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Characterization of clotting factor IX in plasma-derived preparations by electrophoretic techniques

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Abstract

Clotting factor IX preparations from human plasma (pdFIX) have been characterized using electrophoretic methods like sodium dodecyl sulfate–polyacrylamide gel electrophoresis, isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis. Factor IX prior to and after activation with factor XIa was separated by one- and two-dimensional polyacrylamide gel electrophoresis and on isoelectric focusing gels. The main differences between the band patterns of the two pdFIX preparations are due to their purity. Vitronectin was identified by immunological techniques as major accompanying plasma protein, separated from factor IX and characterized by isoelectric focusing and two-dimensional polyacrylamide gel electrophoresis.

Keywords: Factor IX; Vitronectin; Proteins

1. Introduction

Human clotting factor IX is one of the proteins in the clotting cascade which plays a most important part for hemostasis. In the intrinsic system of blood coagulation factor IX, a zymogen, is activated by factor XIa and triggers together with activated factor VIII the activation of factor X [1]. In patients suffering from hemophilia B [2,3], factor IX is missing or defective and has to be substituted by infusion. Concentrates of factor IX used for this therapy are prepared either from human plasma [4] or by recombinant technology [5–7].

By comparison with other vitamin K-dependent glycoproteins the amino acid sequence of factor IX can be divided into various domains [8]. Factor IX consists of one gamma-carboxyglutamic acid (GLA)-domain and two epidermal growth factor like domains which together represent the factor IX light chain (LC), the catalytic domain referred to as the factor IX heavy chain (HC) and the activation peptide (AP) in between, that is cut off by activated clotting factor XIa (factor XIa) during activation of factor IX [9]. The schematic structure of factor IX is shown in Fig. 1. Heavy and light chain are connected by an intramolecular disulfide bridge that is conserved during activation, but is cleaved upon adding a reducing agent [9,11].

Factor IX is known to be posttranslationally modified comprising γ-carboxylation of 12 glutamic acid residues [12], β-hydroxylation on aspartic residue 64 [13], O-linked oligosaccharides on Ser^{53}, Ser^{61}, Thr^{159} and Thr^{169} [14], N-linked glycans on

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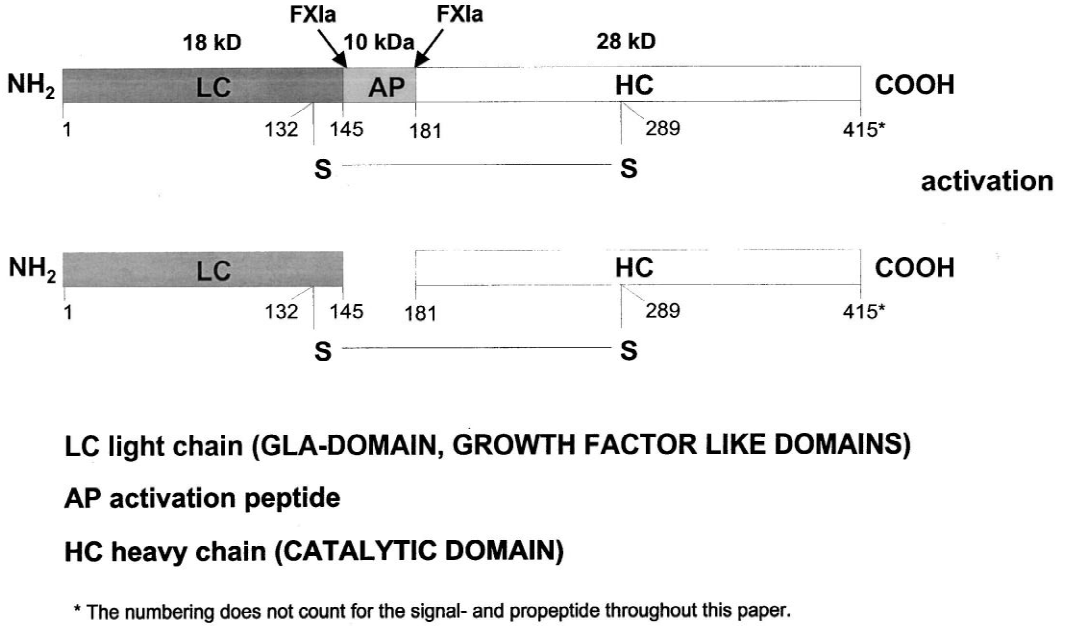


Fig. 1. Schematic structure of factor IX intact glycoprotein as well as heavy and light chain after removal of the activation peptide by factor XIa (kDa=kD=kilodalton).

Asp^{157} and Asp^{167} [15], the sulfation on Tyr^{155} [7,10] and the phosphorylation on Ser^{158} [10]. The presence of carbohydrate moieties, γ-carboxylation, sulfation and phosphorylation results in an increased molecular mass and shift in isoelectric point (p*I*) towards more acidic values compared to the non-modified protein. The protein exists therefore in various isoforms in which the pattern of modification is different to some extent.

This paper describes the use of electrophoretic techniques for the characterization of therapeutic factor IX concentrates. This includes molecular mass estimation of the active ingredient and the concomitant protein load as well as isoform separation by slab gel techniques and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) following in vitro activation with factor XIa.

