Prevention of the influence of Fibrin and α_2-Macroglobulin in the continuous measurement of the thrombin potential

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Prevention of the Influence of Fibrin and α_2-Macroglobulin in the Continuous Measurement of the Thrombin Potential: Implications for an Endpoint Determination of the Optical Density

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Abstract

We proposed the endogenous thrombin potential (ETP) as an overall function test of the coagulation system. We recently introduced a routine test which requires defibrinated plasma. In order to develop an assay in which the ETP-value can be directly obtained by measuring the optical density, we investigated two methods to inhibit fibrinogen clottability and to inactivate α_2-macroglobulin. The first method makes use of hydroxylamine to inactivate α_2-macroglobulin and H-Gly-Pro-Arg-Pro-OH to inhibit fibrin polymerization. At pH 7.35, plasma incubated with 25 mM hydroxylamine and 1.5 mg/mL H-Gly-Pro-Arg-Pro-OH for 5 minutes at 37°C resulted in a reduced endlevel of the amidolytic activity on small chromogenic substrates. The second method uses a metalloprotease purified from *Crotalus basiliscus* to remove α_2-macroglobulin from plasma in combination with H-Gly-Pro-Arg-Pro-OH. Herein plasma is incubated with 3.5 μM protease during 15 minutes at 37°C in the presence of 1 mg/mL polymerization inhibitor. The enzymatic method results in a zero endlevel of the amidolytic activity and this would imply that measurement of the ETP is reduced to an endpoint determination of the optical density. We show that the endpoint determination of the optical density correlates well with the calculated ETP in plasmas with different degrees of anticoagulation. © 1998 Elsevier Science Ltd.

Key Words: Fibrin polymerization inhibitor; Hydroxylamine; α_2-macroglobulin; Snake venom; Thrombin generation assay; Thrombin potential

The time course of thrombin developing in a triggered plasma sample can be continuously monitored in a spectrophotometer. A slow reacting but specific chromogenic substrate is added and the conditions are chosen such that the velocity of product formation is proportional to the concentration of the enzyme present. The first derivative of the optical density signal renders the time course of the amidolytic activity in the sample. This method allows the measurement of the area under the thrombin generation curve (endogenous thrombin potential, ETP) without subsampling [1]. In plasma, thrombin is mainly inhibited by antithrombin III (AT-III) and α_2-macroglobulin (α_2M) [2]. The reaction of free thrombin with α_2M yields a complex (α_2M-IIa) that has no known biological activity but retains amidolytic activity on small chromogenic substrates. The free thrombin curve

Abbreviations: ETP, endogenous thrombin potential.

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therefore is not identical to the amidolytic activity curve and can only be obtained by mathematical processing of the signal [3,4]. We tried to circumvent this problem by synthesizing substrates that, like the biological substrates, were too bulky to be hydrolyzed by α_2M-IIa. Neither the introduction of space demanding groups nor extending binding interactions with thrombin (P5-P′3 [5]) and the fibrinogen recognition exosite [6,7] led to full selectivity toward free thrombin only, even in a 18 amino acid substrate [8]. Alternatively we tried to inactivate α_2M before triggering the plasma [9].

Human α_2M is a plasma glycoprotein that functions as an unspecific protease inhibitor [10]. It consists of four equal subunits of 180 kDa held together by disulfide bridges. Each subunit contains a protease sensitive region, the so-called bait region. When a protease splits a peptide bond in this region, α_2M undergoes a conformational change which makes it trap the protease [11]. The hydrolysis exposes a nucleophile-sensitive β-cysteinyl-γ-glutamyl thiol ester bond, which reacts with ϵ-amino groups of lysine residues of the protease.

In this article we describe two methods to inactivate α_2M in human plasma exploiting its inhibition mechanism. First, native α_2M is sensitive to small nucleophiles such as methylamine (CH_3-NH_2) [12–14]. The methylamine-induced thiol ester cleavage results in a conformational change of α_2M in which it cannot bind a protease. Denaturation of α_2M by methylamine is not suitable in plasma, due to its high basicity. Hydroxylamine (HO-NH_2), however, is a weak base and extremely useful to aminolyse thiol esters [15] under neutral conditions. Protease binding capacity of hydroxylamine-treated α_2M is significantly reduced but its applicability in plasma was not studied [16].

