Factor X Activating Enzyme from Russell's Viper Venom: Isolation and Characterization†

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ABSTRACT: The protease from Russell's viper venom that activates factor X (Stuart factor), factor IX (Christmas factor), and protein C was purified by gel filtration on Sephadex G-150 and QAE-Sephadex A-50 column chromatography. The purified enzyme migrated as a single band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis with an apparent molecular weight of 79 000. A minimal molecular weight of 78 500 ± 800 was determined by sedimentation equilibrium in the presence of 6 M guanidine hydrochloride. Upon reduction with 2-mercaptoethanol, a heavy chain (mol wt 59 000) and a light chain were observed. The light chain migrated as a single band (mol wt 19 000) in 7.5% polyacrylamide–sodium dodecyl sulfate gels but appeared as a doublet (mol wt 18 000 and 20 000) in 10% polyacrylamide–sodium dodecyl sulfate gels. The amino-terminal end of the heavy chain was heterogeneous and contained isoleucine, valine, and serine. The amino-terminal sequence of the light chain was Val-Leu-Asp. The factor X activator contained 13% carbohydrate including 6.0% hexose, 1.7% *N*-acetyleneuraminic acid, and 5.3% galactosamine. Most of the carbohydrate was found to be present in the heavy chain, although some was also observed in both forms of the light chain. The factor X activator had no esterase activity toward benzoyl-Phe-Val-Arg-*p*-nitroanilide or benzoylarginine ethyl ester and was not inhibited by 0.05 M diisopropyl phosphorofluoridate. These data indicate that factor X activator from Russell's viper venom is a highly specific protease composed of one heavy chain and one light chain, and these chains are held together by a disulfide bond(s).

The effect of Russell's viper venom (RVV)[1] on blood coagulation has been recognized for over four decades (Macfarlane and Barnett, 1934). Evidence from several laboratories supports the concept that the clot-promoting effect of RVV arises from its ability to activate factor X (Stuart factor), factor V (proaccelerin), and factor IX (Christmas factor) (Williams and Esnouf, 1962; Markwardt and Walsmann, 1962; Schiffman et al., 1969; Fujikawa et al., 1972; Lindquist et al., 1976).[2]

Purification of the factor X activating enzyme from RVV (RVV-X) has been reported from several different laboratories. Williams and Esnouf (1962) employed DEAE-cellulose chromatography in their isolation procedure. These preparations contained strong coagulant activity as well as esterase activity toward tosylarginine methyl ester. Esterase activity was also observed in the preparations of Furie et al. (1970), and the coagulant activity of these preparations was readily inhibited by diisopropyl phosphorofluoridate (DFP). More recently, Furie and Furie (1975) reported a purification of the coagulant protein from RVV by affinity chromatography on columns of Sepharose–factor X in the presence of lanthanide ions. This preparation was essentially homogeneous and exhibited a molecular weight of 62 000 by gel electrophoresis in the presence of sodium dodecyl sulfate. Markwardt and Walsmann (1962) described a preparation of coagulant protein from RVV which did not possess esterase activity and was not inhibited by DFP. More recently, Jackson et al. (1971) reported the separation of esterase activity from the factor X activating enzyme by gradient elution from DEAE-cellulose columns. This factor X activator was not inhibited by DFP or phenylmethylsulfonyl fluoride, while the esterase activity was readily inhibited by both inhibitors.

In the course of our studies on the activation of protein C by the factor X activator from RVV (Kisiel et al., 1976), milligram quantities of purified enzyme were required. Preliminary studies indicated significant differences in the properties of this preparation and those reported by other investigators. The present work describes the isolation of the factor X activator from RVV in the presence of benzamidine and a partial characterization of the enzyme.