2. Materials and methods

2.1. Materials

Two plasma-derived factor IX preparations (PD1 and PD2) were used as samples. They were prepared according to the protocol given by Hoffer et al. [4]. PD1 and PD2 differ by an additional virus filtration step, applied in the preparation PD2. Due to a 50% decrease of the total protein content, factor IX of higher specific activity is obtained (150 vs. 80–100). Aliquots of PD1 were fractionated by preparative size-exclusion chromatography (SEC) yielding two major peaks from vitronectin (VN) and factor IX. The concentration of factor IX after dissolution was 100 or 500 international units (IU) factor IX/ml, corresponding to 400 and 2000 μg factor IX/ml, respectively. Human FXIa was purchased from Enzyme Research (South Bend, IN, USA). All chemicals of analytical grade were purchased from E. Merck (Darmstadt, Germany) and Sigma (St. Louis, MO, USA).

2.2. Methods

2.2.1. Testing and activation

Factor IX activity was determined in a one-stage coagulation assay using an Amelung KC4A coagulometer (AVL, Vienna, Austria) or alternatively with an ACL 300 apparatus from Instrumentation Laboratory (IL, Vienna, Austria).

The VN enzyme-linked immunosorbent assay (ELISA) was purchased from Takara (Shiga, Japan) and Technoclone (Vienna, Austria) and was performed according to the manufacturer's instructions.

For the activation of factor IX by adding factor XIa the optimum conditions were as follows: The factor IX concentrate (100 or 500 IU factor IX/ml) was incubated with FXIa (0.8 mg/ml) and 50 m*M* $CaCl_2$ for 3 h at 37°C at a ratio of 7:3 and 7:1, respectively. This mixture was directly applied or further prepared for electrophoretic analysis.

2.2.2. Size-exclusion chromatography

HPSEC was performed as described by Josic and co-workers [16,17]. Factor IX and VN were separated from PD1 on a 900×16 mm I.D. column packed with Superdex 200 prep grade (Amersham Pharmacia Biotech, Uppsala, Sweden) using 20 m*M* sodium citrate, 200 m*M* NaCl, pH 7.2 as running buffer.

2.2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting

SDS–PAGE was performed according to the method of Laemmli [18]. Sample preparation included the addition of reducing sample buffer and boiling for 10 min. Then, 0.4 IU (~1.6 μg) factor IX were applied per lane for SDS–PAGE and blotting. The electrophoresis chamber, blot module and the 4–20% precast polyacrylamide gradient gels were from Novex (San Diego, CA, USA). The precision protein standard was from Bio-Rad (Hercules, CA, USA). Gels were silver stained according to the method of Heukeshoven and Dernick [19] or electroblotted for 35 min onto a 0.2-μm nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using Tris–glycine blotting buffer. The primary antibodies used were ESN1 (American Diagnostica, Greenwich, CT, USA) and F0652 (Sigma) against factor IX and V7881 (Sigma) against VN. The respective secondary antibodies were alkaline phosphatase conjugated. Bands were visualized using nitrobluetetrazolium and 5-bromo-4-chloro-3-indolylphosphate as substrates. For N-terminal sequencing of polypeptide bands found in factor IXa, approximately 1.4 IU of activated factor IX were applied on several lanes of an SDS gel and the gel blotted onto a 0.2-μm hydrophobic membrane (Pall, Portsmouth, UK) using a borate buffer. After blotting the membrane was activated with ethanol, stained for 1 min with Coomassie Brilliant Blue (CBB) R and destained with water.

2.2.4. Isoelectric focusing

The complete instrumentation for gel-casting and focusing as well as the low and broad p*I* calibration kits and the strips with immobilized pH gradient (IPG) and IPG buffers were from Amersham Pharmacia Biotech. The 2D-PAGE standard in 9 *M* urea, 5% (v/v) 2-mercaptoethanol, 2% (v/v) Bio-Lyte 5/7 was from Bio-Rad. Different IEF techniques were applied using either self-cast IPG gels or precast IPG strips as outlined below.

2.2.4.1. IPG gels covering the pH range 3.5 to 5.0

These gels were self-cast according to the procedure described by Westermeier [20]. Rehydration of the gels was done overnight in an u-frame cassette under non-reducing conditions with IPG buffer in water, 0.5% (v/v) buffer type 4–7L and 0.5% (v/v) buffer type 3–10NL (L=linear; NL=non-linear). The samples were desalted prior to focusing using PD-10 columns or microspin columns (both from Amersham Pharmacia Biotech). Then, 2 IU factor IX in water were applied per lane on the cathodic end of the strip. The gels were run in a Multiphor II chamber at 10°C cooling temperature in 3 steps using the separation conditions given below, fixed with 20% (w/v) trichloro acetic acid (TCA) in water, stained with CBB G according to the manufacturer and destained with 25% (v/v) ethanol in water.

Subsequent steps	Period (h) (h)	Voltage (kV) (kV)	Current (mA) (mA)	Power (W) (W)
First	1	0.3	3	3
Second	1	1.0	3	7
Third	6.5	4.0	5	15

Voltage was fixed, current and power were maximum tolerable values.

2.2.4.2. IPG Strip covering the pH range 3.0 to 10.0 linearly

2.2.4.2.1. *Factor IXa under non-reducing conditions using water–IPG buffer* IPG strips, pH 3–10L,

18 cm were rehydrated overnight in water, 1% (v/v) IPG buffer, pH 3–10L. The samples containing activated factor IX (2 IU each) were applied at the cathodic end of the strip and focusing at 20°C was done according to the instructions of the manufacturer using a Multiphor II apparatus. The strips were fixed, stained and destained like the IEF gels or used as first dimension for 2D-PAGE.

2.2.4.2.2. *VN under reducing conditions in presence of chaotrops* IPG strips, pH 3–10L, 18 cm were rehydrated overnight in 8 *M* urea, 4% (w/v) 3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS), 2% (v/v) IPG buffer, pH 3–10, 65 m*M* dithioerythritol (DTE) and bromophenol blue. The sample containing 8 μg VN was applied cathodically and focusing at 20°C was done according to the instructions of the manufacturer using a Multiphor apparatus. The strips were fixed, stained and destained like the IEF gels or used as first dimension for 2D-PAGE.

