

# Characterization of thrombin derived from human recombinant prothrombin

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Thrombin (FIIa) is the key enzyme in haemostasis and acts on several substrates involved in clot formation, platelet activation and feed-back regulation of its own formation. During activation of blood coagulation, FIIa is formed by proteolytic cleavage of prothrombin (FII). In the production of recombinant human FII (rhFII), a key question is whether the thrombin formed has the same properties as endogenous thrombin. We have investigated whether FIIa formed from rhFII and plasma-derived human FII (pdhFII) have the same enzymatic and haemostatic properties against a number of substrates and the same haemostatic capacity in plasma, whole blood and on platelets. Pure FIIa was isolated from rhFII and pdhFII cleaved by recombinant ecarin, and analytical methods were developed to compare the activity of FIIa against different substrates. FIIa derived from rhFII and pdhFII were found to have very similar properties in activating FVIII, FXIII, protein C, platelet aggregation and plasma or whole blood coagulation. Further, the same turnover for S-2366 was found with similar  $K_M$ . However, activation of FV with rhFIIa was approximately 25% more effective than with pdhFIIa and heparin-enhanced inhibition of rhFIIa by antithrombin was

significantly more efficient compared with pdhFIIa with 10% higher inhibition both at steady state and at initial rate conditions. Although differences between the two FIIa preparations using ecarin cleavage were observed, FIIa derived from rhFII administered to human would likely be very similar in activity and function as FIIa formed from endogenous FII. *Blood Coagul Fibrinolysis* 26:545–555 Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.

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## Introduction

Prothrombin (FII) is an essential zymogen circulating in the blood, which becomes activated to the serine protease thrombin in response to injury and bleeding, to allow clot formation and haemostasis. Thrombin (EC 3.4.21.5) is generated from FII through proteolytic cleavage at two sites (Arg 271 and Arg320) by factor Xa in the prothrombinase complex [1]. The resulting active 37-kDa serine protease  $\alpha$ -thrombin (FIIa) is no longer associated to the Gla domain and the two Kringle domains of FII and is thus free to diffuse away from the surface on which it was generated.

FIIa has structural features that make it possible to recognize a broad range of diverse protein substrates that includes both procoagulant and anticoagulant functions. FIIa is a trypsin-like serine proteinase, which preferentially cleaves polypeptide substrates at Arg/Lys-Xaa bonds. Most studies have established that FIIa recognition of substrates invariably involves engagement of the active site cleft and either one or both of the two exosites, exosite I and exosite II [2]. But, unlike trypsin, it has a highly restricted specificity toward certain protein substrates and a number of polypeptide hormones [3]. Platelet aggregation is a functional response of platelet activation effected through cleavage by FIIa of

protease-activated receptors (PARs) involving exosite I [4]. Cleavage of fibrinogen to form fibrin monomers and activation of FXIII are other key procoagulant functions of FIIa in the formation of a fibrin-stabilized platelet plug at the site of injury [5]. The formation of a stable fibrin clot also depends on the ability of FIIa to stimulate its own generation through feedback activation of the protein cofactors V and VIII [6] and by activation of FIX on the platelet surface resulting in the local burst of thrombin [7].

FIIa is finally irreversibly inhibited and cleared from the circulation by specific serine protease inhibitors, primarily the serpin antithrombin [8]. One common feature of serpins that inhibits FIIa is the ability to bind to and be activated by heparin-like glycosaminoglycans (GAGs). GAGs bind to exosite II on FIIa with micromolar affinity and have been shown to significantly accelerate the inhibition of FIIa by antithrombin through a bridging mechanism [9]. The rate of FIIa inhibition by antithrombin is enhanced 1000-fold in the presence of heparin [8,10].

Thrombomodulin is expressed on the surface of endothelial cells and binds FIIa with high affinity. Binding to thrombomodulin alters FIIa specificity for procoagulant

substrates toward activation of protein C into the anti-coagulant protease-activated protein C (APC). FIIa interacts with thrombomodulin primarily through exosite I [1,11–13], yet exosite II can also be involved in the presence of a chondroitin sulfate moiety found within thrombomodulin and induces a change in FIIa specificity by blocking the binding of procoagulant substrates, which depend on an interaction with exosite I. Thus, in addition to clot-promoting activities, FIIa also has the ability to downregulate its own formation by inactivation of cofactors Va and VIIIa by APC.

In situations with severe bleeding and concomitant coagulopathy, FII is lost because of bleeding, dilution and consumption, and may become rate-limiting for blood coagulation. The promising haemostatic potential of this rhFII has previously been investigated in a pig bleeding model [14]. In the present study, we confirm that recombinant human thrombin (rhFIIa) prepared from rhFII has similar interactions as plasma-derived human FIIa (pdhFIIa) with a representative selection of different thrombin substrates, both in isolated systems as in more complex plasma and blood models. FIIa derived from rhFII administered to human would therefore likely be very similar in activity and function as FIIa formed from endogenous FII.

## Materials and methods

### Reagents

Human coagulation factors FIIa, FV, FIXa, FX, FXa and rabbit thrombomodulin were from Hematologic Technologies (Essex Junction, Virginia, USA), recombinant human FVIII from Bayer Healthcare (Berkeley, California, USA), and human antithrombin III from Chromogenix (Milan, Italy). Human Protein C was purified by use of the calcium-dependent monoclonal antibody HPC-4 [15]. Unfractionated heparin was from Kabi Pharmacia (Stockholm, Sweden). The chromogenic substrates S-2238, S-2366 and S-2765 and the reversible thrombin inhibitor I-2581 were from Chromogenix. Bovine serum albumin (BSA), 20% (w/v) solution in water, and the phospholipid emulsion used, Phospholipid-TGT, comprising a mixture of synthetic phosphatidyl serine, phosphatidyl choline and purified egg sphingomyelin, were both from Rossix, Mölndal, Sweden.

### New method to purify thrombin from prothrombin

Lyophilized pdhFII was from Enzyme Research Laboratories, (South Bend, Indiana, USA) and rhFII was produced in CHO cells by AstraZeneca, Sweden. In the first step, recombinant ecarin [16] was used to convert FIIa into FII. The FIIa obtained was purified in two steps. First, a Hitrap SP-Sepharose column (GE Healthcare, Uppsala, Sweden) was equilibrated in buffer A1: 50 mmol/l sodium phosphate buffer, 0.1% Tween-80, pH 6.5. The crude FIIa was adjusted to the same condition as buffer A1 and loaded. The column was then

washed with buffer A2: 50 mmol/l sodium phosphate, pH 6.5, and FIIa was subsequently eluted with buffer B: 50 mmol/l sodium phosphate buffer containing 500 mmol/l NaCl, pH 6.5. Then, eluted FIIa was further purified on an HP Hitrap Butyl column (GE Healthcare), which was equilibrated in buffer A3, 50 mmol/l sodium phosphate and 3 mol/l NaCl, pH 6.5. The pooled FIIa samples from the ion exchange chromatography were adjusted to the higher sodium chloride concentration in buffer A3 and were loaded on the column. The column was washed with buffer A3 and subsequently FIIa was eluted with buffer A2 (Fig. 1). The eluate from the butyl column was concentrated at 10°C on a spin filter (Amicon Ultra-15) and buffer exchanged into 50 mmol/l Tris-HCl pH 7.4, 142 mmol/l NaCl buffer using a PD10 column (GE Healthcare), according to the manufacturer's instructions. The protein concentration was determined from the absorbance at 280 nm,  $\epsilon_{280} = 66.8$  l/mmol per cm [17], the samples were aliquoted and then rapidly frozen and stored at  $-80^{\circ}\text{C}$ . The purified FIIa was analyzed by SDS-PAGE (Fig. 1) and by N-terminal sequencing. With the purification method used, ecarin was not detectable in the final thrombin preparations using an ecarin antiserum [16].

