Immunoaffinity Purification of Bovine Factor VII

By Ronald Bach, John Oberdick, and Yale Nemerson

Factor VII has been purified to homogeneity from bovine plasma by a procedure that includes affinity purification on an immunoadsorbent column. Recovery was determined by both coagulant assay and liquid scintillation counting, using $^3$H-factor VII as an internal standard. The purification factor calculated by both methods was ~120,000-fold, with a final yield of ~18%. Homogeneity was assessed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The material migrated as a single polypeptide chain of 53,000 daltons, and following activation by factor Xa, the one-chain zymogen was quantitatively converted to two-chain factor VIIa. Conversion of affinity-purified factor VII to factor VIIa resulted in up to a 119-fold activation of the coagulant activity, which is 2.7-4 times greater than the activatability reported for factor VII prepared by other methods. Zur et al. calculated that pure factor VII, uncontaminated by traces of factor VIIa, would be activated 123-fold upon conversion to factor VIIa. The close agreement between observed activatability of affinity-purified factor VII and the theoretical prediction suggests that we have isolated factor VII essentially free of factor VIIa. The purification data from three lots of bovine plasma yield an estimate for the plasma concentration of factor VII from 10.1 nM to 18.5 nM.

Factor VII is a vitamin K-dependent clotting factor that has been purified to homogeneity from bovine and human plasmas. The isolated zymogen form of bovine factor VII is a single polypeptide chain that exhibits sequence homology with other mammalian serine proteases. Molecular weight estimates of this molecule range from 45,000 to 54,000, as judged by sedimentation equilibrium and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, respectively. The purification required to achieve homogeneity, as determined by coagulation assay, is 200,000–500,000-fold.

Factors Xa and XIla and thrombin have been shown to cleave an Arg–Ile bond in factor VII, converting it to a 2-chain molecule composed of disulfide-linked polypeptides of about 29,500 and 23,500 daltons. The rate of factor Xa-catalyzed conversion of factor VII (1-chain) to factor VIIa (2-chain) is significantly greater than that of the other enzymes that catalyze this reaction. Recently, it has been demonstrated by a variety of techniques, independent of the measurement of coagulant activity, that both molecular forms of factor VII are enzymatically active. The estimated activity of the 1-chain molecule is 0.8% of the more active 2-chain form. In other words, conversion of factor VII to factor VIIa will result in a 120-fold stimulation of the coagulant activity.

Since factor VIIa is more active than its one-chain precursor, measurement of the coagulant activity of factor VII is very sensitive to trace contamination by factor VIIa. During the purification of this protein, small amounts of factor VIIa may be generated, which are below the level of detection by direct physical characterization, such as SDS polyacrylamide gel electrophoresis. This trace contamination by factor VIIa may account for a significant fraction of the observed coagulant activity. One consequence of factor VIIa in preparations of factor VII is an overestimate of yield, specific activity, and fold purification based on the measurement of coagulant activity. Likewise, the activatability of such a mixture of one and two-chain molecules would be decreased. Published purification data for bovine factor VII support the contention that traces of factor VIIa were generated by these procedures. In particular, the recovery of activity in excess of 100% has been observed, and a decrease in activatability from 85-fold to 45-fold during the course of purification has been reported. Thus, it is likely that published estimates of the purification and activatability of bovine factor VII are in error.

In revising the purification of factor VII, we had two main objectives: first, to directly estimate the difference in activity between factor VII and factor VIIa, which requires preparation of essentially pure zymogen, and second, to obtain an accurate estimate of the plasma concentration of factor VII. Previous experience with the immunoaffinity purification of tissue factor indicated that this approach to the purification of a labile trace protein like factor VII has the desired properties of speed and high yield. Immunoaffinity purification was therefore adopted for the isolation of bovine factor VII. To measure directly the recovery of this molecule, factor VII, labeled with tritium, was added to the starting plasma, and recovery of the radioactivity was determined throughout the purification. Thus, by isotope dilution, an accurate estimate of the plasma concentration of factor VII was determined.
MATERIALS AND METHODS

