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ACTIVATION OF DUCK PROTHROMBIN BY FACTOR Xa AND THROMBIN*

Prothrombin from duck sodium citrate plasma, was isolated according to the method of Grant and Suttie [6]. The activation of duck prothrombin in a homologous system containing duck Factor X and calcium ions induced the appearance of the final product-thrombin and activation products of molecular weight in the range 21 500-52 000 as it was estimated by polyacrylamide gel electrophoresis.

Introduction

Prothrombin is a glycoprotein, consisting of a single polypeptide chain with molecular weight of about 70 000. This protein plays a pivotal role in the final stages of blood coagulation by converting fibrinogen to fibrin.

Complete activation of prothrombin by activated Factor Xa, Factor Va (Fig. 1) phospholipids and calcium ions results in the formation of thrombin and two activation products (Fragment 1 and Fragment 2), (Seegers et al. [11]; Magnuseon et al. [7]).

Factor Xa in the presence of calcium ions is also able to activate prothrombin to thrombin. This factor is responsible for split of two polypeptide bonds Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} to form Fragment 1.2 and Intermediate 2, a direct pre-

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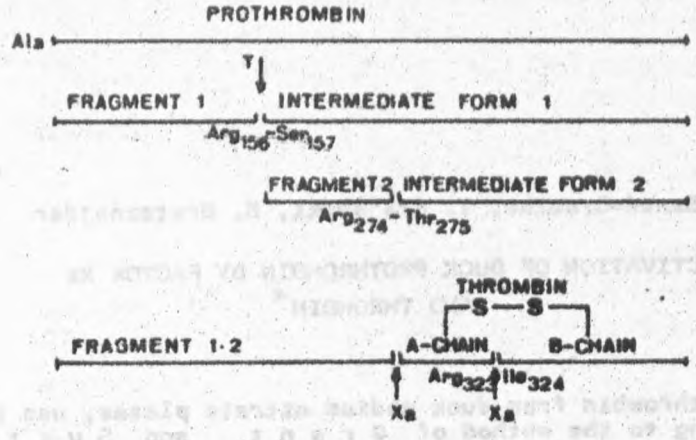

Fig. 1. Scheme of prothrombin activation by factor Xa and thrombin

Schemat aktywacji protrombiny czynnikiem Xa i trombiną

cursor of thrombin (Owen et al. [10]; Esmon et al. [2, 3]; Banaś-Gruszka et al. [1]).

Generated thrombin cleaves the bonds Arg_{156}-Ser_{157} both in the prothrombin and Fragment 1.2 releasing Fragment 1 and Intermediate 1 as well as Fragment 1 and Fragment 2, respectively (Grant, Suttie [6]; Suttie et al. [12]; Malhotra [9]).

Materials and methods

Prothrombin was isolated from fresh duck sodium citrate plasma (9 : 1) according to the method of Grant and Suttie [6]. To isolate duck prothrombin we applied enzyme adsorption of barium citrate. The barium citrate precipitate was dissolved in 0.2 M EDTA (pH 7.0) and then some contaminating proteins were precipitated by the addition of an equal volume of saturated ammonium sulfate. The precipitate was removed by centrifugation and prothrombin was separated from the supernatant by addition of the same volume of saturated ammonium sulfate. The precipitated prothrombin was dissolved in 0.05 M imidazole-HCl (pH 7.8) containing 0.02 M sodium citrate and 0.2 M

ammonium chloride and dialyzed against the same buffer for 3--4 h. The crude prothrombin preparations were chromatographed on DEAE Sephadex A-50 using a 200 ml linear (0.2 to 0.45 M) ammonium chloride gradient in 0.02 M Tris-HCl buffer at pH 7.5. In the course of separation the main peak containing purified prothrombin (elution between 0.24 to 0.26 M ammonium chloride) and 2-3 peaks being most probably impurity proteins and prothrombin degradation products were obtained. Next the main peak was rechromatographed on an identical DEAE Sephadex A-50 column. Every preparation and purification step was carried out in the presence of inhibitors such as: soybean tripsin inhibitor 20 μg/ml, heparin 10 U/ml, phenylmethylsulfonylfluoride 0.001 M and DFP 10^{-4}M.

Factor X was also isolated from duck blood according to the method of E s n o u f et al. [4]. The conversion to the active form (Xa) was performed with Russell's viper venom protease (ratio of enzyme to substrate 1 : 100) in 0.025 M Tris-HCl buffer, pH 7.2 in the presence of calcium ions (0.01 M)(F u j i-k a w a et al. [5]).

