Amino Acids (2010) 39:481–488
DOI 10.1007/s00726-009-0464-z

ORIGINAL ARTICLE

Peptide analogues of 1811–1818 loop of the A3 subunit of the light chain A3-C1–C2 of FVIII of blood coagulation: biological evaluation

K. Patsialas · C. Koutsas · P. Makris · Maria Liakopoulou-Kyriakides

Received: 9 October 2009 / Accepted: 22 December 2009 / Published online: 22 January 2010

Abstract Factor VIII, the plasma protein deficient or defective in individuals with hemophilia A, is a critical member of the blood coagulation cascade. Recent studies have identified the FVIII light chain region Glu1811-Lys1818 as being involved in FIXa binding and in the assembly of the FX-activating FIXaz–FVIIIa complex. Based on this, a series of 12 peptides, analogues of the 1811–1818 loop of the A3 subunit of the light chain A3-C1–C2 of FVIIIa, were synthesized and evaluated for their anticoagulant activity. Only peptide Ac-ETKTYFWK-NH_2 showed significant anticoagulant activity by inhibiting about 40% factor VIII at a concentration of 0.43 mM. It also showed a prolongation of activated partial thromboplastin time of 6.1 s, whereas its effect on prothrombin time measurements was meaningless. All the other peptides did not show any measurable effect at the concentration of 0.43 mM. These findings are encouraging though further investigation of the effect of this active peptide in different biological settings is needed in order to evaluate its possible clinical applications.

Keywords Factor VIII · Synthetic peptides · Anticoagulant activity · aPTT · PT

Introduction

Blood coagulation represents an important defence mechanism against bleeding. The coagulation system consists of a series of stepwise, coordinated reactions involving specific plasma proteins and blood cells that culminate in the formation of an insoluble clot (Hirsh 2003; Hee-Yeon et al. 2008). The physiological function of the blood coagulation system is the prevention of blood loss after injury. However, certain events such as damage to the vessel wall or changes in blood flow can produce changes in the processes of coagulation that result in the pathological event of thrombosis (Hirsh 2003). Thromboembolic diseases are a major cause of mortality worldwide (Spiegel et al. 2004). Traditional antithrombotic treatments mostly consist of low-molecular weight heparins as well as inhibitors of vitamin K, such as coumarin. Both of these anticoagulant families have shown undisputed efficacy in thromboembolism treatment but display a range of undesirable clinical side effects (as hemorrhagic events) which have created the need for new and improved antithrombotic agents (Hirsh 2003; Spiegel et al. 2004; Howard et al. 2007). These include inhibitors of the factor VIIa, Xa, IXa, and VIIIa. Factor XIIIa is a transglutaminase that catalyzes gamma-glutamyl–lysyl bonds between fibrin and other proteins involved in haemostasis. Peptides from various regions of factor XIII A-chain have been synthesized and studied for their effects on cross linking fibrin and other relative substrates (Achyuthan et al. 1993). Other peptide inhibitors of factor XIIIa such as tridegin (Finney et al. 1997) have also been reported. The blood coagulation mechanism consists of two pathways (extrinsic and intrinsic) that are initiated by the exposure of tissue factor groups of activated platelet membranes to circulating protein factors, respectively. Factor VIII in the presence of thrombin is activated and with FIXa, phospholipids and calcium forms the intrinsic tenase complex that proteolytically activates factor X to factor Xa. The activated factor X serine-protease then assembles with cofactor Va and II to form the

K. Patsialas · C. Koutsas · P. Makris · M. Liakopoulou-Kyriakides (✉)
Section of Chemistry, Department of Chemical Engineering, Aristotle University of Thessaloniki, 54006 Thessaloniki, Greece
e-mail: markyr@eng.auth.gr

prothrombinase complex, which in turn cleaves factor II to release thrombin (factor IIa) and finally results in clot formation (Spiegel et al. 2004). The inhibition of the formation of the FVIIIaz–FIXa complex by blocking the interaction sites of the two factors could be a possible mechanism for anticoagulant activity of novel drugs. Recent studies have identified the FVIII region Glu1811-Lys1818 of the A3 subunit of the light chain A3-C1–C2 as being involved (Lenting et al. 1996; Rodgers et al. 2006) in FIXa binding and in the assembly of the FX-activating FIXaz–FVIIIa complex. Here, we synthesized peptide analogues of that 1811–1818 region and evaluated them for anticoagulant activity.

