Activation and Control of Factor VII by Activated Factor X and Thrombin

ISOLATION AND CHARACTERIZATION OF A SINGLE CHAIN FORM OF FACTOR VII*

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SUMMARY

Factor VII purified as previously described, was found to consist of two polypeptide chains joined by disulfide bridges. We now report the isolation and 200,000-fold purification of a single chain form of Factor VII. This was accomplished by protecting the molecule against proteolysis by including benzamidine during the entire purification. The purification was essentially as previously reported except that barium citrate was substituted for barium sulfate as an adsorbent for Factor VII as it resulted in a 4-fold increase in yield.

Single chain Factor VII is rapidly hydrolyzed by Factor X, in the presence of calcium ions and phospholipids, and by thrombin, to a two-chain form which possesses at least 85 times the Factor VII clotting activity of the single chain species. The two-chain form of the enzyme requires tissue factor in order to activate Factor X.

From the observed rates of activation of Factor VII by Factor X, in the presence of calcium ions and phospholipids, it was calculated that at approximately physiological concentration, Factor VII activity would increase at an initial rate of 20-fold per min; this reaction is sufficiently rapid to constitute a feedback control mechanism. The action of thrombin is approximately 40-fold slower under these conditions.

Diisopropylphosphorofluoridate inactivates the single chain and two-chain forms of Factor VII at approximately equal rates. After inhibition, the single chain species could be cleaved but not activated by proteolysis.

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Materials

Sodium dodecyl sulfate, p-aminobenzamidine·HCl, Tris (2-amino-2-hydroxymethyl-1,3-propanediol) (Trizma base), Coomassie blue, soybean trypsin inhibitor, ovalbumin, bovine serum albumin, trypsin, and myoglobin were obtained from Sigma Chemical Co., St. Louis, Mo. Benzamidine·HCl was a product of Aldrich, Milwaukee, Wis. Guanidine·HCl (ultrapure) was a product of Aldrich, Milwaukee, Wis. Guanidine·HCl (ultrapure) was a product of Heico, Delaware Water Gap, Pa. [32P]DFP was a product of Amersham-Searle, Arlington Heights, Ill., and was used as a 40 mM solution in dry 2-propanol (2.4 mCi per ml). DEAE-Sephadex A-50, CM-Sephadex C-50, and Sephadex G-100 were obtained from Pharmacia, Piscataway, N. J. Urea (Fisher Scientific Co.) was deionized before use. Acrylamide monomer and N,N'-methylenebisacrylamide were products of Eastman Scientific Co. (Eastman Kodak Co.) was deionized before use. Sodium dodecyl sulfate was prepared from Micro-Test, St. Louis, Mo. Other chemicals were reagent grade from Fisher or Baker.

In a previous paper (1) it was reported that Factor VII could be purified up to 330,000-fold from bovine plasma by anion exchange chromatography, affinity chromatography on benzamidine coupled to Sepharose, and gel filtration. Purified Factor VII as well as Factor VII in bovine plasma was inhibited by diisopropylphosphorofluoridate (DFP). Electrophoresis of the purified enzyme, inhibited with [32P]DFP, on acrylamide gels in the presence of sodium dodecyl sulfate produced a single band with an apparent molecular weight of 55,000 which coincided with the autoradiographic band. After reduction with 2-mercaptoethanol, a band visualized by autoradiography migrated with an apparent molecular weight of 37,000. These data suggest a multichain protein cross-linked by disulfide bridges.

In the present study, bovine Factor VII was prepared by the same basic procedure but in the presence of benzamidine, an inhibitor of Factor VII and other trypsin-like serine proteases (2). This purified protein had an apparent molecular weight of 53,000 in both the native and reduced states, whether bands were visualized by staining for protein or by autoradiography of the [32P]DFP-protein. We also show that Factor Xa and thrombin can cleave this single chain form of Factor VII to a two-chain form. This proteolysis is accompanied by an increase of at least 85-fold in the specific Factor VII coagulant activity. This activating proteolysis does not alter the requirement for tissue factor for expression of Factor VII activity. After its rapid activation, Factor VII is inactivated more slowly by a further peptide-bond cleavage.

We suggest that the activation and subsequent inactivation of Factor VII by Factor Xa are mechanisms for the control of the tissue factor pathway of coagulation.

