Cupincin: A Novel Cupin Domain Containing Protease from Rice (*Oryza sativa* L.) Bran Comprising of Procoagulant and Fibrinogenolytic Activity

Roopesh Sreedhar¹ · Purnima Kaul Tikur¹

Received: 24 March 2017 / Accepted: 1 August 2017 / Published online: 11 August 2017
© Indian Society of Haematology & Transfusion Medicine 2017

Abstract The current study was carried out to evaluate the pharmacological properties of cupincin- A novel cupin domain containing metalloprotease with limited proteolysis from rice bran on blood coagulation and hydrolysis of human fibrinogen. Cupincin preferentially hydrolyzed the A α chain of fibrinogen and then the B β -chain, but not the γ -chain. Cupincin reduced the re-calcification time of citrated human plasma dose dependently. Analysis of citrated whole blood in the presence of cupincin by rotem showed a decrease in coagulation time and clot formation time. Sonoclot analysis indicated that cupincin cleaved fibrinogen of whole citrated blood. SDS-PAGE and sonoclot analysis (LI-30) indicated that cupincin lacked plasmin-like activity. Global hemostasis tests like rotem and sonoclot analysis determined cupincin as a procoagulant enzyme. Cupincin did not show any effect on prothrombin time and activated partial thromboplastin time tests suggesting its action on the common pathway of coagulation. The involvement of proteases from rice (*Oryza sativa* L.) in haemostasis has never been exploited before. This study could provide the basis for the development of new procoagulant agents from a nontoxic source like rice.

Keywords Rice bran · Protease · Procoagulant · Fibrinogenolytic · Haemostasis · Thromboelastometry

Introduction

Our preceding study reported the purification and characterization of a novel cupin domain containing metalloprotease with limited proteolysis from rice bran named as cupincin. Investigation of its sequence disclosed that the protein belonged to cupin superfamily. It was the earliest report of a cupin domain containing protease. Cupincin was also biochemically characterized and to explore its hydrolysing mechanism its 3D structure was built. Cupincin was found to be a zinc metalloprotease and was specific in its action, cleaving at the Leu₁₅-Tyr₁₆ position in oxidized B chain of insulin and Glu₁-Tyr₂ position when the specificity was determined using neurotensin as a substrate [1].

Proteases have varied applications in the pharmaceutical industries. Plant proteases can be exploited for various cellular and molecular activities, like antibacterial and anticancer therapies, for the treatment of snakebites by inhibiting snake venom activities for instance blood-clotting, defibrinogenation, and fibrin(ogen)olytic and hemorrhagic actions [2]. Plant proteases have been used in traditional medicine from primeval time. Proteases interfering in blood coagulation and fibrin hydrolysis have been isolated and characterized from several plant latexes. A number of plant latex proteases are reported to interfere in haemostasis as procoagulants, signifying their distinctive substrate preference over other proteases [3, 4]. *Asclepiadaceae* plants latex cysteine proteases were reported to demonstrate thrombin and plasmin-like activities [5]. A cysteine protease from *Ficus carica* (*ficin*) and a serine protease from *Synadenium grantii* were demonstrated to play a role in blood coagulation and fibrin hydrolysis [4, 6]. Bromelain, derived from pineapple, was shown to be capable of preventing edema, platelet aggregation and

✉ Purnima Kaul Tikur
purnima@cftri.res.in

¹ Department of Protein Chemistry and Technology, CSIR-Central Food Technological Research Institute, Mysuru, Karnataka 570020, India

metastasis due to its capacity of modifying cell surface structures by peptide cleavage [7]. *M. oleifera* leaf and root aqueous extracts were determined as procoagulant which possessed fibrinogenolytic and fibrinolytic activity [8]. So far, the involvement of a protease from rice in haemostasis has never been reported earlier.

Both fibrinogenolytic and fibrinolytic property of proteases needs to be assessed respectively for ascertaining them as either procoagulant or anticoagulant. The procoagulant activity also known as the haemostatic activity is vital to prevent bleeding and smoothen the progress of wound healing process by aiding in blood clot or fibrin clot formation. Fibrin(ogen)olytic metalloproteases are used in therapeutic or clinical applications due to their potential of reversing the effects of thrombosis. This has revealed as an alternative approach to the prevention and treatment of cardiovascular disorders [2]. The anticoagulant activity also known as fibrinolytic activity possessing proteases aid in degradation of the thrombus formed or fibrin clot in blood circulation [9].

