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Separation of human vitamin K-dependent coagulation proteins using hydrophobic interaction chromatography

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Abstract

A rapid and simple method was developed to separate human vitamin K-dependent plasma proteins from each other, yielding virtually homogeneous pools. The purification technique is based on the single use of hydrophobic interaction chromatography, starting from prothrombin concentrate (PC or DEFIX, also termed factor IX concentrate) as initial material. Phenyl-sepharose HP demonstrated optimal separation by comparing several hydrophobic resins as well as resins used in standard procedures like immobilised heparin and Cibacron blue. Under ideal conditions, factor X could be separated in a single step as well as prothrombin. Factor IX co-eluted with other minor proteins. Focus was given only on these three proteins due to their relative abundance. Complete separation of all proteins present in the starting material was achieved by MonoQ anion-exchange chromatography following the phenyl-sepharose run. The resulting purified material could be demonstrated to be of equal or higher purity than using described methods. This strategy employing hydrophobic interaction chromatography for blood macromolecules could be of immense value for purifying the human vitamin K-dependent proteins and represents a considerable simplification over other purification schemes. It not only involves minimal sample handling but also can be readily up-scaled and is a cost-efficient alternative.

Keywords: Vitamin K-dependent coagulation proteins; Coagulation proteins; Vitamin K-dependent

1. Introduction

The purification of human serum proteins gained wide interest in the last 40 years, leading to a much better understanding of how blood clotting occurs [1]. Of central importance in the process of blood coagulation is the family of proteins which are dependent on vitamin K for their biosynthesis. Seven vitamin K-dependent proteins are known to exist in blood. They all contain gamma carboxy-glutamic acid (Gla) residues, formed by the post-translational carboxylation of glutamic acid by a liver vitamin K-dependent carboxylase [2]. The presence of Gla residues in the amino terminal region of these proteins is essential for the interaction of these vitamin K-dependent proteins with phospholipid membranes and for their enzymatic activities [3]. There are two other well known classes of Gla-containing proteins, namely the Gla-containing pro-

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teins in the bone matrices [4] and the Gla-containing neuropeptides from predatory cone snails venom [5].

Four of the vitamin K-dependent plasma proteins (prothrombin, factors VII, IX and X) posess procoagulant activity, whereas protein C and protein S are anti-coagulant proteins [6]. An additional vitamin K-dependent protein, namely protein Z, has been isolated, but its biological function has not yet been defined [7]. Of all vitamin K-dependent blood coagulation factors, prothrombin is present at >10 times the concentration of factors VII, IX, X, Proteins C, S and Z, thus this molecule has been more intensively investigated than the other factors.

All seven proteins are calcium-binding proteins and they require Ca^{2+} to express their physiological function. The Gla-domain is the actual Ca^{2+} binding site and it undergoes conformational changes by binding to divalent cations [8], though a second Ca^{2+}-binding site was proposed at least in prothrombin [9]. Directly after the Gla-domain, with the exception of prothrombin, each of the other coagulation proteins has one or more epidermal growth factor (EGF)-like domains. Prothrombin has two kringle domains instead. Activation of the vitamin K-dependent proteins occurs by specific cleavage of one or two peptide bonds between the EGF- (or kringle-) like domain and the carboxy domain which contains the active site which is homologous to the pancreatic serine protease trypsin.

Vitamin K-dependent blood clotting proteins have been purified from a vast amount of species, ranging from human [10] to dog [11], rat [12], horse [13], pig [14], bovine [15], sheep [16], rabbit [17] and chicken [18], and even more are known nowadays based on their DNA sequence. All successful protein purification procedures described use as a first step the affinity of the coagulation proteins of interest from plasma for divalent cations [19–22]. Differences in the various protocols start after this point. The major problem with the known systems is very often their limited use on an industrial scale, which not only requires reasonable purity but also cost-efficiency and simplicity.

We describe here a methodology for the separation of human blood coagulation proteins tackling these particular points. The emphasis is given on minimal sample handling, reproducibility and ease of the strategy. This study shows the efficient separation of human coagulation factors X, IX and prothrombin from each other by using standard hydrophobic interaction chromatography.

2. Materials and methods

2.1. Materials

Heparin–sepharose, phenyl-sepharose HP, phenyl-sepharose low and high substitution grade (low and high sub), butyl- and octyl-sepharose and MonoQ anion-exchange resin were obtained from Pharmacia, St. Albans, UK. Affigel Blue was from BioRad, Hemel Hempstead, UK. Prothrombin concentrate (PC, trade name DEFIX) was obtained as a lyophilised and aliquoted product intended for human use, a protein pool of highly enriched factor X and thrombin were from the Scottish Blood Transfusion Service, Edinburgh, UK. Russell's viper venom was obtained from Sigma, Dorset, UK, and Chromozym TH was from Roche Diagnostics, Lewes, UK. Other chemicals were of highest grade available.

2.2. Protein purification

All purification steps were performed at 4°C using FPLC or Gradifrac systems (Pharmacia, St. Albans, UK). The principal buffer was 20 m*M* sodium phosphate pH 7.2 for all procedures, unless stated otherwise. Starting material (prothrombin concentrate) containing 120 mg of total protein per vial was reconstituted in cold distilled water and processed without delay.

