Characterization of Bovine Factor XII_a (Activated Hageman Factor)†

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ABSTRACT: Factor XII_a (activated Hageman factor) was isolated from bovine plasma by ammonium fractionation followed by heparin–agarose, carboxymethylcellulose, and arginine–agarose column chromatography. It was separated from factor XII in the final step by chromatography on benzamidine–agarose. Factor XII_a has a molecular weight of approximately 74 000 and is composed of a heavy and light chain held together by a disulfide bond(s). The amino-terminal sequence of the heavy chain is Thr-Pro-Pro-Trp-Lys-Gly-Pro-Lys-Lys-His-Lys-Leu- which is the same as the precursor protein. The carboyl-terminal residue in this polypeptide chain is arginine. The amino-terminal sequence of the light chain is Val-Val-Gly-Gly-Leu-Val-Ala-Leu-Pro-Gly-Ala-?-Pro-Tyr-Ile-. This latter sequence is homologous with the amino-terminal sequence of a number of plasma serine proteases when compared with the chain containing the active-site serine residue. These data suggest that factor XII is converted to factor XII_a by the cleavage of a specific internal arginyl–valine peptide bond. Factor XII_a, in contrast to factor XII, has hydrolase activity toward arginine-containing substrates and is readily inhibited by antithrombin III and diisopropyl phosphorofluoridate. The inhibitors, in each case, are bound to the light chain of factor XII_a which contains the active-site serine residue.

Factor XII (Hageman factor)[1] is present in plasma in a precursor or inactive form (see Davie and Fujikawa, 1975, for a recent review). Griffin and Cochrane (1976) and Chan et al. (1976) reported that human factor XII is converted to factor XII_a in the presence of kallikrein, high-molecular-weight (HMW) kininogen, and kaolin. Factor XII_a, in turn, participates in the initiation of blood coagulation in the intrinsic pathway (Ratnoff and Colopy, 1955; Ratnoff and Rosenblum, 1958), the extrinsic pathway (Soulier and Prou-Wartelle, 1960; Altman and Hemker, 1967; Gjonnaess, 1972; Laake and Osterud, 1974; Saito and Ratnoff, 1975; Kisiel et al., 1977), as well as fibrinolysis (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961; Ogston et al., 1969) and kinin formation (Margolis, 1959).

We have described a method for the isolation of bovine factor XII from plasma and have characterized this protein as to its size, composition, and amino acid sequence of the amino-terminal and active-site regions (Fujikawa et al., 1977). In these experiments, factor XII was shown to be a glycoprotein with a molecular weight of 74 000 as determined by sedimentation equilibrium. It contains a single polypeptide chain with an amino-terminal sequence of Thr-Pro-Pro-Trp-Lys-Gly-Pro-?-Lys-His-, and this sequence shows some homology with a number of serine protease inhibitors. It also contains a sequence of -Gly-Asp-SER-Gly-Gly- in the carboxyl-terminal region of the molecule which is characteristic of all serine proteases. During the isolation of factor XII, it was observed that a two-chain molecule often was formed when a DEAE[2]-Sephadex column chromatography step was omitted during the early stages of protein fractionation. In the present communication, we wish to describe the isolation and characterization of this two-chain molecule which has been identified as factor XII_a.

Experimental Section

Materials. Bovine antithrombin III was prepared by affinity chromatography employing a modification of the method of Thaler and Schmer (1975) as described by Kurachi et al. (1976). Tosyl-L-arginine methyl ester hydrochloride was obtained from Mann Research Lab., New York, N.Y. *N*-Benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide hydrochloride (S-2160) was a product of Kabi Diagnostica, Stockholm, Sweden. *N,N'*-Methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and 2-mercaptoethanol were obtained from Eastman Kodak Co., Rochester, N.Y. Sephadex G-25 and G-100 were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Diisopropyl phosphorofluoridate (DFP) was obtained from Aldrich Chemical Co., Milwaukee, Wis. A stock solution was prepared by diluting 1 g (one ampule) with 4.5 mL of anhydrous 2-propanol and stored at −20 °C. Tritium-labeled DFP (0.25 mCi/0.051 mg in 0.25 mL) and Aquasol were purchased from New England Nuclear, Boston, Mass. Rabbit brain cephalin was purchased from Sigma Chemical Co., St. Louis, Mo., and a stock suspension was made by suspending the contents of one vial in 100 mL of 0.15 M NaCl. Aliquots (1 mL) were stored at −20 °C. Lithium heparin (154 units/mg) was purchased from Riker Lab., Inc., Northridge, Calif. Human factor XII deficient plasma was obtained from a Hageman trait patient in the Seattle area. This sample was kindly provided by the Puget Sound Blood Center, Seattle. Human Fletcher factor deficient plasma (GK-1702) and human Fitzgerald factor deficient plasma (GK-1601) 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. Benzamidine–agarose with an ε-aminocaproic acid spacer was prepared by a modification of the procedure of Schmer (1972) as previously described (Fujikawa et al., 1977). Carboxypeptidase A and B were products of Worthington Biochemical Corp., Freehold, N.J. Prior to use, the carboxypeptidase was treated with DFP by the method of Fraenkel-Conrat et al. (1955). All other chemicals were commercial preparations of the highest quality available.

