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Linear Analogues Derived from the First EGF-like Domain of Human Blood Coagulation Factor VII: Enhanced Inhibition of FVIIa/TF Complex Activity by Backbone Modification through Aspartimide Formation

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Abstract: Coagulation factor VII bound to its cofactor tissue factor is the physiological initiator of blood coagulation. The interaction between factor VII and tissue factor involves all four of the structural modules found in factor VII, with the most significant contribution coming from the first EGF-like domain. In this study, the synthesis and biological activity of several analogues derived from the first EGF-like domain of FVII comprising the sequence 45–83 are reported on. The six cysteine residues found in the native protein were replaced by Abu. The peptides were isolated from a multicomponent mixture following standard Fmoc solid phase synthesis. Purification and characterisation of the heterogeneous product showed that aspartimide formation was a major side-reaction, occurring predominantly at the Asp^{46}-Gly^{47} and Asn^{57}-Gly^{58} dipeptides. Although relatively common in peptide synthesis, the extent to which this side-reaction had taken place was considered surprising. Reported herein are the analytical methods used to isolate and characterise several of the modified products. Also, the inhibitory effect of these peptides on the formation and enzymatic activity of the factor VIIa/tissue factor complex have been compared. Surprisingly, the peptide containing an *iso*-Asp residue at position 57 possessed 66-fold higher inhibitory activity compared with the original target peptide. A possible explanation for this increase in observed activity is presented.

Keywords: peptide synthesis; human FVII; EGF1-like domain; aspartimide formation; FX activation

INTRODUCTION

Tissue factor, a 45 kD *trans*-membrane glycoprotein, functions as a first line of defence against haemorrhage following tissue injury. Strategically located on a variety of cells within and around blood vessels [1], exposure of TF to the serum protein FVII, induces conversion of the zymogen FVII to the activated form FVIIa [2,3]. The TF–FVIIa complex then initiates blood coagulation in the presence of Ca^{2+} and negatively charged phospholipids [4]. The 50 kD serum serine protease FVIIa in combination with TF in turn catalyses the conversion of factors X and IX to their activated forms. This is an event that ultimately leads to the formation of an insoluble fibrin clot [5]. FVII consists of a light chain with a *N*-terminal γ-glutamic acid-rich (Gla) domain, two EGF-like domains and a heavy chain including the serine protease domain [6]. Although several studies have shown that all four domains of FVII are involved in the interaction with TF [7], the EGF-1

Abbreviations: Acm, acetamidomethyl; DIEA, diisopropylethylamine; ES-MS, electrospray ionisation mass spectrometry; FVII, coagulation factor VII; FVIIa, activated factor VII; FX, coagulation factor X; FXa, activated factor X; TF, tissue factor; IC_{50}, peptide concentration at which 50% inhibition of biological activity is observed; Abu, α-aminobutyric acid; NMP, *N*-methylpyrrolidone; EGF, epidermal growth factor; *iso*-Asp, β-Aspartic acid; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl.

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domain appears to make the most significant contribution to binding [8]. The role of the EGF-2-like domain in FVII–TF binding and coagulant activity has previously been investigated [9,10] and we have recently reported on synthetic peptides from the EGF-2-like domain that were potent inhibitors of FVIIa/TF activity [11]. In a similar manner the present study sets out to examine the inhibitory activity of a peptide sequence spanning the EGF-1 domain. During the synthesis of a linear peptide analogue, comprising the amino acid residues 45–83 of FVII, several side-reactions took place resulting in a complex mixture of products. Isolation and characterisation of approximately 90% of the by-products identified them as sequences modified through aspartimide formation. Deamidation of asparaginyl and aspartyl peptides is a well-studied side-reaction in peptide synthesis [12–14]. We report on the analytical methods used to isolate and identify two of the ring-modified peptides as well as the products formed from their hydrolytic ring opening. A comparative study of their biological properties highlighted a product consisting primarily of *iso*-Asp at position 57, which possessed a 66-fold increase in inhibition of the FVII/TF activity as compared with the target peptide. The significance of this modification with regard to structure–activity will be addressed. It is hoped that a better understanding of the critical interactions involved in TF/FVII binding may eventually facilitate the rational design of new inhibitor molecules for anticoagulant therapy.

