Isolation and Characterization of Bovine Factor VII†

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ABSTRACT: Factor VII (proconvertin) has been purified approximately 5 × 10^5-fold from bovine plasma with an overall yield of 30%. The isolation procedure involves barium sulfate adsorption and elution, DEAE-Sephadex batchwise adsorption and elution, benzamidine-agarose column chromatography, heparin-agarose column chromatography, and preparative polyacrylamide gel disc electrophoresis. The final product was homogeneous when examined by gel electrophoresis in the presence of sodium dodecyl sulfate. A minimal molecular weight of 45,500 was determined by sedimentation equilibrium. The molecular weight estimated by sodium dodecyl sulfate gel electrophoresis was 54,000. Factor VII is composed of a single polypeptide chain possessing an amino-terminal sequence of Ala-Asn-Gly-Phe-Leu-. The amino acid and carbohydrate compositions of factor VII are also reported.

Factor VII (proconvertin, precursor of serum prothrombin conversion accelerator) is a plasma protein which participates in the extrinsic pathway of blood coagulation (Davie and Fujikawa, 1975).[1] In these reactions, factor VII, in the presence of tissue factor, converts factor X to factor X_a (Straub and Duckert, 1961; Williams and Norris, 1966; Nemerson and Pitlick, 1970; Osterud et al., 1972; Jesty and Nemerson, 1974; Fujikawa et al., 1974a). Factor X_a in turn then converts prothrombin to thrombin in the presence of factor V (proaccelerin), calcium ions, and phospholipid (Milstone, 1964; Papahadjopoulos and Hanahan, 1964; Jobin and Esnouf, 1967; Stenn and Blout, 1972; Heldebrant and Mann, 1973; Heldebrant et al., 1973; Kisiel and Hanahan, 1973; Owen et al., 1974; Esmon et al., 1974).

The isolation of factor VII has been difficult since this coagulation factor occurs in plasma at extremely low levels and has been hard to separate from prothrombin, factor IX, and factor X. Duckert et al. (1953) separated factor VII from prothrombin by chromatography on a barium sulfate–Hyflo Super-Cel mixture starting with human plasma or serum. Alexander (1959) employed barium sulfate adsorption and elution followed by ammonium sulfate fractionation. His starting material was oxalated serum which contains less than 5% of the original prothrombin but retains full factor VII activity. Gladhaug and Prydz (1970) used a combination of column chromatography on barium sulfate-Sephadex G-25, DEAE-Sephadex chromatography, gel filtration, and preparative disc gel electrophoresis. The overall purification obtained by this method was about 10,000-fold. The molecular weight of this preparation was estimated at 48,000. Williams and Norris (1966) purified bovine factor VII about 500-fold by barium sulfate adsorption and elution, DEAE-cellulose chromatography, gel filtration, and sucrose gradient centrifugation. Factor VII activity was associated with a fraction with an s_{20} value of 4.8. Nemerson (1966) employed barium citrate adsorption and elution followed by ammonium sulfate fractionation and DEAE-cellulose column chromatography. He observed that factor VII eluted on the descending peak of prothrombin. In 1973, Nemerson and Esnouf added a preparative disc gel electrophoresis step to this procedure and obtained a preparation purified about 2000-fold with a final yield of 12%. More recently, Jesty and Nemerson (1974) and Radcliffe and Nemerson (1975a) purified bovine factor VII approximately 200,000-fold by methods which included chromatography on benzamidine-Sepharose and gel filtration. The overall yield was about 10%. The apparent molecular weight of bovine factor VII was 53,000 as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Fujikawa et al. (1974a) extensively purified bovine factor VII by barium sulfate adsorption and elution, DEAE-Sephadex chromatography, heparin-agarose chromatography, and benzamidine-agarose chromatography. The present work describes an additional purification step for this preparation and a detailed characterization of the final product.

Experimental Section

Materials

Heparin sodium salt (grade I, 170 USP units/mg), soybean trypsin inhibitor (Type I-S and Type II-S), bovine serum albumin, ovalbumin, carbonix anhydrase, diisopropyl phosphorofluoridate, imidazole (grade I), *p*-aminobenzamidine hydrochloride, dithiothreitol, and morpholinoethanesulfonic acid were obtained from Sigma Chemical Co., St. Louis, Mo. Benzamidine hydrochloride and cyclohexylmorpholinoethyl carbodiimide were purchased from Aldrich Chemical Co., Milwaukee, Wis. Barium sulfate (X-ray grade) was obtained from Picker Corp., Cleveland, Ohio. DEAE-Sephadex A-50 was a product of Pharmacia Fine Chemicals, Piscataway, N.J., and Bio-Gel A 15m (agarose) was obtained from Bio-Rad Laboratories, Richmond, Calif. 2-Mercaptoethanol, acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine was purchased from Eastman Kodak Co., Rochester, N.Y. Acrylamide was recrystallized from chloroform according to Loenig (1967). Sodium dodecyl sulfate was obtained from British Drug House, Poole, England. Guanidine hydrochloride (ultrapure) was purchased from Schwarz/Mann, Van Nuys, Calif. Phosphorylase *b* was kindly provided by Dr. Edmond Fischer. Bovine brain