Materials and methods

Reagents

All amino acids and derivatives used in this work were of the S-configuration and were purchased from CBL, Patras, Greece. 2-Chlorotrityl-chloride resin (2-CLTR), and the Rink amide MBHA resin were also from CBL, Patras, Greece. The solvents used were of analytical or HPLC grade.

Methods

TLC was performed on pre-coated plates of silica gel 60 F_{254} (Merck) using AcCN:H_2O (5:1) as solvent system, and the spots were visualized by UV light and with ninhydrin. The reaction products were purified by reverse-phase HPLC (LABALLIANCE Q-GRANT pump and Thermo Finnigan Spectra System UV 6000LP Photodiode Array Detector) and identified by Electron Spray Mass Spectra (ESI–MS) with a Micromass (Platform LC) mass spectrometer.

The biological experiments were conducted in vitro with the analyzer ACL Advance (Instrumentation Laboratory). APTT-SP Kit, ELECTRACHROME Factor VIII and PT-Fibrinogen Recombinant were used for the determination of activated partial thromboplastin time, factor VIII activity and prothrombin time.

Synthesis

The peptides were synthesized using 2-chloro-trityl-chloride (Fig. 1a) or ring amide MBHA resins (Fig. 1b) by Fmoc solid-phase peptide synthesis protocol. The attachment of the first amino acid to the 2-chloro-trityl-chloride resin was carried out in the presence of diisopropylethylamine (DIPEA). 2-CLTR resin (1.0 g) (Barlos et al. 1991; Koutsas et al. 2007) was swelled in DCM (10 ml) for 10 min; then 1 mmol of the C-terminal amino acid was added and the mixture was stirred gently. Subsequently, 2.5 mmol DIPEA were added; the mixture was left to react for 40 min and finally for another 10 min after the addition of 1 ml MeOH. The mixture was transferred to a solid-phase reactor, where it was filtered and washed three times with DCM:MeOH:DIPEA (85:10:5), three with dimethylformamide (DMF), two with i-PrOH, two with DMF, three with i-PrOH and two times with diethylether. Subsequently, the Fmoc group was removed with 20% (v/v) piperidine in DMF.

For the coupling reactions the method of carbodiimides was applied. Specifically, Fmoc-amino acid (threefold excess of the resin substitution) and 1-hydroxybenzotriazole (HOBt, 4.5-fold excess of the resin substitution) were dissolved in DMF (2–4 ml, depending on the amino acid) and diisopropylcarbodiimide (DIC, 3.3-fold excess of the resin substitution) was added. The solution was stirred thoroughly for 1–2 min and then transferred to the solid-phase reactor. The mixture was left, stirring periodically, to react for 3 h, and then it was filtered and washed. The progress of the reaction was followed by Kaiser's test (Kaiser et al. 1970). The Fmoc group was removed as mentioned earlier and the next amino acid was coupled. This procedure was repeated until the eight amino acids were coupled.

For the peptides attached to the Rink amide resin, the activation and coupling of the C-terminal amino acid was accomplished as reported by Tae-Kyung et al. (2008).

The Fmoc protecting group was removed from the resin as mentioned earlier. For the coupling reactions we used also the method of carbodiimides as described earlier.

Removal of protecting groups and cleavage of the peptides from the resins

For the 2-CLTR resin the cleavage was performed in two stages. First, the peptide–resin ester was suspended in a mixture of DCM:trifluoroethanol (TFE):AcOH (7:2:1) for 1 h at room temperature. The resin was filtered off and the filtrate was concentrated in vacuo. The crude protected peptide was precipitated by adding cold Et_2O, stored in the fridge for 24 h, collected by filtration, washed with ether on the filter and dried in vacuo over P_2O_5. At the second stage, the peptide was dissolved in 5 ml solution of trifluoroacetic acid (TFA):DCM:triethylsilane (TES) (75:20:5) and stirred gently for 2 h as to remove the side-protecting groups of the amino acids. The crude free peptide was then precipitated by adding cold Et_2O, stored in the fridge for 24 h, collected by filtration, washed with ether on the filter and dried in vacuo over P_2O_5.