2.3. Heparin–sepharose chromatography

A small scale experiment was performed as follows: 40 mg PC sample was applied to a 6 ml heparin column at 1 ml/min and the column was washed with 20 m*M* phosphate buffer pH 7.2 until a stable baseline was reached. Elution of bound protein was done by applying a linear gradient of ten column volumes from 0 *M* to 1.5 *M* NaCl in phosphate buffer. The fraction size was 2 ml throughout the whole run. A bigger preparation with the same column material was achieved by applying 1.2 g of starting material to a 30 ml heparin–sepharose

column and eluting bound proteins with a single step from 0 *M* to 1.0 *M* NaCl in phosphate buffer.

2.4. Affigel Blue chromatography

Six mililitres Affigel Blue agarose were loaded with 40 mg PC sample at 1 ml/min, and the column was washed with phosphate buffer until a stable baseline was obtained. A gradient of 0 to 1.5 *M* NaCl in phosphate buffer over 10 column volumes efficiently eluted bound proteins. Two mililitre fractions were taken during the run.

2.5. Hydrophobic interaction chromatography

Small scale experiments using different hydrophobic resins were carried out by applying 9 mg PC onto 1 ml columns. NaCl was added to 3 *M* prior to chromatography. Bound proteins were displaced from the various resins with a flow-rate of 1 ml/min after reaching a stable baseline with a linear gradient from 3.0 to 0 *M* NaCl in phosphate. The gradient volume corresponded to 30 column volumes for these chromatographies. Fractions were taken at 1 ml intervals. Medium sized preparations (40 mg PC) were also fractionated on 6 ml phenyl-sepharose HP columns. The resin was equilibrated in 3.0 *M* NaCl in phosphate buffer and eluted with a linear gradient over ten column volumes to 0 *M* NaCl. The fraction size was 2 ml during the whole run. Preparative runs were done by loading the column (30 ml phenyl-sepharose HP) with 480 mg PC protein sample at 1 ml/min and, after washing the column with 3 *M* NaCl in phosphate buffer to baseline, eluting bound proteins with a linear gradient to 0 *M* NaCl in ten column volumes. Fractions were taken every 10 min. A different sample obtained from heparin–sepharose elution (approximately 200 mg of protein) was made up to 3 *M* NaCl and was also applied to the same column and eluted as described above. The fraction size was 5 ml in this case.

2.6. MonoQ chromatography

Protein pools were desalted by repetitive concentration and dilution with phosphate buffer until the salt concentration was below 10 m*M*. Samples were applied to a 8 ml MonoQ column at 1 ml/min and elution occurred within a linear gradient of 0 *M* to 0.5 *M* NaCl in phosphate buffer within ten column volumes.

2.7. Identification of prothrombin

Prothrombin is known to be the major constituent of our starting material (approximately 70% of total protein), therefore it was straightforward to monitor prothrombin elution points by simple SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) analysis, where prothrombin runs as a 81 kDa band [23]. Further proof was found by cleaving the molecule with thrombin to generate prethrombin-1 and fragment 1, as described [24]. Another test was to incubate samples with Russell's viper venom, as described [25], and analysing the resulting proteolytic breakdown by SDS–PAGE and incubation with Chromozym TH as recommended by the manufacturer. Dynamic light scattering results indicated that at pH$<$5, this molecule tends to form aggregates (dimers of 150 kDa and higher) as described elsewhere [26].

2.8. Identification of factor X

Factor X was monitored by its appearance on SDS–PAGE gels, where the protein migrates either as a single band at 75 kDa under non-reducing conditions or in the presence of reducing agents as a double band at 50 and 25 kDa. Comparative analysis with a highly enriched factor X sample obtained as written above led to the identification of this molecule as well. Incubation of factor X with thrombin resulted in no proteolytic cleavage [17].

2.9. Identification of factor IX

Factor IX was identified by its strong interaction with heparin [27] and its molecular weight of 66 kDa based on SDS–PAGE. Since factor IX is a single chain molecule, both reducing and non-reducing gel analysis resulted in the same protein band. It is also known that our starting material contains about 3% factor IX based on clotting assays. Another proof was the elution point from anion-exchange resins, where factor IX eluted right before prothrombin as described in the literature [28]. Furthermore, throm-

bin cleavage tests by adding thrombin to purified factor IX did not result in the breakdown of the molecule.

2.10. Gel electrophoresis

Samples were analysed by SDS–PAGE [29] and gels were stained with Coomassie brilliant blue R-250. Approximate molecular weight determinations were estimated from SDS–PAGE.

2.11. Measurement of protein concentration

Protein concentrations were determined using a dye-binding assay and bovine serum albumin as a standard [30].

2.12. Concentration and storage of proteins

Protein solutions were concentrated using Amicon ultrafiltration cells with YM10 membranes or Amicon Centricon 10 centrifuge units and stored at −20°C.