MATERIALS AND METHODS

Reagents

Amino acid derivatives and general peptide synthesis reagents were purchased from Novabiochem AG, Switzerland and Applied Biosystems Inc., USA. Amino acid cartridges (1 mmol) were used with side-chain protecting groups as follows: *t*-butyl for Asp, Glu, Ser, Thr and Tyr, Trt for Asn, Gln and His, Pmc for Arg and Boc for Lys. All solvents were either peptide synthesis or HPLC grade and were used without further purification.

General Procedures

Analytical reversed phase HPLC was carried out on a Vydac, C18, 218TP54 column with gradient elution using a mobile phase where $A = 0.1\%$ TFA in H_2O and $B = 0.1\%$ TFA in acetonitrile (pH 2) or where $A = 0.1\%$ ammonium acetate in H_2O, and $B = 80\%$ acetonitrile in H_2O (pH 6). Preparative HPLC was performed on a Vydac C_{18}, 218TP1022 column using a gradient of 20–40% B over 120 min where $A = 0.1\%$ ammonium acetate in H_2O, and $B = 80\%$ acetonitrile in H_2O. The crude peptide was purified in 20 mg aliquots and fractions containing pure material combined and lyophilised following analysis by analytical HPLC. The pure peptides **1**, **2** and **3** were obtained in 22, 11 and 4.6% isolated yields, respectively. ES-MS analyses were carried out by M-Scan Ltd., Ascot, Berkshire, UK. Peptide sequencing analyses were carried out at the Biotechnology Centre, University of Oslo, Norway. Amino acid compositions were determined at the Amino Acid Analysis Laboratory, University of Uppsala, Sweden.

Peptide Synthesis

The peptide was assembled on Fmoc-AA-[Tenta Gel S AC] resin supplied by Rapp Polymere Gmbh, Tubingen, Germany using an Applied Biosystems 433A synthesiser on a 0.1 mmol scale. Each coupling cycle comprised: Fmoc deprotection using 20% piperidine in NMP, activation and coupling using 10-fold molar excess of Fmoc amino acid, HBTU, DIEA, HOBT with a coupling time of 45 min and capping of unreacted amino functions with a solution of acetic anhydride 4.7% v/v, DIEA 2.2% v/v, HOBt 0.2% w/v in DMF. The peptidyl resin was washed with dichloromethane and diethyl ether then dried *in vacuo*. The peptides were side-chain deprotected and cleaved from the resin using Reagent K [15] for 4 h. The mixture was filtered and the peptide solution evaporated *in vacuo*. The peptide was then precipitated and washed well with diethyl ether before air drying. The crude material was dissolved in 0.1% TFA and lyophilised.

Hydrolysis of the Peptide Aspartimide Intermediates 2 and 3

The pure peptides **2** and **3** (0.1 mg) were dissolved in a 0.25% ammonia solution (0.5 ml) and left at ambient temperature for 4 h. The hydrolysis was monitored by analytical HPLC until no starting material remained. The samples were then lyophilised and analysed for peptide content by amino acid analysis.

Inhibition of TF/FVIIa Complex Formation and Function: FX Activation Assay

Peptides **1**–**5**, at selected concentrations spanning the dose range 0.012–1 mM were incubated in Tris buffer (pH 7.5) at 37°C for 30 min. Lipidated TF (5 pM, American Diagnostica, Inc., Greenwich, USA) and calcium chloride (5 mM) were then added and the mixture left at room temperature for 40 min. FVIIa (5 pM; Novo Nordisk A/S, Gentofte, Denmark) and FX (50 nM; Enzyme Research Laboratory, South Bend, IN, USA) were then added along with the chromogenic FXa substrate S2765 (0.4 mM; Chromogenix, Mølndal, Sweden) used to monitor indirectly the formation of FXa. Readings were taken at 405 nm using a microtitre plate reader. The reaction was terminated by the addition of EDTA (0.3 M) and the IC_{50} values calculated from the dose response curves.

RESULTS AND DISCUSSION

The present study was undertaken in order to investigate the inhibition of FVIIa/TF complex formation by synthetic peptides selected from the EGF-1 domain of FVII. The major goal was to identify sequences possessing antithrombotic activity with a view to furthering our understanding of the key interactions that occur during binary complex formation.

A study has previously been reported on a group of Norwegian FVII-deficient patients homozygous for a mutation in the EGF-2 domain [9]. The mutation, Gln100Arg, was found to reduce the efficiency of binding of FVII to TF. Furthermore, results from an *ex vivo* model of human thrombus formation using the mutant protein gave only partial protection against acute thrombus formation at stenotic lesions [10]. Subsequent synthesis of peptides from this domain containing the native Gln at position 100 gave several sequences that selectively inhibited FVIIa/TF-dependent FX activation and coagulation of plasma [11].

