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On the variation of glycosylation in human plasma derived antithrombin

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Abstract

The paper presents data on the primary structure of the glycan variants present in human antithrombin (AT) isoforms obtained from a plasma pool. The analysis is conducted on the level of glycopeptides gained by tryptic digestion. The glycopeptides were pre-separated by lectin-affinity chromatography and analyzed by means of electrospray ionization–tandem mass spectrometry involving collision-induced dissociation. Variations of the canonical biantennary complex-type structure were present with relative abundances of about 1–5% and most of them were found site-specifically. Core fucosylation was observed at one single glycopeptide only (peptide containing N^{155}), triantennary glycan structures with two glycopeptides (containing N^{155} and N^{135}). Deficiency of one terminal sialic acid was observed as not site-specific. Fucosylation was not yet reported to be present in human AT from plasma, opposite to recombinant human AT from baby hamster kidney cells, which was reported as fully core fucosylated. In total, the variability in the carbohydrate structure of plasma derived AT appears as being quite limited. This might be of significance in the context of the reported correlation between glycosylation and physiological activity.

Keywords: Antithrombin; Glycoform analysis; Glycoproteins; Lectin-affinity chromatography; Tandem mass spectrometry

1. Introduction

Antithrombin (AT) is a glycoprotein importantly involved in the regulation of blood coagulation. It belongs to the family of serine-protease-inhibitors and its regulatory effect is based on the inhibition of thrombin, clotting factors IXa, Xa, and XIa as well as kallikrein and plasmin [1,2]. AT is synthesized in the liver and is normally present in human plasma at a concentration of approximately 0.15 g/L [3,4]. Patients with a reduced AT level are subjected to an increased risk of thromboembolic complications reaching even widespread thrombosis. Preparations of AT thus serve as therapeutic agents with anticoagulant activity. Despite this main regulatory function in blood coagulation several other types of activities, like anti-inflammatory, apoptosis inducing as well as angiogenesis controlling properties are described or under discussion, respectively, as recently reviewed by Römisch et al. [2].

AT is a glycoprotein with a molecular weight of approximately 58.000 Da [5]. It contains four *N*-glycosylation sites at the asparagine residues N^{96}, N^{135}, N^{155} and N^{192} [6,7]. No O-linked glycosylation or other posttranslational modifications are present, at least not with abundances higher than 1% [5]. The glycoprotein exists in a number of isoforms as result of a microheterogeneity in the attached carbohydrates. Two groups of isoforms, designated as α-AT and β-AT, can be isolated by heparin-affinity chromatography thanks to differences in their binding strength to this glycosaminoglycan [8–10]. Whereas the predominant (approximately 93 to 94% abundance) α-isoforms are glycosylated on all four possible glycosylation sites [6], the much lower abundant β-isoforms

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(approximately 6–7% abundance) lack the entire carbohydrate side chain on N^{135} [7]. Both groups of isoforms consist of several isoforms themselves [11] differing in the carbohydrate structure.

The binding strength of β-AT to heparin was reported to be higher by a factor of 13, compared to α-AT [9]. As the binding of AT to heparin results in a more than 300-fold increase in the anticoagulant activity [12], the strength and kinetics of this binding are thus of major importance [7,13], and β-AT, exhibiting the higher affinity to heparin, was therefore considered to be the physiologically most important form of AT [7,14], although being present with rather low relative abundance. It was suggested that the oligosaccharide at N^{135}, present only in α-AT, is located close to the putative heparin binding site impeding in this way and to a certain extent the interaction with heparin [15,16]. Whereas this correlation between AT glycosylation and heparin binding, and eventually its anticoagulant activity, have attracted broad interest and were subject of extensive investigations in the past [9,12–14,17,18], an analogous correlation with the other types of activities (anti-inflammatory, apoptosis inducing, angiogenesis controlling) is not yet established.

