Purification and Some Characteristics of the Coagulation Factor IX from Human Plasma

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Non-activated coagulation factor IX was purified approx. 10000-fold from human plasma. The final product was electrophoretically homogeneous and comprised a single polypeptide chain with a molecular weight of about 70000 and a pI of 4.3–4.45. The *N*-terminal amino acid was glycine. The amino acid and the carbohydrate contents were analysed and a monospecific antiserum to the factor was raised in rabbits.

Factor IX is a plasma protein which takes part in the intrinsic pathway of blood coagulation. Like most of the coagulation factors, factor IX exists in a zymogen form. After activation of the zymogen form by activated factor XI (XIa), the resultant activated factor IX (IXa), together with thrombin-exposed factor VIII (VIIIt), phospholipid and Ca^{2+} ions, causes the generation of the proteolytic enzyme, factor Xa (EC 3.4.21.6), from factor X (Østerud & Rapaport, 1970). Barton (1967) demonstrated that factor IXa, factor VIII, phospholipid and Ca^{2+} must be present in order to activate factor X and that the mixing of these four components did not lead to irreversible changes in any of them. It remains, however, to be clearly demonstrated that the activator is a macromolecular complex, similar to the complex of factor Xa, factor V, phospholipid and Ca^{2+} that converts prothrombin into thrombin.

To study the activation of factor IX by factor XIa, and to examine in more detail the mechanism by which factor IXa participates in the formation of factor X activator, highly purified factor IX preparations with well-defined characteristics are required. We describe here a method for the purification of human factor IX. The preparations obtained were homogeneous in disc-gel electrophoresis and sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The main difficulties in the purification were the prevention of activation of factor IX and the separation of factors IX and X. Some characteristics of the purified factor IX are reported.

Materials and Methods

Materials

Chemicals and biochemicals were obtained as follows: glucose oxidase was from Boehringer Corp., Mannheim, Germany; galactose oxidase was from Kabi, Stockholm, Sweden; barium sulphate

(Baker-analysed) was from Baker Chemicals, Deventer, The Netherlands; Sepharose 4B was from Pharmacia, Uppsala, Sweden; Whatman cellulose DE-11 was from Balston Ltd., Maidstone, Kent, U.K.; polyacrylamide was from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; *NN'*-methylene-bisacrylamide and soya-bean trypsin inhibitor were from Merck, Darmstadt, Germany; rabbit immunoglobulin against albumin was from Dacopatts, Copenhagen, Denmark; freeze-dried Russell's-viper venom was from Wellcome Reagents Ltd., Beckenham, Kent, U.K.; bovine serum albumin was from Sigma Chemical Co., St. Louis, Mo., U.S.A.; fluorescamine was from Hoffmann-La Roche, Basel, Switzerland. Polyamide sheets were obtained from BDH, Poole, Dorset, U.K.

Methods

Protein concentration. This was determined by the Lowry method (Lowry *et al.*, 1951) or by the method of Böhlen *et al.* (1973) with bovine serum albumin as standard.

Electrophoretic methods. Analytical polyacrylamide-gel electrophoresis was performed at pH 9.4 as described by Ornstein (1964) and Davis (1964). The gels were stained with Coomassie Brilliant Blue R 250 as described by Bjørklid *et al.* (1973). Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was carried out by the methods of Weber & Osborn (1969), Laemmli (1970) and Neville (1971). The running pH was 7.0, 8.9 and 9.2 respectively.

Preparative polyacrylamide-gel electrophoresis (model 200; Buchler Instruments, Fort Lee, N.J., U.S.A.) was carried out as described by the manufacturers, by using 7.5% (w/v) gels. The upper buffer was 0.052M-Tris–glycine buffer, pH 8.9, the lower buffer was 0.2M-Tris–HCl buffer, pH 8.1, and the elution buffer was 0.1M-Tris–HCl, pH 8.1. Samples (30–60 ml) containing about 20 mg of protein in 10% (w/v) sucrose were applied. A constant current of

50mA was used. The column was eluted at a rate of 1 ml/min. When the preparative electrophoresis had to be repeated to obtain pure factor IX, the appropriate fractions were pooled, dialysed against the upper buffer and sucrose was added.

Electrofocusing was carried out by the method of Catsimpoolas (1968).

For molecular-weight determination (Laemmli, 1970), standards of ovalbumin (mol.wt. 45000), bovine serum albumin (mol.wt. 68000) and heavy (mol.wt. 51500) and light (mol.wt. 23500) chains of γ -globulin were used.