Second, we describe an enzymatic method to inactivate α_2M by means of a protease from a snake venom. From the literature it is known that several snake venom proteases are inhibited by α_2M [17, 18]. In vivo it is important to neutralize venom proteolytic activity to minimize hemorrhagic effects after snake envenomation. Recently, the isolation and purification of an α_2M-specific metalloprotease from *Crotalus basiliscus* has been published by Svoboda et al. [19]. This enzyme has no thrombin-like activity and does not activate factor X and plasminogen.

In this article, we describe the application of hydroxylamine and the metalloprotease in the continuous thrombin generation assay to inactivate α_2M. The enzymatic method results in a zero endlevel of the amidolytic activity on small chromogenic substrates. This implies that measuring the thrombin potential is simplified to an endpoint determination of the optical density.

1. Materials and Methods

1.1. Reagents

The chromogenic substrate used in the continuous thrombin generation experiments was 2-(methylsulfonyl)ethyloxycarbonyl-valyl-arginine *p*-nitroanilide monohydrochloride (Msc-Val-Arg-pNA) [8]. H-D-Phe-Pip-Arg-pNA (S2238) was used as substrate to measure thrombin activity in subsampling experiments. The substrates were dissolved in distilled water. The concentration was determined at 316 nm using a molar extinction coefficient of 12,500 $Lmol^{-1}cm^{-1}$. Stock solutions were stored in the dark at 4°C. The fibrin polymerization inhibitor H-Gly-Pro-Arg-Pro-OH (GPRP, Pefabloc® FG) was obtained from Pentapharm (Basel, Switzerland). Heparin (4th International Standard Heparin) was obtained from the National Institute for Biological Standards and Control (London, UK). Hydroxylamine was a 40% solution in water and diluted in buffer A and was purchased from Fluka (Büchs, Switzerland). Small unilamellar phospholipid vesicles were prepared as described by Rosing et al. [20]. The vesicles composed of a mixture (on molar basis) of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) / 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) 80/20. The phospholipids were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Buffer A: 20 mM hepes-NaOH, 0.15 M NaCl, pH 7.35 containing 0.5 g/L bovine serum albumin (essentially fatty acid free; Sigma, Bornem, Belgium). Buffer B: buffer A containing additional EDTA (20 mM), pH 7.90.

1.2. Plasma

Plasma was prepared by collecting nine parts of blood (from 10 healthy volunteers) on one part of 0.13 M trisodium citrate. Following centrifugation at 900*g* (15°C, 10 minutes) and 10,000*g* (15°C, 10 minutes) the obtained plasma was pooled and centrifuged at 4°C for 1 hour at 23,000*g*; this plasma

was aliquoted and stored at −80°C. Plasma was defibrinated by mixing an aliquot of plasma with 1/50 volume of 50 U/mL of Arwin® (Knoll; Ludwigshafen, Germany), letting a clot form for 10 minutes at 37°C. The fibrin clot was removed by winding it on a plastic spatula.

1.3. Proteins

Recombinant human tissue factor (180 ng/mL) (Dade; Düdingen, Switzerland) was used as a trigger for coagulation. Purified α_2M inactivating protease (protease A [19]) from the venom of *Crotalus basiliscus* was a gift of Drs. M. Janssen and M. Gempeler (Pentapharm; Basel, Switzerland). A stock solution was prepared by dissolving 1.488 mg of lyophilized protein in 1 mL distilled water, aliquots of 100 μL were stored at −80°C. Plasma was incubated with 1/15 volume of protease A for 15 minutes at 37°C.

1.4. Manual Determination of Thrombin Generation in Plasma

This method is described in full detail by Hemker et al. [3]. Briefly, to 240 μL plasma were added 40

