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Studies on Interaction between Coagulation Factor X and Plasmin*)

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ABSTRACT

Coagulation factor X was isolated from normal human plasma by a two-step sequential affinity column chromatography method. The concentration of factor X was confirmed to be 115 units per litter of plasma. In the present study, it was first confirmed that factor X could be activated to factor Xa by Lys-plasmin.

INTRODUCTION

Coagulation factor X circulate in plasma in an inactive or zymogen form, and must be converted to an active form, factor Xa, by components of the intrinsic or extrinsic pathway. Factor Xa, in turn, hydrolyzes prothrombin to thrombin in the presence of calcium ions, phospholipid, and a co-factor, factor V. Thrombin then converts fibrinogen to fibrin, which becomes insoluble and precipitates in plasma to form a clot. The activation of factor X by means of the intrinsic pathway is catalyzed by factor IXa and VIII in the presence of phospholipid and calcium ions[4,6], and the activation by extrinsic pathway is catalyzed by factors VII and tissue factor in the presence of phospholipid and calcium ions[1,4]. On the other hand, it has been reported that the coagulant protein of the venom from the Russel's viper[7] and trypsin[4] also capable of activating factor X. This paper describes about the activation of factor X by plasmin.

MATERIALS AND METHODS

Reagents:

Pyro-Glu-Gly-Arg-pNA (Kabi, S-2444), H-D-Phe-L-Pip-L-Arg-pNA (Kabi, S-2238), Bz-Ile-Glu-Gly-Arg-pNA (Kabi, S-2222), Aprotinin (Bayer), Zinc-Chelate-Agarose (Pierce) were purchased. ACH-Sepharose 4B was prepared by the methods previously described[12]. Lys-plasmin (specific activity; 30 IU/mg protein) was a gift from Professor Kenneth C, Robbins (Michael Reese Research Foundation, Chicago, USA).

Preparation of plasma:

Venous blood samples were drawn from normal persons, volunteers and a 52-years-old man who was diagnosed as acute pancreatitis followed by disseminated intravascular coagulation (DIC). One volume of 3.8% sodium citrate was added to nine volumes of blood sample, and plasma was obtained by centrifugation of the mixture at 1580 g for 10 min at 4°C. Plastic equipment and previously siliconized glassware and syringe needles were used to prevent the activation of coagulation or fibrinolysis factors.

Determination of amidolytic activity by factor Xa:

Factor Xa amidolytic activity was determined by a modification of the endpoint method described by Claeson et al.[2] with Bz-Ile-Glu-Gly-Arg-pNA, measuring released p-nitroaniline (pNA). The system contained 0.75 ml of 0.05 M Tris-HCl buffer, pH 7.5 containing 0.15 M NaCl and 10 KIU/ml of aprotinin, 0.1 ml of sample solution, 0.05 ml of 0.1% gelatine solution containing 10 KIU/ml of aprotinin and 0.1 ml of 3 mM S-2222 solution; after incubation for 5 min at 37°C, 0.1 ml of 50% acetic

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acid was added to stop the reaction; the absorbance was read at 405 nm, S-2222 is a substrate for factor Xa, is cleaved by factor Xa ten times as rapidly as by thrombin, and six times as rapidly as by αVIIa[13].

Factor X, a zymogen form of factor Xa, was measured by enhancement of S-2222 amidolytic activity after activation of factor X to factor Xa by Lys-plasmin treatment. Treatment with Lys-plasmin was carried out by mixing 0.1 ml of the sample solution and 0.1 ml of Lys-plasmin (0.1 mg/ml, 3 IU/ml), and incubating at 37°C for 15 min. The reaction was stopped by adding 100 KIU of aprotinin to inhibit plasmin. S-2444 and S-2238 amidolytic activities were determined by quite the same method as S-2222.

RESULTS AND DISCUSSIONS

Isolation of factor X from normal human plasma and activation of factor X to Xa by Lys-plasmin:

Zinc-chelate-Sepharose 4B affinity chromatography was introduced by Porath et al.[9] and has been used as the isolation method of tissue-type plasminogen activator[10]. In the present study, the authors first tried to isolate urokinase (UK)-zymogen[11,15] from normal human plasma using this method as follows; 20 ml of citrated human plasma was passed through the Zinc-chelate-Sepharose 4B column (2×5 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.5 containing 1 M NaCl. After washing with excess amounts of the equilibration buffer until absorbance at 280 nm was zero, adsorbed fractions were eluted with the same equilibration buffer containing 0.05 M Imidazole. Adsorbed and eluted factions were collected and dialyzed against the phosphate buffer saline (PBS) at 4°C overnight. By this affinity chromatography method, about 1.7 mg of protein was isolated in adsorbed and eluted fractions from 20 ml of original plasma. To confirm the presence of UK-zymogen in the adsorbed and eluted fractions, the changes of S-2444 amidolytic activity in Zinc-chelate adsorbed and eluted fractions was about 4-5 times increased after incubation with 3 IU of Lys-plasmin for 15 min at 37°C. The authors considered that the increase of the S-2444 amidolytic activity might be a phenomenon induced by the activation of UK-zymogen to active UK, because S-2444 is useful for determining UK activity, and plasmin has been confirmed to activate the UK-zymogen to active UK[11,15]. To further confirm about this, the authors applied the Zinc-chelate adsorbed and eluted fractions on a monospecific anti-UK-IgG-Sepharose 4B column. Unexpectedly, UK-zymogen-like protein in adsorbed and eluted fractions failed in binding to anti-UK-IgG-Sepharose 4B column, indicating that this protein is not UK-zymogen, because UK-zymogen possess the same antigenicity with active UK[11,15].

