Purification and Properties of Human Coagulation Factor VII*

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Blood coagulation Factor VII was purified 100,000-fold from fresh frozen human plasma to apparent homogeneity with a yield of 30% based on coagulation assay. The molecular weight estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was 48,000. Factor VII is composed of a single polypeptide chain with the NH2-terminal sequence Ala-Asn-Ala-Phe-Leu-(Gla)-(Gla-Leu-(Arg)-Pro. It is converted to a two-chain form (Factor VIIa) connected by disulfide bonds by the action of Factor X, in the presence of phospholipids and calcium, and by Factor XII, without additional cofactors. This conversion is associated with a 20- to 25-fold increase in coagulation assay activity. Factors VII and VIIa were inhibited by 15 mM diisopropyl fluorophosphate with 50% inactivation in 160 and 60 min, respectively. The presence of tissue factor and CaCl2 accelerated the inactivation by approximately 5-fold. Neither Factor VII nor VIIa were inhibited by antithrombin III in the absence of heparin. However, with the addition of heparin, Factor VIIa was inhibited at a rate approximately 25 times that of Factor VII.

Coagulation Factor VII, a vitamin K-dependent coagulation factor, is a single-chain glycoprotein connected by disulfide bonds by the action of Factor X, in the presence of CaCl2 and phospholipids or by thrombin or Factor XII, without additional cofactors. Associated with this conversion, an apparent 40-fold increase in activity was observed as measured in a one-stage coagulation assay. Further, studies have shown that bovine Factor VII is inactivated by diisopropyl fluorophosphate (DFP) (50% inhibition by 2 mM DFP in 20 min) (5, 8) but unaffected by antithrombin III in the absence or presence of heparin (9).

Attempts to purify human Factor VII have been reported previously, but in these studies the material produced was only partially pure or was obtained in such small quantities that it was characterized as activity without detectable protein.

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The abbreviation used is: DFP, diisopropyl fluorophosphate.

(10-15). Here we report the isolation of human Factor VII in sufficient quantities for preliminary characterization.

EXPERIMENTAL PROCEDURES

Materials

Quaternary aminophethyl (QAE) Sephadex A-50, Sephadex G-25-80, and Sephadex G-100-120 were purchased from Pharmacia and low molecular weight standards for polyacrylamide gel electrophoresis were from Bio-Rad. Diisopropyl fluorophosphate, soybean trypsin inhibitor type I, bovine serum albumin, Trizma base, and benzamidine were obtained from Sigma. Factor VII-deficient plasma was purchased from George King (Overland Park, KS), and heparin from Organon (Liquemine, 1000 USP units/ml). Phenyl-ethyl sulfonamide was obtained from Amersham. All other chemicals were of reagent grade or better from J. T. Baker or Fisher.

Methods

Factor VII coagulation assay was performed in a fibrometer (Becton-Dickinson) at 37°C. The sample to be assayed was appropriately diluted in assay buffer which consisted of 0.15 M NaCl, 5 mM sodium citrate, 0.05 M Tris-HCl (pH 7.4), and 5 mg/ml of bovine serum albumin. Sixty microliters of Factor VII-deficient plasma, 60 μl of a 1: 5 dilution of stock human brain thromboplastin, and 60 μl of the sample to be assayed were incubated 30 s at 37°C. The reaction was then started by addition of 60 μl of 25 mM CaCl2 at 37°C.

Standard curves were constructed with dilutions of normal, pooled, citrated human plasma and were linear in the range of from 0.025 to 0.00025 units/ml with clotting times from 25 to 100 s. One unit of Factor VII was defined as that present in 1 ml of pooled, citrated human plasma.

Human brain thromboplastin was used as a source of tissue factor and was a gift from Dr. Heinz Joist (Thrombosis Center CORE Hemostasis Laboratory). It was a saline (0.9% NaCl) extract of fresh human brain prepared after the method of Hjort (16). A human brain was obtained within 24 h postmortem, and meninges and cerebellum were removed under running tap water. Care was taken to remove as many small blood vessels as possible. It was cut into approximately four equal sections, and each was homogenized in a Waring Blender with 250 ml of saline (warmed to 45°C) for 2 min at maximum speed. The brain homogenates were combined and stirred at 37°C for 30 min and then centrifuged at 27,000 × g for 6 min at room temperature. The sediment was resuspended in 500 ml of saline (warmed to 45°C) and incubated an additional 30 min at 37°C with stirring, followed by centrifugation at 27,000 × g for 6 min. The two supernatants were combined, the pH was adjusted to 7.35 with 1 N HCl, and the mixture was stored in 2-ml portions at −70°C. Under these conditions the tissue factor activity was stable at least 1 year. The clotting time of a mixture of 120 μl pooled human plasma, 60 μl of a 1:5 dilution of stock human brain thromboplastin, and 60 μl of 25 mM CaCl2 was 15 s.

