

Quantification of Plasmatic Thrombin Generation by Trypsin

Thomas W. Stief; Institute of Clinical Chemistry, University Hospital, Marburg, Germany;
 thstief@med.uni-marburg.de

Abstract

Active trypsin in blood could severely modulate hemostasis. In the present study citrated blood or plasma of healthy donors was pre-incubated with trypsin. The plasma was recalcified and specific thrombin generation was measured. 1-10 ng/ml trypsin enhances intrinsic thrombin generation > 2fold. Trypsin might activate intrinsic coagulation factors or prothrombin (100 ng/ml trypsin generates within 60 min 15 mIU/ml thrombin from 100 µg/ml prothrombin), causing a pathologic disseminated intravascular coagulation. The recalcified coagulation activity assay (RECA) allows to investigate the action of trypsin on intrinsic thrombin generation. The RECA is a very sensitive method to detect prothrombotic changes of blood.

Key words: Trypsin, Thrombin, RECA, Chromogenic Test, Laboratory Medicine

Introduction

Trypsin is an aggressive serine protease that may alter the hemostasis system in human blood [1-5]. Enhanced generation of intravascular thrombin threatens the life of the patient. In the present medical laboratory work the action of purified trypsin on intrinsic thrombin generation in blood or plasma was investigated with a new specific and sensitive thrombin generation assay: in a first near-physiologic assay phase plasma is recalcified and the generated thrombin is quantified in a second assay phase [6].

Material and Methods

Thrombin generation in blood or plasma pre-incubated with trypsin

a) Pre-analytic:

2 x 4.5 ml venous blood of n=4 healthy donors who gave informed consent were mixed with 2 x 0.5 ml 106 mM sodium-citrate. 500 µl citrated whole blood, 250 µl citrated plasma (obtained by centrifugation of blood at 2800g (4000 rpm) for 10 min at 23°C) or 250 µl 6.7 % human albumin (Kabi, Stockholm, Sweden) were pipetted into 1 ml Eppendorf®-polypropylene-cups, using an Eppendorf-Multipette® with 0.9 % NaCl-rinsed and completely emptied new disposable tips. Unrinsed tips may release some plastic material that behaves as a coagulation inhibitor. The Eppendorf-cups had been prefilled with 25 µl 0.5 % human albumin, 0.9 % NaCl containing 0-11 µg/ml porcine pancreatic trypsin type II (Sigma, Deisenhofen, Germany; Article Nr. T 7168; final sample conc. = 0-1 µg/ml for supplemented plasma or albumin or about 0-873 ng/ml for supplemented blood of a mean hematocrit value of 0.42) or 220 ng/ml lipopolysaccharide (LPS; from *Escherichia coli* 055:B5 chromatographically purified by gel filtration, protein content < 1 %; Sigma; Article Nr. L 2637, 5 mg/ml stabilized by 5 % human albumin), resulting in a final supplemented plasma LPS reactivity of about 17.5 ng/ml. LPS served as a positive control for intrinsic coagulation activation [6]. After 10 min at 23°C the whole blood - cups were centrifuged at 11000 rpm for 5 min at 23°C, using an Eppendorf-centrifuge. 50 µl plasma samples (from preincubated blood or preincubated plasma), 6.7 % albumin, or 1 IU/ml bovine thrombin (DadeBehring, Marburg, Germany) in 6.7 % human albumin were pipetted into polystyrol flat bottom wells (Polysorp®, NUNC, Wiesbaden, Germany; article nr. 446140) at 23°C.

b) Analytic:

Phase 1: Thrombin generation

5 µl 250 mM CaCl₂ were added in duplicate to the 50 µl samples (pH 7.4), using a 0.9 % NaCl-rinsed new disposable polypropylene tip (Table 1). The wells were incubated for 0, 5, 10, or 20 min coagulation reaction time (CRT at 37°C). 0 min CRT = basal thrombin activity in citrated plasma (first addition of stop reagent then addition of Ca²⁺ - reagent).

Phase 2: Thrombin detection

100 µl 2.5 M arginine, pH 8.6, 0.16 % Triton X 100® (Sigma; stop reagent) were added, to stop hemostasis activation and to depolymerize generated fibrin. The basal absorbance was measured at 405 nm,

using a microtiterplate reader with a 1 mA resolution (Milenia; DPC, Los Angeles, USA). 25 μ l 1 mM chromogenic thrombin substrate HD-CHG-Ala-Arg-pNA (Pentapharm, Basel, Switzerland; substrate reagent) in 1.25 M arginine (final conc.: 0.14 mM chromogenic substrate and 1.56 M arginine) were added and $\Delta A/t$ was measured at 23°C. Of major importance are the thrombin generation values in the ascending part of the thrombin generation curve, i.e. at a CRT point where thrombin is still not significantly entrapped into the nascent fibrin.