The investigation described herein was designed to compare the biological activity of linear analogues derived from the EGF-1 domain of FVII. RP-HPLC analysis of a crude synthetic 39-mer comprising residues 45–83 of FVII initially revealed an apparently homogeneous product when eluted with a shallow H_2O/acetonitrile gradient containing 0.1% TFA, (pH 2) (Figure 1(a)). However ES-MS analysis of the crude material clearly showed that the peptide was contaminated with a second major product of molecular weight 18 Da lower than expected. Re-analysis using H_2O/acetonitrile gradients containing 0.1% ammonium acetate (pH 6) showed the crude product to be a multicomponent mixture with the major peak comprising ca. 50% of the total calculated peak area (Figure 1(b)).

The presence of several Asp-Gly and Asn-Gly dipeptides in the target peptide were believed to be the main cause of product heterogeneity, due to the fact that these sequences take part readily in aspartimide formation [16]. During Fmoc solid phase synthesis, aspartimide formation most commonly occurs when the resin bound peptide is treated with the base piperidine (Scheme 1) [17–19]. Under aqueous conditions the resulting cyclic intermediates can be converted to either the α- or β-peptide and their epimers [20]. The extent of this side-reaction has been shown to depend on a variety of factors, including amino acid sequence, solvent polarity and conformational preferences of the resin-bound peptide [21].

Following preparative HPLC of the crude peptide, three products were isolated in sufficient yield for biological evaluation. The major component was identified as the all L-amino acid peptide **1**, with the minor products, **2** and **3**, being made up of the

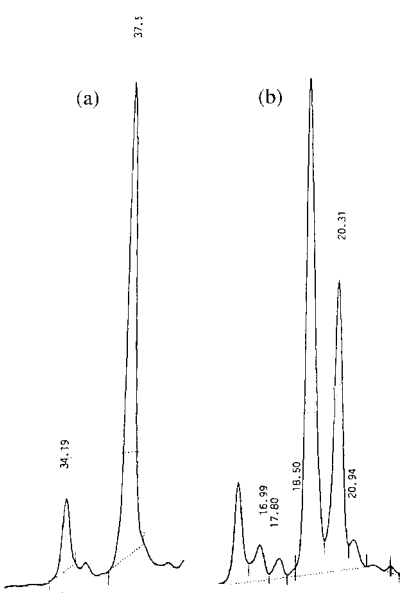
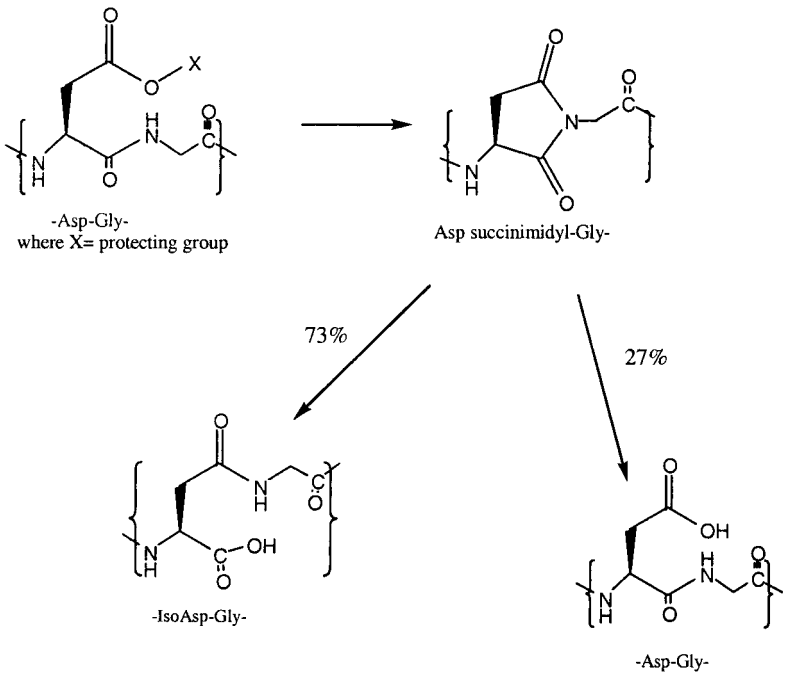


Figure 1 (a) Shows the crude peptide run on a RP C_{18} HPLC column eluted with a gradient of 20–35% *B* over 40 min, where $A = 0.1\%$ TFA/H_2O and $B = 0.1\%$ TFA/acetonitrile. (b) Shows the crude product run on a RP C_{18} HPLC column and eluted with a gradient of 30–40% *B* over 20 min where $A = 0.1\%$ ammonium acetate/H_2O (pH 6) and $B = 20\%$ H_2O/acetonitrile.



