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Short communication

Purification of factor X by hydrophobic interaction chromatography

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Abstract

Human factor X has been purified to homogeneity by hydrophobic interaction chromatography on phenyl-sepharose. The coagulation protein did not interact with the resin in the presence of 2–3 *M* NaCl whereas contaminants were retained. This single purification step, in conjunction with classical purification strategies, is a powerful tool in generating high purity factor X and is based on resins which are readily available.

Keywords: Purification; Factor X

1. Introduction

Factor X is a critical enzyme in the blood coagulation cascade [1]. It is a glycoprotein with an approximate molecular mass of 75 kDa consisting of two subunits of 50 and 25 kDa, which are linked by a disulphide bridge. Activation occurs by a single cleavage by the factor VIIa–tissue factor complex liberating a 52 amino acid peptide from the heavy chain of factor X, generating factor Xa [2]. This polypeptide then forms a one-to-one complex with factor Va in the presence of calcium ions and phospholipid [3], which in turn converts prothrombin to thrombin leading to clot formation.

Purification of the vitamin K-dependent factor X was extensively described in the past. Hydroxyapatite was initially used for the purification of the human isoform [4], which was later substituted by diethyl aminoethyl (DEAE) chromatography where the bovine factor X was purified to homogeneity in the inactive [5] as well as the active [6] form. Most promising results were obtained by using affinity-resins like antibody columns [7,8] or binding to immobilised heparin [9]. Other solid supports employed in factor X purification strategies were Cibacron Blue resins [10,11] or sulphated dextran, where the protein of interest was not retained but contaminants were. Hydrophobic interaction chromatography was also described as an alternative method, where in contrast to the work described here, immobilised phenylalanine retained factor X and other macromolecules [12]. These traditional factor X purification schemes suffer from low yields, low capacity, lengthy dialysis steps, heterogeneity of the final product, and contamination by autoproteolytic activated factor Xa. Furthermore another obstacle is,

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that some methods which are suitable for the purification of factor X from one species, do not seem to work for other species.

The work presented here focuses on the purification of human factor X using a novel strategy which does not include any inhibitors during the separation process and at the same time prevents autoproteolytic activation.

2. Materials and methods

2.1. Materials

Heparin-sepharose and phenyl-sepharose HP were obtained from AmershamPharmacia Biotech, Amersham, UK. Human prothrombin concentrate (PC, trade name DEFIX) was obtained as a lyophilised and aliquoted product intended for clinical use from the Scottish Blood Transfusion Service, Edinburgh, UK. PC consists of ~80% prothrombin and 20% all other blood proteins. All other chemicals and reagents were of the highest grade available.

2.2. Protein purification

All purification steps were performed at 4°C using FPLC or Gradifrac systems and XK 26/20 columns (AmershamPharmacia Biotech, Amersham, UK). 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 1.2-g PC sample was applied to a 30-ml heparin column at 1 ml/min and the column was washed with 20 m*M* citrate buffer pH 7.5 until a stable baseline was reached. Elution of bound protein was done by applying a linear gradient of four column volumes from 0 to 1.0 *M* NaCl in citrate buffer. The fraction size was 7 ml throughout the whole run.

2.4. Hydrophobic interaction chromatography

Heparin-sepharose bound proteins (~150 mg total protein) were made up to 3.0 *M* NaCl and were applied to a 50-ml phenyl-sepharose HP column in 50 m*M* Tris, pH 7.2, containing 3.0 *M* NaCl. Elution was achieved by applying a linear gradient over 70 ml from 3.0 to 0 *M* NaCl. The flow-rate was 1 ml/min and the fraction size was 10 ml during the whole run.

2.5. 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.

2.6. Gel electrophoresis

Samples were analysed by SDS–PAGE [13] and gels were stained with Coomassie brilliant blue R-250. Approximate molecular mass determinations were estimated from SDS–PAGE.

2.7. Measurement of protein concentration

Protein concentrations were determined using a dye-binding assay and bovine serum albumin as a standard [14].