Previously cupincin was shown to be a metalloprotease with limited proteolysis. The Present study was carried out to exploit the limited proteolysis of cupincin, which perhaps will aid in the development of new procoagulant agents from non-toxic source like rice.

Experimental Procedures

Materials

Human plasma fibrinogen was obtained from Sigma-Aldrich, (USA). Neoplastine® CL plus and APTT reagent were obtained from Stago (France), Glass beads gbACT + kit was obtained from Sienco, Inc. (USA) and all other reagents used were of analytical grade and were either from Merck or Sigma-Aldrich, (USA). Citrated whole blood was used for re-calcification experiments using rotational thromboelastometry (Rotem®, Pentapharm GmbH diagnostic division, Munich, Germany). All the experiments involving human blood were carried out in “Vijayashree Diagnostics” (Coagulation lab and ultrasound scanning) Kormangala, Bengaluru, Karnataka-560043.

Determination of protein concentration

Total protein concentration of cupincin was determined by the dye-binding method of Bradford [10], using Bovine serum albumin (BSA) as the standard protein.

Fibrinogenolytic Activity

The fibrinogenolytic activity was assessed according to the method of Ouyang and Teng [11]. The reaction mixture of

150 μ L contained 5% of human fibrinogen in 50 mM Tris–HCl buffer, pH-7. 0. This was incubated with the 20 μ g (30 μ L) of cupincin from 0 min to 4 h at 37 °C. The reaction was terminated by adding 20 μ L of denaturing buffer containing 1 M urea, 4% SDS and 4% β -mercaptoethanol. The reaction mixtures were analysed using 10% (w/v) SDS-PAGE under reduced conditions according to the method of Laemmli [12]. The protein banding pattern was analysed by coomassie brilliant blue R-250 staining.

Fibrinolytic Activity

Platelet poor plasma (50 μ L) prepared as per the procedure described in [13] was mixed with equal volume of 0.25 M CaCl_2 for 15 min to form soft fibrin clot, fibrin clot was washed thoroughly with Tris–HCl buffer (50 mM, pH-7) and was incubated with 0–40 μ g of cupincin for 2 h at 37 °C. The reaction was stopped by adding 50 μ L of reducing sample buffer and the samples were kept in boiling water bath for 5 min and then centrifuged for settling plasma clot debris. Aliquots (25 μ L) of the samples were analyzed in 10% SDS-PAGE for fibrin degradation analysis.

Fibrinolytic Activity (Quantitative Method)

Quantitative assay was determined according to plate method as described in [14]. A mixture consisting of 2 mL of platelet poor plasma, 3 mL of 1.2% agarose in 10 mM Tris–HCl (pH-7), 0.15 M NaCl, 0.05% sodium azide and 0.25 M CaCl_2 was poured into flat petri dish and left for 2 h at 37 °C. Cupincin (10–40 μ g) in 10 mM Tris–HCl buffer, pH 7.0 and 2.5 units of Urokinase was independently placed on the surface and incubated overnight. Trichloro acetic acid (TCA; 0.01%) was added over the surface and the diameter of the clear translucent zones due to lyses of fibrin clot (plaque) was measured in mm.

In vitro Coagulant Assays

Re-Calcification Time

Re-calcification time was carried out according to the previously described procedure of Condrea et al. [15]. Human citrated plasma was pre-warmed at 37 °C. Citrated plasma (200 μ L) was mixed with Tris–HCl buffer (50 mM, pH-7) and pre-incubated for 5 min with varying concentrations of cupincin (0–60 μ g). 20 μ L of 0.25 M CaCl_2 was added to initiate clot formation and the time taken for the visible clot to appear from the time of addition of CaCl_2 was recorded in seconds (s). Tris–HCl buffer alone was

Fig. 1 **a** Fibrinogenolytic activity of cupincin: 120 μ L of 5% human fibrinogen was incubated with 20 μ g of cupincin at 37 $^{\circ}$ C. 20 μ L of the reaction mixture was taken at different time interval and the reaction was terminated by adding 20 μ L of reducing sample buffer and subjected for 10% SDS-PAGE. Lane 1: Cupincin alone, Lane 2: 0 min, Lane 3: 1 h, Lane 4: 2 h, Lane 5: 3 h, Lane 6: 4 h, Lane 7:

Low range molecular mass marker. **b** Plasma re-calcification time of cupincin increasing concentration of cupincin (0–60 μ g) in 30 μ L volume containing 50 mM Tris–HCl, pH-7, was pre incubated with 200 μ L human plasma at 37 $^{\circ}$ C for 5 min. The clotting time was determined visually after adding 20 μ L of 0.25 M CaCl_2 . The results are expressed as mean $\pm$ SD, $n = 3$

used as a control instead of cupincin. The results were expressed as mean $\pm$ SD of three experiments.