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

thromboplastin was prepared according to Quick (1966). Four grams of bovine brain acetone powder was extracted with 50 ml of 0.15 *M* NaCl at 48–50° for 20 min, centrifuged, and stored at −20° in 2-ml aliquots. Bovine factor VII deficient plasma was prepared as described by Nemerson and Clyne (1974) from pooled blood collected in 0.1 *M* sodium citrate (pH 7.0) (nine parts blood, one part 0.1 *M* sodium citrate). Dialysis tubing was treated according to McPhie (1971) prior to use. All other chemicals were commercial preparations of the highest quality available.

Methods

Protein concentrations were determined from the absorbance at 280 nm assuming an $E^{1\%}$ value of 10.0 and correcting for Rayleigh scattering according to Shapiro and Waugh (1966). For carbohydrate analyses, protein mass was determined by amino acid analysis after hydrolyzing the sample in 6 *N* HCl for 24 hr at 110° in evacuated tubes.

Amino acid analyses were performed according to the methods of Moore and Stein (1963) and Spackman et al. (1958) employing a Durrum Model D 500 amino acid analyzer. Samples were hydrolyzed in 6 *N* HCl at 110° for 24, 48, 72, and 96 hr in evacuated tubes. Threonine and serine content was determined by extrapolation to zero hydrolysis time. Valine and isoleucine values were those determined for the 96-hr hydrolysis. Tryptophan was estimated according to Goodwin and Morton (1946), and half-cystine was determined as cysteic acid by the method of Hirs (1967).

Neutral sugar was determined with anthrone according to Spiro (1966) using a 1:1 mixture of mannose–galactose as a standard. 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* trifluoroacetic acid for 2 hr at 120° as described by Albersheim et al. (1967).

Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn (1969). Samples were subjected to electrophoresis at room temperature for 3 hr in 10% acrylamide gels at a current level of 6 mA/gel. The electrophoresis buffer was 0.1 *M* Tris-H_3PO_4–0.1% sodium dodecyl sulfate (pH 7.0). Gels were stained for protein with Coomassie Brilliant Blue R according to Fairbanks et al. (1971). The molecular weight of factor VII was obtained by interpolation from a linear semilogarithmic plot of apparent molecular weight vs. migration distance using four standard proteins: phosphorylase *b* (95,000), bovine serum albumin (68,000), ovalbumin (45,000), and bovine carbonic anhydrase (29,000).

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. Rayleigh patterns were recorded on Kodak II-G photographic plates, and the plates were subsequently read on a Nikon microcomparator automated as described by DeRosier et al. (1972). By this procedure, the entire fringe envelope is read at 50-μ intervals and the calculated Fourier transform parameter employed to compute the actual fringe displacement using the program developed by DeRosier et al. (1972). Point-by-point molecular weight averages were then calculated from the data utilizing a computer program developed by Teller (1973).

Sedimentation equilibrium measurements were performed with three different concentrations (1.2, 0.6, and 0.3 mg/ml) of diisopropyl phosphoryl factor VII in 6 *M* guanidine hydrochloride–0.1 *M* dithiothreitol–0.05 *M* sodium acetate (pH 5.5). Salt-free, lyophilized diisopropyl phosphoryl factor VII was dissolved in 6 *M* guanidine hydrochloride and dialyzed against this solvent for 48 hr at room temperature prior to ultracentrifugation. The appropriate sample concentration was obtained by dilution with the diffusate. Ultracentrifugation was carried out at 20° at a rotor speed of 26,000 rpm. A partial specific volume of $\bar{v}$ = 0.706 ml/g was determined from the amino acid analyses and corrected for 13% carbohydrate content (Cohn and Edsall, 1943; Longsworth, 1953). The solvent density at 20° was obtained from its index of refraction at 23.5° measured with an Abbe refractometer (Kielley and Harrington, 1960).