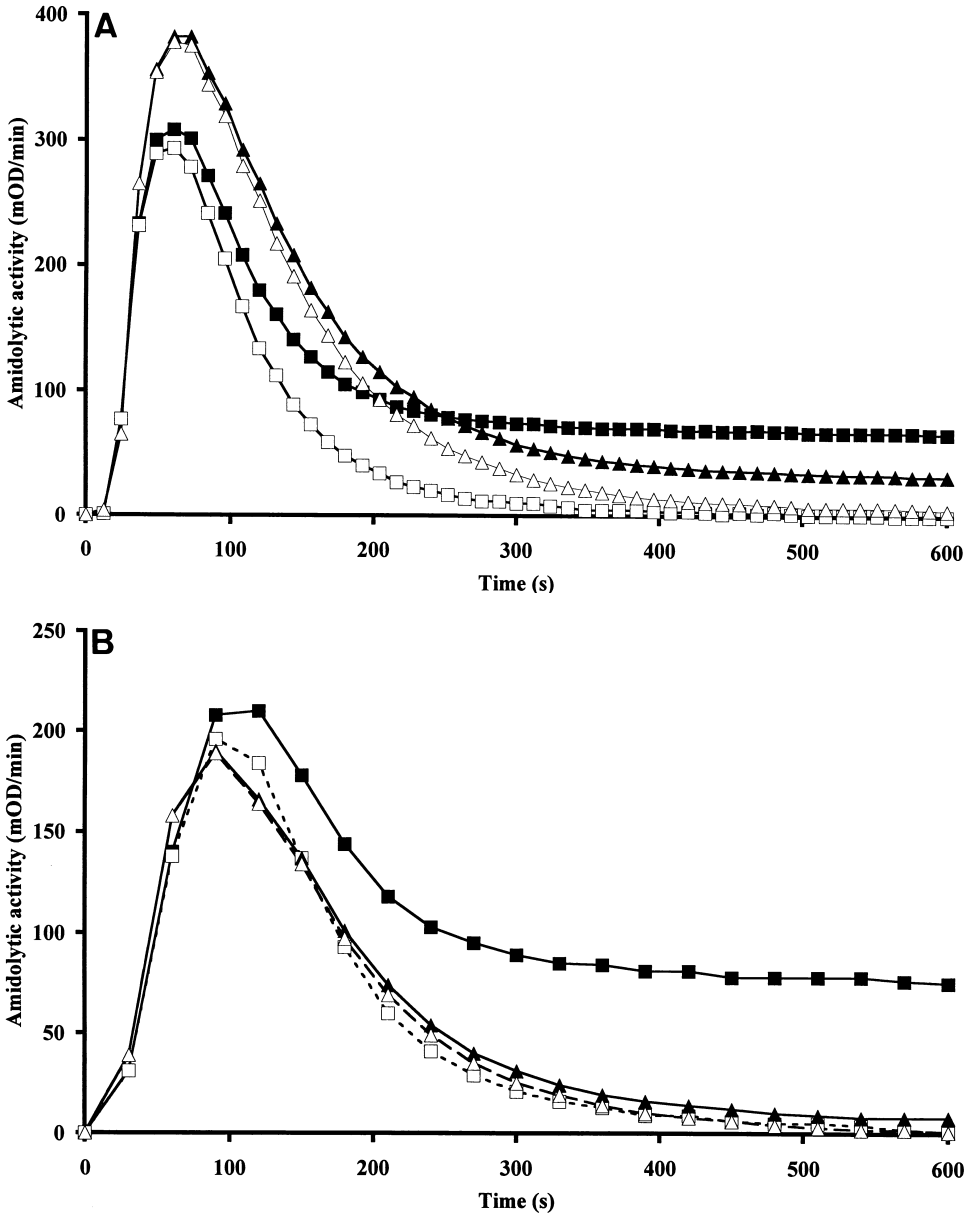

Fig. 1. Thrombin generation curves obtained by subsampling. (A) The influence of 25 mM HO-NH_2 on thrombin generation. (Filled squares, measured amidolytic activity curve (control); open squares, calculated free thrombin curve (control); filled triangles, measured amidolytic activity curve (25 mM HO-NH_2); open triangles, calculated free thrombin curve (25 mM HO-NH_2). (B) The influence of 3.5 μM protease A on thrombin generation. (Filled squares, measured amidolytic activity curve (control); open squares, calculated free thrombin curve (control); filled triangles, measured amidolytic activity curve (3.5 μM protease A); open triangles, calculated free thrombin curve (3.5 μM protease A). The curves shown in A were measured in plasma triggered with a tissue factor concentration of 6 ng/mL. Every 12 seconds a sample was drawn to measure thrombin activity. At high thrombin activity the reaction was stopped after 30 seconds to 1 minute, to prevent exhaustion of the substrate. Curves in B were measured as described in Materials and Methods.

