade) was a product of J. T. Baker Chemical Co., Phillipsburg, N.J., and further purified by vacuum distillation. *N,N'*-Methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, guanidine hydrochloride, and 2-mercaptoethanol were obtained from Eastman Kodak Co., Rochester, N.Y. Cyanogen bromide was a product of Pierce, Rockford, Ill. Sephadex G-100, G-50, G-25, and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. CM-cellulose (CM52) was a product of Whatman Biochemical Ltd., Springfield Mill, Maidstone, Kent, England. Guanidine hydrochloride (Ultra Pure) was obtained from Schwarz/Mann, Van Nuys, Calif. All other chemicals were commercial preparations of the highest quality available.

Methods

Heparin–agarose was prepared by the cyanogen bromide method as previously described (Fujikawa et al., 1973). Benzamidine–agarose and arginine–agarose each with an ϵ-aminocaproic acid spacer were prepared by a modification of the procedure of Schmer (1972). Freshly activated agarose (250 mL, settled volume) was coupled with 25 g of ϵ-aminocaproic acid in 500 mL of 0.1 M $NaHCO_3$ (pH 8.3) and gently stirred overnight at 4 °C. It was then washed successively with water and 0.1 M Mes–NaOH buffer (pH 4.75) and stored in sodium azide (10^{-4} M) at 4 °C until use. ϵ-Aminocaproic acid–agarose (100 mL) was suspended in 100 mL of 0.1 M Mes–NaOH buffer (pH 4.75) and water-soluble carbodiimide (10 g in 20 mL of 0.1 M Mes–NaOH buffer (pH 4.75)) was added to the suspension and the mixture was then stirred for 30 min at room temperature. Five grams of *p*-aminobenzamidine or 10 g of L-arginine in 10 mL of 0.1 M Mes–NaOH buffer (pH 4.75) was added to the suspension and stirring was continued for 15 h. The remaining active groups were blocked by the addition of 10 g of glycine ethyl ester and stirring was continued for an additional 4 h. Each of the final products was thoroughly washed with water and stored at 4 °C in the presence of 10^{-4} M sodium azide.

Human factor XII deficient plasma was obtained from two different Hageman trait patients from the Seattle area. These samples were kindly provided by Dr. Gottfried Schmer of this laboratory and the Puget Sound Blood Bank, Seattle, Wash. Fitzgerald factor deficient plasma (GK-1601), Hageman factor deficient plasma (GK-1202), and Fletcher factor deficient plasma (GK-1702) from human sources were purchased from George King Biomedicals, Salem, N.H. Bovine factor XI deficient plasma was kindly provided by Dr. G. Kociba of Ohio State University.

A stock solution of 1 M iPr_2FP was prepared by diluting 1 g of iPr_2FP (one ampule) with 4.5 mL of anhydrous 2-propanol and stored at −20 °C. A stock suspension of phospholipid was made by suspending one vial of rabbit brain cephalin in 100 mL of saline, and 1-mL aliquots were stored at −20 °C. A stock solution of polybrene was made by dissolving 10 g of polybrene in 100 mL of water and stored in a refrigerator.

The protein concentration of solutions of purified factor XII was determined from the absorbance employing $E_{280}^{1\%} = 14.2$ and correcting for Rayleigh scattering according to Shapiro and Waugh (1966). The $E_{280}^{1\%}$ value was determined in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl in an analytical ultracentrifuge by the method of Babul and Stellwagon (1969).

For amino acid analysis, samples were hydrolyzed at 110 °C for 24, 48, 72, and 96 h in evacuated sealed tubes in 6 N HCl. Analyses were carried out by the method of Moore and Stein (1963) using a Durrum Model D 500 amino acid analyzer. The values for serine and threonine were determined by extrapolation to zero-time hydrolysis and the values of 96-h hydrolysis were taken for valine and isoleucine. Half-cystine was determined as cysteic acid according to the procedure of Hirs (1967) and *S*-carboxymethylcysteine by the method of Crestifield et al. (1963). Tryptophan content was determined as a relative molar ratio to histidine after base hydrolysis as described by Hugli and Moore (1972). The averages of four hydrolyses were used for the other amino acids.

Neutral sugar was determined by the phenol–sulfuric acid method of Dubois et al. (1956) using an equimolar mixture of mannose and galactose as a standard. Hexosamine was analyzed by the Elson and Morgan method as described by Gardell (1957) after the samples were hydrolyzed in evacuated sealed tubes for 18 h at 110 °C with 2 N HCl. Galactosamine was used as a standard and hexosamine was expressed as *N*-acetylhexosamine. Neuraminic acid was determined by the method of Warren (1959) using *N*-acetylneuraminic acid as the standard. Protein concentrations of samples for carbohydrate analysis were determined by amino acid analysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (1969) using 7.5% gels. Samples (5–10 μg in 10–50 μL) were incubated for at least 4 h at 37 °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 h. Gels were stained for 1–2 h with 2% 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) employing a six-channel Kel-F centerpiece as described by Kisiel and Davie (1975).