Experimental Section

Materials

Bovine serum albumin, ovalbumin, carbonic anhydrase, dithiothreitol, lyophilized *Vipera russelli* venom, *N*-acetylneuraminic acid, and thiobarbituric acid were obtained from Sigma Chemical Co., St. Louis, Mo. Diisopropyl phosphorofluoridate, cyclohexanone, and benzamidine hydrochloride were purchased from Aldrich Chemical Co., Milwaukee, Wis. Sephadex G-25, Sephadex G-150, and QAE-Sephadex A-50 were products of Pharmacia Fine Chemicals, Piscataway, N.J. *N*-Benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide hydrochloride was obtained from AB Bofors, Mölndal, Sweden. 4-Vinylpyridine was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Bio-Rad Laboratories, Richmond, Calif. 2-Mercaptoethanol and *N,N,N',N'*-tetramethylethylenediamine were products of Eastman Kodak Co., Rochester, N.Y. Sodium dodecyl sulfate was obtained from British Drug House, Poole, England. Urea (Ultra-Pure) was

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[1] Abbreviations used are: RVV, Russell's viper venom; RVV-X, the factor X activating enzyme from Russell's viper venom; DFP, diisopropyl phosphorofluoridate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; [^{3}H]BAEE, ^{3}H-labeled benzoylarginine ethyl ester.

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

purchased from Schwarz/Mann, Van Nuys, Calif. Guanidine hydrochloride was obtained from Heico, Inc., Delaware Water Gap, Pa. Phosphorylase *b* and benzoylarginyl ethyl ester labeled with [^{3}H]ethanol were kindly provided by Dr. Edmond Fischer and E. Fodor, respectively, of this department. Bovine brain phospholipid (cephalin) was prepared according to Bell and Alton (1954). 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 $E_{280}^{1\%} = 10.0$ and correcting for Rayleigh scattering according to Shapiro and Waugh (1966). For the carbohydrate analyses, protein mass was determined by amino acid analysis following hydrolysis in 6 N HCl for 24 h at 110 °C in sealed tubes.

Neuraminic acid was determined according to Warren (1959) using *N*-acetylneuraminic acid as a standard. Neutral hexose was determined with anthrone using a 1:1 mixture of mannose and galactose as a standard (Spiro, 1966). Hexosamine was quantitated with an amino acid analyzer on samples previously hydrolyzed in 2 N HCl for 20 h at 110 °C.

Amino acid analyses were performed according to the methods of Spackman et al. (1958) and Moore and Stein (1963) employing a Durrum Model D-500 amino acid analyzer. Samples were hydrolyzed at 110 °C in 6 N HCl for 24, 48, and 72 h in evacuated tubes. Isoleucine, leucine, and valine values were those determined by extrapolation to zero hydrolysis time on semilogarithmic graph paper. Tryptophan was measured according to Hugli and Moore (1972), and half-cystine was determined as cysteic acid by the method of Hirs (1967).

Sodium dodecyl sulfate gel electrophoresis was performed according to Weber and Osborn (1969) with the following modifications. Samples were subjected to electrophoresis at room temperature for 3 h in 7–10% acrylamide gels at a current level of 5 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 brillant blue R according to Fairbanks et al. (1971), or for carbohydrate with Schiff's reagent according to Glossmann and Neville (1971). The molecular weight of the factor X activator was obtained by extrapolation from a linear semilogarithmic plot of apparent molecular weight vs. distance of migration using the following proteins as standards: bovine serum albumin (68 000), ovalbumin (45 000), bovine carbonic anhydrase (29 000), and myoglobin (17 000).

Sedimentation equilibrium experiments were performed at three different protein concentrations at 20 °C at a rotor speed of 20 000 rpm. Salt-free, lyophilized RVV-X was dissolved in 0.05 M Tris-HCl–6 M guanidine-HCl (pH 7.5) and dialyzed against this solvent for 60 h at room temperature. The protein sample was diluted with the diffusate to produce three solutions with an absorbance of 1.4, 0.7, and 0.5 at 280 nm. A partial specific volume of $\bar{v} = 0.677$ was determined for the coagulant enzyme from the amino acid analysis and corrected for 13% carbohydrate content (Gibbons, 1966; Lee and Timasheff, 1974). The density of the solvent at 20 °C was computed from its index of refraction at 23.5 °C measured with an Abbe refractometer (Kielley and Harrington, 1960).

Ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control. Sedimentation equilibrium studies were performed according to Yphantis (1964) utilizing a six-channel Kel-F centerpiece. Rayleigh patterns were recorded on Kodak II-G photographic plates, and the plates were analyzed on an automatic microdensitometer as described by DeRosier et al. (1972). Point-by-point molecular weight averages were calculated with a computer program developed by Teller (1973).

Amino-terminal analysis was performed with a Beckman Sequenator Model 890A. Methods of sequence analysis and operations of the instrument were adaptations (Hermodson et al., 1972) of the technique of Edman and Begg (1967).

The procoagulant activity of the factor X activator was measured essentially according to Jackson et al. (1971) employing pooled, citrated bovine plasma. One unit of procoagulant activity was arbitrarily defined as the amount of activity present in 1 μg of crude venom. A calibration curve of log of clotting time vs. log of the venom concentrations was constructed for each purification table. Prior to assay, preparations of the enzyme were diluted in 0.05 M Tris–HCl–0.1 M NaCl (pH 7.5) buffer containing 1 mg/ml bovine serum albumin. In this assay system, 100 pg of the purified enzyme produced a clot in about 25 s. The esterase activity of purified RVV-X was measured with benzoyl-Phe-Val-Arg-*p*-nitroanilide according to Svendsen et al. (1972) and with ^{3}H-labeled benzoylarginine ethyl ester ([^{3}H]BAEE) according to Roffman et al. (1970) and Anderson et al. (1975). In each assay system, approximately 200 μg of RVV-X was employed.

Purification of the Factor X Activator. Lyophilized venom (1 g) was dissolved in 5 ml of 0.05 M sodium acetate buffer (pH 5.5) containing 0.5 M NaCl and 5 mM benzamidine-HCl. Insoluble material was removed by centrifugation for 5 min at 1000*g* at 20 °C. The supernatant was applied to a Sephadex G-150 column (2.6 × 96 cm) previously equilibrated with 0.05 M sodium acetate buffer (pH 5.5) containing 0.5 M NaCl–5 mM benzamidine hydrochloride. Chromatography was performed at room temperature with a flow rate of approximately 0.5 ml/min. Those fractions containing coagulant activity were pooled and dialyzed at 4 °C against 4 l. of 0.05 M Tris-H_3PO_4 buffer (pH 7.5) containing 1 mM benzamidine hydrochloride. The dialyzed sample was applied to a QAE-Sephadex A-50 column (1.6 × 30 cm) previously equilibrated at 4 °C with 0.05 M Tris-H_3PO_4 buffer (pH 7.5) containing 1 mM benzamidine hydrochloride. The column was then washed with approximately 30 ml of equilibrating buffer and further washed with 1–2 column volumes of 0.05 M Tris-H_3PO_4 buffer (pH 6.0) containing 1 mM benzamidine hydrochloride. Coagulant activity was then eluted from the column with a linear gradient of NaCl prepared from 100 ml of 0.05 M Tris-H_3PO_4 buffer (pH 6.0) containing 1 mM benzamidine hydrochloride and 100 ml of 0.05 M Tris-H_3PO_4 buffer (pH 6.0) containing 0.5 M NaCl and 1 mM benzamidine hydrochloride (Figure 1). The fractions containing coagulant activity were pooled (shown by solid bar), made 10 mM in benzamidine hydrochloride and 0.02% in sodium azide, and stored at 4 °C. Salt-free protein was obtained after desalting by gel filtration through a Sephadex G-25 column (2.6 × 50 cm previously equilibrated with 0.1 M NH_4HCO_3) followed by lyophilization. In some experiments, the enzyme was isolated from venom purchased from Miami Serpentarium. The yield of factor X activator from this source was usually about half that observed from venom purchased from Sigma. No significant differences were observed, however, in the properties of the final preparation isolated from these two sources.