For peptide amides attached to the Rink amide MBHA resin, the cleavage was performed at room temperature and

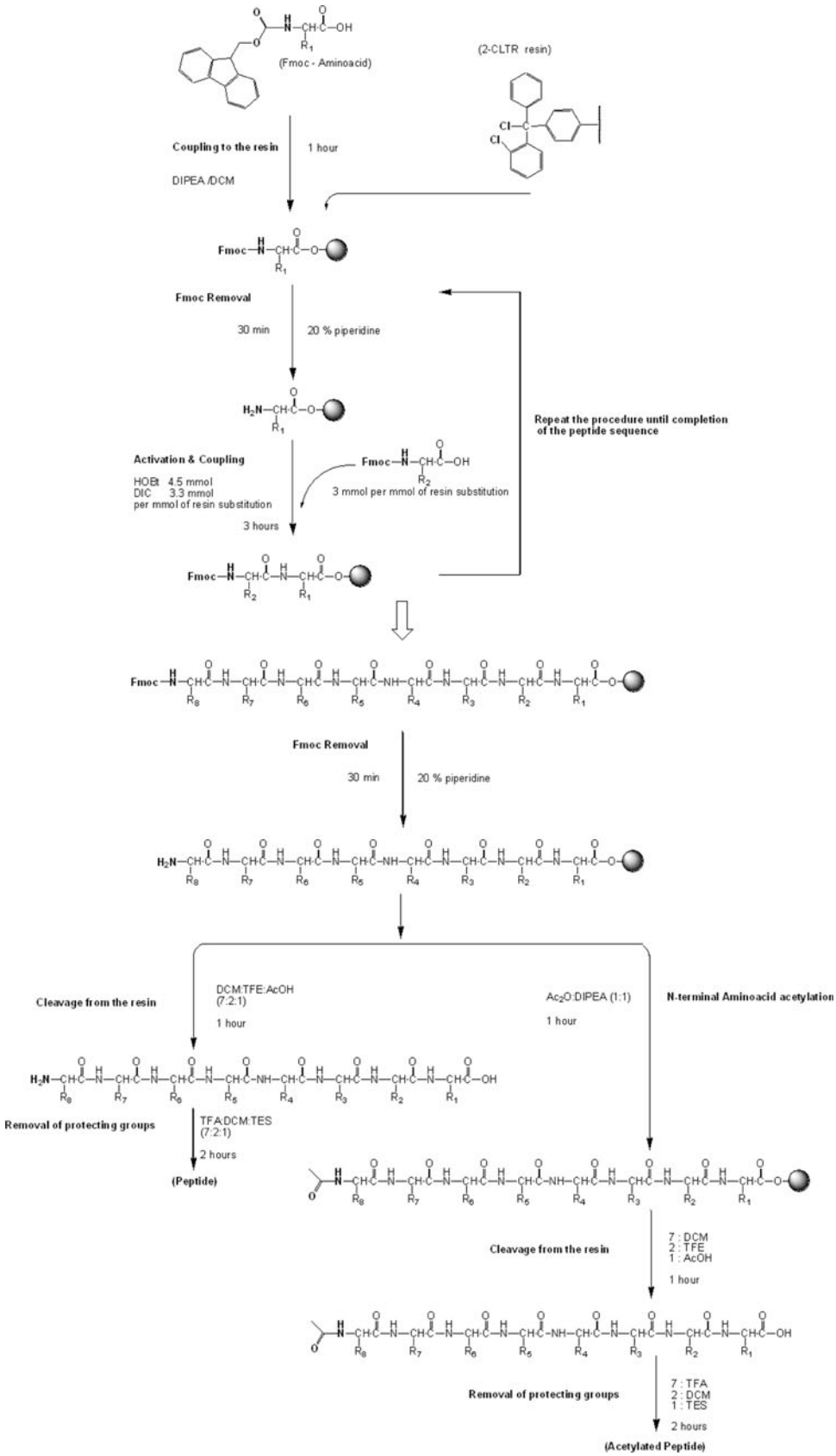
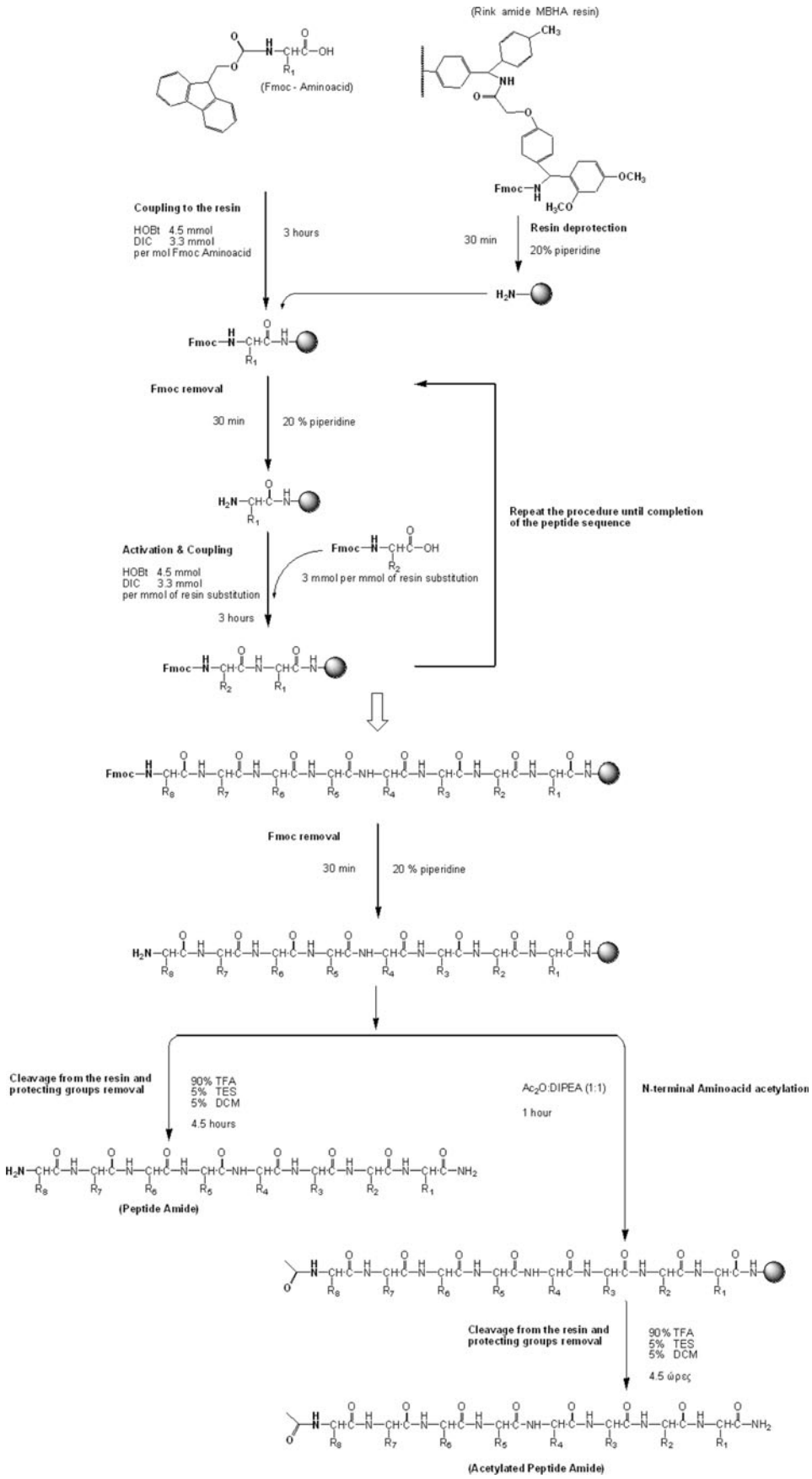