Sedimentation equilibrium studies were carried out in 0.05 M sodium acetate (pH 5.5), containing 6 M guanidine-HCl (Ultra Pure) using *S*-pyridylethyl factor XII. Analyses were performed at three different protein concentrations (1.2 mg, 1.0 mg, 0.6 mg/mL) at 20 °C at a rotor speed of 20 000 rpm. A partial specific volume of 0.704 was calculated from the amino acid composition and corrected for 13.5% carbohydrate content according to Gibbons (1966) and Lee and 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 and Harrington, 1960).

Amino-terminal sequence analysis was performed with a Beckman sequencer, Model 890A, using *S*-pyridylethyl and *S*-carboxymethyl derivatives. The operation of this instrument and the methods employed are adaptations (Hermodson et al., 1972) of the original technique of Edman and Begg (1967). The specific program employed was the DMBA 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 analysis of the intact protein was carried out on two different preparations of *S*-pyridylethyl factor XII employing 8-mg samples. The analysis of the cyanogen bromide fragment containing the active site was carried out on 2 mg of *S*-carboxymethyl peptide and 3.0 mg of *S*-pyridylethyl peptide.

S-Pyridylethyl and *S*-carboxymethyl derivatives of factor XII were made according to Friedman et al. (1970) and Crestfield et al. (1963), respectively. The protein was separated from the salt and excess reagents by gel filtration on Sephadex G-50 (1.8 × 50 cm column). Formic acid (9%) was employed for the elution of the *S*-pyridylethyl derivative and 5% formic acid in 20% acetic acid for elution of the *S*-carboxymethyl derivative.

Cyanogen bromide digestion was carried out by dissolving 20 mg of salt-free *S*-carboxymethyl or *S*-pyridylethyl factor XII and 40 mg of cyanogen bromide in 20 mL of 72% formic acid, and the reaction was allowed to proceed for 24 h at 4 °C. After lyophilization, the digest was fractionated by gel filtration on Sephadex G-100 (2.5 × 90 cm column) in the presence of 6 M urea and 9% formic acid. The flow rate was 4.3 mL/h.

For the assay of factor XII, the sample was diluted 100- to 1000-fold with Michaelis buffer (3.6×10^{-2} M sodium acetate, 3.6×10^{-2} 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 2 min with 0.05 mL of factor XII deficient plasma and 0.05 mL of kaolin suspension (50 mg of kaolin/10 mL of saline). One-tenth milliliter of an equal mixture of the stock cephalin suspension and 0.025 M $CaCl_2$ was then added and the clotting time was determined. Activity was calculated from a calibration curve where the log of factor XII concentration was plotted against the log of the clotting time. This plot was linear from 165 to 500 s when fresh oxalated normal bovine plasma was used as a source of factor XII. One unit of activity is defined as the amount of activity present in 1 mL of normal bovine plasma. For the assay of factor XII_a, 0.05 mL of saline was added to the test tube instead of the kaolin suspension. Fletcher factor, Fitzgerald factor, and factor XI activities were determined by a procedure identical with that of the factor XII assay, except that the respective deficient plasma was used. Factor XII was tested for esterase activity by the method of Roberts (1958) employing tosyl-L-arginine methyl ester as substrate.

Purification of Bovine Factor XII

(a) *First Heparin–Agarose Column Chromatography.* Fresh bovine blood was collected and rapidly mixed with 0.1 vol of anticoagulant solution containing 0.1 M oxalate, heparin (100 mg/L), and crude soybean trypsin inhibitor (100 mg/L). Plasma was obtained by centrifugation at room temperature with a continuous flow separator (DeLaval Model BLE 519). Subsequent steps were performed at 4 °C employing plastic containers. The plasma was mixed with $BaSO_4$ (20 g/L) for 30 min and the slurry was centrifuged for 10 min at 7800*g* in a Sorvall RC3 centrifuge. The supernatant was brought to 20% saturation by the slow addition of solid ammonium sulfate. After centrifugation (15 min at 7800*g*), the supernatant was brought to 50% saturation by the addition of more solid ammonium sulfate. The solution was centrifuged again (60 min at 7800*g*) and the precipitate was dissolved in 4.5 L of cold distilled water. Polybrene (50 mg/L), soybean trypsin inhibitor (22 mg/L), and 1 M iPr_2FP (0.2 mL/L) were added to this sample, and the solution was dialyzed overnight against distilled water followed by 0.02 M Tris-HCl buffer (pH 7.2) containing 0.05 M NaCl. The dialyzed sample was applied to a heparin–agarose column (8.0 × 20 cm) with a flow rate of less than 200 mL/h. The column was then washed with 2.5 to 3 L of the same Tris buffer containing 1 M iPr_2FP (0.1 mL/L) and the protein was eluted by a salt gradient formed by 3 L of 0.15 M NaCl in 0.02 M Tris-HCl buffer (pH 7.2) and 3 L of 0.6 M NaCl in 0.02 M Tris-HCl buffer (pH 7.2). Both solutions also contained polybrene (50 mg/L) and 1 M iPr_2FP (0.2 mL/L). Fractions (200 mL) were collected in plastic bottles employing a Pharmacia preparative fraction collector. Polybrene (20 mg) and soybean trypsin inhibitor (40 mg) in 2 mL of water were added to each bottle before the gradient was started. Factor XII usually eluted from the column in the fractions between 1.2 and 2.0 L of eluate (see accompanying paper by Koide et al., 1977).