Amino acid analysis. The N-terminal amino acid was determined qualitatively by the dansyl method (Gros & Labouesse, 1969). The dansyl amino acids were identified by using both silica-G thin-layer plates and polyamide sheets (Deyl & Rosmus, 1965; Woods & Wang, 1967). Amino acid analyses were done by the method of Spackman *et al.* (1958). Samples were hydrolysed in redistilled 6M-HCl under N_2 at 100°C for 24 or 48 h, dried *in vacuo*, washed and dried once more, and analysed in a Jeol JLC-6AH amino acid analyser. No correction for amino acid destruction during hydrolysis was introduced.

Tyrosine and tryptophan were determined by the method of Edelhoch (1967).

Carbohydrate analyses. Glucose oxidase and galactose oxidase reagents were used as indicated by the manufacturers. Sialic acid, hexosamines and total hexoses were determined as described by Warren (1959), Kabat & Mayer (1961) and Winzler (1955) respectively. Sialic acid was liberated by hydrolysis with 0.05M- H_2SO_4 for 1 h at 80°C. The other carbohydrates were analysed after hydrolysis with 50ml of 3M-HCl under N_2 in a 100ml flask for 5 h at 104°C. HCl was removed *in vacuo*, and the residue was dissolved in a small volume of water and dried again.

Clotting assays. The factors VII, IX, X and Xa were estimated by one-stage assays as described by Østerud & Rapaport (1970), except that in the factor Xa assay, factor VIII-deficient plasma was used instead of the mixture of $BaSO_4$ -treated ox plasma and bentonite-adsorbed plasma. Factor II was estimated as described by Prydz & Gladhaug (1970). Pooled normal plasma was taken to contain 100 units/ml. Veronal-buffered saline (Hjort, 1957) was used for all dilutions. Two plasma samples from factor IX-deficient patients were used as substrates. When normal plasma was replaced by veronal-buffered saline their clotting times were 87–90s and 72–74s respectively.

Standard curves based on dilutions of pooled normal plasma were established for all substrates and used for the conversion of clotting times into units (Hjort, 1957). The assay for factor IXa (Østerud & Rapaport, 1970) was modified since factor IXa, completely devoid of factor Xa, failed to shorten the

clotting time of factor IX-deficient plasma and cephalin in plastic tubes. This confirmed our earlier results (Østerud *et al.*, 1971) that factor VIII must be exposed to thrombin to become active in the formation of factor X activator. The factor IX-deficient plasma was therefore preincubated with thrombin (final concentration 0.02 NIH unit/ml) for 2 min at 37°C before addition of test substance and Ca^{2+} .

Purification of factor IX. Human blood from healthy fasting donors was collected into 250ml polycarbonate centrifuge bottles containing 25ml of 2% (w/v) EDTA (pH adjusted to 7.35 with NaOH). The blood was centrifuged twice for 10 min at 12000g at 4°C. The plasma (usually from two donors) was then absorbed with $BaSO_4$ (100mg/ml) for 20 min at 4°C with constant stirring. The $BaSO_4$ pellet was washed three times with an equal volume of 0.15M-NaCl, eluted with 180ml 0.14M-trisodium citrate, and then re-eluted with 30–40ml of the citrate solution. The eluates were immediately combined and diluted to 2 litres with 0.01M-sodium phosphate buffer, pH 8.0; regenerated DE-11 Whatman cellulose (corresponding to 20g dry weight), equilibrated with the phosphate buffer, was added, and the mixture was stirred for 30 min at 4°C and filtered through Whatman filter paper on a Büchner funnel. The cellulose was resuspended by stirring for 10 min in 2 litres of 0.01M-sodium phosphate buffer containing 0.10M-NaCl, then filtered and resuspended once more in 0.01M-sodium phosphate buffer containing 0.13M-NaCl. The cellulose was stirred and filtered, then eluted with 200ml of 0.18M-NaCl in the same phosphate buffer as above. This time the suspension was stirred for 20 min before the eluate was collected by filtration. This elution step was repeated. The eluates were pooled, concentrated to about 50ml by ultra-filtration (PM 30 Amicon filters) and dialysed against upper electrophoresis buffer before being subjected to preparative polyacrylamide-gel electrophoresis. All solutions used throughout the purification procedure until the last step (the affinity chromatography) contained soya-bean trypsin inhibitor (20 μ g/ml). Occasionally, factor IX was not eluted with 0.18M-NaCl. In these cases elution was effected by 0.23M-NaCl. These elution problems may arise from the exact technique used to regenerate the DE-11 cellulose.