Methods. The protein concentration of purified bovine factor XII was determined by absorbance employing an

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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: DFP, diisopropyl phosphorofluoridate; CM, carboxymethyl; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

$E_{280nm}^{1\%}$ = 14.2 (Fujikawa et al., 1977), and the same value of $E_{280nm}^{1\%}$ was assumed for factor XII$_a$. A value of $E_{280nm}^{1\%}$ = 6.0 was employed for bovine antithrombin III (Kurachi et al., 1976).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments (as shown in Figure 2) were carried out by the method of Weber and Osborn (1969) using 7.5% gels. Samples (10-15 μg of protein 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 2 h with 2% Coomassie brilliant blue and destained electrophoretically. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis experiments (as shown in Figure 8) were carried out by a modification of the method of Weber and Osborn (1969) as previously described (Kurachi et al., 1976). The buffer solution for these experiments was 0.1 M Tris-phosphate (pH 7.0) containing 0.1% sodium dodecyl sulfate, and 7.5% polyacrylamide gels were employed.

S-Carboxymethyl derivatives of the heavy and light chain of factor XII$_a$ were prepared as follows. Factor XII$_a$ (22 mg) was reduced and carboxymethylated according to the method of Crestfield et al. (1963). Salt and excess reagent were removed by gel filtration on Sephadex G-25 (1.6 × 27 cm column) with 20% acetic acid in 5% formic acid followed by lyophilization. The lyophilized material (22 mg) was dissolved in 5 mL of 3 M urea-9% formic acid solution and applied to a Sephadex G-100 column (1.8 × 83 cm). Elution was carried out with 3 M urea-9% formic acid solution at a constant flow rate of 10 mL/h. Each of the two peaks was pooled, desalted on a Sephadex G-25 column, and lyophilized. The first peak contained 9.9 mg and the second peak contained 5.6 mg of material.

Amino-terminal sequence analysis was performed with a Beckman sequencer, Model 890C, using the *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 peptide system of Hermodson et al. (1972). Phenylthiohydantoin amino acid residues were identified by gas chromatography after silylation or directly by high-pressure liquid chromatography (Bridgen et al., 1976). Protein concentration of the sample for the sequence study was determined by amino acid analysis. The yield for each residue was not corrected for losses that occurred during the conversion to and extraction of the phenylthiohydantoin amino acids. The sequence analysis on the polypeptide chains was carried out on 75 nmol of the *S*-carboxymethyl heavy chain of factor XII$_a$ assuming a molecular weight of 46 000 and 77 nmol of the *S*-carboxymethyl light chain of factor XII$_a$ assuming a molecular weight of 28 000. Carboxyl-terminal amino acids were determined by the method of Fraenkel-Conrat et al. (1955) employing pancreatic carboxypeptidase A and B. The carboxymethylated heavy chain of factor XII$_a$ (19.5 nmol) was incubated at 37 °C with carboxypeptidase A (10 μg) or carboxypeptidase B (7.2 μg) in 0.05 M Tris-HCl buffer (pH 8.2) containing 4 M urea. After incubation for 24 h, the amino acids released were analyzed by a Durrum Model D-500 amino acid analyzer. The control experiments without the substrate were run simultaneously.

For an experiment of the inhibition of factor XII$_a$ by radioactive DFP, factor XII$_a$ (120 μg) was incubated with 10 μL of 0.1 M DFP and 40 μL of [^{3}H]DFP (total 24.8 × 10^6 cpm) in 0.2 mL of 0.05 M Tris-HCl buffer (pH 7.5). After incubation for 1 h, the sample was dialyzed extensively against 0.05 M phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulfate and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis before and after reduction with 2-mercaptoethanol. The gels containing about 30 μg of factor XII or factor XII$_a$ were then stained, sliced, and solubilized in 0.5 mL of 30% H_2O_2 at 80 °C. The solubilized samples were mixed with 10 mL of Aquasol and radioactivities were counted in a Beckman Model LS-100C liquid scintillation counter.

