

Mass spectrometric characterization of N- and O-glycans of plasma-derived coagulation factor VII

François Fenaille · Catherine Groseil ·
Christine Ramon · Sandrine Riandé · Laurent Siret ·
Sami Chtourou · Nicolas Bihoreau

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Abstract Factor VII (FVII) is a vitamin K-dependent glycoprotein which, in its activated form (FVIIa), participates in the coagulation process by activating factor X and factor IX. FVII is secreted as single peptide chain of 406 residues. Plasma-derived FVII undergoes many post-translational modifications such as γ -carboxylation, N- and O-glycosylation, β -hydroxylation. Despite glycosylation of recombinant FVIIa has been fully characterized, nothing is reported on the N- and O-glycans of plasma-derived FVII (pd-FVII) and on their structural heterogeneity at each glycosylation site. N- and O-glycosylation sites and site specific heterogeneity of pd-FVII were studied by various complementary qualitative and quantitative techniques. A MALDI-MS analysis of the native protein indicated that FVII is a 50.1 kDa glycoprotein modified on two sites by diantennary, disialylated non-fucosylated (A2S2) glycans. LC-ESIMS/MS analysis revealed that both light chain and heavy chain were N-glycosylated mainly by A2S2 but also by triantennary sialylated glycans. Nevertheless, lower amounts of triantennary structures were found on Asn₃₂₂ compared to Asn₁₄₅. Moreover, the triantennary glycans were shown to be fucosylated. In parallel, quantitative

analysis of the isolated glycans by capillary electrophoresis indicated that the diantennary structures represented about 50% of the total glycan content. Glycan sequencing using different glycanases led to the identification of triantennary difucosylated structures. Last, MS and MS/MS analysis revealed that FVII is O-glycosylated on the light chain at position Ser₆₀ and Ser₅₂ which are modified by oligosaccharide structures such as fucose and Glc(Xyl)_{0–1–2}, respectively. These latter three O-glycans coexist in equal amounts in plasma-derived FVII.

Keywords Coagulation factor VII · N-glycosylation · O-glycosylation · Mass spectrometry

Abbreviations

A2S1	diantennary monosialylated glycan
A2S2	diantennary disialylated glycan
A2S2F	diantennary disialylated fucosylated glycan
A3S3	triantennary trisialylated glycan
A3S3F	triantennary trisialylated fucosylated glycan
DHB	2,5-dihydroxybenzoic acid
Endo H	endoglycosidase H
Fuc	fucose
FVII	factor VII
FVIIa	activated factor VII
Glc	glucose
GN	N-acetylglucosamine
H	hexose
HPCE-LIF	high performance capillary electrophoresis-laser induced fluorescence
HC	heavy chain of activated factor VII
HCCA	α -cyano-4-hydroxycinnamic acid

F. Fenaille · C. Groseil · C. Ramon · S. Riandé · L. Siret ·
S. Chtourou · N. Bihoreau (✉)
Laboratoire Français du Fractionnement et des Biotechnologies,
3 avenue des Tropiques, BP 305, Les Ulis,
91958 Courtaboeuf cedex, France
e-mail: bihoreau@lfb.fr

F. Fenaille
e-mail: francois.fenaille@cea.fr

Present address:

F. Fenaille
CEA/iBiTec-S, Service de Pharmacologie et d'Immunoanalyse,
91191 Gif-sur-Yvette, France

LC	light chain of activated factor VII
LC–ESIMS/MS	liquid chromatography coupled to electrospray ionization tandem mass spectrometry
MALDI-TOFMS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
PNGase F	peptide-N-glycosidase F
PTMs	post-translational modifications
Q-TOF	quadrupole time-of-flight mass spectrometer
TFA	trifluoroacetic acid
THAP	2,4,6-trihydroxyacetophenone
Xyl	xylose

Introduction

Blood coagulation factor VII (FVII) is a vitamin K-dependent glycoprotein which in its activated form, factor VIIa (FVIIa), participates in the coagulation process by activating factor X and/or factor IX in the presence of calcium and tissue factor [1]. FVII is secreted in the form of a single peptide chain of 406 residues (~50 kDa). This protein has four distinct structural domains: a N-terminal γ -carboxylic domain (Gla), two “epidermal growth factor (EGF)-like” domains as well as a serine protease domain. The activation of FVII into FVIIa is characterized by the cleavage of Arg₁₅₂-Ile₁₅₃ bond, resulting in a two-chain molecule consisting of a light chain (LC) of 152 amino acids (~20 kDa) and a heavy chain (HC) of 254 amino acids (~30 kDa) linked to each other by a single disulfide bond (Cys₁₃₅-Cys₂₆₂) [1, 2]. Plasma-derived FVIIa undergoes many post-translational modifications: the first ten glutamic acids of the N-terminal moiety are γ -carboxylated, Asp₆₃ is partially hydroxylated, Asn₁₄₅ and Asn₃₂₂ are N-glycosylated, while Ser₅₂ and Ser₆₀ are O-glycosylated [1, 3].

Characterization of N- and O-linked glycans is mandatory to further investigate whether oligosaccharides have structural and/or functional roles. For instance, recent data from pulse-chase analysis of N-glycosylation site knockout mutants demonstrated cotranslational glycosylation of Asn₁₄₅ but primarily or exclusively post-translational glycosylation of Asn₃₂₂ [4]. Thus, it was speculated that the cotranslational conformation(s) of FVII are unfavorable for glycosylation of Asn₃₂₂, whereas during post-translational folding, the protein obtains a conformation that makes Asn₃₂₂ accessible to the oligosaccharyltransferase complex [4]. Protein folding and N-glycosylation of FVII are likely to influence one another, while it seems that O-glycosylation contributes more directly to the biological activity of FVII. By use of site-specific mutants, it was suggested

that the O-glycosylations could provide structural elements that are of importance for the association of FVIIa with tissue factor [5].

Although the N-glycans of recombinant FVIIa have been fully characterized [6], the only available information on the composition of plasma-derived FVIIa N-linked glycans is from the analysis of its monosaccharide composition [7]. Indeed, nothing is reported on the N-glycans present and on their structural heterogeneity at each N-glycosylation site.

Regarding O-glycosylation, the occupancies of Ser₅₂ and Ser₆₀ have been previously assessed in two separate studies using a combination of different time-consuming biochemical and biotechnological approaches. The technique used to map the O-fucosylation site of FVIIa involved the production of recombinant FVIIa by baby hamster kidney cells cultured in a medium containing radiolabeled fucose [8]. This method lacks sensitivity, necessitating purification of relatively large amounts of protein. The technique is also labor intensive, usually involving purification of the radiolabeled peptides and Edman sequencing. In a parallel approach and using a combined strategy of amino acid sequencing, carbohydrate and amino acid composition analysis, and plasma desorption mass spectrometry, three other different O-glycan structures consisting of either glucose, glucose-xylose, or glucose-(xylose)₂ were also detected in plasma and recombinant factor VII [8]. The linkage of the glucose-xylose and glucose-(xylose)₂ motifs to Ser₅₂ has been previously demonstrated by other authors [9].

Glycosylation analysis has been greatly influenced by methodological developments, especially those regarding mass spectrometric techniques [10–13]. Currently, the study of site-specific protein glycosylation involves proteolytic digestion of the glycoprotein of interest, and further analysis of the resulting peptide by mass spectrometry. Such “bottom-up” approach has been widely used to characterize numerous glycoproteins using electrospray (ESI) and/or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry techniques [14, 15]. Unfortunately, enzymatic digestion, liquid chromatography on-line to ESIMS/MS, or off-line to MALDI-TOFMS are time-consuming approaches and frequently produce small (glyco)peptides that elute in the column dead volume. Thus, characterization of complex glycoproteins can greatly benefit from preliminary mass measurement of the intact, fully modified protein by mass spectrometry. The mass resolution obtained by MALDI is not sufficient to distinguish isoforms closely related by mass, but can provide useful information on protein glycosylation when combined with specific enzymes [16, 17]. The use of electrospray mass spectrometry (ESIMS) using a quadrupole time-of-flight mass spectrometer (Q-TOF) with high mass resolution, high sensitivity, and broad mass range (up

to hundreds of kilodaltons), has been successfully used for accurate mass measurement of protein isoforms [18–20].

In the current study, N- and O-glycosylations of FVIIa were studied by a combination of “top-down” and “bottom-up” approaches. First, MALDI-TOFMS was used in combination with enzymatic deglycosylation to estimate the carbohydrate content of FVIIa and the main glycan structures. Intact light and heavy chains were then analyzed by ESIMS using a Q-TOF instrument to obtain more insight on glycosylation heterogeneity of each chain by accurate mass measurement. At this point, “top-down” sequencing of intact protein species by mass spectrometry was investigated in terms of information brought on N- and O-glycans present on each chain.

