Volume 317, number 3, 245–249 FEBS 12105 February 1993

FVIIa derivatives obtained by autolytic and controlled cathepsin G mediated cleavage

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Received 10 December 1992

The heavy chain of coagulation factor VII contains a serine esterase entity. A partial cleavage in the heavy chain occurs during purification and activation of the single-chain zymogen, presumably as a result of autolysis. Neutrophil cathepsin G initially generates a Gla-domainless FVIIa without coagulant activity. However, on extended exposure cleavage also occurs in the heavy chain, resulting in a complete loss of enzyme activity. Four cleavage sites on the heavy chain, two susceptible to trypsin-like autolysis and two susceptible to chymotrypsin-like cathepsin G-mediated catalysis have been identified. The hydrolysis of peptide bonds in the heavy chain might contribute to regulation of the coagulation process in vivo.

FVIIa; Cathepsin G; SDS-PAGE; Amino acid sequence analysis; Electrospray mass spectrometry

1. INTRODUCTION

Coagulation factor VII consists of 406 amino acids with a serine esterase domain in the C-terminal part of the molecule [1]. The trypsin-like serine esterase becomes active towards its substrates FX and FIX after cleavage of a single peptide bond between Arg-152 and Ile-153, leaving two chains, covalently linked by one disulfide bridge. Several coagulation enzymes are capable of activating FVII, a process which is greatly enhanced by the binding to tissue factor and negatively charged phospholipids [2,3]. Single-chain FVII is converted to the two-chain form (FVIIa), when bound in sufficient density to charged surfaces [4,5], and evidence for autoactivation has been presented [6]. However, after activation, a minor degree of cleavage in the heavy chain of the purified plasma and recombinant FVIIa has been found [7]. Heavy chain cleavage of FVIIa was also observed after incubation with cathepsin G from human neutrophilic granulocytes [8]. We have identified four sites on the heavy chain, which are susceptible to proteolytic attack under non-denaturing conditions, and which might possibly be exposed to enzymes present during hemostasis and inflammation. Cleavage at these sites might be a first step in the in vivo degradation of FVIIa.

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Abbreviations: Gla, γ-carboxyglutamic acid; GD-L, Gla-domainless light chain; L, light chain; H, heavy chain; r, recombinant; RP, reverse phase; RT, retention time; ES-MS, electrospray mass spectrometry.

2. EXPERIMENTAL

Recombinant FVIIa [7], 1 mg/ml was dialysed against 10 mM Tris-HCl, pH 8.6, containing 75 mM NaCl and 5 mM $CaCl_2$. Calcium ions were complexed with EDTA, added in an excess of 5 mM, before the incubation with cathepsin G at 37°C. The final pH of the incubation mixture was 7.6. Cathepsin G, purified from human neutrophiles according to the method of Baugh and Travis [9] was a gift from Drs. H.J. Flodgaard and O. Nordfang (Novo Nordisk, Copenhagen).

Specific clotting activity was determined in a one-stage FVII clotting assay, based on FVII immunodepleted human plasma and rabbit thromboplastin.

The substrate for amidolytic activity was S-2288 Ile-Pro-Arg-*p*-nitroanilide (KABI Vitrum, Sweden). The rFVIIa was diluted to 0.15 μM in 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, pH 8.3, containing 0.1% PEG-6000, and substrate was added to a final concentration of 1 mM. The absorption at 405 nm was read after 30 min incubation at 37°C.

Reduction with DTT was performed overnight at room temperature in 0.3 M Tris-HCl, pH 8.1 containing 6 M guanidine hydrochloride. Iodoacetamide was added for carboxymethylation of free sulfhydryl groups. Excess reagents were removed by dialyses in tubing with a molecular cut-off of 3,500.

RP-HPLC, SDS-PAGE and amino acid sequence analysis were performed as previously described [7,8,10].

ES-MS analysis was performed using an API III LC/MS/MS system (Sciex, Thornhill, Ontario, Canada). The triple quadrupole instrument has a mass-to-charge (*m/z*) range of 2,400 and is fitted with a pneumatically assisted electrospray (also referred to as ion-spray) interface [11,12]. Sample introduction was done by a syringe infusion pump (Sage Instruments, Cambridge, MA) through a fused capillary (75 μm i.d.) with a liquid flow-rate set at 0.5–1 μl/min. The instrument *m/z* scale was calibrated with the singly charged ammonium adduct ions of poly(propylene glycols) under unit resolution.