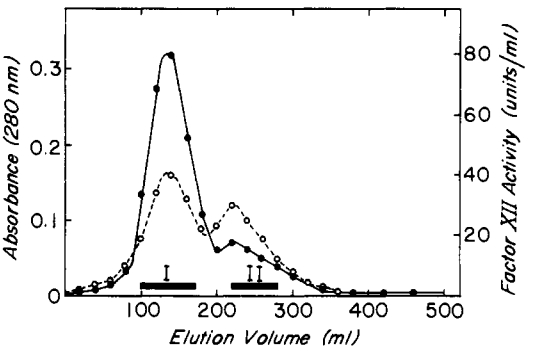

FIGURE 1: The separation of factors XII and XII$_a$ by benzamidine-agarose column chromatography. About 19 mg of protein from the arginine-agarose column chromatography (as previously described, Fujikawa et al., 1977) was applied to a benzamidine-agarose column (2.2 × 13 cm). The column was previously equilibrated with 0.02 M imidazole hydrochloride buffer (pH 6.0) containing 0.02 M naCl and polybrene (0.5 mg/L). The protein was eluted from the column by a linear gradient composed of 250 mL of 0.02 M NaCl in 0.02 M imidazole hydrochloride buffer (pH 6.0) containing polybrene (0.5 mg/L) and 250 mL of 1 M NaCl in 0.02 M imidazole hydrochloride buffer (pH 6.0) containing polybrene (0.5 mg/mL). Fractions (10 mL) were collected at a flow rate of about 100 mL/h. Factor XII activity was assayed as described under Methods. Absorbancy due to protein was calculated by subtracting the basal absorbance of the imidazole buffer. (○-○) Activity; (●-●) absorbancy at 280 nm.

Factor XII$_a$ was prepared in a manner identical with that described for the isolation of factor XII (Fujikawa et al., 1977), except that the DEAE-Sephadex column chromatography step was omitted. This resulted in the conversion of 5-60% of the factor XII to factor XII$_a$ during the isolation procedure. Factor XII$_a$ was separated from factor XII in the final column chromatography step employing benzamidine-agarose as described under Results.

Factor XII or factor XII$_a$ was assayed with kaolin by the same method previously described (Fujikawa et al., 1977). Amidase activity of factor XII or factor XII$_a$ was measured according to Svendsen et al. (1972) using the chromogenic substrate *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide (S-2160). Esterase activity employing tosyl-L-arginine methyl ester as substrate was measured by the method of Roberts (1958).

Results

Separation of Bovine Factor XII and Factor XII$_a$. The isolation procedure developed in our laboratory for factor XII employed ammonium sulfate fractionation of the starting plasma followed by heparin-agarose, DEAE-Sephadex, CM-cellulose, arginine-agarose, and benzamidine-agarose column chromatography (Fujikawa et al., 1977). This isolation procedure yielded a single-chain factor XII with a molecular weight of 74 000 as determined by sedimentation equilibrium centrifugation. The DEAE-Sephadex step gave a purification of less than twofold and was occasionally omitted. When this step was not included in the isolation procedure, a double peak of factor XII activity was often observed in the final step which was benzamidine-agarose column chromatography (Figure 1). The specific activity of the second peak was about 1.5- to

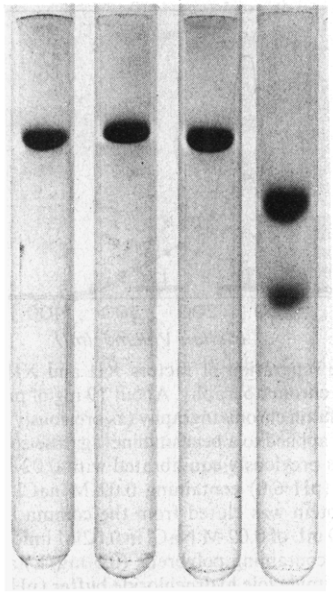

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of bovine factors XII and XII_a. Gel 1, factor XII; gel 2, factor XII reduced with 2-mercaptoethanol; gel 3, factor XII_a; gel 4, factor XII_a reduced with 2-mercaptoethanol. Each gel contained about 10 to 15 μg of protein. The anode was at the bottom of the gels.

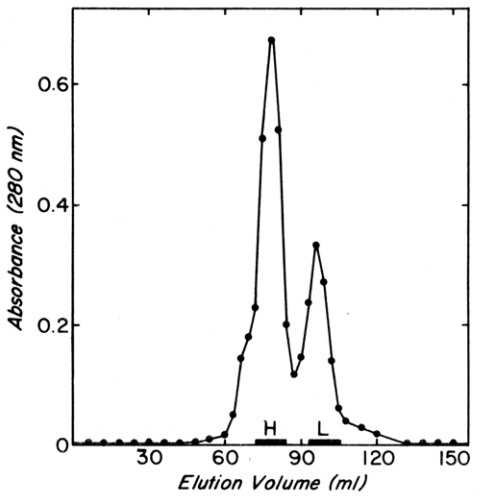

FIGURE 3: Separation of the heavy and light chains of factor XII_a on Sephadex G-100. The *S*-carboxymethyl factor XII_a (22 mg) was applied to a Sephadex G-100 column (1.8 × 83 cm) and elution was carried out with 3 M urea–9% formic acid at a flow rate of 10 mL/h. Fractions, shown by the solid bars, containing peaks I (heavy chain) and II (light chain) were pooled separately, desalted, and lyophilized. The yield of polypeptide was 9.9 mg from peak I and 5.6 mg from peak II.