Bovine plasma containing 0.38% trisodium citrate and 5 mM benzamidine – HCl as anticoagulants was obtained from Irvine Scientific (Santa Ana, CA). Tris (trizma base), bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, Coomassie brilliant blue R-250, and diisopropylfluorophosphate (DFP) were from Sigma Chemical Co. (St. Louis, MO). Benzamidine – HCl was a product of Aldrich (Milwaukee, WI). Guanidine – HCl (ultrapure) was purchased from Heico (Delaware Water Gap, PA). Aqueous and sodium H3-borohydride (5–10 Ci/m mole) were from New England Nuclear (Boston, MA). Sodium dodecyl sulfate, acrylamide, N,N'-methylenebisacrylamide, temed, ammonium persulfate, and Affi-Gel 10 were purchased from Bio-Rad (Richmond, CA). Grand Island Biological Co. (Grand Island, NY) was the source of Freund’s adjuvant (complete and incomplete). Protein A-Sepharose, Sephacryl S-200, Sephadex G-25, and DEAE-Sephadex A-50 were the products of Pharmacia (Piscataway, NJ). Diaflo YM-10 ultrafiltration membranes were purchased from Amicon (Danvers, MA). All other chemicals were reagent quality.

Factor VII Assay

Factor VII was assayed by the method of Nemerson and Clyne, with the exception that bovine plasma was depleted of factor VII by adsorption with an immunoaffinity column specific for factor VII. An immunoabsorbent column was constructed as described below and used exclusively for the preparation of deficient plasma. Bovine plasma, containing 0.38% trisodium citrate, was thawed at room temperature and centrifuged for 10 min at 5,000 g to remove insoluble material. The plasma was then passed over the affinity column at room temperature, and fractions were collected into plastic tubes. Each fraction was assayed for factor VII by combining 0.1 ml of sample, 0.1 ml 100 mM NaCl, 50 mM Tris, pH 7.5, containing 0.1% bovine serum albumin (TBSA), and 0.1 ml thromboplastin in a borosilicate glass tube. After incubation for 20 sec at 37°C, 0.1 ml 25 mM CaCl2 was added and the time elapsed to formation of a fibrin clot recorded. Plasma fractions with clotting times in excess of 90 sec were pooled for use as substrate plasma and stored in plastic tubes at –20°C. Samples of the normal citrated bovine plasma were saved and stored at –20°C for use as the assay standard, with 1 ml defined as containing 1.0 U of factor VII activity. Serial dilutions of this material in TBSA were assayed using the factor VII-deficient substrate plasma as described above. A log-log plot of clotting time versus plasma dilution (factor VII U/ml) yielded a straight line from 0.2 factor VII U/ml (–25 sec) to 0.0125 factor VII U/ml (–60 sec) and was used as the standard curve for determining the activity of samples generated during the purification.

Protein Determinations

Protein concentrations were estimated by absorbance at 280 nm, assuming an A1% of 10 for protein mixtures and an A1% of 12.9 for the purified factor VII. Samples containing benzamidine - HCl were either dialyzed against 100 mM NaCl, 50 mM Tris, pH 7.5 (TBS), or diluted into TBS and read against a blank containing the same concentration of benzamidine - HCl.

Factor VII Tritiation

The sialic acid residues on factor VII were labeled with tritium by oxidation with sodium periodate followed by reduction with NaBH3H2O, following the protocols previously described for tritiation of bovine factors IX and X.11-13 It was assumed that the molecule contains 10 mole of sialic acid per mole of protein. The specific radioactivity of the labeled protein was determined to be ~150,000 cpm/µg. The covalent association of the label with the protein was demonstrated by the precipitability of the tritium (>99%) with 5% trichloroacetic acid and the comigration of the tritium and the protein on SDS polyacrylamide gels (see reference 8, Fig. 7).