2.2.5. 2D-PAGE

After IEF the strips were cut to size and reequilibrated in a solution containing 50 m*M* Tris–HCl, pH 6.8, 6 *M* urea, 30% (v/v) glycerol, 2% (w/v) SDS and 2% (w/v) DTE for 10 min. Disulfide groups were subsequently blocked for 10 min with a solution of the same composition but using 2.5% (w/v) iodacetamide instead of DTE. The strips were fixed on the top of precast 4–20% polyacrylamide gels, 8×8×0.1 cm (Novex) using hot agarose. The gels were run for 10 min at 200 V/40 mA followed by 2 h at 200 V/80 mA, Coomassie stained or silver stained according to Heukeshoven and Dernick [19] or electroblotted as outlined above for one-dimensional gels.

3. Results

3.1. SDS–PAGE and N-terminal sequencing

One-dimensional SDS–PAGE was adequate for a rough estimation of the purity of factor IX preparations (Fig. 2). Under reducing conditions factor IX appears as a broad band with an apparent molecular mass of approximately 70 000 (cf. arrow). In the plasma-derived clotting factor IX (pdFIX) concentrate, containing considerable amounts of VN (PD1) [17], this factor IX-band is flanked by two bands with an apparent molecular mass of 65 000 and 75 000, which result from the two known forms of VN in human plasma [21]. In these pdFIX samples some high-molecular-mass bands are stained, indicating the presence of high-molecular-mass plasma proteins and/or plasma protein aggregates formed despite the presence of SDS and the reducing conditions. These high-molecular-mass bands are markedly reduced in the more pure pdFIX preparation (PD2), because during the filtration step VN and high-molecular-mass proteins are removed to a great extent [17].

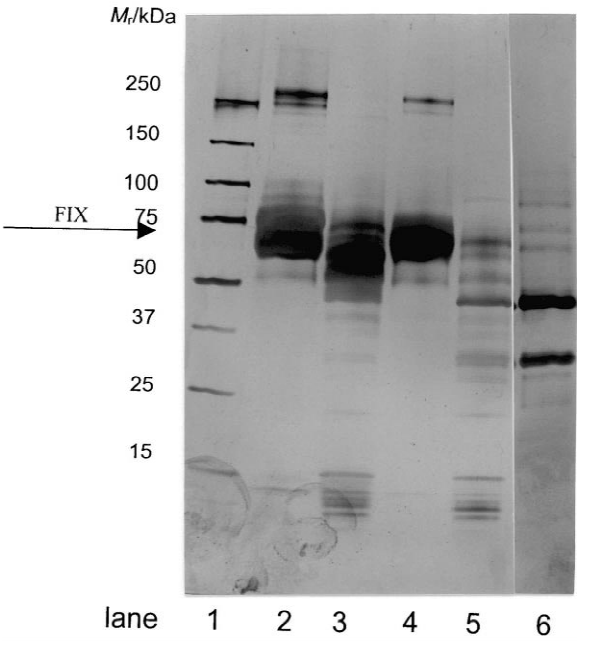


Fig. 2. A total of 0.4 IU factor IX (FIX)of factor XIa-activated and non-activated factor IX preparations were applied after reducing sample preparation onto a 4–20% polyacrylamide gel. Separation was followed by silver staining. Lanes 1=molecular mass standard (kD=kilodalton); 2 and 4=non-activated factor IX samples: PD1, PD2; 3 and 5=activated factor IX samples: PD1, PD2; 6=factor XIa.

Upon activation of factor IX by incubation with factor XIa, the separate chains of factor IX and factor XIa are expected as the main bands under reducing conditions, i.e. the separate factor IX-HC (M_r~26 000 [1]), the factor IX-LC (~16 000 [1]) and the factor IX-AP (~10 000 [1]), as well as the factor

XIa-HC (~41 000) and -LC (~27 000). However, as shown below, some of these polypeptides and various further cleavage products of factor IX and accompanying proteins were detected. To identify the polypeptides obtained by activation, the most intense bands in a Coomassie stained blot of factor IXa from PD2 (indicated by arrows in Fig. 3a) were submitted to N-terminal sequence analysis. The determined amino acid sequences and their positions within the proteins factor IX, factor XI and VN are given in Table 1. The two bands at apparent molecular masses of 50 000 and 30 000 were identified as the factor XIa-HC and -LC. The six other identified bands near M_r 10–15 000 could be assigned to one VN fragment and to five polypeptides originating from the factor IX heavy chain. In four of these cases the N-terminus was identical to the factor IX-HC (amino acid 181). Due to obvious molecular mass differences, the presence of heavy chain fragments of different lengths is assumed. One band had another N-terminus, the amino acid sequence being part of the factor IX-HC starting from amino acid number 320. The factor IX-LC and the AP could be not found and identified.