Automated Edman degradations were performed with a Beckman sequenator Model 890A. Methods of sequenator analysis and operation of the instrument are adaptations (Hermodson et al., 1972) of the technique of Edman and Begg (1967). For the amino-terminal analysis, 3 mg of diisopropyl phosphoryl factor VII was employed. The terminal residues were quantitated from the protein concentration previously determined in the amino acid analyzer after hydrolysis.

Factor VII was assayed according to the method developed by Nemerson and Clyne (1974). Factor VII samples were diluted for assay with 0.05 *M* Tris-HCl–0.1 *M* NaCl (pH 7.5) containing 1 mg/ml of bovine serum albumin. Citrated plasma, pooled from five cows, was used as a reference standard factor VII. One unit of factor VII activity is arbitrarily defined as that activity present in 1 ml of pooled bovine plasma.

Factor X and prothrombin were assayed by one-stage methods according to Bachmann et al. (1958) and Hjort et al. (1955), respectively. Factor IX was measured by a one-stage assay as described by Fujikawa et al. (1973).

Heparin-agarose was prepared by a modification of the procedure described by March et al. (1974); 500 ml (settled volume) of agarose A 15m (100–200 mesh) was activated with 100 g of cyanogen bromide dissolved in 50 ml of acetonitrile. The pH of the suspension was maintained at pH 11 by the dropwise addition of 6 *N* NaOH. The temperature of the suspension was maintained at 15–18° by the addition of ice chips. The activation reaction was conducted for 20 min and the temperature was rapidly lowered to 4° by the addition of ice chips. The slurry was immediately filtered under vacuum and washed with 4 l. of ice-cold 0.1 *M* $NaHCO_3$. The activated, washed agarose was transferred to a 500-ml solution of 0.1 *M* $NaHCO_3$ containing 6.4 g of heparin and stirred for 18 hr at 4°. After coupling, the heparin-agarose was washed exhaustively with 0.1 *M* Tris-HCl–2 *M* NaCl (pH 9.0).

Benzamidine-agarose was prepared according to Schmer (1972) using ϵ-aminocaproic acid as an arm between benzamidine and the agarose beads.

The soybean trypsin inhibitor was purified according to Frattali and Steiner (1969) from a commercial preparation of soybean trypsin inhibitor (Sigma; Type I-S).

Purification of Bovine Factor VII. Bovine blood was collected and rapidly mixed with oxalate, heparin, benzamidine hydrochloride, and soybean trypsin inhibitor as previously described (Fujikawa et al., 1972). The plasma was isolated at room temperature with a continuous flow separator (De Laval Model BLE 519). Subsequent steps were performed at 4° unless otherwise specified. The plasma was

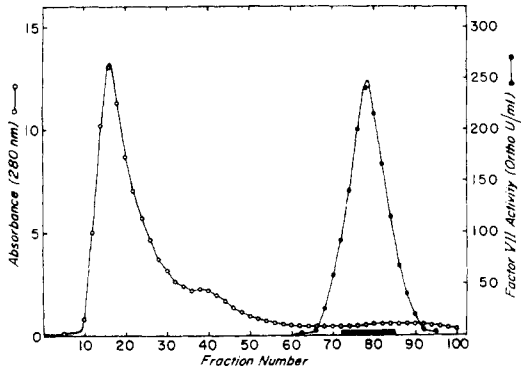

FIGURE 1: Elution pattern from bovine factor VII from the benzamidine-agarose column. Protein was eluted from the column (2.6 × 50 cm) with a linear gradient arising from 500 ml each 0.3 and 0.9 *M* guanidine hydrochloride in 0.05 *M* imidazole-HCl–0.1 *M* NaCl (pH 6.5). Fractions (8 ml) were collected at a flow rate of 120 ml/hr. Factor VII activity was determined as described under Methods. (○) Absorbance at 280 nm; (●) clotting activity. The solid bar shows those fractions which were combined.