Immunoadsorbent Column

Antiserum was prepared against bovine factor VII by immunizing a 6-wk-old male white New Zealand rabbit with factor VII prepared by the previously published protocol.1 One hundred micrograms of homogeneous factor VII in 150 mM NaCl, 100 mM NaH2PO4, pH 7.6, was emulsified with an equal volume of Freund’s complete adjuvant. The emulsion was injected subcutaneously into multiple sites bilaterally along the back of the animal. After 4 wk, the rabbit was boosted with 10 µg of factor VII emulsified in Freund’s incomplete adjuvant by the same inoculation routine. Subsequently, the boosts were repeated every 2 wk. Following the second boost, the animal was bled and the serum was evaluated by using Ouchterlony double immunodiffusion1 against bovine plasma and pure factor VII.

Once the titer of the antiserum reached a plateau, sufficient antiserum was accumulated for construction of an immunoadsorbent column. Freshly drawn blood was allowed to clot in a glass tube at room temperature. After removing the clot, the sample was centrifuged at 5,000 g for 15 min and the serum withdrawn. The IgG fraction was prepared by passing the serum over a protein A-Sepharose column equilibrated with TBS. The column was then washed extensively with TBS until the absorbance of the eluate at 280 nm returned to baseline. The bound IgG was then eluted with 1 M acetic acid and dialyzed immediately against TBS at 4°C. The IgG concentration of the dialyzed material was determined by absorbance at 280 nm, assuming an A1% of 14. The IgG was further dialyzed against 100 mM NaHCO3 at 4°C. Affi-Gel 10 was prewashed with 2-propanol and H2O at 0°C. The protein and Affi-Gel 10 were combined (10 mg IgG/ml bed volume). Coupling continued overnight at 4°C with gentle mixing. Unreacted sites were then blocked by the addition of 1/10 vol of 1 M glycine ethyl ester, pH 8.0, for 1 hr at 25°C. Coupling efficiency was determined by measuring the absorbance at 280 nm of the supernatant following dialysis. Prior to use, the column was washed with the buffers used during the immunopurification as described below. When not in use, the column was stored at 4°C in TBS, 1 mM Na3HPO4.

Factor VII Purification

Ten liters of bovine plasma, containing 0.38% trisodium citrate and 5 mM benzamidine - HCl as anticoagulants, was thawed at room temperature and centrifuged at 5,000 g for 10 min to remove insoluble material. 3H-factor VII (~6 x 106 cpm) was added as an internal standard for calculation of protein recovery by liquid scintillation counting. Following withdrawal of a sample for counting and assay for factor VII activity, the vitamin K-dependent factors were isolated by barium citrate adsorption, as previously described.1,5 The barium citrate eluate, containing factors II, VII, IX, and X, was dialyzed extensively against TBS, 25 mM benzamidine - HCl at 4°C, centrifuged at 5,000 g for 30 min at 4°C to remove precipitated material, and chromatographed on DEAE-Sephadex A-50. The DEAE-Sephadex column (2.5 cm x 45 cm) was equilibrated with TBS, 25 mM benzamidine - HCl. The protein was applied at 4°C, and the column was then washed with 250 ml of starting buffer and eluted with a continuous 1.6-liter gradient, ranging from 0.12 M NaCl to 0.58 M NaCl with 50 mM Tris, pH 7.5, 25 mM benzamidine - HCl. The factor VII elution profile was monitored by counting aliquots for tritium. The tritum peak emerged on the trailing edge of the peak of eluted protein (absorbance at 280 nm), which is predominantly prothrombin (not shown). Factor VII was pooled and applied directly to the affinity column.
The immunoadsorbent column (125 ml bed volume) contained factor VII-specific IgG coupled to Affi-Gel 10 at a concentration of 7.2 mg IgG/ml bed volume. The column was prepared as described above and equilibrated with TBS, 25 mM benzamidine - HCl. The factor VII pool from ion-exchange chromatography was applied directly to the affinity column at 4°C. If binding of tritium to the column was less than 90%, the breakthrough was recycled over the column. The affinity column was washed with 2 column volumes of TBS, 10 mM benzamidine - HCl, followed by 1 column volume 0.5 M NaCl, 50 mM Tris, pH 7.5, 10 mM benzamidine - HCl and 1 column volume 1 M guanidine - HCl, 50 mM Tris, pH 7.5, 10 mM benzamidine - HCl. The factor VII was then eluted from the column with 4 M guanidine - HCl, 50 mM Tris, pH 7.5, and 10 mM benzamidine - HCl. Elution of factor VII from the immunoadsorbent column was monitored by liquid scintillation counting. The protein was fully eluted in approximately 1 column volume. The factor VII pool was immediately dialyzed against 2 x 4 liters of TBS, 10 mM benzamidine - HCl at 4°C.

