

B. Bretsznajder, T. Krajewski, Z. Banaś-Gruszka

ISOLATION AND CHARACTERIZATION OF BIRD FACTOR X*

Factor X was obtained from oxalate plasma of duck blood by E s n o u f et al. method [4]. The molecular weight estimated by polyacrylamide gel electrophoresis was 52 000. Following reduction with β -mercaptoethanol two polypeptide chains of molecular weights: 39 000 and 13 000 have been found. During the conversion of Factor X to the active form (Factor Xa) decrease of molecular weight was observed.

Introduction

Factor X is a plasma zymogen protease responsible for conversion of prothrombin to thrombin. Molecule of bovine Factor X (mol. wt. 55 000) consists of two polypeptide chains: a heavy chain (mol. wt. 39 300) and a light chain (mol. wt. 16 500) held together by a disulfide bond (E s n o u f et al. [4]). The protein contains carbohydrate moiety constituting about 10% of the molecule which binds mainly with the heavy chain (M i z o u c h i et al. [13]). N-terminal region of the light chain rich in γ -carboxyglutamic acid is homologous to those of bovine prothrombin and Factor IX (D a v i e, F u j i k a w a [2]). Factor X can be fractionated chromatographically by DEAE Sephadex A-50 into two fractions: Factor X₁ and Factor X₂. However, both the forms neither differ in their electrophoretic mobility nor biological properties (J a c k s o n, H a n a h a n [8]; F u j i k a w a et al. [6]; J a c k s o n [7]). In vivo

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Factor X is activated by Factor IX at the presence of Factor VIII (intrinsic pathway) or by Factor VII and tissue activate factor (extrinsic pathway). Both the pathways require the presence of calcium ions and phospholipids. Factor X is also activated by protease from Russell's viper venom, as well as trypsin (Fujioka et al. [5]; Jesty, Esnouf [9]; Jesty et al. [10]). During the activation process the glycopeptide of mol. wt. 11 000 is split off from N-terminal region of the heavy chain of zymogen. This reaction gives rise to activate Factor X which is able to convert prothrombin to thrombin (Esmon et al. [3]; Kissel, Hanahan [11]; Owen et al. [15]).

Little literature data on structure and properties of bird Factor X encouraged us to isolate and characterize duck Factor X in our laboratory.

Materials and methods

Factor X was isolated from fresh oxalate duck plasma (9 : 1) according to the method of Esnouf et al. [4]. The purification of Factor X was carried out by three-step technique:

1. Adsorption of plasma vitamin-K-dependent proteins on barium sulfate and elution of adsorbed factors by 0.1 M citrate buffer, pH 6.8.

2. Selective adsorption and chromatography separation of Factor X and prothrombin on DEAE Sephadex A-50. The linear gradient of 0.14-0.47 M NaCl in 0.1 M HCl-Tris buffer, pH 7.5 was applied for protein elution.

3. Rechromatography of Factor X on the same ion exchanger with linear gradient of 0.24-0.5 M NaCl in 0.1 M HCl-Tris buffer, pH 7.5. In some experiments rechromatography of Factor X on CNBr-Sepharose-heparine with linear gradient of 0.005-0.5 M KCl in 0.01 M triethanolamine-HCl buffer, pH 6.35 was done. The solutions used during the preparation of Factor X contained PMSF 10^{-4} M (or 10^{-3} M).

Protein concentration was determined by Bradford method [1]. Homogeneity of Factor X samples was tested in 7% polyacrylamide gel electrophoresis according to the method of We-

ber and Osborn [17]. Following acid hydrolysis of a protein sample (5.5 M HCl at 110°C for 24, 48 and 72 hr in vacuum) amino acid composition of duck Factor X was established on an automated amino acid analyzer (JLC 6AH, Jeol).

Factor X was activated by protease from Russell's viper venom at the presence of calcium ions at 37°C for 15 min. In a typical experiment the activation mixture contained: 250 µl Factor X (0.5 mg protein per ml), 500 µl 0.4 M Tris-HCl buffer, pH 7.5 containing 0.04 M CaCl₂ and 0.8 M NaCl, 3 ml of water and 50 µl Russell's viper venom (0.2 mg per ml). The reaction was stopped with 100 µl 0.4 M EDTA.