EXPERIMENTAL PROCEDURE

Materials

Sodium dodecyl sulfate, p-aminobenzamidine·HCl, Tris (2-amino-2-hydroxymethyl-1,3-propanediol) (Trizma base), Coomassie blue, soybean trypsin inhibitor, ovalbumin, bovine serum albumin, trypsin, and myoglobin were obtained from Sigma Chemical Co., St. Louis, Mo. Benzamidine·HCl was a product of Aldrich, Milwaukee, Wis. Guanidine·HCl (ultrapure) was a product of Heico, Delaware Water Gap, Pa. [32P]DFP was a product of Amersham-Searle, Arlington Heights, Ill., and was used as a 40 mM solution in dry 2-propanol (2.4 mCi per ml). DEAE-Sephadex A-50, CM-Sephadex C-50, and Sephadex G-100 were obtained from Pharmacia, Piscataway, N. J. Urea (Fisher Scientific Co.) was deionized before use. Acrylamide monomer and N,N'-methylenebisacrylamide were products of Eastman Scientific Co. (Eastman Kodak Co.) was deionized before use. Sodium dodecyl sulfate was prepared from Micro-Test, St. Louis, Mo. Other chemicals were reagent grade from Fisher or Baker.

Bovine fibrinogen, Factors VII- and X-deficient bovine plasma, Factors II-, VII-, and X-deficient bovine plasma, Russell's viper venom (Vipera russelli), Australian Taipan venom (Ozyuranus...
Factor VII-deficient plasma was prepared from citrated bovine plasma by the method of Nemerson and Clyne (3). Bovine brain thromboplastin was prepared by the method of Quick (4). Human brain phospholipid (Cephalin) was prepared by the method of Bell and Alton (5).

Coagulation Assays—Factor VII was assayed by the method of Nemerson and Clyne (3). Factor X was assayed by the method of Bachmann et al. (6), using Sigma Factors VII- and X-deficient bovine plasma. Activated Factor X was assayed identically but with Russell's viper venom omitted. Prothrombin was assayed by the method of Johnin and Esnouf (14). Thrombin was assayed by its ability to clot an equal volume of fibrinogen (2 mg per ml) and was calibrated against National Institutes of Health (NIH) standard thrombin (Lot 3B). Factor V was assayed by the method of Lewis and Ware (8). The coagulant protein of Russell's viper venom was assayed by the method of Williams and Esnouf (9). Citrated normal bovine plasma was defined as containing 100 units per ml of prothrombin, Factor V, Factor VII, and Factor X. Thrombin concentration is expressed in NIH units per ml. An arbitrary unit of Factor X was defined such that 1 unit of Factor X generated 1 unit of Factor VII after activation with the coagulant protein from Russell's viper venom. In this unit 1 ml of normal bovine plasma contains a potential of 100 units of Factor X.

Determination of Protein—Protein concentrations were estimated from absorbance at 280 nm assuming an A_280 of 10. Solutions containing 10 mM benzamidine required 10-fold dilution before measurement and were read against buffer containing 1 mM benzamidine.

Concentration of Protein Solutions—Protein solutions were concentrated with an Amicon Diaflo cell using PM-10 membranes.

Sodium Dodecyl Sulfate Gel Electrophoresis—Electrophoresis was performed and the gels were stained for protein as previously described (1), except that urea was omitted from the gels. Gels were stained for carbohydrate by the periodic acid-basic fuchsin method of Kapitany and Zebrowski (10). Gels were dried to a film on filter paper before autoradiography on Kodak RP/R54 x-ray film.

Preparation of Factor Xa—Bovine Factor X was prepared by the method of Esnouf et al. (11) as modified by Jesty et al. (12). Coagulant protein from Russell's viper venom and Factor Xa were purified as previously described (9, 13). Factor Xa was stored at -20°C in 50% glycerol.

Preparation of Thrombin—Bovine prothrombin was purified by the method of Esnouf et al. (11). Taipan venom coagulant protein was purified by a modification of the method of Lanchantin et al. (14). Prothrombin (1 mg per ml) was reacted at 37°C for 5 min with Taipan coagulant protein (7 µg per ml) in 0.15 M NaCl, 50 mM Tris-Cl, pH 7.5, 5 mM CaCl_2. The coagulant protein was largely removed from the reaction by batch adsorption to DEAE-Sephadex (10 mg per ml) and removal of the DEAE-Sephadex by filtration. The thrombin-containing filtrate was applied to a column of CM-Sephadex (1.8 X 30 cm) and developed with a linear gradient, from 0.15 to 0.45 M NaCl, in 50 mM Tris-Cl, pH 7.5 (500 ml each column). The resulting thrombin had a specific activity of 1600 NIH units per mg and was stored at -20°C in 50% glycerol.