Scheme 1

cyclic aspartimide-containing sequences shown in Table 1. Hydrolytic ring opening of peptides **2** and **3** resulted in the generation of a further two products **4** and **5** consisting predominantly of the *iso*-Asp containing peptides derived from **2** and **3**, respectively.

This side-reaction was not observed in the synthesis of the EGF-2 domain of FVII [11], though the primary structure of this domain also contained several potential sites for aspartimide formation. This was in direct contrast to the EGF-2-like domain of FX, which has been reported earlier as a problem synthesis mainly due to aspartimide formation [17].

In order to fully characterise the sites of aspartimide formation, *N*-terminal sequencing was carried out on peptides **1**, **4** and **5**. Since *iso*-Asp residues are refractory to Edman degradation, significant drops in sequencing yield were expected to occur at the Asp or Asn residues where aspartimide formation had taken place. The sequencing results for peptide **4** showed a clear drop between Ser^{45} and Asp^{46} indicating the presence of an *iso*-Asp residue at this site (Table 2). Furthermore, from the sequencing data it was calculated that around 23% of the product contained L-aspartic acid an observation in agreement with previously published data [22]. A similar drop was observed during the sequencing of peptide **5** between the residues Gln^{56} and Asn^{57} with around 14% of the ring opening resulting in the all L-amino acid peptide **1**.

All products were further analysed by amino acid analysis, ES-MS and HPLC. The results were in agreement with the predicted sequences and are summarised in Table 3.

Table 1 Peptide Sequences Synthesised, Human FVII, EGF-1-like Domain 45–83

Peptide	Human FVII, EGF-1-like domain 45–83
1	NH_2-SDGDQUASSPUQNGGSUKDQLQSYIUFULPAFEGRNUET-OH
2	NH_2-SSu*GDQUASSPUQNGGSUKDQLQSYIUFULPAFEGRNUET-OH
3	NH_2-SDGDQUASSPUQSu[a]GGSUKDQLQSYIUFULPAFEGRNUET-OH
4	NH_2-S[Iso-D]GDQUASSPUQNGGSUKDQLQSYIUFULPAFEGRNUET-OH
5	NH_2-SDGDQUASSPUQ [Iso-D]GGSUKDQLQSYIUFULPAFEGRNUET-OH

[a] Aspartimide ring product.

Table 2 *N*-Terminal Sequencing Data for Peptides **1**, **4** and **5**, Residues 45–59

Yield (pM)	Ser	Asp	Gly	Asp	Gln	Abu	Ala	Ser	Ser	Pro	Abu	Gln	Asn	Gly	Gly
Peptide **1**	998	530	983	270	696	nd[b]	995	376	348	504	nd	455	352	492	427
Peptide **4**	752	54[a]	61	15	54	nd	70	15	28	38	nd	28	22	5	10
Pernde **5**	120	105	50	103	100	nd	55	20	20	26	nd	50	6[a]	2	nd

[a] Denotes significant drops caused by *iso*-aspartic acid unable to propagate the Edmans degradation further. The yields are given in pmol after background subtraction.

Peptides **1**–**5** were evaluated for inhibition of FVIIa/TF catalysed activation of FX. The results presented in Table 4 show that the parent peptide **1** possessed only weak inhibitory activity with an IC_{50} of around 70 μM. This was in contrast to the peptide from the EGF-2 like domain that possessed an IC_{50} value in the same assay of around 1.6 μM [11]. We found the comparitive drop in activity of peptide **1** a surprise due to the well-documented contribution to the total binding energy made by the EGF-1 like domain in the complex with TF [8]. The result may, however, reflect the loss of critical elements of structure on replacement of the cysteine residues with Abu, such as the key hydrogen bond between Asn^{57} and Cys^{81} [23,24].