3.2. Immunoblotting with specific antibodies

Immunoblots with specific antibodies are commonly used for the detection of plasma proteins as well as their activated forms presumed that after activation the protein epitopes are still reactive with the antibody. In case of factor IX almost all commercially available antibodies are directed against the heavy chain [22], two thereof (i.e. ESN1 and F0652) were employed in this work. The two antibodies revealed a slightly different staining pattern on the

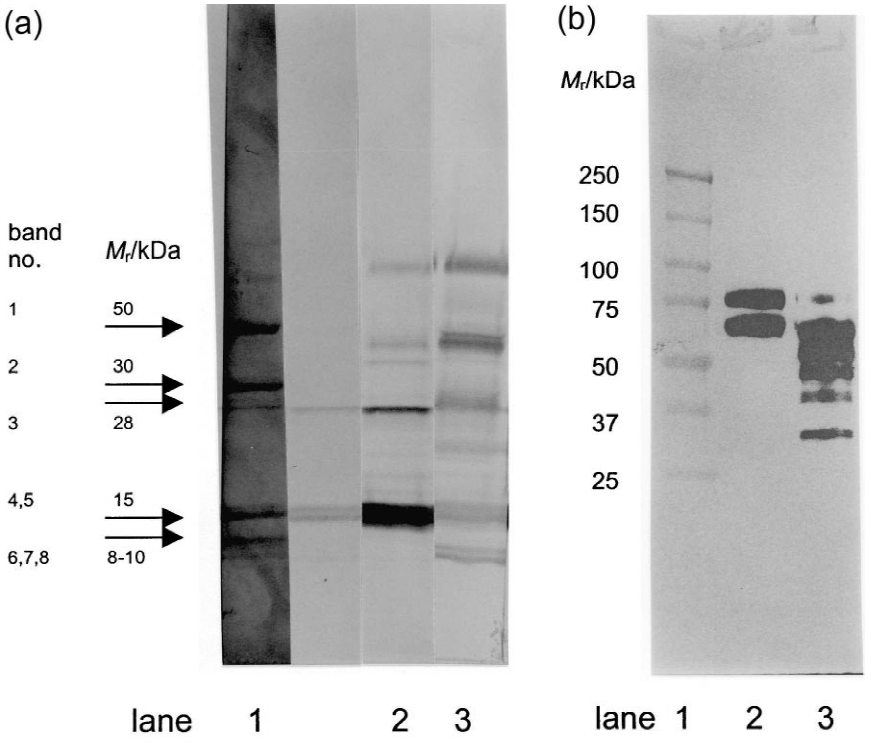


Fig. 3. (a) 0.4 IU factor IXa (PD 2) were separated after reducing sample preparation on several lanes of a 4–20% polyacrylamide gel and electroblotted onto nitrocellulose. The blot was cut and the single lanes color- or immunostained. Lanes 1=Coomassie stain; 2=monoclonal antibody ESN1; 3=polyclonal antibody F0652. (b) 0.4 IU factor IX (PD1) were applied without and after factor XIa incubation after reducing sample preparation. The immunoblot was stained with the monoclonal antibody V7881 against VN. Lanes 1=molecular mass standard; 2=PD1; 3=PD1+factor XIa.

Table 1
N-Terminal sequences of the bands 1–8 in Fig. 3a

Band number	Amino acid sequence determined	Assigned chain fragments number of amino acids	Protein chain	Approx. molecular mass according to SDS–PAGE
1	EXVTQLLD[b]	Factor XI N-terminus of HC	Factor XI-HC	50 000
2	IVGGTASVRG	Factor XI N-terminus of LC	Factor XI-LC	30 000
3	VVGGEDAKPG	Factor IX 181[a] — ≤415	Factor IX-HC	28 000
4	BBGGEDAKPG	Factor IX 181[a] — ≤415	Factor IX-HC	15 000
5	VVGGEDAKPG	Factor IX 181[a] — ≤415	Factor IX-HC	15 000
6	VVGGEDAKPG	Factor IX 181[a] — ≤415	Factor IX-HC	10 000
7	XALVLQYLRV[b]	Factor IX 320 — ≤415	Factor IX-HC	8000
8	TSAGTRQ	VN-325 — ≤475	VN-75/VN-65	

[a] Reported cleavage site of factor XIa.
[b] X indicates a not identified amino acid.

blot membrane as shown for activated PD2 in Fig. 3a. Both antibodies react with the bands identified as the factor IX-HC polypeptide with the apparent molecular mass of about 28 000, and the bands around M_r 10 000 which were identified as HC fragments are stained with different intensity. Antibody F0652 is a polyclonal one and may therefore visualize even tiny amounts of residual intact factor IX due to its higher sensitivity. It further reacts with all activation products and may crossreact with other plasma proteins. ESN1 is a monoclonal antibody against epitopes located on the factor IX heavy chain and is able to stain the intact factor IX, its HC as well as various fragments thereof.

The presence of VN in preparations of pd factor IX was proven by immunoblotting with a monoclonal antibody against VN (i.e. V7881) (Fig. 3b). The two bands above and below M_r 70 000 are stained in full agreement with the finding in the silver stained gel. When treating VN containing samples by factor XIa in vitro, several bands below M_r 65 000 are found which can be stained by V7881 (Fig. 3b).

3.3. Isoelectric focusing and 2D-PAGE

From standard proteins separated in water as well as in 9 *M* urea the IEF calibration curves were established for the determination of the sample p*I*. The values obtained under these separation conditions for factor IX and VN are summarized together with published and calculated data in Table 2.

Table 2
Comparison of theoretical and determined p*I* values of factor IX- and VN polypeptides

	Amino acids	p*I* range determined		p*I*
		This paper	Literature	calculated[d]
Factor IX				
Total protein	1–415	4.03–4.62[a]	4.00–4.65 [23–28]	5.20
HC-LC pdFIX	1–145 to 181–415	4.57–4.96[a]	n.d.	n.d.
VN				
VN-75	20–478	4–5[b]	4.25–5.25[c] [21]	5.47
VN-65	20–398	4–5[b]	4.25–5.25[c] [21]	5.54
V-10	399–478	n.d.	4.58[c] [29]	5.08

[a] Using water–IPG buffer.
[b] 8 *M* urea, calibration standards in 9 *M* urea.
[c] 8 *M* urea.
[d] Calculated from Swiss Prot database (http://www.expasy.ch/pi_tool.html); n.d., not determined.