Human Factor X and Xa were prepared as described previously (17). Human antithrombin III was prepared by Dr. Marc Shuman (University of California, San Francisco) using a modification (18) of the method of Yin (19). The characteristics of this preparation have been previously reported (17).

Bovine Factor XII was a gift from Dr. Earl Dawe (University of Washington, Seattle, WA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 10% gels (ratio acrylamide/bis, 37:5:1) employing the method of Laemmli (4% stacking gel) (20) or of Weber and Osborn (21). Gels were stained for protein with Coomassie brilliant blue R.
Molecular weights were estimated by interpolation from linear semilogarithmic plots of apparent molecular weights vs migration distance, using the following protein standards: phosphorylase b, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 22,000; and lysozyme, 14,300.

Amino Acid Composition

A sample of the final Factor VII preparation was dialyzed 48 h against 1.0 M acetic acid. Portions containing 200 to 300 μg of protein were lyophilized and then hydroyzed in 6 N HCl at 100°C in sealed, evacuated tubes for 24, 48, and 72 h. Amino acid analysis was performed in duplicate using a Durrum model D 500 amino acid analyzer. The values for serine and threonine were determined by extrapolation to zero hydroyzation time. Isoeucine and valine values were calculated from the 72-h hydroyzation times. Half-cystine was calculated from the 72-h hydrolysis times. Half-cystine was determined as cystic acid by the method of Hirsh (22). The NH₂-terminal sequence analysis was performed on a Beckman 890C Se-

Factor VII Purification

Starting Material—Human, citrated, fresh frozen plasma (with platelets removed) was kindly provided by Dr. William Miller of the Missouri-Illinois Regional Red Cross Blood Program. One hundred and ten units were thawed at 37°C, pooled (19.5 liters), and transferred to the cold room. All subsequent steps were performed at 4°C and in plastic containers. Benzamidine 156 g, and 500 mg of soybean trypsin inhibitor was added and the plasma was stirred for 30 min.

Barrium Citrate Adsorption and Elution and Ammonium Sulfate Fractionation (24) —One liter of  BaCl₂, 1.0 M, was added dropwise over 1 h and the mixture was stirred an additional 30 min. The barium citrate precipitate was collected by centrifugation at 3000 Xg for 15 min and the supernatant plasma was decanted. The precipitate was resuspended in 10 liters of 0.1 M NaCl, 0.02 M BaCl₂, 0.025 M benzamidine, 0.02 M Tris-HCl, pH 8.0, and again collected by centrifugation. This washing procedure was repeated three more times using 6 liters each of the same buffer. The barium citrate precipitate then was resuspended in 4 liters of 0.15 M sodium citrate, 0.025 M benzamidine, 0.05 M Tris-HCl (pH 8.0), and 10 mg/liter of soybean inhibitor, and the mixture was stirred 2 h to elute the adsorbed proteins. The barium citrate was removed by centrifugation at 3000 Xg for 30 min and dry ammonium sulfate, 600 g (25% saturation), was added slowly to the supernatant with stirring. The precipitate was removed by centrifugation at 3000 Xg for 30 min and an additional 1000 g of ammonium sulfate (70% saturation) was added slowly to the super-

Sephadex G-100 Chromatography—The sample then was applied to a column (1.5 x 95 cm) of Sephadex G-100 equilibrated with 0.15 M NaCl, 0.02 M benzamidine, 0.02 M Tris-HCl, pH 7.5, at a flow rate of 10 ml/h, and 1.85-m1 fractions were collected (Fig. 1). Fractions 36 to 44 were pooled and concentrated (Amicon PM-10) to approxi-

FIG. 1. Elution pattern of human Factor VII from QAE-Sephadex. Protein was eluted from the column (6 x 34 cm) with a linear gradient from 0.1 to 0.55 M NaCl in 0.025 M benzamidine, 0.05 M Tris-HCl, pH 8.0, at a flow rate of 15 ml/h. Fractions (14 ml) were collected at a flow rate of 200 ml/h. Factor VII eluted early in an area of relatively little protein, while the activities of prothrombin, Factor X, and Factor IX eluted under the large protein peak which followed. Only the Factor X activity is shown in the figure. A has been determined on samples diluted 20-fold from which the A₃₄₀ of a 20-fold dilution of the elution buffer was subtracted. O--O, absorbance at 280 nm; Δ--Δ, Factor VII activity; O- - -O, Factor X activity; ---, conductivity.