It is well known that S-2444 can be hydrolyzed not only by UK, but also by plasmin, kallikrein, thrombin and factor Xa. Since plasma plasmin and kallikrein can be inhibited by aprotinin, there remains the possibility that UK-zymogen-like protein may be clotting factors, such as thrombin and factor Xa. Therefore, the authors examined whether this protein is thrombin or factor Xa using their specific substrates, S-2238 and S-2222, respectively. As shown in Table 1, UK-zymogen-like enzyme did not hydrolyze S-2238, but S-2222, indicating that this protein might be factor X or Xa. In the present study, the authors further purified factor X from Zinc-chelate adsorbed and eluted fractions using ACH-Sepharose 4B as follows; 30 ml of Zinc-chelate adsorbed and eluted fractions was passed through the ACH-Sepharose 4B column (2×5 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4 containing 2 M NaCl. Sixty ml of un-

Table 1. Amidolytic activity of Zinc-chelate adsorbed-eluted fraction

	S-2444 amidolytic activity	S-2238 amidolytic activity	S-2222 amidolytic activity
		ΔA405/5 min/0.1*	
before Lys-plasmin treatment	0.011	0.007	0.053
after Lys-plasmin treatment	0.050	0.006	0.256

* 0.1 ml of sample solution is proportionate to 2 ml of orginal plasma

Table 2. Purification of factor X from normal human plasma by a two-step sequential affinity chromatography

	Zinc-chelate-Sepharose 4B		ACH-Sepharose 4B	
	unadsorbed fractions	adsorbed-eluted fractions	unadsorbed fractions	adsorbed-eluted fractions
total protein (mg)	1360	1.7	1.1	0.6
S-2222 amidolytic activity (U)**	ND*	2.6	2.3	0

Data is average of 11 purification experiments.
Total volume of original plasma sample is 20 ml.
*ND; not determined,
**U; 1 unit was defined as the amount of enzyme which produces ΔA405/5 min=1.0 after Lys-plasmin treatment under standard condition described in Meterials and Methods.

adsorbed fractions were collected with equilibration buffer. After washing with equilibration buffer until absorbance at 280 nm was zero, adsorbed fractions were eluted with the equilibration buffer containing 8 M urea and 30 ml fractions were collected. Both unadsorbed and adsorbed and eluted fractions were dialyzed against PBS (2L×2) overnight at 4°C. The precipitates formed during dialysis were removed out by centrifugation 10, 000 rpm for 30 min and concentrated to 3 ml by Carbowax 20, 000. Table 2 summarizes the purification of factor X by this two-step sequential affinity chromatography method. As shown in Table 2, factor X could not bind to ACH-Sepharose 4B, and 2, 3 units of factor X was isolated from 20 ml of original plasma. On the other hand, factor Xa also could not bind to ACH-Sepharose 4B. This indicates that factor X or Xa can be isolated from thrombin, because thrombin can be bound to ACH-Sepharose 4B. There are many reports on purification method of factor X[3,5,8,14]. DiScipio et al.[3] succeeded in purifying factor X using Benzamidine-Sepharose and poly-(homoarginine)-Sepharose columns and reported that the total amounts of factor X in human plasma was about 8 mg/l. In the present study, although factor X cannot be purified finally, the authors confirmed that the total amounts of factor X in human plasma was about 155 units/l. The authors also consider that Zine-chelate-Sepharose 4B is a useful resin for the purification of factor X or Xa. It was of particular interest that plasmin can activate factor X to factor Xa as well as the activation of UK-zymogen to active UK. Although the activation mechanism of factor X to factor Xa is still unclear, this phenomenon makes us speculate the possibility that plasmin may play an important role in maintenance of the hemostatic balance as the factor of feed back from fibrinolysis to coagulation.

Isolation of factor X from patient plasma with acute pancreatitis followed by DIC:

The authors tried to isolate factor X from patient plasma with acute pancreatitis followed by DIC, and confirmed that no factor X-like clotting factors could be isolated from patient plasma by a two-step sequential affinity chromatography. Serial studies of the level of factor X in DIC show significant changes in activity, but in view of the many circumstances that can alter clotting factor activity, definite conclusions concerning the level of factor X cannot be drawn from the data. This data is thought to be the result of consumption of factor X during the process of DIC.

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