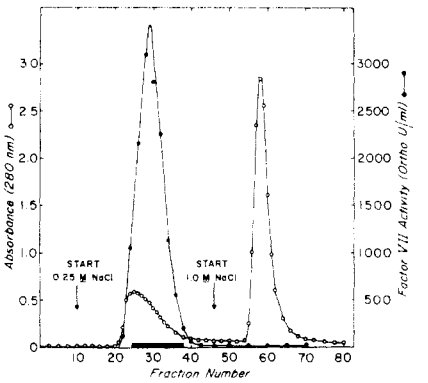

FIGURE 2: Elution pattern for bovine factor VII from the heparin-agarose column. Protein was eluted from the column (1.6 × 20 cm) in a stepwise manner with 100 ml of 0.25 *M* NaCl followed by 100 ml of 1.0 *M* NaCl as described under Methods. Fractions (3 ml) were collected at a flow rate of 60 ml/hr. (○) Absorbance at 280 nm; (●) clotting activity. The solid bar shows those fractions which were combined.

mixed with barium sulfate (40 g/l.) for 30 min, and the slurry was centrifuged for 20 min at 1500*g* in a Sorvall RC3 centrifuge. The barium sulfate precipitate was resuspended in approximately 3.5 l. of 0.005 *M* sodium acetate in a 4-l. Waring Blendor followed by centrifugation. The 0.005 *M* sodium acetate washing was repeated three times. Factor VII was eluted from the barium sulfate by resuspending the precipitate in 0.2 *M* sodium citrate (pH 7.0) containing 10^{-2} *M* benzamidine and 200 mg of crude soybean trypsin inhibitor followed by stirring for 30 min. The slurry was centrifuged at 1500*g* for 30 min. The supernatant was made 0.025 *M* in Na_4EDTA and an additional 100 mg of crude soybean trypsin inhibitor added to the supernatant. The pH of the barium sulfate eluate was then adjusted to 7.0 with HCl and the eluate dialyzed overnight against 60 l. of distilled water. The solution was centrifuged for 15 min at 5000*g* and the supernatant was stirred for 30 min with 1 l. DEAE-Sephadex A-50 (settled volume). The DEAE-Sephadex was previously equilibrated with 0.05 *M* sodium citrate–5 m*M* benzamidine (pH 7.0). The suspension was allowed to settle and the supernatant decanted from the DEAE-Sephadex. The DEAE-Sephadex was then packed into a 7.5 × 30 cm column and the column washed with 4 l. of 0.05 *M* sodium citrate–5 m*M* benzamidine (pH 7.0). Factor VII was eluted from the column with 0.11 *M* sodium citrate–5 m*M* benzamidine (pH 7.0). Those fractions containing factor VII were combined and the pH of the eluate was adjusted to 6.5 with HCl. Crude soybean trypsin inhibitor (50 mg) was added to the DEAE-Sephadex eluate and the solution was concentrated to approximately 100 ml by ultrafiltration. Ultrafiltration was performed with an Amicon ultrafiltration apparatus utilizing a PM-10 membrane The solution was then dialyzed overnight against 4 l. of 0.05 *M* imidazole-HCl–0.1 *M* NaCl (pH 6.5) and applied to a benzamidine-agarose column (2.6 × 50 cm) previously equilibrated with 0.05 *M* imidazole hydrochloride–0.1 *M* NaCl (pH 6.5). After application of the sample, the column was washed with 250 ml of 0.05 *M* imidazole hydrochloride–0.1 *M* NaCl (pH 6.5) containing 0.3 *M* guanidine hydrochloride. Factor VII was then eluted from the column with a linear gradient prepared from 500 ml each of 0.3 and 0.9 *M* guanidine hydrochloride in 0.05 *M* imidazole hydrochloride–0.1 *M* NaCl (pH 6.5) (Figure 1). The fractions containing factor VII activity were pooled (shown by the solid bar) and dialyzed overnight against 4 l. of 0.05 *M* imidazole hydrochloride–1 m*M* benzamidine (pH 6.0). The dialyzed sample was made 2.5 m*M* in $CaCl_2$ and applied to a heparin-agarose column (1.6 × 20 cm) previously equilibrated with 0.05 *M* imidazole hydrochloride–2.5 m*M* $CaCl_2$–1 m*M* benzamidine (pH 6.0). After the sample was applied, the column was washed with 50 ml of equilibrating buffer. Factor VII was eluted from the column with 100 ml of 0.25 *M* NaCl dissolved in the equilibrating buffer (Figure 2). Factor IX, and other adsorbed proteins, were eluted from the column with 1.0 *M* NaCl dissolved in the equilibrating buffer. The fractions containing factor VII activity (as shown with the solid bar) were pooled and 3 mg of soybean trypsin inhibitor was added to the eluate. The solution was then dialyzed overnight against 4 l. of 0.05 *M* Tris-HCl–0.025 *M* boric acid–5 m*M* EDTA–10 m*M* benzamidine (pH 8.0). This buffer was selected in preference to the upper buffer or 0.05 *M* Tris-H_3PO_4 (pH 7.5) since a better resolution of factor VII from factor II was observed in analytical disc electrophoresis if the sample had previously been dialyzed against this buffer. The dialyzed sample was concentrated to approximately 10 ml by ultrafiltration and subjected to preparative discontinuous electrophoresis at 2° in a Buchler Poly-Prep 200 apparatus. The gel concentration and buffer compositions were identical with those previously described (Kisiel and Hanahan, 1973). The sample was subjected to electrophoresis at 60 mA constant current for approximately 12 hr. The proteins were eluted from the resolving gel with 0.1 *M* Tris-HCl–1 m*M* benzamidine (pH 8.0) at the rate of 0.8–1.0 ml/min. Each fraction of the electrophoresis eluent containing factor VII activity was concentrated by ultrafiltration and a portion subjected to sodium dodecyl sulfate gel electrophoresis. Only those fractions exhibiting homogeneity by this criteria were pooled and employed in the characterization studies. Soybean trypsin inhibitor is highly anionic and was well resolved by this procedure. Inasmuch as benzamidine absorbs strongly at 280 nm, a 1-ml aliquot of the pooled sample was dialyzed overnight against 1 l. of 0.05 *M* Tris-HCl–0.1 *M* NaCl (pH 7.5) to remove the benzamidine in order to obtain a more accurate protein concentration value spectrophotometrically. The pooled sample from the preparative electrophoresis was routinely made 5 m*M* in diisopropyl phosphorofluoridate to reduce the possibility of factor VII autolysis during certain characterization procedures. The