Factor XII_a	Val	Val	Gly	Gly	Leu	Val	-	Ala	Leu	Pro	Gly	Ala	?	Pro	Tyr	Ile						
Factor IX_a[a]	Val	Val	Gly	Gly	Glu	Asp	-	Ala	Glu	Arg	Gly	Glu	Phe	Pro	Trp	Gln						
Factor X_a[b]	Ile	Val	Gly	Gly	Arg	Asp	Cys	Ala	Glu	Gly	Glu	Cys	-	Pro	Trp	Gln						
Thrombin[c]	Ile	Val	Glu	Gly	Gln	Asp	-	Ala	Glu	Val	Gly	Leu	Ser	Pro	Trp	Gln						
Protein C[d]	Ile	Val	Asp	Gly	Gln	Glu	-	Ala	Gly	Trp	Cys	Glu	Ser	Pro	Trp	?						

FIGURE 4: Amino-terminal sequence of the light chain of factor XII_a and the active-site chain of several other bovine plasma serine enzymes. Amino acid residues that are identical to factor XII_a are shown in blocks. Dashes refer to spaces that have been inserted to bring the five proteins into alignment for better homology. Amino acids that are not known are shown as a question mark. (a) From Enfield et al., 1974; (b) from Titani et al., 1975; (c) from Magnusson et al., 1975; (d) from Kisiel et al., 1976.

2.0-fold greater than the first peak. When the protein under each of the two peaks (shown by the bars) was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, single sharp bands were observed (Figure 2). Peak I (gel 1) has the same migration as peak II (gel 3), indicating that the two proteins have essentially the same molecular weight. In the presence of 2-mercaptoethanol, peak I also migrated as a single sharp band (gel 2) with an apparent molecular weight of 78 000, while peak II gave rise to a heavy and a light polypeptide chain (gel 4). The heavy chain has an apparent molecular weight of about 52 000, and the light chain has an apparent molecular weight of about 31 000. The true molecular weights for these polypeptide chains are probably somewhat lower than those obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Accordingly, we have tentatively assumed molecular weights of 46 000 for the heavy chain and 28 000 for the light chain (see Discussion). These data indicate that peak I is single-chain factor XII which was previously characterized in our laboratory (Fujikawa et al., 1977) and peak II is composed of a heavy and a light chain held together by a disulfide bond(s). These data also suggest that the second peak is factor XII_a which has been activated by limited proteolysis. This was confirmed by showing that the second peak has esterase and amidase activities, and the esterase activity is inhibited by DFP as described below.

Isolation and Characterization of the Heavy and Light Chains of Factor XII_a. Factor XII_a was reduced, *S*-carboxymethylated, and subjected to gel filtration on Sephadex G-100 to separate the two chains. Two major fractions were obtained (Figure 3). The first peak corresponded to the heavy chain of factor XII_a and migrated as a single sharp band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The second peak also migrated as a single band and corresponded to the light chain of factor XII_a.

An amino-terminal analysis was then carried out on the heavy and light chains of factor XII_a. The amino-terminal residue of the heavy chain was threonine, and no other amino acids were detected. The amino acid sequence of the first 12 residues was Thr-Pro-Pro-Trp-Lys-Gly-Pro-Lys-Lys-His-Lys-Leu-. This sequence is identical with and extends that found originally in the amino-terminal end of factor XII (Fujikawa et al., 1977). Also, a lysine residue was clearly identified in position 8 in the present experiments. The yields for residues 2 through 12 were 0.2, 0.3, not determined, 0.7, 0.2, 0.2, 0.3, 0.5, not determined, 0.4, and 0.3 equiv per 46 000 g of protein, respectively. Some overlap occurred after residue 3, presumably due to incomplete cleavage at the previous proline residues (Hermodson et al., 1972). Histidine in position 10 was identified by the spot test of Hermodson et al. (1972).

The amino-terminal sequence of the light chain is shown in Figure 4 along with the amino-terminal sequences of a number of plasma serine proteases. Valine (approximately 0.4 equiv per 28 000 molecular weight) was identified as the amino-terminal residue, and no other amino acids were detected. The repetitive yields for the degradations in this chain were 97% based upon the yield of valine at positions 1 and 6. The yields for residues 2 through 15 were 0.4, 0.2, 0.2, 0.3, 0.2, 0.3, 0.3, 0.1, 0.1, 0.3, unknown, 0.1, 0.1, and 0.1 equiv, respectively. No residue was identified in position 12. The amino-terminal sequence present in the light chain of factor XII_a is homologous with many of the other plasma serine proteases. Indeed, Val, Gly, Ala, and Pro in positions 2, 4, 8, and 14, respectively, are identical to the corresponding position of a number of these plasma enzymes (Figure 4). Furthermore, many of the residues

TABLE I: Effect of Factors XII and XII$_a$ on Various Deficient Plasmas.[a]

Plasma	Clotting time (s)			
	Plus kaolin		Minus kaolin	
	Factor XII	Factor XII$_a$	Factor XII	Factor XII$_a$
Factor XII deficient (Hageman factor deficient)	138, 144	129, 128	>600	243, 251
Prekallikrein deficient (Fletcher factor deficient)	122, 120	130, 128	>600	351, 354
Fitzgerald deficient (HMW kininogen deficient)	>600	212, 211	>600	309, 310
Factor XI deficient (plasma thromboplastin antecedent)	543, 528	465, 422	>600	>600

[a] Assay conditions are described under Methods. Each assay contained 65 ng of factor XII or XII$_a$. Kaolin (0.25 mg) was added where indicated. The clotting times for two different sets of experiments are shown.

that are not identical are very similar such as valine and isoleucine in position 1.