Table 1. Inhibition of the endogenous thrombin potential by international standard heparin in defibrinated and non-defibrinated plasma

Heparin ISH U/mL	Defibrinated plasma Calculated from curve control (25 mM HO-NH2) Inhibition (%)	Non-defibrinated plasma	
		Calculated from curve protease A/GPRP Inhibition (%)	Calculated from curve HO-NH2/GPRP Inhibition (%)
0	0 (0)	0	0
0.2	18.8 (23.6)	20.1	8.1
0.3	30.2 (30.6)	28.8	16.4
0.5	51.2 (51.7)	49.4	29.4
0.8	69.2 (72.1)	66.7	44.9
0.1	72 (79.4)	73.3	52.9
0.125	80.2 (86.1)	78	59.9

	Non-defibrinated plasma	
	Measured as endpoint control Inhibition (%)	Measured as endpoint protease A/GPRP Inhibition (%)
0	0	0
0.2	18.7	22.5
0.3	30.2	30.5
0.5	51.5	51.6
0.8	69.2	67.5
0.1	71.9	71.6
0.125	80.1	76.7

HO-NH_2/GPRP: plasma incubated with hydroxylamine (25 mM) and polymerization inhibitor H-Gly-Pro-Arg-Pro-OH (1.5 mg/mL), protease A/GPRP: plasma incubated with protease A (3.5 μM) and polymerization inhibitor H-Gly-Pro-Arg-Pro-OH (1.0 mg/mL).
The inhibition of the ETP by heparin in the presence of hydroxylamine (25 mM) in defibrinated plasma is given by the values between brackets.

μL buffer A (containing the substance to be tested) followed by 20 μL phospholipid vesicles DOPC/DOPS 80/20 (20 μM). Thrombin generation was started at zero time by adding 60 μL tissue factor (1/40) in 0.1 M $CaCl_2$. At regular time intervals samples of 10 μL were subsampled into 490 μL buffer B containing S2238 (200 μM) to determine thrombin activity. After 2 minutes the reaction was stopped by adding 300 μL 1M citric acid. The optical density was read at 405 nm and the amidolytic activity was calculated.

1.5. Continuous Registration of Thrombin Generation Curves

The continuous thrombin assay was adapted to a Cobas Fara laboratory automaton as described by Wielders et al. [21]. In this study the following conditions were used: 80 μL plasma sample (75 μL plasma and 5 μL buffer A containing the substances to be tested) and 20 μL of hydroxylamine (150 mM)/GPRP (9 mg/mL) or protease A (0.40 mg/mL)/GPRP (6 mg/mL) were pipetted into a cuvette ring and incubated at 37°C for 5 and 15 minutes, respectively. Then 20 μL of start reagent (10 μL tissue factor (undiluted), 5 μL 0.4 M $CaCl_2$ and 5 μL 12 mM Msc-Val-Arg-pNA) was added. The change in optical density was monitored at 405 nm every 30 seconds for 15 minutes.

2. Results and Discussion

The first purpose of this study was to determine whether hydroxylamine or protease A were able to inactivate α_2M in plasma to reduce the residual amidolytic activity of the α_2M-IIa complex and to test their applicability in the continuous thrombin assay, and thereby to check any influence on thrombin generation and inhibition processes. A further goal was to evaluate whether hydroxylamine or protease A could be used in non-defibrin-