3.3.1. IEF under non-reducing conditions using water–IPG buffer

3.3.1.1. IPG gels pH 3.5–5.0 for intact factor IX and VN

Isoelectric focusing of factor IX is very sensitive to the focusing protocol. Whereas IEF of factor IX from water or urea solution did not work successful focusing of non-activated factor IX was achieved by IPG gels when rehydrated with water–IPG buffers without further additives. The complete pdFIX preparations and SEC-separated VN (SEC-VN) were run on IPG gels ranging from pH 3.5–5.0 (Fig. 4a). Both, factor IX as well as VN, are separated into series of bands, indicating strong heterogeneity with respect to the p*I*. The p*I* values of the various isoforms are found between 4.03 and 4.62 in case of factor IX and 4.38 to ≥5 for VN using the calibration curve shown in Fig. 4b. The p*I* values of these compounds are thus overlapping to a minor extent.

The accordance of the given p*I* values with published values for factor IX ranging from 4.0 to 4.65 [23–28] is excellent. These values were determined in one case by means of high performance capillary electrophoresis (HPCE) and Ampholine PAGPlates pH 4–5 [23], or by chromatofocusing [24–28].

3.3.1.2. 2D-PAGE of activated factor IX using IPG strips pH 3–10L

Activated factor IX (PD2) was applied to IPG strips pH 3–10L rehydrated with water–IPG buffer without reducing agent following the protocol for non-activated factor IX. Under these conditions, HC and LC are still connected by one disulfide bond [9]. Then, 7 cm out of the 18 cm strip covering the region of the stained bands between pH 4.57 and 7.27 were taken for subsequent second dimension SDS–PAGE separation.

The silver-stained two-dimensional gel of PD2 (Fig. 5) exhibits particularly at the high-molecular-mass region stained lines rather than spots. Two-dimensional immunoblots incubated with anti-factor IX directed antibodies exhibited no reactivity of these high-molecular-mass spot-lines, neither with the monoclonal nor with the polyclonal factor IX antibody (not shown). A better separated spot pattern is obtained at the molecular mass of the factor IX heavy chain, which can be identified by comparison with the parallel one-dimensional run of factor IXa on the two-dimensional gel (cf. arrow in Fig. 5). By their reaction with the anti-HC-directed antibodies these spots are in fact identified as factor IX-HC (not shown). Some discrete spots at low-molecular-mass in the two-dimensional gel are stained. The absence of an isoform pattern in these cases indicate that these fragments probably carry no modifications. They can be stained by antibody ESN1 and F0652 (not shown).

Although VN is present in the pdFIX samples, no VN spot could be detected applying an anti-VN directed antibody (V7881). It is not yet clear whether VN was precipitated during the IEF separation at the basic end of the strip under the chosen buffer conditions without chaotropics. As VN does not migrate under these conditions the presence of VN in the sample has no influence on the factor IX sample pattern, being the same for PD1 and PD2.

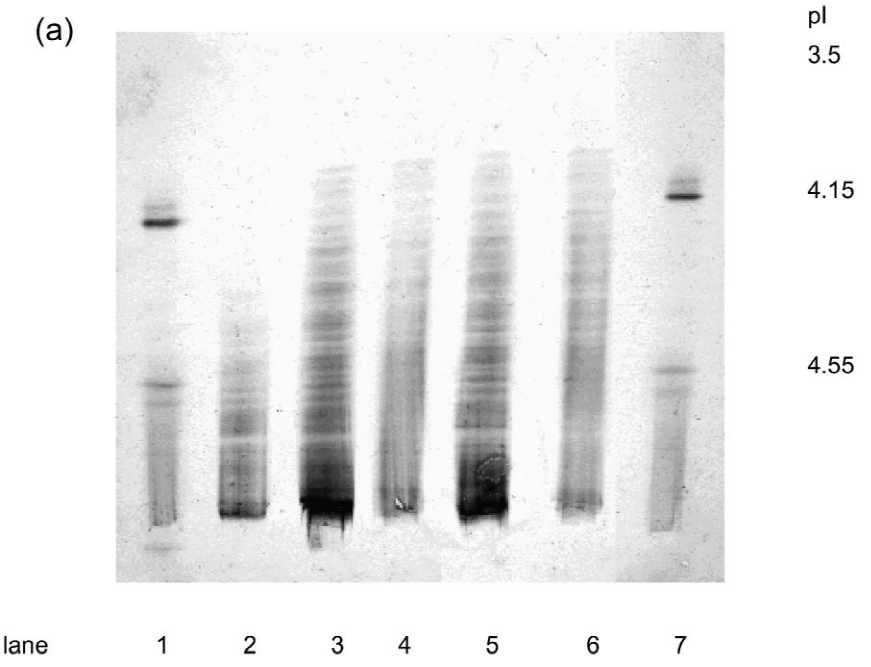
3.3.2. Two-dimensional-PAGE of VN using IPG strips pH 3–10L under reducing conditions and 8 M urea

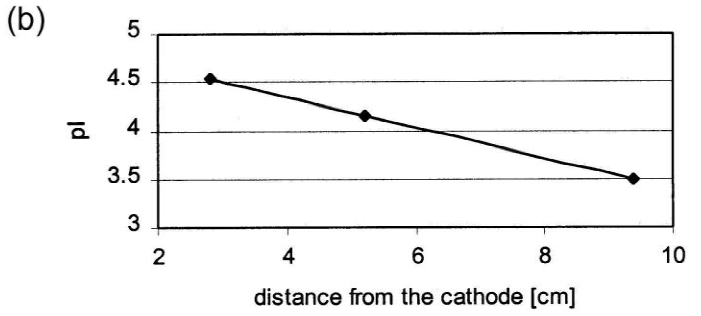
Focusing of SEC-VN in 8 *M* urea worked well and resulted in the separation into a series of isoforms between p*I* 4 and 5 as calibrated by the position of standard proteins in 9 *M* urea.

