The Purification and Properties of Factor X from Pig Serum and its Role in Hypercoagulability *in vivo*

By ROBERT J. DUPE and ROY M. HOWELL

Department of Biochemistry, Queen Elizabeth College, Campden Hill, London W8 7AH, U.K.

(Received 28 December 1972)

The molecular weights or shapes of Factor X preparations determined by gel filtration were dependent on the density of the BaSO_4 used for the initial adsorption from serum. One form obtained with BaSO_4 of density 2 g/ml behaved as if it had a molecular weight of 39000 and possessed preformed clotting activity (Factor X_a), whereas that of the form adsorbed with BaSO_4 of density 1 g/ml had a molecular weight of 69000 and consisted of inactive Factor X precursor. Thus degradation accompanied by activation seems to occur as a result of surface adsorption on high-density BaSO_4 and is associated with an interchange of protein between the two bands observed electrophoretically. The clotting and esterase activities measurable *in vitro* after complete activation were not matched by a corresponding ability to induce thrombus formation and 'lethality' *in vivo*. The most effective preparations of Factor X in this respect possessed preformed activity, which was enhanced in the presence of phospholipid. Factor X lost activity more rapidly in dilute solution, and its concentration at the surface of phospholipid micelles probably decreases less by dilution in circulating blood.

Factor X occupies a central place in the final pathways of coagulation leading to prothrombin activation, and there is some evidence to suggest that, owing to this position, it may be involved in the generation of a hypercoagulable state *in vivo* (Barton *et al.*, 1970). It is generally prepared from bovine plasma together with prothrombin by adsorption on BaSO_4 followed by ion-exchange chromatography, and the product from this source was shown by Esnouf & Williams (1962) to possess esterase activity towards tosylarginine methyl ester. In contrast, Lundblad & Davie (1965) reported that, when prepared from bovine prothrombin-poor serum, Factor X possessed no activity towards this substrate.

Serum also contains an agent referred to as serum thrombotic accelerator, which may be related to Factor X and which is capable of inducing a state of hypercoagulability in experimental animals (Reimer *et al.*, 1960; Howell & Scott, 1964). However, earlier work on the isolation of serum thrombotic accelerator from pig serum (Howell & Scott, 1966) showed that it has a molecular weight ten times that of Factor X; therefore on this basis they are not comparable.

The present paper presents a scheme for the further purification of Factor X from pig serum after its initial adsorption on various grades of BaSO_4 (Howell & Dupe, 1972). In addition to describing some of its properties from this source, an attempt is made to clarify the nature of the thrombotic state by defining its relationship to serum thrombotic accelerator in terms of clotting activity *in vivo*.

Materials and Methods

Materials

Pig blood was used exclusively and was collected from the abattoir in glass containers. It was left to clot spontaneously and then kept for 20 h at 4°C to destroy thrombin before being centrifuged at 2500g for 5 min to separate serum. In the case of plasma the separation was identical except that for the collection of blood, sodium oxalate (0.1 M) was placed in the glass containers (1 vol. to 9 vol. of blood). BaSO_4 for X-ray analysis and soil testing respectively was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Methods

Preparation of BaSO_4 -adsorbable protein. Proteins adsorbable on BaSO_4 were prepared by the method of Howell & Scott (1964) except that the citrate concentration used for protein elution was decreased to 0.06 M at pH 5.8. The resulting eluates are referred to according to whether X-ray- or soil-testing-grade BaSO_4 was used. However, owing to its lower content of protein and absence of lipoprotein the main purification was attempted from soil-testing grade BaSO_4 , and X-ray-grade BaSO_4 was used in some preparations for comparative purposes only. The importance of defining the density of the solid BaSO_4 used for the adsorption of clotting factors was discussed by Howell & Dupe (1972). The bulk density of the solid X-ray-grade BaSO_4 normally used

for clotting-factor adsorption is 1.0 g/ml, whereas the value for the soil-testing-grade material should be at least 2.0 g/ml. Natural barytes of this density was also kindly supplied by Barium Chemicals Ltd., Widnes, Lancs., U.K.

Precipitation. Precipitation with $(\text{NH}_4)_2\text{SO}_4$ was done by the method of Howell & Scott (1964). The fraction precipitated between 33 and 60% saturation was dialysed against 25 mM-Tris-HCl buffer, pH 8.0.