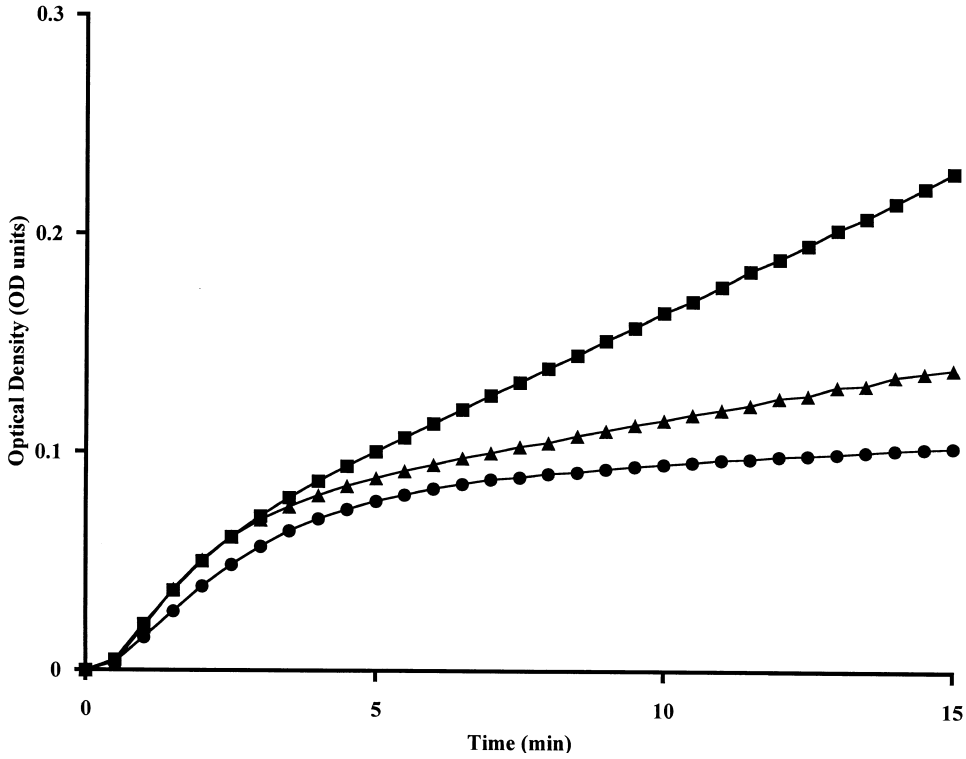

Fig. 2. Optical density curves measured in defibrinated plasma. (Filled squares, control; filled triangles, 25 mM HO-NH_2; filled circles, 3.5 μM protease A).

ated plasma, in this way making the time consuming defibrination of plasma redundant.

Plasma was incubated with hydroxylamine (25 mM, 5 minutes at 37°C) and a significant reduction of the residual amidolytic activity of α_2M-IIa was observed (Figure 1A). The interaction between hydroxylamine and α_2M was studied by Lambin et al. [16] in a purified system. They found that hydroxylamine-treated α_2M partially retained its activity. However, complex formation between thrombin and α_2M was not prevented by hydroxylamine as observed by the reduced but persistent amidolytic activity. Increasing the hydroxylamine concentration (100 mM) or the incubation time (2 hours) did result in a prolonged lag phase and alterations of the thrombin peak instead in a zero amidolytic endlevel.

Hydroxylamine, however, increases the half-life of thrombin in plasma since it disturbs the interaction of AT-III with thrombin. This results in a higher thrombin peak compared to the control plasma (Figure 1A). AT-III acts as a pseudosubstrate for thrombin. The arginine393 residue at P1 of the reactive-site loop interacts with the active site of thrombin to meet its primary specificity [22]. The formed intermediate, either tetrahedral [23] or an acyl-enzyme [24,25], can be dissociated in the presence of nucleophiles [26]. Fish and Björk [27] described that incubation of the thrombin-AT III complex with 2 M hydroxylamine resulted after 30 minutes in 15% to 20% free active thrombin and reactive-site-loop-cleaved AT-III. We found that AT-III activity in the presence of heparin is not influenced by hydroxylamine (Table 1). The bridging of AT-III to thrombin by heparin creates additional binding sites that enhance the stability of the AT-III thrombin complex.

The optical density curve measured in hydroxylamine treated plasma is shown in Figure 2 and differs only in a reduction of the amidolytic endlevel from the control plasma.

When plasma was incubated with the metalloprotease purified from the venom of *Crotalus basiliscus* (protease A) [19], the free thrombin curve was obtained directly (Figure 1B) (this was also observed by Hemker et al. [3] in α_2M-deficient plasma obtained by immunodepletion). Protease A titrates α_2M from plasma without disturbing thrombin generation or AT-III mediated inhibition of thrombin, and the measured thrombin generation curve overlaps the calculated free thrombin curve. The optical density curve obtained from pro-