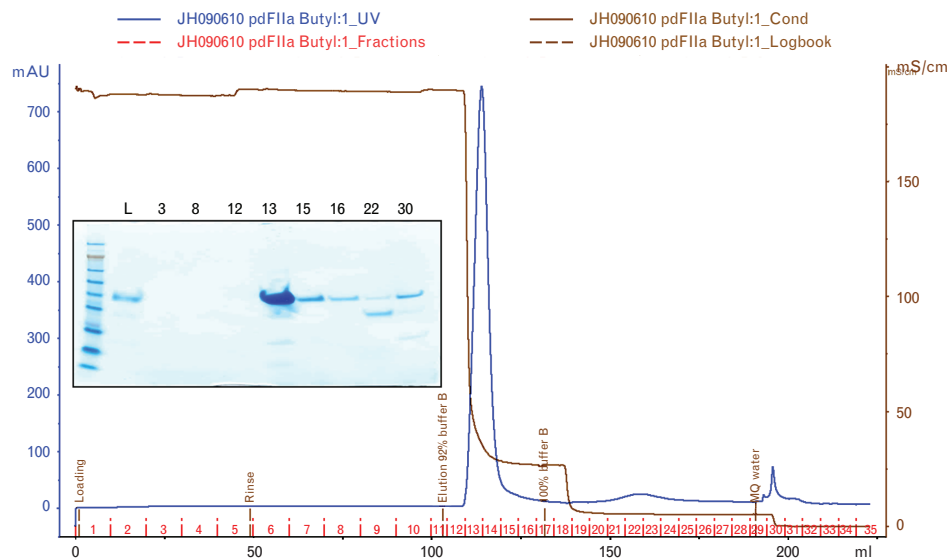
### Blood collection and preparation of a plasma pool

Blood samples were drawn, after approval from the local ethical committee and informed consent, from healthy volunteers employed by AstraZeneca R&D (Mölndal, Sweden). Human whole blood was collected with minimal trauma into polypropylene tubes from Sarstedt (Nümbrecht, Germany) by free flow from a 17-gauge Venflon needle from Becton Dickinson Infusion Therapy AB (Helsingborg, Sweden), to minimize platelet activation (nine volumes blood with one volume 0.11 mol/l trisodium citrate). The first 2 ml of the collected blood was discarded. Analysis of whole blood samples was performed within 4 h after collection. A platelet-poor plasma pool was prepared by collecting blood from 30 healthy volunteers. Plasma was prepared by centrifugation of the citrated blood at 2000g in a swing-out rotor for 20 min at 20°C. The plasma supernatant was put on ice, pooled, aliquoted and stored at  $-80^{\circ}\text{C}$ .

### Washed human platelets

The platelet suspension was prepared on the day of the experiment as previously described [18]. First citrated blood was centrifuged for 15 min at 240g and 37°C, the platelet-rich plasma (PRP) was collected and prostaglandin I<sub>2</sub> (PGI<sub>2</sub>, BD Pharmingen, San Diego, California, USA) was added to a final concentration of 0.8  $\mu\text{mol/l}$ . Remaining red blood cells were pelleted and discarded by a 10-min centrifugation at 125g. The PRP was centrifuged for 10 min at 640g and resuspended in PBS supplemented with 10 mmol/l HEPES, 2.7 mmol/l KCl, 1 mmol/l MgCl<sub>2</sub>, 0.1% D-glucose (37°C) and 0.8  $\mu\text{mol/l}$  PGI<sub>2</sub>. Platelets were pelleted and resuspended in PBS

Fig. 1



Representative example from the preparation of FIIa. The chromatogram shown is from the second chromatography step, described in Materials and Methods. The inserted picture shows a 4–12% SDS-PAGE gel without reducing agent present, showing the loaded material (L) and indicated chromatography fractions. The fractions 13–16 were subsequently pooled and further processed as described in Materials and Methods.

without PGI<sub>2</sub> to  $200 \times 10^9$  cells/l after platelet count in a Sysmex blood cell counter (Kobe, Japan). The washed platelet suspension of  $200 \times 10^9$  cells/l was kept at 4°C for 2 h prior to the experiments for the inhibitory effect of remaining PGI<sub>2</sub> to ablate, and was then supplemented with CaCl<sub>2</sub> to 1 mmol/l.

#### Plate readers

For measurement of the change in optical absorbance at 405 nm, A<sub>405</sub>, both a SpectraMax and a ThermoMax Reader (Molecular Devices Corporation, Sunnyvale, California, USA) were used. Fluorescence was recorded with an Infinite M200 plate reader from Tecan (Männedorf, Switzerland)

#### FIIa amidolytic activity end-point determination

On the day of the experiments, the FIIa samples were rapidly thawed and diluted in Tris buffer [50 mmol/l Tris-HCl pH 7.3 (20°C), 0.19 mol/l NaCl buffer]. Before each assay series, the concentration of active FIIa of the diluted FIIa samples was determined by measuring the rate of hydrolysis from the change in absorbance at 405 nm,  $\Delta A_{405}$ , of 1.5 mmol/l S-2238 in Tris buffer in an end-point assay at room-temperature. The criterion of equivalence was <5% deviation of the rate of hydrolysis. The amidolytic activity of FIIa was found to be unchanged if FIIa was kept stored on ice during the day of the experiment.

#### Hirudin titration to determine the specific activity of FIIa

The specific activity of the purified FIIa was measured by titration of the FIIa with a known concentration of hirudin as previously described [19]. Hirudin inhibits the

active site of  $\alpha$ -FIIa one-to-one ( $K_D$  20 fmol/l [20]) but only weakly inhibits hydrolyzed FIIa,  $\gamma$ -thrombin ( $K_D$  125 nmol/l). Thus,  $\gamma$ -thrombin might give a background activity, which has to be compensated for by subtracting the remaining hydrolytic activity from the data points.