3. Results

Our starting material was chosen as a commercially available prothrombin (or factor IX) concentrate, since the initial steps in all successful purification strategies diverged only after obtaining a protein pool similar to this product.

A comparative analysis using different resins for subfractionation of human vitamin K-dependent proteins is shown in Fig. 1. Heparin–sepharose chromatography of PC samples showed that the major bulk of proteins did not interact with the column material (Fig. 1A). SDS–PAGE analysis (Fig. 1D, lane A7) revealed that most of the constituents are prothrombin. Elution with NaCl resulted in one peak which contained factor X in early fractions, followed by factor IX (Fig. 1D, lanes A27 and A31 respectively), as expected based on published results [27]. Another protein species of approximately 150 kDa, which could not be identified in the course of this work, was eluting together with the factor X containing sample. This macromolecule is readily visible in the initial material by gel analysis (Fig. 1D, lane PC). It is quite likely that this protein is part of complement factor C4 since this polypeptide is known to be present in the used starting material, but further evidence could not be obtained.

Another well known procedure of coagulation protein purification is the use of immobilised Cibacron blue resins. Affigel Blue–agarose chromatography was performed as described in the Methods section. The resulting chromatogram is shown in Fig. 1B. The first peak visible in the chromatogram trace contained no protein with a molecular weight above 21 kDa, as seen by Coomassie staining. Application of a salt gradient resulted in a very broad peak which contained factor X and some prothrombin in the early fractions (Fig. 1D, lane B31), followed by the bulk of prothrombin (lane B36). The aforementioned protein of 150 kDa was found across the whole peak section. Factor IX could not be visualised in any sample from this run, which might be due to the low amount present or that this column material might not enrich this protein.

Another resin tested for its ability to bind vitamin K-dependent proteins was phenyl-sepharose HP. Chromatography of an identical amount of starting material over an identically sized column like in the two runs mentioned above resulted in a separation as shown in Fig. 1C. The first peak corresponding to the breakthrough fraction could be demonstrated to contain all factor X present in the applied sample (Fig. 1D, lane C7). Prothrombin eluted as the major peak at about half the gradient (lane C41) and factor IX was present in later fractions, seen as a band migrating slightly faster on SDS–PAGE (lane C53). Surprisingly, the contaminating 150 kDa protein species was partially eluted together with factor X, and the main amount of the same molecule was found as a homogeneous preparation in fractions 10 to 30.

The resolving power of hydrophobic interaction chromatography for our purposes was tested further by screening several commercially available resins. Butyl-sepharose chromatography resulted in a poor peak pattern as seen in Fig. 1E. Prothrombin could be found almost in the first fractions and eluted quite early from the resin, implying a weak interaction of the protein with the resin. Octyl-sepharose (Fig. 1F) displayed an opposite behaviour by binding prothrombin tightly, and displacement from the column