Results and discussion

Figure 1 shows a typical DEAE Sephadex A-50 chromatography profile of proteins previously selectively adsorbed on the same bed. As we can see fractions containing prothrombin and Factor X were eluted as two separate peaks. The Factor X fraction was then rechromatographed on DEAE Sephadex A-50 (Fig. 2) or CNBr-Sepharose-heparine (Fig. 3). In both cases Factor X was eluted as two partly overlapping peaks defined: Factor X₁ and Factor X₂. This heterogeneity may be due to the presence of an additional sulfate group in duck Factor X₂ as it is suggested for bovine Factor X (Morita, Jackson [14]; Titani et al. [16]). Both the forms possess identical electrophoretic mobility and during SDS electrophoreses migrate as one band of mol.wt. 52 000 (see Fig. 4a, end of this volume, p. 193). The molecular weight estimated by this method is in good agreement with that reported by Esnouf et al. [4], for bovine Factor X (1973). After reduction with β-mercaptoethanol two bands of mol.wts 39 000 and 13 000, corresponding to the heavy and light chains of bovine Factor X appear (see Fig. 4b, end of this volume, p. 193). The amino acid analysis demonstrates that in duck Factor X about 30% of acid and 14% of basic amino acid residues are present. In comparison with bovine Factor X duck protein contains less basic amino acids and almost the same amount of acid amino acids

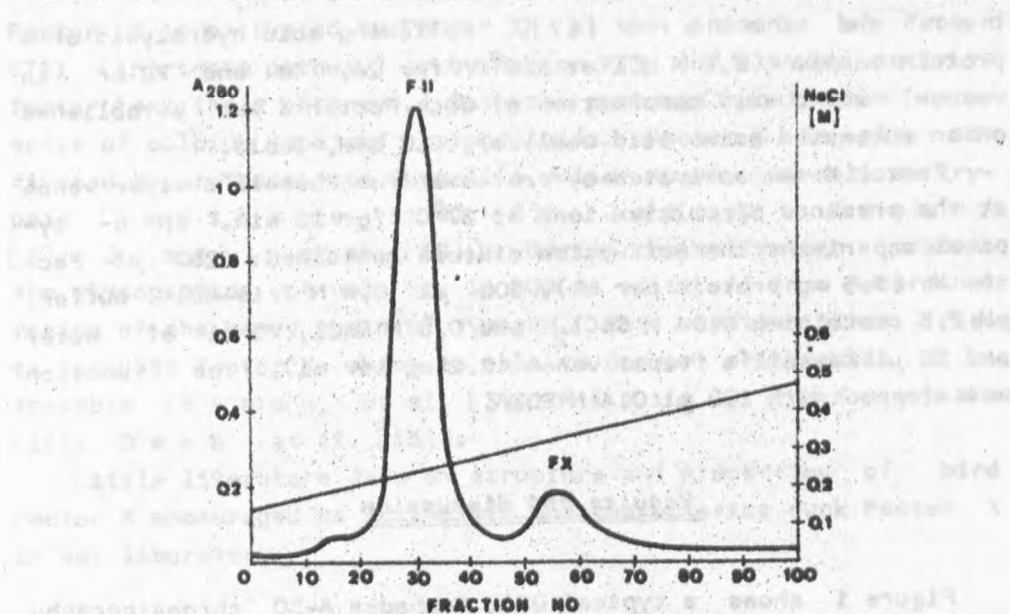


Fig. 1. Separation of duck Factor II and Factor X on a column (3.5 x 20 cm) of DEAE Sephadex A-50. The protein was adsorbed on 2.5 g of DEAE Sephadex, which was poured onto 6 g of DEAE Sephadex equilibrated in 0.14 M NaCl in 0.1 M HCl-Tris buffer, pH 7.5. The column was eluted with a 1.8 litre linear gradient of 0.14-0.47 M NaCl in 0.1 M HCl-Tris buffer, pH 7.5 containing 10^{-4} M PMSF. 18 ml fractions were collected.