Purification of Factor V—Bovine Factor V was prepared by the method of Dombrose et al. (15) and stored at -20°C in 50% glycerol.

Purification of Factor VII—Bovine Factor VII was purified by the method of Nemerson and Clyne (16) with the modification that 6.5 mM benzamidine was present at all stages. Barium citrate eluate was prepared at the New England Enzyme Center, Boston, Mass., by the method of Nemerson and Esnouf (16) with the modification that 6.5 mM benzamidine was present at all stages. Barium citrate eluate was prepared at the New England Enzyme Center by a modification of the procedure of Aronson and Ménaché (17). Fifty liters of bovine plasma were collected into 0.10 volume of 3.85% trisodium citrate containing 65 mM benzamidine. Barium citrate was precipitated by the addition of 4 liters of 1 M BaCl_2. The barium citrate was collected by centrifugation and washed once with ice-cold 5 mM BaCl_2. Protein was eluted with 55% saturated ammonium sulfate containing 50 mM benzamidine and then precipitated with 65% saturated ammonium sulfate containing 50 mM benzamidine. At this stage, the precipitated proteins can be stored as a suspension at 0°C for at least 24 hours. The 65% ammonium sulfate precipitate from the barium citrate eluate was dissolved in 20 mM Tris-Cl, pH 7.5, and 10 mM benzamidine before adsorption onto DEAE-Sephadex.

Barium sulfate eluate or barium citrate eluate was chromatographed on DEAE-Sephadex as described by Nemerson and Clyne (10), with the modification that all solutions used contained 10 mM benzamidine. Further purification by affinity chromatography on benzamidine coupled to Sepharose, gel filtration on Sephadex G-100, and rechromatography on benzamidine coupled to Sepharose were by the method of Jesty and Nemerson (1) with the following modifications: (a) all solutions contained 10 mM benzamidine; and (b) gel filtration was performed last and the 0.2 M guanidine was omitted from the buffer. This produced a product free of guanidine so that the final dialysis step could be avoided. The solution was concentrated by ultrafiltration and stored frozen at -20°C with 10 mM benzamidine present. Immediately before use, samples were thawed and freed of benzamidine on a column of Sephadex G-25.

RESULTS

Purification of Factor VII—It was observed that during the purification of bovine Factor VII by the method of Jesty and Nemerson (1), the yields of Factor VII activity could be increased severalfold by performing the procedure slowly. This suggested that during processing either (a) a zymogen was being converted to active Factor VII, (b) a cofactor was being activated, or (c) Factor VII was being rendered more active. Proteolytic cleavage by Factor VII itself or by a contaminating enzyme seemed likely. Since activation was most rapid during the chromatography on DEAE-Sephadex, the effect of two proteolytic inhibitors on this reaction was investigated (Fig. 1).

Plasma was processed by the method of Nemerson and Esnouf (16) through the stage of adsorption to DEAE-Sephadex and washing off loosely bound material. Aliquots of the slurry were then incubated at 22°C and protein was eluted at intervals and assayed for Factor VII (Fig. 1). Activity approximately tripled in 2 days. In contrast, an aliquot eluted at zero time and incubated in solution activated at less than 10% the rate of the material bound to DEAE-Sephadex. Various amounts of benzamidine or p-aminobenzamidine were added to other aliquots of DEAE-Sephadex slurry as indicated in Fig. 1. Activation was largely eliminated by 10 mM benzamidine or 5 mM p-aminobenzamidine.

The enhancement of the rate of "spontaneous" activation of partially purified Factor VII by adsorption to DEAE-Sephadex may reflect concentration of the reactants on a surface. As the cofactor effects of the phospholipid-calcium ion complex in the activation of prothrombin by Factor Xa may reflect a similar phenomenon, the effect of concentration and cofactors on spontaneous activation of Factor VII was studied. Factor VII purified by the method of Jesty and Nemerson (1) to Step 4 (benzamidine Pool 1) was incubated at several concentrations at 20°C with 3 mM CaCl_2 and 0.1 mg of phospholipids per ml. At a concentration of 300 units of Factor VII per ml, no activation was observed in 1 hour. An increase of 30% per hour was observed at 15,000 units per ml, and 200% per hour at 125,000 units per ml. To ascertain whether Factor VII itself or contaminating enzymes were responsible for the observed activation, various concentrations of soybean trypsin inhibitor (STI) were included in solutions of partially purified Factor VII to which CaCl_2 and phospholipids were added (Fig. 2). At a Factor VII concentration of approximately 200 µg per ml, 3-fold activation was complete in 115 hours. Activation was essentially eliminated by 10 µg of STI per ml, and greatly slowed by even 0.1

scutellatus scutellatus), and Tiger snake venom (Notechis scutellatus occidentalis) were obtained from Sigma.