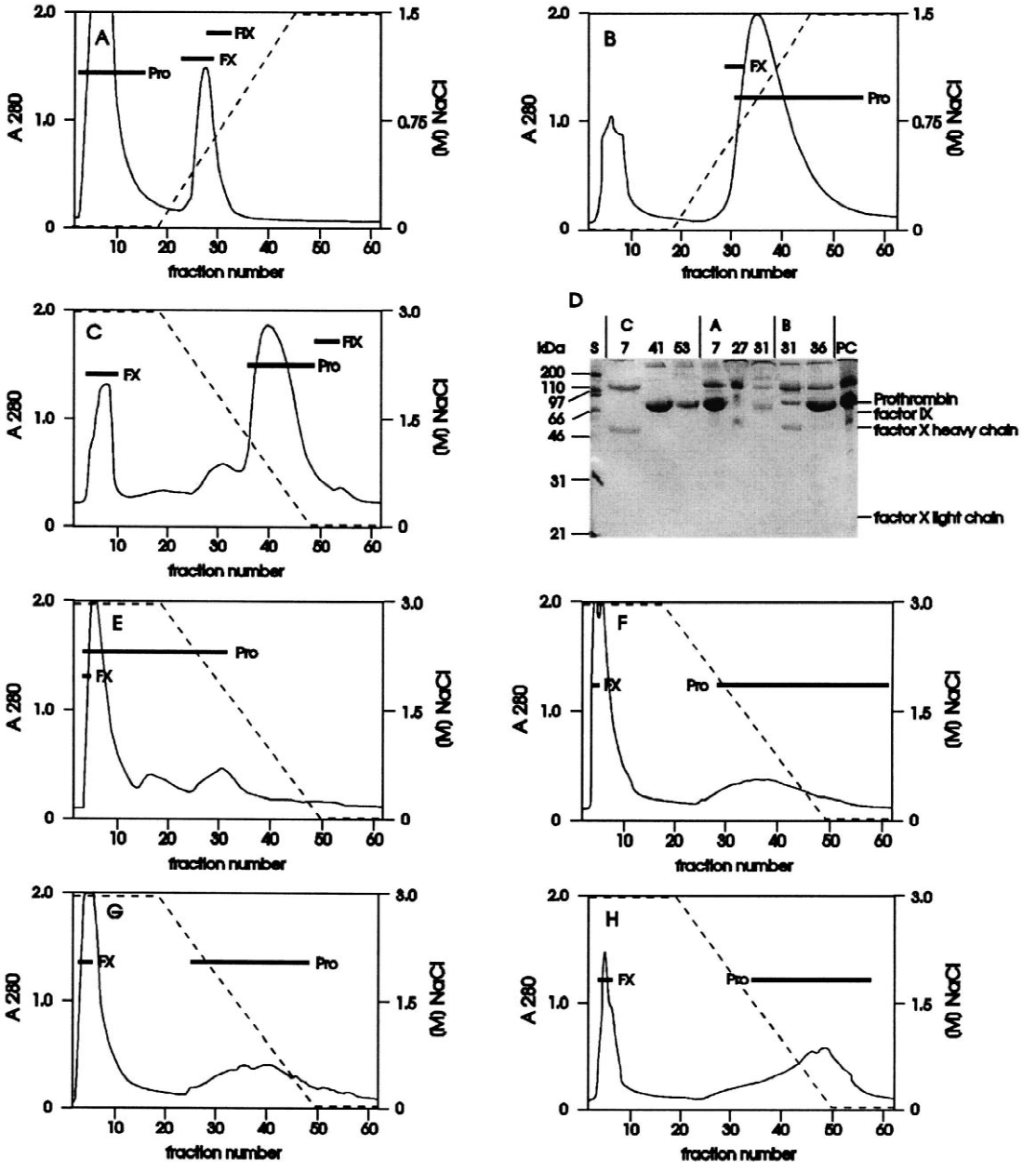

Fig. 1. Comparative chromatography analysis of human prothrombin complex (PC) on different resins. 40 mg PC in phosphate buffer were chromatographed on heparin–sepharose (A), Affigel Blue (B) and phenyl-sepharose HP (C). The screening for optimal hydrophobic interaction resins included, apart from phenyl-sepharose HP also butyl-sepharose (E), octyl-sepharose (F), phenyl-sepharose low sub (G) and phenyl-sepharose high sub (H). The absorbances at 280 nm (solid lines) were monitored throughout the whole runs and are indicated to the left of the different panels. Salt concentrations (dashed lines) are depicted on the right. Solid bars indicate the elution points of prothrombin (Pro), factor IX (FIX) and factor X (FX) as visualised by SDS–PAGE analysis. 10 μl each of selected samples from three chromatographies were analysed on reducing 12% SDS–PAGE (D). Lane numbers correspond to sample numbers of the runs shown in panels A, B and C. PC depicts 60 μg of the starting material. S is the molecular weight marker, and the corresponding migration points in kDa are indicated on the left hand side of the gel. The migration points of prothrombin, factor IX, the heavy chain of factor X and the light chain of factor X are marked on the right.

material occurred over a broad range where protein kept leaching off even after extensive washing with buffer not containing NaCl. A range of phenyl-sepharoses was also subjected to the screening procedure. Low substitution grade phenyl-resin shifted the prothrombin elution peak to the middle of the gradient (Fig. 1G), and high substitution grade of the same resin type displayed a slightly tighter binding of prothrombin (Fig. 1H). Factor X did not interact with any of the hydrophobic resins. Based on gel analysis of all the different runs performed with the mentioned solid supports, phenyl-sepharose HP yielded the best results and was used for all further protein separations.

Chromatography of 1.2 g of PC complex was carried out using heparin–sepharose in order to identify both factors IX and X unambiguously by direct comparison of these proteins to eluted material from different runs, and to evaluate the elution pattern of the phenyl-sepharose HP chromatography. The samples interacting with heparin, containing both factors IX and X, were pooled and subjected to a phenyl-sepharose HP column, as shown in Fig. 2. Factor X could be found in the breakthrough fractions (Fig. 2, lane 14), and factor IX eluted at around 0.6 *M* NaCl (samples 70 to 78), as seen in the Coomassie stained gel. The 150 kDa protein, which co-purified in the heparin-chromatography step,