#### Enzyme kinetics for activated factor II with S-2366

The enzymatic constants of FIIa at 37°C were determined from the time course of hydrolysis of the small tripeptide chromogenic substrate, S-2366 in a plate assay. First, 100  $\mu$ l Tris buffer was added to the wells and incubated at 37°C, hereafter 50  $\mu$ l S-2366 diluted in water to 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mmol/l was added. The enzyme reaction was then started by addition of 50  $\mu$ l 2 nmol/l rhFIIa or pdhFIIa, and reading of A<sub>405</sub> (every 10 s for 10 min) was initiated as soon as possible. Before the S-2366 assay, the FIIa dilutions were analyzed according to their activity with S-2238. Six independent series, three for each FIIa preparation, with for all assay series the same S-2366 dilutions, were run. Final assay concentrations were thus 0.5 nmol/l FIIa and 0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mmol/l S-2366. The initial rate,  $v$ ,  $dA_{405}/dt$  was determined by linear regression of the first minute of the reaction. The  $K_M$  and  $V_m$  were subsequently determined by nonlinear regression using the Michaelis–Menten equation:  $v = V_m/[1 + (K_M/\{S-2366\})]$ .

#### FXIII activation kinetics

All reagents for the FXIII activation experiments were from Zedira GmbH, Darmstadt, Germany. The FXIIIa peptide substrate Abz-NE(Cad-Dnp)EQVSPLTLLK has a reported  $K_M$  value of 20  $\mu$ mol/l [21] and can be used in an assay concentration of 50  $\mu$ mol/l at 37°C

providing linear concentration–response curves over a wide range of FXIIIa concentrations [21]. A stock solution of FXIII, 5 mg dissolved in 2.5 ml water, was divided in aliquots and stored dark at  $-20^{\circ}\text{C}$ . Prior to the experiment, the substrate was diluted by addition of  $20\ \mu\text{l}$  to 18 ml FXIII buffer to a final concentration of  $56\ \mu\text{mol/l}$ . FXIII was diluted to  $10\ \text{mg/l}$  with 0.1% BSA and FIIa to  $54\text{--}435\ \text{pmol/l}$ , according to the specific activities of the batches. For the experiment at room temperature ( $24^{\circ}\text{C}$ ), all reagents and plates were kept at the bench, whereas for experiments at  $37^{\circ}\text{C}$ , all reagents and plates were warmed up at  $37^{\circ}\text{C}$ . First,  $90\ \mu\text{l}$  per well of  $56\ \mu\text{mol/l}$  FXIIIa substrate/buffer (final assay concentration  $50\ \mu\text{mol/l}$ ) was added to Greiner 96 Flat Bottom Black Polystyrol GRE96fb-half area plates, followed by  $5\ \mu\text{l}$  of FIIa, diluted to obtain final assay concentrations of 0, 2.7, 5.4, 10.9 and  $21.8\ \text{pmol/l}$ . Finally  $5\ \mu\text{l}$  of FXIII diluted to  $10\ \text{mg/l}$  was added and  $\Delta F\ \lambda_{\text{em}}\ 418\ \text{nm}$  was measured each 15 or 30 s for 25–40 min [Monitor with  $\lambda_{\text{ex}}=313\ \text{nm}$  ( $5\ \text{nm}$ )/ $\lambda_{\text{em}}=418\ \text{nm}$ , ( $20\ \text{nm}$ ); 25 flashes, 1 s shake, top reading].

#### FVIII activation kinetics

The activation of FVIII by FIIa was monitored in a chromogenic assay wherein activated FVIII serves as a cofactor in an ensuing FXa generation step with FIXa as trigger in the presence of phospholipid emulsion and  $\text{CaCl}_2$ . The activity of FXa was measured from the hydrolysis of the chromogenic FXa substrate S-2765.

The study comprised three independent assay series in which activation of FVIII over time was monitored and three independent assay series in which three FIIa concentrations were used at one single activation time, 10 min. For both studies, two reagents denoted Reagents 1 and 2 were prepared and introduced in the FVIII activation phase (Reagent 1) and FX activation phase (Reagent 2), respectively. Reagent 1 comprised FVIII, phospholipid emulsion and  $\text{CaCl}_2$ , whereas Reagent 2 comprised FIXa, FX, phospholipid emulsion and  $\text{CaCl}_2$ . FIXa was added to Reagent 2 just prior to the assay start. Both reagents were dispensed into suitable plastic tubes and stored at  $-70^{\circ}\text{C}$ . One vial of each reagent was thawed in water ( $20\text{--}25^{\circ}\text{C}$ ) prior to each assay series.

In the FVIII activation kinetics study, that is the first study,  $100\ \mu\text{l}$  Reagent 1 was brought to  $37^{\circ}\text{C}$  and then mixed with  $100\ \mu\text{l}$  FIIa or buffer and incubated at  $37^{\circ}\text{C}$  for 0, 1, 1.5, 2, 2.5 and 3 min. Final conditions during FVIII activation were  $0.65\ \text{nmol/l}$  FVIII,  $200\ \text{pmol/l}$  FIIa,  $12.5\ \mu\text{mol/l}$  phospholipid emulsion and  $7.5\ \text{mmol/l}$   $\text{CaCl}_2$ . FIIa activity was quenched at the different time points by addition of  $50\ \mu\text{l}$  hirudin (final concentration  $4\ \text{nmol/l}$ ). Forty microliters was then subsampled into  $100\ \mu\text{l}$  Tris buffer ( $37^{\circ}\text{C}$ ) in another well and after mixing,  $40\ \mu\text{l}$  was again subsampled and transferred to an empty well followed by addition of  $200\ \mu\text{l}$  Reagent 2. Final concentrations during FX activation were  $25\ \text{pmol/l}$  FVIII,

$5.7\ \text{nmol/l}$  FIXa,  $60\ \text{nmol/l}$  FX,  $10.6\ \mu\text{mol/l}$  phospholipid emulsion and  $5.8\ \text{mmol/l}$   $\text{CaCl}_2$ . After 60 s,  $50\ \mu\text{l}$   $3\ \text{mmol/l}$  S-2765 was added, also containing ethylenediaminetetraacetic acid (EDTA) to stop further FX activation, and the activity of formed FXa was determined after 10-min substrate hydrolysis, which was terminated by addition of  $50\ \mu\text{l}$  2% citric acid.

The same assay conditions as used in the first study were used in the second study with the following modifications: the three FIIa concentrations were 80, 125 and  $200\ \text{pmol/l}$  and a fixed FVIII activation time of 2 min was used. Furthermore, the hydrolysis of  $3\ \text{mmol/l}$  S-2765 was extended to 15 min.

In both studies, Reagent 1 and FIIa were kept on ice before addition to the microplate at  $37^{\circ}\text{C}$ . Both pdhFIIa and rhFIIa were assayed at the same time in one microplate, as single replicates at each time point in first study and as duplicates of each dilution in the second study.

#### Factor V activation kinetics

The activation of FV by FIIa was monitored in a chromogenic assay in which activated FV serves as a cofactor in an ensuing FIIa generation step with FXa as trigger in the presence of phospholipid emulsion and calcium ions to initiate the prothrombinase reaction. The activity of FIIa was measured from the hydrolysis of  $3\ \text{mmol/l}$  of the chromogenic FIIa substrate S-2238.