(b) *DEAE-Sephadex Column Chromatography.* The pooled fraction containing factor XII from the first heparin–agarose column was diluted with cold 0.05 M Tris-HCl buffer (pH 7.5) to obtain a conductivity of 6.5 $m\Omega^{-1}$ using a Radiometer conductivity meter, type CDM2e. This usually required about 1.0 L of Tris buffer for 800 mL of sample, and 1 M iPr_2FP (0.2 mL/L) was added to the sample. A small precipitate was removed by centrifugation for 15 min at 7800*g*. The sample was then applied to a DEAE-Sephadex column (6 × 15 cm) which had been previously equilibrated with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.05 M NaCl and polybrene (100 mg/L). After washing the column with 2 L of the same buffer, the protein was eluted with a salt gradient consisting of 1.5 L of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.05 M NaCl and polybrene (100 mg/L) and 1.5 L of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and polybrene (100 mg/L). Factor XII eluted with the main protein peak which appeared between 300 and 1200 mL of eluate.

(c) *Second Heparin–Agarose Column Chromatography.* Polybrene (100 mg/L) and 1 M iPr_2FP (0.2 mL/L) were added to the factor XII from the DEAE-Sephadex column, and the sample was dialyzed overnight against 40 L of 0.05 M imidazole (pH 6.0) containing 0.06 M NaCl. A small precipitate which formed was removed by centrifugation (15 min at 7800*g*) and the sample was applied to a second heparin–agarose column (6 × 18 cm) at a flow rate of 200 mL/h using a peristaltic pump. The second heparin–agarose column was previously equilibrated with the imidazole buffer described above. After applying the sample, the column was washed with 7 to 8 L of 0.05 M imidazole-HCl buffer (pH 6.0), containing 0.25 M NaCl at a flow rate of 400 mL/h. The protein was eluted from the column by a linear salt gradient formed by 1.8 L of 0.05 M imidazole-HCl buffer (pH 6.0) containing 0.25 M NaCl and polybrene (100 mg/mL) and 1.8 L of 0.05 M imidazole-HCl buffer (pH 6.0) containing 1.0 M NaCl and polybrene (100 mg/L). The flow rate was maintained at 400

TABLE I: Purification of Bovine Factor XII.

Purification Step	Vol (mL)	Total Protein (mg)[a]	Total Act. (Units)	Sp Act. (Units/mg)	Recovery (%)	Purification (Fold)
Plasma	15 000	10.5×10^5	15 000	0.014	100	1
$AmSO_4$ fractionation	6 000	4.6×10^5	13 200	0.029	88	2.1
First heparin-agarose	1 220	11 000	12 200	1.11	81	79.3
DEAE-Sephadex	830	4 550	7 470	1.64	50	117
Second heparin-agarose	450	240	4 050	16.9	27	1 200
CM-cellulose	125	64	3 150	49.2	21	3 510
Arginine-agarose	240	15	2 930	195	20	13 900
Benzamidine-agarose	118	14	2 760	197	18	14 100

[a] Protein concentration was determined by absorption employing $E_{280}^{1\%} = 10.0$ for plasma and subsequent steps up to the CM-cellulose column; an $E_{280}^{1\%} = 14.2$ was employed for the arginine-agarose column and the benzamidine-agarose column.

mL/h. Factor XII was eluted from the column in the second protein peak. The active fractions were pooled and polybrene was added to a final concentration of 100 mg/L. The heparin-agarose column was reused after washing with 500 mL of 0.1 M Tris base (pH 10.6) containing 2 M NaCl.

(d) *CM-Cellulose Column Chromatography.* The factor XII sample from the second heparin-agarose column was dialyzed overnight against 18 L of 0.05 M sodium acetate buffer (pH 5.2) containing 0.06 M NaCl. A small precipitate that formed was removed by centrifugation (15 min at 7800*g*) and the sample was applied to a CM-cellulose column (3.3 × 13 cm) at a flow rate of 300 mL/h. The column was then washed with 150 mL of the same buffer and the protein was eluted with a gradient composed of 500 mL of 0.05 M sodium acetate buffer (pH 5.2) containing 0.06 M NaCl and polybrene (100 mg/L) and 500 mL of 0.05 M sodium acetate buffer (pH 5.2) containing 0.5 M NaCl and polybrene (100 mg/L). Factor XII consistently appeared in the second peak from this column.

(e) *Arginine-Agarose and Benzamidine-Agarose Column Chromatography.* Polybrene (100 mg/L) was added to the pooled fractions of factor XII from the CM-cellulose column and the solution was dialyzed overnight against 10 L of 0.02 M imidazole-HCl buffer (pH 6.0) containing 0.02 M NaCl. The dialyzed solution was applied to an arginine-agarose column (2.5 × 6 cm) which had been previously equilibrated with 0.02 M imidazole-HCl buffer (pH 6.0) containing 0.02 M NaCl and polybrene (50 mg/L). Factor XII appeared at the column breakthrough and the column was then washed with the same buffer. All protein fractions, as determined by absorbance at 280 nm, were pooled and the sample was applied to the benzamidine-agarose column (3.3 × 13 cm) which was previously equilibrated with 0.02 M imidazole-HCl buffer (pH 6.0) containing 0.02 M NaCl and polybrene (50 mg/L). The column was eluted by a linear gradient consisting of 250 mL of 0.02 M imidazole-HCl buffer (pH 6.0) containing 0.02 M NaCl and polybrene (50 mg/L) and 250 mL of 0.02 M imidazole-HCl buffer (pH 6.0) containing 1.0 M NaCl and polybrene (50 mg/L). Factor XII activity was eluted from the column with the single protein peak. The arginine-agarose and benzamidine-agarose columns were reused after washing with 100 mL of 0.1 M Tris base (pH 10.6) containing 2 M NaCl and 1 M guanidine-HCl.