Fig. 1 Synthetic strategy for peptides PA1, PA3, PA5 and PA7 (**a**) and PA2, PA4, PA6, PA8-PA12 (**b**)

Fig. 1 continued



the protecting groups of the amino acids were removed simultaneously as the cleavage solution was acidic enough. Specifically, peptide–resin complex was suspended in a solution of TFA:TES:DCM (90:5:5) and left to react for 4.5 h, stirring periodically. The resin was filtered off and the filtrate was concentrated in vacuo. The crude peptide amide was precipitated by adding cold Et_2O, stored in the fridge for 24 h, collected by filtration, washed with ether on the filter and dried in vacuo over P_2O_5.

Acetylation

The resin-bound peptide (after the removal of Fmoc group) was suspended in equimolar mixture of (Ac_2O):DIPEA (tenfold excess of the resin substitution) for 1 h at room temperature. After that time the mixture was filtered and washed. The cleavage from the resin was performed as described earlier.

Purification of peptides

The end products in all cases were analysed and purified by preparative RP-HPLC. Elution was performed using water and acetonitrile, acidified with 0.05% trifluoroacetic acid. The column used was an RP-C_{18} and linear gradient program was developed. Fractions containing the peptides were pooled and lyophilized. The peptides were determined to be homogenous by analytical HPLC and identified by ESI–MS.