The N-glycosylation sites and site-specific oligosaccharide heterogeneity were precisely characterized using a combination of enzymatic digestions, HPLC separation, HPCE-LIF, MALDI-TOF and TOF/TOF experiments in conjunction with ESIMS and MS/MS analyses.

Experimental section

Materials

Dithiothreitol (DTT) and iodoacetamide were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Trypsin, Asp-N and pepsin were purchased from Roche (Mannheim, Germany). Peptide-N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) were from Prozyme (San Leandro, USA) and Sigma-Aldrich, respectively. The MALDI matrices α -cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Laser BioLabs (Sophia Antipolis, France), whereas 2,4,6-trihydroxyacetophenone (THAP) was from Fluka (Saint Quentin Fallavier, France). FVIIa was in-house purified from a pool of human plasma, as previously described [21]. Other chemicals and reagents were obtained from commercial sources at the highest level of purity available. All buffers were prepared using ultrapure water (MilliQ, Millipore).

Estimation of N-glycan composition by endoglycosidase digestion of FVIIa and MALDI-TOFMS analysis

FVIIa was enzymatically digested using PNGase F and Endo H in organic-aqueous solvent systems [22]. Briefly, 1 μ L of a FVIIa aqueous solution (*i.e.* \sim 2 μ g) was mixed with 6 μ L of acetonitrile/water (37.5:62.5, *v/v*, final concentration ACN: 30%) and 0.5 μ L of a PNGase F (*i.e.* \sim 1.25 mU) or Endo H solution (*i.e.* \sim 2.5 mU). The resulting mixture was incubated 1 h at 37°C and the deglycosylated FVIIa further analyzed by MALDI-TOFMS

to estimate the contribution and nature of N-glycosylation on protein (see below).

HPCE-LIF oligosaccharide mapping

The characterization and quantification of different N-linked sugars were performed by HPCE-LIF, as previously described [23]. Briefly, after denaturation and reduction of FVII, the sugars were released by PNGase F treatment (E/S 5 mU/200 μ g) at 37°C for 18 h and isolated by protein precipitation with ice-cold ethanol. The glycans were treated with exoglycosidases (sialidase, ratio E/S 1 mU/10 μ g), galactosidase, HexNAcase (kit Prozyme), fucosidase (ratio E/S: 1 mU/10 μ g) in order to quantify and characterize each isolated structure. All the glycosidases used were purchased by Prozyme. The glycans were labeled with a fluorophore (APTS) and separated by HPCE-LIF in a N-CHO coated capillary (dimension 50 cm \times 50 μ m ID, Beckman Coulter) mounted into a CE system, ProteomeLab PA800 (Beckman Coulter). The electrophoresis was performed at 25 kV, 20 min at 20°C using a separation buffer “gel buffer-N” (Beckman Coulter). The labeled glycans were detected by LIF at λ_{ex} 488 nm and λ_{em} 520 nm.

Proteolytic digestion of FVIIa

Approximately 100 μ g of lyophilized FVIIa were dissolved in 50 μ L of 8 M urea, 0.4 M ammonium bicarbonate solution pH 8.0. Disulfide bridge reduction was accomplished by adding 10 μ L of a 100 mM DTT solution in water and incubating the resulting mixture for 45 min at 56°C. The solution was allowed to cool at room temperature, after which 10 μ L of a 200 mM iodoacetamide solution in water were added, and the solution was incubated at RT for 30 min in the dark. After an eight-fold dilution with water, digestion with trypsin (1:25, *w/w*) or Asp-N (1:100, *w/w*) was performed by an overnight incubation at 37°C.

Fractionation of FVIIa digests by HPLC

The peptides obtained after trypsin or Asp-N digestion were fractionated using a (3 mm \times 250 mm, 5 μ m) Uptisphere C18 column (Interchim, Montluçon, France) mounted on an Akta Purifier FPLC system (GE Healthcare, Saclay, France). Detection was performed at 214 and 280 nm, and the flow rate was set to 0.5 mL/min. Solvent A was 0.12% (*v/v*) TFA and solvent B was acetonitrile containing 0.1% (*v/v*) TFA. The gradient used was: 0% B for 14 min, 0–5% B in 2 min, 5–20% B in 24 min, 20–30% B in 34 min, 30–60% B in 50 min, and finally 60–90% B in 20 min. Six hundred microliter-fractions were collected in the range 16–120 min and further evaporated to dryness. Fractions were further reconstituted in 20 μ L of water prior to analysis.

Enzymatic digestions performed on the MALDI target

On-target deglycosylation of glycopeptides was performed according to the procedure previously described [23]. Briefly, 0.5 μ L of the LC fraction dried and resuspended in water, 0.5 μ L of PNGase F solution (*i.e.* \sim 1.25 mU), and 1.5 μ L of water were thoroughly mixed on the MALDI target. The reaction was allowed to proceed for 15 min at RT, and further stopped by adding 0.5 μ L of HCCA matrix solution.

Sialic acid linkage analysis was also performed on-target on 2-AB labeled N-glycans, following an adaptation of a previously published work [24]. Briefly, 0.5 μ L of the oligosaccharide solution, 0.5 μ L of sialidase solution (either specific of α 2-3,6 or α 2-3 linkages, \sim 2.5 mU), and 1.5 μ L of water were mixed on the MALDI target. The desialylation reaction was allowed to proceed for 15 min at RT, and further stopped by adding 0.5 μ L of HCCA matrix solution.

MALDI-TOFMS analyses

Analyses were carried out on an Autoflex II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a nitrogen laser. Analyses were carried out using different matrix solutions: (a) HCCA, 0.3 mg/mL in ethanol/acetone (2:1, *v/v*); (b) DHB, 20 mg/mL in acetonitrile; (c) THAP, 10 mg/mL in acetonitrile/water (1:1, *v/v*) containing 0.1% TFA. HCCA and DHB matrices were used for the analysis of proteolytic peptides and glycopeptides, respectively; THAP was used for the analysis of native and deglycosylated proteins as well as released oligosaccharides [22]. In each case, 0.5 μ L of analyte solution and 0.5 μ L of matrix solution were deposited and thoroughly mixed on the target. For protein analysis, ions were analyzed in the linear mode after acceleration at 20 kV, with an extraction delay of 500 ns. Laser energies were 30–45% depending on the protein. Peptide analysis was performed in the reflectron mode with an acceleration of 19 kV and a delayed extraction time of 110 ns, whereas laser energy ranged from 20% to 30% depending on the peptide and matrix. External calibration was achieved by use of standard mixtures, purchased from Bruker Daltonik GmbH. A total of 100–200 single spectra were added to produce a MALDI mass spectrum.

LC–ESIMS analysis of FVIIa heavy and light chains

Reversed phase chromatography was performed using an Agilent 1100 HPLC system (Palo Alto, CA, USA). Reduced and alkylated heavy and light chains were separated by injecting an aliquot of the reaction mixture (\sim 20 μ g) onto a Prosphere C4 (150 \times 2.1 mm, 5 μ m) equilibrated at 40°C at a flow rate of 350 μ L/min. Buffer A

was 0.1% trifluoroacetic acid (TFA), while buffer B was acetonitrile containing 0.1% TFA. After 3 min of isocratic elution at 25% B, the percent B was raised to 60% in 15 min using a linear gradient and further to 80% in 2 min. After a 5 min stay at 80% B, the % B was decreased to 25% B in 2 min and the column was re-equilibrated using starting conditions for 13 min. After passing through the UV-detector operating at 214 nm, the chromatographic flow was electrosprayed into a Q-TOF instrument (QSTAR XL, Applied Biosystems, Toronto, Canada). The TOF region was calibrated daily using standard peptides. The instrument was scanned from *m/z* 700 to *m/z* 2,000 in 1.5 s. The ionspray voltage was 5 kV, and the source block temperature was set at 350°C. For “top-down” experiments, the declustering potential was raised from 60 to 110 V. The protein mass spectra were deconvoluted using the Bio-Analyst software.

LC–ESIMS/MS analysis of FVIIa digests

Peptide separation was achieved using a 2 mm \times 150 mm Uptisphere C18 column at a flow rate of 250 μ L/min and maintained at 40°C. Buffer A was 0.1% formic acid, while buffer B was acetonitrile containing 0.1% formic acid. The gradient separation was 5–60% B over 90 min. The peptides eluted from the column were introduced on line into the QSTAR instrument. The ionspray voltage was 5 kV, and the source block temperature was set to 300°C. All LC-MS/MS analyses were performed using data-dependent switching between MS and MS/MS modes. MS cycles comprised a TOFMS survey scan with an *m/z* range of 300–1,800 Th for 1 s, followed by two product ion scans with an *m/z* range of 50–1,800 Th for 2 s each. The collision energy was automatically set according to the mass and charge state of the peptides chosen for fragmentation. After a given precursor was selected, dynamic exclusion was used for the next 60 s to prevent its subsequent reselection.