2.8. Concentration, salt removal and storage of proteins

Protein solutions were concentrated by using Amicon ultrafiltration cells with YM10 membranes or Amicon Centricon 10 centrifuge units. Desalting of samples was done by dialysis against 20 m*M* Tris, pH 7.5, at 4°C and final protein pools were stored at −20°C.

3. Results and discussion

Vitamin K-dependent proteins from plasma samples are now being produced using chromatographic fractionation and purification methods [15]. The prothrombin concentrate (PC) we used for our purposes was additionally purified by DEAE ion-exchange chromatography prior to dispensation into vials. This additional chromatography step is of limited benefit for the sub-fractionation of blood

clotting factors since factor X and other coagulation proteins are known not to separate on DEAE-sepharose if they are of human origin [16], in contrast to bovine material where separation can be achieved readily [5,17].

Enrichment of both factor IX and X according to published results [18] was used as the first fractionation step involving heparin-sepharose (Fig. 1). The main bulk of protein, which consists mainly of prothrombin, does not interact with the resin (Fig. 1, inset, fraction 5). Factor X elutes slightly before factor IX (fractions 35 and 38, respectively). Another protein of ~150 kDa is also co-eluting with both proteins. Its nature is currently unknown, though it was described that anticoagulants like antithrombin-III tend to form tight complexes with vitamin K-dependent proteins in the presence of heparin [19], thereby co-eluting from immobilised heparin-resins. NaCl was added to the pool consisting of factor X to 3M and loaded onto a phenyl-sepharose HP column as shown in Fig. 2. Factor X was found in the flow-through fractions as seen by SDS–PAGE analysis depicted in the inset of Fig. 2 (fraction 6). The contaminant 150-kDa protein shows a slight retardation in elution and was separated from the major peak of factor X in this way (fraction 8). Factor IX, which is present in the initial pooled material, eluted from the column very late (fraction 18). Phenyl-sepharose chromatographic runs in the presence of 4.0 *M* NaCl resulted in complete binding of all proteins present in the starting material to the resin, and a reasonable separation could not be obtained (result not shown). Partition of the contaminating 150-kDa protein and factor X was readily achieved by addition of NaCl to a final concentration of 1.0–3.0 *M* to the sample and equilibration of the column material in 2.0–3.0 *M* NaCl. Optimal conditions were elucidated as described in the Materials and methods section. Final yields were ~10 mg of high-purity factor X from an initial 1.2 g starting material. The purified factor X sample can be stored as a lyophilised solid at 4°C and has been shown to be stable for at least 6 months. It was used to specifically cleave recombinant proteins with factor X cleavage sites (data not shown).

The purification scheme described in this report has yielded electrophoretically homogeneous human blood clotting factor X which is free of other vitamin K-dependent clotting factors (Fig. 3) by a relatively simple and rapid method. The advantage is that only