The heavy chain of factor XII$_a$ was then incubated with carboxypeptidase A or B in an attempt to identify the carboxyl-terminal residue. The amino acids released by these enzymes were then analyzed by the amino acid analyzer. Arginine (0.9 mol/mol of heavy chain) was the only basic amino acid found after incubation with carboxypeptidase B for 24 h at 37 °C. Only traces of amino acids were released by carboxypeptidase A treatment under essentially identical conditions. These data indicate that bovine factor XII$_a$ contains a heavy and light chain held together by a disulfide bond(s) and the heavy chain originates from the amino-terminal portion of the precursor molecule. These experiments also suggest that the light chain originated from the carboxyl-terminal portion of the precursor molecule following hydrolysis of an internal arginyl–valine peptide bond.

Clotting and Hydrolase Activity of Factor XII and Factor XII$_a$. Factor XII and factor XII$_a$ were then tested against a number of deficient plasmas that lack activity for proteins which participate in the early phase of the coagulation process. Factor XII and factor XII$_a$ readily correct human factor XII deficient plasma (Table I). The specific activity of factor XII$_a$ was 1.5 to 2.0 times greater, however, than factor XII when assayed in factor XII deficient plasma in the presence of kaolin. In the absence of kaolin, factor XII has no detectable clotting activity, while factor XII$_a$ has activity equivalent to about 10% of that found in the presence of kaolin. These data indicate that factor XII$_a$ as well as factor XII require kaolin for maximal activity in the coagulant assay.

Factor XII and factor XII$_a$ also correct human prekallikrein deficient plasma (Table I). Furthermore, the specific activity of factors XII and XII$_a$ is very high in this assay. Factor XII$_a$ also has substantial clotting activity in human Fitzgerald deficient plasma in the presence of kaolin. Factor XII and factor XII$_a$, however, have little or no activity in bovine factor XI deficient plasma.

Factor XII$_a$ has considerable esterase activity towards tosyl-L-arginine methyl ester. The specific activity was 12 μmol of substrate hydrolyzed per min per mg of protein when the enzyme was assayed with 0.04 M substrate in 0.20 M Tris buffer (pH 8.0) in a final volume of 0.25 mL at 37 °C. The esterase activity of factor XII$_a$ was not influenced by the addition of kaolin (2.5 mg per 0.25 mL). No esterase activity was detected with factor XII in the presence or absence of kaolin. Esterase activity of factor XII$_a$ toward substrates such as benzoyl-L-arginine ethyl ester was noted earlier by Schoenmakers et al. (1965) and Komiya et al. (1972).

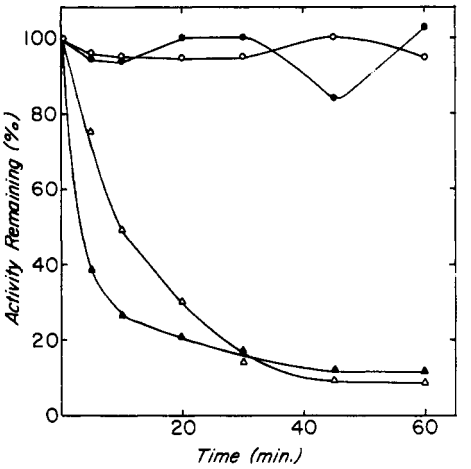

FIGURE 5: Effect of DFP on factors XII and XII$_a$. Reaction mixtures contained 50 μg of factor XII or XII$_a$, 1×10^{-3} M DFP in 0.04 M Tris-HCl buffer (pH 8.0) and 0.15 M NaCl. The final volume was 1.0 mL. Reaction mixtures were incubated at room temperature in the presence or absence of 10 mg of kaolin. Aliquots were removed after mixing at the desired times, diluted 100-fold with Michaelis buffer, and assayed for coagulant activity as described under Methods. (○-○) Factor XII; (●-●) factor XII plus kaolin; (△-△) factor XII$_a$; (▲-▲) factor XII$_a$ plus kaolin.

Factor XII$_a$ also has weak amidase activity toward *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide. The specific activity toward this substrate was 0.036 μmol of substrate hydrolyzed per min per mg of enzyme employing a substrate concentration of 1×10^{-4} M in 0.05 M Tris-HCl and 0.05 M imidazole hydrochloride buffer (pH 8.3) in a final volume of 1.0 mL at 30 °C. No amidase activity was detected for factor XII employing the same conditions.