For performing the two-dimensional-PAGE 7 cm from the acidic end of the 18 cm strips were cut and submitted to the second dimension separation. A series of spots at M_r 75 and 65 000 and three additional spots at M_r 15 000 were obtained in the Coomassie stained gel (Fig. 6a). On the immunoblot only the M_r 75 and 65 000 spots were stained (Fig. 6b). High-molecular-mass aggregates of VN are not found when using urea and DTE.

4. Discussion

The main purpose of characterizing and comparing different factor IX products suited for therapeutic application were (i) establishing of factor IX isoform pattern as separated by IEF and by 2D-PAGE and as found without and after activation by factor XIa and





x	y
2.8	4.55
5.2	4.15
9.4	3.50

Fig. 4. (a) Coomassie stained IEF of different factor IX samples and SEC-VN on 3.5–5 IPG gel. Rehydration was in water, 1% (v/v) IPG buffer. Samples were applied cathodically. Lanes 1,7=low p*I* standard; 2=SEC-VN; 3,5=PD1; 4,6=PD2. (b) Using the standard proteins, an calibration curve for the IPG gel ranging from pH 3.5 to 5.0 was established. By measuring the distance from the cathode the p*I* region for factor IX was calculated from the curve.

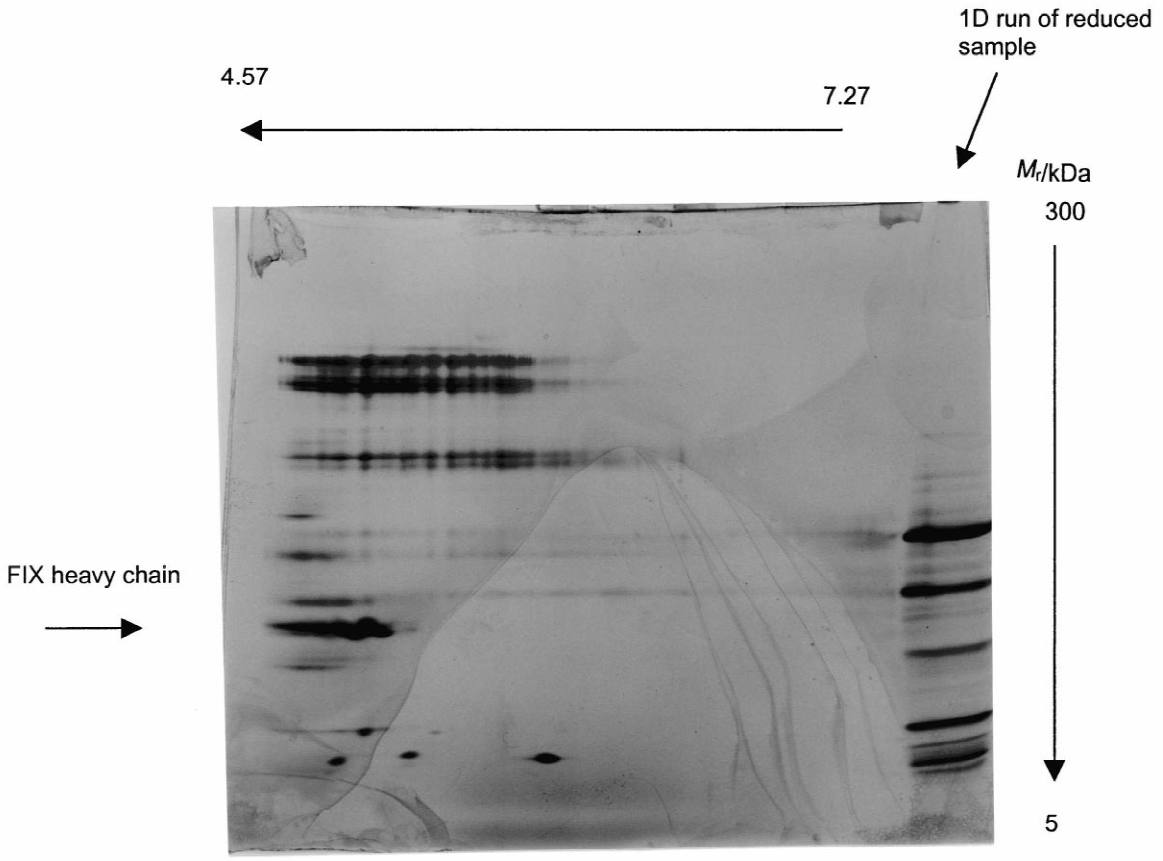


Fig. 5. Silver stained two-dimensional gel of PD2. IEF with a linear IPG, pH 3–10 in water, 1% (v/v) IPG buffer 3–10L, cathodic sample application. The second dimension electrophoresis was run on 4–20% gradient gels. On the one-dimensional lane the reduced sample was separated. The arrow indicates the factor IX heavy chain.

(ii) the evaluation of the presence of accompanying plasma proteins, particularly VN.