Similar as for FVIII, FV activation comprised two studies: three independent assay series in which activation of FV versus time was monitored; and three independent assay series in which three FIIa concentrations were used at one single activation time; however, in this case the time is 7 min. For both studies, two reagents denoted Reagents A and B were prepared and introduced in the FV activation phase (Reagent A) and FII activation phase (Reagent B), respectively. Reagent A comprised FV and  $\text{CaCl}_2$ , whereas Reagent B comprised FXa, FII, PL and  $\text{CaCl}_2$ . FXa was added to Reagent B just prior to the start of the assay. Both reagents were dispensed into suitable plastic tubes and stored at  $-70^{\circ}\text{C}$ . One vial of each reagent was thawed in water ( $20\text{--}25^{\circ}\text{C}$ ) prior to each assay series.

In the FV activation kinetics experiment, that is in the first study,  $150\ \mu\text{l}$  Reagent A or buffer was brought to  $37^{\circ}\text{C}$  and then mixed with  $100\ \mu\text{l}$  FIIa or buffer and incubated at  $37^{\circ}\text{C}$  up to 10 min. Final conditions during FV activation were  $10.4\ \text{nmol/l}$  FV,  $300\ \text{pmol/l}$  FIIa,  $12.5\ \mu\text{mol/l}$  PL and  $6\ \text{mmol/l}$   $\text{CaCl}_2$ . After each minute, FIIa activity was quenched by addition of  $50\ \mu\text{l}$  hirudin (final concentration  $1.3\ \text{nmol/l}$ ). Forty microliters was then subsampled into  $240\ \mu\text{l}$  Tris buffer ( $37^{\circ}\text{C}$ ) in another well and after mixing,  $40\ \mu\text{l}$  was again subsampled and transferred to an empty well, followed by addition of  $200\ \mu\text{l}$  Reagent B. Final concentrations during FII activation were  $124\ \text{pmol/l}$  FV,  $4.2\ \text{pmol/l}$  FXa,  $200\ \text{nmol/l}$

FII, 10  $\mu\text{mol/l}$  phospholipid emulsion and 6.1 mmol/l  $\text{CaCl}_2$ . After 60 s, 50  $\mu\text{l}$  3 mmol/l S-2238 was added, also containing EDTA to stop further FII activation, and the activity of formed FIIa was determined after 15-min substrate hydrolysis, terminated by addition of 50  $\mu\text{l}$  2% citric acid.

The same assay conditions as used in the first study were used in the second study with the following modifications: the three FIIa concentrations were 120, 200 and 300 pmol/l and a fixed FV activation time of 7 min was used. FIIa used in the FV activation phase was completely inhibited after addition of hirudin and thus did not contribute to FIIa generation in the second phase. Similarly, the low residual concentration of hirudin during the FII activation phase resulted in only a minor lag phase of FIIa generation and did not affect comparisons of formed FIIa.

In both experiments, Reagent A and FIIa were kept on ice before addition to the microplate at 37°C. Both pdhFIIa and rhFIIa were assayed at the same time in one microplate, as single replicates at each time point in the first study and as duplicates of each dilution in the second study.

#### Activation of Protein C by FIIa and thrombomodulin

Activation of Protein C by FIIa in the presence of thrombomodulin was monitored in a chromogenic assay in which APC activity was determined with the substrate S-2366. The study comprised a Protein C activation kinetics study for which three independent assay series were performed and in which activation of Protein C over time was monitored and three independent assay series in which three FIIa concentrations were used at one single activation time, 10 min.

In the Protein C activation study, 100  $\mu\text{l}$  Protein C was incubated with 50  $\mu\text{l}$  FIIa/thrombomodulin for 1–20 min where after 50  $\mu\text{l}$  hirudin was added to quench FIIa activity. One hundred microliters S-2366 (3 mmol/l) was then added and the absorbance was determined after 5-min hydrolysis. Final assay conditions during Protein C activation were 100 nmol/l Protein C, 1.33 nmol/l FIIa and 6.67 nmol/l thrombomodulin. The solution of S-2366 also contained the thrombin inhibitor I-2581, 72  $\mu\text{mol/l}$ , as a safe-guard against any residual FIIa activity. All protein components were stored in Tris-BSA buffer. Both pdhFIIa and rhFIIa were assayed at the same time in one microplate, as single replicates at each time point. To minimize preanalytical variables, the positioning of the FIIa preparations on the microplate was altered between the assay series. Thrombomodulin was freshly diluted with each FIIa dilution for each assay series. For further comparison of the FIIa batches, 100 nmol/l Protein C was activated during 10 min with 0.42, 0.83 and 1.33 nmol/l of FIIa in the presence of 6.67 nmol/l thrombomodulin. FIIa was then quenched with hirudin and the activity of

generated APC determined by hydrolysis of 2.2 mmol/l S-2366 as described above. In these assays, both pdhFIIa and rhFIIa were assayed at the same time in one microplate, as duplicates of each dilution.

#### Interaction of antithrombin and FIIa in presence of excess heparin

The inhibition of FIIa by antithrombin was determined by adding FIIa to antithrombin in the presence of heparin and measuring the initial and steady state residual FIIa activity versus time from hydrolysis of the FIIa substrate S-2238. After preheating all reactants to 37°C, 100  $\mu\text{l}$  FIIa was mixed with an equal volume of a reagent comprising antithrombin, heparin and S-2238 and initial recording was started. Final assay conditions were: FIIa 1 nmol/l, antithrombin 200 nmol/l, heparin 1 IU/ml and S-2238 0.4 mmol/l. Both pdhFIIa and rhFIIa were assayed simultaneously in the same microplate as triplicates with buffer (=blank) and triplicates with antithrombin + heparin. Three independent assay series were run.

#### Determination of the plasma coagulation time induced by FIIa

Plasma coagulation time was investigated by coagulometry using an Amelung coagulometer (Heinrich Amelung GmbH, Lemgo, Germany). Plasma was preincubated (37°C,  $\geq 30$  min) before addition of the reagents to the Amelung KC 10 micro cups: 25  $\mu\text{l}$  saline or FIIa diluted to 4.5–15 nmol/l FIIa, followed by 25  $\mu\text{l}$  citrated plasma, using two samples for each dilution of FIIa. Two experimental series were used. Coagulation time was measured and the coagulation rate (1/coagulation time) plotted against the FIIa concentration and the slopes were calculated by linear regression. The slopes were then used to compare the different FIIa samples.

#### Whole blood coagulation by rotational thromboelastometry

Whole blood coagulation initiated with FIIa was analyzed with rotational thromboelastometry (ROTEM) (TEM International GmbH, Munich, Germany). All solutions and the citrated whole blood were prewarmed to 37°C. Then 290  $\mu\text{l}$  whole blood was mixed with 10  $\mu\text{l}$  FIIa 200 nmol/l and 40  $\mu\text{l}$  62.5 mmol/l  $\text{CaCl}_2$  in saline. The clot formation was followed for 60 min and coagulation time, the clot formation time (CFT) and maximum clot firmness (MCF) were determined. Four independent assay series were run for each FIIa preparation for each of the six blood donors.