Methods

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0.1 M NaCl, 5 mM Tris-Cl, pH 7.5, and divided into nine plastic tubes. The slurry was washed with 3 x 40 ml of 0.1 M NaCl, 50 mM Tris-Cl, pH 7.5, on a Buchner funnel, made to a final volume of 30 ml in 0.1 M NaCl, 5 mM Tris-Cl, pH 7.5, and stirred for 20 min, and filtering. The filtrate was incubated at 20°C in a plastic tube and assayed at intervals for Factor VII.

The filtrate was incubated at 20°C in a plastic tube and assayed at intervals for Factor VII. Benzamidine-HCl and p-aminobenzamidine-HCl were added to seven of the nine tubes as indicated. A: Factor VII adsorbed to 3 ml of wet DEAE-Sephadex by stirring for 30 min. The slurry was washed with 3 X 40 ml of 0.7 M NaCl, 50 mM Tris-Cl, pH 7.5, to one of the tubes containing no inhibitor, stirring for 20 min, and filtering. The filtrate was incubated at 20°C in a plastic tube and assayed at intervals for Factor VII (A - . A.). The other tubes were incubated at 20°C, and at intervals aliquots were combined with 2 volumes of 0.7 M NaCl, 50 mM Tris-Cl, pH 7.5, stirred for 20 min, and filtered, and the filtrates were assayed for Factor VII. A-----A, no inhibitor added. O---O, 1 mM p-aminobenzamide. |||, 2.5 mM p-aminobenzamide; []--[], 5 mM p-aminobenzamide. B: •---•, no STI; •-----•, 0.1 µg per ml of STI; •-----•, 1 µg per ml of STI; O----O, 10 µg per ml of STI.

When purified Factor VII which had been prepared from a barium sulfate eluate in the presence of 10 mM benzamidine was inhibited with 1 mM [32P]DFP and studied by sodium dodecyl sulfate gel electrophoresis, nonreduced samples migrated as single bands of about 53,000 daltons. However, electrophoresis of reduced samples indicated heterogeneity. The bulk of the material was present in a 53,000-dalton band but several per cent were present in the two-chain form with bands at 29,500 and 23,500 daltons. Autoradiography indicated that DFP had been incorporated by the 53,000- and 29,500-dalton bands. In contrast, the previous Factor VII prepared in the absence of inhibitor (1) was over 95% in the two-chain form after reduction with 2-mercaptoethanol. The explanation is apparent from the right-hand column of Table I, which indicates that this preparation of Factor VII containing primarily the single chain form can be activated a further 11-fold by Factor X, or thrombin (discussed below). Two-chain Factor VII prepared in the absence of benzamidine (1) cannot be further activated. As discussed later and shown in Fig. 8, the 53,000-dalton form is cleaved to the two-chain form during activation. It can be noted from Table I that during purification the material is losing "activatability," i.e., being activated despite the presence of 10 mM benzamidine; column yields of over 100% activity were often observed (Table I).

When Factor VII was prepared identically but beginning with a barium citrate adsorption, two significant differences were seen. First, 9 mg of Factor VII were obtained from 50 liters of plasma in contrast to the 2.5 mg obtained using the barium sulfate adsorption procedure (Table I). Second, the material obtained was almost entirely in the single chain form (Fig. 8) and could be further activated 45-fold, despite partial activation having occurred during purification (Table I). Factor VII present in the barium citrate eluate before chromatography could be activated 85-fold (Table I).

The various chromatographic profiles obtained by the above procedure do not differ significantly from those obtained by Jesty and Nemerson (1) except for the relative amount of prothrombin seen in the Sephadex G-100 gel filtration step (Fig. 3), which merely reflects the altered sequence of chromatographic procedures. From this it can be concluded that both the activated form and single chain form of Factor VII chromatograph very similarly.