Prothrombin Time (PT) Test and Activated Partial Thrombin Time (aPTT) Test

PT and aPTT test was carried out as previously described [13]. Briefly for PT various amount of cupincin in 50 μ L of Tris–HCl (50 mM, pH- 7) was pre-incubated with 50 μ L of human PPP at 37 $^{\circ}$ C for 1 min and 100 μ L of PT reagent was added to initiate the clot formation. The clotting time with Tris–HCl was considered as normal clotting time. For APTT test the varying concentration of cupincin in 50 μ L Tris–HCl was incubated with 50 μ L of human PPP and 50 μ L of APTT reagent for 3 min at 37 $^{\circ}$ C. The clot formation was initiated by adding 50 μ L of 25 mM CaCl_2 . The clot formation time with Tris–HCl alone was considered as normal clotting time.

Thromboelastometry Analysis

CT (clotting time, in seconds), CFT (clot formation time, in seconds) and MCF (maximum clot firmness, in mm) of the citrated whole blood was quantified by using Rotem® analyzer (Rotem® Pentapharm GmbH diagnostic division; Munich, Germany), where various concentration of cupincin in 20 μ L of Tris–HCl (50 mM, pH-7) was mixed with 20 μ L of 200 mM CaCl_2 . To this reaction mixture, 300 μ L of citrated whole blood was added and clot formation was observed for over 50 min. Clot formation function with only Tris–HCl was considered as control.

Sonoclot analysis

Sonoclot coagulation and platelet function analyzer (Sienco, Inc, USA) was used to monitor clot detection, clot rate and platelet function (clot retraction) where a glass bead activated test tube (gbACT + Kit obtained from Sienco, Inc, USA) was used. Cupincin in 20 μ L of Tris–HCl (50 mM, pH-7) was added to 300 μ L of citrated whole blood followed by 20 μ L of 200 mM CaCl_2 . Subsequently, after 10 s of switching on the analyzer, the head assembly of it was closed and Signature viewer software (Sienco, Inc.) was used to analyze the acquired data.

Results

Cupincin preferentially hydrolysed the A α -chain of human fibrinogen upon prolonged incubation B β -chain of fibrinogen was also hydrolysed, while γ -chain of fibrinogen was resistant irrespective of the incubation time of cupincin (Fig. 1a). Cupincin exhibited procoagulant activity in dose-dependent manner. Cupincin reduced the re-calcification time of human citrated plasma, the clotting time reduced from 212 $\pm$ 6 to 30.5 $\pm$ 2 s at 50 μ g cupincin concentration (Fig. 1b). However, cupincin was not able to clot human plasma in the absence of CaCl_2 . Cupincin did not hydrolyze the partially cross-linked fibrin clot or soft clot, α -chain, β -chain, and γ - γ dimer of fibrin clot remained intact in the SDS-PAGE under reducing conditions (Fig. 2a). Fibrinolytic activity of cupincin was further clarified by semi-quantitative agarose plate method where

Fig. 2 Fibrinolytic activity of cupincin: **a** 50 µL of platelet poor plasma was mixed with equal volume of 0.25 M CaCl_2 for 15 min to form soft fibrin clot. Fibrin clot was incubated with 0–40 µg of cupincin for 2 h at 37 °C. Reaction was stopped by adding 50 µL of reducing sample buffer. Lane 1: Plasma clot alone, Lane 2: Plasma + 10 µg cupincin, Lane 3: Plasma + 20 µg cupincin, Lane 4: Plasma + 30 µg cupincin, Lane 5: Plasma + 40 µg cupincin, Lane 6: Plasma + 50 µg cupincin, Lane 7: Plasma + Urokinase (2.5U).