Table 1: RECA scheme

Phase 1: Physiologic thrombin generation

50 μ l citrated plasma into flat bottom-wells
5 μ l 250 mM CaCl_2 (Ca^{2+} - reagent)

0 - 40 min coagulation reaction time (37°C)

Phase 2: Specific detection of generated thrombin

100 μ l 2.5 M arginine, pH 8.6, 0.16 % Triton X 100® (Stop-reagent)

3 min (37°C or 23°C)

25 μ l 1 mM CHG-Ala-Arg-pNA, 1.25 M arginine, pH 8.7 (Substrate-reagent)
 $\Delta A/t$ at 405 nm

Calibrator = 50 μ l 1 IU/ml bovine thrombin instead of plasma (\rightarrow about 16 mA/min at 23°C);
max. ΔA = 900 mA, linear ΔA range = up to 40 % of 900 mA = 360 mA

Kinetic of thrombin generation by action of trypsin on plasma vs. on purified prothrombin

a) Plasmatic thrombin generation by trypsin

2 μ l 0, 26, 260, 2600 ng/ml trypsin (0, 1, 10, 100 ng/ml final) in 0.5 % human albumin/0.9 % NaCl were placed in duplicate into F-wells at 23°C. 50 μ l unfrozen citrated plasma of n=3 healthy donors, who gave informed consent, or 1 IU/ml bovine thrombin standard in 6.7 % human albumin were added. Immediately thereafter, 5 μ l Ca^{2+} - reagent were added. After 0-60 min CRT 100 μ l stop-reagent were added. After 3 min (23°C), 25 μ l substrate - reagent were added and the linear $\Delta A/t$ was determined at 23°C.

b) Activation of purified human prothrombin by trypsin

2 μ l 0, 26, 260, 2600 ng/ml trypsin (0, 1, 10, 100 ng/ml final) in 0.5 % human albumin/NaCl were placed in duplicate into F-wells at 23°C. 50 μ l 100 μ g/ml purified human prothrombin (EnzymeResearch-Haemochrom, Essen, Germany) in 6 % human albumin, or 1 IU/ml bovine thrombin in 6 % human albumin were added, and immediately thereafter, 5 μ l Ca^{2+} - reagent were added. After 0-60 min CRT 100 μ l stop-reagent were added. After 3 min (23°C), 25 μ l substrate-reagent were added and the linear $\Delta A/t$ was determined, prolonging the incubation time up to 18 h (23°C).

A control experiment consisted in incubation of plasminogen with trypsin, kallikrein, or plasminogen activators: 5 μ l 0 or 100 μ g/ml human plasma kallikrein (Haemochrom, Essen, Germany), 10, 100, or 1000 ng/ml trypsin, 10 ng (1 IU)/ml urokinase (medac, Hamburg, Germany), or 12.6 ng/ml tissue type plasminogen activator (t-PA; Boehringer Ingelheim, Germany), all in 6.7 % human albumin were incubated with 45 μ l 0 or 111.1 μ g/ml human Glu-plasminogen (Chromogenix, Mölndal, Sweden) in 6.7 % human albumin in presence and absence of 5 μ l Ca^{2+} - reagent for 0-120 min (37°C) in U-wells. Then 100 μ l stop-reagent and 25 μ l 1.3 mM chromogenic plasmin substrate HD-Val-Leu-Lys-pNA (Chromogenix) in 1.2 M KCl, 1.6 M arginine, pH 8.7 were added and $\Delta A/t$ was determined at 405 nm. The results were standardized against 100 μ l 2.5 M arginine, 0.16 % Triton X 100, pH 8.6, + 45 μ l 6.7 % human albumin + 5 μ l 0.5 U/ml human plasmatic plasmin + 25 μ l plasmin substrate in presence and absence of calcium ions, i.e. against about 50 mU/ml plasmin final activity in the fibrinolysis reaction time.

Differential blood count after trypsin incubation

To exclude major changes on the differential blood count, 1 ml citrated whole blood of 3 healthy donors was incubated with 50 μ l 0-110 μ g/ml porcine pancreatic trypsin for 30 min at 23°C. Then the blood cells were automatically counted by a XE2100 (Sysmex, Hamburg, Germany).

Statistics

The intra-assay coefficients of variation (CV), as defined as standard deviation divided by mean value and multiplied by 100 %, of the thrombin generation values measured were < 5 %. The data points were compared to the respective controls and tested for significance ($p < 0.05$) by the two-fold Yates-corrected chi-square comparison against the control-mean (χ^2_{\times} Test) [7].

Results

Preincubation of whole blood with about 100 ng/ml plasmatic trypsin increases the 10 min CRT thrombin generation (in the important ascending part of the thrombin generation curve) from about 7 mIU/ml generated thrombin to about 12 mIU/ml generated thrombin, the basal thrombin activity (no CRT) was about 5 mIU/ml (Fig. 1a).