Table I: Purification of Bovine Factor VII.

Purification Step	Volume (ml)	Total Protein[a] (mg)	Total Act.[b] (units)	Sp. Act. (units/mg)	Recovery (%)	Purification (fold)
Plasma	48×10^3	3.4×10^6	4.8×10^4	0.014	100	1
$BaSO_4$ eluate	4.7×10^3	14.8×10^3	5.9×10^4	3.97	120	284
Batchwise DEAE-Sephadex	1.0×10^3	3.04×10^3	4.50×10^4	14.8	94	1,060
Benzamidine-agarose column	120	70.4	2.40×10^4	341	50	24,300
Heparin-agarose column	36	18.8	4.50×10^4	2390	94	171,000
Preparative electrophoresis	45	2.03	1.44×10^4	7110	30	508,000

[a] Protein concentration was determined from the absorbance at 280 nm using $A_{280}(1\%) = 10.0$. [b] Activity of factor VII was assayed as described under Methods.

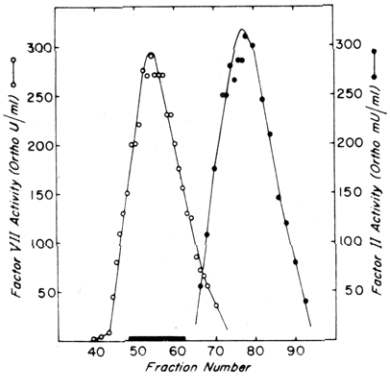

FIGURE 3: Elution pattern for bovine factor VII and prothrombin (factor II) from preparative electrophoresis. Protein was eluted from the resolving gel with 0.1 *M* Tris-HCl–1 m*M* benzamidine (pH 8.0). Fractions (3 ml) were collected at a flow rate of 60 ml/hr. (○) factor VII activity; (●) prothrombin activity. The solid bar shows those fractions which were combined.

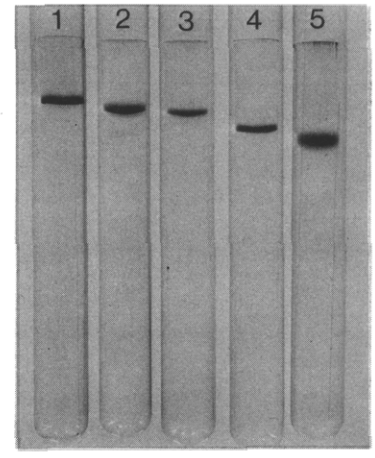

FIGURE 4: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of bovine factor VII, prothrombin, factor IX, and factor X. Sample 1, prothrombin; sample 2, factor IX; sample 3, factor X_1; sample 4, factor VII; and sample 5, reduced factor VII. Samples 1–4 contained 10 μg of unreduced protein and sample 5 contained 20 μg of unreduced protein. All samples were stained for protein with Coomassie Brilliant Blue R. The anode was at the bottom of the gel.

resulting diisopropyl phosphoryl factor VII was concentrated by ultrafiltration and desalted by gel filtration through a Sephadex G-25 column (1.6 × 50 cm) equilibrated with 0.1 *M* NH_4HCO_3. Salt-free protein was obtained by lyophilization.