#### Washed platelet aggregation induced by FIIa

Aggregation of washed human platelets was monitored at 37°C as the increase in turbidity and used to evaluate the potency of rhFII as described [18]. Light absorption was measured at 650 nm in 150  $\mu\text{l}$  platelet suspension before (R0) and after a 5-min plate-shake (R1). Ten microliters active FII 0.1–10 nmol/l was added and after an

additional 5-min plate shake, light absorption was measured again (R2). Percentage aggregation was calculated from R2-R0. If spontaneous aggregation (R1-R0) was more than 15% of maximal R2-R0, the platelet suspension was not used.

### Data analysis

The data from the plate readers were analyzed with the program Softmax pro 4.8 provided by Molecular Devices Corporation and subsequently transferred to Excel. XLfit4 Excel Add-In Version 4.2.2, GraFit version 5.0.13 (Erithacus, Software Limited) and R (version 2.10.1) from The Foundation for Statistical Computing were used for analysis of the raw data. The CT, CFT and MCF were evaluated from the data from ROTEM using the ROTEM 05 version 2.98 software from TEM International GmbH (Munich, Germany).

### Results

In this article, we report the characterization and comparison of the properties of  $\alpha$ -thrombin (FIIa) prepared from pdhFII and from rhFII. Thrombin of high purity could conveniently be produced by the use of recombinant ecarin as described in Materials and Methods (Fig. 1). For characterization purposes, a range of new methods were developed or adapted. To allow comparison of different FIIa preparations from plasma or recombinant sources, we first determined the specific activity of the batches by determination of the amidolytic activity and by titration with hirudin as described in Material and Methods. The specific activities obtained by these methods were for each FIIa batch used highly similar, 0.84 (pdhFII) and  $0.72 \pm 5\%$  (rhFIIa) respectively, and there was a strict linear relation between the amidolytic activity and the specific activity, suggesting that although a fraction of FIIa was not active, there were negligible amounts of  $\gamma$ -thrombin present, as  $\gamma$ -thrombin hydrolyses the short chromogenic or fluorogenic peptide substrates used but has a much lower affinity to hirudin. All FIIa concentrations were therefore based on the concentration of active thrombin.

### Enzyme properties, $K_M$ and $V_m$ of FIIa for S-2366

We have chosen to determine the enzymatic properties of the thrombin preparations using the chromogenic substrate S-2366 rather than S-2238 because thrombin has a very high affinity toward S-2238 ( $K_M < 10$  nmol/l), which would make it more difficult to obtain an accurate comparison between rhFIIa and pdhFIIa. As the  $K_M$  of S-2366 is approximately 20-times that of the more potent substrate S-2238 [22,23], this allows the use of micromolar substrate concentrations instead of nanomolar making it a more reliable assay. Our results show that  $K_M$  and  $V_m$  values versus S-2366 are very similar for rhFIIa and pdhFIIa. The kinetic constants calculated are listed in Table 1. Statistical analysis of data indicates that there was no significant difference between FIIa prepared from

**Table 1** Michaelis–Menten constants at 37°C for FIIa with S-2366

	$K_M$ ( $\mu$ mol/l)	CV (%)	$V_m$ (arb. units)	CV (%)
rhFIIa	464	5.7	72	3.5
pdhFIIa	435	5.4	72	3.2

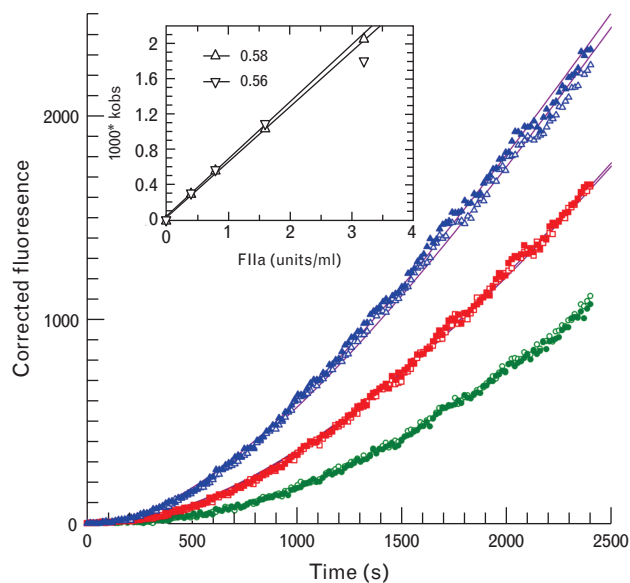
pdhFIIa, plasma derived human FIIa at final 0.5 nmol/l, based on specific activity by hirudin titration; rhFIIa, recombinant human FIIa. Results are from weighted non-linear regression analysis.

the pdhFII or from rhFII in the activity versus the small peptide substrate S-2366. Also, in assays with S-2238, there was no difference between rhFIIa and pdhFIIa (results not shown).

### Activation kinetics of FXIII by FIIa

Activation of FXIII was measured by the activity of the formed FXIIIa on a fluorogenic substrate, Abz-NE(Cad-Dnp)EQVSPILTLLK-OH, over time. In the absence of added FXIII, the fluorogenic substrate was not cleaved by added FIIa. However, all tested preparations of human FXIII contained approximately 10% of FXIIIa, giving rise to a linear increase in fluorescence in the absence of added FIIa (data not shown). In the kinetic assay, each time curve was therefore corrected by subtraction of the preexisting FXIIIa from the sample curves (Fig. 2). To be able to differentiate between rhFIIa and pdhFIIa, the proteins were diluted according to their specific activity so that final assay concentrations of 0, 2.7, 5.4, 10.9 and 21.8 pmol/l for both rhFIIa and pdhFIIa were obtained. The assay was run both at room temperature (results not shown) and at 37°C (Fig. 2). At room temperature, steady state was only reached for the highest FIIa concentrations. As shown in Fig. 2, the instrumental noise increased in the later phase of the time curves. At all FIIa concentrations, all FXIII will eventually be converted by FIIa into FXIIIa, giving rise to a steady state rate, the linear phase, which would be identical for each curve, assuming that the generated FXIIIa is stable during the duration of the assay. From Fig. 2, it is clear that with the lowest FIIa concentration, 2.72 pmol/l, the steady state is not reached during the assay time. However, it was still possible to apply non-linear curve fitting to the data points according to  $V_s * t - V_s * (1 - \exp(-k_{obs} * t/1000)) / (k_{obs}/1000)$  with the final steady state  $V_s = 1.52$  for the data at 37°C, obtained from linear regression of the final phase. In the insert in Fig. 2, the apparent rate constant,  $k_{obs}$ , is shown to be linearly related to the FIIa concentration, indicating that initial-rate conditions are fulfilled. Evaluation of the data at room temperature confirmed the similarity of rhFIIa and pdhFIIa (not shown). At room temperature, the rate of FXIIIa with the fluorogenic substrate was 0.75, which is approximately half that at 37°C. The initial rates,  $k_{obs}/(FIIa)$ , at room temperature were 0.34 and 0.32 for rhFIIa and pdhFIIa, respectively. At 37°C, the corresponding values were 0.58 and 0.56.