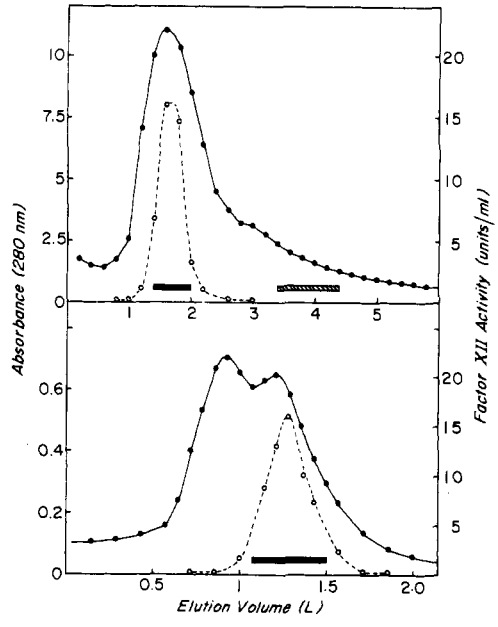

FIGURE 1: Elution pattern for heparin-agarose column chromatography. The top panel shows the elution pattern for factor XII activity from the first heparin-agarose column. Protein was eluted from the column (8.0 × 20 cm) with a linear gradient formed by 3 L of 0.02 M Tris-HCl buffer (pH 7.2), containing 0.15 M NaCl and 3 L of 0.02 M Tris-HCl buffer (pH 7.2), containing 0.6 M NaCl. Both solutions also contained polybrene (50 mg/L) and 2×10^{-4} M iPr_2FP. The flow rate was 300 mL/h. Factor XII activity was assayed as described under Methods: (●—●) absorbance at 280 nm; (○- - -○) clotting activity. The fractions shown with the solid bar were pooled. The bottom panel shows the elution pattern for the second heparin-agarose column. The column (6 × 18 cm) was washed with 7 L of 0.05 M imidazole-HCl buffer (pH 6.0) containing 0.25 M NaCl, and the protein was eluted with a linear gradient formed by 1.8 L of 0.05 M imidazole-HCl buffer (pH 6.0) containing 0.25 M NaCl and polybrene (100 mg/L) and 1.8 L of 0.05 M imidazole-HCl buffer (pH 6.0) containing 1.0 M NaCl and polybrene (100 mg/L). The flow rate was 400 mL/h: (●—●) absorbance at 280 nm: (○- - -○) clotting activity. The fractions shown with the solid bar were pooled. The absorbance due to protein was calculated by subtracting the basal abosrbance of the imidazole buffer.

Results

Purification of Bovine Factor XII. A summary of the purification of factor XII is shown in Table I. Fourteen to eighteen milligrams of purified factor XII was obtained from 15 L of plasma with an overall yield of 18 to 20%. The increase of specific activity was 13 000- to 14 000-fold, and the specific activity of the final preparation was nearly 200 units/mg of protein. The purification procedure consists of eight steps including adsorption of contaminants on barium sulfate, ammonium sulfate fractionation, two heparin-agarose column chromatography steps in addition to chromatography on DEAE-Sephadex A-50, CM-cellulose, arginine-agarose, and benzamidine-agarose.