Prothrombin activation by Factor Xa in the presence of calcium ions was performed according to G r a n t and S u t-t i e [6]. Prothrombin samples (50 μg, 1 mg per ml) were incubated with Factor Xa (5 μg) in the presence of calcium ions (5 mM) for 0, 5, 15, 30, 60, 120 and 240 min. at 37°C. The reaction was stopped by adding sodium dodecylsulfate (SDS) to a final concentration of 1% and heating to 70°C (water bath) for 5--10 min.

Prothrombin activation products appearing during the conversion of prothrombin into thrombin were examined by SDS polyacrylamide gel electrophoresis (7.5%) by W e b e r and O s-b o r n method [13].

Results and discussion

The preparation of duck prothrombin after purification on DEAE Sephadex A-50 according to G r a n t and S u t t i e [6] was chromatographically homogenic and moved in polyacrylamide gel as a single band(see Fig. 2, 3, end of this volume,p. 191).

Table 1

Molecular weight of prothrombin and prothrombin activation products

Ciężar cząsteczkowy protrombiny i produktów jej aktywacji

Protein	Molecular weight	Incubation time (min.) 0	5	15	30	60	120	240
Prothrombin (duck)	77 000	+	+	+	+	±	±	-
Intermediate form 1	52 000	-	-	-	±	+	+	+
Fragment 1.2	45 000	-	-	±	+	+	+	+
Thrombin	33 000	-	±	+	+	+	+	+
Fragment 1	23 500	-	-	+	+	+	+	+
Fragment 2	21 500	-	-	-	±	+	+	+

+ presence, - absence, ± trace amount of fraction

Table 2

Molecular weight of prothrombin and prothrombin activation products (activation in the presence of DFP)

Ciężar cząsteczkowy protrombiny i produktów jej aktywacji (proces aktywacji prowadzono w obecności DFP)

Protein	Molecular weight	Incubation time (min.) 0	5	15	30	60	120
Prothrombin (duck)	77 000	+	+	+	+	+	±
Fragment 1.2	45 000	-	±	+	+	+	+
Thrombin	33 000	-	+	+	+	+	+

+ presence, - absence, ± trace amount of fraction

The activation of duck prothrombin in a homologous system containing Factor Xa and calcium ions induces the appearance of degradation products. The analysis carried out by the PAGE-SDS (7,5%) revealed the presence of an intermediate and the final product-thrombin of molecular weight in the range of 21 500--52 000 (Tab. 1; Fig. 4 - end of this volume, p. 192 and Fig. 5). On the basis of the results obtained we can assume that similarly to

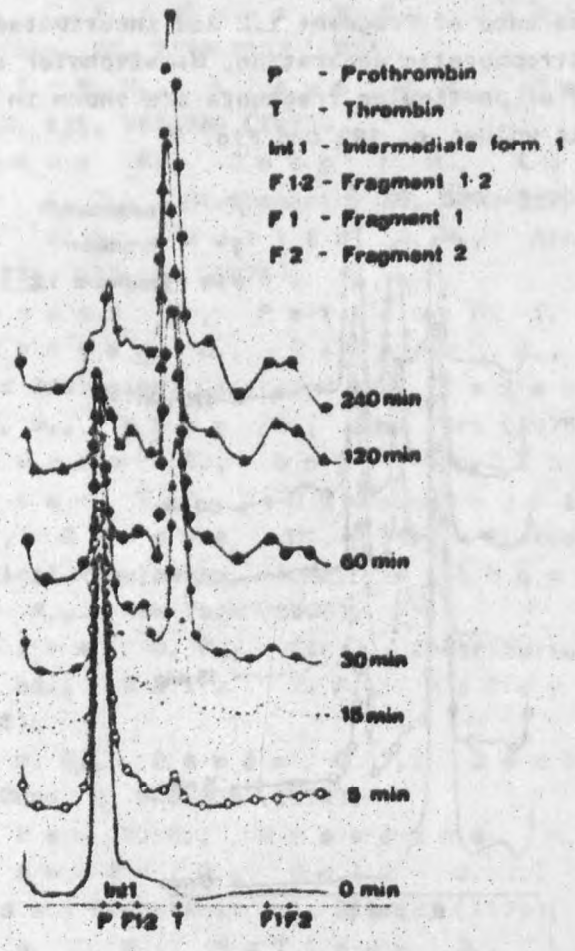

Fig. 5. Densitometer scans of stained 1% SDS polyacrylamide gel electrophoresis (7.5%), 8 mA per gel, of the time course of the activation of duck prothrombin by Factor Xa. Incubation times were 0, 5, 15, 30, 60, 120, 240 min.