Fig. 2



Generation of FXIII by FIIa at 37°C. Example of experimental time curves at 37°C corrected for FIIa = 0: recombinant human FIIa (open symbols) and plasma-derived human FIIa (closed symbols): inverted triangles: 21.8; triangles: 10.9; squares: 5.4 and rhomboids: 2.7 pmol/l. Insert shows  $k_{obs}$  values from nonlinear regression for mean values of three experiments.

### Activation of FVIII

FVIIIa is a very unstable protein [24], which was apparent from the decline in activity at longer activation times (Fig. 3) and only the data collected during the first 3 min were used to calculate the slope by linear regression in the plot of mean FVIII activation as a function of activation time with FIIa (Fig. 3a). Based on slope ratios, rhFII was slightly more (8%) active than pdhFII.

### Activation of FV

As shown in Fig. 4, FV is more easily activated by rhFIIa than by pdhFIIa. Moreover, similar results were obtained if FV was activated at different FIIa concentrations (Fig. 4). Although the conditions for steady state enzyme kinetics were apparently not fulfilled, *id est* a linear relation between the added FIIa concentration and the response, our results suggest that the rhFIIa was more active than pdhFIIa. The increase in activity during the course of the reaction is because of the generation of FIIa in the prothrombinase reaction, generating more FVa. The difference between rhFIIa and pdhFIIa ranged from 20 to 31% depending on FIIa concentration in the experiment.

### Activation of Protein C by the complex of FIIa and thrombomodulin

As shown in Fig. 5, the generation of APC by the complex of FIIa and thrombomodulin was linear during the first 10 min, indicating that the concentration of Protein C is

high enough to neglect the consumption during activation, and that the generated APC is stable during the first 10 min. Therefore, a 10-min incubation of Protein C with the different FIIa concentrations has been chosen for the conditions used in Fig. 5. The results shown in Fig. 5 indicate that Protein C was more easily activated by rhFIIa than by pdhFIIa, although the difference was small (8%). The results from additionally tested FIIa concentrations were in agreement with the results shown in Fig. 5 (results not shown).

### Inhibition of FIIa by antithrombin in presence of heparin

Our results indicate that the inhibitory effect of antithrombin facilitated by heparin is more pronounced for rhFIIa than for pdhFIIa, suggesting a slight increase in association rate constant. In the initial rate phase, the rhFIIa activity is reduced to 49% of the original as a result from inhibition with antithrombin, and the pdhFIIa activity is reduced to 68% of the original activity. The inhibitory effect of antithrombin and heparin (Fig. 6 and Table 2), is more pronounced for rhFIIa than for pdhFIIa, although similar  $k_{obs}$  values were obtained. Compared with pdhFIIa, the inhibition of rhFIIa by antithrombin in the presence of heparin was statistically significant and more efficient ( $P < 0.01$ ). The difference in inhibition at steady state was 10%.

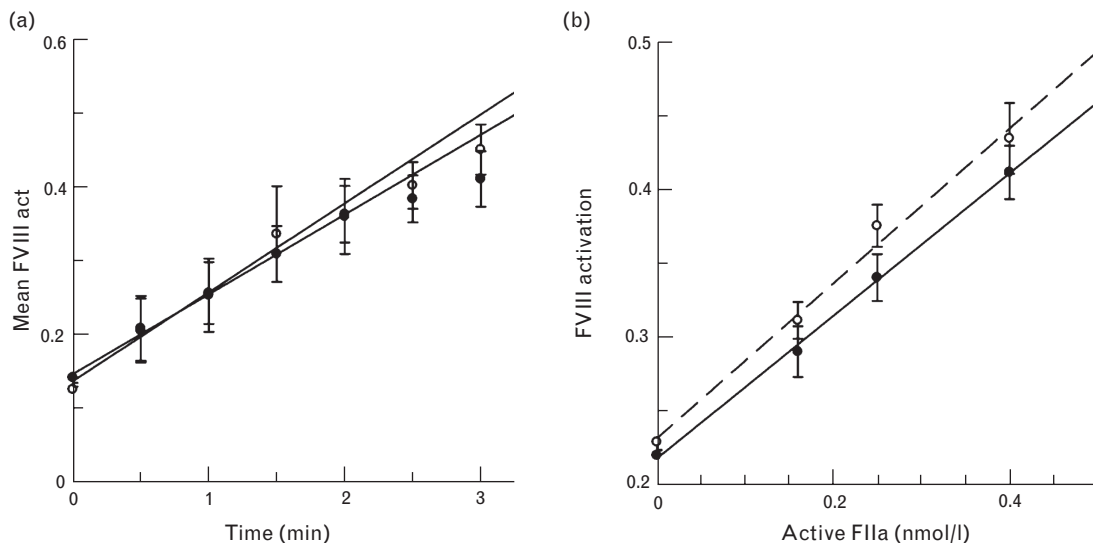
### Plasma coagulation induced by FIIa measured by coagulometry

After addition of rhFIIa or pdhFIIa to citrated plasma containing its natural substrate fibrinogen, the coagulation time was measured by coagulometry. In this assay, coagulation depends primarily on the thrombin added, as calcium-dependent generation of additional coagulation factors (*i.e.* thrombin propagation) will not occur in the absence of free calcium ions. A linear relation was found between the coagulation rate =  $1/\text{coagulation time}$  versus the active FIIa concentration, as shown in Fig. 7. Statistical analyses of data showed a small but significant ( $P < 0.01$ ) difference in activity; comparing slopes, rhFIIa was approximately 6% more potent than pdhFIIa.

### FIIa-activated whole blood coagulation measured by rotational thromboelastometry

In the absence of added FIIa, the coagulation time by recalcification was more than 10 min, whereas coagulation time decreased to approximately 2 min in the presence of 0.25 mg/l FIIa corresponding to 6.8 nmol FIIa/l blood. The mean values  $\pm$  standard deviations are collected in Table 3 for buffer, rhFIIa and pdhFIIa. The same relation was found for CFT, related to the rate of fibrin polymerization and MCF, a measure for clot elasticity dependent on fibrinogen, FXIIIa and platelets. There was no significant difference between rhFIIa and pdhFIIa in CFT or MCF, whereas the CT for rhFIIa was 20% shorter than for pdhFIIa.