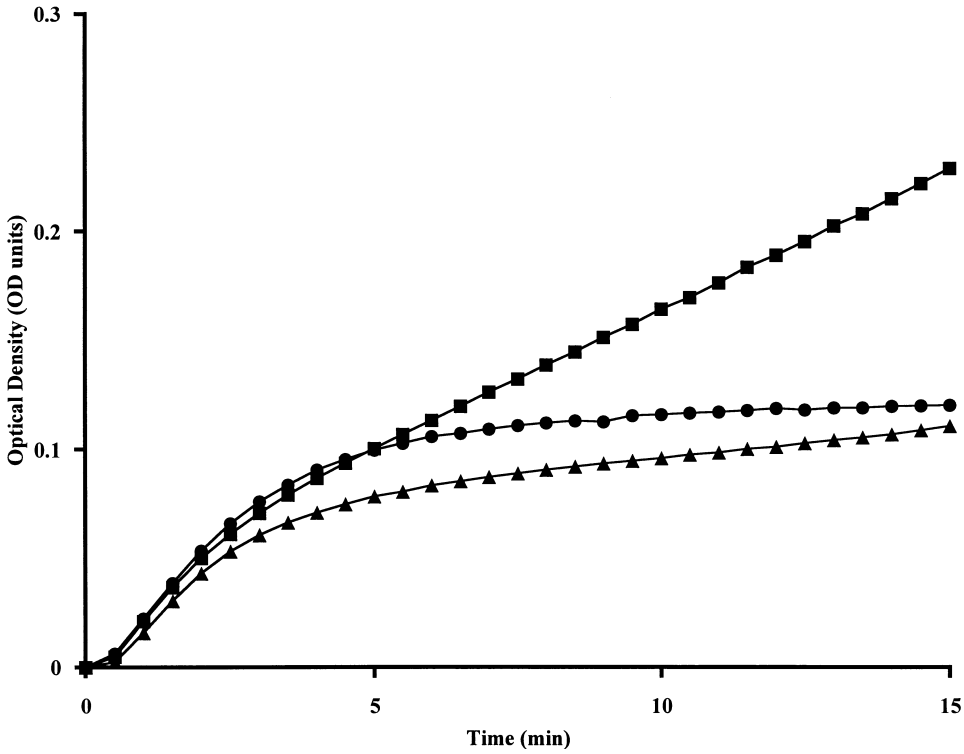

Fig. 3. Optical density curves measured in non-defibrinated plasma. (Filled squares, control (defibrinated); filled triangles, HO-NH_2 (25 mM)/GPRP (1.5 mg/mL); filled circles, protease A (3.5 μM)/GPRP (1.0 mg/mL)). The slope of the optical density curve calculated after 15 minutes is: 12.9 mOD/min, control; 2.9 mOD/min, hydroxylamine; and 0.8 mOD/min in the presence of protease A.

tease A-treated plasma shows an almost zero endlevel of the amidolytic activity (Figure 2). In principal, the jump in optical density could be used as a measure for the amount of thrombin formed and thus representing the ETP.

These results prompted us to test hydroxylamine and protease A in non-defibrinated plasma. Since hydroxylamine could be used as pseudosubstrate for factor XIIIa and protease A was said to hydrolyze the fibrinogen Aα and Bβ chain [19], it was hoped that fibrin polymerization could be postponed to the end of the optical density recording. An earlier report from Retzios and Markland describes the purification and characterization of three fibrino(geno)lytic enzymes from the venom of *Crotalus basiliscus basiliscus* [28]. They describe a metalloprotease, Cbfib 1.2, with a M_w of 23,500 and pI 4.7 which could be identical to protease A (M_w: 23,400 and pI 4.8). An activity on α_2M was, however, not described. Defibrination of plasma is a time-consuming process and we sought ways to circumvent this extra step. Our first attempt was to measure thrombin generation in non-defibrinated plasma in the presence of hydroxylamine or protease A alone, but this did not lead to correct thrombin generation curves. The fibrin polymerization inhibitors such as H-Gly-Pro-Arg-Pro-OH [29,30] and H-Gly-Pro-Arg-Pro-Ala-NH2 [31] prevent the occurrence of turbidity in non-defibrinated plasma during thrombin generation at a concentration of 4 mg/mL (~10 mM). The successful application of H-Gly-Pro-Arg-Pro-OH as fibrin polymerization inhibitor was recently reported by Prasa et al. [32]. In the presence of hydroxylamine or protease A the concentration of these polymerization inhibitors could be reduced to 1.5 and 1.0 mg/mL, respectively.