Effect of DFP on Factors XII and XII$_a$. When factor XII was incubated with 1×10^{-3} M DFP in the presence or absence of kaolin, no loss in coagulant activity occurred (open and solid circles, respectively, Figure 5). Factor XII$_a$, however, was readily inactivated by 1×10^{-3} M DFP in the presence or absence of kaolin (open and solid triangles, respectively, Figure 5). These data indicate that factor XII$_a$ is a typical serine protease. These experiments are also consistent with our previous data showing the presence of an active-site serine sequence in factor XII (Fujikawa et al., 1977). Factor XII$_a$ was then incubated for 1 h at room temperature with 5×10^{-4} M radiolabeled DFP leading to 90% inhibition of coagulant activity. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Methods. The protein band corresponding to factor XII$_a$

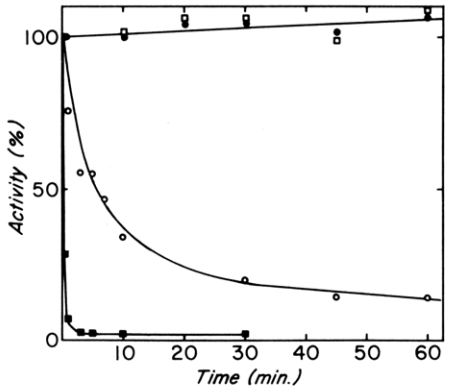

FIGURE 6: Time curve showing the inhibition of factor XII_a by antithrombin III. Factor XII_a (0.38 mg/mL) was incubated at 37 °C with antithrombin III (0.48 mg/mL) in the presence or absence of heparin (74 units/mL). Reaction mixtures also contained 0.025 M Tris-HCl with 0.15 M NaCl (pH 7.5). Aliquots were removed at the times shown, diluted 1000-fold with Michaelis buffer, and assayed for residual coagulant activity as described under Methods. (●-●) Factor XII_a; (□-□) factor XII_a plus heparin; (○-○) factor XII_a plus antithrombin III; (■-■) factor XII_a plus antithrombin III plus heparin.

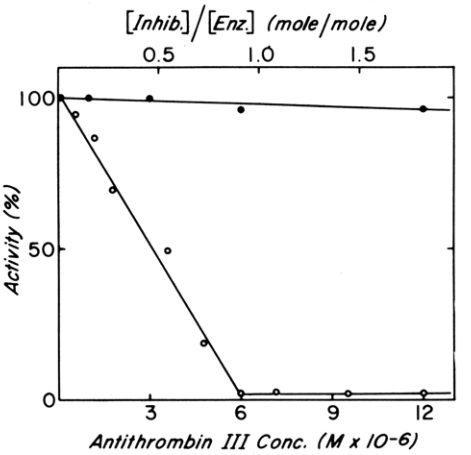

FIGURE 7: Inhibition of factor XII_a as a function of antithrombin III concentration. Factor XII_a or XII (0.42 mg/mL) was incubated with antithrombin III (0.42 mg/mL) in the presence of heparin (74 units/mL) in 0.025 M Tris-HCl containing 0.15 M NaCl (pH 7.5) for 5 min and assayed for residual factor XII_a as described in the legend to Figure 6. (●-●) Factor XII plus antithrombin III plus heparin; (○-○) factor XII_a plus antithrombin III plus heparin.

contained 4660 cpm. In the reduced gels, 4790 cpm was incorporated into the light chain, whereas only 220 cpm was found in the heavy chain. These data indicate that the inhibition of factor XII_a is due to the covalent binding of the inhibitor to the enzyme, and this binding occurs in the light chain which contains the active-site serine residue.

Effect of Antithrombin III on Factors XII and XII_a. When factor XII_a was incubated at 37 °C in the presence or absence of heparin, no loss of coagulant activity was observed (solid circles, open squares, respectively, Figure 6). When factor XII_a, however, was incubated with antithrombin III, a rapid inhibition occurred (open circles). Furthermore, the rate of inactivation of factor XII_a by antithrombin III was greatly accelerated in the presence of heparin (solid squares).

The effect of increasing concentrations of antithrombin III in the presence of heparin is shown in Figure 7. No effect of antithrombin III on factor XII was observed (solid circles). Factor XII_a, however, was inhibited by antithrombin III and formed a one to one molar complex with the inhibitor (open circles). In these studies, a molecular weight of 74 000 was employed for factor XII_a and a molecular weight of 56 000 for bovine antithrombin III (Kurachi et al., 1976). The apparent association constant for antithrombin III and factor XII_a was calculated to be about 5×10^7 per mol.