Column chromatography. DEAE-cellulose (Whatman DE-23) was obtained from Reeve Angel and Co. Ltd., London E.C.4, U.K. Precycling was carried out as recommended by the manufacturer with final equilibration in 25 mM-Tris-HCl buffer, pH 8.0. The ion exchanger was originally packed to a height of 24 cm in glass columns (2.0 cm $\times$ 30 cm), but later the packed height was limited to 16 cm, as this gave improved separations. The fraction precipitated by $(\text{NH}_4)_2\text{SO}_4$ (10–15 ml) was washed on to the column with 10–20 ml of 0.025 M-Tris-HCl, pH 8.0, and was then eluted with a linear gradient consisting of NaCl and trisodium citrate in increasing concentration. The reservoir contained 300 ml of 0.36 M-NaCl and 0.096 M-sodium citrate in 0.025 M-Tris-HCl buffer, pH 8.0, and the mixing chamber contained 300 ml of the same buffer but without NaCl and citrate. The flow rate was maintained at 60 ml/h and the E_{280} of the effluent was monitored continuously with a Uvi-cord II absorptiometer (LKB Instruments Ltd.). Fractions (5 ml) were collected and those containing Factor X were pooled and concentrated for the subsequent stage by ultrafiltration.

Gel filtration. Sephadex G-200 (medium grade; Pharmacia, London W.5, U.K.) was sieved to obtain the 200–300-mesh fraction and was allowed to swell for 3 days at room temperature before packing it in a Lucite column (3 cm $\times$ 35 cm) with no dead space. For gel filtration the column was used at a flow rate of 15 ml/h and the eluting buffer was 0.025 M-Tris-HCl, pH 8.0, with 0.2 M-NaCl. In later preparations 0.025 M- CaCl_2 was added to the eluting buffer to obtain a high-molecular-weight component in an active coagulable state. The eluate was collected in 3 ml fractions and those containing coagulant activity were pooled and concentrated. The Sephadex G-200 column was calibrated with Blue Dextran 2000, Yellow Dextran, vitamin B₁₂, fibrinogen, bovine serum albumin, immunoglobulin G, trypsin and cytochrome c, which were passed through the column sequentially to obtain a plot of the ratio of V_e to V_0 against the logarithm of their molecular weight (Andrews, 1964).

Concentration of samples. Ultrafilters obtained from LKB Instruments Ltd. (LKB 6300a) were used for the concentration of protein at 4°C. Occasionally isolated Factor X was also concentrated by acetone precipitation: 20 vol. of cold acetone (4°C) to 1 vol. of sample. The precipitated protein was separated

immediately by centrifugation at 15000g for 15 min and the supernatant was then treated with further acetone to ensure complete precipitation. The acetone-precipitated powder was dried in a gentle stream of air and redissolved in a little Tris-HCl buffer, pH 8.0, before storing it at -18°C.

Phospholipid and digitonin. L- α -Phosphatidylcholine from soya beans was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. and was prepared as a 5% (w/v) emulsion in 0.15 M-NaCl by ultrasonic treatment for 1 min at a frequency of 20 kHz in an M.S.E. ultrasonic disintegrator. The emulsion was stored before use for not more than 3–4 days at 4°C. It was used either alone or in combination with digitonin (BDH Chemicals Ltd.) and was added to solutions of Factor X immediately before infusion into animals as a substitute for phospholipid normally supplied by platelets (Howell & Dupe, 1971a).

Clotting assays in vitro. Total Factor X was determined by the technique of Bachmann *et al.* (1958) by using Russell's-viper venom combined with a mixed phospholipid preparation from brain as a complete thromboplastin. The partial thromboplastin time was a modification of this assay in which Russell's-viper venom was omitted from the above thromboplastin reagent. This enabled preformed Factor X activity (Factor Xa) to be measured, 1 unit of activity being defined as the amount present in 1.0 ml of serum or plasma fully activated with Russell's-viper venom. Unknown samples were measured by reference to a Factor Xa dilution curve obtained by using the Bachmann *et al.* (1958) assay. All clotting reagents were obtained from Diagnostic Reagents Ltd., Thame, Oxon, U.K. Russell's-viper venom was obtained from The Wellcome Foundation, Beckenham, Kent, U.K., and was reconstituted as recommended.

Thermal stability. This was investigated for Factor X activity by preincubating the sample for various time-intervals up to 1 h at 50° and 60°C before assaying for clotting activity at 37°C in the usual manner.

Clotting assays in vivo. These were performed as described by Howell & Dupe (1971a) by using male Sprague-Dawley rats weighing 140–160 g. Test material either alone or with platelet substitute was injected into the inferior vena cava. After 10 min clot production in the isolated portal vein was estimated as 1 (+) where up to one-half of the vessel was occupied with clot, to 2 (+) where the whole length of it was filled. More potent infusions that killed the animal within 5 and 2 min were assayed as 3 (+) and 4 (+) respectively. In the latter cases additional thrombin could be identified at the site of injection and elsewhere in the venous system.

Protein determination. The E_{280} , or the colorimetric procedure of Goa (1955) was employed with bovine serum albumin as a standard.

Carbohydrate. This was measured by the method of

Björnesjö (1955) except that the ethanol precipitation step was omitted owing to the very low amounts of protein present.