Anticoagulant assay

Anticoagulant activity was determined by the activated partial thromboplastin time (aPTT) and prothrombin time (PT) clotting assays. Human blood was collected from healthy individuals into plastic tubes containing anticoagulant (1:9, 0.129 M trisodium citrate). The citrated blood was immediately centrifuged at 2,000×*g* for 10 min at room temperature to obtain the platelet-poor plasma (PPP). A 300-μL aliquot of PPP was transferred into the measuring cell of the instrument, and 300 μL of peptide solution (in Owren-Koller buffer, pH 7.4) was added to a final concentration of 0.43 mM, and the cell was incubated at 37°C for 30 min. As control solution we used 300 μl of PPP and 300 μL of Owren-Koller buffer incubated under the same conditions. After incubation the samples were measured in the analyzer.

The assay of aPTT includes the addition of contact activator at 37°C for a specific period of time. Calcium is then added to trigger further reactions, and the time required for clot formation was measured as it is reported (Hee-Yeon et al. 2008; ten Boekel et al. 2007). The APTT-SP kit contains synthetic phospholipids and silica to ensure a highly reproducible and stable product (Van den Besselaar et al. 1993; Ray and Hawson 1989).

The assay for PT includes the addition of recombinant rabbit tissue factor (RTF) relipidated in a synthetic phospholipid blend and combined with calcium chloride, buffer and preservatives. The addition of the reagent to the plasma initiates the activation of the extrinsic pathway. This results ultimately in the conversion of fibrinogen to fibrin, with formation of a solid gel. Fibrinogen is quantitated by relating the absorbance or light-scatter during clotting to a calibrator as reported (Hee-Yeon et al. 2008).

Inhibition of blood coagulation factor VIII assay

Factor VIII is a high-molecular-weight plasma protein which serves as a cofactor to factor IXa in the activation of factor X to factor Xa. By using optimal amounts of Ca^{2+}, phospholipids, factor IXa and excess factor X, the rate of activation of factor X is linearly related to the amount of factor VIII. Factor Xa hydrolyses a chromogenic substrate, liberating the chromophoric group, *p*-nitroaniline (pNA). The colour is then read photometrically at 405 nm (Barrowcliffe et al. 2002). The factor Xa generated, and thus the intensity of colour, is proportional to the factor VIII activity in the sample (Mertens and Bertina 1985; Cinotti et al. 1991; Van der Velde and Poller 1995).

The measurements were made using the automatic analyzer ACL Advance and the results were automatically translated into % activity. Standard solutions of factor VIII, at various concentrations, were made for a reference curve of absorbance versus % activity of FVIII. For the inhibition studies, peptides (in Owren-Koller buffer, pH 7.4) and recombinant FVIII (1 U mL^{-1} in Owren-Koller buffer, pH 7.4) and 300 μL of each solution were transferred into a measuring cell to final concentration 0.43 mM and incubated at 37°C for 30 min. For control, 600 μL solution of recombinant FVIII (1 U ml^{-1} in Owren-Koller buffer) was used and incubated under the same conditions.

Results and discussion

A series of 12 peptides were synthesized and evaluated for their anticoagulant activity. A general synthetic pathway of these peptides is shown in Fig. 1a and b. The first one, peptide PA1, resembles the 1811–1818 sequence of the A3 subunit of the light chain A3-C1–C2 of FVIIIa. The other six (PA2–PA7) include peptide analogues of PA1 with N-terminal amino group either free or protected with acetyl-group and the C-terminal amino acid either with free carboxyl group or modified to amide. Peptide PA8 was obtained by replacing glutamic with aspartic acid and peptides PA9 and PA10 by substituting Tyr and Phe,

respectively, with Ile. Two shorter *N*-acetylated peptides PA11 and PA12 were also obtained and examined for anticoagulant activity. The amino acid sequence of the synthesized peptides and their masses are given in Table 1. All peptides were purified by HPLC and identified by ESI–MS. These peptides were tested for anticoagulant activity in vitro at concentrations ranging from 1 to 10 mM. More specifically, activated partial thromboplastin time, prothrombin time and the inhibition of factor VIII were measured. In Fig. 2 the results of HPLC analysis and ESI–MS of peptide PA4 (the main peak in the chromatogram) are given, which, as will be shown in the following, presented the most significant values, as far as these biological activities are concerned.

The results of the measurement of the activated partial thromboplastin time of the 12 peptides at concentration of 0.43 mM are depicted in Fig. 3. The measurement of aPTT is used in general as a screening procedure for the evaluation of the intrinsic coagulation pathway. The prolongation of it against the normal values indicates inhibition of the coagulation process. As can be seen in Fig. 3, all peptides presented a tendency to prolongate aPTT; peptide PA4 presented the highest value 6.10 s on estimated concentration of 0.43 mM, thus indicating inhibition of coagulation process. The effect of PA4 at various concentrations, ranging from 0.2 to 8 mM on the prolongation of aPTT is shown in Fig. 4. The IC_{50} value as it was calculated by non-linear regression fitting is 0.59 mM.