Fig. 1b demonstrates the LPS – control, i.e. the thrombin generating capacity of citrated whole blood that had been preincubated for 10 min (23°C) with a plasma concentration of about 20 ng/ml LPS. This is about 20fold the normal LPS reactivity in our blood and represents a typical LPS reactivity of intensive-care patients with gram-negative sepsis. Here, intrinsic thrombin generation at 10 min CRT is about 30 mIU/ml in presence of about 20 ng/ml LPS as compared to only about 10 mIU/ml without addition of LPS. The thrombin generating capacity of about 20 ng/ml LPS in whole blood is comparable to that of about 1000 ng/ml trypsin in whole blood or 1 ng/ml trypsin in plasma (Fig. 2). In trypsin-supplemented plasma, 1-10 ng/ml trypsin results into maximal thrombin activity (about 50 mIU/ml at 10 min CRT (compared to about 10 mIU/ml at 0 min CRT), higher trypsin concentrations result into decreasing specific amidolytic thrombin activity.

Healthy donor plasma, pre-reacted for 10 min with 1000 ng/ml trypsin, showed a thrombin activity of 15 mIU/ml at 0 min CRT, i.e. 5 mIU/ml basal activity of thrombin plus 10 mIU/ml thrombin-like activity caused by trypsin; 1000 ng/ml trypsin in 6.7 % human albumin had a thrombin like activity of 25 mIU/ml, i.e. about 60 % of the amidolytic activity of added trypsin was inactivated by plasmatic trypsin inhibitors. 10 µg/ml trypsin in 6.7 % albumin cleaved the chromogenic substrate HD-CHG-Ala-Arg-pNA comparable to about 0.2 IU/ml thrombin in albumin (= about 0.1 µg/ml thrombin; 1 mg IIa = 2525 IU), i.e. in the RECA-test conditions the chromogenic substrate is an about 100fold better substrate for thrombin than for trypsin.