Determination of the minimum amount of Russell's-viper venom required to activate Factor X for assays *in vivo*. Russell's-viper venom (10 μ l, diluted 1/150000) was added to 20 ml of Factor X solution containing about 0.2 mg of protein. After incubation for 10 min at room temperature 0.1 ml was removed and Factor X activity was measured in a partial thromboplastin clotting-time test. Further additions of venom (10 μ l) were made until the Factor X preparation was fully activated (Fig. 1). The same amount of venom necessary to cause activation *in vitro* was then used to activate preparations for assay *in vivo*.

Electrophoresis. This was done in 0.5% agarose in sodium barbitone buffer, pH 8.4 (1.05). The gel was dissolved in the buffer by heating them over a steam bath, and then the solution was cooled to 50°C before being poured into an immunoelectrophoresis tray (Shandon Scientific Co. Ltd., London N.W.10, U.K.) containing eight microscope slides. Electrophoresis was done at 30–40 mA and 160 V for 40–60 min, and the protein was then stained for 5–10 min in Trichrome (Fischl & Gabor, 1963) followed by destaining in 5% and 2% (v/v) acetic acid respectively.

Fig. 1. Determination of the minimal amount of Russell's-viper venom required to activate 0.25 mg of purified Factor X originating from protein eluted from soil-testing-grade BaSO₄.

The partially activated Factor X was activated fully by progressive addition of 10 μ l samples of Russell's-viper venom at intervals of 10 min. The activity generated by each addition of the venom was assayed by using the partial thromboplastin clotting-time test. The incubation was carried out at 20°C.

Esterase activity. This was determined towards two amino acid esters, Tos-Arg-OMe* (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) and Z-Tyr-ONp (BDH Chemicals Ltd.), on a Unicam SP. 800 spectrophotometer equipped with scale expansion and a recorder, by measuring the E_{250} and E_{400} respectively. Comparative studies were performed at a single substrate concentration of 7 and 0.3 mM respectively to ensure zero-order kinetics; for kinetic studies with Z-Tyr-ONp alone the substrate concentrations were varied from 0.1 to 1.0 mM. The K_m and V_{max} were calculated by the method of Lineweaver & Burk (1934). The reaction cuvette was maintained at 35°C and contained 2.7 ml of 0.05 M-Tris-HCl buffer, pH 8.0, 0.1 ml of substrate and 0.2 ml of a solution containing purified Factor X (1.0 mg/ml); water replaced Factor X in the control cuvette. Esterase activity was measured by following the rate of product formation continuously for a given time-period, and the initial rates of reaction were determined by noting the change in slope of extinction relative to the water blank. The rate of any spontaneous hydrolysis in buffer alone was determined separately against a water blank and subtracted. The change in absorbance per unit time was related to standard curves prepared by using known amounts of pure *p*-nitrophenol at pH 8.0.

In an attempt to characterize further the relationship between esterase and Factor X activity the effect of both substrates as competitive inhibitors of the latter were tested by means of the Bachmann *et al.* (1958) assay.

Results

Isolation of pig Factor X

Pig serum was used, since other studies had shown it to be nearest to human serum in terms of ability to induce intravascular coagulation and it was more readily available. The progress of a typical purification of Factor X starting with adsorption to soil-testing-grade BaSO₄ is shown in Table 1.

Adsorption on BaSO₄. This confirmed previous work showing that if a grade of BaSO₄ used for soil testing was substituted for the X-ray grade normally used, both Factor X and serum thrombotic accelerator could be eluted by trisodium citrate in a partially active form associated with less protein and no lipoprotein (Howell & Scott, 1964; Howell & Dupe, 1972). However, the soil-testing-grade BaSO₄ must have a bulk density of at least 2.0 g/ml and when recent batches failed to meet this specification the substitution of ground natural barytes of this density was found to yield eluates with a low protein

* Abbreviations: Tos-Arg-OMe, *N*^α-tosyl-L-arginine methyl ester; Z-Tyr-ONp, *N*^α-benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester.

content (approx. 1.0mg/ml) and with no detectable lipoprotein. BaSO₄ at this density gave a 160-fold purification (Table 1).

(NH₄)₂SO₄ precipitation. A 2–3-fold purification was obtained (Table 1).

DEAE-cellulose chromatography. The separations for proteins originating from soil-testing and X-ray grades of BaSO₄ on a column of bed length 16cm is shown in Figs. 2(a) and 2(b) respectively. Factor X in all cases was eluted towards the trailing edge of the run in fractions 90–110 for the former separation and 110–130 for the latter. Factor X required the presence of citrate ions in addition to NaCl for elution, otherwise recovery was low, indicating irreversible adsorption. It was eluted at the same gradient strength regardless of whether protein eluted from soil-testing-grade BaSO₄ or X-ray-grade BaSO₄ was used as the starting material.