Optical density curves in non-defibrinated plasma are given in Figure 3. Under these conditions, the optical density curve of protease A-treated plasma in the presence of 1 mg/mL H-Gly-Pro-Arg-Pro-OH showed a zero amidolytic endlevel. In hydroxylamine treated plasma, a small residual amidolytic activity persists. More important, however, hydroxylamine/GPRP reduces the anticoagulant activity of heparin/AT-III significantly (Table 1) in contrast to the protease A/GPRP system (Table 1, Figure 4). The latter system allows the ETP determination as an endpoint measurement of the optical density. We are currently investigating the feasibility of measuring the ETP under these experimental conditions.

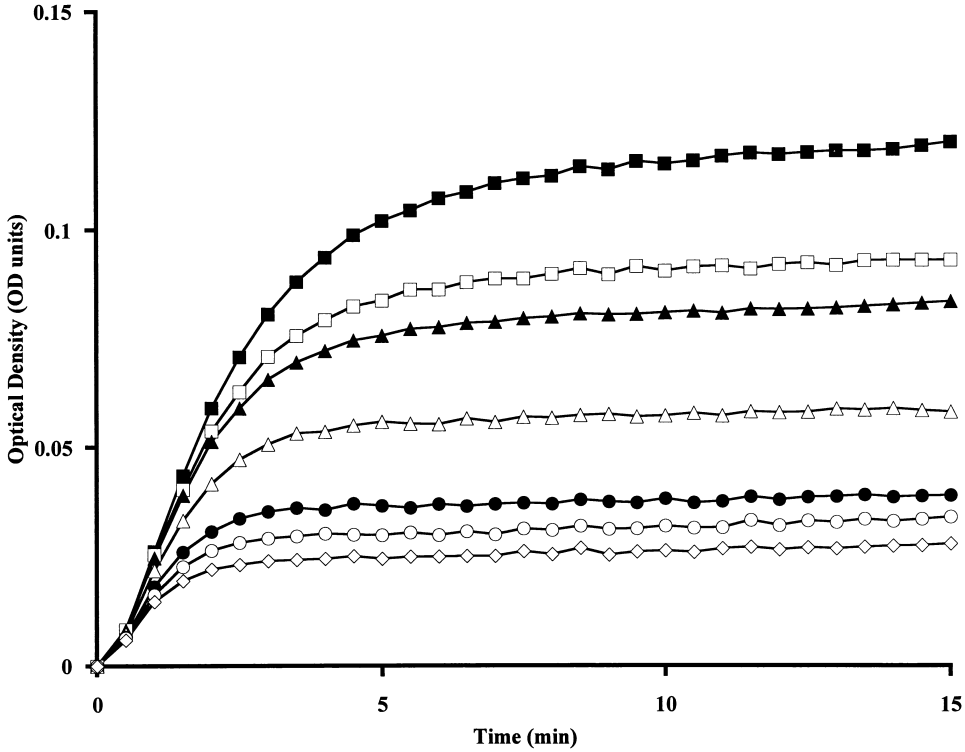

Fig. 4. Optical density curves in non-defibrinated heparinized plasma in the presence of protease A (3.5 μM)/GPRP (1.0 mg/mL). Thrombin generation is measured in plasma with an increasing amount of standard heparin. The jump in optical density is a measure for the ETP (Table 1). (Filled squares, control; open squares, 0.02; filled triangles, 0.03; open triangles, 0.05; filled circles, 0.08; open circles, 0.10; open diamonds, 0.125 U ISH/mL.) The slope of the optical density curve calculated after 15 minutes is: 0.8 mOD/min, control; 0.5, 0.5, 0.2, 0.3, 0.4, and 0.3 mOD/min at the several heparin concentrations.

In conclusion, we found two methods to inactivate α_2M in plasma to reduce the residual amidolytic activity. In protease A-treated plasma, the measured thrombin generation curve leads directly to the free thrombin curve. We extended the method of the continuous thrombin generation assay for measurement in non-defibrinated plasma. Finally, we show that the determination of the ETP could be reduced to an endpoint determination of the optical density in different degrees of anticoagulation.

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