Results

Preparation of Bovine Factor VII. Factor VII was purified 5×10^5-fold from bovine plasma by a five-step procedure in approximately 30% yield (Table I). These steps included barium sulfate adsorption and elution, DEAE-Sephadex batchwise adsorption and elution, benzamidine-agarose column chromatography, heparin-agarose column chromatography, and preparative polyacrylamide gel disc electrophoresis. The overall recovery of factor VII ranged from 22 to 35% and about 2 mg of protein was routinely obtained from 50 l. of plasma.

In this purification procedure, nearly all of the factor X separated from factor VII in the batchwise DEAE-Sephadex step and the eluate from the DEAE-Sephadex consisted mainly of prothrombin, factor IX, and factor VII. The major portion of prothrombin, factor IX, and the remaining traces of factor X were separated from factor VII on the benzamidine-agarose column (Figure 1). Although factor VII eluted from this column in a sharp, symmetrical peak, prothrombin and factor IX trailed into this peak. Consequently, the eluate from the benzamidine-agarose still contained approximately 70% contaminating proteins as judged by sodium dodecyl sulfate gel electrophoresis.

Factor VII was completely resolved from factor IX on the heparin-agarose column where factor VII and prothrombin are eluted with the 0.25 *M* NaCl wash, and factor IX is eluted with the 1.0 *M* NaCl wash (Figure 2). The eluate from the heparin-agarose column contained approximately equal amounts of factor VII and prothrombin and several other minor contaminants. The overall yield of factor VII activity after heparin-agarose chromatography was consistently higher than that observed after the benzamidine-agarose step. The reason for this phenomenon is not apparent at this time. It is unlikely that it is due to inhibition by trace amounts of benzamidine hydrolyzed from the agarose since the factor VII samples are diluted about 10,000-fold before assay.

Factor VII was effectively resolved from prothrombin by preparative polyacrylamide gel disc electrophoresis (Figure 3). The low recovery of activity from the preparative electrophoresis may be due to denaturation of factor VII caused by the dilute protein concentration upon elution from the resolving gel. This is suggested by the fact that the marked instability of factor VII activity upon dilution was entirely avoided by the inclusion of 1 mg/l. of bovine serum albumin in the diluent. Factor VII from the preparative electrophoresis step exhibited less than 0.001 Ortho U/ml of prothrombin and undetectable levels of factors IX and X when assayed at 0.5 mg/ml.

Sodium Dodecyl Sulfate Gel Electrophoresis of Bovine Factor VII. A single protein band was observed by sodium dodecyl sulfate gel electrophoresis for factor VII before and after reduction with 2-mercaptoethanol (Figure 4, samples

Table II: Molecular Weight of Bovine Factor VII by Sedimentation Equilibrium.[a]

M_1	M_n	M_w	M_z
45,500 ± 1800	52,100 ± 2700	58,700 ± 3600	67,100 ± 3300

[a] M_1 represents the smallest molecular weight species calculated according to Teller (1973). M_n, M_w, M_z represent the number average molecular weight, the weight average molecular weight, and the Z-average molecular weight, respectively.

Table III: Amino Acid and Carbohydrate Compositions of Bovine Factor VII.

Components	Factor VII (residues/45,500)
Amino Acid	
Lysine	9.5
Histidine	8.7
Arginine	25.4
Aspartic acid	28.4
Threonine	14.2
Serine	17.0
Glutamic acid	47.5
Proline	23.5
Glycine	39.8
Alanine	27.7
Half-cystine[a]	28.0
Valine	25.8
Methionine	1.3
Isoleucine	7.7
Leucine	33.1
Tyrosine	7.3
Phenylalanine	16.0
Tryptophan[b]	5.0
Carbohydrate	
Hexose	14.1
N-Acetylglucosamine	6.2
N-Acetylneuraminic acid[c]	7.9
Protein (%)	87
Carbohydrate (%)	13

[a] Determined as cysteic acid according to Hirs (1967). [b] Determined spectrophotometrically by the method of Goodwin and Morton (1946). [c] Determined as neuraminic acid but reported as *N*-acetylneuraminic acid since all naturally occurring neuraminic acid is substituted (Warren, 1959).