Wykresy densytometryczne rozdziałów w 7,5% żelu poliakryloamidowym, 8 mA na rurkę, po aktywacji kaczej protrombiny czynnikiem Xa. Czas inkubacji: 0, 5, 15, 30, 60, 120, 240 min.

mammalian prothrombin. Factor Xa cleaves the same kinds of binding sites in duck prothrombin between Arg_{274}-Thr_{275} and Arg_{323}-Ile_{324} (S e e g e r s et al. [11]; M a g n u s s o n et al. [8]; G r a n t et al. [6]). The appearance of Fragment 1 and Fragment 2 as well as Fragment 1 and Intermediate 1, probably results from the fact that during the activation of duck prothrombin, generated thrombin splits the bonds between Arg_{156}-Ser_{157}. Addition of DFP into incubation mixture resulted in appearance of Fragment 1.2 and inactivated form of thrombin. Electrophoretic separation, densitometer scans and molecular weight of particular fragments are shown in Tab. 2, Fig. 6 - end of this volume, p. 192 and Fig. 7.

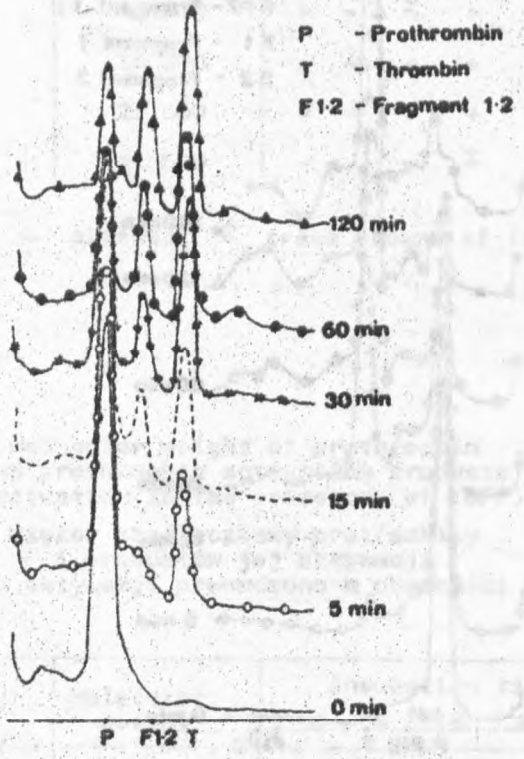

Fig. 7. Densitometer scans of stained 1% SDS polyacrylamide gel electrophoresis (7.5%), 8 mA per gel of the time course of activation duck prothrombin by Factor Xa in the presence of DFP (inhibition of thrombin action). Incubation times were 0, 5, 15, 30, 60, 120 min.

Wykresy densytometryczne rozdziałów w 7,5% żelu poliakryloamidowym, 8 mA na rurkę, po aktywacji kaczej protrombiny czynnikiem Xa w obecności DFP (hamuje działanie trombiny). Czas inkubacji 0, 5, 15, 30, 60, 120 min.

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AKTYWACJA PROTROMBINY KACZEJ CZYNNIKIEM Xa I TROMBINĄ

Protrombinę otrzymywano ze świeżego osocza cytrynianowego kaczek (9 : 1) zgodnie z metodą G r a n t a i S u t t i e [6]. Uzyskane preparaty poddawano rechromatografii na DEAE Sephadexie A-50, uzyskując jednorodne pod względem elektroforetycznym białko. Aktywację prowadzono czynnikiem Xa w obecności jonów wapniowych. Czynnik X aktywowano enzymem proteolitycznym z jadu węża (Russell's viper venom). Próbki protrombiny inkubowano z preparatem czynnika Xa i jonami wapniowymi w czasie 0,5, 15, 30, 60, 120, 240 min. w 37°C. Reakcję przerywano przez dodanie SDS do końcowego stężenia 1% i umieszczenie mieszaniny reagującej w temp. 70°C na okres 5-10 min. Analizę produktów pośrednich pojawiających się podczas konwersji protrombiny w trombinę przeprowadzano za pomocą rozdziału w 7,5% żelu poliakryloamidowym z SDS. Analiza przeprowadzona za pomocą PAGE-SDS wykazała obecność w określonych przedziałach czasowych frakcji białkowych o masie cząsteczkowej około 21 500-52 000.