With respect to accompanying proteins, the difference between pdFIX preparations of different purity (resulting from an additional virus-filtration step [17]) was obvious. From the three VN polypeptides described in the literature [21,29], the presence of M_r 75 000 and 65 000 VN (VN-75 and VN-65, respectively) in the pdFIX products was confirmed. M_r 10 000 VN (VN-10) was not identified so far, neither by immunoblotting nor by sequencing. It may not be present as a free polypeptide or does not react with the antibody used. VN was separated from factor IX by SEC, its elution volume corresponding to an apparent molecular mass range between 700 000 and 160 000 [17]. From this mass range the presence of VN in polymeric forms under the SEC conditions applied (no chaotrops, low ionic strength) can be concluded. This VN-fraction is separated into a series of isoforms with p*I* values between 4 and 5 by IEF in 8 *M* urea. These values differ to a certain extent from the published values, 4.75–5.25 [21]. It is not likely, that the different urea concentration in the standard protein solutions might be responsible for the difference to the published values. After second dimension separation VN-75 and VN-65 are detected indicating that high-molecular-mass multimers are not present under this condition or do not migrate into the strip during isoelectric focusing.

The molecular mass of factor IX as derived from

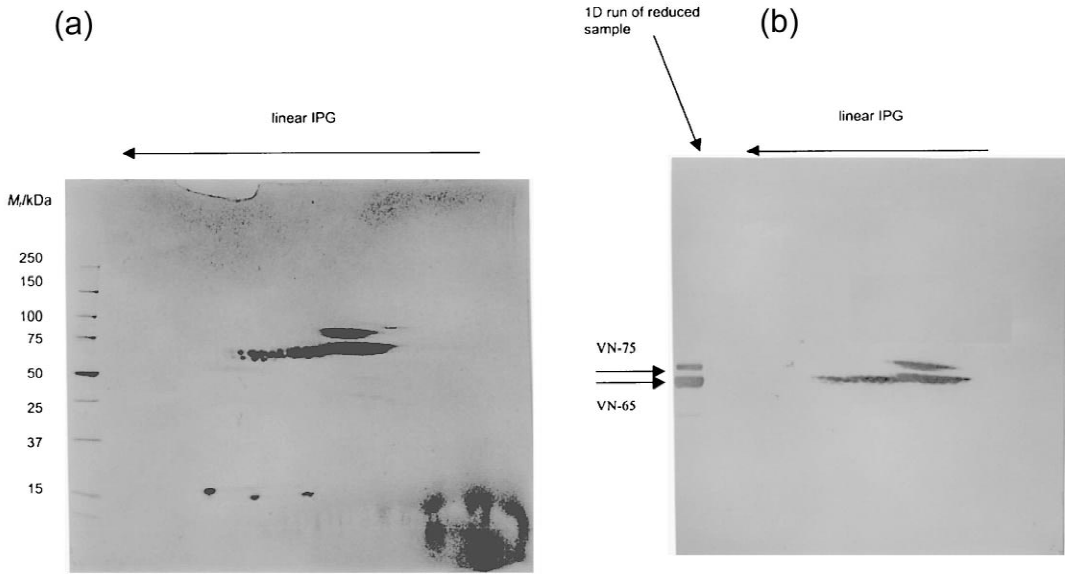


Fig. 6. Two-dimensional-PAGE and immunoblot of SEC-VN. IEF with a linear IPG, pH 3–10 in 8 *M* urea, 4% (w/v) CHAPS, cathodic sample application. (a) Coomassie stained two-dimensional gel. On the one-dimensional lane the protein standard was applied. (b) Immunoblot against VN. On the one-dimensional lane the reduced sample was separated. The arrows indicate VN-75 and VN-65.

SDS–PAGE was about 70 000, in agreement with previously published values, varying between 55 000 and 71 000 [7,30–32].

Factor IX activation by factor XIa results in cleavage and the generation of various separate polypeptides. Within the eight major bands found, only the factor IX-HC and fragments thereof could be identified using immunoblotting or N-terminal sequencing, the light chain and activation peptide could not be found. From the presence of various factor IX-HC fragments we conclude that factor XIa, at least in vitro and in the ratio factor XIa to factor IX applied, does not only cleave off the activation peptide but additionally cleaves unspecificly after arginine residues resulting in several factor IX heavy chain fragments of different molecular masses (and predominantly with the same N-terminus). It is not yet clear why the factor IX light chain and activation peptide could not be identified. Both chains are heavily posttranslationally modified and highly negatively charged [7,10,12–15]. One might speculate whether these features result in a reduced susceptibility to staining (in gels and on blots) and to an atypical migration behavior in electrophoresis. The activation peptide with a total mass of about 10 000 carries two N-glycans, and the mass results therefore to about 60% from the carbohydrate moieties and to only 40% from amino acids, a proportion not commonly found in proteins. This might influence the electrophoretic mobility in SDS–PAGE in an hardly predictable way and may adversely affect the blotting yield. VN is also degraded by factor XIa and this evidence was confirmed by the sequencing result and by IEF experiments (data not shown here).

The microheterogeneity of factor IX due to posttranslational modifications was confirmed by IEF and 2D-PAGE particularly with respect to the p*I* values. At least 20 isoforms with p*I* differences of about 0.02 pH units could be identified in the range between pH 4.02 and 4.62. The selection of the IEF conditions turned out as a very critical parameter. For factor IX the mixture of water and IPG buffer (a mixture of carrier ampholytes) yielded best separations, the presence of urea and reducing agents affected the separation adversely. Maybe due to denaturation and unfolding the highly purified protein precipitates already at the application point.