Fig. 1a

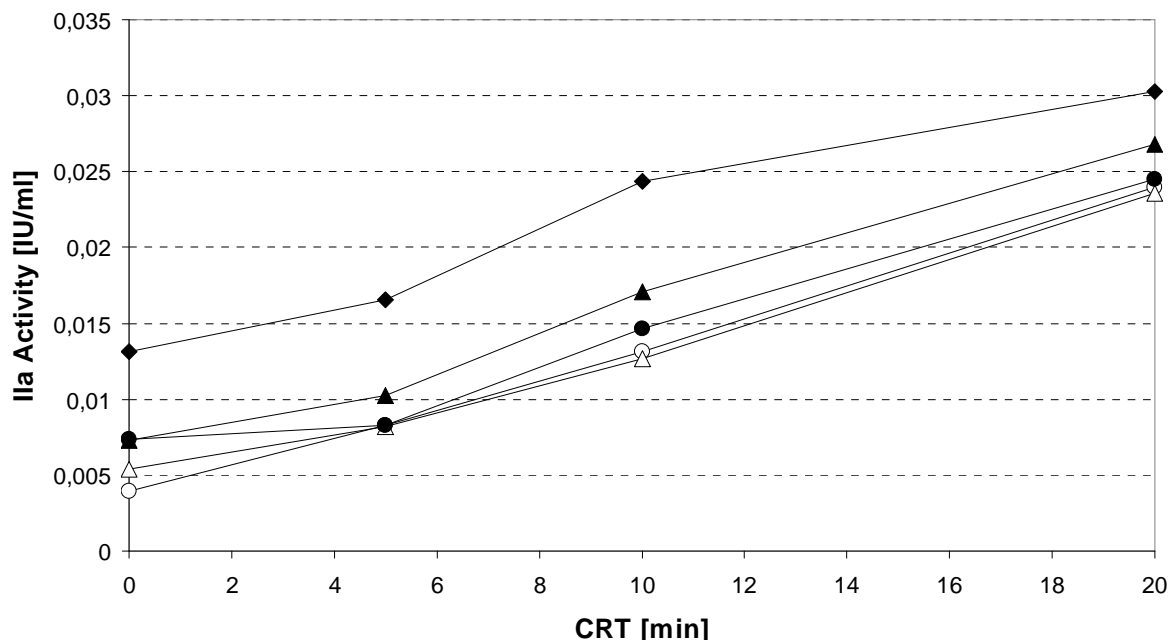


Fig. 1b

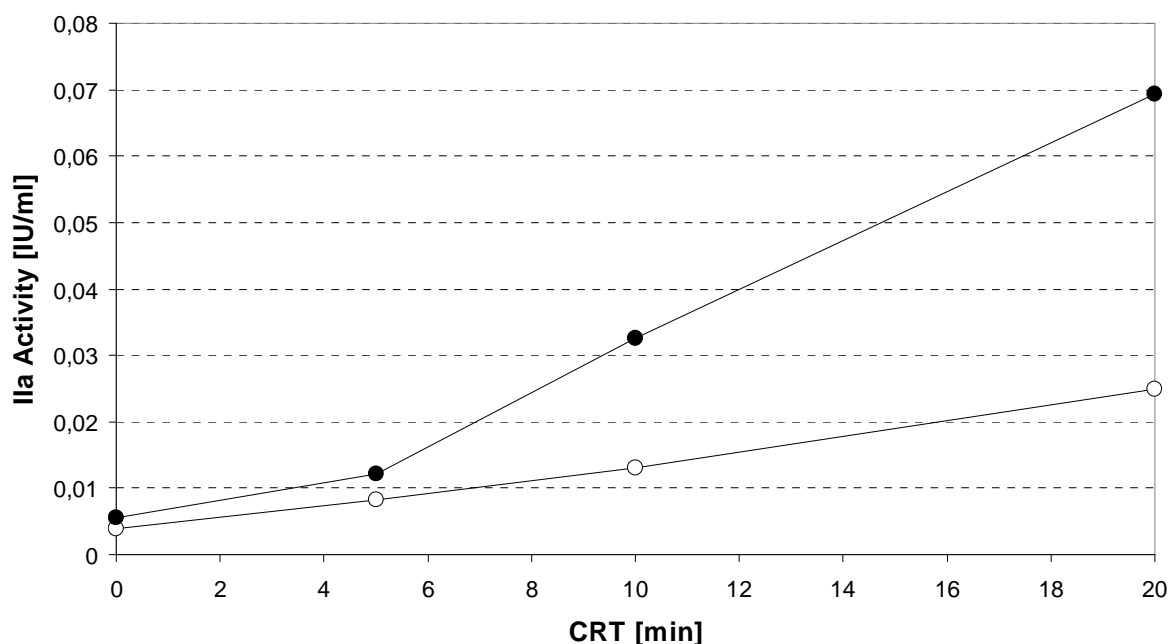


Fig. 1: Thrombin generation in citrated whole blood preincubated with trypsin or LPS

500 μ l citrated whole blood of $n=4$ healthy donors were preincubated for 10 min at 23°C at final trypsin concentrations of 0 ng/ml (O), 0.87 ng/ml (Δ), 8.7 ng/ml (\bullet), 87 ng/ml (\blacktriangle), and 873 ng/ml (\blacklozenge) (Fig. 1a) and final LPS concentrations of 0 ng/ml (O) or 17.5 ng/ml (\bullet) (Fig. 1b). 50 μ l preincubated plasma samples were pipetted into F-wells. 5 μ l 250 mM CaCl_2 were added. After 0, 5, 10, or 20 min coagulation reaction time (CRT at 37°C), 100 μ l 2.5 M arginine, pH 8.6, 0.16 % Triton X 100® and then 25 μ l 1 mM HD-CHG-Ala-Arg-pNA in 1.25 M arginine were added and $\Delta A/t$ was measured at 23°C. Shown are the obtained mean values.