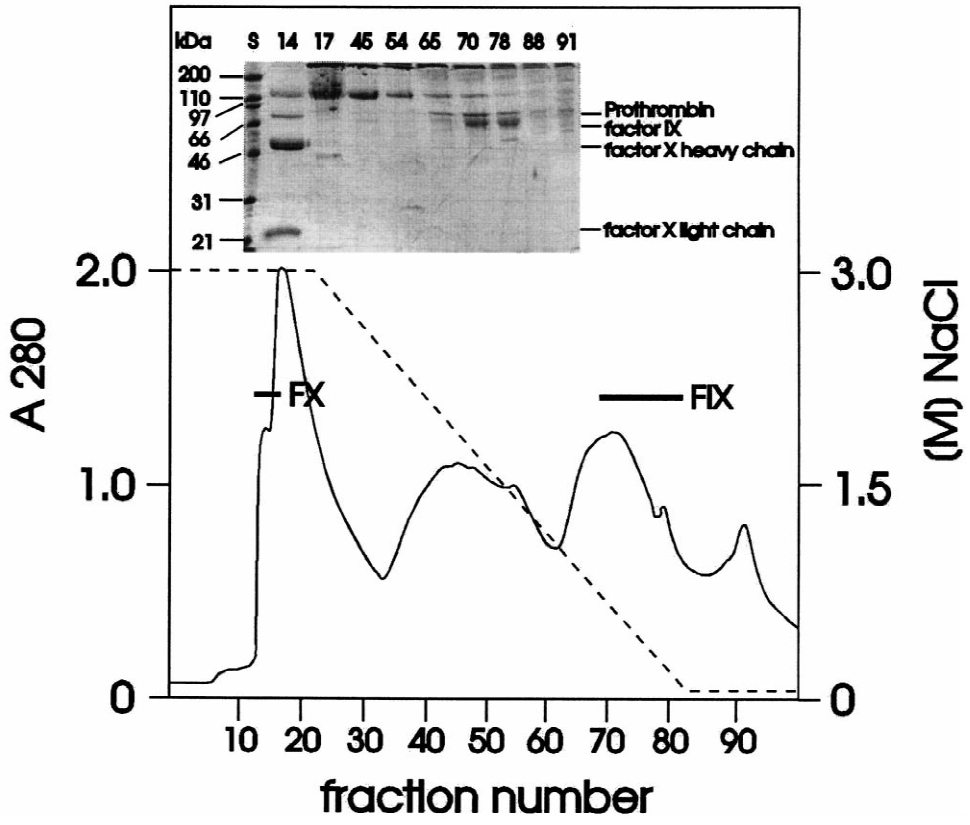

Fig. 2. Isolation and separation of human factors IX and X. Protein pools which were eluted from a heparin–sepharose column were applied to phenyl-sepharose HP chromatography, as described in the text. The absorbance at 280 nm was monitored during the whole run (solid line), and the scale is indicated on the left. The salt gradient is depicted as a dashed line and is shown on the right of the panel. The elution points of factor IX and X are indicated as solid bars in the chromatogram. The inset shows a reducing 12% SDS–PAGE analysis of selected samples. The numbering of the lanes correspond to sample numbers. S is the molecular weight marker. The corresponding migration points in kDa are indicated on the left of the gel. The migration points of factor IX, the heavy chain and the light chain of factor X, are indicated on the right.

could be separated this way almost totally from factor X.

Preparative runs of PC samples on phenyl-sepharose HP, were performed based on the experience from the previous chromatographies (Fig. 3). Up to 500 mg of total protein were resolved on a 30 ml column without a loss in resolution. Variations of NaCl concentrations in either the starting material or the mobile phases showed that 3 *M* was an optimal amount in both the sample and the running buffer. At 4 *M* NaCl, all the protein bound including factor X, whereas 2 *M* NaCl resulted in a flow-through fraction containing factor X and all of the 150 kDa macromolecule. Prothrombin and factor IX resolutions were unaffected by these variations. Another parameter tested was the buffer composition. The change of the principal buffer from phosphate to citrate, Tris or Hepes set at pH 7 to 8, had no influence on the elution pattern.

Protein pools were fractionated further by ion-exchange chromatography. The strategy is summarised in Fig. 4A and the SDS–PAGE analysis of the