FIG. 2. Gel filtration on Sephadex G-100. The Factor VII after elution from QAE-Sephadex using CaCl₂ was concentrated to 2 ml and applied to a column (1.5 x 95 cm) of Sephadex G-100-120. Fractions (1.85 ml) were collected at a flow rate of 15 ml/h. A₃₄₀ was determined on samples diluted 20-fold from which the A₃₄₀ of a 20-fold dilution of the elution buffer was subtracted. O--O, absorbance at 280 nm; O- - -O, Factor VII activity.
TABLE I

Purification of human Factor VII

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Activity</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1.4 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>19,500</td>
<td>0.014</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Barium citrate ammonium sulfate</td>
<td>2.68 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>15,000</td>
<td>5.60</td>
<td>77</td>
<td>400</td>
</tr>
<tr>
<td>QAE-Sephadex 1</td>
<td>115.0</td>
<td>15,600</td>
<td>135</td>
<td>80</td>
<td>9,700</td>
</tr>
<tr>
<td>QAE-Sephadex 2</td>
<td>5.1</td>
<td>7,900</td>
<td>1,550</td>
<td>41</td>
<td>111,000</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>2.6</td>
<td>5,900</td>
<td>2,300</td>
<td>30</td>
<td>163,000</td>
</tr>
<tr>
<td>Sephadex G-25</td>
<td>0.5</td>
<td>4,000</td>
<td>8,000</td>
<td>30</td>
<td>132,000</td>
</tr>
</tbody>
</table>

<sup>a</sup> Protein concentration was estimated by absorbance at 280 nm, assuming ε<sub>280</sub> = 10.

RESULTS

Purification of Factor VII—Three major factors made the purification of human Factor VII difficult: 1) it is a trace protein in human plasma; 2) activation and subsequent degradation of the Factor VII occurs during the purification procedure; 3) loss of activity occurs at protein concentrations of less than approximately 30 μg/ml. For these reasons, relatively large volumes of fresh frozen plasma were used as starting material, and benzamidine, which had been used successfully by Radcliff and Nemerson in the purification of bovine Factor VII (5), was added at concentrations of from 20 to 50 mM in all buffers. In addition, soybean trypsin inhibitor was added prior to each chromatography step except the last (12).

Human Factor VII was purified approximately 100,000-fold by a five-step process with an approximate 30% yield based on activity in a coagulation assay (Table I). In this purification procedure, the vitamin K-dependent coagulation factors were removed from plasma by adsorption to barium citrate. Factor VII was separated from Factors II, IX, and X by chromatography upon QAE-Sephadex (Fig. 1). With the addition of CaCl<sub>2</sub> to the buffer of the second QAE-Sephadex column, Factor VII was eluted and represented approximately 50% of the protein as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. We found that rapid concentration of this Factor VII pool, as it eluted from the column, was required to prevent significant loss in activity. The remaining contaminants, which were without detectable activity by coagulation assay, were removed by gel filtration on Sephadex G-100. The final preparation had a specific activity of 2.3 units/μg of protein<sup>a</sup> and was without detectable Factor X, Factor IX, or prothrombin activity.

Properties of Factor VII—The final preparation of Factor VII appeared to be homogeneous as judged by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate. There was a single protein seen before or after reduction with 2-mercaptoethanol (see Fig. 3). In the Laemml system (20) of the Factor VII following Sephadex G-100 gel filtration. Each gel contains 12.5 μg of protein; left sample, unreduced; right sample, following reduction with 5% 2-mercaptoethanol.