b Semi-quantitative method of fibrinolytic activity of cupincin a mixture consisting of 2 mL of Platelet Poor Plasma, 3 mL of 1.2% agarose in 10 mM Tris-HCl, 0.15 M NaCl, 0.05% sodium azide and 0.25 M CaCl_2 was poured into flat Petri dish and left for 2 h at 37 °C. Cupincin (10–40 µg) in 10 mM Tris-HCl buffer, pH 7.0 and 2.5 units of Urokinase was independently placed on the surface and incubated overnight. Then 0.01% TCA was added

Table 1 Summary of APTT and PT analysis of cupincin

Sample	APTT (s)	PT (s)
Control	27.5 ± 0.5	12.0 ± 0.6
Cupincin (20 µg)	28.5 ± 1	12.7 ± 0.2
Cupincin (40 µg)	26 ± 2	13 ± 0.4
Cupincin (60 µg)	26 ± 3	13 ± 0.4
Cupincin (80 µg)	30 ± 2	13.2 ± .06
Cupincin (100 µg)	31.5 ± 3	13.3 ± 0.8

The results are expressed as mean ± SD

only urokinase (positive control) showed activity (Fig. 2b, c).

Cupincin did not show any effect on APTT test even at the concentration of 100 µg and was similar to the control clotting time of 27.5 ± 0.5 s. Similarly, 100 µg of cupincin was not able to show any significant effect on prothrombin time, where control clotting time was 12.0 ± 0.6 s (Table 1).

Procoagulant effect of cupincin was reconfirmed by using citrated whole blood in Rotem® analyzer by evaluating parameters such as CT, CFT and MCF. Cupincin reduced the clotting time (CT) to 218 ± 3 and 183 ± 1 s respectively at 24 and 40 µg concentration in comparison with the control CT of 252 ± 4 s. Cupincin reduced the clot formation time (CFT) to 259 ± 4 and 210 ± 2 s respectively at 24 and 40 µg concentration in comparison with the control CFT of 266 ± 3 s. However, cupincin did not cause any significant change on maximum clot firmness (MCF) and was similar to the control value of 53 ± 1 mm at the tested concentrations of cupincin and also the lysis

index-LI30 and LI45 values of cupincin were similar to the control value (Table 2; Fig. 3a–d).

Analysis of coagulation status using sonoclot analyzer indicated ACT in the presence of 40 µg of cupincin was 136 ± 3 s and clot rate was found to be 35 ± 0.82 s. Whereas when buffer alone was used as the control, ACT was recorded to be 166 ± 6 s and clot rate was determined to be 21 ± 0.64 s. Cupincin did not cause any significant change to the platelet function (Table 3; Fig. 4).

Discussions

Cupincin a metalloprotease with limited proteolysis was isolated purified and characterized in depth in our previous report [1]. In the current study, the possible mechanism(s) of action of cupincin in blood coagulation cascade and other haemostatic effects have been investigated. Cupincin hydrolysed human fibrinogen time-dependently; the corresponding band of Aα-fibrinogen vanished with the appearance of low molecular weight bands in SDS-PAGE under reducing conditions, classifying cupincin as α fibrinogenase. Generally, proteases isolated from plant latex exhibiting procoagulant activity are also reported to show fibrinolytic activity (plasmin-like activity) [4–8]. However, unlike procoagulant enzymes from plant latex proteases, cupincin did not hydrolyze plasma clot as evident in SDS-PAGE and in the semi-quantitative fibrinolytic assay on an agarose plate method indicating that it lacks ‘plasmin-like’ activity. Lack of ‘plasmin-like’ activity of cupincin was also evident by no change in the LI30 and LI45 values in sonoclot analyzer. Selective fibrinogenase activity and lack of ‘plasmin-like’

Table 2 Thromboelastometric analysis of cupincin by Rotem

Sample	Clotting time (s)	Clot formation time (s)	MCF (mm)	Lysis index (LI 30) (%)	Lysis index (LI 45) (%)
Control	252 ± 4	266 ± 3	53 ± 1	100	100
Cupincin (24 µg)	218 ± 3	259 ± 4	53 ± 2	100	100
Cupincin (40 µg)	183 ± 1	210 ± 2	55 ± 2	100	100

The results are expressed as mean ± SD

Fig. 3 Re-calcification time of cupincin using citrated whole blood by Rotem: varying concentrations of cupincin was mixed with Citrated whole blood (300 µL). Clotting was initiated by adding 50 µL of 25 mM CaCl₂ and subjected to Rotem analysis. The parameters such as clotting time (CT), clot formation time (CFT)

were recorded in seconds and maximum clot firmness (MCF) was express in mm. **a** control, **b** in presence of 24 µg cupincin, **c** in presence of 40 µg cupincin, **d** overlay of control and tested concentrations of cupincin

activity can prove to be pharmacologically important and can be exploited for treatment and management of wide array inherited or acquired bleeding disorders.