The formation of a stable one to one molar complex between factor XII_a and antithrombin III was demonstrated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 8). Gels 1 and 2 show factor XII_a before and after reduction with 2-mercaptoethanol, respectively. Gels 3 and 4 show antithrombin III before and after reduction with 2-mercaptoethanol, respectively. Gel 5 shows a mixture containing factor XII_a and antithrombin III (1.3 molar excess) incubated for 10 min in the presence of heparin. Two protein bands are observed in this gel. The fast-moving protein band corresponds to residual antithrombin III. The band corresponding to factor XII_a has disappeared, but a new slow-moving band is present, and this band corresponds to a complex of approximately 120 000. When the mixture containing the enzyme–inhibitor complex is reduced with 2-mercaptoethanol, three protein bands are observed (gel 6). The fast-moving band corresponds to the heavy chain of factor XII_a. The middle band corresponds to the residual antithrombin III (also seen in gel 5). The slow-moving band corresponds to a complex of antithrombin III and

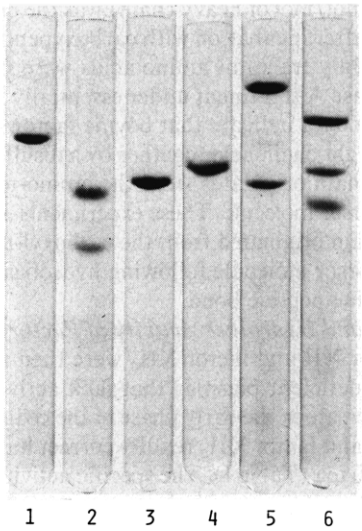

FIGURE 8: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of factor XII_a incubated with antithrombin III and heparin. Gels 1 and 2 contain 10 μg of factor XII_a before and after reduction with 2-mercaptoethanol, respectively. Gels 3 and 4 contain 10 μg of antithrombin III before and after reduction with 2-mercaptoethanol, respectively. Gels 5 and 6 contain factor XII_a and antithrombin III plus heparin before and after reduction with 2-mercaptoethanol, respectively. In the experiments shown in gels 5 and 6, factor XII_a and antithrombin III were incubated for 10 min in the presence of heparin as described in the legend to Figure 6, and 20-μL aliquots were removed and added to 1 μL of 1 M DFP and 10 μL of 0.1 M Tris-phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate. The samples containing approximately 20 μg of protein were then heated for 1 min at 100 °C in the presence and absence of 10 μL of 2-mercaptoethanol and then applied to 7.5% polyacrylamide gels. Electrophoresis and staining of the proteins were carried out as described under Methods. The anode was at the bottom of the gels.

the light chain of factor XII_a. These data indicate that a very stable complex is formed between factor XII_a and antithrombin III, and this complex directly involves the light chain of factor XII_a which contains the active-site serine residue.

In control experiments with factor XII and antithrombin III in the presence or absence of heparin, no complex was formed as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis or loss in coagulant activity.

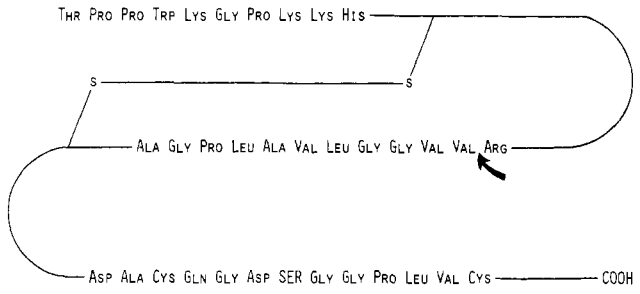

FIGURE 9: Partial structure of bovine factor XII. The active-site serine residue is shown in large caps. The arrow indicates the tentative site of cleavage in the protein during its conversion to factor XII$_a$. The resulting heavy and light chains of factor XII$_a$ are held together by a disulfide bond(s). Amino acid sequences for the amino-terminal and active-site region were taken from Fujikawa et al. (1977) and confirmed and extended in the present experiments.

Discussion

The present data indicate that bovine factor XII is a single-chain protein present in plasma in a precursor form lacking hydrolase activity and sensitivity to serine protease inhibitors. The single-chain molecule is converted to a two-chain molecule by an unidentified plasma protease. The two-chain molecule has esterase and amidase activity toward arginine-containing peptide substrates. It is also readily inhibited by serine protease inhibitors such as DFP and antithrombin III. Factor XII$_a$ also has activity toward protein substrates and readily activates bovine factor VII (Kisiel et al., 1977). Accordingly, the two-chain molecule was identified as factor XII$_a$ or activated factor XII.

The nature of the enzyme(s) that converts factor XII to factor XII$_a$ during the isolation procedure has not been established. A number of plasma enzymes including plasmin (Kaplan and Austen, 1971; Revak et al., 1974), kallikrein (Cochrane et al., 1973; Bagdasarian et al., 1973b; Revak et al., 1974; Griffin and Cochrane, 1976; Chan et al., 1976) and factor XI$_a$ (Revak et al., 1974) have been reported to activate human factor XII. Whether kallikrein or factor XI$_a$ was involved in the activation of bovine factor XII in the present experiments is not known. It is unlikely that it was activated by traces of plasmin, since bovine factor XII is very resistant to this enzyme (K. Fujikawa and E. W. Davie, unpublished results). The enzyme contaminant which converts factor XII to factor XII$_a$ during the isolation procedure has varied from one preparation to another, and some preparations yielded only factor XII even when the DEAE-Sephadex step was omitted. Thus, the nature and identity of the converting enzyme will require further investigation.