The dialyzed pool from the affinity column was then concentrated on an Amicon YM10 membrane to approximately 2 ml. The concentrate was then gel filtered on Sephacryl S-200 (0.9 cm x 95 cm) in TBS, 5 mM benzamidine - HCl at 4°C, and 1-ml fractions were collected. Each fraction was then assayed for factor VII activity, absorbance at 280 nm, and tritium. The fractions comprising the coincident peaks of tritium and factor VII activity were pooled and stored at -20°C. The factor VII concentration of the purified pool was determined by absorbance at 280 nm. The homogeneity of the material was determined by SDS polyacrylamide gel electrophoresis and activation by factor Xa, as described in Results, Figs. 1 and 2.
RESULTS

The purification data from a single preparation of factor VII are summarized in Table 1. Factor VII was purified ~120,000-fold from bovine plasma as judged by both coagulant activity and tritium recoveries, with a final yield of ~18%. Purification based on the recovery of tritium and activity are in close agreement (114,000-fold versus 125,000-fold). These data yield an estimate for the plasma concentration of factor VII of 580 μg/liter (10.9 nM). This plasma concentration was corrected for the ^1H-factor VII added as an internal standard.

In this purification scheme, factor VII was completely resolved from factor X, partially separated from prothrombin, and copurified with factor IX on DEAE-Sephadex A-50. Factor VII, which comprises <2% of the protein in the DEAE pool, was then separated from factor IX and prothrombin on the immunoadsorbent column as judged by specific coagulant assays and SDS polyacrylamide gel electrophoresis. Washing the affinity column with 0.5 M NaCl and 1 M guanidine · HCl prior to elution removed nonspecifically adsorbed protein from the column. In addition to factor VII, the affinity column eluate contains a high molecular weight protein. This material appears to be IgG, based on its electrophoretic mobility on SDS polyacrylamide gels with and without reduction.

Gel filtration of the IgG-Affi-Gel pool on Sephacryl S-200 separated factor VII and the high molecular weight contaminant. The peaks of tritium and factor VII activity were coincident. This pool from gel filtration contained chromatographically pure factor VII, as judged by SDS polyacrylamide gel electrophoresis (Fig. 2). In fractions preceding the factor VII peak, a low level of tritium counts is detected. However, no factor VII activity is observed in these fractions. This suggests that some inactive factor VII has been generated by the affinity column step, which is then removed by gel filtration. This inactive material may be factor VII denatured by the 4 M guanidine · HCl elution of factor VII complexed with antibodies stripped from the affinity column. The presence of IgG and a molecule with the same molecular weight as factor VII in these fractions was confirmed by SDS polyacrylamide gel electrophoresis. This supports the hypothesis that the inactive factor VII in the affinity column eluate is complexed with IgG.