At this stage the specific activity was increased 2500 times compared with that of the original pig serum (Table 1).

Gel filtration. As the material from the previous stage contained another protein that was eluted in the same position as Factor X, further purification was attempted by filtration through Sephadex G-200. This procedure also provided information on the physical nature of the various Factor X preparations obtained (Fig. 3).

The protein that was eluted before Factor X during the Sephadex G-200 stage was similar to material observed by Howell & Scott (1966) when protein eluted from soil-testing-grade BaSO₄ was separated

on cellulose phosphate. However, gel filtration of the other fractions eluted from the DEAE-cellulose revealed that the bulk of this high-molecular-weight protein had been eluted before Factor X in the preceding stage. Thus filtration of the fraction containing Factor X through a Sephadex G-200 column merely provided a further small yield of this material, which possesses a molecular weight of approx. 380 000 and activity in a one-stage prothrombin assay towards pig plasma artificially depleted of Factor V.

Properties of isolated Factor X

Table 2 summarizes the properties of purified Factor X obtained from serum and plasma by using different grades of BaSO₄, and shows that the assay methods differentiated between non-activated and preactivated forms. In this study eluates prepared from serum by using BaSO₄ of density 2g/ml in the first stage yielded preparations of Factor X that possessed preformed activity and which was eluted from Sephadex G-200 in approximately the same position as Yellow Dextran (marker 4, mol.wt. 40 000). In contrast, when BaSO₄ of density 1g/ml was used Factor X possessed little preformed activity and was eluted from Sephadex G-200 very close to bovine serum albumin (marker 5, mol.wt. 68 000).

The relationship between V_e/V_0 and the logarithm of the molecular weight of the various Factor X preparations is shown in Fig. 3. The position of markers 4 and 5, which were nearest to the Factor X obtained with each grade of BaSO₄ specified, is

Table 1. *Summary of a typical purification of Factor X from pig serum*

One unit of Factor X activity is taken as the amount present in 1 ml of normal pig serum as determined by the Bachmann *et al.* (1958) assay. Factor X assays were carried out on the individual column fractions at 1/10 dilution. Of the total protein applied to the DEAE-cellulose column 80–90% was recovered. Soil-testing-grade BaSO₄ was used for the preliminary adsorption in stage (1).

Purification stage	Vol. (ml)	Total Factor Xa activity (units)	Specific activity (units/mg of protein)	Purification factor	Recovery of Factor X (%)
Pig serum	1000	1500	0.0187	0	100
(1) BaSO ₄ eluate	240	720	3.0	160	48
(2) 30–60%-satd.- (NH ₄) ₂ SO ₄ -pre- cipitable fraction	12	960	7.6	405	64
(3) DEAE-cellulose fractions 90–110 pooled and con- centrated	22	925	46.5	2500	61
(4) Sephadex G-200 fractions 38–48 pooled and con- centrated	14	840	60.0	5330	55

Fig. 2. Fractionation on DEAE-cellulose of the proteins adsorbed by different grades of BaSO_4 from pig serum

(a) Gradient elution of 33–60%–satd.-(NH_4) $_2\text{SO}_4$ -precipitable protein from a soil-testing-grade BaSO_4 eluate. The linear gradient of trisodium citrate (0–0.096M) and NaCl (0–0.36M) started at fraction 20. The column was equilibrated with Tris–HCl buffer, 0.025M, pH 8.0; —, protein (E_{280}); ●, Factor X activity as measured in a Bachmann *et al.* (1958) assay; units as defined in Table 1. (b) 33–60%–satd.-(NH_4) $_2\text{SO}_4$ -precipitable protein from an X-ray-grade BaSO_4 eluate separated and assayed as for (a), except that the gradient was applied at fraction 38.

shown. The anomalous clotting behaviour of impure Factor X obtained by using batches of BaSO_4 with densities outside the specified limits has been reported (Howell & Dupe, 1972) and was apparent as an alteration in the ratio of Factor X/ X_a . The preparation which extended the upper limit to 80000 was the only exception in that this was obtained from pig plasma by using X-ray-grade BaSO_4 with a normal specification of 1 g/ml. In contrast to the preparations eluted around marker 4, none of those eluted around marker 5 exhibited preformed clotting activity.

The altered elution pattern caused by adsorption to higher-density BaSO_4 could be due to a change in shape of the molecule to a more compact form and/or a decrease in the molecular weight. It is noteworthy

that dehydration of a Factor X preparation during acetone precipitation, although not altering the apparent size of the molecule, did increase the preformed clotting activity (Table 2). The presence of significant amounts of carbohydrate ranging from 7 to 9% (Table 2) remained constant and independent of the grade of BaSO_4 used.