Activation of Factor VII by Factor Xa, CaCl2, and Phospholipids—The activation of Factor VII purified from a barium sulfate eluate by Factor Xa with and without CaCl2 and phospholipids is shown in Fig. 4. Factor Xa alone (4 units per ml) caused minimal activation over 4 hours. The addition of
Table I
Purification of Factor VII

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein Activity</th>
<th>Yield</th>
<th>Specific Activity</th>
<th>Purification</th>
<th>Activityb</th>
<th>Fold</th>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>units x 10^4</td>
<td>%</td>
<td>units/mg</td>
<td>-fold</td>
<td>-fold</td>
</tr>
<tr>
<td>A. Fractionation of barium sulfate eluate from 50 liters of bovine plasma</td>
<td></td>
<td></td>
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<tr>
<td>1. Plasma</td>
<td>3.8 x 10^6</td>
<td>5</td>
<td>100</td>
<td>1.4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2. Barium sulfate eluate</td>
<td>1.3 x 10^6</td>
<td>2.3</td>
<td>46</td>
<td>177</td>
<td>120</td>
<td>2</td>
</tr>
<tr>
<td>3. DEAE pool</td>
<td>2.3 x 10^6</td>
<td>2.8</td>
<td>56</td>
<td>1200</td>
<td>720</td>
<td>177</td>
</tr>
<tr>
<td>4. Benzamidine Pool I</td>
<td>5.5</td>
<td>1.2</td>
<td>23</td>
<td>210,000</td>
<td>150,000</td>
<td>11</td>
</tr>
<tr>
<td>5. Sephadex G-100 pool</td>
<td>2.7</td>
<td>0.87</td>
<td>17</td>
<td>320,000</td>
<td>230,000</td>
<td>11</td>
</tr>
<tr>
<td>6. Benzamidine Pool II</td>
<td>3.6 x 10^6</td>
<td>5</td>
<td>100</td>
<td>1.4</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

B. Fractionation of barium citrate eluate from 50 liters of bovine plasma |
| 1. Plasma | 3.6 x 10^6 | 5.0 | 100 | 1.4 | 1 | 1 |
| 2. Barium citrate eluate | 2.8 x 10^6 | 2.0 | 40 | 72 | 52 | 65 |
| 3. DEAE pool | 3.0 x 10^6 | 2.8 | 56 | 950 | 680 | 65 |
| 4. Benzamidine Pool I | 75 | 2.8 | 55 | 37,000 | 27,000 | 60 |
| 5. Benzamidine Pool II | 21 | 3.2 | 63 | 151,000 | 107,000 | 60 |
| 6. Sephadex G-100 pool | 9 | 2.4 | 48 | 270,000 | 193,000 | 45 |

* Calculated from absorbance at 280 nm assuming A:?, = 10.
* In an activation system containing Factor X, CaCl2, and phospholipids.
* These materials activated too slowly for the derivation of meaningful data.

Fig. 3. Gel filtration of benzamidine Pool II. Benzamidine Pool II (Table I-B) was concentrated to 2 ml and applied to a column (1.5 X 90 cm) of Sephadex G-100 (medium). Protein was eluted with 0.2 M NaCl, 50 mM Tris-Cl, pH 7.5, 10 mM benzamidine at a flow rate of 15 ml per hour (23-mm pressure head). Fractions (1.3 ml) were assayed for prothrombin and Factor VII. Absorbance were read on aliquots diluted 10X and blanked against a 10X dilution of the elution buffer. These values X 10 are shown. Fractions indicated by arrows were pooled and concentrated.

phospholipids to the Factor Xa was without effect. Inclusion of CaCl2 led to slow activation. The combination of Factor Xa, CaCl2, and phospholipids, however, caused a rapid 11-fold activation at a rate over 500 times that with Factor Xa alone. Inclusion of Factor V at a concentration of 100 units per ml had no effect on the rate of activation by the complete system. The presence of 0.1% ovalbumin in this and all other experiments with dilute Factor VII should be noted. Factor VII solutions more concentrated than about 20 µg per ml may be stored for several hours at 20°, chromatographed, frozen, and thawed with no detectable loss of activity, but dilute solutions are markedly unstable to any manipulations in the absence of carrier protein. Bovine serum albumin, ovalbumin, myoglobin, and prothrombin were all satisfactory for this purpose.

Fig. 4. Activation of Factor VII (benzamidine Pool II, Table I-A) by combinations of Factor Xa (4 units per ml), CaCl2 (3 mM), and phospholipids (0.1 mg per ml) in 0.1 M NaCl, 50 mM Tris-Cl, pH 7.5, 1 mg per ml of ovalbumin at 20°. ✿ Factor Xa, CaCl2, phospholipids; ○, CaCl2, phospholipids; ■ Factor Xa, CaCl2; □ Factor Xa, CaCl2, phospholipids.