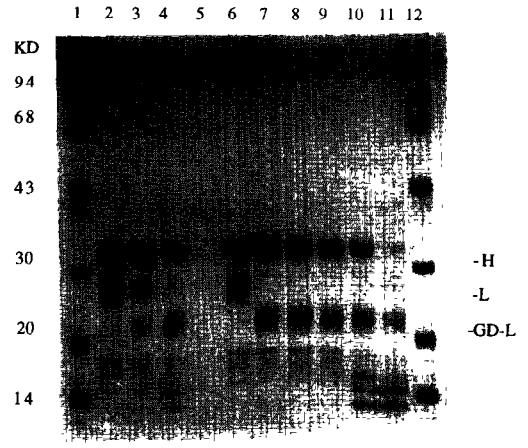
3. RESULTS

3.1. *Heavy-chain cleavage, present after purification and activation*

3.1.1. *SDS-PAGE, RP-HPLC, amino acid sequence.* Recombinant FVII is activated to rFVIIa during purification [5,7]. SDS-PAGE of the reduced molecule shows the H- and L-chain, and in addition traces of four bands in the region 16–19 kDa (Fig. 1, lane 2). RP-HPLC of the purified rFVIIa revealed two minor peaks eluted in front of the main protein [7]. The SDS-PAGE analysis of the first of these minor peaks showed a two-chain structure of 36 kDa, consisting of a 16 kDa band, covalently linked to the L-chain, and N-terminal amino acid sequence analysis confirmed the sequences for the intact L- and H-chain, starting at Ala-1 and Ile-153, respectively (data not shown). The second minor peak contained a single peptide chain of 17 kDa, with an N-terminal sequence starting at Gly-291 (Table I, sequence A).

The remaining two bands of 19 and 17 kDa (Fig. 1, lane 2) were present in the main protein peak from RP-HPLC. When the predicted residues for the L- and H-chain were disregarded, N-terminal amino acid sequencing revealed a sequence, starting at Lys-316, and corresponding to a cleavage in the third loop of the heavy chain (Table I, sequence B).

The observed SDS-PAGE pattern is consistent with a cleavage after Arg-290 (γ cleavage site in Fig. 4), resulting in an L-linked fragment 153–290 (lowest band of 16 kDa) and a non-covalent fragment 291–406 containing the N-linked carbohydrate at Asn-322 (band at 17 kDa), and a cleavage after Arg-315 (β cleavage site in Fig. 4), resulting in three covalently linked chains, the L chain, the fragment 153–315 (upper band of 19 kDa) and the fragment 316–406 carrying the *N*-glycan (band at 17 kDa). Since the heavy-chain cleaved forms could not be isolated under non-denaturing conditions, the clotting activity of the individual forms could not be analysed.



Time (min)	0	20	120	0	5	10	20	60	120
$CaCl_2$ (5 mM)	+	+	+	0	0	0	0	0	0
Clot activity (%)	100	35	9	100	0	0	0	0	0
Amidolytic act. (%)	100	ND	ND	100	ND	92	96	60	21

Fig. 1. SDS-PAGE and activity of cathepsin G degraded rFVIIa. rFVIIa (1 mg/ml) was incubated with cathepsin G (1/50 w/w) at 37°C in the presence and absence of calcium ions. At the indicated intervals, aliquotes were either diluted into 1% SDS, 1% DTT and boiled for SDS-PAGE, or diluted into Ca^{2+} containing buffer on ice for clotting analysis or diluted into substrate containing buffer for measuring amidolytic activity. Molecular weight markers were applied in lanes 1 and 12. ND, not determined.

3.2. *Heavy-chain cleavage by cathepsin G mediated catalysis*

3.2.1. *SDS-PAGE and enzymatic activity.* In the absence of calcium ions, and a ratio of cathepsin G to rFVIIa of 1:500 w/w, rFVIIa is completely converted to a Gla-domainless form within five minutes. At a higher enzyme/substrate ratio (1:50 w/w), a des (1–44) rFVIIa was identified as the main product after 20 min of incubation [8]. Heavy-chain cleavage is detectable by SDS-PAGE after 60 min of incubation, generating three chains (Fig. 1, lane 10), and the H-chain is completely degraded after 120 min of incubation, giving rise to