Fig. 2

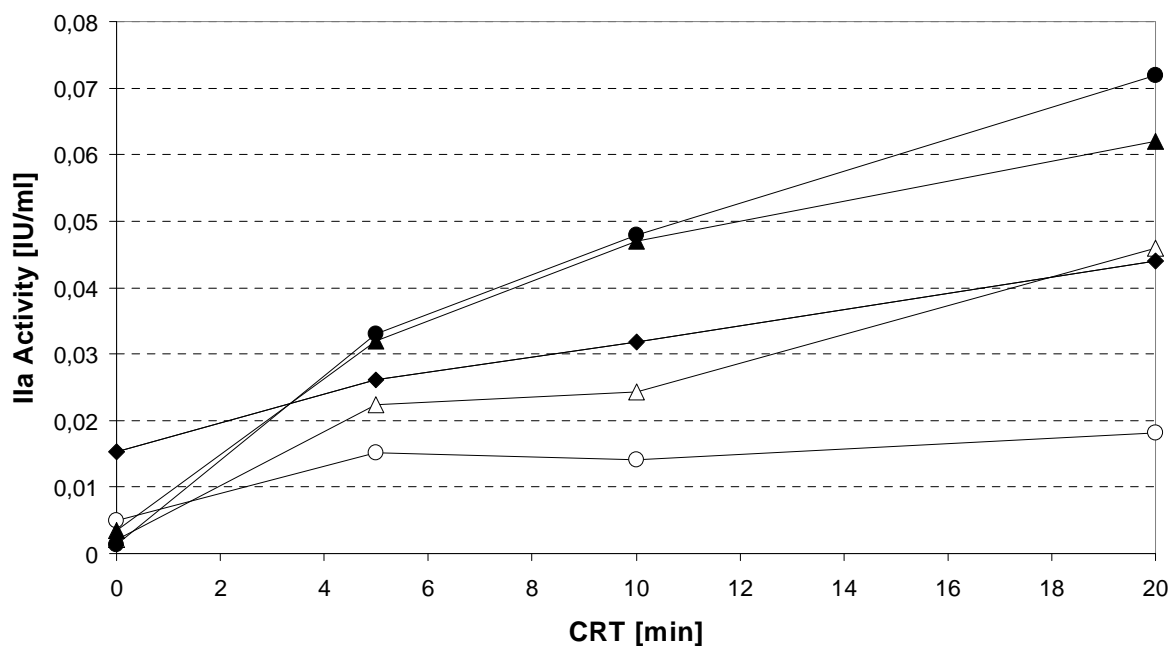


Fig. 2: Thrombin generation in citrated plasma preincubated with trypsin

250 μ l citrated plasma of the above $n=4$ healthy donors were preincubated for 10 min at 23°C at final plasmatic trypsin concentrations of 0 ng/ml (O), 1 ng/ml (Δ), 10 ng/ml (\bullet), 100 ng/ml (\blacktriangle), and 1000 ng/ml (\blacklozenge) in preincubated plasma. The preincubated plasma samples were analyzed as indicated in Fig. 1.

Fig. 3

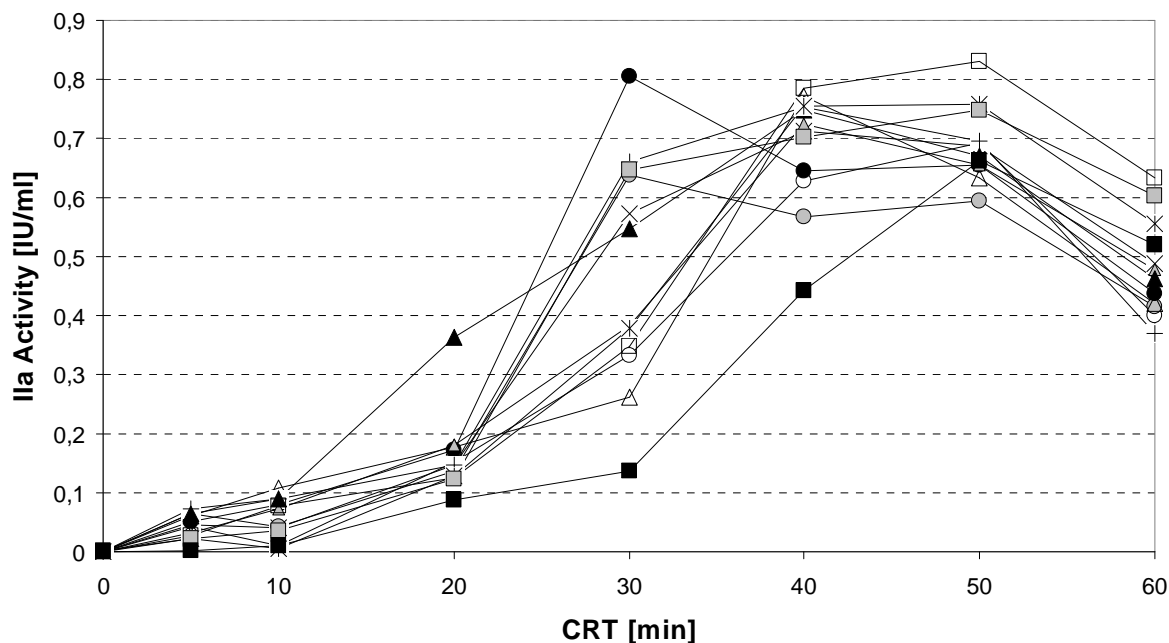


Fig. 3: Kinetic of thrombin generation in plasma by 0-100 ng/ml trypsin

2 μ l trypsin (final concentrations: 0 ng/ml (white symbols), 1 ng/ml (cross symbols), 10 ng/ml (grey symbols), 100 ng/ml (black symbols)) were placed in duplicate into F-wells at 23°C. 50 μ l unfrozen citrated plasma of n=3 healthy donors (donor 1 = circles or x, donor 2 = triangles or +, donor 3 = squares or *) were added, and immediately thereafter, 5 μ l 250 mM CaCl_2 were added. After 0-60 min CRT 100 μ l stop-reagent were added. After 3 min (23°C), 25 μ l substrate-reagent were added and the linear $\Delta A/t$ was determined at 23°C.