Fig. 5 depicts the time course of activation of our most highly purified Factor VII (G-100 pool, Table I-B) by Factor Xa, CaCl2, and phospholipids at 37° at a Factor VII concentration approximating that in plasma (defined as containing 100 units per ml). The initial rates of activation are proportional to the concentration of added Factor Xa. Five units of Factor Xa per ml caused a 48-fold activation at an initial rate of about 10-fold per min. The effect of higher concentrations of Factor Xa could not be examined due to interference of the Factor Xa in the Factor VII assay. In the units used in this study, complete activation of the Factor X in plasma would generate 100 units of Factor Xa per ml.

The rate of destruction of activated Factor VII by Factor Xa,
FIG. 5. Activation of Factor VII (Sephadex G-100 Pool, Table IB) by Factor X, CaCl₂ (3 mM) and phospholipids (0.1 mg per ml) at 37°C in 0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 1 mg per ml of ovalbumin. Factor X, was present at the following concentrations: O--O, 5 units per ml; □ --□, 2.5 units per ml; ■ --■, 0.6 unit per ml; □ --□, 0.2 unit per ml; ▲ --▲, no Factor X, added.

CaCl₂ and phospholipids is shown in Fig. 6. After activation with 1 unit of Factor X, per ml, inactivation was studied by adjusting several aliquots to final concentrations of 1, 10, and 33 units of Factor X, per ml. The rate of destruction was proportional to the concentration of Factor X, and occurred at a rate approximately 5% of that of the activation at equivalent concentrations of Factor X,.

The time course of activation and inactivation of Factor VII by Factor X, CaCl₂, and phospholipids as visualized by bio assay and sodium dodecyl sulfate gel electrophoresis is shown in Figs. 7 and 8. At intervals the mixture was assayed for Factor VII (Fig. 7) and aliquots were removed and reacted with 0.05 volume of [³²P]DFP (40 mM in 2-propanol) at 0°C. The samples were then prepared for electrophoresis, one-half of each being reduced with 2-mercaptoethanol. The left half of Fig. 8 depicts the changes seen with nonreduced samples and the right half the changes seen with reduced samples. No change is observed in the electrophoretic behavior of the nonreduced samples until activation is complete and destruction has begun. During inactivation the initial band of 53,000 daltons is cleaved to two species with apparent molecular weights of 41,000 and 12,500 (the molecular weights of these nonreduced bands were estimated by comparison with the mobilities of the reduced standards and are therefore only approximate). The autoradiogram indicates that the DFP-sensitive catalytic site is in the smaller fragment. This fragment, while retaining its ability to incorporate DFP, has lost its coagulant activity. Electrophoresis of the reduced samples indicates that Factor VII activity increases in parallel with cleavage of the 53,000-dalton chain to a form containing two chains of molecular weights 29,500 and 23,500 cross-linked by disulfide bridges. At peak activity, almost all of the material has been converted to the two-chain form, and autoradiography indicates that the active site is on the heavier chain. During the inactivation phase of the reaction the 29,500-dalton chain is cleaved to two chains with apparent molecular weights of 17,000 and 12,500. As in the nonreduced samples, the smaller fragment incorporates [³²P]DFP. It is of note that the 23,500-dalton light chain of the activated molecule is not degraded during the time course of Fig. 7.

The above experiment was repeated with single chain Factor VII that had been inhibited with [³²P]DFP and unreacted DFP and hydrolysis products removed on a Sephadex G-25 column. This inhibited Factor VII was reacted with Factor X, CaCl₂, and phospholipids under the same conditions as in Fig. 7, and samples were subjected to sodium dodecyl sulfate gel electrophoresis as in Fig. 8. No Factor VII activity was generated, but the resultant electrophoretic gel revealed the same patterns after staining for protein and autoradiography as seen in Fig. 8. This indicates that the changes seen in Fig. 8 are due to the action of Factor X, not Factor VII.