Preparation of the Heavy and Light Chains. Salt-free lyophilized factor X activator was reduced and S-pyridyl-

TABLE I: Purification of the Factor X Activator from Russell's Viper Venom.

Purification Step	Volume (ml)	Total Protein (mg)	Total Act.[a] (units)	Sp. Act.[b] (units/mg)	Recovery (%)	Purification (fold)
Crude venom	5	1000	1×10^6	1000	100	1.0
Sephadex G-150	50	118[c]	27.5×10^4	2330	27.5	2.33
QAE-Sephadex	24	51[c]	26.4×10^4	5164	26.4	5.16

[a] Activity of the factor X activator was assayed as described under Methods. [b] The specific activity of the crude venom sample was not assayed prior to gel filtration and was assumed to be 1000 units/mg of protein. [c] Protein concentration was determined from the absorbance at 280 nm assuming $A_{280}(1\%) = 10.0$.

ethylated by the method of Friedman et al. (1970). Salt and excess reagents were removed by gel filtration in a Sephadex G-25 column (2.6 × 60 cm) in the presence of 9% formic acid. Void volume fractions from the column were combined and lyophilized. The heavy and light chains were separated by gel filtration on a Sephadex G-150 column (2.6 × 90 cm) previously equilibrated with 9% formic acid containing 3 M urea. Salt-free protein was obtained by dialysis against distilled water followed by lyophilization.

Results

Preparation of the Coagulant Enzyme. The factor X activator (RVV-X) was purified fivefold from Russell's viper venom by a two-step procedure in approximately 25% yield (Table I).[3] About 50 mg of enzyme was obtained from 1 g of crude venom by this procedure.

Chromatography of crude venom in Sephadex G-150 columns resulted in an activity and protein elution profile similar to that reported by Schiffman et al. (1969). RVV-X activity was found to elute in the descending portion of the first major peak. Gel filtration was carried out at a relatively low pH in the presence of benzamidine hydrochloride to minimize proteolysis of the enzyme. The preparation from Sephadex G-150 chromatography contained two major proteins and three to four minor proteins as shown by sodium dodecyl sulfate gel electrophoresis.

The factor X activator was completely resolved from contaminating proteins by QAE-Sephadex A-50 chromatography (Figure 1). The yield of activity after QAE-Sephadex chromatography was virtually quantitative.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of RVV-X. A single protein band was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for RVV-X under nonreducing conditions in 7.5 and 10% gels (Figure 2). Molecular weight estimates of 90 000 and 79 000 were observed for unreduced RVV-X in 7.5 and 10% gels, respectively. Upon reduction with 2-mercaptoethanol, a heavy chain of 67 000 and a light chain of 19 000 were observed in

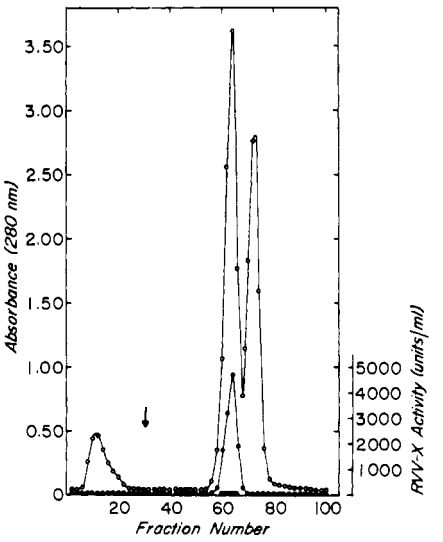

FIGURE 1: Elution pattern for the QAE-Sephadex A-50 column. RVV-X was eluted from the column (1.6 × 30 cm) with a linear gradient formed from 100 ml of 0.05 M Tris-phosphate buffer (pH 6.0) containing 1 mM benzamidine and 100 ml of 0.05 M Tris-phosphate buffer (pH 6.0) containing 0.5 M NaCl and 1 mM benzamidine. Fractions (3 ml) were collected at a flow rate of 30 ml/h. RVV-X activity was determined as described under Methods. (O—O) Absorbance at 280 nm; (●—●) RVV-X activity. The solid bar shows the fractions which were combined.