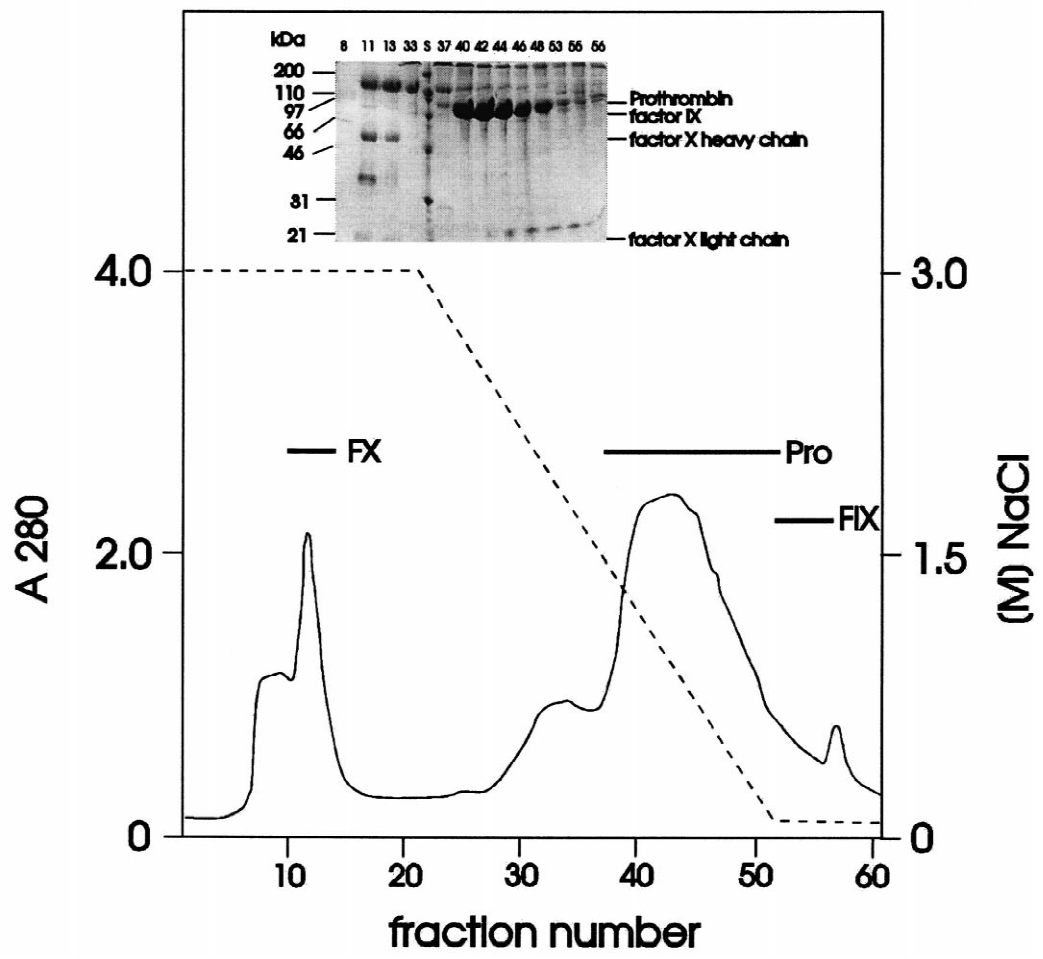

Fig. 3. Chromatography of human prothrombin complex on phenyl-sepharose HP. This shows the results of an example run. Fraction numbers can be related to pool letters A to O using NaCl concentrations in Table 1. The solid line shows the absorbance at 280 nm during the run, and the salt gradient is shown as a dashed line. The elution points of prothrombin, factor IX and X are indicated as solid bars in the chromatogram. A reducing 12% SDS–PAGE analysis of selected samples is shown in the inset. The numbering of the lanes correspond to sample numbers. S is the molecular weight marker. The corresponding migration points in kDa are indicated on the left of the gel. The migration points of prothrombin, factor IX, the heavy chain of factor X and the light chain of factor X are indicated on the right.

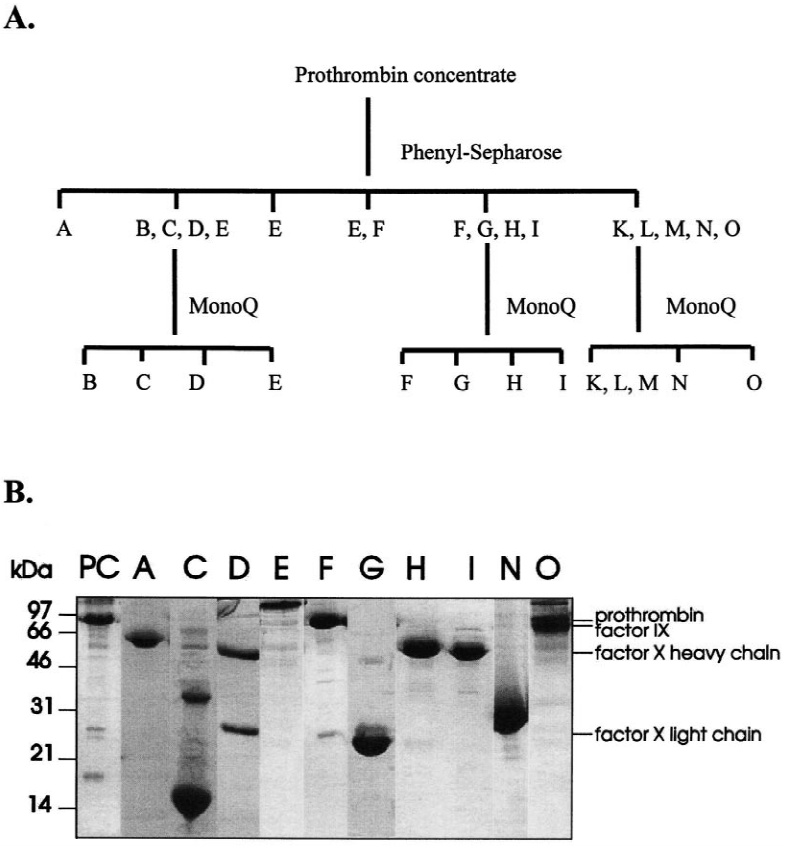

Fig. 4. Analysis and summary of protein samples obtained after fractionation of human prothrombin concentrate. A.) A systematic scheme for purification of human proteins present in a commercially available prothrombin concentrate product. The detailed procedures are described in the text. The letters correspond to the different protein molecular weight species as outlined in Table 1, and analysed in Fig. 5B.) 15% reducing SDS–PAGE analysis of several purified protein pools. The letters correspond to the different fractions as described in Table 1 and Fig. 4A. The samples were resolved on several individual gels. PC is the starting material of the purification procedure. Molecular weights in kDa are indicated on the left. The migration points of prothrombin, factor IX, the heavy chain of factor X and the light chain of factor X are marked on the right.