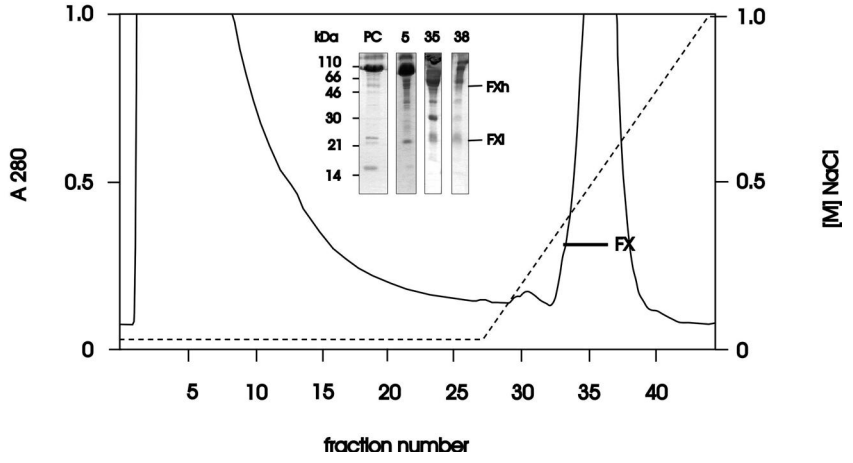

Fig. 1. Heparin-sepharose chromatography of human prothrombin concentrate (PC). The absorbance at 280 nm (solid line) was monitored throughout the whole run and is indicated to the left. The salt concentration (dashed line) is depicted on the right. The solid bar indicates the elution point of factor X (FX) as visualised by SDS–PAGE analysis. A 10-μl amount of each of selected samples was analysed on a reducing 12% SDS–PAGE (inset). Lane numbers correspond to sample numbers of the run. PC depicts 60 μg of the starting material. Molecular weight markers in kDa are indicated on the left hand side of the gel. The migration points of the heavy chain of factor X (FXh) and the light chain of factor X (FXl) are marked on the right.

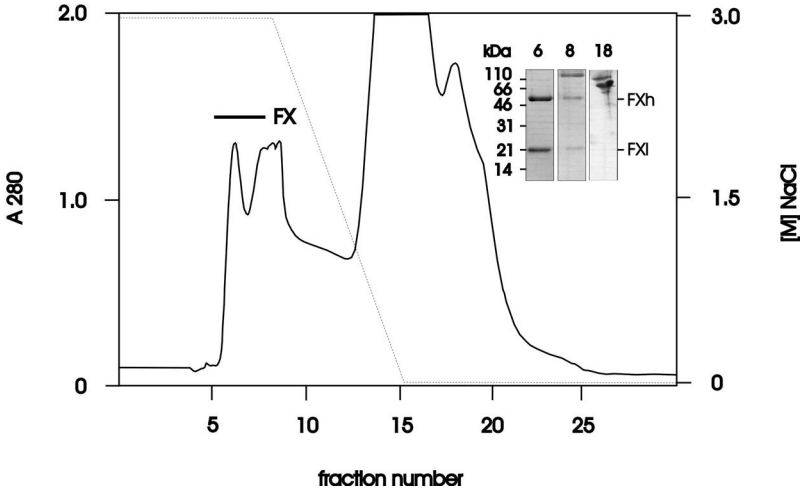

Fig. 2. Isolation and separation of human factor X. Protein pools which were eluted from a heparin-sepharose column were applied to phenyl-sepharose HP chromatography as described in the text. The absorbance at 280 nm was monitored during the whole run (solid line) and the scale is indicated on the left.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

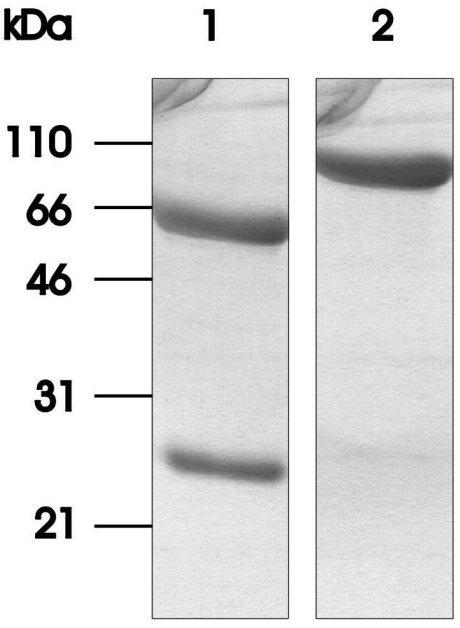

Fig. 3. A 15% SDS–PAGE analysis of human factor X. A 5-μg sample of purified factor X was subjected under reducing (lane 1) and non-reducing (lane 2) conditions to gel analysis. Molecular weight markers are indicated on the left.

resins are used which are readily available, well characterised and cost-efficient. The strategy using hydrophobic interaction chromatography for the purification of human factor X is a good alternative to other methodologies and could lead to products which might be used clinically.

4. Nomenclature

DEAE	Diethyl aminoethyl
PAGE	Polyacrylamide gel electrophoresis
PC	Prothrombin concentrate
SDS	Sodium dodecyl sulphate

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