7.5% gels (Figure 2). In 10% gels in the presence of 2-mercaptoethanol, a heavy chain of 59 000 and two light chains with apparent molecular weights of 20 000 and 18 000 were observed. The heavy chain stained very strongly for carbohydrate, while the light chain stained positively for carbohydrate only when quantities of 100 μg or more of protein were applied to the gel.

The observation that RVV-X is a glycoprotein offers one possible explanation for the apparent dependence of its molecular weight on gel concentration (Segrest and Jackson, 1972). Moreover, the variation in molecular weight of the heavy chain as a function of gel concentration is probably due to its higher carbohydrate content.

Molecular Weight of RVV-X by Sedimentation Equilibrium. Sedimentation equilibrium experiments with RVV-X in 6 M guanidine hydrochloride indicated the protein was homogeneous at the concentrations tested (Table II). The minimal molecular weight calculated for the denatured protein by the method of Teller (1973) was 78 500 ± 800.

Amino Acid and Carbohydrate Compositions of RVV-X. The amino acid and carbohydrate compositions of RVV-X are shown in Table III. RVV-X is a glycoprotein containing approximately 13% carbohydrate including hexose, galactosamine, and neuraminic acid. Thus, the molecule is composed

[3] The low recovery of RVV-X activity following gel filtration in part may be due to denaturation of the enzyme at pH 5.5. Another possible explanation of the low recovery is the apparent separation by gel filtration of RVV-X and the factor V activating enzyme (RVV-V). This enzyme has been reported to be a DFP-sensitive enzyme and presumably accounts for half the total coagulant activity of the crude venom (Esmon and Jackson, 1973). Attempts to inhibit this enzyme in crude venom samples (1 mg/ml in 0.05 M Tris-HCl-0.1 M NaCl, pH 7.5) with DFP at a final concentration of 20 mM revealed that the apparent RVV-X activity in the crude venom was not appreciably reduced (less than 15%) by this compound after incubation for 3 h at room temperature. Consequently, no further attempt was made to correct for the contribution of RVV-V activity in the crude venom samples.

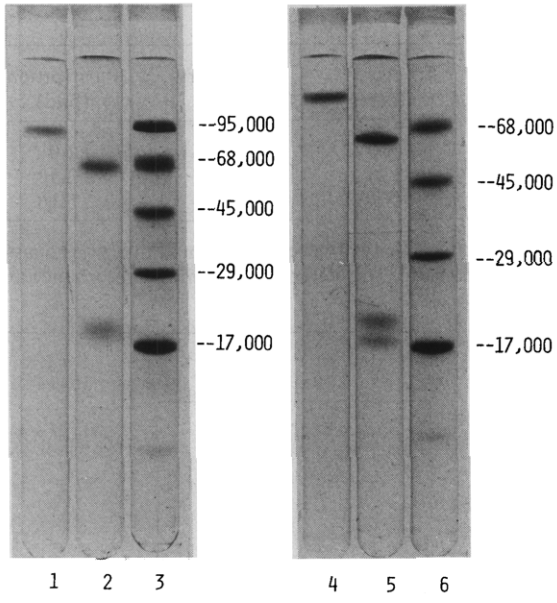

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of RVV-X in 7.5 and 10% gels. Samples 1 and 4 were unreduced RVV-X (10 μg); samples 2 and 5 were reduced RVV-X (20 μg); sample 3 was a mixture of standard proteins including phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and myoglobin; sample 6 was a mixture of standard proteins consisting of bovine serum albumin, ovalbumin, carbonic anhydrase, and myoglobin. Samples 1–3 are 7.5% gels and samples 4–6 are 10% gels. All samples were stained for protein with Coomassie brilliant blue R. The anode was at the bottom of the gel.