Rozdział chromatograficzny kaczego czynnika II i czynnika X na kolumnie (3,5 x 20 cm) z DEAE Sephadex A-50. Białka zaadsorbowane na 2,5 g DEAE Sephadex A-50 nanoszone na kolumnę uformowaną z 6 g DEAE Sephadex A-50, zrównoważoną 0,14 M NaCl w 0,1 M buforze HCl-Tris, pH 7,5. Elucję prowadzono wobec tego samego buforu (1,8 l) o wzrastającym stężeniu od 0,14 do 0,47 M NaCl. Rozdział prowadzono w obecności PMSF o stężeniu 10^{-4} M. Zbierano 18 ml frakcje

and hence the protein is more acidic. The duck Factor X was activated by protease from Russell's viper venom at the presence of calcium ions and the molecular changes of this factor during the activation reaction by polyacrylamide gel electrophoresis were examined (see Fig. 5, end of this volume, p. 193). At zero time a single protein band corresponding to Factor X (52 000) was observed. After 5 min. of incubation a second faster moving band corresponding to Factor Xa (40 000) appear-

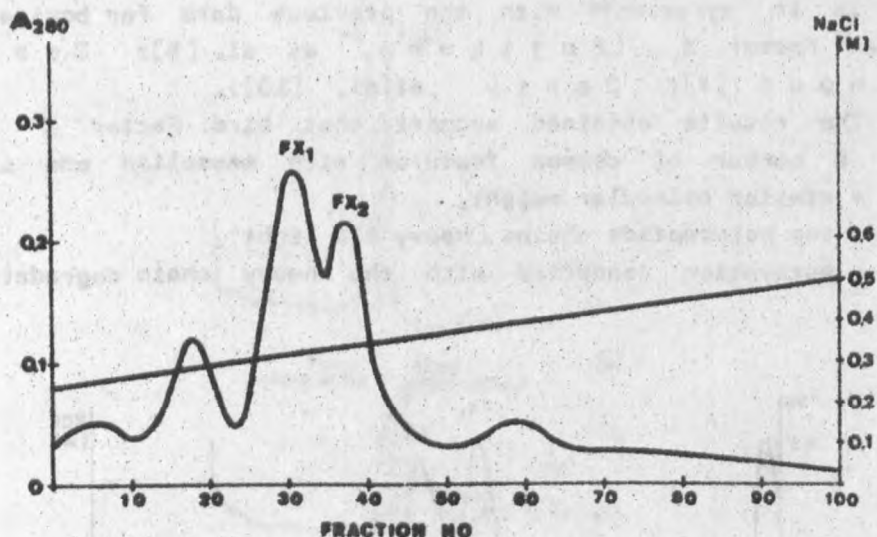


Fig. 2. Rechromatography of duck Factor X on a column (3 x 28 cm) of DEAE Sephadex A-50 equilibrated in 0.24 M NaCl in 0.1 M HCl-Tris buffer, pH 7.5. The column was eluted with a 1 litre linear gradient of 0.24-0.5 M NaCl in 0.1 M HCl-Tris buffer, pH 7.5 containing 10^{-4} M PMSF. 10 ml fractions were collected

Rechromatografia kaczego czynnika X na kolumnie (3 x 28 cm) z DEAE Sephadex A-50 zrównoważonej 0,24 M NaCl w 0,1 M buforze HCl-Tris, pH 7,5. Elucję prowadzono wobec tego samego buforu (1 l) o wzrastającym stężeniu od 0,24 do 0,5 M NaCl. Rechromatografię prowadzono w obecności PMSF o stężeniu 10^{-4} M. Zbierano 10 ml frakcje

ed. Under the experimental conditions complete conversion of Factor X to Factor Xa occurs after 15 min. Figure 6a shows the densitometric scanning of gel after electrophoresis of a nonreduced sample of Factor X and Factor Xa. Two peaks corresponding to nonactive and active forms of Factor X are present. Figure 6b shows these proteins after reduction with β -mercaptoethanol. Three distinct protein peaks are obtained. The first and the second correspond to the heavy chains of Factor X and Factor Xa, respectively. The third one corresponds to the light chains of both forms of Factor X as they possess identical electrophoretic mobility. These results indicate that molecular weight of only the heavy

chain of Factor X decreased during the activation process. It is in agreement with the previous data for bovine and human Factor X (Fujikawa et al. [5]; Jesty, Esnouf [4]; Jesty et al. [10]).

The results obtained suggest that bird Factor X has got a number of common features with mammalian one i.e.:

- similar molecular weight,
- two polypeptide chains (heavy and light),
- activation connected with the heavy chain degradation.