Two-dimensional-PAGE of factor IXa (PD2) revealed a diffuse silver stained pattern at high molecular mass, that was not expected from the one-

dimensional separation. One can speculate whether irreversible protein aggregation and denaturation takes place during the IEF run as described by Ji and Simpson [33] for non-reducing IEF and in the absence of chaotrops. On the other hand, factor IX HC derived spots are obtained, proved by apparent molecular mass of about 30 000 and immunoblotting. However, the presence of several factor IX HC spots does not necessarily indicate the presence of various isoforms of the HC. As the isoelectric focusing was done under non-reducing conditions under which HC and LC were still connected, the seeming p*I* heterogeneity of the spots attributed to the HC probably results from the heterogeneity of the LC. The determined p*I* values thus refer to the HC-LC polypeptide fragment. During reequilibration the disulfide binding between the HC and LC is reduced and therefore the HC migrates at the expected molecular mass. Combining immunostaining of two-dimensional blots and sequencing results there is great evidence that factor IX-HC fragments present further discrete spots stained on two-dimensional-PAGE at M_r 10–15 000 (Figs. 3a and 5).

The presented panel of analytical methods was valuable to detect differences between factor IX preparations due to VN. Factor IX as well as VN were characterized with respect to molecular mass, isoelectric points and factor XIa cleavage.

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References

[1] A.P. Reiner, E.W. Davie, in: A.L. Bloom, C.D. Forbes, D.P. Thomas, E.G.D. Tuddenham (Eds.), Haemostasis and Thrombosis, Churchill Livingstone, New York, 1994, p. 309.
[2] E. Berntorp, Vox Sang. 70 (1996) 61.
[3] S.A. Limentani, K.P. Gowell, S.R. Deitcher, Acta Haematol. 94 (1995) 12.
[4] L. Hoffer, H. Schwinn, D. Josic, J. Chromatogr. A 844 (1999) 119.
[5] R.J. Kaufman, L.C. Wasley, B.C. Furie, B. Furie, C.B. Shoemaker, J. Biol. Chem. 261 (1986) 9622.
[6] L. Bush, C. Webb, L. Bartlett, B. Burnett, Semin. Hematol. 35 (1998) 18.
[7] G.C. White, A. Beebe, B. Nielsen, Thromb. Haemost. 78 (1997) 261.
[8] B. Furie, B.C. Furie, Cell 53 (1988) 505.
[9] S.P. Bajaj, J.J. Birktoft, Methods Enzymol. 222 (1991) 96.
[10] M. Bond, M. Jankowski, H. Patel, S. Karnik, A. Strang, B. Xu, J. Rouse, S. Koza, B. Letwin, J. Steckert, G. Amphlett, H. Scoble, Semin. Hematol. 35 (1998) 11.
[11] R.J. Kaufman, Thromb. Haemost. 79 (1998) 1068.
[12] S. Gillis, B.C. Furie, B. Furie, H. Patel, M.C. Huberty, M. Switzer, W.B. Foster, H.A. Scoble, M.D. Bond, Protein Sci. 6 (1997) 185.
[13] C. Derian, W.C.T. VanDusen, J. Biol. Chem. 264 (1989) 6615.
[14] K.L. Agarwala, S.-I. Kawabata, T. Takao, H. Murata, Y. Shimonishi, H. Nishimura, S. Iwanaga, Biochemistry 33 (1994) 5167.
[15] J.C. Rouse, A.-M. Strang, W. Yu, J.E. Vath, Anal. Biochem. 256 (1998) 33.
[16] D. Josic, H. Horn, P. Schulz, H. Schwinn, L. Britsch, J. Chromatogr. A 796 (1998) 289.
[17] D. Josic, C. Kannicht, K. Löster, K. Pock, G. Iberer, A. Buchacher, Hemophilia, in press.
[18] U.K. Laemmli, Nature 227 (1970) 680.
[19] J. Heukeshoven, R. Dernick, Electrophoresis 6 (1985) 103.
[20] R. Westermeier, in: H.-J. Kraus (Ed.), Electrophoresis in Practice, VCH, Weinheim, 1997, p. 195.
[21] K.T. Preissner, R. Wassmuth, G. Müller-Berghaus, Biochem. J. 231 (1985) 349.
[22] K.J. Smith, K. Ono, Thromb. Res. 33 (1984) 211.
[23] A. Buchacher, P. Schulz, J. Choromanski, H. Schwinn, D. Josic, J. Chromatogr. A 802 (1998) 355.
[24] S. Chandra, L. Pechet, Biochim. Biophys. Acta 328 (1973) 456.
[25] L. Pechet, J.A. Smith, Biochim. Biophys. Acta 200 (1970) 475.
[26] H. Suomela, Thromb. Res. 7 (1975) 101.
[27] H. Suomela, Eur. J. Biochem. 71 (1976) 145.
[28] H. Suomela, Thromb. Haemost. 35 (1976) 211.
[29] O. Golaz, G.J. Hughes, S. Frutiger, N. Paquet, A. Bairoch, C. Pasquali, J.-C. Sanchez, J.-D. Tissot, R.D. Appel, C. Walzer, L. Balant, D.F. Hochstrasser, Electrophoresis 14 (1993) 1223.
[30] T. Burnouf, C. Michalski, M. Goudemand, J.J. Huart, Vox Sang. 57 (1989) 225.
[31] P.A. Feldman, P.I. Bradbury, J.D. Williams, G.E. Sims, J.W. McPhee, M.A. Pinnell, L. Harris, G.I. Crombie, D.R. Evans, Blood Coagul. Fibrinolysis 5 (1994) 939.
[32] C. Lutsch, P. Gattel, B. Fanget, J.-L. Véron, K. Smith, J. Armand, M. Grandgeorge, Biotechnol. Blood Proteins 227 (1993) 75.
[33] H. Ji, R.J. Simpson, Methods Mol. Biol. 112 (1999) 255.