Results and discussion

MALDI-TOFMS of intact and N-deglycosylated FVIIa

Figure 1 shows the MALDI mass spectra obtained for native FVIIa, FVIIa deglycosylated with PNGase F in 30% ACN and the released oligosaccharides. The molecular mass of native FVIIa was determined to be 50.16 kDa (Fig. 1a). The broad shape of this peak indicates high structural heterogeneity related to the numerous post-translational modifications (PTMs) described as N- and O-glycosylation at two sites, and 10 γ -carboxyglutamic acids in plasma-derived FVIIa [1]. For the fully de-N-glycosylated protein, an average

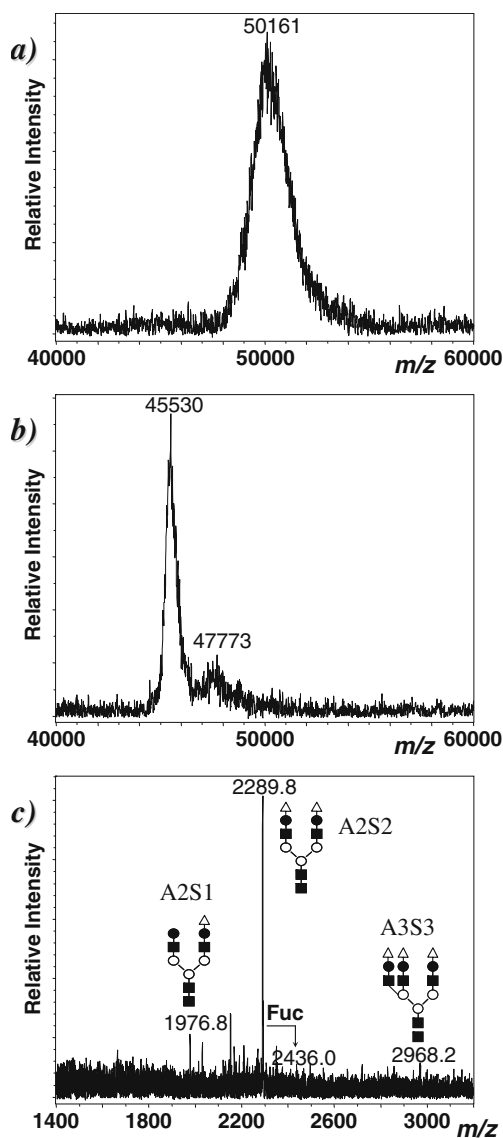


Fig. 1 Deglycosylation of FVIIa studied by MALDI-TOF mass spectrometry. **a** Native FVIIa, **b** PNGase F-treated FVIIa, and **c** released oligosaccharides

molecular mass of 45.53 kDa was measured (Fig. 1b). The N-glycan content is therefore of ~4.6 kDa, thus accounting for ~10% of the mass of native FVIIa. The mass difference of ~0.5 kDa from the theoretical average molecular mass of 46.07 kDa (calculated from the amino acid sequence and considering 12 disulfide bridges) indicates the presence of the other PTMs, namely γ -carboxylations, and O-glycosylations. It should be noticed that this mass difference could be slightly underestimated as a consequence of partial fragmentation of γ -carboxylic acid residues under MALDI conditions [25].

N-deglycosylation was voluntarily performed in 30% ACN to obtain partial deglycosylation [22]. An intermediate form appeared as a broad and badly resolved peak with an apex mass of 47.77 kDa (Fig. 1b). This peak differs from

the native FVII and the completely de-N-glycosylated FVIIa by ~2.2 kDa suggesting the presence of a diantennary disialylated glycan (A2S2, 2,204 Da) as major form on the two glycosylation sites. This is corroborated by the analysis of the released N-glycans (Fig. 1c). Beside the predominant A2S2 structure, minor A2S1 and A3S3 species along with the corresponding fucosylated forms can also be observed.

FVIIa was also treated with Endo H to estimate the contribution of high mannose and hybrid glycans. FVIIa proved to be insensitive to Endo H (data not shown), which was consistent with previous data indicating that FVIIa contains complex-type glycans.

Accurate molecular weight determination of FVIIa heavy and light chains by LC-ESI-MS

Analysis of FVIIa was performed after reduction and alkylation by LC-ESI-QTOFMS to obtain more insight on site-specific glycosylation heterogeneity on both light (N- and O-glycosylation) and heavy chains (N-glycosylation).

The HPLC elution profile of reduced and alkylated plasma-derived FVIIa is shown in Fig. 2a. LC and HC elute at 15.40 and 23.04 min, respectively. The corresponding deconvoluted ESI mass spectra are represented in Fig. 2b and c, respectively. The LC of FVIIa showed a high level of structural heterogeneity, due to the presence of ten γ -carboxylation, two O-glycosylation and one N-glycosylation sites. The lower-mass region of the corresponding spectrum (Fig. 2b) revealed three peaks at 20,837, 20,969 and 21,101 Da separated by 132 Da, mass increment consistent with the addition of xylose residues (theoretical average mass increment: +132.1 Da). These three peaks of roughly similar intensities are likely to represent O-glycosylation isoforms bearing 0, 1, and 2 xylose residues, respectively [8]. The theoretical average mass of FVIIa LC was calculated at 20,835 Da, considering ten γ -carboxyglutamic acids, a A2S2 motif on Asn₁₄₅, and O-glycosylation and O-fucosylation of Ser₅₂ and Ser₆₀, respectively [1]. This value is in good agreement with the measured value of 20,837 Da (Table 1) and perfectly corroborates the previous hypothesis regarding O-glycosylation heterogeneity. At higher mass values, other peaks also appeared as triplets separated by 132 Da: 21,493/21,625/21,757 Da, and 21,639/21,771/21,903 Da (Fig. 2b). These peaks account for N-glycosylation heterogeneity and represent the substitution of the A2S2 by A3S3 (+656 Da) and A3S3F (+802 Da) structures, respectively. These data show that approximately equal amounts of bi- and tri-antennary structures are present on Asn₁₄₅. MALDI-TOFMS measurement of native and partially de-N-glycosylated FVIIa rather gives A2S2 as major structure on the two N-glycosylation sites, which is due to the broad and badly resolved shape of the peak corresponding to the intermediate glycoform

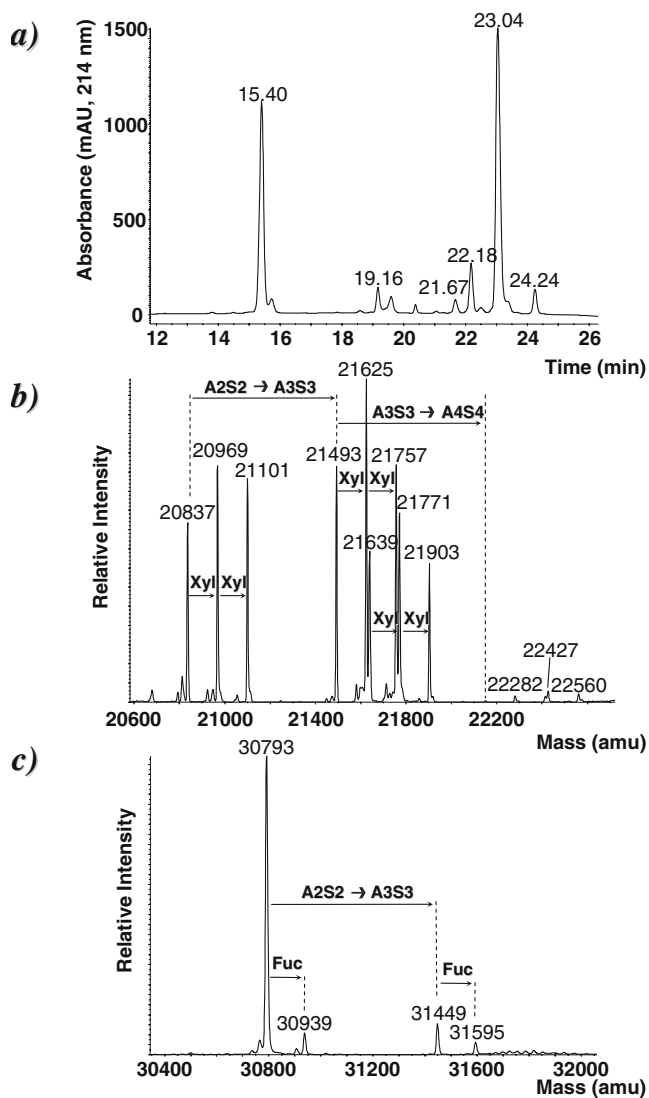


Fig. 2 LC-UV/ESI-MS analysis of reduced and alkylated LC and HC of FVIIa. **a** LC-UV chromatogram, and deconvoluted ESI mass spectra of **b** LC (peak at 15.40 min) and **c** HC (peak at 23.04 min)

(Fig. 1b) thus rendering difficult a precise mass measurement. It should be noted that a considerable fraction of triantennary glycans showed a high level of fucosylation. This trend is reinforced on tetra-antennary tetra-sialylated structures (A4S4). Indeed, the low levels of tetra-antennary structures observed exhibit a dominant peak at 22,427 Da corresponding to A4S4F structures with one O-linked xylose residue.