As the last step, the factor IX-containing eluate from the preparative polyacrylamide-gel electrophoresis was submitted to affinity chromatography on a Sepharose 4B column to which antibodies against factor X and albumin were bound. Solid NaCl (final concentration 0.3M) was added to the eluate from the preparative polyacrylamide-gel electrophoresis, and the affinity column was equilibrated and eluted with 0.3M-NaCl.

Purification of factor Xa. The three first steps in the purification procedure were identical with those in

the procedure for factor IX, including the preparative polyacrylamide-gel electrophoresis. The active fractions from the latter contained factors IX and X and albumin. They were pooled, Russell's-viper venom and Ca^{2+} were added (final concentrations 5 $\mu\text{g}/\text{ml}$ and 3 mM respectively) and the mixture was incubated at 22°C for 10 min. Under these conditions Russell's-viper venom destroys factor IX activity (B. Østerud, unpublished work).

The mixture was submitted to preparative polyacrylamide-gel electrophoresis after dialysis against the upper buffer. The eluted fractions were tested for factor Xa and their purity was monitored by analytical polyacrylamide-disc-gel electrophoresis. Some preparations had to be rerun in the preparative electrophoresis system to show only one band on the analytical gels. The preparations were stored at -20°C.

Immunological methods. Antisera against purified factor IX and factor Xa were raised by dissolving 100–200 μg of purified protein in 1–1.5 ml of 0.15M-NaCl, adding 1 vol. of Freund's incomplete adjuvant and injecting the mixture into the foot-pads of a rabbit. At 3 and 4 weeks later similar mixtures were injected subcutaneously at multiple sites and the rabbits were bled 7–8 days later. The antisera were treated with BaSO_4 as described by Hjort (1957) and inactivated at 56°C for 30 min.

The neutralization titre was estimated as described by Østerud & Rapaport (1970). The immunoglobulin fraction was prepared from the anti-(factor Xa) serum (30 ml) by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-Sephadex A-50 batch absorption as described by Skotland *et al.* (1974). The preparation obtained was dialysed against 0.15M-NaCl and together with an anti-albumin preparation (2 ml) coupled to Sepharose 4B (20 ml packed volume) which had been activated by the CNBr method of Cuatrecasas (1970). The mixture was stirred for 24 h at 4°C. The substituted Sepharose was washed as described by Pharmacia and packed into columns of 10–20 vol.

Immunodiffusion in 1% agarose was carried out by the standard method (Skotland *et al.*, 1974).

Immunoelectrophoresis was carried out at 5 V/cm as described by Scheidegger (1955), by using 1%

agarose and 0.06M-sodium barbital buffer, pH 8.6, containing 1 mM-calcium lactate. The air-dried gels were stained with 0.05% Coomassie Brilliant Blue in 12% (w/v) trichloroacetic acid and destained with 10% (w/v) trichloroacetic acid. Polyethylene glycol 6000 (Koch-Light, Colnbrook, Bucks., U.K.) was added to the agarose at 3% final concentration in the immunodiffusion and immunoelectrophoretic experiments.

Results

Quantitative data for a representative experiment (Table 1) show that a purification of about 8000-fold was obtained by the first three steps in the procedure. The preparations at this stage gave two bands in analytical disc-gel electrophoresis (Plate 1a). The first band (m 0.71) contained albumin and the second band (m 0.67) contained factor X as well as factor IX. Factor IX had a slightly higher mobility than factor X, but extensive overlapping was observed. When fractions from the preparative gel electrophoresis were submitted to affinity chromatography (see the Materials and Methods section) a further 1.7–2-fold purification was obtained, giving a final specific activity of about 15 000 units/mg of protein. (Normal plasma contains 100 units/ml.) The recovery in this (final) step was greater than 92%. The final product contained no activated factor IX. A trace of factor IXa was found in some eluates from the preparative polyacrylamide-gel electrophoresis, but this was always removed on the affinity column, probably by binding to α_2 -macroglobulin present in the immunoglobulin fractions coupled to the Sepharose. With certain batches of BaSO_4 an almost complete activation of factor IX was observed. This was prevented by adding soya-bean trypsin inhibitor (20 $\mu\text{g}/\text{ml}$) to plasma and to all solutions used throughout the purification, except those used for the affinity chromatography.