Table I

Amino acid sequence analysis of rFVIIa fractions from RP-HPLC

A. Minor peak

Cycle no.	1	2	3	4	5	6	7	8	9	10	11	12
PTH-aa	Gly	Ala	Thr	Ala	Leu	Glu	Leu	Met	Val	Leu	Asn	Val
Yield in pmol	169	164	46	140	124	117	125	117	124	87	128	121

*B. Main peak**

Cycle no.	1	2	3	4	5	6	7	8	9	10	11	12
PTH-aa	Lys	Val	Gly	Asp	Ser	Pro	Asn	Ile	Thr	Glu	Tyr	Met
Yield in pmol	92	–	–	168	77	136	–	203	149	160	177	84

*In addition to the NH_2-terminal sequences for the L-chain (Ala-Asn-Ala-Phe-Leu-) and the H-chain (Ile-Val-Gly-Gly-Lys-), the sequence shown was recovered. In cycles 2 and 3 the heavy chain and the derivative release the same amino acids. The Asn in cycle 7 is glycosylated.

bands of molecular weight close to and below 15 kDa (Fig. 1, lane 11). In the presence of calcium ions, cleavage of both the L- and H-chain is delayed (Fig. 1, lane 4).

While the tissue factor-dependent clotting activity is reduced in parallel with the formation of Gla-domainless rFVIIa, the enzymatic activity towards a low-molecular substrate is lost with the cleavage of the H-chain, and 5% of the amidolytic activity of the control remains after 3 h of incubation with cathepsin G.

From Fig. 1 is seen that the H-chain degradation fragments (16–19 kDa), present before addition of cathepsin G changes position in the SDS-PAGE gel, indicating at least two cleavage sites, one in between residues 153 and 290 and another in between residues 316 and 406.

3.2.2. *RP-HPLC and N-terminal sequence analysis.* The pattern on RP-HPLC after extended cathepsin G exposure (1:50, 120 min), is shown in Fig. 2. As the GD-rFVIIa peak (Rt 27 min) diminish, two main peaks with Rt 24 and 26 min, appear. Analysed by SDS-PAGE, the former peak contained the GD-L chain with the 15 kDa band covalently linked, and N-terminal amino acid sequence analysis confirmed the Ser-45 sequence from the GD-L-chain and the Ile-153 sequence from the H-chain. The fragment eluted at 26 min had an N-terminal sequence starting at Ser-333 (Table II), confirming the cleavage in between residues 316 and 406, predicted from SDS-PAGE (δ cleavage site in Fig. 4).

3.2.3. *ES-MS.* The fragments, isolated by RP-HPLC were applied to ES-MS. The mass obtained for the fragment starting at Ser-333 was 8,152 (Fig. 3a), confirming an intact C terminus of the rFVIIa. The theoretical value, calculated from amino acid sequence of 333–406 is 8,153. As the L-chain contains *O*- and *N*-linked carbohydrate, the mass for the L-linked heavy chain fragment could not be calculated from the amino acid content. In order to characterize this fragment, the cathepsin G-treated rFVIIa was reduced and alkylated before fractionation on RP-HPLC (data not shown). Analysis by ES-MS gave beside the mass for the alkylated 333–406 fragment of 8,268 (the theoretical mass is 8,267), a fraction containing the mass of 14,259 (Fig. 3b). The

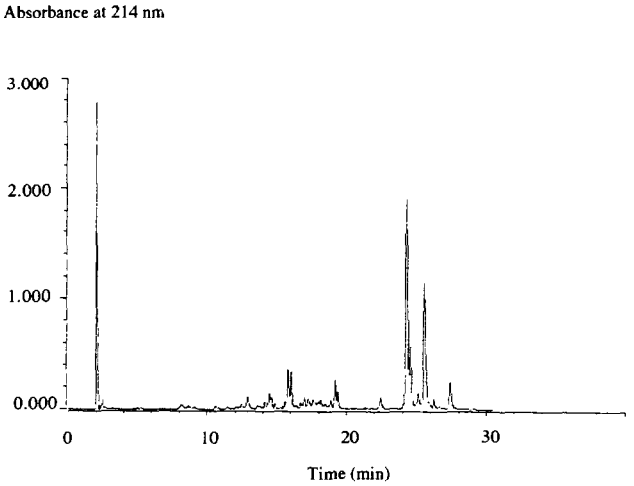


Fig. 2. RP-HPLC of cathepsin G degraded rFVIIa. 0.5 mg of rFVIIa incubated 120 min at 37°C with cathepsin G (1/50 w/w) were applied. Fractions, absorbing at 214 nm were collected and concentrated by evaporation.

calculated value for the alkylated fragment 153–278 is 14,260. The cleavage between 153 and 290, predicted from the SDS-PAGE pattern, therefore occurs after Phe-278 (ε cleavage site in Fig. 4).