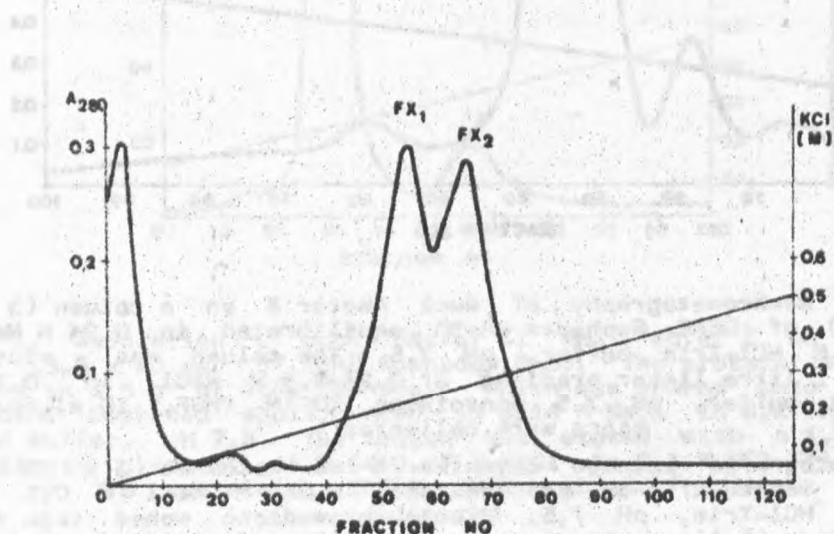


Fig. 3. Rechromatography of duck Factor X on a column of CNBr-Sepharose 4B-heparine equilibrated in 0.005 M KCl in 0.01 M triethanolamine-HCl buffer, pH 6.35. The column was eluted with a 0.5 litre linear gradient of 0.005-0.5 M KCl in 0.01 M triethanolamine-HCl buffer, pH 6.35 containing 10^{-4} M PMSF. 4 ml fractions were collected

Rechromatografia kaczego czynnika X na kolumnie z CNBr-Sepharose 4B-heparyna zrównoważonej 0,005 M KCl w 0,01 M buforze trójetanolamina-HCl, pH 6,35. Elucję prowadzono wobec tego samego buforu (0,5 l) o wzrastającym stężeniu od 0,005 do 0,5 M KCl, Rechromatografię prowadzono w obecności PMSF o stężeniu 10^{-4} M. Zbierano 4 ml frakcje

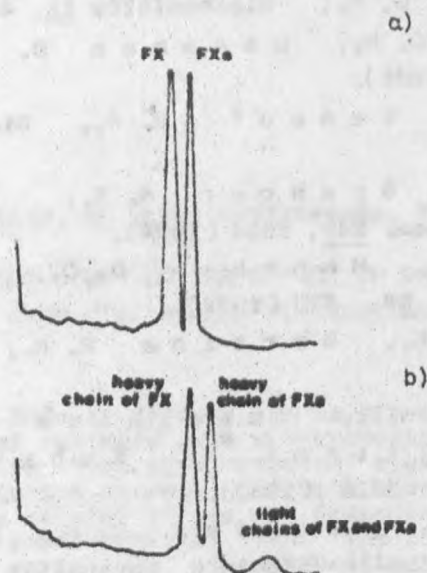


Fig. 6. Densitometer scans of stained SDS polyacrylamide gel electrophoresis of duck Factor X and Factor X_a. a) non-reduced protein; b) reduced protein

Wykresy densytometryczne po elektroforezie w żelu poliakrylamidowym z SDS kaczego czynnika X i czynnika X_a. a) białka przed redukcją; b) białka zredukowane

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Department of Biochemistry
Institute of Biochemistry and Biophysics
University of Lodz

B. Bretsznajder, T. Krajewski, Z. Banaś-Gruszka

OTRZYMYWANIE I CHARAKTERYSTYKA PTASIEGO CZYNNIKA X

Czynnik X otrzymywano ze świeżego osocza kaczego zgodnie z metodą Eanoufa i wcp. [4]. Masa cząsteczkowa określona za pomocą metody elektroforezy w żelu poliakryloamidowym wynosi 52 000. Po redukcji β -merkaptoetanolem otrzymano dwa łańcuchy polipeptydowe o masie cząsteczkowej 39 000 i 13 000. Podczas przejście czynnika X do aktywnej formy (czynnika Xa) obserwuje się spadek masy cząsteczkowej łańcucha ciężkiego zymogenu.