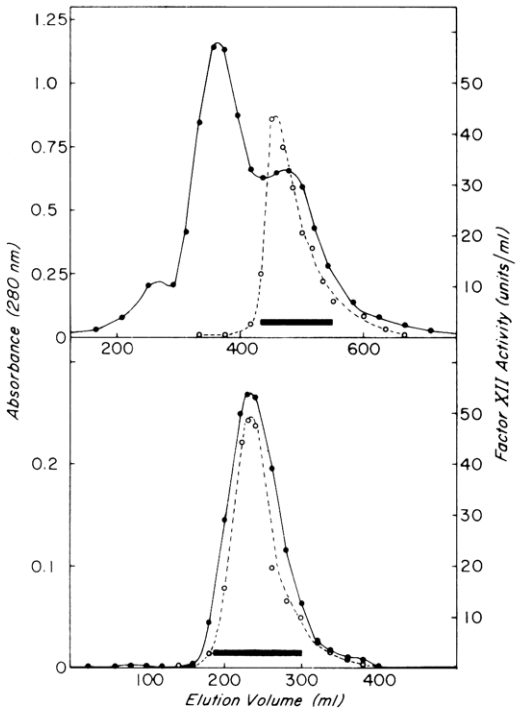

FIGURE 2: Elution pattern for CM-cellulose and benzamidine-agarose column chromatography. The top panel shows the elution pattern for factor XII from the CM-cellulose column. Protein was eluted from the column (3.3 × 13 cm) with a linear gradient formed by 500 mL of 0.05 M sodium acetate buffer (pH 5.2) containing 0.06 M NaCl and polybrene (100 mg/L) and 500 mL of 0.05 M sodium acetate buffer (pH 5.2) containing 0.5 M NaCl and polybrene (100 mg/L). The flow rate was 300 mL/h. Factor XII activity was assayed as described under Methods: (●—●) absorbance at 280 nm; (O- - -O) clotting activity. The fractions shown with the solid bar were pooled. The bottom panel shows the elution pattern for factor XII from the benzamidine-agarose column. Protein was eluted from the column (3.3 × 13 cm) by a linear salt gradient formed by 250 mL of 0.02 M imidazole-HCl buffer (pH 6.0) containing 0.02 M NaCl and polybrene (50 mg/L) and 250 mL of 0.02 M imidazole-HCl buffer (pH 6.0) containing 1.0 M NaCl and polybrene (50 mg/L): (●—●) absorbance at 280 nm; (O- - -O) clotting activity. The fractions shown with the solid bar were pooled. The absorbance due to protein was calculated by subtracting the basal absorbance of the imidazole buffer.

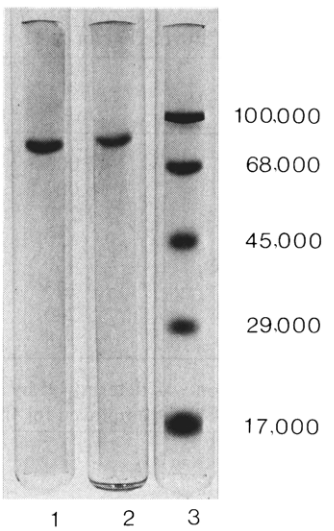

FIGURE 3: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of bovine factor XII. Electrophoresis was carried out in 7.5% polyacrylamide gels at 8 mA/tube for 4 h as described under Methods. Gel 1 is 10 μg of factor XII before reduction; gel 2 is 10 μg of factor XII after reduction; gel 3 includes the following standards: phosphorylase (mol wt 100 000), bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (29 000), and myoglobin (17 000). The anode was at the bottom of the gel.

Protease inhibitors, including soybean trypsin inhibitor and iPr_2FP, were added to the various protein solutions during the early stages of purification to protect factor XII from proteolytic degradation. Polybrene, which prevents the surface contact activation of factor XII, was also added to the various buffers throughout the entire procedure.

The steps employed up to the first heparin-agarose column were developed for the simultaneous purification of factor XII and factor XI. Factor XII eluted from the first heparin-agarose column in the fractions between 1.2 and 2.0 L of eluate (solid bar, top panel, Figure 1) and was readily separated from factor XI which eluted in a later fraction (shown by the hatched bar).

A small purification of factor XII was obtained by DEAE-Sephadex column chromatography. Approximately half of the protein appeared in the breakthrough fraction. Factor XII eluted in the next peak along with the bulk of the protein. This step was important, however, since a protease(s) which splits factor XII into two chains was removed in this step.

In the second heparin-agarose column chromatography step, about 95% of the contaminant protein was removed by extensive washing of the column with buffer containing 0.25 M NaCl. Factor XII was then eluted in the second protein peak as shown in the bottom panel, Figure 1. Extensive washing of the second heparin-agarose column was possible since factor XII binds rather tightly to the heparin-agarose at pH 6.0 in contrast to pH 7.2 which was employed in the first heparin-agarose column.

Factor XII was purified about threefold on CM-cellulose (top panel, Figure 2). On this column, factor XII eluted in the descending edge of the second protein peak.CM-Sephadex or CM-cellulose columns have been used by other investigators in an early stage of purification of factor XII. The use of CM-cellulose in the early stage of purification of bovine factor XII should be avoided, however, since some activation of factor XII has been observed under these conditions.

All of the factor XII activity appeared in the breakthrough peak of the arginine-agarose column, while three-fourths of the contaminant protein remained bound to the column. Accordingly, the assay of factor XII was usually omitted and the entire breakthrough peak was applied to the benzamidine-agarose column. Factor XII was eluted from the benzamidine-agarose column as a single peak and the clotting activity paralleled the protein (lower panel, Figure 2). This final step was usually omitted, unless the factor XII preparation was contaminated with small amounts of two-chain factor XII which has tentatively been identified as activated factor XII. In this situation, the two-chain molecule appeared as a second peak slightly overlapping the major protein peak.

Purified factor XII was stable at −20 °C for several months when stored in 0.02 M imidazole-HCl buffer (pH 6.0) containing 0.15 M NaCl and polybrene (50 mg/L).