Kolarich *et al.* have observed a similar fucosylation pattern on human α_1 -antitrypsin [26]. Diantennary structures were shown to contain only marginal amounts of fucose, whereas a considerable fraction of tri- and tetra-antennary glycans showed a mass increment corresponding to one deoxyhexose, indicating an increased fucosylation on tri- and tetraantennary glycans. Moreover, the authors have highlighted the presence of α 2-6-linked sialic acids on diantennary disialylated N-glycans, whilst triantennary trisialylated structures contained one α 2-3 sialic acid per N-glycan, which, together with a fucose, formed a sialyl Lewis X determinant on the β 1-4-linked *N*-acetylglucosamine [26]. Preliminary structural investigations were performed to assess whether FVIIa bears the same kind of sialylated N-glycans (see paragraph below).

The deconvoluted ESI mass spectrum of FVIIa HC was easier to interpret (Fig. 2c), because this peptidic chain bears less post-translational modifications than LC, with only one N-glycosylation site located at Asn₃₂₂. The average molecular mass of the main isoform was determined at 30,793 Da (Fig. 2c), value in excellent agreement with the theoretical average mass of 30,793 Da considering an A2S2 glycan on Asn₃₂₂. Low amounts of A2S2F, A3S3 and A3S3F containing isoforms could also be observed at 30,939, 31,449, and 31,595 Da, respectively. Thus considerably lower amounts of triantennary trisialylated structures were found on Asn₃₂₂ compared to Asn₁₄₅. The fucosylation level was also lower on the HC glycosylation site. The different glycoforms observed on light and heavy chains are summarized in Table 2.

Table 1 FVIIa light and heavy chains analysis by LC-ESI-MS

Peak retention time (min)	Theoretical average mass (Da) ^a	Observed average mass (Da) ^a	Attribution
15.40	20,835	20,837	LC (Ala ₁ -Arg ₁₅₂)
19.16	12,409	12,410	HC fragment (Lys ₃₁₆ -Pro ₄₀₆)
19.59	15,237	15,237	HC fragment (Gly ₂₉₁ -Pro ₄₀₆)
20.38	15,572 ^b	15,573 ^b	HC fragment (Ile ₁₅₃ -Arg ₂₉₀)
21.67	30,809	30,809	HC ox. (Met ₂₉₈)
22.18	30,809	30,809	HC ox. (Met ₃₀₆)
23.04	30,793	30,793	HC (Ile ₁₅₃ -Pro ₄₀₆)
24.24	18,399 ^b	18,400 ^b	HC fragment (Ile ₁₅₃ -Arg ₃₁₅)

^a Masses corresponding to the first glycoform

^b Non glycosylated protein species

Table 2 Results of site-specific N- and O-glycosylation analysis of FVIIa

N- or O-Glycosylation site	Main glycan structures observed	Abbreviated name	Relative distribution of the main glycan structures at each glycosylation site ^a (%)	
O-glycosylation				
Ser ₅₂	Glc		30	
	Glc-Xyl		39	
	Glc-Xyl-Xyl		31	
Ser ₆₀	Fuc		100	
N-glycosylation				
Asn ₁₄₅	(HexNAc) ₄ (Hex) ₅ (Sial) ₁	A2S1	2	
	(HexNAc) ₄ (Hex) ₅ (Sial) ₁ (Fuc) ₁	A2S1F	<1	
	(HexNAc) ₄ (Hex) ₅ (Sial) ₂	A2S2	29	
	(HexNAc) ₄ (Hex) ₅ (Sial) ₂ (Fuc) ₁	A2S2F	1	
	(HexNAc) ₅ (Hex) ₆ (Sial) ₂	A3S2	<1	
	(HexNAc) ₅ (Hex) ₆ (Sial) ₂ (Fuc) ₁	A3S2F	<1	
	(HexNAc) ₅ (Hex) ₆ (Sial) ₃	A3S3	40	
	(HexNAc) ₅ (Hex) ₆ (Sial) ₃ (Fuc) ₁	A3S3F	23	
	(HexNAc) ₅ (Hex) ₆ (Sial) ₃ (Fuc) ₂	A3S3F2	<1	
	(HexNAc) ₆ (Hex) ₇ (Sial) ₄	A4S4	<1	
	(HexNAc) ₆ (Hex) ₇ (Sial) ₄ (Fuc) ₁	A4S4F	1	
	Asn ₃₂₂	(HexNAc) ₄ (Hex) ₅ (Sial) ₁	A2S1	<1
		(HexNAc) ₄ (Hex) ₅ (Sial) ₂	A2S2	81
		(HexNAc) ₄ (Hex) ₅ (Sial) ₂ (Fuc) ₁	A2S2F	6
(HexNAc) ₅ (Hex) ₆ (Sial) ₂		A3S2	<1	
(HexNAc) ₅ (Hex) ₆ (Sial) ₃		A3S3	9	
(HexNAc) ₅ (Hex) ₆ (Sial) ₃ (Fuc) ₁	A3S3F	3		

^a Approximate abundances were determined using MS signal intensities observed on spectra from Fig. 2

These data are in good agreement with the lower degree of sialylation of Asn₃₂₂ in recombinant FVIIa produced in baby hamster kidney cells [6]. Moreover, Bolt *et al.* who have recently demonstrated by pulse-chase analysis of N-glycosylation site knockout mutants that glycosylation of Asn₁₄₅ was cotranslational while glycosylation of Asn₃₂₂ was primarily or exclusively post-translational [4]. Thus, it was speculated that the cotranslational conformation of FVIIa is unfavorable for glycosylation of Asn₃₂₂, whereas during post-translational folding, the protein conformation renders Asn₃₂₂ accessible to the oligosaccharyltransferase complex [4]. The differences between the glycosylation pattern of the two N-glycosylation sites of FVIIa could also be related to their position in the peptide chain. Indeed, it has been suggested that, with increasing distance of the glycosylation site from the C-terminal, the complexity of the oligosaccharides increases [27].

The other peaks visible in the chromatogram of Fig. 2a, in the 18–24 min time window, correspond to HC degradation products formed during protein purification [7]. For instance, protein species eluting at 21.7 and 22.2 min exhibited molecular masses shifted by +16 Da compared to native HC (Table 1), which suggests the presence of an oxidized amino acid residue. Complementary experiments confirmed this assumption and showed that these two peaks correspond to heavy chains bearing an

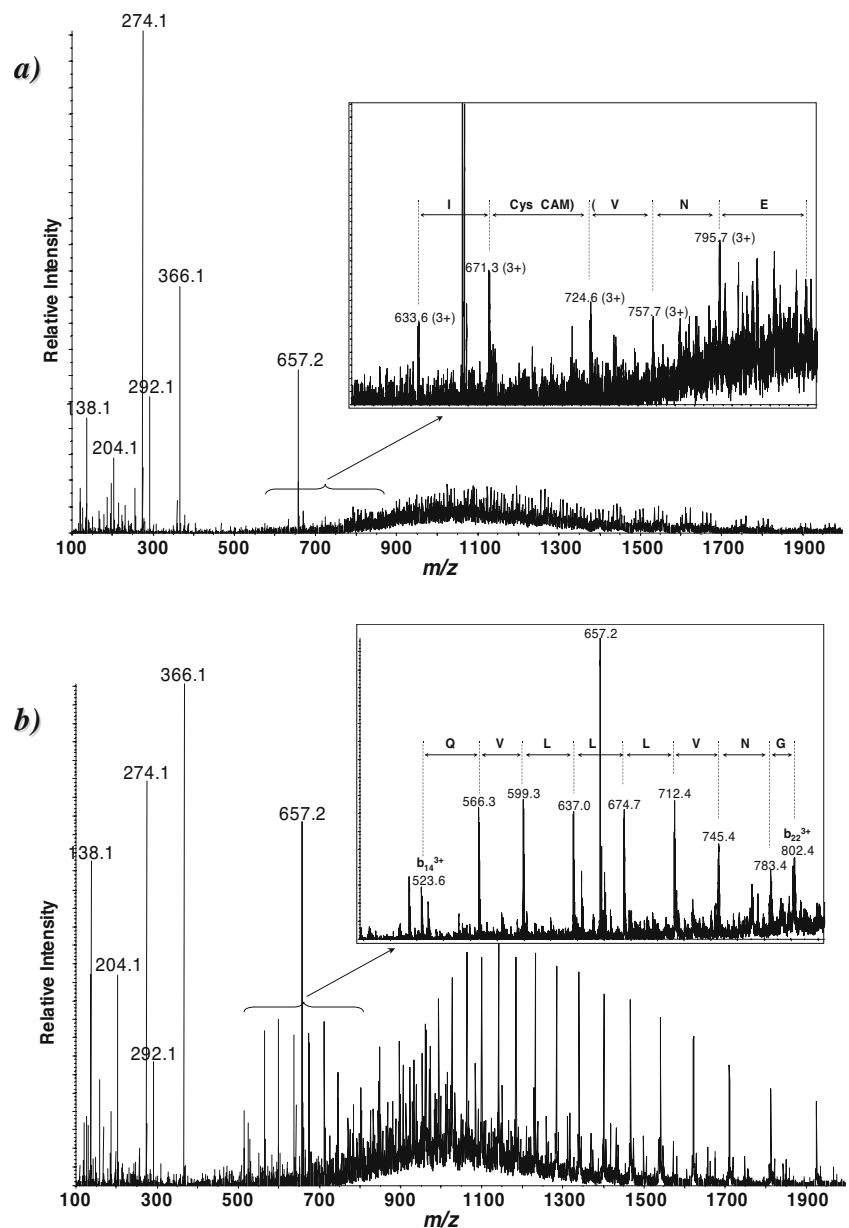
oxidized methionine residue at position 298 and 306, respectively. This is in agreement with previously published data [28]. The other peaks originate from hydrolysis of HC either after Arg₂₉₀ or Arg₃₁₅ as deduced from accurate mass measurement (Table 1). These observations are also corroborated by previous data [29].