4 and 5). The molecular weight of factor VII estimated by this technique was 56,000 ± 2600 for the unreduced protein and 54,000 ± 2600 for the reduced protein. The apparent molecular weight was independent of gel concentration when electrophoresis was carried out in polyacrylamide gels ranging from 5 to 12.5% (Segrest and Jackson, 1972). A molecular weight of 60,000 was determined by gel filtration and this value is identical with that found for factor VII in plasma. This is consistent with the conclusion that little or no degradation has occurred during the isolation procedure. These molecular weight values are considerably higher than the minimal molecular weight determined by sedimentation equilibrium as described below.

The sodium dodecyl sulfate polyacrylamide gel patterns for bovine prothrombin (sample 1), factor IX (sample 2), and factor X (sample 3) are also shown in Figure 4 for comparison. Of the four vitamin K dependent clotting factors, only prothrombin exhibits an apparent molecular weight by this technique which is in good agreement with 68,000 calculated by sedimentation equilibrium (Cox and Hanahan, 1970) and 72,000 by amino acid sequence (S. Magnusson,

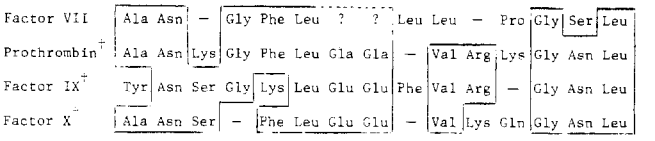

FIGURE 5: Amino-terminal sequences of bovine factor VII, prothrombin, factor IX, and the light chain of factor X_1. Amino acids that are identical are shown in blocks. Dashes refer to spaces that have been inserted to bring the four proteins into alignment for better homology. Gla refers to γ-carboxyglutamic acid. Dagger indicates results from Fujikawa et al. (1974b).

personal communication). In contrast, factor IX has a molecular weight of 68,000 by the sodium dodecyl sulfate polyacrylamide gel technique and 55,400 by sedimentation equilibrium (Fujikawa et al., 1973). Factor X has a molecular weight of 67,000 by the sodium dodecyl sulfate polyacrylamide gel technique and 55,000 by sedimentation equilibrium (Fujikawa et al., 1972) and 55,100 by amino acid sequence (Enfield et al., 1975; Titani et al., 1975; Fujikawa et al., 1975).

Sedimentation Equilibrium Studies on Bovine Factor VII. Sedimentation equilibrium experiments with diisopropyl phosphoryl factor VII in 6 *M* guanidine hydrochloride indicated the protein was heterogeneous at the concentrations tested (Table II). Inasmuch as the M_n, M_w, and M_z vs. concentration plots are superimposable at the protein concentration studied, the observed heterogeneity most probably reflects monomer aggregation rather than a contaminating protein species. The minimal molecular weight calculated for the diisopropyl phosphoryl factor VII by the method of Teller (1973) was 45,500 ± 1300.

Amino Acid and Carbohydrate Compositions of Bovine Factor VII. The amino acid and carbohydrate compositions of factor VII are shown in Table III. The amino acid compositions represent the average composition of three preparations of factor VII, while the carbohydrate composition was derived from one factor VII preparation. Factor VII is a glycoprotein containing approximately 13% carbohydrate by weight which includes hexose, glucosamine, and neuraminic acid. Thus, the factor VII molecule is composed of about 6000 g of carbohydrate and 39,500 g of protein/45,500 g of glycoprotein.

Amino-Terminal Sequence of Bovine Factor VII. The amino-terminal sequence of diisopropyl phosphoryl factor VII is shown in Figure 5, along with the amino-terminal sequences of bovine prothrombin, factor IX, and the light chain of factor X_1 (Fujikawa et al., 1974b). The yield of amino-terminal alanine was approximately 0.5 equiv/mol of protein assuming 39,500 g of protein/mol of factor VII. The low yield of amino-terminal alanine is probably due to the low but constant amount of phenylthiohydantoin amino acid which is destroyed in the gas chromatography column. This destruction becomes magnified when small amounts of protein are employed as was the case in these experiments.

No amino acids in factor VII were detected in turns 6 and 7. These positions probably contain γ-carboxyglutamic acid as noted in the Discussion.

Discussion

The present data indicate that bovine factor VII is a single-chain glycoprotein, confirming the recent results of Radcliffe and Nemerson (1975a). No two-chain molecule was observed in the present studies. Furthermore, incubation of the highly purified factor VII at neutral pH at a con-

centration of 0.5 mg/ml for 48 hr at 4° did not give rise to any two-chain factor VII as determined by sodium dodecyl sulfate gel electrophoresis. This indicates that the final preparation is essentially free of contaminating proteases such as factor X_a and thrombin which hydrolyze factor VII (Radcliffe and Nemerson, 1975a).