Cupincin reduced the plasma re-calcification time of citrated human plasma dose-dependently and was dependent on calcium ion for coagulation indicating that this

Fig. 3 continued

metalloprotease needs calcium ion for coagulation. Cupincin did not show any significant effect on APTT and PT time indicating its non-interference in extrinsic and intrinsic pathways of coagulation.

Qualitative and quantitative assessment of functional status of coagulation in citrated whole blood with respect to variations in coagulation time, clot formation, strength of the clot and clot lysis are analysed by using Rotem® in vitro [16]. The limitations of routine coagulation tests using PPP are overcome by whole blood coagulation analyses. The condition mimics the physiological status of blood by involving interaction among coagulation system, platelets and RBC's. Stages of development and resolution

of clot (clotting time, clot formation time, strength and stability of clot, fibrinolysis) are monitored by Rotem® analysis [17, 18]. The results of experiments using citrated whole blood by Rotem® were in correlation with the results of re-calcification experiments using PPP. Reduction in CT (CT-Is time from the start of measurement until the initiation of clotting) and CFT (CFT-Is time from initiation of clotting until clot of 20 mm thickness is formed) indicated that cupincin exhibited positive procoagulant effect towards citrated whole blood. Cupincin did not show any significant effect on MCF, MCF depends on the increasing stabilization of the clot by the polymerized fibrin, thrombocytes as well as factor XIII [19].

Table 3 Sonoclot analysis of cupincin

Sample	Activated clotting time (ACT) (s)	Clot rate (CR)	Platelet function
Control	166 ± 6	21 ± 0.64	4.2 ± 0.032
Cupincin (40 µg)	136 ± 3	35 ± 0.82	3.3 ± 0.068

The results are expressed as mean ± SD

Fig. 4 Sonoclot analysis of cupincin: parameters such as ACT, CT and platelet function was determined using sonoclot analyser

The analysis by sonoclot confirmed that cupincin is procoagulant in nature. Decrease in ACT (ACT-is the measure of change in viscosity of the sample from the initiation of the test where the liquid sample is converted to fibrin gel by cleaving fibrinogen) indicates that cupincin is cleaving fibrinogen resulting in the formation of fibrin gel. This was in correlation with the decrease in CT (CT-is the automated result of sonoclot analyzer. The first rise in Sonoclot Signature is the period during which fibrinogen forms a fibrin gel. During initial gel formation, the maximum slope of the sonoclot signature is clot rate) indicating a stable fibrin gel formation.

Conclusions

The objective of thrombolytic therapy is rapid and specific fibrinolysis thereby lowering the attendant hemorrhagic risk of the administration of such an agent. Several proteases affecting blood coagulation and fibrinolysis have been isolated and well characterized from snake venoms, spider venoms, leeches, annelids, insects, caterpillar, algae and from other microbial sources. So far proteases from a nontoxic source like rice which affect coagulation and fibrinolysis, have not been isolated and well studied. Based on the clotting time of re-calcified plasma, cupincin exhibits procoagulant activity. Global haemostasis tests like

Sonoclot and Rotem® analysis also demonstrated that cupincin was procoagulant in nature. More precisely, cupincin is a α -fibrinogenase cleaving human fibrinogen. The results obtained also suggest the potential action of cupincin is in the common pathway of blood coagulation. It would be interesting to further elucidate the role of cupincin in the blood coagulation cascade which will pave a way for further studies in understanding the applicability of this protease related to the development of diagnostic reagents and in the treatment of haemostatic disorders. Nevertheless, this study demonstrates the potential of cupincin as a new source of bioactive molecules for therapeutic purposes with particular emphasis on the development of procoagulant agents from non-toxic source like rice.