Fig. 4

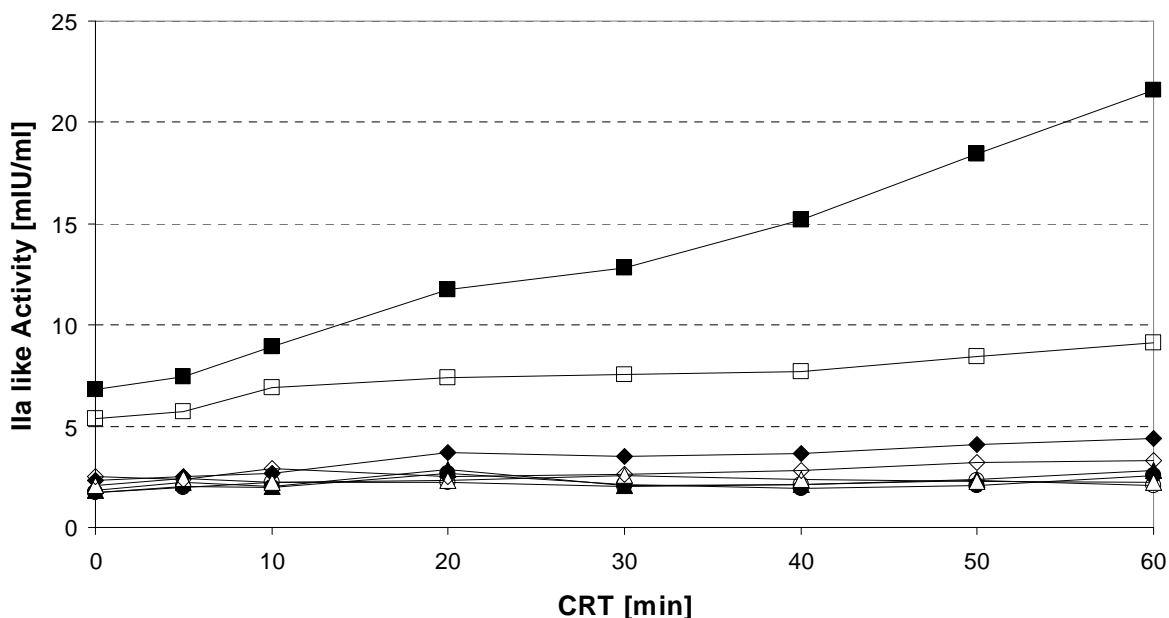


Fig. 4: Kinetic of activation of purified human prothrombin by 0-100 ng/ml trypsin

2 μ l trypsin (final concentrations: 0 ng/ml (circles), 1 ng/ml (triangles), 10 ng/ml (diamonds), 100 ng/ml (squares)) were placed into F-wells. 50 μ l 100 μ g/ml purified human prothrombin in 6 % human albumin, or 1 IU/ml bovine thrombin in 6 % human albumin were added, and immediately thereafter, 5 μ l 250 mM CaCl_2 (black symbols) or physiol. NaCl (white symbols) were added. After 0-60 min CRT 100 μ l stop-reagent and 25 μ l substrate-reagent were added and the linear $\Delta A/t$ was determined at 23°C.