The minimal molecular weight for factor VII was found to be 45,500 by sedimentation equilibrium. This value is much lower than that found by gel filtration and sodium dodecyl sulfate gel electrophoresis. These methods, however, tend to give high molecular weights for glycoproteins due to aggregation, nonspecific binding to Sephadex, and decreased binding of the detergent to the protein (Segrest and Jackson, 1972).

Protease and coagulation inhibitors were routinely added at various steps during the isolation of factor VII. Benzamidine was first employed in our laboratory for the isolation of bovine factor VIII (Schmer et al., 1972) and has also been extremely helpful in the isolation of bovine factor IX and factor X (Fujikawa et al., 1972, 1973). The present procedure also employs heparin, soybean trypsin inhibitor, and oxalate to inhibit various plasma proteases. In the absence of protease inhibitors, considerable degradation of factor VII occurs (Radcliffe and Nemerson, 1975a).

The last three steps employed in the isolation of factor VII include benzamidine-agarose column chromatography, heparin-agarose column chromatography, and preparative polyacrylamide gel disc electrophoresis. Benzamidine-agarose was developed in our laboratory by Schmer (1972) for the isolation of thrombin. It has also been used successfully by Jesty and Nemerson (1974) and Radcliffe and Nemerson (1975a) in the preparation of their factor VII. Heparin-agarose column chromatography has been extensively employed for the isolation of bovine factor IX (Fujikawa et al., 1973) and was useful in the separation of this protein from factor VII in the present procedure.

The highly purified bovine factor VII prepared by Jesty and Nemerson (1974) appeared to be a multichain glycoprotein containing a heavy chain of 37,000 daltons. More recently, however, Radcliffe and Nemerson (1975a) isolated a single-chain factor VII with a molecular weight of 53,000 as estimated by sodium dodecyl sulfate gel electrophoresis. This preparation contains an amino-terminal sequence of Ala-Asx-Gly-Phe-Leu- (Radcliffe and Nemerson, 1975b). In the presence of factor X_a, phospholipid, and calcium ions, the single-chain factor VII was activated about 45-fold and was cleaved into a two-chain molecule held together by a disulfide bond(s). Upon reduction, this molecule yields two chains with apparent molecular weights of 29,500 and 23,500. Factor VII was also inactivated by conversion to fragments with molecular weights of 41,000 and 12,500 in the presence of factor X_a. The relationship of these various molecules to the factor VII with a heavy chain of 37,000 molecular weight (Jesty and Nemerson, 1974) is not known.

Factor VII shows a great deal of homology in its amino-terminal region with prothrombin, factor IX, and the light chain of factor X (Figure 5). These data provide further evidence that these four vitamin K dependent proteins have evolved from a common ancester (Titani et al., 1972; Fujikawa et al., 1974b; Enfield et al., 1974). No amino acids were found in positions 6 and 7 in factor VII. It appears probable, however, that γ-carboxyglutamic acid occupies these two positions since this amino acid has been identified in prothrombin in positions 6 and 7 (Stenflo et al., 1974; Magnusson et al., 1974; Nelsestuen and Zytkovicz, 1974) and probably also occurs in the same position in factor VII, factor IX, and factor X. In the presence of dicumarol, an antagonist of vitamin K, inactive coagulation factors are found in the plasma. In abnormal prothrombin, the γ-carboxyglutamic acid is replaced by glutamic acid (Stenflo et al., 1974) indicating that vitamin K is directly or indirectly involved in a carboxylation reaction.

The overall yield of factor VII was about 2 mg/50 l. of bovine plasma. This is equivalent to 6–7 mg of factor VII assuming a 100% recovery of activity. These data suggest that factor VII occurs in bovine plasma at a concentration of about 0.13 mg/l. This value is much lower than the 1 mg/l. estimated by Radcliffe and Nemerson (1975a). Whether this difference is due to errors in the clotting assay is not known.

In the extrinsic coagulation system, factor VII activates factor X in the presence of tissue factor (Fujikawa et al., 1974a; Jesty and Nemerson, 1974). During this reaction, a specific peptide bond is cleaved in the amino-terminal region of the heavy chain. This cleavage occurs between Arg-51 and Ile-52 giving rise to factor $X_{a\alpha}$ (Fujikawa et al., 1975). Factor VII isolated by the present procedure has an absolute requirement for tissue factor in this reaction. A similar finding has been reported by Radcliffe and Nemerson (1975a) for their highly purified factor VII or activated factor VII. The role of tissue factor in this reaction remains to be clarified.

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