resulting protein samples as listed in Table 1 is shown in Fig. 4B. Of all the different protein pools isolated this way, only factor X (pool D), factor IX (pool O), prothrombin (pool F) and its breakdown products prethrombin-1 (pool H and I) and fragment 1 (pool I), could be identified. The reason why prethrombin-1 eluted at two different NaCl concentrations from the MonoQ column is unknown. The same behaviour was observed by fractionating thrombin-cleaved prothrombin on the same column. An explanation could be that thrombin cleaves prothrombin at two different sites which are in close proximity, resulting in two protein species with different charge ratios but almost identical (and hence not detectable) molecular weights. An interesting protein is the 18 kDa macromolecule (pool C) since the high purity sample showed a brown-yellow colouration and tended to form dimers as seen by calibrated gel filtration on Sephacryl-S200. Some coagulation inhibitors like α-1 microglobulin contain a covalently linked chromophore of unknown nature, and it is possible that this 18 kDa protein is identical to the 20 kDa molecule described earlier [31], or at least belonging to the family of microglobulins. The same assignment based on molecular weight could be made for the 24 kDa protein sample (pool G),

Table 1
Summary of different protein samples obtained after phenyl-sepharose HP and MonoQ fractionation of human prothrombin concentrate[a]

Protein pools	Mol. wt. (SDS–PAGE)	Phenyl [*M*] NaCL	MonoQ [*M*] NaCl	% of total	Possible protein
A	60	3.1	0.14	0.03	
B	75	3	0.17	0.15	
C	18	3	0.20	1.125	inhibitor
D	75 (50+25)	3	0.28	2.92	factor X
E	150	3+2.4	0.31	22.5	inhibitor
F	81	1.5	0.26	62.1	Prothrombin
G	24	0.8	0.07	0.18	inhibitor
H	50	1.5	0.12	0.59	Prethrombin-1
I	50	1.5	0.17	1.53	Prethrombin-1
K	70	0.4	0.01	0.05	inhibitor
L	40	0.4	0.01	0.05	
M	100	0.1	0.21	1.6	
N	30	0.4	0.24	1.6	Fragment 1
O	66	0.6	0.25	1.6	factor IX

[a] Protein pools were labelled corresponding to their retention on phenyl-sepharose HP and MonoQ. Their approximate molecular weights (in kDa), based on SDS–PAGE analysis, their peak elution points from the hydrophobic resin and from the MonoQ anion-exchanger are included. The % of total corresponds to the amount of purified protein of the according pool compared to the initial amount of PC protein used at the starting point prior to fractionation. The putative proteins assigned to the appropriate protein pools are included. Letters A to O refer to fractions described in Fig. 4A and B.

identifying it as putative protein HC, which is known to have an approximate molecular weight of 28 kDa [32]. Another possible protease inhibitor present in the PC sample could be antithrombin III, with an approximate molecular weight of 65 kDa [33], which could be assigned to pool K. It is also fair to assume that the high molecular weight macromolecule of about 150 kDa (pool E), which is present at a level of approximately 22.5% in the starting material, corresponds to either α2-macroglobulin or complement factor C4, which have a molecular weight of 186 and 196 kDa, respectively [34]. The latter is known to form dimers and even multimers, which might explain the elution behaviour from the column materials.

Another finding is also noteworthy: it was described that fragment 1 is less hydrophobic than prothrombin [24], therefore an earlier elution from the phenyl-column would be expected, but in this work fragment 1 seems to elute after prothrombin from the hydrophobic resin, implying that the former protein is more hydrophobic than the latter.

The possibility that some of the unidentified proteins are in fact breakdown products of other macromolecules can not be excluded since potent protease inhibitors were not included in the purification schemes described in this paper. This would at least explain why proteolytic fragments of prothrombin, like prethrombin-1 and fragment 1, could be detected and even successfully purified to homogeneity during the course of this work.

4. Discussion

The aim of this work was to establish a purification scheme of human coagulation proteins which is based on the usage of cost efficient conventional chromatographic purification steps. The main complication with all the coagulation proteins is their relatively close isolelectric points and their similar molecular masses, making it particularly difficult to employ either ion-exchangers or size exclusion resins for any separation or purification of such molecules. Another difficulty arises in the versatility of a working methodology where one purification protocol is suitable for proteins of one species but not another. Of all the many procedures described in the last 30 years, affinity resins seemed the only way to achieve any reasonable separation [35,36]. Antibody columns were highly favoured since the binding of the different antibodies and their cognate antigens

were demonstrated to be very specific (as reviewed by [37]). The disadvantage though lays in the nature of the resin which is not very robust, and the elution of any bound material very often requires harsh conditions like low or high pH or with chaotropic agents such as KSCN. Furthermore availability of such antibodies is rather limited and very often coupled with high costs.