Peptide **2**, containing the aspartimide ring between the residues Asp^{46} and Gly^{47}, was regarded inactive. Both Asp^{46} and Gly^{47} are known Ca^{2+} coordinating residues [8,25] and in peptide **2** this ability was lost due to the aspartimide ring formation. Loss of metal ion binding may, therefore, explain the abrogation in biological activity of this peptide. The activity of the ring opened product **4** derived from **2** containing primarily the *iso*-Asp residue at position 46 resulted in the concomitant re-introduction of an anionic oxygen atom. This appears to be a critical functionality at this position and may possibly involve metal ion coordination.

In direct contrast to the loss of activity of peptide **2** due to aspartimide formation we observed enhanced anticoagulant activity for pure peptide **3** containing the aspartimide ring between Asn^{57} and Gly^{58}. In this case, ring formation via the neutral side-chain of the Asn residue may help in the stabilisation of a set of active conformations not available to the parent peptide **1**. When this product was hydrolysed yielding product **5**, a 66-fold in-

Table 3 Analytical Data

Peptide	Amino acid analysis	ES-MS $(M+H)^+$	[a]HPLC/R_T (min)	Purity (%)
1	Asx 5.02(5); Thr 1.00(1); Ser 4.97(5); Glx 6.00(6); Pro 2.08(2); Gly 4.02(4); Ala 2.04(2); Ile 0.97(1); Leu 2.00(2); Tyr 0.94(1); Phe 2.01(2); Lys 0.98(1); Arg 1.01(1); Abu 5.96(6)	Calc. 4056.3; obtained 4056.5 ± 0.64	17.4	>98
2	Asx 4.95(5); Thr 0.99(1); Ser 4.94(5); Glx 5 92(6); Pro 2.04(2); Gly 4.06(4); Ala 2.04(2); Ile 1.02(1); Leu 2.05(2); Tyr 0.94(1); Phe 2.04(2); Lys 0.99(1); Arg 1.01(1), Abu 6.02(6)	Calc. 4038.3; obtained 4038.1 ± 0.11	18.7	>95
3	Asx 4.90(5); Thr 1.07(1); Ser 4.87(5); Glx 5.87(6); Pro 1.98(2); Gly 4.13(4); Ala 2.03(2); Ile 1.04(1); Leu 2.11(2); Tyr 1.05(1); Phe 2.08(2); Lys 1.01(1); Arg 1.11(1); Abu 5.76(6)	Calc. 4039.3; obtained 4038.2 ± 0.32	14.0	>98
4	Asx 4.81(5); Thr 1.04(1); Ser 4.85(5); Glx 5.85(6); Pro 2.06(2); Gly 4.00(4); Ala 1.95(2); Ile 1.01(1); Leu 2.04(2); Tyr 1.00(1); Phe 2.06(2); Lys 1.03(1); Arg 1.01(1); Abu 5.85(6)	Calc. 4056.3; obtained 4056.8 ± 0.14	17	>95[b]
5	Asx 5.02(5); Thr 0.99(1); Ser 5.03(5); Glx 5.94(6); Pro 20.03 (2); Gly 4.08(4); Ala 1.85(2); Ile 1.00(1); Leu 2.04(2); Tyr 1.04(1); Phe 1.99(2); Lys 1.04(1); Arg 1.05(1); Abu 5.95(6)	Calc. 4057.3; obtained 4057.1 ± 0.15	14.0	>95[c]

[a] HPLC gradient of 30–40% *B* over 20 min where *A* = 0.1% ammonium acetate in H_2O, and *B* = 80% acetonitrile in H_2O.
[b,c] Peptides **4** and **5** contained primarily an *iso*-Asp residue. Calculated values for the L-Asp analogue were obtained from sequencing and were 23 and 14%, respectively.

Table 4 Comparison of Anticoagulant Activity of Peptides **1**–**5** in a FX Activation Assay

Peptide	IC_{50} (μM)[a]
1	73 ± 8.7
2	450 ± 54
3	18 ± 2.2
4	50 ± 6.0
5	1.1 ± 0.13

[a] All values are corrected for peptide content by amino acid analysis. The results are given as the mean of at least three experiments.

crease in activity compared with the parent peptide was observed.

The ring opened product **5** was more hydrophilic due to the replacement of the neutral side-chain of Asn at position 57 with the acid function of the *iso*-Asp residue.

Furthermore, product **5** was found by analytical HPLC to be the most hydrophilic of all the products tested in the assay. It remains to be investigated whether the acidic function at position 57 of peptide **5** provides enhanced activity by stabilisation of active conformers through salt bridge formation or metal binding interactions. It is, however, hoped that the knowledge gained from these modified sequences will help in the design of more potent inhibitors of blood coagulation.

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