Factor VIII is a high-molecular-weight plasma protein which serves as a cofactor to factor IXa in the activation of factor X to factor Xa. Factor VIII is usually tested by one- or two-stage clotting assays. Here, the chromogenic method which is more precise and is the reference method of the European Pharmacopoeia and the International Society on Thrombosis and Haemostasis was used. By using optimal amounts of Ca^{2+}, phospholipids, factor IXa and excess factor X, the rate of activation of factor X is linearly related to the amount of factor VIII. Factor Xa hydrolyses a chromogenic substrate, liberating the chromophoric group, *p*-nitroaniline (pNA). The colour is then read photometrically at 405 nm (Barrowcliffe et al. 2002). The factor Xa generated, and thus the intensity of colour, is proportional to the factor VIII activity in the sample (Mertens and Bertina 1985; Cinotti et al. 1991; Van der Velde and Poller 1995).

The estimation of the inhibition of FVIII activity by these peptides is given in Fig. 5. As it can be seen in this figure, peptide PA4 presented the most significant inhibitory effect compared with the other analogues examined. This assay is indicative of the influence of the peptides in the FVIIIa and more specifically in the interactions between FIXa and FVIIIa and the formation of the intrinsic tenase complex. Interestingly, this finding is in agreement with the results of the aPTT measurement, presenting evidence for the involvement of this peptide in the intrinsic pathway of blood coagulation. As shown in Figs. 3 and 5, respectively, tetra-peptide PA11 and hexa-peptide PA12 did not show any activity, suggesting that PA4 represents the minimal amino acid sequence required for biological activity.

On the other hand, all peptides including PA4 did not show any significant prolongation of prothrombin time as it can be seen from Fig. 6. This in combination with the above results further enhances the evidence for the role of peptide PA4 in the intrinsic blood coagulation cascade. In vitro assays and studies have shown that coagulation cascade comprises two somewhat independent pathways (extrinsic and intrinsic) that converge on a common pathway with thrombin generation as the end point. The currently available anticoagulants are limited by modest therapeutic benefits, increased bleeding risk and drug-reduced thrombophilia (Howard et al. 2007). It has been

Table 1 Peptide analogues of the l811–1818 loop of the A3 subunit of the light chain A3-C1–C2 of FVIIIa

a/a	Peptides	MW
PA1	Glu^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-COOH	1,102.2
PA2	Glu^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-NH_2	1,101.2
PA3	Ac-Glu^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-COOH	1,144.3
PA4	Ac-Glu^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-NH_2	1,143.3
PA5	Asp^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-COOH	1,088.2
PA6	Asp^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-NH_2	1,087.2
PA7	Ac-Asp^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-COOH	1,130.3
PA8	Ac-Asp^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-NH_2	1,129.3
PA9	Ac-Glu^{1811}-Thr^{1812}-Lys^{1813}-Thr1^{814}-Ile^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-NH_2	1,093.3
PA10	Ac-Glu^{1811}-Thr^{1812}-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Ile^{1816}-Trp^{1817}-Lys^{1818}-NH_2	1,109.3
PA11	Ac-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-NH_2	683.8
PA12	Ac-Lys^{1813}-Thr^{1814}-Tyr^{1815}-Phe^{1816}-Trp^{1817}-Lys^{1818}-NH_2	913.1