Fig. 3



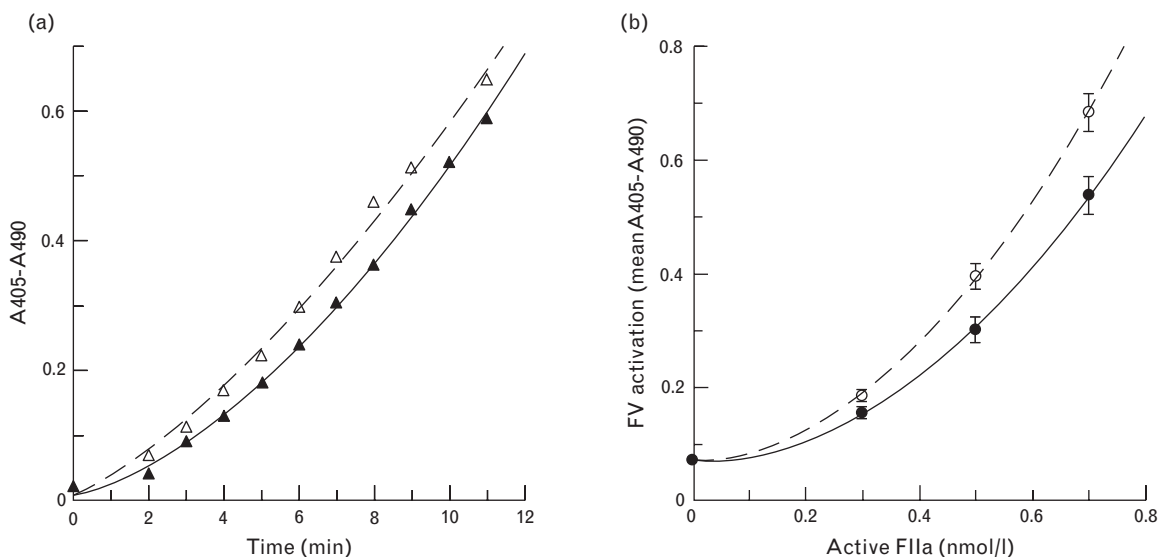
(a) FVIII activation kinetics. Linear regression is done to the first 2 min of FVIII activation. Data values are mean values  $\pm$  standard deviation for the three series. Recombinant human FIIa (rhFIIa, open symbols) and plasma-derived human FIIa (pdhFIIa, closed symbols). The S-2238 activity of rhFIIa and pdhFIIa prior to the three FVIII activation kinetic series was analyzed as triplicates. The difference in S-2238 activity of the rhFIIa and pdhFIIa dilutions was found to be less than 5% for all six assay series. (b) FVIII activation at different FIIa concentrations. FVIII was incubated with FIIa for 2 min before stop with hirudin. Data values are mean values  $\pm$  standard deviation for the three series. By linear regression ( $R^2 = 0.999$ ) for rhFIIa (open symbols), a slope was calculated of  $0.523 \pm 0.014$  and for pdhFIIa (closed symbols) of  $0.481 \pm 0.005 \Delta A^*/\text{nmol}$ .

**FIIa-induced platelet aggregation**

Platelets prepared from six donors were tested for FIIa-induced aggregation. There was a large variation in the concentration of FIIa needed to obtain half-maximal

aggregation ( $EC_{50}$ ) depending on the donor (Table 4), but median  $EC_{50}$  values were comparable for rhFIIa and pdhFIIa (89 and 92 pmol/l, respectively), and the difference was not statistically significant.

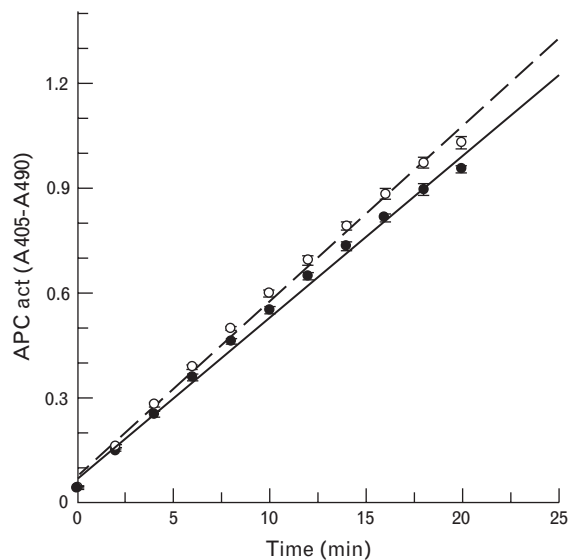
Fig. 4



(a) FV activation kinetics. Mean values for the three series. A subsample was taken each minute from the activation solution, containing 0.3 nmol/l FIIa, and stopped with hirudin: recombinant human FIIa (rhFIIa, open symbols) and plasma-derived human FIIa (pdhFIIa, closed symbols). (b) FV activation at different FIIa concentrations. FV was activated by FIIa for 7 min before stopping with hirudin and subsampling; rhFIIa (open symbols) and pdhFIIa (closed symbols). Prior to each experiment, the used dilutions for all series were compared regarding the activity with S-2238, and analyzed as triplicates. Both FIIa preparations had similar activity versus S-2238 prior to the FV activation experiment, with a variation of  $<5\%$ .



Fig. 5

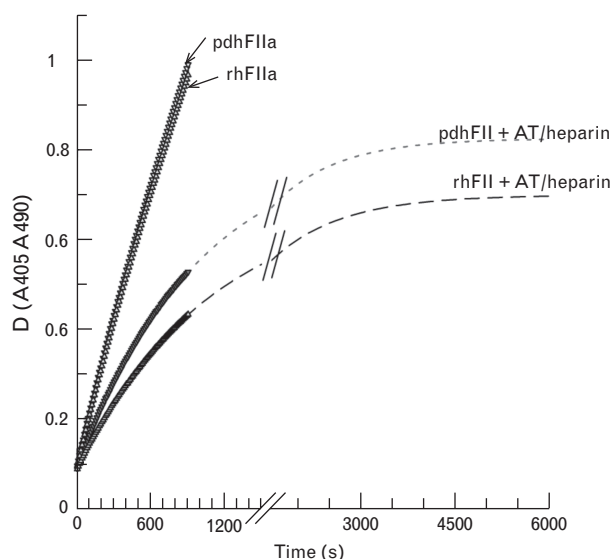


Protein C activation kinetics. Mean activated protein C activity  $\pm$  standard deviation with hydrolysis of S-2366 from the increase in A405 from all three assay series at each time point for recombinant human FIIa (open circles) and plasma-derived human FIIa (closed circles) generated with 1.33 nmol/l FIIa/6 nmol/l thrombomodulin.

## Discussion

A key question in producing recombinant human prothrombin, rhFII, is whether this proenzyme will be converted into an active enzyme, rhFIIa, with the same

Fig. 6



Kinetics of inactivation of FIIa by antithrombin + heparin. The increase in A405 in time for 1 nmol/l FIIa with 200 nmol/l antithrombin and 1 IU/ml heparin and 0.4 mmol/l S-2238. Curve fitting (dashed lines) to the initial progress phase of inhibition, mean values of the time curves/split axis.