Homogeneity. Electrophoretically the purified fraction obtained from protein eluted from soil-testing-grade BaSO_4 separated as a single band in the β -globulin region together with a much fainter band detectable in the albumin region. Factor X from protein eluted from X-ray-grade BaSO_4 showed the same qualitative pattern, except that the secondary band in the albumin position was more intense.

Fig. 3. Plot of V_c/V_0 against molecular weight for gel filtration of Factor X preparations and marker proteins on Sephadex G-200

The column was equilibrated with 0.025M-Tris-HCl buffer, pH8.0 containing 0.2M-NaCl. The hatched areas, \\\ (for ten determinations) and /// (for five determinations), indicate preparations from a soil-testing-grade-BaSO₄ eluate and an X-ray-grade-BaSO₄ eluate respectively. The widths indicate the range of variation obtained with batches of BaSO₄ whose densities were later found to be outside the specified limits of 2.0 and 1.0g/ml respectively. The mean value in each case was 39000 and 69000. Total volume of the column (V_c) was 180ml and void volume (V_0) was 55ml. Flow rate was 15ml/h and 3.0ml fractions were collected. Numbers indicate positions of markers: 1, vitamin B₁₂; 2, cytochrome c; 3, trypsin; 4, Yellow Dextran; 5, bovine serum albumin; 6, γ -globulin; 7, fibrinogen; 8, Blue Dextran ---; shows non-linear region of the graph.

Table 2. Properties of Factor X preparations, excluding kinetic data

Total clotting activity was measured and units are defined as in Table 1. Preformed clotting activity was measured in a partial thromboplastin clotting-time test. —, Not determined.

Starting material	Grade of BaSO ₄ used	Apparent molecular weight	Carbohydrate (% of total protein)	Clotting activity (units/mg of protein)	
				Total	Preformed
Serum	X-ray	68-70000	7-9	10	0.3×10^{-4}
Plasma	X-ray	80000	7-9	20	0.5×10^{-4}
Serum	Soil-testing	30-40000	7-9	60	5.0
Serum*	Soil-testing	35-40000	—	15	10.0

* Factor X after separation from serum underwent acetone precipitation and was redissolved in water to a final concentration of 3.0mg/ml.

These separations are shown in Fig. 4 and in both cases a diffuse band was observed on the cathode side of the origin immediately after staining and before all excess of stain had been removed. On complete destaining this band was no longer visible, and it was thought to consist of low-molecular-weight peptide material which could not be fixed and which was therefore removed in the washing stage. It may well correspond to the small fragment (mol.wt. <1000)

observed in elution profiles after Sephadex G-200 filtration. Precipitation of Factor X preparations with acetone did not remove this non-fixable peptide material, nor did it change the electrophoretic mobility of the Factor X.

Activation studies. Although the Factor X prepared from protein eluted from soil-testing-grade BaSO₄ possessed considerable preformed activity as measured by the partial thromboplastin clotting time when

compared with material obtained from protein eluted from X-ray-grade BaSO₄, activation with either Russell's-viper venom or insolubilized trypsin was required to activate the preparation fully (Table 4).

Fig. 4. Electrophoretic mobility of Factor X preparations from (a) a soil-testing-grade-BaSO₄ eluate and (b) an X-ray-grade-BaSO₄ eluate in agarose gel with barbitone buffer (pH 8.4, I 0.05)

The arrows indicate the origin (O) for each sample and the migration of albumin under identical conditions. Hatching (▨; ▩) indicates the relative intensity of the stained bands and the broken line outlines those bands which disappeared on destaining.

The use of insolubilized trypsin for the activation of Factor X in impure eluates from X-ray-grade and soil-testing-grade BaSO₄ was described by Howell & Dupe (1971b), but it was sometimes difficult to activate fully the purified Factor X obtained in the present work by this means.

Clot production in vivo. The results of experiments to determine the ability of Factor X preparations to produce red coagulation thrombus *in vivo* both before and after proteolytic activation is shown in Table 3. Neither Factor X nor phospholipid was thrombogenic when injected alone, whereas 1.0 unit of Factor X in combination with 1.0mg of phospholipid/ml induced sufficient clot formation to fill the isolated portal vein. Also, addition of the surfactant digitonin to the L- α -phosphatidylcholine in a molar ratio of 1:2 respectively (Howell & Dupe, 1971a) increased the potency of the infusate, which killed the animal (4+), and post-mortem examination revealed a very extensive clot at the site of injection as well as in both sides of the heart and elsewhere in the venous system. Similar extensive clot formation that killed the animal could be induced when the amount of Factor X infused was doubled while the concentration of the phospholipid was kept at a constant value of 1.0mg/ml. However, a decrease of the Factor X infusate to 0.5 unit at this concentration of phospholipid brought its concentration below the threshold necessary for clot promotion, as did the serial dilution of the phospholipid at a constant Factor X concentration

Table 3. Activity of purified Factor X *in vivo*

The preparations used for the assays were obtained from soil-testing-grade BaSO₄ (density 2.0g/ml) and therefore contained preformed Factor Xa activity. The bioassay was performed as described in the text. The mean clot formation score is shown and the number of rats used in each determination is shown in parentheses. In preparation (3), complete activation of Factor X was achieved by preincubation with 5.0 μ l of Russell's-viper venom (see the text).