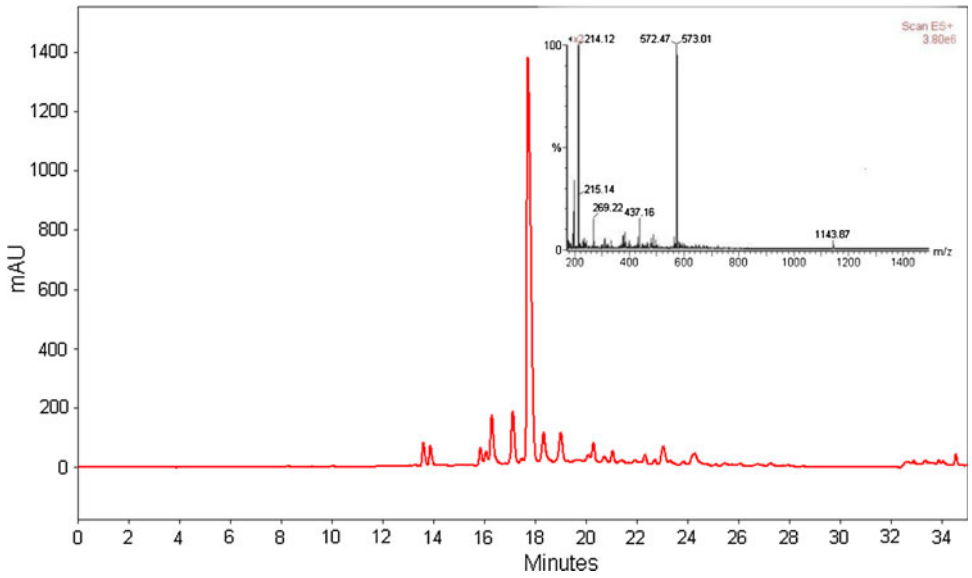


Fig. 2 HPLC analysis and ESI–MS of peptide PA4

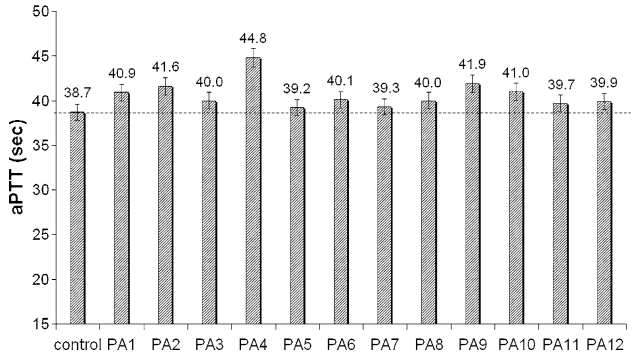


Fig. 3 Activated partial thromboplastin time measurements (s) of the peptides PA1–PA8

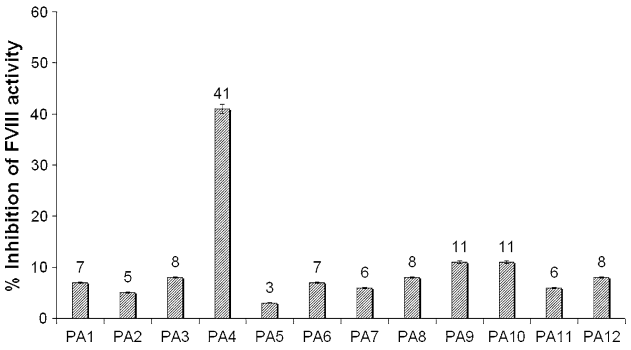


Fig. 5 Inhibition of FVIII by peptides (PA1–PA12) at concentration of 0.43 mM

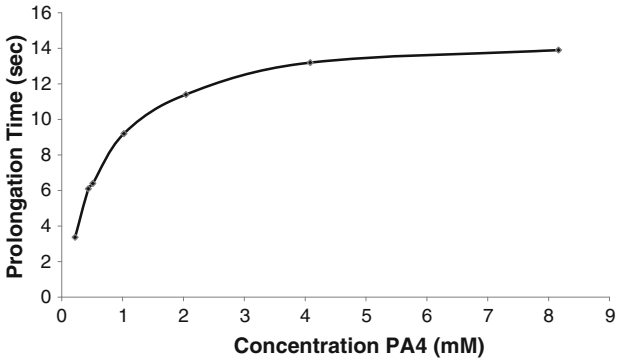


Fig. 4 aPPT times (s) of PA4 at various concentrations

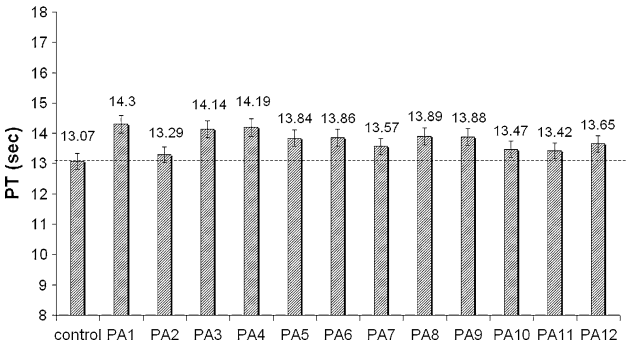


Fig. 6 Prothrombin time measurements (s) of peptides PA1–PA12

also found that anticoagulants such as low-molecular weight heparins and specifically fontaparinux inhibit both FXa and FIIa through interaction with thrombin. In the circumstances, intrinsic anticoagulants may present the advantage to evade hemorrhagic effects in case of injury. Taking into consideration that thrombosis and hemostasis in vivo are rather complicated events, further investigation of the anticoagulant effect of peptide Ac-ETKTYFWK-NH_2 (PA4) in relative biological settings is needed before we make any rationalization.