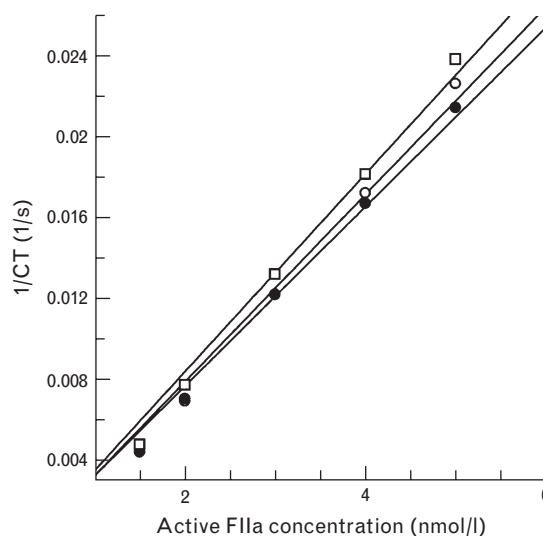
Table 2 Initial FIIa activity in buffer or in presence of antithrombin + heparin

Initial rate	Buffer (mAbs/min)	Buffer (CV%)	antithrombin + heparin (mAbs/min)	antithrombin + heparin (CV%)	$k_{obs}$ ( $s^{-1}$ )
rhFIIa	71	3.1%	35	0.6%	0.0009
pdhFIIa	71	1.8%	48	5.3%	0.0010

pdhFIIa, plasma derived human FIIa; rhFIIa, recombinant human FIIa Mean values for initial rate (first 60 s) from three independent series. Both rhFIIa (recombinant human FIIa) and pdhFIIa (plasma-derived human FIIa) were included in each assay series. Final conc.: 200 nmol/l antithrombin, 1 IU/ml heparin, 1 nmol/l FIIa, 0.4 mmol/l S-2238.

enzymatic properties as the endogenously generated thrombin. In comparing the enzymatic activities of rhFIIa and pdhFIIa, a number of known substrates need to be taken into account. We have studied how FIIa prepared and isolated from either pdhFII or rhFII using a novel method affected the different thrombin substrates, both small chromogenic substrates as well as the isolated proteins FV, FVIII, FXIII and Protein C. We have also compared the activity of rhFIIa and pdhFIIa in complex systems such as washed platelets, plasma and whole blood. The platelet aggregation assay showed that both FIIa activated the PAR receptor with the same activity. With some of the zymogen substrates, FV and Protein C, there was a small but statistically significant higher activity of rhFIIa compared with pdhFIIa. However, for most assays, two to three preparations of thrombin have been compared and the small differences observed may be a result of differences inherent to these

Fig. 7



Plasma coagulation induced by FIIa. Plasma coagulation time was determined by coagulometry. The coagulation rate = 1/ coagulation time is plotted versus the concentration of active FIIa and the lines were drawn by linear regression: recombinant human FIIa (open circles) and plasma-derived human FIIa (closed circles) and recombinant human FIIa batch rhFIIa 070816HM (open squares) from a previous study were included for comparison.

**Table 3 FIIa-activated whole blood coagulation measured by ROTEM**

Recalcification	Whole blood	+rhFIIa 6.8 nmol/l blood	+pdhFIIa 6.8 nmol/l blood
Coagulation time	734 ± 72 s	137 ± 28 s	172 ± 40 s
CFT	387 ± 91 s	143 ± 45 s	146 ± 26 s
MCF	43 ± 4.3 mm	66 ± 6.1 mm	65 ± 5.4 mm

pdhFIIa, plasma-derived human FIIa; rhFIIa, recombinant human FIIa; ROTEM, rotational thromboelastometry. ROTEM analysis was performed as described in Material and Methods. Mean values ± SD are shown in the table.

preparations or reflect small errors in the determination of protein concentration. We did not observe any differences in enzyme kinetic properties. Therefore, we conclude that the small difference observed in the FIIa activity, related to the protease activity, is unlikely to be of any biological relevance. This is further supported by the fact that the more complex assays using platelets, plasma or whole blood suggested that rhFIIa and pdhFIIa gave a similar coagulation process.

The only protein for which we observed a substantial difference in interacting with pdhFIIa or rhFIIa was antithrombin. Inhibition of rhFIIa by antithrombin in the presence of heparin shows a significantly more efficient inhibition of rhFIIa compared with pdhFIIa. Interestingly, a further interaction site for antithrombin is located in exosite II, a different part of thrombin than the active site [8].

In preparing FIIa from FII, it was not possible to obtain thrombin with the same specific activity each time. These differences could not be explained by formation of  $\gamma$ -FIIa or by other impurities (Fig. 1). To be able to compare the FIIa preparations, we have therefore adjusted for the differences in specific activity in our experiments by ways that depend on access to a functional active site (calibration with a chromogenic substrate and titration with hirudin). The differences adjusted for might, in that case, be related to a conformational change of the active site, without effect on exosite II. It is possible that if heparin is bound to exosite II, the native state of the active site recovers, which might explain the observed differences between rhFIIa and pdhFIIa in the interaction with antithrombin. However,

**Table 4 EC<sub>50</sub> for active FIIa for washed platelet aggregation**

Donor EC <sub>50</sub> (pmol/l)	rhFIIa	pdhFIIa
M245	94	82
M259	109	102
M375	47	37
K112	173	192
M241	52	69
K246	59	67
Median	77	76
Mean	89	92

EC<sub>50</sub> = concentration of FIIa needed to obtain half-maximal aggregation at 37°C; pdhFIIa, plasma-derived human FIIa; rhFIIa, recombinant human FIIa.

we have not further explored this assumption. No obvious reason for a difference in conformation or access to the active site has been determined, but we have observed that the procedure in changing the buffer of the purified thrombin did affect the specific activity as rapid changes in buffer conditions were not well tolerated. Glycan analysis of rhFII used to produce the rhFIIa did not reveal any major differences to pdhFII (not shown). In the process of generating FIIa from rhFII or pdhFII by ecarin, we did not observe any major differences that could be observed by SDS-PAGE or by a chromogenic activity assay; FIIa was generated approximately with the same speed and the same observed intermediates. In addition, it has previously been shown that rhFII equivalent to the material we used for generating rhFIIa behaved similar as pdhFII in prothrombin time (PT) and activated partial thromboplastin time (APTT) assays [25,26].

Recombinant FIIa produced from pre-FII (FII lacking the Gla-domain,) by a method different from ours, has previously been characterized [27]. Also in this case, the obtained FIIa was very similar to pdhFIIa, although the FIIa activity on FV, FVIII, FXIII and Protein C was not evaluated [27].

We have chosen to only compare inhibition of FIIa by the serpin antithrombin, its principal inhibitor in plasma, thereby assuming that also other serpins are similarly affected. In this work, we have not aimed to investigate specifically the interaction of FIIa with all possible isolated components known to be modulated, as many play a role in hemostasis. With the complex assays used, washed platelet aggregation involving the PAR receptors [28], plasma coagulation and whole blood coagulation measured by ROTEM, it is unlikely that large differences involving some of these factors playing a role in haemostasis would have been missed, although this possibility cannot be completely excluded. In the plasma and whole blood coagulation assays, not only activation is studied but also the effect of many of the irreversible inhibitors, for example the serpins and  $\alpha$ -macroglobulin. In all the complex assays used in the work presented here, rhFIIa and pdhFIIa were similar. We therefore conclude that rhFIIa formed from in-vivo administration of rhFII is likely to have a biological activity comparable with FIIa generated from endogenous FII.

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## Conflicts of interest

The authors declare no conflict of interest.

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