Preparation	Amount of Factor X injected (μ g of protein)	Units of Factor X injected	Concentration of phospholipid injected (mg/ml)	Clot formation <i>in vivo</i>
(1)	60	0.5	0	0 (2)
	120	1.0	0	0 (2)
	60	0.5	1.0	0 (2)
	120	1.0	1.0	2+ (5)
	120	1.0	1.0*	4+ (2)
	200	3.0	1.0	4+ (2)
(2)	100	1.5	1.0	2+ (3)
	50	0.75	1.0	2+ (2)
	50	0.75	0.1	Trace (2)
	0	0	1.0	0 (2)†
(3)	0.6	2.0	1.0	0 (2)
	0.6	3.0	1.0	0 (2)

* The surfactant digitonin (0.4mg/ml) was added to the platelet substitute soya-bean phospholipid. The total volume injected was maintained at 2.0ml by dilution with 0.15M-NaCl.

† Infusion of venom and phospholipid alone.

Table 4. Activity of Factor X from pig serum and plasma towards Z-Tyr-ONp ester at pH8.0 and 35°C together with kinetic parameters for activity derived from eluates from soil-testing-grade BaSO₄

Total Factor X activity and preformed activity were determined and expressed in units as in Table 2. Esterase activity is expressed as μmol of Z-Tyr-ONp hydrolysed/h/mg of Factor X protein. In preparation (2) Factor X obtained was concentrated by acetone precipitation.

Preparation	Grade of BaSO ₄ used	Starting material	Factor X activity (units/mg of protein)		Esterase activity	$10^3 \times V_{\text{max}}$. (μmol of <i>p</i> -nitrophenol/min)	K_m (mM)
			Total	Preformed			
(1)	Soil-testing	Serum	50	3.0	0.23	4.5	0.038
(1)*	Soil-testing	Serum	—	5.0	0.30	5.2	0.037
(2)	Soil-testing	Serum	—	2.0	3.60	—	—
(3)	Soil-testing	Serum	30	1.0	0.06	3.3	0.037
(4)	X-ray	Serum	10	1.0×10^{-4}	0.020	—	—
(5)	X-ray	Plasma	20	2.4×10^{-4}	0.013	—	—

* Factor X obtained was assayed after activation with insolubilized trypsin.

of 1 unit. The infusion of 2 units of Factor Xa obtained after complete activation with Russell's-viper venom failed to produce any thrombus *in vivo* even in the presence of phospholipid, which suggests that the assay *in vivo* is more discriminating than the test *in vitro* in measuring the total physiological effectiveness.

Esterolytic action of Factor X. Activity towards amine esters was studied by using Tos-Arg-OMe and Z-Tyr-ONp, since the latter was found most useful in work with impure preparations (Howell & Dupe, 1972). In agreement with these earlier studies, we could not detect activity of purified Factor Xa from pig serum towards Tos-Arg-OMe, as reported by Esnouf & Williams (1962) for Factor Xa separated from bovine plasma. Preparations from protein eluted from soil-testing-grade BaSO₄ that possessed preformed clotting activity caused higher rates of hydrolysis of Z-Tyr-ONp and also acetone precipitation increased the activity towards Z-Tyr-ONp (Table 4). V_{max} and K_m were calculated with this substrate for preparations of Factor X from protein eluted from soil-testing-grade BaSO₄; the values were close to each other.

Both Tos-Arg-OMe and Z-Tyr-ONp inhibited clotting activity *in vitro*, the latter ester (Fig. 5) being most effective; *p*-nitrophenol, the product of any hydrolysis, did not inhibit coagulation.

Thermal denaturation studies on Factor X preparations. After 1 h at 50°C there was no loss in clotting activity, whereas at 60°C decay of activity occurred (Fig. 6).

Loss of esterase activity towards Z-Tyr-ONp was also measured at 60°C to compare it with the corresponding decrease in clotting activity. After 20 min at 60°C a typical sample of Factor X had lost 90% of its clotting activity and 25% of its esterase activity.

Fig. 5. Inhibition of Factor Xa in a partial thromboplastin time test by increasing concentrations of amine ester substrates, measured as a percentage of the normal by using a standard dilution curve

For details see the text; 0.1 mg of Factor Xa was used. ●, Z-Tyr-ONp; △, Tos-Arg-OMe.

However, an acetone-precipitated sample was more resistant, since it lost only 70% of its clotting activity and 16% of its esterase activity after 60 min.