A heavy chain fragment, corresponding to 279–332, and containing the *N*-linked carbohydrate was not recovered. It might have been further degraded or lost during RP-HPLC.

4. DISCUSSION

A single peptide bond, between Arg-152 and Ile-153, is cleaved during the activation of FVII, converting the single-chain proenzyme to the coagulant active two-chain FVIIa. In the presence of calcium and phospholipid, a second slower cleavage followed the activation of human plasma FVII by human FXa, or by FIXa and FIIa at higher concentrations, producing a third band of lower molecular weight than the light chain, which could be seen as a triplet on stained SDS-PAGE [13]. The third band appeared after the activation of FVII was completed, and concomitant with the increasing amount of this third chain, the clotting activity de-

Table II

Amino acid sequence analysis of cathepsin G degraded rFVIIa

Cycle no.	1	2	3	4	5	6	7	8	9	10	11	12
PTH-aa	Ser	Asp	Gly	Ser	Lys	Asp	Ser	Cys	Lys	Gly	Asp	Ser
Yield in pmol	269	397	380	161	342	377	126	–	348	380	196	76
	*	*	*		*				*	*		

*The same amino acids are released from the (45–152) L-chain and/or the H-chain. The amount of Gla-domainless rFVIIa present in the RP-HPLC fraction (RT 26 min) corresponded to 10%. PTH-Cys is not determined.

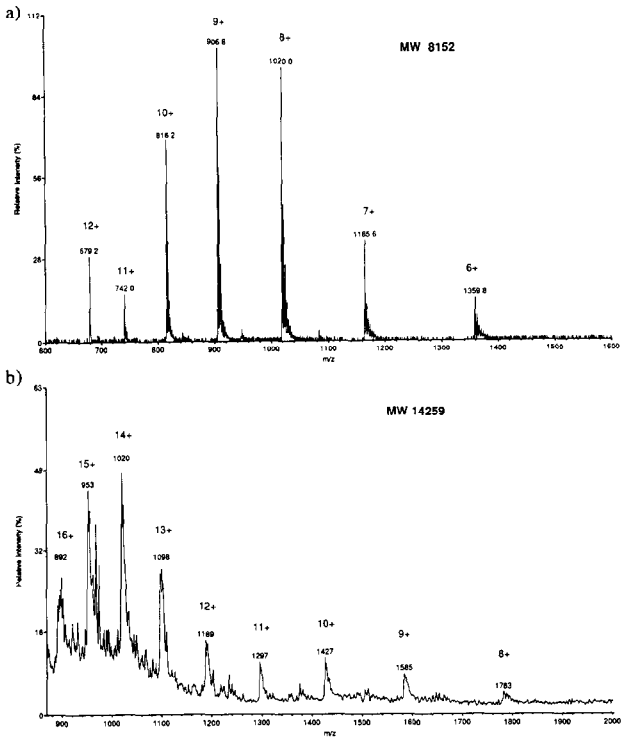


Fig. 3. ES-MS of cathepsin G degraded rFVIIa derivatives. (a) The spectrum of RP-HPLC fraction (RT 26 min) from Fig. 2. (b) The spectrum shows a fraction from RP-HPLC of reduced and alkylated cathepsin G degraded rFVIIa.

creased. When the experiment was repeated in plasma, the third chain was not formed [13].

Also bovine FVII was reported to be cleaved slowly in the heavy chain after the rapid activation by FXa, to give a proteolytic inactive three chain structure [4]. The inactivating cleavage occurred between an Arg–Gly bond [14], which was later identified as the bond between Arg-290 and Gly-291 [15].

We have found that cleavage occurs not only in the same position, after Arg-290 in the human rFVIIa molecule, but also after Arg-315. The heavy chain cleavages could be demonstrated after the activation, which occurred during purification [7]. Several coagulation enzymes have been reported to activate FVII, in addition to FVIIa itself, and although the possibility of trace amount of a contaminating enzyme cannot be excluded, our preliminary data indicate that the heavy-chain cleavage at the two tryptic sites, exposed after the conversion of single chain to double chain can be mediated by FVIIa itself.