Factor XII (25 ng) prepared by this method clotted factor XII deficient plasma in 165 s employing the assay described in Methods. In the absence of factor XII, the clotting time was greater than 600 s. Factor XII contained no detectable factor XII_a as suggested by the fact that factor XII deficient plasma had an absolute requirement for kaolin when clotting was initiated in the presence of factor XII. In the absence of kaolin, the coagulation time for factor XII deficient plasma was greater than 600 s in the presence or absence of factor XII. Furthermore, no esterase activity was detectable for factor XII in the presence or absence of kaolin with tosyl-L-arginine

Factor XII										Thr	Pro	Pro	Trp	Lys	Gly	Pro	?	LYS	His
Pancreatic trypsin inhibitor				Arg	Pro	Asp	Phe	Cys	Leu	Glu	Pro	Pro	Tyr	Thr	Gly	Pro	Cys	LYS	Ala
Bovine Colostrum inhibitor	Phe	Gln	Thr	Pro	Pro	Asp	Leu	Cys	Gln	Leu	Pro	Gln	Ala	Arg	Gly	Pro	Cys	LYS	Ala
Turtle egg white inhibitor	(Lys ,	Glx ,	Asx ,	Gly ,	Arg)	Asp	Ile	Cys	Arg	Leu	Pro	Pro	Glu	Gln	Gly	Pro	Cys	LYS	Gly
Snail inhibitor K		Pyr	Gly	Arg	Pro	Ser	Phe	Cys	Asn	Leu	Pro	Ala	Glu	Thr	Gly	Pro	Cys	LYS	Ala
Russell's viper venom inhibitor		His	Asp	Arg	Pro	Thr	Phe	Cys	Asn	Leu	Ala	Pro	Glu	Ser	Gly	Arg	Cys	ARG	Gly

FIGURE 4: Amino-terminal sequence of bovine factor XII. The amino-terminal sequences of several protease inhibitors are also shown (Laskowski et al., 1974). Residue 8 in factor XII was not identified and is shown as a question mark. The lysine shown in capital letters is in the active site of the protease inhibitors. Amino acids that are identical with factor XII are shown in blocks.

TABLE II: Amino Acid and Carbohydrate Compositions of Bovine Factor XII.

Component	Bovine Factor XII (Residues/74 000)
Amino acid	
Lysine	25.5
Histidine	26.5
Arginine	33.1
Aspartic acid	44.1
Threonine	31.9
Serine	34.7
Glutamic acid	65.6
Proline	48.5
Glycine	47.5
Alanine	47.7
Half-cystine	25.7[a]
Valine	30.2
Methionine	1.4
Isoleucine	13.3
Leucine	50.4
Tyrosine	13.3
Phenylalanine	21.3
Tryptophan	14.0[b]
Carbohydrate	
Hexose	13.9 (3.4%)
N-Acetylhexosamine	15.6 (4.7%)
N-Acetylneuraminic acid	12.9 (5.4%)
Protein (%)	86.5
Carbohydrate (%)	13.5

[a] The same value was obtained when determined as cysteic acid or *S*-carboxymethylcysteine. [b] Determined in the amino acid analyzer after base hydrolysis as described under Methods.

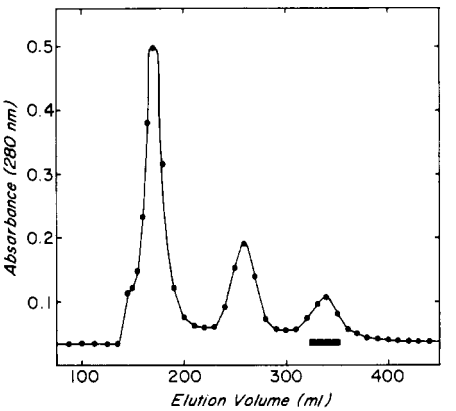

FIGURE 5: Gel filtration of a cyanogen bromide digest of *S*-carboxymethyl bovine factor XII. The lyophilized digest (20 mg) was dissolved in 5 mL of 9% formic acid containing 6 M urea and applied to a Sephadex G-100 column (2.5 × 92 cm). The cyanogen bromide peptides were eluted with 9% formic acid in 6 M urea. The flow rate was 4.3 mL/60 min; fractions were collected in 5 mL/tube. The fractions shown by the solid bar were pooled and used for sequence analysis.

methyl ester as substrate employing an enzyme concentration of 48 μg of factor XII per 0.25 mL of reaction mixture by the method of Roberts (1958). In this assay, 0.4 μg of trypsin per 0.25 mL hydrolyzed 5 μmol of substrate in 30 min.

Factor XII was free of other coagulation factors such as factor XI and Fitzgerald factor (high molecular weight kininogen). It showed substantial activity in Fletcher factor deficient plasma. This activity was probably due in part to an artifact of the assay system, however, since the Fletcher factor deficient plasma clotted in 260 s in the presence of kaolin. Thus, it seems likely that high levels of factor XII interfere with the specificity of the Fletcher factor clotting assay. In contrast, factor XII deficient plasma, factor XI deficient plasma, and Fitzgerald factor deficient plasma had clotting times of greater than 600 s in the presence of kaolin and appeared to be nearly free of their respective coagulant protein.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis of Bovine Factor XII. A single, sharp protein band was observed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis for factor XII before and after reduction with 2-mercaptoethanol (samples 1 and 2, Figure 3). The apparent molecular weight estimated by this technique was 78 000 for the reduced protein. These experiments indicate that bovine factor XII is a single polypeptide chain. A mol wt of 78 000 for factor XII is slightly higher than the minimal molecular weight determined by sedimentation equilibrium as described below and is probably due to decreased binding of sodium dodecyl sulfate to the glycoprotein (Segrest and Jackson, 1972).