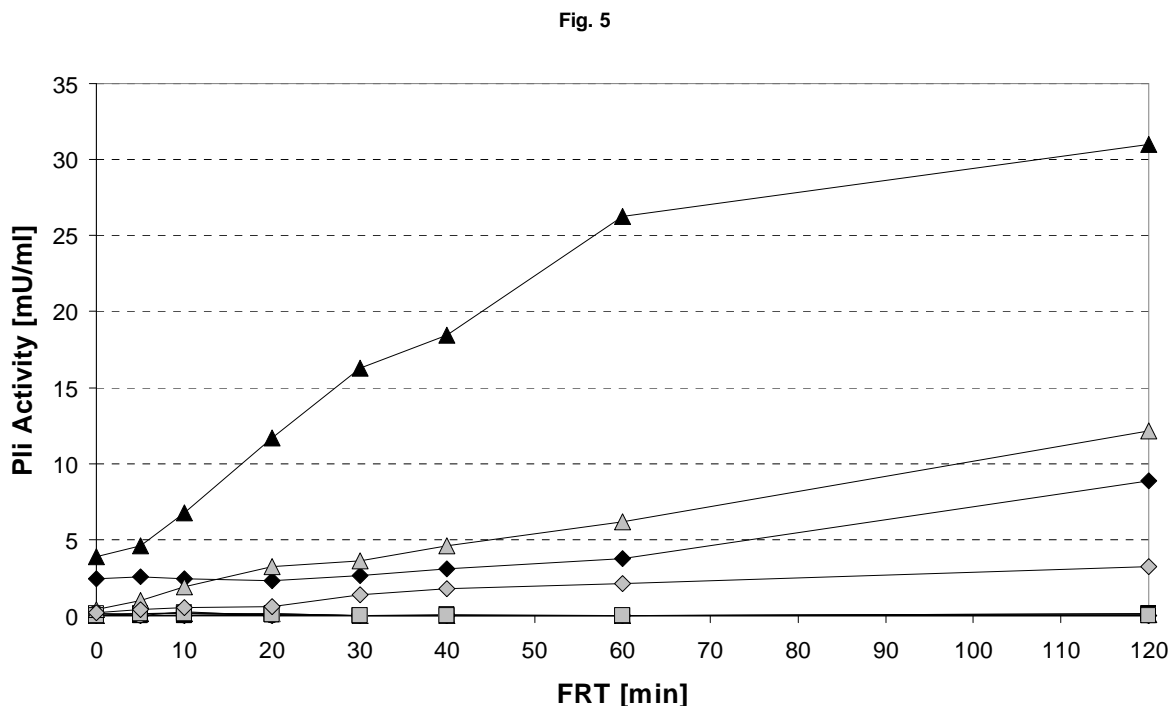


Fig. 5: No activation of purified human plasminogen by trypsin or kallikrein

5 µl 0 (circles) or 100 µg/ml human plasma kallikrein (squares), or 1000 ng/ml trypsin (crosses), 10 ng/ml urokinase (triangles), or 12.6 ng/ml t-PA (diamonds), all in 6.7 % human albumin were incubated with 45 µl 0 (white symbols or -) or 111.1 µg/ml human Glu-plasminogen in 6.7 % human albumin in presence (grey symbols or +) or absence (black symbols or *) of 5 µl 250 mM CaCl₂ for 0-120 min fibrinolysis reaction time (FRT at 37°C). Then 100 µl stop-reagent and 25 µl 1.3 mM HD-Val-Leu-Lys-pNA in 1.2 M KCl, 1.6 M arginine, pH 8.7 were added and ΔA/t was determined at 405 nm. Not visible symbols are nearly identical to the x-axis.

Figure 3 again demonstrates that 1-10 ng/ml trypsin added immediately before recalcification enhance thrombin generation about 2fold to about 0.65 IU/ml thrombin at 30 min CRT (ascending part of the thrombin generation curve). 100 ng/ml trypsin in 2 of 3 donor samples increase and in 1 of 3 donor samples decrease thrombin generation.

Figure 4 shows that the unspecific serine protease trypsin can activate prothrombin directly: within 60 min (37°C) 100 ng/ml trypsin generate about 15 mIU/ml thrombin from 100 µg/ml purified prothrombin (100 % physiological plasma concentration) in presence of free Ca⁺⁺ ions. Absence of calcium ions results only into generation of about 5 additional mIU/ml thrombin at 60 min CRT, when compared to 0 min CRT. The prothrombin preparation used contained about 2 mIU/ml basal thrombin. Trypsin does not inactivate thrombin under the experimental conditions used. Fig. 5 demonstrates that even 100 ng/ml final trypsin activity did not cleave the chromogenic plasmin substrate in the present technique at supra-molar concentrations of arginine, that acts as a competitive inhibitor for serine proteases.

Up to final plasmatic trypsin concentrations of about 1 µg/ml trypsin no hemolysis appeared by 10 min (23°C) preincubation of citrated blood of healthy donors. Final plasmatic trypsin concentrations up to about 10 µg/ml do not change significantly the number of erythrocytes, neutrophils, lymphocytes, monocytes, eosinophils, or basophils. There appeared a slight decrease (< 10 %) in the number of thrombocytes when incubating whole blood at about 10 µg/ml plasmatic trypsin.

Discussion

Trypsin is an aggressive serine protease of broad specificity. Trypsin cleaves proteins at arginyl- or lysyl-residues. Depending on the exact protein localization of these arginyl- or lysyl- targets, trypsin can activate or inactivate hemostasis factors. Of particular importance seems to be the activation of pre-kallikrein to kallikrein and that of factor XII to factor XIIa, both trypsin-like enzymes in the contact phase of coagulation [8,9]: 1-10 ng/ml trypsin enhances intrinsic plasmatic thrombin generation > 2fold. Elevated blood concentrations of trypsin can be found in patients with pancreatitis [10] or inflammatory diseases [11] like adult respiratory distress syndrome (ARDS): in severe ARDS plasmatic trypsin is about 100-1000 ng/ml [12].

Trypsin can also inactivate the two main coagulation accelerators factor V and factor VIII [13-22]. This could account for some trypsin-activity dependent decrease in intrinsic thrombin generation. The presence of blood cells strongly decreases the contact pathway activating action of trypsin. This might be due to interaction of hydrophobic phospholipids of the cell membranes with hydrophobic parts of the trypsin molecule. Thus, in the microcirculation of the capillaries the erythrocytes might not protect the plasma against trypsin from alveolar macrophages or pancreas cells, which could result in enhanced intrinsic hemostasis activation. Additionally, free trypsin can enhance inflammation and might possess some hormone like actions on protease cleavable cell receptors [23,24].

In conclusion, trypsin in ng/ml quantities in circulating blood can result into an enhanced intrinsic phase triggered coagulation activation that may cause a pathologic disseminated intravascular coagulation. Further clinical studies on intrinsic hemostasis activation in fresh citrated blood of patients suffering from pancreatitis or ARDS are indicated.