TABLE II

Amino acid composition of Factor VII

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/40,000 g of protein&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>32</td>
</tr>
<tr>
<td>Threonine</td>
<td>20</td>
</tr>
<tr>
<td>Serine</td>
<td>31</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>44</td>
</tr>
<tr>
<td>Proline</td>
<td>21</td>
</tr>
<tr>
<td>Glycine</td>
<td>37</td>
</tr>
<tr>
<td>Alanine</td>
<td>21</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>20</td>
</tr>
<tr>
<td>Valine</td>
<td>19</td>
</tr>
<tr>
<td>Methionine</td>
<td>2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>12</td>
</tr>
<tr>
<td>Leucine</td>
<td>32</td>
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<tr>
<td>Tyrosine</td>
<td>10</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>11</td>
</tr>
<tr>
<td>Histidine</td>
<td>10</td>
</tr>
<tr>
<td>Lysine</td>
<td>17</td>
</tr>
<tr>
<td>Arginine</td>
<td>20</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>N.D.&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assumes that human Factor VII has 40,000 g of protein, the remainder being carbohydrate (6).

<sup>b</sup> N.D., not determined.

was detected at cycles 6 and 7, and we assume that these residues are γ-carboxyglutamic acids.

Formation of Factor VII—Activated Factor VII (Factor VII<sub>a</sub>) was produced by incubating Factor VII with 1.0% (w/w) Factor X, in the presence of 100 μg/ml of phospholipid and 4 mM CaCl<sub>2</sub>. A 20- to 25-fold increase in activity by coagulation

FIG. 3. Ten per cent polyacrylamide gel electrophoresis in sodium dodecyl sulfate (20) of the Factor VII following Sephadex G-100 gel filtration. Each gel contains 12.5 μg of protein; left sample, unreduced; right sample, following reduction with 5% 2-mercaptoethanol.
Human Factor VII

Comparison of the NH₂ terminal sequences of human prothrombin, Factor X, and Factor IX (24), bovine Factor VII (6), and human Factor VII.

Gla refers to γ-carboxyglutamic acid. Parentheses surround Gla residues which are suspected but not yet proven. Dashes refer to spaces which have been inserted to bring the sequences into alignment for better homology. See note added in proof.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Sequence position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human prothrombin</td>
<td>Ala Asn Thr - Phe Leu Gla Gla - Val Arg Lys Gly Asn Leu</td>
</tr>
<tr>
<td>Human Factor IX</td>
<td>Tyr Asn Ser Gly Lys Leu (Gla) (Gla) Phe Val Gin - Gly Asn Leu</td>
</tr>
<tr>
<td>Human Factor X</td>
<td>Ala Asn Ser - Phe Leu Gla Gla - Met Lys</td>
</tr>
<tr>
<td>Bovine Factor VII</td>
<td>Ala Asn - Gly Phe Leu (Gla) (Gla) Leu Leu - Pro Gly Ser Leu</td>
</tr>
<tr>
<td>Human Factor VII</td>
<td>Ala Asn - Ala Phe Leu (Gla) (Gla) Leu - (Arg) Pro</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table III</th>
<th>Comparison of the NH₂ terminal sequences of human prothrombin, Factor X, and Factor IX (24), bovine Factor VII (6), and human Factor VII.</th>
</tr>
</thead>
</table>

The effect of DFP upon Factor VII and Factor IX was assessed in the presence of heparin. The effect of antithrombin III upon Factor VII and Factor VII in the presence of heparin was more complex. Following the initial loss of approximately 30% of the Factor VII activity, a much slower rate of inactivation of Factor VII was found, with a half-life of 270 min. We postulate that the initial rapid loss of activity is due to contamination of the Factor VII preparation.

Figure 4. Ten percent polyacrylamide gel electrophoresis in sodium dodecyl sulfate (20) of activated Factor VII (Factor VII). Each gel contains 5 μg of protein; left sample, unreduced; right sample, reduced with 5% 2-mercaptoethanol.

Figure 5. Inhibition of Factor VII and VII by diisopropyl fluorophosphate (15 mM). Reaction mixtures (500 μl) containing 3.5 μg/ml of Factor VII or Factor VII in 0.1 M NaCl, 0.125 M Tris-HCl (pH 7.5), and 5 mg/ml of bovine serum albumin were constructed at room temperature. Reaction mixtures designated tissue factor + CaCl₂ also contained human brain thromboplastin (final dilution, 1:20) and 4 mM CaCl₂. Following removal of a 10-μl sample for zero time analysis, 10 μl of 0.75 mM DFP in 2-propanol was added. Samples then were removed at the indicated time, immediately diluted, and assayed for Factor VII activity.

- Figure 4: Ten percent polyacrylamide gel electrophoresis in sodium dodecyl sulfate (20) of activated Factor VII (Factor VII). Each gel contains 5 μg of protein; left sample, unreduced; right sample, reduced with 5% 2-mercaptoethanol.