Acknowledgements We are grateful to Director, CSIR-Central Food Technological Research Institute (CFTRI), Mysuru, for his support and encouragement. The authors wish to thank Vilas Hiremath of Vijayashree diagnostic centre, Bengaluru for his help in Thromboelastometry and Sonoclot analysis. Roopesh S acknowledges the award of Senior Research Fellowship from Indian Council of Medical Research, New Delhi.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Sreedhar R, Kaul TP (2016) Cupincin: a unique protease purified from rice (*Oryza sativa* L.) bran is a new member of the cupin superfamily. PLoS ONE 11(4):e0152819. doi:[10.1371/journal.pone.0152819](https://doi.org/10.1371/journal.pone.0152819)
2. Costa JO, Fonseca KC, Garrote FMS, Cunha CC, de Freitas MV, Silva S, Araújo RB, Penha SN, de Oliveira F (2010) Structural and functional comparison of proteolytic enzymes from plant latex and snake venoms. Biochimie 2:1760–1765
3. Rajesh R, Shivaprasad HV, Gowda RC, Nataraju A, Dhananjaya BL, Vishwanath BS (2007) Comparative study on plant latex proteases and their involvement in hemostasis: a special emphasis on clot inducing and dissolving properties. Planta Med 73:1061–1067
4. Richter G, Hans PS, Friedrich D, Peter L (2002) Activation and inactivation of human factor X by proteases derived from *Ficus carica*. Br J Haematol 119:1042–1051
5. Shivaprasad HV, Riyaz M, Kumar VR, Dharmappa KK, Tarannum S, Siddesha JM (2008) Cysteine proteases from the Asclepiadaceae plants latex exhibited thrombin and plasmin like activities. J Thromb Thrombolysis 28:304–308
6. Rajesh R, Gowda CD, Nataraju A, Dhananjaya BL (2006) Purification and characterization of a 34-kDa, heat stable glycoprotein from *Synadenium grantii* latex: action on human fibrinogen and fibrin clot. Biochimie 88:1313–1322
7. Salas CE, Gomes MTR, Hernandez M, Lopes MTP (2008) Plant cysteine proteinases: evaluation of the pharmacological activity. Phytochemistry 69:2263–2269

8. Satish S, Sairam F, Ahmed AU (2012) *Moringa oleifera* Lam: protease activity against blood coagulation cascade. *Pharmacogn Res* 4:44–49
9. Priyanka U, Raghu RA, Poojitha B, Ranimol VR, Bindu J, Zohara N, Nanjunda S (2015) Laticiferous plant proteases in wound care. *Int J Pharm Sci* 7:44–49
10. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
11. Ouyang C, Teng CM (1976) Fibrinogenolytic enzymes of *Trimeresurus mucrosquamatus* venom. *Biochim Biophys Acta* 420:298–308
12. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
13. Diganta D, Nanjaraj U, Vilas H, Bannikuppe SB, Robin D (2013) Biochemical and biological characterization of *Naja kaouthia* venom from North-East India and its neutralization by polyvalent antivenom. *J Venom Res* 4:31–38
14. Devaraja S, Girish KS, Devaraj VR, Kemparaju K (2010) Factor Xa-like and fibrin(ogen)olytic activities of a serine protease from *Hippasa agelenoides* spider venom gland extract. *J Thromb Thrombolysis* 29:119–126
15. Condrea E, Mammon Z, Aloof S, Devries A (1964) Susceptibility of erythrocytes of various animal species to the hemolytic and phospholipid spitting action of snake venom. *Biochim Biophys Acta* 84:365–375
16. Ferraris VA, Ferraris SP, Saha SP, Eugene AH, Constance KH, Royston BD, Charles RB, Higgins RSD, George D, Jeremiah RB (2007) Perioperative blood transfusion and blood conservation in cardiac surgery: The Society of Thoracic Surgeons and The Society of Cardiovascular Anesthesiologists clinical practice guideline. *Ann Thorac Surg* 83(5 Suppl):S27–S86
17. Hett DA, Walker D, Pilkington SN, Smith DC (1995) Sonoclot analysis. *Br J Anaesth* 75:771–776
18. Luddington R, Baglin T (2004) Clinical measurement of thrombin generation by calibrated automated thrombography requires contact factor inhibition. *J Thromb Haemost* 11:1954–1959
19. Lang T, Bauters A, Braun SL, Pötzsch B, Von Pape KW, Kolde HJ, Lakner M (2004) Multi-centre investigation on reference ranges for ROTEM thromboelastometry. *Blood Coagul Fibrinolysis* 16:301–310