Sedimentation Equilibrium Studies on Bovine Factor XII. Sedimentation equilibrium experiments on factor XII in 6 M guanidine hydrochloride indicated the protein was heterogeneous at the concentrations tested. The smallest molecular weight species calculated according to Teller (1973) was 76 600 ± 2 400 for the *S*-pyridylethyl factor XII. The number average molecular weight, the weight average molecular weight, and the *Z* average molecular weight were 83 100 ± 2000, 89 700 ± 6 300, and 107 700 ± 12 100, respectively. A minimal mol wt of 74 000 was obtained for factor XII after correction for 25.7 *S*-pyridylethyl residues. Whether the observed heterogeneity of the protein is due to monomer aggregation is not clear since the experiments were carried out in 6 M guanidine hydrochloride. It was not due to the formation of stable aggregation products since the protein still migrated on sodium dodecyl sulfate–polyacrylamide gels as a single, sharp band after centrifugation.

Amino Acid and Carbohydrate Compositions of Bovine Factor XII. The amino acid and carbohydrate compositions of factor XII are shown in Table II. Bovine factor XII is composed of 86.5% protein and 13.5% carbohydrate. This

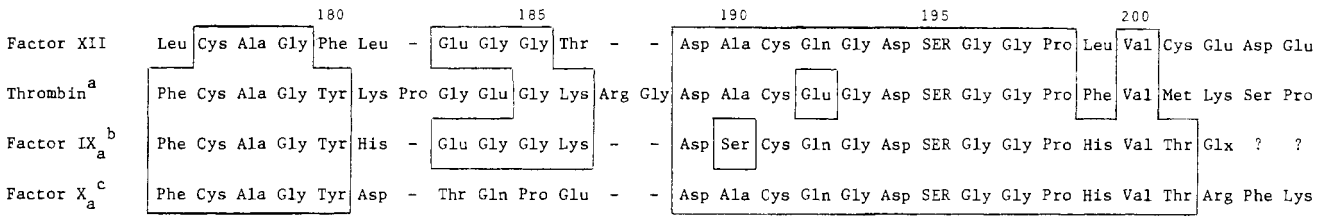

FIGURE 6: Active-site sequences of bovine factor XII and several other coagulation enzymes. The amino acid sequence of the active-site region of factor XII was determined from two different cyanogen bromide peptide preparations. One was prepared from *S*-carboxymethyl factor XII and the other from *S*-pyridylethyl factor XII. Amino acid residues in factor XII that are identical with other serine proteases are shown in blocks. Dashes refer to spaces that have been inserted to bring the four proteins into alignment for better homology. Amino acids that are not known are shown as a question mark. The number system is similar to that of chymotrypsin where the active-site serine is residue 195. Literature references are: a, from Magnusson et al. (1975); b, from Enfield et al. (1974); c, from Titani et al. (1975).

corresponds to 64 000 g of protein and 10 000 g of carbohydrate per 74 000 g of glycoprotein. The methionine content of bovine factor XII was 1.4 residues, suggesting the presence of two methionine residues per mol of protein. The carbohydrate in factor XII includes 3.4% hexose, 4.7% *N*-acetylhexosamine, and 5.4% *N*-acetylneuraminic acid.

Amino-Terminal and Active-Site Sequences of Bovine Factor XII. The amino-terminal sequence for the first 10 residues of factor XII is shown in Figure 4 along with the amino-terminal sequences for several protease inhibitors. Threonine was identified as the amino-terminal residue of factor XII and no other amino acids were detected. The yield of the first five residues was not readily determined due to the difficulty in quantitating these residues. The yield of glycine in position 6 was 0.63 equiv per mol of protein. No amino acid was found in position 8.

It was then of interest to examine the amino acid sequence of factor XII in another region of the molecule which might contain an active-site sequence similar to other serine proteases. Furthermore, the amino acid analysis of factor XII indicated the presence of two methionine residues per mol of protein. It seemed likely that one of the two methionines might be located 15 to 20 residues prior to an active-site serine since this methionine is highly conserved in various serine proteases. Accordingly, a cyanogen bromide digestion was made of *S*-carboxymethyl factor XII and *S*-pyridylethyl factor XII, and the polypeptide fragments were fractionated by gel filtration on Sephadex G-100 (Figure 5). Three major polypeptide peaks were obtained with each preparation. The third polypeptide peak migrated as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a molecular weight of approximately 8000–10 000. The amino-terminal sequence of this polypeptide chain is shown in Figure 6 along with the active-site regions of several other plasma proteases. Twenty-six residues were identified in this peptide employing either the *S*-carboxymethyl or the *S*-pyridylethyl peptide. Leucine (about 0.7 equiv per 10 000 mol wt) was identified as the amino-terminal residue and no other amino acids were detected. The repetitive yields for the degradations were about 92%. The active-site serine corresponding to Ser-195 in chymotrypsin appeared in position 17 of the peptide. It is clear from these data that bovine factor XII contains an amino acid sequence which is characteristic of the active-site region of a number of serine proteases.

Discussion

The present data indicate that bovine factor XII is a single-chain glycoprotein with a mol wt of 74 000. This molecular weight is slightly smaller than those reported for bovine, human, and rabbit factor XII by other investigators including Komiya et al. (1972), Revak et al. (1974), and Chan and Movat (1976). The amino acid composition of bovine factor XII is fairly similar to that of human factor XII (Revak et al., 1974), although some differences are quite apparent. Human factor XII is also a single-chain molecule (Revak et al., 1974; Chan and Movat, 1976). Thus, the human and bovine preparations are quite similar, but differ from the three-chain molecule reported for factor XII from rabbit plasma (Cochrane et al., 1972).