When whole antisera were coupled to Sepharose, factor IX was activated during passage through the column. No loss (<8%) was observed when the immunoglobulin fraction was used. The final product was homogeneous in analytical disc-gel

Table 1. *Purification of factor IX*

Units are calculated by assuming that there are 100 units in 1 ml of normal human plasma.

	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)
Plasma	35700	52500	1.5	100
BaSO_4 eluate	152	30490	200	58
DE-11 DEAE-cellulose eluate	19	24750	305	47
Preparative electrophoresis	2.5	20130	8060	38

electrophoresis with a mobility relative to that of Bromphenol Blue of 0.67 (Plate 1b). Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of factor IX in the presence or absence of 2-mercaptoethanol gave one band irrespective of the method used. The mobility corresponded to a molecular weight of $70\,000 \pm 4\,000$.

Some factor IX preparations seemed, however, to undergo spontaneous proteolysis during the first three steps of the purification procedure even though soya-bean trypsin inhibitor was present, since in some cases new protein bands with molecular weights of about 63 000 and 53 000 appeared in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. These preparations gave, however, a single band in regular disc electrophoresis. After the final affinity chromatography factor IX was stable in the form with molecular weight 70 000. Fujikawa *et al.* (1973) observed a similar degradation of bovine factor IX during its purification.

In 0.3M-NaCl (pH 6.8) factor IX had a molar extinction coefficient, ϵ , of $57\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 280 nm. The pI was 4.30–4.45 as determined by electrofocusing.

Amino acid and carbohydrate analysis

Glycine was the only N-terminal amino acid found. The amino acid composition (Table 2) showed no remarkable features. A molecular weight of the

Table 2. Amino acid analysis of factor IX

Analyses were the mean of three hydrolysates (24 and 48 h). Residues/molecule were calculated on the basis of a polypeptide chain of mol.wt. 57 000. Values in parentheses are nearest whole integers. No corrections were introduced for loss during hydrolysis.

	Residues/molecule	
Lysine	30.7	(31)
Histidine	12.3	(12)
Arginine	16.1	(16)
Aspartic acid	43.0	(43)
Threonine	28.3	(28)
Serine	34.6	(35)
Glutamic acid	75.1	(75)
Proline	8.8	(9)
Glycine	60.8	(61)
Alanine	34.3	(34)
Cystine (half)	15.2	(15)
Valine	30.0	(30)
Methionine	1.3	(2)
Isoleucine	18.2	(18)
Leucine	36.8	(37)
Tyrosine	9.8	(10)
Phenylalanine	16.6	(17)
Tryptophan*	9.9	(10)

* Determined spectrophotometrically.

Table 3. Carbohydrate composition of factor IX

Values (%) are percentage of total weight (polypeptide plus carbohydrate). Mol/mol ratios were calculated on the basis of mol.wt. 70 000.

	Composition	
	(%)	(mol/mol)
Total hexoses	11.7	54.4
Galactose	3.9	
Glucose	< 0.1	
Hexosamines	1.6	6.3
Sialic acid	3.1	8.9

polypeptide chain of factor IX of about 57 000 was estimated by subtracting the estimated molecular weight of the carbohydrate portion from the total molecular weight of the factor (about 70 000) obtained by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. When this molecular weight of the polypeptide chain was used to calculate the molarity of the factor IX samples hydrolysed, a total of 483 residues per molecule, adding up to a molecular weight of about 52 400, was found. The discrepancy between the two estimates of the molecular weight of the polypeptide chain may be due to (a) destruction of amino acids during hydrolysis, (b) too low an estimate for the molecular weight of the carbohydrate component owing to loss of carbohydrate during hydrolysis, (c) too high an estimate of the total molecular weight of factor IX, or (d) a systematic overestimation of the amount of protein hydrolysed, caused by differences in the colour yield or fluorescence obtained with factor IX and the bovine serum albumin used as standard. No corrections were introduced. A molecular weight of 52 400 for the polypeptide chain may therefore be too low.

Basic amino acids were about 10% of the total, whereas glutamic acid and aspartic acid were 24% of the total. No attempt was made to determine how many of these residues were present as glutamine and asparagine before hydrolysis. Spectrophotometric determination of tryptophan gave 9.9 (10 residues/molecule). The carbohydrate analyses (Table 3) showed the presence of hexoses, hexosamine and sialic acid. Glucose was present at less than 0.3 residue/molecule of protein.