Autolysis of coagulation enzymes is known from human FIIa, where the α-form is converted to β and γ forms by cleavage of the B-chain. The latter cleavage occurs in a region [16], equivalent to the 290–291 cleavage in the FVIIa molecules. When compared to other vitamin K-dependent coagulation enzymes, this region has a minimal conservation of amino acid sequence; it is located on the surface of the enzymes near the active site and it is believed to be essential for defining the substrate specificity [17,18]. The conservation of the sequence in this region of human (L-D-R-G-A-T-A) and bovine FVII (L-E-R-G-V-T-A) supports this hypothesis.

The cleavage after 315 occurs in a region, which on the crystal structure of trypsin is opposing the 290 region, and which is unique to FVII in comparison with other vitamin K dependent enzymes, due to the insertion, 311–315. While the human FVII contains two adjacent basic residues (L-Q-Q-S-R-K-V-G-D-S-P), the bovine FVII has a Gln residue in between the two basic residues (L-Q-Q-S-R-Q-R-P-G-G-P), and cleavage in the bovine FVII in this position was not reported [4]. In that sense, the 315 cleavage might be unique for the human FVIIa, in the same way as the autolysis of FIIa, producing the γ-form is obtained for human FIIa, but not for bovine FIIa.

The conformational change induced by surface binding, rendering the FVII susceptible to autoactivation, might expose areas on the molecule, which are essential for the substrate specificity, but at the same time susceptible for proteolytic attack. The in vitro situation with concentrated pure enzyme, bound to charged surface matrices, might be parallelled to a certain degree in vivo, by the tissue factor binding of FVII on the cell surface, creating a localized high concentration of bound enzyme. Regulation by inhibitors TFPI and ATIII is probably predominant immediately after the initiation of the coagulation, before a possible slower inactivation by proteolysis plays a significant role.

Cathepsin G has been shown to remove the Gla-domain of vitamin K-dependent coagulation factors at a position, corresponding to Phe-40–Trp-41 in plasma FVII [19]. Using a higher ratio of enzyme to substrate, we found cleavage at Tyr-44–Ser-45 in the L-chain [8],

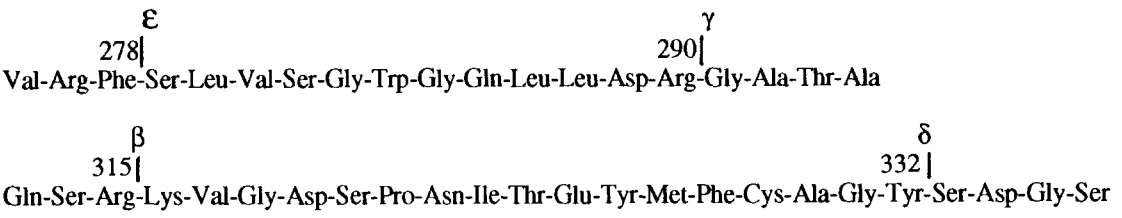


Fig. 4. Cleavage sites in the heavy chain of rFVIIa, β and γ exposed by activation, and δ and ε susceptible to cathepsin G catalysis.

and on prolonged exposure, H-chain cleavage. Two cleavage sites on the H-chain have been identified, one, Tyr-332–Ser-333, occurring at a sequence identical to the L-chain cleavage at Tyr-44 (Y-S-D-G), was determined by N-terminal sequence analysis, while a second site, Phe-278–Ser-279 could be deduced from analysis of recovered fragments by ES-MS.

The accuracy of mass measurements on a triple quadrupole instrument is generally better than 0.02%, although low-intensity spectra may result in poorer mass precision. The spectra, shown in Fig. 3a and b, both display relatively broad peaks, due to the formation of a sodium-adduct ion. However, the adduct-ions are clearly resolved from the protonated molecular ion, on which the mass determination is based, and they do not interfere with the precision of the molecular weight determination. ES-MS is a very powerful tool for structural characterization, and the analysis reported here confirmed an intact C-terminus of the recombinant FVIIa molecule.

The 279–332 fragment was not recovered and might have been further degraded.

In vitro, calcium ions were found to have a protective role in the L-chain and H-chain cleavages, while heparin potentiated the heavy chain cleavage by cathepsin G (data not shown). Other coagulation enzyme degradation patterns are influenced by heparin, which might act as a template for the catalysis mechanism [20] and cell surface proteoglycans, presumably have a modulating effect, in directing the proteolysis rate, in vivo. Cathepsin G is released from stimulated neutrophils and might be a possible link between the hemostasis and inflammation processes.

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