Decay of Factor X with time in dilute solutions at 20°C. Factor X activity was far more stable in con-

Fig. 6. Thermal denaturation of Factor X activity with time

Factor X (1.0mg/ml) prepared from protein eluted from soil-testing-grade BaSO_4 was incubated at 60°C . ●, Loss of activity measured in a Bachmann *et al.* (1958) assay; ▲, loss of activity in a partial thromboplastin clotting-time test; ○, a one-point assay after a 60min incubation of an acetone-precipitated sample (3.0mg/ml) of Factor X. Each point is the mean of duplicates. No loss in activity was recorded at 50°C for any of the samples.

Fig. 7. Effect of dilution on the decay of Factor X activity with time at 20°C

Dilutions were carried out in 0.15 M-imidazole buffer, pH 7.3, and the remaining Factor X was determined in a Bachmann *et al.* (1958) assay at suitable time-intervals. Concentrations: ●, 260 μg of protein/ml; ▲, 26 μg of protein/ml; △, 2.6 μg of protein/ml; ○, 0.26 μg of protein/ml; □, 0.026 μg of protein/ml. Results are the mean of duplicates.

centrated solutions (Fig. 7). Therefore, we kept the volume of column eluates as low as possible to minimize losses of Factor X.

Discussion

The information in the present paper shows that the properties and activity of isolated Factor X depend not only on whether it is derived from serum or plasma, but also on the grade of BaSO_4 used for the preliminary adsorption. Reported differences in the properties of Factor X preparations obtained by various workers may therefore be partially reconciled when factors such as the species of origin, the isolation technique and whether it is derived from serum or plasma are taken into account. For instance the purification procedure for Factor X from plasma devised by Jackson *et al.* (1968) could not be applied successfully in this study, as the citrate used in the pre-chromatographic stages prevented adsorption of Factor X to the DEAE-cellulose. Previous work (Howell & Dupe, 1972) had shown that the physical properties of the BaSO_4 adsorbent used in the first stage could influence the properties of the Factor X preparations obtained and in particular the presence or absence of preformed Factor Xa. The delayed elution of the latter from Sephadex G-200 compared with the inactive form obtained from protein eluted from X-ray-grade BaSO_4 indicated a considerable decrease in size or alteration in shape, the apparent molecular weight of this preformed Factor Xa being similar to the value of 38000 reported for fully activated Factor X by Tishkoff *et al.* (1968).

The activation of Factor X by adsorption to high-density BaSO_4 is advantageous when partially activated material is required without use of proteolytic activators. This method of activation has not been reported previously, and although its exact mechanism is unknown it seems to be caused by adsorption to a suitable surface, the physical nature of which was discussed by Howell & Dupe (1972).

The apparent molecular weight of 80000 observed for the inactive form of Factor X obtained from plasma with X-ray-grade BaSO_4 corresponds to the value obtained by Jackson & Hanahan (1968). In the present work the comparatively small size decrease from 80000 for plasma Factor X to 69000 for that from serum, when the same X-ray grade of BaSO_4 was used, suggests that limited proteolysis during clotting decreases the size of precursor Factor X, the bulk of which nevertheless remains unactivated. A value of 85000 for plasma Factor X as well as a component of molecular weight 40000 was reported by Esnouf & Williams (1962) for Factor X from bovine serum. However, the grade of BaSO_4 used was not stated, and these authors suggested that the low-molecular-weight entity may be produced by degradation during purification. We obtained a

satisfactory yield of Factor X by performing the whole purification in 3 days without using protease inhibitors such as di-isopropyl fluorophosphate, used by Jackson *et al.* (1968) to prevent autolysis. However, it is likely that some degradation occurred during the present purification procedure, in view of the low-molecular-weight peptide material that migrated to the cathode on agarose gel (Fig. 4).

The electrophoretic mobilities of the two major bands (Fig. 4) agree with similar observations of Papahadjopoulos *et al.* (1964), who noted the presence of two components in purified Factor X from human plasma together with a transfer of protein from the fast to the slower moving band on activation with trypsin. Similarly we found that substitution of low- for high-density BaSO₄ caused a corresponding interchange of protein between the two bands. These results together with those from differential clotting assays and gel filtration suggest that the two bands represent precursor (X) and activated (Xa) forms. Thus the differences reported in the literature for the molecular weight of Factor X could arise from experimental variations involving use of different grades of BaSO₄.