During the isolation procedure, bovine factor XII was separated from an unknown protease(s) that splits the single-chain molecule into two chains held together by a disulfide bond(s). Separation of factor XII from the protease(s) was achieved primarily by the DEAE-Sephadex column chromatography step. Polybrene and other protease inhibitors were also added at various steps of the isolation procedure to restrict the degradation of the single-chain factor XII. The two-chain molecule has the same molecular weight as the precursor molecule. Furthermore, it has one and a half times the specific activity of the single-chain factor XII in the coagulation assay in the presence of kaolin. It has essentially no coagulant activity, however, in the absence of kaolin. The two-chain factor XII contains considerable esterase activity toward tosyl-L-arginine methyl ester, while the single-chain factor XII has no esterase activity in the presence or absence of kaolin. Accordingly, the two-chain molecule has been tentatively identified as factor XII$_a$. Structural studies on this preparation are presently being carried out to clarify this important question.

The activation of human factor XII by proteolytic enzymes such as plasmin, trypsin, and kallikrein has been reported by a number of different investigators (Kaplan and Austen, 1971; Burrowes et al., 1971; Wuepper, 1972; Cochrane et al., 1973; Bagdasarian et al., 1973). During this reaction, human factor XII is cleaved into fragments with molecular weights of 52 000, 40 000, and 28 000 (Revak et al., 1974). In preliminary experiments, we have observed that bovine factor XII is extremely resistant to trypsin and plasmin in the presence or absence of kaolin. Furthermore, bovine factor XII was neither degraded nor converted to an activated coagulation factor by these proteolytic enzymes. In addition, we have been unable to show an activation of bovine factor XII in the presence of kaolin as measured by an esterase assay indicating that kaolin alone does not activate factor XII directly. Recently, Griffin and Cochrane (1976) reported that the activation of human factor XII by kallikrein is stimulated by high molecular weight kininogen in addition to kaolin. Thus, it appears likely that the role of kaolin and other surfaces in the contact activation of blood coagulation might be merely to concentrate several proteins, including factor XII, and thus permitting them to interact and trigger the coagulation process.

Bovine factor XII contains an active-site region which shows considerable homology with that found in the carboxyl-terminal region of thrombin, factor IX_a, and factor X_a (Figure 6). This sequence is also homologous to that found in pancreatic trypsin (Walsh and Neurath, 1964) and plasmin (Groskopf et al., 1969). These data suggest that factor XII_a participates in various physiological reactions, such as blood coagulation, by splitting protein substrates with a peptide bond containing a basic amino acid. Indeed, factor XII_a has substantial esterase activity toward benzoyl-L-arginine ethyl ester (Schoenmakers et al., 1965). A specificity toward basic amino acids is also consistent with the fact that residue 189 in factor XII (employing the chymotrypsin numbering system) is aspartic acid. This residue is located in the bottom of the binding pocket in trypsin (Stroud et al., 1971) and forms an ion pair with an arginine or lysine residue in the substrate (Mares-Guia and Shaw, 1965; Ruhlmann et al., 1973; Blow et al., 1974; Sweet et al., 1974; Krieger et al., 1974). In chymotrypsin, residue 189 is serine which is uncharged at neutral pH, and this residue gives rise to a different substrate specificity. Factor XII also contains an aspartic acid residue in position 194 next to the active-site serine. In the activation of chymotrypsinogen and trypsinogen, a new positively charged α-amino group of isoleucine is generated, and this new amino-terminal amino acid folds into the interior of the protein forming an ion pair with the carboxyl group of Asp-194 (Sigler et al., 1968; Stroud et al., 1975). It seems probable that a similar mechanism may occur in the activation of factor XII when it is converted to factor XII_a by limited proteolysis.

Factor XII contains an amino-terminal sequence which shows considerable homology with the reactive-site region of a number of protease inhibitors (Figure 4). Whether this portion of factor XII has inhibitory activity toward serine proteases is not known. It is possible that the amino-terminal region of factor XII masks the active-site region of this coagulation protein. Accordingly, one possible effect of kaolin on factor XII could be the unfolding of the amino-terminal portion of the molecule making the catalytic site region available for a substrate. This could lead to the conversion of a small amount of factor XII to factor XII_a in the absence of proteolysis. If this occurs, however, it is a very minor pathway of factor XII activation since no esterase activity was detected for factor XII in the presence of kaolin. It is also possible that the amino-terminal portion of factor XII is cleaved from factor XII during blood coagulation, and this fragment participates as an inhibitor of one or more serine proteases that are involved in the coagulation process.

Bovine factor XII described in the present paper readily corrected factor XII deficient plasma employing assay plasma from three unrelated patients with Hageman trait. Bovine factor XII also showed substantial clotting activity in Fletcher factor deficient plasma (Hathaway et al., 1965), a deficiency characterized by the absence of prekallikrein (Wuepper, 1972). The bovine preparation described in the present investigations was free, however, of prekallikrein as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In this system, prekallikrein migrates much slower than factor XII (R. Heimark of our laboratory, unpublished data). The reasons for prekallikrein-like activity for the pure factor XII are not understood. A similar effect has also been noted by Saito et al. (1975) for human factor XII.

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