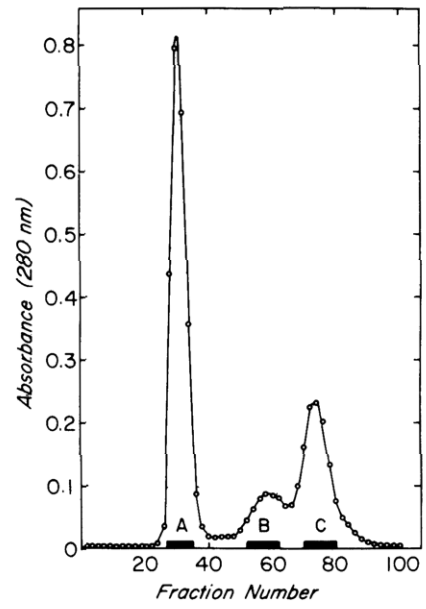

FIGURE 3: Separation of the heavy and light chains of RVV-X by gel filtration. Reduced and pyridylethylated RVV-X (30 mg) was subjected to gel filtration on a column of Sephadex G-150 (2.6 × 95 cm). Elution was carried out with 9% formic acid in 3 M urea, as described in Methods. Peak A contained the heavy chain and peaks B and C contained the light chains. Fractions that were pooled are shown by the bars.

TABLE II: Molecular Weight of the Factor X Activator from Russell's Viper Venom by Sedimentation Equilibrium.[a]

M_1	M_n	M_W	M_Z
78 500 ± 800	79 600 ± 800	80 700 ± 1400	82 700 ± 1250

[a] M_1 represents the smallest molecular weight species calculated according to Teller (1973). M_n, M_W, and M_Z refer to 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 RVV-X.

Components	RVV-X (residues/78 500)
Amino acid	
Lys	40.8
His	16.8
Arg	28.6
Asp	75.7
Thr	24.9
Ser	41.7
Glu	69.1
Pro	25.6
Gly	33.6
Ala	31.6
Half-Cystine[a]	36.8
Val	35.9
Met	13.9
Ile	28.8
Leu	39.8
Tyr	15.3
Phe	33.9
Trp[b]	7.9
Carbohydrate	
Hexose	29.1
N-Acetylgalactosamine	19.0
N-Acetylneuraminic acid	4.6
Protein (%)	87
Carbohydrate (%)	13

[a] Determined as cysteic acid according to Hirs (1967). [b] Determined by the procedure of Hugli and Moore (1972).

of approximately 10 200 g of carbohydrate and 68 300 g of protein per 78 500 g of glycoprotein.

Separation of the Heavy and Light Chains of RVV-X. The heavy and light chains of RVV-X are readily separated by gel filtration on Sephadex G-150 equilibrated with 9% formic acid and 3 M urea (Figure 3). In addition, the two forms of the light chain are partially resolved by this procedure. Protein from pool A (Figure 3) was found to be pure heavy chain when examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Densitometry scans of the gels of the protein from pool B (Figure 3) indicated the sample contained approximately 70% of the 20 000 species and 30% of the 18 000 species. Likewise, densitometry scans of the gels of the protein from pool C (Figure 3) revealed the sample contained 35% of the 20 000 form and 65% of the 18 000 form. While partial separation of the two forms of the S-pyridylethylated light chain was observed under these conditions, no separation of the two forms of the light chain was observed when RVV-X was carboxymethylated according to Crestfield et al. (1963) and subjected to gel filtration on Sephadex G-150. Under these conditions, two well-resolved peaks of protein were observed. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that the first peak consisted of pure heavy chain while the second, smaller peak contained the two forms of the light chain in a proportion of 1:1.5 (20 000:18 000) as estimated by densitometry.