It was also noted that resins such as benzamidine–sepharose and arginine–sepharose could be of advantage in separating vitamin K-dependent proteins [38,39], though we could not obtain any results which were superior or even equivalent to the data we generated using different methodologies. The benzamidine-resin showed a preference for activated plasma factors but did not render any fractionation of inactive material, whereas the arginine–sepharose displayed some affinity for prothrombin (results not shown).

Another possibility in separating blood coagulation proteins was the use of immobilised Cibacron blue resins [40]. Blue Dextran agarose chromatography was used for the separation of human factor X from other coagulation proteins, exploiting the lower affinity of factor X towards the resin than the other proteins (namely prothrombin and factors VII and IX), whereas Cibacron Blue agarose had almost no resolving power. An extensive use of this column material was described for the efficient separation of bovine coagulation proteins [28]. Our results indicate clearly the major problem encountered in employing this kind of resin, which is a rather limited separation of human vitamin K-dependent proteins, thus supporting the previous observations.

Heparin–sepharose was described in the past to retain specifically factors IX and X, and thrombin with high affinity [20,27,41], though other proteins like antithrombin-III, an anticoagulant, tend to form a tight complex with these proteins in the presence of heparin [42], thereby co-purifying with the eluted material. This problem was also encountered in our work. An unidentified protein of approximately 150 kDa was also retained on the solid support and co-eluted mainly with factor X. It seems reasonable to assume that this high molecular weight macromolecule is forming a tight complex with other proteins like factor X. Unfortunately prothrombin, which does not interact with heparin, and was found therefore in the flow-through fractions, was also not pure and did contain a considerable amount of other proteins as visualised by SDS–PAGE. Re-chromatography of unbound prothrombin-containing fractions over heparin–sepharose did also reveal that at least some factor X was still present (results not shown), implying that the chromatography using heparin–sepharose is a powerful tool for obtaining partially pure factors IX and X, but not prothrombin.

The interaction of the seven vitamin K-dependent proteins with hydrophobic biomaterials such as phospholipids in the presence of divalent cations is well known (e.g. [26]). An application for purification purposes using this knowledge was also reported [43], and more recently the specific Ca^{2+} mediated interaction of coagulation proteins with several resins (as reviewed in [44]). Previously octyl- and phenyl-sepharose were used in the purification of bovine factor V [45]. Protein C was also described to bind to hydrophobic resins both in the presence and absence of calcium ions, though with different mobilities [46], and phenyl-sepharose was used in the separation of human prothrombin fragment 1 from uncleaved prothrombin [24].

Comparative chromatographic runs of PC with several resins, as carried out in this report, showed a strong interaction of almost all proteins present in a human plasma product with hydrophobic resins and an immobilised Cibacron blue matrix. The fact that interaction of the proteins with phenyl-sepharose did occur in the absence of calcium or any other divalent cations is consistent with data obtained by studies with prothrombin and its degradation products [24,47], though controversial reports were published in the past as well [9].

The main difference is that we substituted the added salt from ammonium sulfate to basic saline which is beneficial for any intended therapeutic use of isolated material and might prevent the accidental precipitation of proteins of interest. Several hydrophobic resins were tested under identical conditions, and phenyl-sepharose HP demonstrated the highest resolving power. It should also be noted that the protein elution patterns from phenyl-sepharose HP chromatographic runs were all identical, regardless whether the column size was 1, 6 or 30 ml. This

finding proposes the feasibility of even further upscaling, leading to levels needed in industry applications.

In the presence of 3 *M* NaCl, factor X did not interact with the resin, whereas both prothrombin and factor IX did. The separation of prothrombin and factor IX using phenyl-sepharose is rather limited; both proteins elute directly after each other from this type of column material. A combination of hydrophobic interaction chromatography with one of the other established methodologies might lead even to higher purities needed for therapeutic purposes. Indeed, as it could be demonstrated in this report, a combination of heparin-chromatography, where factors IX and X are specifically retained on the column material, followed by phenyl-sepharose chromatography, leads to very homogenous pools of both factor IX and X as well as prothrombin.

An additional advantage of the use of phenyl-sepharose in the purification of human vitamin K-dependent proteins is that lipoproteins from plasma have been found to be quantitatively bound to this matrix and not eluted except by the addition of an organic modifying agent such as ethylene glycol or propylene glycol [48]. Thus any lipoproteins which may be present as contaminants in the coagulation protein preparations would be removed.

Multiple post-translational modifications occur on plasma vitamin K-dependent proteins, and these modifications are absolutely required for their full biological activities. As a result, it has been well documented that finding a mammalian cell line that can produce a high level of fully active recombinant vitamin K-dependent coagulation proteins has not been easy and post-translational modifications, like glycosylation patterns, differ significantly from the original proteins (as reviewed in [49]). This novel application of hydrophobic interaction chromatography for the separation of human coagulation proteins not only avoids such problems by using human biomaterial, but also generates pure end-products in large quantities which can be used either in therapeutics or as a research tool, making this separation strategy an elegant alternative to other procedures. Furthermore, identification of the various proteins described in this work which were separated by hydrophobic interaction and ion-exchange chromatography will lead to a much clearer picture in the fractionation power of human serum samples of this particular resin.

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