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with Factor VII\textsubscript{a} (see "Discussion"). In reaction mixtures containing heparin, but without antithrombin III, no inactivation of either Factor VII or VII\textsubscript{a} was detected (data not shown). Further, the addition of tissue factor and CaCl\textsubscript{2} to the reaction mixtures did not inhibit the inactivation of either Factor VII or Factor VII\textsubscript{a} by antithrombin III and heparin.

**DISCUSSION**

Although purification of bovine Factor VII (5, 6, 8) was reported several years ago, isolation of human Factor VII has been problematic. The successful purification of human Factor VII reported here required the use of relatively large amounts of human plasma as starting material and the inclusion of protease inhibitors throughout the purification procedure. A similar purification scheme was attempted using Cohn Fraction III as starting material; however, this method has yielded only two-chain Factor VII of relatively low specific activity.

The difference in the apparent molecular weight of human Factor VII as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the differing buffer systems (Tris/glycine versus sodium phosphate) is not explained. A similar discrepancy in the molecular weight assessment of human Factor IX has previously been described (27). Molecular weight analysis by the Laemmli method has consistently given higher values for human Factor X and Factor IX, and bovine Factor VII than those obtained by sedimentation equilibrium experiments (6). In the case of Factor IX, the molecular weight determined by electrophoresis in a phosphate buffer system agreed much more closely with the reported sedimentation experiments (27). Therefore, we have assumed the value of 48,000 for human Factor VII.

The properties of the human protein are very similar to those described for bovine Factor VII. Each has been isolated as a single-chain protein of similar molecular weight which can be activated by Factor X, in the presence of phospholipid and calcium ions and by Factor X\textsubscript{a}, without additional cofactors (5, 7). The amino acid compositions indicate considerable homology, and the NH\textsubscript{2}-terminal sequences are very similar, with 8 of the first 10 residues apparently identical.

Confirming the qualitative reports of others (15, 28), we have found Factor VII to be less sensitive to DFP inactivation than the bovine protein and, further, that the rate of inactivation of Factor VII or VII\textsubscript{a} is increased 5-fold in the presence of tissue factor and calcium. A similar acceleration of the rate of inactivation of two-chain bovine Factor VII by DFP has been reported (8).

Although it has been difficult to detect contamination of the Factor VII product with activated Factor VII by polyacrylamide gel electrophoresis, the yield of activity following QAE-Sephadex chromatography has consistently been greater than expected. This suggests that some activation of Factor VII is occurring during the chromatography procedure itself despite the presence of inhibitors. If human Factor VII has 35 times the activity of native Factor VII, as suggested by the studies of Radcliff et al. (30), then the initial drop in activity found following treatment of our Factor VII preparation with antithrombin III and heparin (Fig. 6) could be explained by <1% (w/w) contamination with Factor VII\textsubscript{a}, which would be nearly undetectable by polyacrylamide gel analysis. Further, if one assumes this degree of contamination of the final Factor VII preparation, it would be predicted that its activation would be associated with a 23-fold increase in activity, which agrees well with our findings.

If the above assumptions are correct, then the rate of inactivation of Factor VII by antithrombin III and heparin is approximately 25 times faster than the inactivation of Factor VII. Nevertheless, the rates of inactivation of other activated coagulation factors by antithrombin III and heparin are orders of magnitude faster and, thus, the physiological role, if any, of the inactivation of Factor VII, by antithrombin III and heparin remains to be established. It is notable, however, that the presence of phospholipid and CaCl\textsubscript{2} protect Factor X\textsubscript{a}, from inhibition by antithrombin III (31), whereas the presence of tissue factor and CaCl\textsubscript{2} did not prevent the inactivation of Factor VII or Factor VII\textsubscript{a} by antithrombin III and heparin.

**Acknowledgments**—We wish to thank Dr. Joseph P. Miletich for valuable discussions and Owen J. Bates and Dr. Ralph A. Bradshaw for assistance with the amino acid and sequence analyses.

**Note Added in Proof**—After this paper was accepted for publication, Dr. W. Kisiel, University of Washington, informed us that he had obtained preliminary sequence data on human Factor VII that confirmed our results at every position except cycle 9, where he found Arg while we initially reported Val. Upon analysis of the aqueous phase of sequence cycles 8 to 10 by high pressure liquid chromatography, we also found arginine at cycle 9 and thus have tentatively assigned Arg at this position.

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Human Factor VII

1247

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