There is some controversy as to whether Factor X preparations possess esterase activity, as Lundblad & Davie (1965) reported no esterase activity towards synthetic amine substrates in a preparation from serum. Esnouf & Williams (1962) noted the appearance of activity towards Tos-Arg-OMe in samples obtained from bovine plasma. In the present study, although activity towards Z-Tyr-ONp was established, no esterase activity towards Tos-Arg-OMe was found either in the activated or preactivated state, and it appears that the detection of esterase activity is dependent on both the substrate used and the origin of the Factor X. However, both Z-Tyr-ONp and Tos-Arg-OMe caused inhibition of the clotting activity of Factor X preparations *in vitro*, indicating that with Tos-Arg-OMe binding occurred even though no hydrolysis resulted. The kinetic studies with Z-Tyr-ONp indicated that the K_m value was unaffected, whereas that for V_{max} increased as a result of activation with insolubilized trypsin.

With preparations from protein eluted from X-ray-grade BaSO₄, which possessed limited amounts of preformed Factor Xa activity, specific esterase activity was ten times lower than that from protein eluted from soil-testing-grade BaSO₄, which has a considerable amount of Factor Xa activity. This study indicated that hydrolytic activity towards Z-Tyr-ONp had a good correlation with the coagulant activity of Factor X, except for material concentrated by acetone precipitation, where the esterase activity was increased, in contrast to the clotting activity, which was unchanged. Thermal denaturation studies on Factor X preparations at 60°C also support the idea of closely associated sites for both activities,

since a decrease in clotting activity was also accompanied by decreased esterase activity; however, caution is needed when interpreting the exact relationship between loss of these two activities measured as units, since they are not directly compatible.

Factor X was kept as concentrated as possible during the purification because of the rapid decay of Factor Xa, which occurred in dilute solutions, and which could result in a complete loss in activity over comparatively short periods of time. The dependence of activity on concentration may indicate that decay of activity caused by rapid dilution in the blood could be important *in vivo* as a clearing mechanism for activated Factor X. The optimum production of thrombus which occurred either in the presence of phospholipid (Barton *et al.*, 1970) or with a combination of phospholipid and digitonin (Howell & Dupe, 1971a) could result from Factor Xa attachment to the surface of these micellar-forming substances as described by Jobin & Esnouf (1967). Thus by preventing its removal, activity can be maintained until thrombus accumulates. The high-molecular-weight protein (>350000) associated with a portion of the Factor X was similar to serum thrombotic accelerator separated by Howell & Scott (1966) by using cellulose phosphate chromatography; recent experiments with Sephadex G-200 equilibrated with 0.025 M-CaCl₂ to separate the high-molecular-weight material from Factor X have shown that it possesses an independent clotting activity measurable *in vitro*.

This investigation was supported by a grant from The British Heart Foundation.

References

- Andrews, P. (1964) *Biochem. J.* **91**, 222-233
- Bachmann, F., Duckert, F. & Koller, F. (1958) *Thromb. Diath. Haemorrh.* **2**, 24-38
- Barton, P. G., Yin, E. T. & Wessler, S. (1970) *J. Lipid Res.* **11**, 87-95
- Björnesjö, K. B. (1955) *Scand. J. Clin. Lab. Invest.* **7**, 147-152
- Esnouf, M. P. & Williams, W. J. (1962) *Biochem. J.* **84**, 62-71
- Fischl, J. & Gabor, J. (1963) *Clin. Chim. Acta* **8**, 330-331
- Goa, J. (1955) *Scand. J. Clin. Lab. Invest.* **7**, Suppl. **22**, 19-55
- Howell, R. M. & Dupe, R. J. (1971a) *Brit. J. Exp. Pathol.* **52**, 495-502
- Howell, R. M. & Dupe, R. J. (1971b) *Biochem. J.* **123**, 11P-12P
- Howell, R. M. & Dupe, R. J. (1972) *Thromb. Diath. Haemorrh.* **28**, 306-316
- Howell, R. M. & Scott, G. B. D. (1964) *Brit. J. Exp. Pathol.* **45**, 618-626
- Howell, R. M. & Scott, G. B. D. (1966) *Brit. J. Exp. Pathol.* **47**, 177-185

- Jackson, C. M. & Hanahan, D. J. (1968) *Biochemistry* **7**, 4506–4517
- Jackson, C. M., Johnson, T. F. & Hanahan, D. J. (1968) *Biochemistry* **7**, 4492–4505
- Jobin, F. & Esnouf, M. P. (1967) *Biochem. J.* **102**, 666–674
- Lineweaver, H. & Burk, D. (1934) *J. Amer. Chem. Soc.* **56**, 658–666
- Lundblad, R. L. & Davie, E. W. (1965) *Biochemistry* **4**, 113–120
- Papahadjopoulos, D., Yin, E. T. & Hanahan, D. J. (1964) *Biochemistry* **3**, 1931–1939
- Reimer, S. M., Wessler, S. & Deykin, D. (1960) *Proc. Soc. Exp. Biol. Med.* **105**, 438–442
- Tishkoff, G. H., Williams, L. C. & Brown, D. M. (1968) *J. Biol. Chem.* **243**, 4151–4167