When a gel prepared in the same manner as the gel in Fig. 8 was stained for carbohydrate, the nonreduced bands at 53,000 and 41,000 daltons gave positive reactions; the 12,500-dalton...
FIG. 8. A, sodium dodecyl sulfate gel electrophoresis of the activation and destruction of Factor VII. Aliquots were removed from the reaction described in Fig. 7 at the times indicated, placed on ice, and combined with 0.05 volume of 40 mM [32P]DFP in 2-propanol. After 3 hours, the aliquots were combined with an equal volume of 4% sodium dodecyl sulfate-10 M urea and heated to 100º for 4 min. Samples on the right half of the gel were reduced with 5% 2-mercaptoethanol (+ 2-ME); samples on the left half of the gel are nonreduced (- 2-ME). The gel was stained for protein with Coomassie blue. The molecular weight standards (0.25 mg per ml of bovine serum albumin, ovalbumin, α-chymotrypsinogen, and cytochrome C) were reduced with 5% mercaptoethanol. B, autoradiogram of the gel shown in A.

active site fragment did not. The reduced bands at 53,000, 29,500, 23,500, and 17,000 daltons gave positive carbohydrate stains.

Activation of Factor VII by Thrombin—It has been reported that human and rabbit thrombin can activate human Factor VII at least 2-fold (19). When our bovine Factor VII at approximately physiological concentration was incubated at 37º with various concentrations of bovine thrombin, the rate of activation was found to be proportional to the concentration of thrombin (Fig. 9). The same degree of activation (45-fold) was obtained as during activation with Factor X, CaCl₂, and phospholipids. Nine NIH units of thrombin per ml caused an initial activation rate of 0.7-fold per min. Assuming that complete activation of the prothrombin present in plasma would generate approximately 120 NIH units of thrombin per ml (20), 9 units of thrombin per ml correspond to the activation of 8% of the prothrombin during coagulation.

The possibility that the observed effect was due to contaminants in the thrombin preparation was examined in two ways.

Inhibition of Factor VII with DFP—An experiment was performed to compare the rates of inhibition of the two forms of Factor VII by DFP. Aliquots were removed at time zero and at 30 min from the activation reaction described in Fig. 7 and 0.05 volume of [32P]DFP (40 mM in 2-propanol) was added to each. Fig. 11 indicates that the single chain form and the
The changes in coagulant activity are accompanied by the hydrolysis of only two peptide bonds (Fig. 8). The first cleavage activates the molecule and results in two chains of 29,500 and 17,000 daltons. The active serine is on the smaller fragment and the carbohydrate is on the larger fragment. In contrast, the light chain (23,500 daltons) of activated Factor VII, like the light chain of Factor Xa (12), is not further cleaved by Factor Xa over the time course studied (Fig. 8).

DISCUSSION

The present data show that bovine Factor VII exists in plasma as a single polypeptide chain with an apparent molecular weight of 53,000. This protein can be cleaved by activated Factor X and by thrombin. The initial cleavage to a two-chain species joined by disulfide bridges is accompanied by a marked increase in coagulant activity. A further cleavage by these enzymes leads to lower molecular weight fragments and loss of coagulant activity. Thus two enzymes involved in the tissue factor system activates the molecule and results in two chains of 29,500 and 17,000 daltons. The active serine is on the smaller fragment and the carbohydrate is on the larger fragment. In contrast, the light chain (23,500 daltons) of activated Factor VII, like the light chain of Factor Xa (12), is not further cleaved by Factor Xa over the time course studied (Fig. 8).

The activation of Factor VII by Factor Xa or thrombin reflects the relative rates of the two cleavages catalyzed by these enzymes. In the presence of CaCl2 and phospholipids, the initial activating cleavage occurs approximately 25 times faster than the second inactivating cleavage. Thus, under the conditions we used, a rapid activation and slow decay were observed.

An estimate of the relative abilities of Factor Xa and thrombin to activate Factor VII at similar molar concentrations may be obtained by extrapolating from the data presented in Figs. 4, 5, and 11. In the presence of CaCl2 and phospholipids, 1 µg of Factor Xa per ml would cause 20-fold activation per min and 1 µg of thrombin per ml would cause 0.05-fold activation per min. In the absence of cofactors, 1 µg of Factor Xa per ml would cause less than 0.04-fold activation per min and 1 µg of thrombin per ml would cause 0.2-fold activation per min. Thus, in the presence of CaCl2 and phospholipids, Factor Xa is approximately 400 times as effective as thrombin on a molar basis, while in the absence of cofactors thrombin is severalfold more effective than Factor Xa.