Bovine factor XII$_a$ migrates on sodium dodecyl sulfate-polyacrylamide gel electrophoresis at the same rate as factor XII. The latter protein has a molecular weight of 74 000 as determined by sedimentation equilibrium studies (Fujikawa et al., 1977). This indicates that factor XII$_a$ also has a molecular weight of 74 000. These experiments suggest that factor XII$_a$ is formed by the cleavage of a single internal arginyl-valine peptide bond in factor XII yielding a two-chain molecule held together by a disulfide bond(s). This reaction is illustrated in Figure 9. Since the molecular weight of factor XII and factor XII$_a$ appears to be the same, it seems unlikely that an activation peptide is released during the activation reaction. The release of a very small activation peptide could not be detected, however, in the present experiments. The heavy chain of factor XII$_a$ has an apparent molecular weight of 52 000, while the light chain has an apparent molecular weight of 31 000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Glycoproteins, however, tend to bind less sodium dodecyl sulfate, resulting in higher apparent molecular weight estimates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Segrest and Jackson, 1972). Thus, it seems likely that the true molecular weights for the heavy and light chains of factor XII$_a$ are about 10% lower, which is equivalent to around 46 000 and 28 000, respectively. A summation of these two values would then give a molecular weight of 74 000 for factor XII$_a$.

The size of the two chains in bovine factor XII$_a$ is quite similar to some of the fragments formed when human factor XII is converted to factor XII$_a$ by plasmin, kallikrein, factor XI$_a$, or trypsin (Kaplan and Austen, 1971; Cochrane et al., 1973; Revak et al., 1974; Revak and Cochrane, 1976). In these reactions, human factor XII is cleaved into fragments with molecular weights of 52 000, 40 000, and 28 000. These fragments, however, are not linked together by a disulfide bond(s). The enzymatic activity is associated with the 28 000 molecular weight fragment, while the 52 000 and 40 000 molecular weight fragments contain the kaolin binding sites (Revak and Cochrane, 1976). Thus, human factor XII$_a$ with a molecular weight of 28 000 differs substantially from the high-molecular-weight bovine factor XII$_a$ described in the present studies. Indeed, bovine factor XII$_a$ is more similar to the high-molecular-weight human factor XII$_a$ described by Bagdasarian et al. (1973a,b).

Bovine factor XII also differs from human factor XII in its sensitivity to antithrombin III and DFP. Stead et al. (1976) have recently reported the inactivation of single-chain human factor XII (molecular weight 75 000) by DFP or antithrombin III. Furthermore, a stable one to one molar complex of antithrombin III and single-chain human factor XII was reported by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In contrast, single-chain bovine factor XII does not react with DFP or antithrombin III in the presence or absence of heparin. Bovine factor XII$_a$, however, readily reacted with DFP and antithrombin III, and in each case the inhibitor was bound to the light chain of the enzyme.

As previously noted (Fujikawa et al., 1977), the basic mechanism for the proteolytic activation of factor XII is probably the same as that for the activation of chymotrypsinogen and trypsinogen. In these cases, a new amino-terminal residue is formed which folds into the interior portion of the protein, giving rise to the formation of an ion pair with the carboxyl group of the aspartic acid residue which is adjacent to the active serine (Sigler et al., 1968; Stroud et al., 1975). This leads to the charge relay network which is characteristic of the pancreatic serine proteases (Matthews et al., 1967; Sigler et al., 1968; Blow et al., 1969; Shotton and Watson, 1970). Thus, it seems likely that the newly formed amino-terminal valine residue in factor XII$_a$ also forms an ion pair with the aspartic acid adjacent to the active serine residue in a similar manner giving rise to an active serine protease (Figure 9).

A simple explanation for the effect of bovine factor XII or factor XII$_a$ on prekallikrein-deficient plasma is not evident (Table I). Bovine factors XII and XII$_a$ are free of prekallikrein, as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis where prekallikrein migrates slightly slower than factor XII (R. Heimark, unpublished data). A similar effect has been noted by Saito et al. (1975) for human factor XII. Bovine factor XII$_a$ also shows significant activity in Fitzgerald deficient plasma (Table I). This suggests that HMW kininogen may participate primarily in those reactions leading to factor XII activation rather than the activation of factor XI by factor XII$_a$. Factors XII and XII$_a$ both require kaolin for maximal activity in factor XII deficient plasma

(Table I). Thus, the presence of kaolin does not directly lead to the activation of factor XII but seems to influence the coagulation process by concentrating certain reactants and substrates and thus facilitating their interaction.

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