Acknowledgments This Research Project is co-financed: 80% by European Union-European Social Fund and 20% by General Secretary Research & Technology (PENED 03ED569).

References

Achyuthan KE, Slaughter TF, Santiago MA, Enghild JJ, Geenberg CS (1993) Factor XIIIa-derived peptides inhibit transglutaminase activity. Localization of substrate recognition sites. J Biol Chem 268:21284–21292

Barlos K, Chatzi O, Gatos D, Stavropoulos G (1991) 2-Chlorotrityl chloride resin. Studies on anchoring of Fmoc-amino acids and peptide cleavage. Int J Peptide Protein Res 37:513–520

Barrowcliffe WT, Raut S, Sands D, Hubbard RA (2002) Coagulation and chromogenic assays of Factor VIII activity: general aspects, standardization and recommendations. Semin Thromb Hemost 28:247–255

Cinotti S, Longo G, Messori A, Morfini M, Blomback M, Schimpf K (1991) Reproducibility of one-stage, two-stage and chromogenic assays of factor VIII activity: a multi-center study. Thromb Res 61:385–393

Finney S, Seale L, Sawyer RT, Wallis RB (1997) Tridegin, a new peptidic inhibitor of factor XIIIa, from the blood-sucking leech *Haementeria ghilianii*. Biochem J 324:797–805

Hee-Yeon J, Won-Kyo J, Se-Kwon K (2008) Purification and characterization of a novel anticoagulant peptide from marine echiuroid worm, *Urechis unicinctus*. Process Biochem 43:179–184

Hirsh J (2003) Current anticoagulant therapy—unmet clinical needs. Thromb Res 109:S1–S8

Howard EL, Becker KCD, Rusconi CP, Becker RC (2007) Factor IXa inhibitors as novel anticoagulants. Arterioscler Thromb Vasc Biol 27:722–727

Kaiser E, Colescott R, Bossinger CD, Cook PI (1970) Color test for detection of free terminal amino groups in the solid phase synthesis of peptides. Anal Biochem 34:595–598

Koutsas C, Sarigiannis Y, Stavropoulos G, Liakopoulou-Kyriakides M (2007) Conjugation of resveratrol with RGD and KGD derivatives. Protein Peptide Lett 14:1014–1020

Lenting PJ, van de Loo J-WHP, Donath M-JSH, van Mourik JA, Mertens K (1996) The sequence Glu^{1811}–Lys^{1818} of human blood coagulation Factor VIII comprises a binding site for activated Factor IX*. J Biol Chem 271:1935–1940

Mertens K, Bertina RM (1985) The role of factor VIII in the activation of blood coagulation factor X by activated factor IX. Thromb Haemost 54:654–660

Ray MJ, Hawson GAT (1989) A comparison of two APTT reagents which use silica activators. Clin Lab Haematol 11:221–232

Rodgers SE, Duncan EM, Barbulescu DM, Quinn DM, Lloyd JV (2006) In vitro kinetics of factor VIII activity in patients with mild haemophilia A and a discrepancy between one-stage and two stage-factor VIII assay results. Br J Haematol 136:138–145

Spiegel PC, Kaiser MS, Simon AS, Stoddard LB (2004) Disruption of protein-membrane binding and identification of small-molecule inhibitors of coagulation factor VIII. Chem Biol 11:1413–1422

Tae-Kyung L, Jeong-Hyun C, Jang-Woong B, Yoon-Sik L (2008) Preparation of MBHA resin by benzotriazole-mediated amidoalkylation. Tetrahedron Lett 49:5380–5382

ten Boekel E, Böck M, Vrielink GJ, Liem R, Hemdriks H, de Kieviet W (2007) Detection of shortened activated partial thromboplastin times: an evaluation of different commercial reagents. Thromb Res 121:361–367

Van den Besselaar AMHP, Neuteboom J, Bertina RM (1993) Effect of synthetic phospholipids on the response of the activated partial thromboplastin time to heparin. Blood Coagul Fibrinolysis 4:895–903

Van der Velde EA, Poller L (1995) The APTT monitoring of heparin—the ISTH/ICSH collaborative study. Thromb Haemost 73:73–81