The rate and extent of activation of Factor VII during normal clot formation can be estimated by assuming 10% activation of Factor X and prothrombin (21). At these concentrations Factor Xa would activate Factor VII at a rate of 20-fold per min, whereas conversion of a similar percentage of prothrombin to thrombin would result in an activation rate of less than 0.5-fold per min. This suggests that during clot formation Factor Xa would be expected to cause 40-fold greater activation of Factor VII than would thrombin. This suggests that the feedback of Factor Xa on Factor VII is more likely a control mechanism than is the feedback of thrombin. The rate of activation by Factor Xa is sufficiently rapid to play such a role. It is also possible that destruction of activated Factor VII, which under these approximately physiological conditions would proceed at a rate of 3% per min, constitutes a postactivation damping mechanism. Under conditions where a large amount of tissue factor is available and activation of Factor Xa is nearly complete (12), an activation rate of 200-fold per min would be followed by destruction of activated Factor VII at a rate of 80% per min, producing a transient burst of Factor VII activity. Clearly these calculations take no account of numerous other possibly important factors. In this connection the action of anti-thrombin III on Factor Xa does not appear rapid enough to prevent activation of Factor VII by this enzyme since Maciukiewicz (22) has reported that a half-life of approximately 40 s for Factor Xa, in the presence of physiological levels of anti-thrombin III is increased over 35-fold by the presence of calcium ions, phospholipids, and Factor V.

This study indicates that native bovine Factor VII cannot be purified except in the presence of a proteolytic inhibitor. The lability of Factor VII is indicated by the fact that proteolysis continues slowly in 10 mM benzamidine (Table 1). We have also observed that absorption of plasma by barium citrate gives a severalfold greater yield of Factor VII than does absorption by barium sulfite (Table 1). This has been reported to be the case also in the purification of prothrombin and Factor X (17, 20).

The ability of Factor VII to be activated complicates evaluation of the yields obtained during purification. It is unlikely that the 48% yield reported in Table II is valid, and an estimate of 500 µg of Factor VII per liter of bovine plasma based on that yield may well be an underestimate. A minimum estimate of 1 mg of Factor VII per liter of plasma seems justified. The same problem is encountered in evaluating the specific activities ob-
tained by previous procedures. Jesty and Nemerson (1) reported a specific activity of 207,500 units per mg of Factor VII which cannot be further activated. In this study a specific activity of 270,000 units per mg was obtained for material which could be activated a further 45-fold, giving a maximum activated Factor VII specific activity of 12,000,000 units per mg. The failure to obtain material of this specific activity by the method of Jesty and Nemerson (1) (specific activities up to 1,700,000 units per mg were occasionally obtained) suggests that both activation and destruction of Factor VII occur during purification in the absence of benzamidine. Benzamidine (10 mM) does not appear to prevent either process entirely. In this connection it should be emphasized that benzamidine, besides thrombin and Factor X, or VIII show a 5-fold increase during coagulation, but that this rise in Factor VII activity occurs during clotting of plasma deficient in Factors IX, XI, or XII (27, 28). Consideration must also be given to possible activation by kallikrein (23). Before it is possible to assess the significance of the activation of Factor VII during coagulation, the interaction of Factor VII with the enzymes of the intrinsic pathway, kallikrein, plasmin, and perhaps other enzymes must be examined. It also remains to be decided whether plasma Factor VII possesses coagulant activity before activation.

### REFERENCES


### Additional Background

- **Human Factor VII with tissue factor**: Enzymatically activates Factor X to Xa in the presence of phospholipids and calcium ions, and plasma lipids. This interaction is critical for blood coagulation.
- **Factor VII activation by kallikrein**: Kallikrein, a serine protease, can activate Factor VII, though its role in physiological coagulation is less well understood.
- **Single Chain Factor VII**: Unlike Factor X and Prothrombin, single-chain Factor VII does not appear to be activated by tissue factor. It can be activated in the absence of tissue factor, suggesting other activating mechanisms.
- **Affinity Chromatography**: A powerful method for purifying proteins, allowing for high specificity and purity in the final product.

### Notes

- The term "activated Factor VII" is used differently in this context compared to previous uses.
- Key enzymes for Factor VII activation include thrombin, tissue factor, and Factor X.
- The importance of identifying the full range of enzymes that can activate Factor VII is underscored by the potential implications for understanding blood coagulation.

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*Note: The above text is a concise summary of the original document, focusing on key points regarding the activation of Factor VII.*
Activation and control of factor VII by activated factor X and thrombin. Isolation and characterization of a single chain form of factor VII.
R Radcliffe and Y Nemerson


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