“Top-down” mass spectrometry to characterize FVIIa heavy and light chains

In a “top-down” mass spectrometry approach, intact protein ions are introduced into the gas phase by ESI and are subsequently fragmented in the mass spectrometer. In-source CID can be used on a LC time scale to obtain structural information on specific proteins, as recently demonstrated on different mixtures of nonglycosylated proteins [30]. Here we show that this approach can be “routinely” used to obtain more structural insight on proteins bearing N- and O-glycans.

Figure 3 represents the ESI mass spectra of FVIIa LC and HC that were submitted to in-source CID during LC-MS analysis. Tandem mass spectra of glycopeptides are dominated by carbohydrate fragment ions [31, 32]. As may be observed from Fig. 3, in-source CID mass spectra of both chains exhibit the same characteristic pattern with intense oxonium ions at m/z 204 (HexNAc), m/z 186

Fig. 3 LC–ESIMS analysis with in-source CID of reduced and alkylated LC and HC of FVIIa. ESI mass spectra of **a** LC, and **b** HC

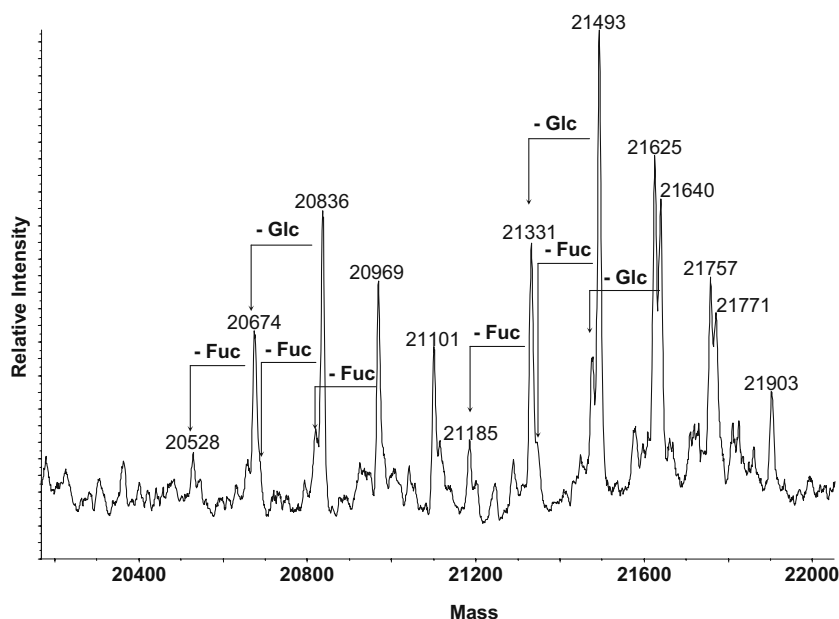


(HexNAc–H₂O), m/z 168 (HexNAc–2H₂O), m/z 138 (HexNAc–H₂O–CH₂O), m/z 274 (Sial–H₂O), m/z 292 (Sial), m/z 366 (HexNAc–Hex), and m/z 657 (HexNAc–Hex–Sial). These data are consistent with the N-glycosylation of both LC and HC by complex-type sialylated glycans, and confirm the attribution performed on the basis of the corresponding deconvoluted mass spectra (Fig. 2). It should be noted that it was not possible to distinguish oxonium ions derived from O-glycans constitutive of FVIIa. In addition, several internal and b-type fragment ions can be observed for LC and HC, respectively (Fig. 3a and b, insert). The triply charged internal fragment ions observed for LC result from a cleavage between Leu₇₃ and Pro₇₄. Cleavage N-terminal to proline is known to be preferential when the preceding residue is leucine [33]. Cleavages at

Pro residues often give intense y-type ions, which can further break down to yield internal cleavage ions [34]. Such limited sequence information is sufficient to confirm protein identity.

As discussed, in-source CID mass spectra show intense carbohydrate and a few peptide-derived fragment ions, but large multiply charged ions corresponding to partially fragmented species still exist above m/z ~900 (Fig. 3). Deconvolution of this mass range can also provide informative data. The spectrum obtained for the LC is shown in Fig. 4. Almost all LC isoforms previously observed (Fig. 2) are still present, but in different relative proportions. Even though the relative proportions of N-linked structures remain more or less unchanged, species that do not possess xylose residues are now the most

Fig. 4 Deconvoluted mass spectrum of LC obtained by LC–ESIMS with in-source CID



intensively detected glycoforms with peaks at 20,836 (bearing A2S2 N-glycan), 21,493 (A3S3), and 21,640 Da (A3S3F). These species further fragment by losing 162 and 146 Da, which are representative of the O-linked glucose and fucose residues, respectively. Interestingly, O-fucose and O-glucose losses are not only consecutive but also concomitant, thus suggesting that the two glycans are linked to different amino acid residues. Last, it should be noted that the O-glucose loss is only observed from glycoforms that do not bear xylose residues, thus potentially indicating that the two xylose residues are linked to the glucose.

Regarding the HC which bears only N-glycans, no significant differences were observed between the deconvoluted spectra obtained with or without in-source fragmentation (data not shown).

Identification and characterization of N-glycopeptides

To determine the oligosaccharide heterogeneity and glycosylation site, reduced and alkylated FVIIa was enzymatically digested into peptides and glycopeptides using either trypsin or Asp-N. Considering both unmodified and post-translationally modified peptides, 100% of the amino acid sequence was covered by LC–ESIMS/MS using these two proteolytic enzymes. LC–ESIMS/MS was used as a specific technique to identify glycopeptides through the monitoring of carbohydrate fragment ions [32]. Figure 5a and b represent the MS/MS spectra of the triply and quadruply charged ions at m/z 1,021.4 and m/z 1,158.6 corresponding to the major glycoform of the tryptic glycopeptides bearing Asn₁₄₅ and Asn₃₂₂, respectively. Although the latter glycopeptide possesses a N-terminal Lys residue not

cleaved by trypsin, it was always observed with the highest intensity. As previously observed for LC and HC (Fig. 3), these MS/MS spectra are dominated by carbohydrate fragment ions. For instance, the presence of product ions at m/z 274 (Sial–H₂O) and m/z 292 (Sial) confirmed the presence of sialylated oligosaccharides. Fragment ions (peptide + 203 + H)⁺, (peptide + 83 + H)⁺ or (peptide + 203 + H)²⁺ are also observed and constitute a good signature of glycopeptides. The 203 and 83 mass unit increments correspond to a GlcNAc and a fragmented GlcNAc residues, respectively [35, 15]. In addition, several fragment ions of the y- and/or b-series were also detected for the peptides (Asn₁₄₅-Arg₁₅₂) and (Lys₃₁₆-Lys₃₃₇) and confirmed peptide assignment. The carbohydrate residue molecular weight was calculated from the observed molecular weight of the peptide and the calculated molecular weight of the glycopeptide (Table 2). Thus, the different glycopeptides carry the same major oligosaccharide structure whose calculated residue mass is ~2,204.8 Da corresponding to a diantennary disialylated glycan (A2S2).

The N-glycosylation sites were then identified using MS/MS sequencing by tracking the Asp residues newly formed upon PNGase F deglycosylation. In the present work, HPLC fractions containing glycopeptides were first analyzed by MALDI-TOFMS before and after deglycosylation with PNGase F, thus confirming the oligosaccharide structures and the number of glycosylation sites. Glycosylation sites were further identified by MALDI-TOF/TOF experiments as shown in Fig. 5c and d for the two glycopeptides of interest. For example, the spectrum for the (Asn₁₄₅-Arg₁₅₂) peptide (Fig. 5c) shows sufficient fragmentation to allow the precise localization of the glycosylation site on position 145.