Considering the structures of the carbohydrate chains attached to the four N-linkage sites, these were reported to be identical in all four glycans, i.e., complex-type, biantennary, with terminal sialic acids (SAs) at the non reducing ends of both antennas, and with linkage positions and configurations as described by Franzén et al. [6]. These structure data were published more than 20 years ago, at that time apprehending only the predominant isoform of α-AT. Later, β-AT was reported to carry three of these carbohydrate chains, the glycan at N^{135} is simply missing [15]. Recently, a certain microheterogeneity with respect to the primary structure of the carbohydrate chains was found in both forms of AT, α-AT and β-AT [11]. By means of various electrophoretic methods, including isoelectric focusing (IEF), capillary zone electrophoresis (CZE), and two-dimensional gel electrophoresis (2D-E), more than seven glycoforms could be distinguished which differ in p*I*-values, electrophoretic mobilities and molecular weight [11,19,20]. In a very recent paper [5], we showed by means of capillary zone electrophoresis connected to electrospray–mass spectrometry (CZE–ESI–MS) that most of these isoforms are present with abundances below 5%, two of them in trace amounts below 2%, whereas the major isoforms of α-AT and β-AT have abundances of about 82 and 4%, respectively. By the accurate determinations of the molecular masses of the intact protein isoforms, we could assign the most probable carbohydrate structures to five minor populated isoforms present in human plasma. This was done on the basis of the assessed total number of different monosaccharides (hexoses, *N*-acetyl-hexoseamines, deoxyhexoses, sialic acids) and assuming a high similarity between the glycan structures of the new isoforms and the canonical structure reported for the major isoforms by Franzén et al. [6]. From these data—together with our previous results [11]—it could also be confirmed that no other posttranslational modifications were present, at least down to abundances of 1% and excluding deamidation.

This present paper continues in the analysis of glycan structures and focuses on two main aims. First, it presents results, which ascertain the glycosylation structures postulated on the base of the accurate molecular masses of the intact proteins. This confirmation is done here on the level of the glycopeptides analyzed by means of electrospray–tandem mass spectrometry (ESI–MS^n) applying collision-induced dissociation (CID) of the glycans and the peptides. Secondly, the paper establishes the site-specific variation found in the glycosylation of AT within a plasma pool and which has not been reported yet.

2. Experimental

2.1. Sample

Human plasma derived AT was obtained as a generous gift from Octapharma Pharmazeutika Poduktions Gesellschaft (Vienna, Austria). It was purified from a plasma pool (comprising about 1500 L) of human blood-donors by means of several chromatographic methods, including heparin-affinity chromatography as reported in [10] and stored in lyophilized form. The sample aliquots contained about 500 IU, corresponding to 60 mg of AT. They were dissolved in 10 mL water and desalted by means of PD-10 columns (Amersham Biosciences, Uppsala, Sweden). The desalted solution was divided in 50 μL fractions, lyophilized and stored at −18 °C for further use.

2.2. Buffer solutions

All chemicals were purchased from Sigma-Aldrich (Vienna, Austria) if not indicated differently, water was of UHQ quality. The denaturing buffer employed prior to the digestion of the protein consisted of 6 M guanidinium hydrochloride and 1 M tris-(hydroxymethyl)-aminomethane hydrochloride (TRIS) and was obtained by dissolving 5.6 g guanidinium hydrochloride in 5.7 mL 1 M TRIS adjusting the pH to 8.5 by 1 M HCl. The digestion buffer consisted of 100 mM TRIS at pH 8.5. For affinity chromatography, the background buffer was 100 mM ammonium acetate at pH 7, the activating buffer 0.1 M ammonium acetate, pH 6, containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, the binding buffer consisted of 0.1 M TRIS with 0.5 M NaCl at pH 7.4, and the eluting solution was 0.3 M α-D-methylmannopyranoside in 20 mM ammonium acetate at pH 8.5. Regenerating buffer consisted of 20 mM ammonium hydrogencarbonate at pH 7.0 with 20% ethanol.

2.3. Enzymatic degradation

The desalted, lyophilized AT samples were re-dissolved in 270 μL denaturating buffer, and after adding 2 μL 50 mM aqueous dithiothreitol (DTT) solution the sample was incu-

bated at 37 °C for 1 h. For the derivatization of the free thiol-groups of the cystein residues 6 μL 0.2 M aqueous iodoacetamide solution was added to the sample, which was kept for 1 h at room temperature in the dark. Afterwards, the sample was again desalted by use of a size-exclusion column (Sephadex G25, 0.9 mL column volume) to remove excessive reagent. Elution of the derivatized protein from this column was achieved by using 0.4 mL of the digestion buffer. To this solution 12.5 μg trypsin (modified, sequence grade, Roche Diagnostics, Mannheim, Germany) dissolved in 50 μL water were added and the enzymatic degradation was carried out at 37 °C for 12 h.

2.4. Affinity chromatography

500 μL Con A Sepharose (Amersham Biosciences, Uppsala, Sweden) suspension was slowly filled into a solvent tubing of 12.5 cm length and 3 mm i.d., whose one side was terminated with filter paper as a frit. After the Sepharose has fully sedimented, the second side of the column was also closed (again using a filter paper as a frit) and applied to an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany). The packed column was rinsed over night with background buffer at a flow rate of 30 μL/min.