Immunology

Immunodiffusion and immunoelectrophoresis (Plate 2) gave only one band against plasma and serum. One ml of the best antiserum neutralized completely the factor IX activity in 7–8 ml of normal human plasma. Two experiments were carried out in order to see if the antiserum cross-reacted with factors II, VII or X, which structurally are rather

EXPLANATION OF PLATE I

Analytical polyacrylamide-gel electrophoresis of factor IX preparations after preparative polyacrylamide-gel electrophoresis (20 μ g) (a) and after affinity chromatography (5 μ g) (b)

For further details see the text.

EXPLANATION OF PLATE 2

Immunoelectrophoresis of normal human plasma (a) and serum (b) (both 10 μ l) against anti-(factor IX) serum

Electrophoresis was run for 150min at 4°C and 5V/cm in 0.06M-veronal buffer, pH 8.6. The gel was photographed before staining.

Table 4. *Effect of anti-(factor IX) serum on factors II, VII, IX and X*

Plasma was incubated with antiserum, normal rabbit serum or buffer for 60 min at 37°C and portions (0.05 ml) of the mixtures were withdrawn for testing. Antiserum and normal rabbit serum were treated with BaSO₄ (see the Materials and Methods section). E = value expected if antiserum, normal rabbit serum or veronal-buffered saline did not interfere with the respective factor activity. F = value found.

Normal human plasma (0.2 ml) incubated with:	Volume (ml)	Activity (units/ml) of incubation mixture in test systems for factor:							
		II		VII		IX		X	
		E	F	E	F	E	F	E	F
Antiserum	0.2	50	40	50	53	50	0	50	48
	0.1	67	66	67	74	67	0	67	64
	0.05	80	80	80	93	80	0.7	80	81
Normal rabbit serum	0.05	80	90	80	100	80	76	80	91
Veronal-buffered saline	0.2	50	67	50	60	50	37	50	48
	0.1	67	78	67	83	67	64	67	71

similar to factor IX. Expt. I (Table 4) showed that there was essentially no decrease in the activity of the factors II, VII and X, whereas factor IX was completely neutralized. The antiserum used in this experiment had a titre of 1:3.5 (1 ml of neutralized factor IX in 3–4 ml of normal human plasma). In Expt. II (not shown) the incubation mixtures were diluted 1:5 before testing. Again, factor IX activity was completely removed (activity in control, 10–16 units/ml, in test sample no detectable activity), whereas the activities of factors II, VII and X showed no detectable differences.

Discussion

The final product of the purification procedure reported here was homogeneous non-activated factor IX, which gave rise to a monospecific antiserum in rabbits. To avoid activation the isolation was carried out within a period of 2 days. Factor IX may be activated with various degrees of effectiveness by commercial BaSO₄ batches (Howell & Dupe, 1972). In the present work, such activation was prevented by the use of soya-bean trypsin inhibitor.

Our results show that, unlike their bovine counterparts (Denson, 1967), the four human vitamin K-dependent factors in their non-activated form are eluted at about the same ionic strength from DEAE-cellulose. Factors IX and X were separated from factors II and VII by the higher electrophoretic mobility of the former factors. The affinity chromatography was necessary to remove non-activated factor X and albumin.

The molecular weight of the final homogeneous preparation was about 70000. This is lower than the value estimated from gel-filtration experiments (Østerud & Schiffman, 1971). The discrepancy probably arises from the anomalous behaviour of glycoproteins in gel filtration.

The present data indicate that factor IX is a glycoprotein consisting of a single polypeptide chain with a pI of 4.30–4.45. Chandra & Pechet (1973) reported the pI of a partially (1082-fold) purified human factor IX to be 4.10–4.37. The pI value is probably strongly influenced by the negative charges introduced into the molecule by the nine sialic acid residues and by the vitamin K-induced modifications of the primary structure of the polypeptide chain. Bovine factor IX (Fujikawa *et al.*, 1973, 1974) is reported to be a glycoprotein with 26% carbohydrate, a molecular weight of 55400 and a pI of 3.7. Tyrosine is the *N*-terminal amino acid and the amino acid composition is also somewhat different. However, there is a considerable similarity between the number of half-cystine, methionine, lysine, arginine, isoleucine, tyrosine and tryptophan residues in factor IX from the two sources.

The similarity between the molecular weight of factor IX as isolated from bovine plasma and the breakdown product of human factor IX observed by us (55400 and 53000 respectively) may suggest that the bovine preparations have already been exposed to proteolytic activity.

The antiserum prepared in rabbits was monospecific without any absorptions. One line was found in immunoelectrophoresis against plasma and serum, confirming the purity of the preparations.

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