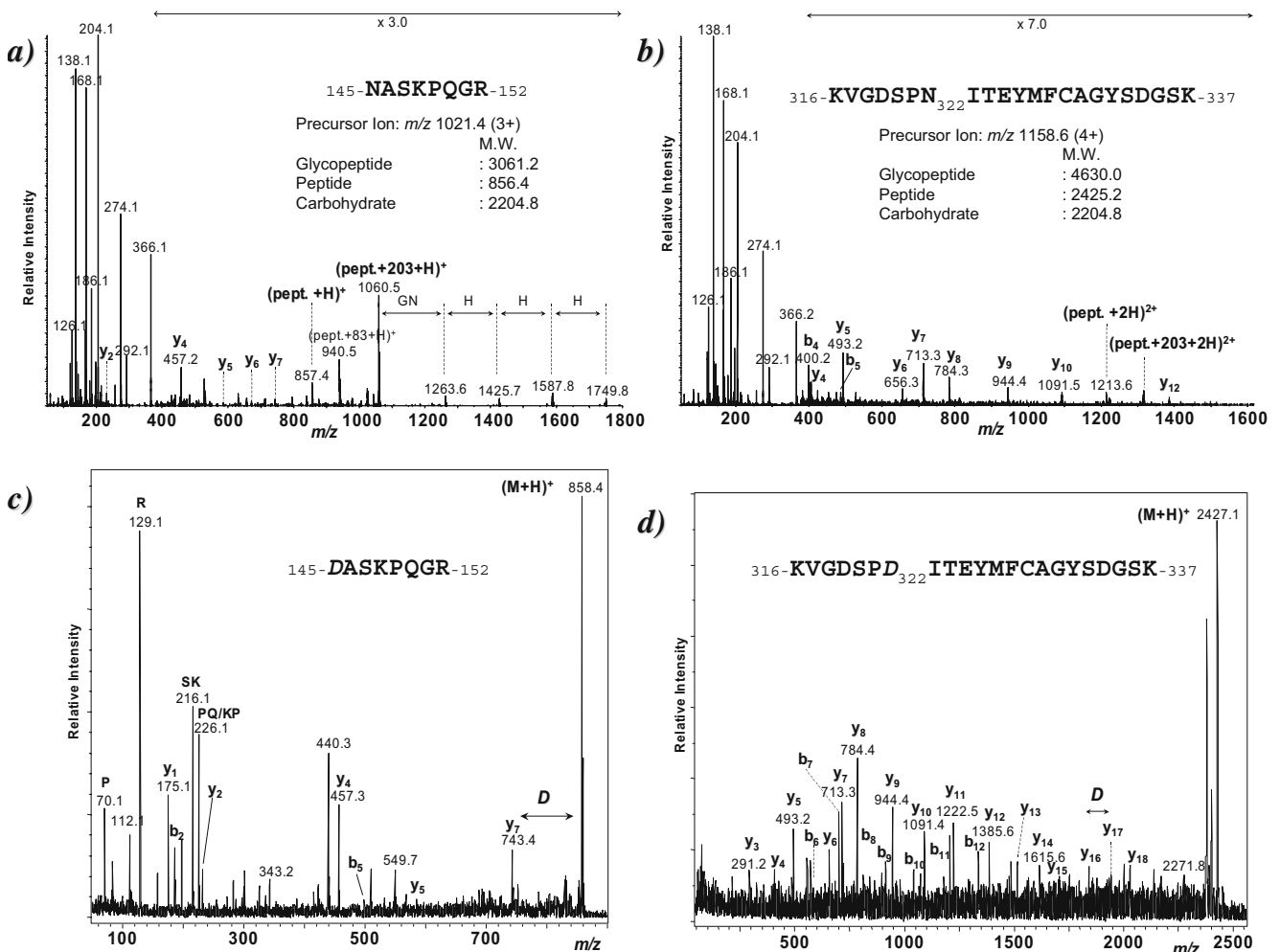


Fig. 5 Identification of glycosylated peptides and glycosylation sites. LCESIMS/MS spectra of **a** m/z 1,021.4 (3+), and **b** m/z 1,158.6 (4+) corresponding to the tryptic glycopeptides bearing Asn₁₄₅, and Asn₃₂₂,

respectively. MALDI-TOF/TOF tandem mass spectra of **c** m/z 858.4, and **d** m/z 2,427.1 corresponding to the same tryptic peptides after PNGase F treatment

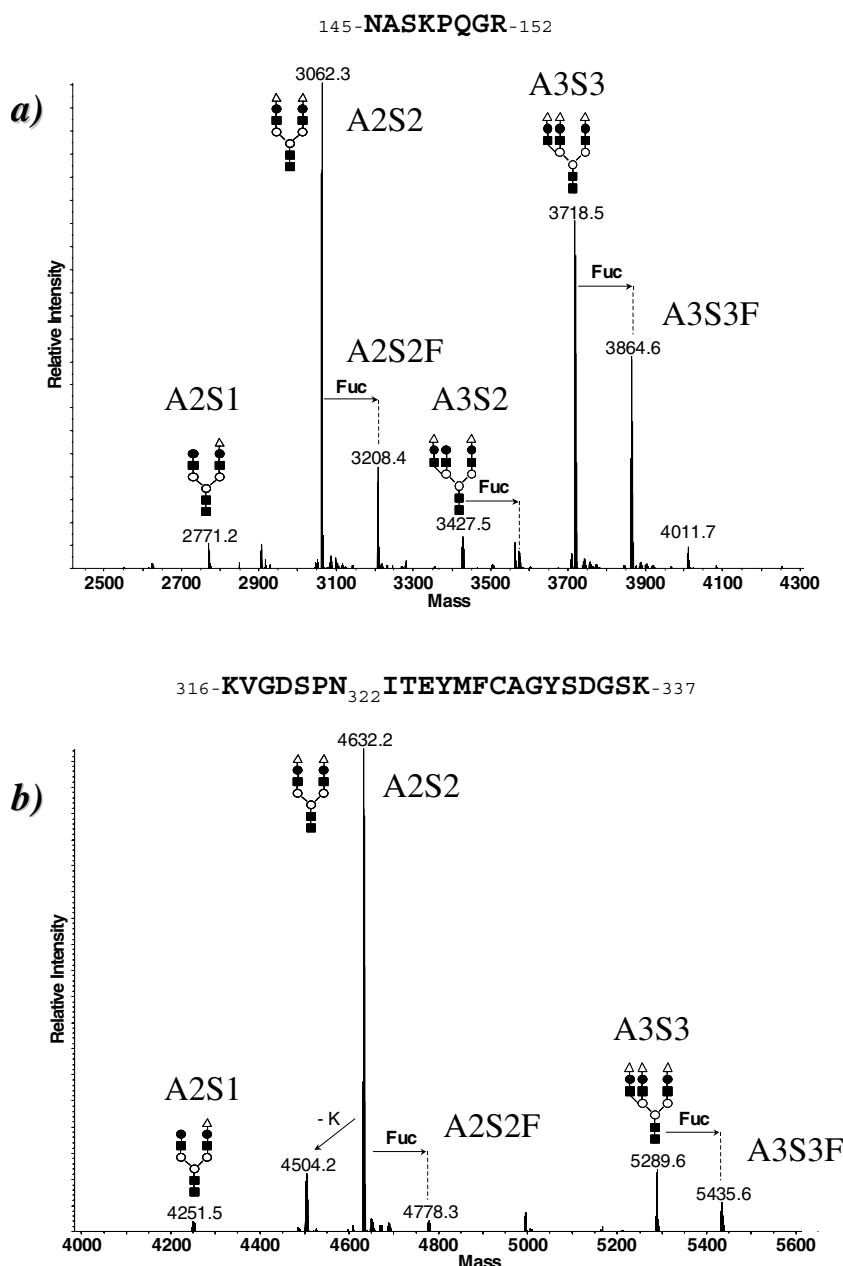
The heterogeneity of carbohydrate structures on each N-glycosylation site was determined by LC-ESIMS as above described and their relative proportions estimated by working on deconvoluted mass spectra. Such an approach, involving glycopeptide analysis, has recently been demonstrated to be accurate and robust for oligosaccharide quantitation [36]. Figure 6 represents the results obtained for the two tryptic glycopeptides. The same major glycoforms were found at the two glycosylation sites, with A2S2 as predominant structure in both cases. However, significant differences were found between Asn₁₄₅ and Asn₃₂₂ in the relative distribution of the different glycoforms. Indeed, similar proportions of A2S2 (peak at 3,062.3 Da) and A3S3 (3,718.5 Da) structures were found at Asn₁₄₅, while the A2S2 (4,632.2 Da) structures show by far the highest signal at Asn₃₂₂. A particularly high level of fucosylation was also observed on triantennary structures present on Asn₁₄₅. These data are in excellent agreement with those previously obtained by LC-ESIMS analysis of intact LC and HC,

which underlines the usefulness of such a technique as a screening tool. However, the traces of A2S1 and A3S2 on one or both sites were not observed with this “top-down” approach. In contrast, tetraantennary structures were not observed at the glycopeptide level when trypsin was used as a digesting agent, but only with Asp-N. This is probably due to the small size of this glycopeptide and its low retention on reversed phase columns, especially when the size of the N-linked glycans increases. This point both demonstrates the necessity of using several digesting agents and the usefulness of measuring intact protein masses to more accurately identify the glycan structures of a given glycoprotein.