Amino-Terminal Sequences of the Heavy and Light Chains of RVV-X. Amino-terminal analyses were then carried out on

carboxymethylated derivatives of the two chains employing a Beckman sequenator. The heavy chain was found to be heterogeneous at the amino-terminal end and contained principally isoleucine with smaller quantities of valine and serine in approximately equal amounts. Valine, leucine, and aspartic acid were observed in cycle 2; threonine and aspartic acid in cycle 3; valine and threonine in cycle 4; alanine, proline, and serine in cycle 5; glutamine in cycle 6; and aspartic acid, glutamine, and phenylalanine in cycle 7.

An amino-terminal sequence of Val-Leu-Asp was observed for the light chain of RVV-X. Only one amino acid was found during each cycle in the sequenator with this sample. This finding indicates that the difference between the two forms of the light chain is not due to heterogeneity at the amino-terminal portion of the molecule.

Other Properties of RVV-X. Purified preparations of RVV-X were also tested for their sensitivity to DFP. Solutions of purified RVV-X (1 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl) were incubated in the presence of 50 mM DFP and 0.65 M isopropyl alcohol. Control samples were treated with identical concentrations of isopropyl alcohol. After incubation for 3 h at room temperature, no differences in clotting activity were observed between the controls and the DFP-treated samples.

During the activation of factor X, factor IX, and protein C by RVV-X, specific Arg-Ile or Arg-Val bonds are cleaved (Fujikawa et al., 1972, 1974, 1975; Kisiel et al., 1976; Lindquist et al., 1976). Thus, it was of interest to reexamine the RVV-X for esterase activity toward benzoyl-Phe-Val-Arg-*p*-nitroanilide and benzoylarginine ethyl ester. With the chromogenic substrate, no esterase activity was detected at enzyme concentrations as high as 100 μg/2.2 ml in the presence or absence of 10 mM Ca^{2+}.

Using the more sensitive radioactive assay, a specific activity of 0.6 nmol per min per mg of protein was obtained for benzoylarginyl ethyl ester. This is approximately 10 000 times less than that observed for trypsin with the same substrate. Furthermore, the hydrolysis of the [^{3}H]BAEE by the purified RVV-X was completely inhibited by prior incubation of the enzyme preparation with 50 mM DFP. These experiments indicate that the weak esterase activity observed in the RVV-X preparation was due to trace contamination by an arginine esterase present in crude venom (Jackson et al., 1971).

The procoagulant activity of purified RVV-X was stable for 2 months at 4 °C when stored in 0.05 M Tris-H_3PO_4 buffer (pH 6.0) containing 5 mM benzamidine and 0.02% NaN_3 at a protein concentration of 1 mg/ml. Some preparations, when frozen at −20 °C, lost 50% of their activity upon thawing, while other preparations were completely stable under these conditions. The reason for this phenomenon is not apparent at this time. Accordingly, purified preparations of the enzyme were routinely stored at 4 °C.

Discussion

The present data indicate that the factor X activator from Russell's viper venom is a glycoprotein consisting of a heavy chain and a light chain, and these chains are linked by a disulfide bond(s). Two forms of the light chain were consistently observed in purified preparations. The addition of a protease inhibitor throughout the isolation procedure, however, had no effect on the relative ratio of the two chains. The two forms of the light chain were also observed in Sephadex G-150 purified RVV-X preparations indicating that the heterogeneity of the light chain occurred either during gel filtration or was present in the crude, lyophilized venom. The low pH and high concentrations of protease inhibitor employed during gel filtration would presumably reduce proteolysis by contaminating proteases during this step. It was not possible to detect the presence of the two forms of the light chain in crude venom since it contains a large proportion of proteins with a molecular weight of 15 000–20 000.