Results of the activation of affinity-purified factor VII by factor Xa are presented in Figs. 1 and 2. In this experiment, factor VII was activated 119-fold from 840 factor VII U/mg to a final specific activity of 10^4 factor VII U/mg. The SDS polyacrylamide gels demonstrate the conversion of the 53,000-dalton zymogen to factor VIIa, composed of a 23,500-dalton light chain and 29,500-dalton heavy chain, as previously described. Prolonged activation (1,200 min) resulted in essentially complete conversion of the one-chain molecule into the two-chain species, along with a trace of the inactive three-chain molecule. Activatability of the crude fractions, barium citrate eluate, and DEAE pool (Table 1) is significantly less than that of the purified material. Since factor VII is a trace component of these pools, activation cannot be followed on SDS gels. Thus, incomplete conversion to factor VIIa or accumulation of the inactive three-chain species may occur in these fractions, and the measured activatability may be less than the true value. The anomalous results with respect to total factor VII units and activatability of these pools is considered further in the Discussion.

The final specific activity of this affinity-purified factor VII is in good agreement with the specific activity of activated factor VII prepared by the conventional protocol (1.25 × 10^5 factor VII U/mg).²

Table 1. Bovine Factor VII Purification

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Total Tritium (cpm x 10^-3)</th>
<th>Tritium (cpm x 10^-3)</th>
<th>Tritium Purification (fold)</th>
<th>Total Activity (Factor VII U x 10^3)</th>
<th>Activity Purification (fold)</th>
<th>Activatability (fold)</th>
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<tbody>
<tr>
<td>Plasma*</td>
<td>6.88 x 10^3</td>
<td>6.07</td>
<td>100</td>
<td>1</td>
<td>4.6</td>
<td>1</td>
</tr>
<tr>
<td>Barium citrate* eluate</td>
<td>3.76 x 10^3</td>
<td>3.63</td>
<td>59.8</td>
<td>109</td>
<td>8.3</td>
<td>180</td>
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<tr>
<td>DEAE pool*</td>
<td>186</td>
<td>2.94</td>
<td>48.4</td>
<td>1,790</td>
<td>5.76</td>
<td>125</td>
</tr>
<tr>
<td>IgG-Affi-Gel pool*</td>
<td>5.33</td>
<td>1.97</td>
<td>32.4</td>
<td>41,900</td>
<td>0.90</td>
<td>19.6</td>
</tr>
<tr>
<td>S-200 pool†</td>
<td>1.03</td>
<td>1.08</td>
<td>17.8</td>
<td>114,000</td>
<td>0.86</td>
<td>18.6</td>
</tr>
</tbody>
</table>

* Radcliffe and Nemerson² define the coagulant activity of citrated bovine plasma as 100 factor VII U/ml. We have adopted the standard convention of 1 factor VII U/ml in this article. Thus, the specific activity for their preparation of factor VIIa has been divided by 100 to conform to the definition of factor VII units used herein.

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Data presented represent the purification of factor VII from 10 liters of bovine plasma, with 0.38% trisodium citrate and 5 mM benzamidine · HCl as anticoagulants. Each sample (1 ml) was combined with 10 ml Aquasol, and total tritium counts per minute (cpm) were determined using a Searle Delta 300 liquid scintillation counter. Plasma total tritium was corrected for quenching empirically. Factor VII activity and activatability were determined as described in Materials and Methods and Fig. 1.
However, the specific activity of affinity-purified factor VII prior to activation is one-third of that of the conventionally purified material. This suggests that affinity-purified factor VII is virtually free of contamination with the more active two-chain species, factor VIIa (see below).

In Table 2, the purification data of three factor VII preparations from different lots of plasma are summarized. Overall yield of $^3$H-factor VII ranges from 13.8% to 18.6%. The calculated plasma concentration of factor VII varies from 533 μg/ml to 978 μg/ml in these different lots. Activation of the purified factor VII under conditions described in Fig. 1 ranged from 98-fold to 119-fold, with final specific activities of $10^2$–$1.09 \times 10^4$ factor VII U/mg.