References

1. Landaburu RH, Barnhart MI, Seegers WH. Prothrombin activation with trypsin as enzyme. *Am J Physiol* 1961; 201: 298-302.
2. Gabryelewicz A, Niewiarowski S. Activation of blood clotting and inhibition of fibrinolysis in acute pancreatitis. *Thromb Diath Haemorrh*. 1968; 20: 409-14.
3. Kwaan HC, Anderson MC, Gramatica L. A study of pancreatic enzymes as a factor in the pathogenesis of disseminated intravascular coagulation during acute pancreatitis. *Surgery*. 1971; 69: 663-72.
4. Satake K, Ha S, Hiura A, Nishiwaki H, Haku A, Umeyama K. Effect of a synthetic protease inhibitor (Fut-175) on coagulation abnormalities during experimental acute pancreatitis in dogs. *Gastroenterol Jpn* 1990; 25: 720-6.
5. Saif MW. DIC secondary to acute pancreatitis. *Clin Lab Haematol* 2005; 27: 278-82.
6. Stief TW. Thrombin generation by exposure of blood to endotoxin: a simple model to study disseminated intravascular coagulation. *Clin Appl Thrombosis/Hemostasis* 2006; 12: 137-161.
7. Stief TW. The fibrinogen antigenic turbidimetric assay (FIATA). The X^2_{∞} test: the corrected chi-square comparison against the control-mean. *Clin Appl Thromb Hemost* 2007; 13: 73-100.
8. Bode W, Schwager P, Huber R. The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding. The refined crystal structures of the bovine trypsinogen-pancreatic trypsin inhibitor complex and of its ternary complex with Ile-Val at 1.9 Å resolution. *J Mol Biol* 1978; 118: 99-112.
9. Takahashi H, Nagasawa S, Suzuki T. Studies on prekallikrein of bovine plasma. II. Activation of prekallikrein with proteinases and properties of kallikrein activated by bovine Hageman factor. *J Biochem* 1980; 87: 23-34.
10. Whitcomb DC, Lowe ME. Human pancreatic digestive enzymes. *Dig Dis Sci* 2007; 52: 1-17.
11. Vassalli JD, Granelli-Piperno A, Reich E. Neutral proteinases of leucocytes and the inflammatory process. *Ciba Found Symp* 1979; 75: 381-95.
12. Deby-Dupont G, Haas M, Pincemail J, Braun M, Lamy M, Deby C, Franchimont P. Immunoreactive trypsin in the adult respiratory distress syndrome. *Intensive Care Med* 1984; 10: 7-12.
13. Colman RW. The effect of proteolytic enzymes on bovine factor V. II. Kinetics of activation and inactivation by papain, plasmin, and other proteolytic enzymes. *Biochemistry* 1969; 8: 1445-50.
14. Vogel CN, Parfitt HE Jr, Kingdon HS, Lundblad RL. Preparation of modified bovine factor VIII with enhanced biological activity using insoluble-trypsin columns. *Thromb Diath Haemorrh* 1973; 30: 229-34.
15. Kirby EP, Martin N, Marder VJ. Degradation of bovine factor 8 by plasmin and trypsin. *Blood* 1974; 43: 629-40.

16. Triantaphyllopoulos DC. Inactivation of factor VIII by a mechanism independent of the generation of thrombin. *Thromb Haemost* 1979; 42: 838-47.
17. Martin SE, Marder VJ, Francis CW, Loftus LS, Barlow GH. Enzymatic degradation of the factor-VIII-von-Willebrand protein: a unique tryptic fragment with ristocetin cofactor activity. *Blood* 1980; 55: 848-58.
18. Rick ME, Popovsky MA, Krizek DM. Degradation of factor VIII coagulant antigen by proteolytic enzymes. *Br J Haematol* 1985 ; 61: 477-86.
19. Pittman DD, Wang JH, Kaufman RJ. Identification and functional importance of tyrosine sulfate residues within recombinant factor VIII. *Biochemistry* 1992; 31: 3315-25.
20. Xue J, Kalafatis M, Mann KG. Determination of the disulfide bridges in factor Va light chain. *Biochemistry* 1993; 32: 5917-23.
21. Pazzagli L, Cecchi C, Cappugi G, Catalani R, Bertini M, Jolles P, Ramponi G. A peptide fraction from factor VIII reduces PKC activity in cultured endothelial cells. *Life Sci* 1998; 62: 829-37.
22. Nogami K, Wakabayashi H, Ansong C, Fay PJ. Localization of a pH-dependent, A2 subunit-interactive surface within the factor VIIIa A1 subunit. *Biochim Biophys Acta* 2004; 1701: 25-35.
23. Coughlin SR. Thrombin signaling and protease-activated receptors. *Nature* 2000; 407: 258-64.
24. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost* 2005; 3: 1800-14.