Heterogeneity was also observed in the amino-terminal portion of the heavy chain of RVV-X. Indeed, a very large number of hydrophobic residues were observed and these were very similar to the terminal residues reported for several precursors for dog pancreas secretory proteins (Devillers-Thiery et al., 1975). It has been suggested that the hydrophobic residues at the amino-terminal end of these proteins may play an important role in their transfer across the microsomal membrane. A similar role may exist for the hydrophobic residues in the heavy chain of RVV-X. In this case, however, the removal of these hydrophobic residues may have been incomplete during the processing of this protein.

The minimal molecular weight for RVV-X was found to be 78 500 under denaturing conditions by sedimentation equilibrium and is in excellent agreement with an apparent molecular weight of 79 000 obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The molecular weight observed for the heavy chain is similar to that reported by Furie and Furie (1975) for their reduced RVV-X preparation.

The RVV-X prepared in this study was completely resistant to high concentrations of DFP and confirms the earlier findings of Markwardt and Walsmann (1962) and Jackson et al. (1971). While DFP readily inhibited a weak arginine esterase activity in the present RVV-X preparation, it had no effect on its coagulant activity toward factor X. RVV-X also cleaves arginine bonds in bovine protein C (Kisiel et al., 1976) and bovine factor IX (Lindquist et al., 1976), and pretreatment of the enzyme with high concentrations of DFP does not inactivate the enzyme toward these substrates.

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Substrate Binding Properties of Converting Enzyme Using a Series of *p*-Nitrophenylalanyl Derivatives of Angiotensin I†

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ABSTRACT: The binding properties of angiotensin I for the active site of rabbit lung converting enzyme (CE) have been investigated. A series of angiotensin I like substrates, all containing the C-terminal tripeptide, (NO_2)Phe-His-Leu, were synthesized by increasing the length of the peptide at the N-terminal end. A total of eight peptides were studied, the largest being [Asn^1,(NO_2)Phe^8]angiotensin I. As determined by thin-layer chromatography, all substrates were hydrolyzed only at the (NO_2)Phe-His bond by purified converting enzyme, with the release of the dipeptide, His-Leu. By using an absorbance increase upon hydrolysis, the Michaelis constants and velocity maxima were determined and used to estimate those amino acids in the angiotensin I molecule that contribute significantly to binding to converting enzyme. It was hypothesized that, upon addition or substitution of one or more amino acids to the N-terminal end, a proportional decrease in both K_M and V_m is needed in order to conclude that the substrate actually increases its affinity for the enzyme. A test of the proportionality for the variation of K_M and V_m was found to be positive for all the substrates, except the N-terminal carbobenzoxy-blocked tripeptide, Z(NO_2)Phe-His-Leu. Substitutions near the bond that is hydrolyzed (e.g., proline for the carbobenzoxy group) appear to alter the catalytic properties of CE, while additions far removed from the site of hydrolysis (e.g., the N-terminal tripeptide Asn-Arg-Val) may enhance binding affinity.

Angiotensin I converting enzyme (CE)[1] (EC 3.4.15.1) catalyzes the hydrolysis of the decapeptide, angiotensin I, to the octapeptide, angiotensin II, and the dipeptide, histidyl-leucine (Page and Bumpus, 1961). The enzyme also inactivates bradykinin by hydrolysis of the C-terminal dipeptide (Yang et al., 1970). The enzyme was originally partially purified from plasma by Skeggs et al., 1956 and later by Yang and Erdös, 1967; Lee et al., 1971a,b. Preparations of purified CE have also been obtained from lung (Lee et al., 1971c; Cushman and Cheung, 1972; Nakajima et al., 1973; Dorer et al., 1972; Igic et al., 1972; Stevens et al., 1972; Lanzillo and Fanburg, 1974; Massey and Micalizzi, 1974), which contains a particularly high concentration of converting enzyme (DePierre and Roth, 1972).

Assays of CE have employed analysis of the hydrolysis

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[1] Abbreviations used are: CE, rabbit lung converting enzyme; Boc, *tert*-butoxycarbonyl; DMF, dimethylformamide; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; TLC, thin-layer chromatography; uv, ultraviolet.