**DISCUSSION**

Since factor VII is cleaved by several plasma proteases, preparation of the one-chain zymogen requires the use of protease inhibitors. Even with this precaution, it appears that previous methods for factor VII purification have produced factor VII contaminated with small amounts of factor VIIa, as judged by recovery of coagulant activity in excess of 100% and decreasing activatability throughout. In this article, we describe a procedure to purify factor VII in high yield and essentially free of factor VIIa.

From the inhibition kinetics of bovine factor VII and factor VIIa by diisopropyl fluorophosphate (DFP), Zur et al. predicted a theoretical maximum activation of 123-fold for the conversion of pure one-chain factor VII to the more active two-chain species. They also present a method for calculating the contamination of factor VII preparations by factor VIIa based on the observed fold activation. Radcliffe and Nemerson prepared factor VII that was 45-fold activatable. Following a similar procedure, Kisiel and Davie prepared factor VII, which was later reported to be 30-fold activatable. The calculated factor VIIa contamination of these preparations is 1.4% and 2.5%, respectively. By contrast, we have prepared factor VII by immunoabsorbent affinity chromatography that can be activated 98–119-fold (Table 2). The estimated factor VIIa contamination of this material (0.028%–0.22%) is significantly less than for the previous preparations.

The close agreement between the predicted theoretical maximum activation and that observed with affinity-purified material suggests that we have prepared essentially pure one-chain factor VII. It also contributes additional support to the substantial body of evidence that the zymogen is proteolytically active.

From the measurement of coagulant activity and activatability (Table 1), it would appear that factor VIIa was generated during the early steps in our purification and that the immunoabsorbent column step eliminated this contamination. By direct comparison of factor VII and factor VIIa for stability in 4 M guanidine·HCl and for binding and recovery from the affinity column, no differences in behavior were observed. It appears, therefore, that the increased coagulant activity and decreased activatability in the barium citrate eluate and DEAE pool are the result of factor VIIa generated during storage at 4°C prior to assay. Likewise, the absence of factor VIIa in the final product may be due to the rapid processing of the material through these steps preceding the affinity column.

A primary objective of our research is a complete kinetic description of the network of reactions that leads ultimately to fibrin clot formation. Once these kinetics parameters are known, it may be possible to derive a mathematical model that precisely describes the dynamic behavior of the system in response to any perturbation. Central to such a description of coagulation is an accurate estimate of the concentration in normal plasma of all the clotting factors. We have described a method for determining the plasma concentration of factor VII, free from the ambiguities inherent in the estimates of coagulant activity. From the recovery of tritium and the final yield of pure factor VII, the calculated concentration of factor VII in bovine plasma ranges from 10.1 nM to 18.5 nM. By way of comparison, the plasma concentrations of the other vitamin K-dependent clotting factors are 2,100 nM, 200 nM, and 60 nM for prothrombin, factor X, and factor IX, respectively.

**ACKNOWLEDGMENT**

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**Table 2. Summary of Bovine Factor VII Preparations**

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Plasma Volume (Liters)</th>
<th>Factor VII Recovered (mg)</th>
<th>Tritium (% Yield)</th>
<th>Plasma [Factor VII] (μg/Liter)</th>
<th>Activatability (fold)</th>
<th>Activity (U/mg x 10$^4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.1</td>
<td>0.74</td>
<td>13.8</td>
<td>533</td>
<td>102</td>
<td>1.09</td>
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<tr>
<td>2</td>
<td>10</td>
<td>1.03</td>
<td>17.8</td>
<td>580</td>
<td>119</td>
<td>1.00</td>
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<tr>
<td>3</td>
<td>9.9</td>
<td>1.80</td>
<td>18.6</td>
<td>978</td>
<td>98</td>
<td>1.04</td>
</tr>
</tbody>
</table>

Plasma [factor VII] was corrected for the $^3$H-factor-VII added as internal standard. Activatability was determined as described in Fig. 1.
REFERENCES

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