N-glycans characterization by HPCE-LIF

Figure 7a represents the electropherograms of the native N-glycans obtained after FVIIa deglycosylation by PNGase F. We noted that FVIIa glycans are of diantennary sialylated

Fig. 6 Deconvoluted ESI mass spectra of tryptic mass spectra of tryptic glycopeptides bearing **a** Asn₁₄₅, and **b** Asn₃₂₂



type and mostly non-fucosylated diantennary disialylated (A2S2), and fucosylated structures (A2S2F), monosialylated diantennary structures (A2S1) could also be detected at low level. Triantennary species also exist but elute as several peaks, mainly due to the presence of several isomers. Following α 2-3,6 desialylation (Fig. 7b), we noted the complete disappearance of the sialylated structures, and the appearance of several peaks corresponding to diantennary, non-fucosylated glycans (A2G2, peak 1) and triantennary forms (A3G3) thus confirming the presence of glycans previously identified. The triantennary non-fucosylated (A3G3) and fucosylated (A3G3F) structures (peaks 3 and 5) were less present. The peaks 4 and 6 correspond to

triantennary fucosylated forms. Complementary experiments conducted with specific neuraminidases revealed complete desialylation of diantennary N-glycans upon treatment with α 2-6-specific neuraminidase, whereas triantennary structures showed partial desialylation, when treated with this enzyme. Triantennary glycans were shown to also contain α 2-3-neuraminic acid residues, with \sim 1 residue per glycan.

Defucosylation of the desialylated glycans by α 1-6 fucosidase (Fig. 7c) was complete, with the disappearance of A2G2F, A3G3F and peak 6, and yielded A2G2, A3G3 structures and peak 4, respectively. The fucose linked to A2G2 and A3G3 was thus bound in an α 1-6 manner.

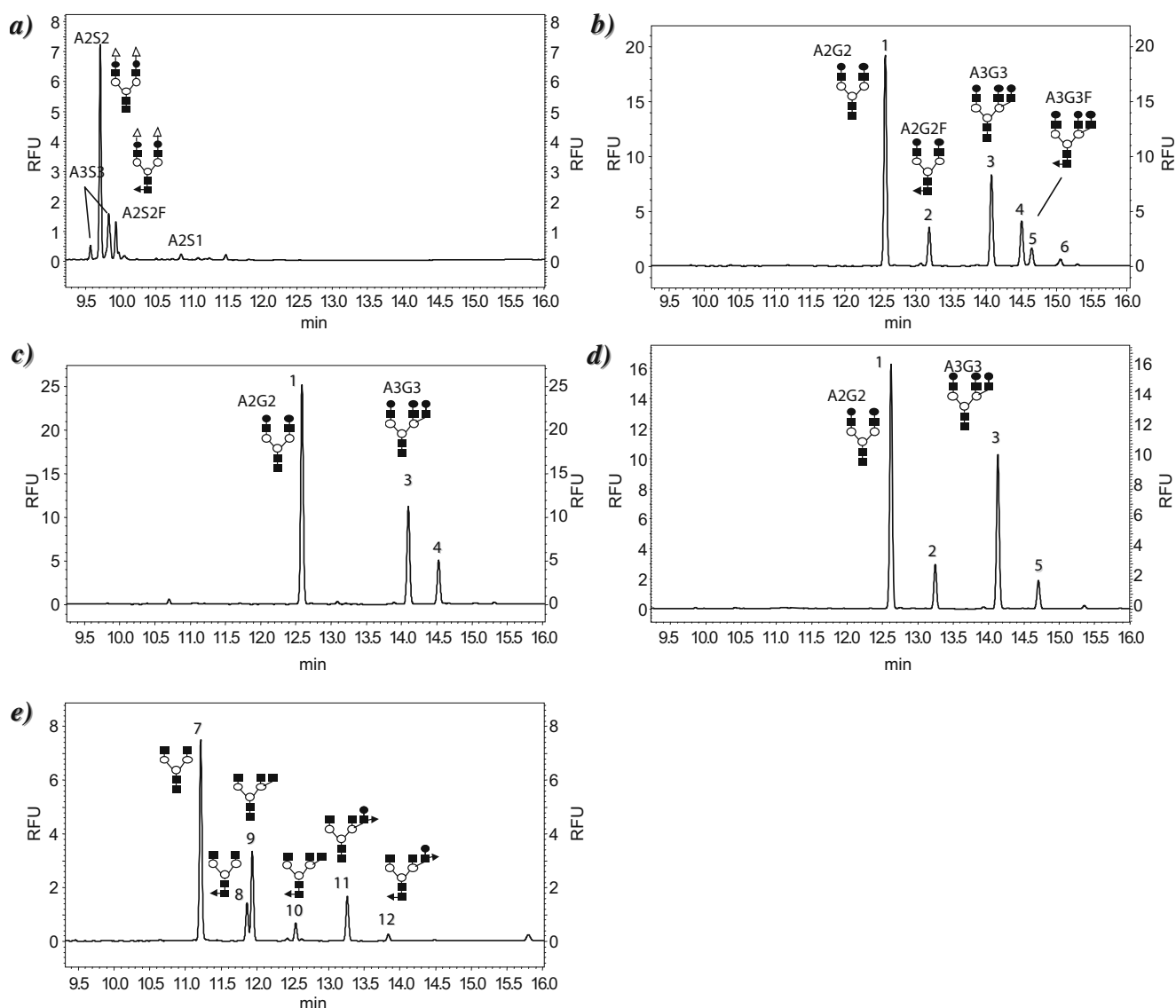


Fig. 7 Preliminary structural analysis of N-glycans from human FVII. APTS-labelled N-glycans derived from FVII were analyzed **a** under their native form, and after treatment with **b** unspecific neuraminidase,

c unspecific neuraminidase and α 1-6 fucosidase, **d** unspecific neuraminidase and α 1-3,4 fucosidase, and **e** unspecific neuraminidase and β 1-4 glucosaminidase

Defucosylation by α 1-2 fucosidase showed identical profiles when compared to the untreated product (data not shown). The profile obtained following α 1-3,4 fucosidase treatment (Fig. 7d) showed a partial defucosylation with the disappearance of peaks 4 and 6 correlated with an increase of A3G3 ($\sim +10\%$) and A3G3F ($\sim +2\%$), respectively.

Deagalactosylation of the desialylated glycans by β 1-4 galactosidase (Fig. 7e) yielded A2G0, A2G0F, A3G0 and A3G0F structures (peaks 7, 8, 9, and 10, respectively). Peaks 4 and 6, which differ respectively by one fucose unit (Fig. 7b), disappeared to generate peaks 11 and 12. We suggest that peaks 11 and 12 corresponded to partially deagalactosylated triantennary glycans due to the presence of the fucose on position α 1-3 on the *N*-acetylglucosamine. These results suggested that peak 5 corresponded to

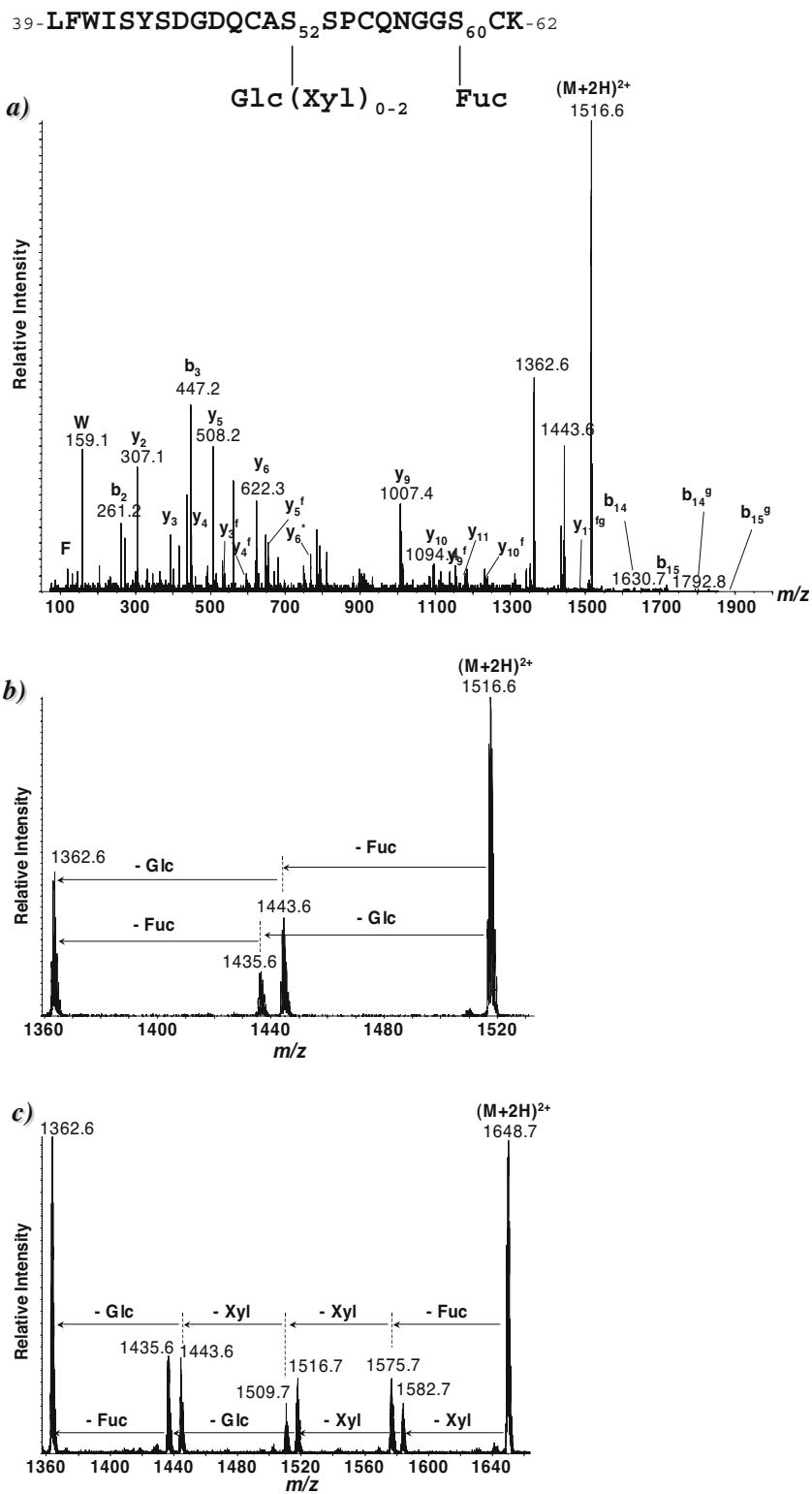
triantennary glycan bearing one fucose on position α 1-3,4 and peak 6 corresponded to the same triantennary glycan bearing two fucoses on positions α 1-3,4 and α 1-6. Together with the results obtained after α 1-3,4 fucosidase treatment, these data tend to confirm the presence of a fucose residue on an *N*-acetylglucosamine of one arm of triantennary structures. Indeed, it has been shown previously that, with such fucosylated structures, the removal of the fucose by α 1-3,4 fucosidase is a prerequisite to enable galactosidase to act on the galactose [26, 37]. The question whether α 2-3-neuraminic acid and α 1-3 fucose residues are linked to the same arm of triantennary structures is still under investigation. Lastly, the remarkable N-glycan similarity between human FVII and antitrypsin must be pointed out.

Identification and characterization of O-glycopeptides

Coagulation FVIIa possesses two O-glycosylation sites located at Ser₆₀ and Ser₅₂ bearing O-fucose, and O-glucose

or O-glucose-(xylose)_{1,2} motifs, respectively [1]. The apparent chemical lability of the O-glycosidic bond makes it very difficult to be analyzed by CID tandem mass spectrometry experiments under conventional operating

Fig. 8 Characterization of O-glycosylation sites. **a** ESIMS/MS spectrum of *m/z* 1,516.6 (2+) corresponding to the tryptic O-glycosylated peptide bearing fucose and glucose residues; **b** zoom of MS/MS spectrum of **(a)**, and **c** zoom of the MS/MS spectrum of *m/z* 1,648.7 (2+) corresponding to the tryptic O-glycosylated peptide bearing fucose and glucose-(xylose)₂ residues



conditions. This is particularly true for the analysis of O-fucosylation, which is a highly labile modification [38].

Figure 8a depicts the MS/MS spectrum of the doubly charged ion at m/z 1,516.6 corresponding to the peptide (Leu₃₉-Lys₆₂) bearing glucose and fucose oligosaccharides on Ser₅₂ and Ser₆₀, respectively. Under the low-energy CID conditions used, the precursor ion was only partially fragmented, and hence was still the most abundant in the MS/MS spectrum. Detachment of the sugar moieties was obviously the favored process (Fig. 8a and b), thus giving rise to doubly charged ions at m/z 1,443.6 (loss of fucose), 1,435.6 (loss of glucose) and 1,362.6 (losses of both fucose and glucose). These losses of fucose and glucose occurred concomitantly, thus indicating that they are located on two different sites of attachment. The fragment ions of the b- and y-series deglycosylated during CID were prominent, but glycosylated fragment ions were still sufficiently abundant to allow the precise location of the two glycans on Ser₅₂ and Ser₆₀ (Fig. 8a).

Linkage of xylose residues to the glucose located on Ser₅₂ can be confirmed using the same MS/MS approach. Figure 8c represents a zoom of the MS/MS spectrum of the doubly charged ion at m/z 1,648.7 corresponding to the glycopeptide bearing both the fucose and glucose-(xylose)₂ motifs. This ion showed both concomitant losses of fucose and xylose, and consecutive losses of two xylose and one glucose residues. These data indicated that the two xylose residues are linked to the glucose residue bound to Ser₅₂, and not to the fucose residue on Ser₆₀, which corroborates the previous hypothesis made on the basis of the “top-down” results.

Last, it should be noted that under our experimental conditions, none of the O-glycopeptides studied exhibited intense carbohydrate fragment ions as previously observed for N-glycopeptides. This is probably due to the chemical nature of the sugars.

Deconvolution of the mass spectra obtained for the different O-glycoforms of this particular peptide showed three peaks at 3,033.3, 3,165.4, and 3,297.4 Da corresponding to O-glycans consisting of glucose, glucose-(xylose)₁, and glucose-(xylose)₂, respectively (Fig. 9, Table 2). According to this spectrum, approximately equal amounts of these three glycan structures coexist in plasma-derived FVII, which corroborates the data obtained on entire LC by LC-ESI/MS.

Conclusions

Although the N-linked carbohydrate structures linked to recombinant FVIIa have been studied, nothing has been reported on N-glycans constitutive of plasma-derived

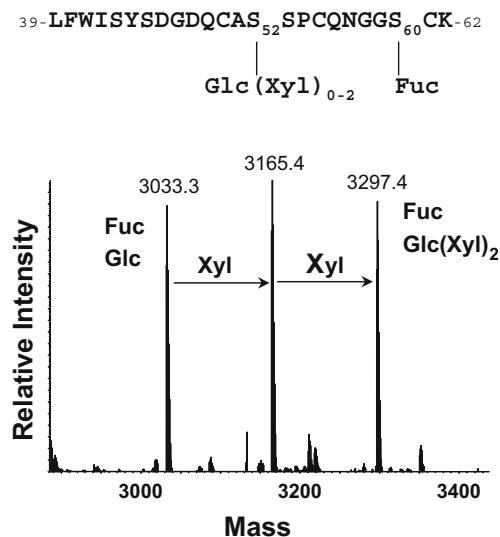


Fig. 9 Deconvoluted ESI mass spectrum of the tryptic glycopeptide bearing O-glycosylation sites

FVIIa. Moreover, O-glycosylation of Ser₅₂ and Ser₆₀ has been established through two separate studies that are time-consuming and poorly sensitive. In the present article, N- and O-glycosylation of plasma-derived FVIIa has been thoroughly characterized using modern mass spectrometry techniques by combining “top-down” and “bottom-up” approaches. Data obtained by these approaches appeared to be similar regarding the extent of N- and O-glycosylation and structures of the main oligosaccharide residues. Accurate mass measurements and protein fragmentation experiments provided solid foundation to complete the characterization of the proteome using “bottom-up” tactics involving peptide analysis.

Our data successfully provided comprehensive information on the site-specific N-linked oligosaccharides in plasma-derived FVIIa. A2S2 structures were shown as the main structures on the two N-glycosylation sites Asn₁₄₅ and Asn₃₂₂. The triantennary structures were found more abundantly on the “N-terminal site” Asn₁₄₅ and were shown to be highly fucosylated. A particular attention was devoted to the study of this high fucosylation level. Exoglycosidase digests confirmed the particular fucosylation of triantennary structures with the presence of a fucose residue on an *N*-acetylglucosamine of one arm of triantennary structures. In the opposite, fucosylation of diantennary structures was demonstrated to be of the core α 1-6 type.

We also showed that tandem mass spectrometry enables both the simultaneous assessment of the O-glycosylation of the two serine residues, and the nature and sequence of the glycans present on each O-glycosylation site. Such an approach is faster and easier to handle compared to the approaches that were used before to elucidate O-glycosylation of FVII.

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