For chromatography the column was flushed with 200 μL activating buffer, 200 μL binding buffer and, afterwards, 50 μL of the tryptic digest sample were loaded. The column was then washed for 1 h with background buffer. Afterwards, 200 μL elution buffer were injected and the eluting glycopeptides were collected in a vessel. After each run the column had to be regenerated by flushing two times with 200 μL regenerating buffer. The elution of the compounds was controlled with an UV-detector (Agilent VWD 1100 series, Agilent, Waldbronn, Germany) at 214 nm.

2.5. Electrospray ionization ion trap mass spectrometry (ESI–QIT–MS)

ESI–MS was performed on a Bruker Esquire 3000+ mass spectrometer equipped with a quadrupole ion trap (QIT) mass analyzer (Bruker Daltonik, Bremen, Germany). The eluate solution from the lectin-AFC was diluted 1:1 with acetonitrile, containing 1% formic acid. No further desalting was carried out. Sample was introduced by direct infusion using a Cole Palmer 74900 syringe pump (Cole-Palmer Instrument, London, United Kingdom) at a flow rate of 3 μL/min. The scan range was adjusted from *m/z* 400 to 3000 with a scan rate of *m/z* 13,000 s^{-1}. Mass spectra were typically obtained by averaging the unselected scans over 60 s.

2.6. MALDI-TOF-MS

MALDI-TOF experiments were performed on a Kratos Axima LNR (Kratos Analytical, Manchester, UK) instrument equipped with a linear time-of-flight tube. On a stainless steal target 0.4 μL of 0.2 M diammonium hydrogen citrate were applied, afterwards 0.6 μL of the sample solution obtained from the lectin-AFC and then 0.8 μL of matrix solution (a solution of 10 mg/mL 2,4,6-trihydroxyacetophenon in acetonitrile). The droplet was dried at room temperature [21]. Spectra were obtained by averaging 100–400 single, unselected laser shots.

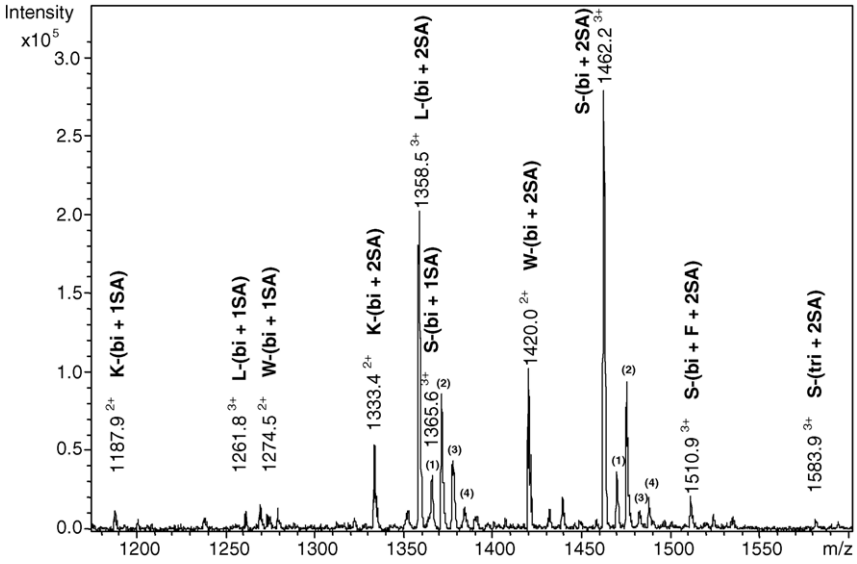


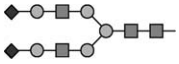
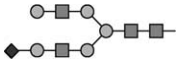
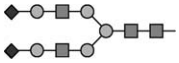
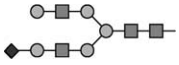
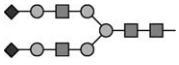
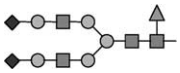
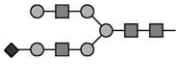
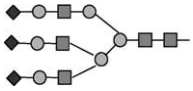
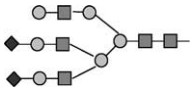
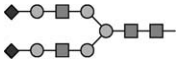
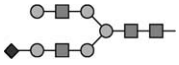
Fig. 1. Positive ion ESI–QIT–MS^1 spectrum of AT glycopeptides after pre-separation by ConA - AFC. Average masses are given for the $[M + nH]^{n+}$ ions of the glycopeptides specified, with $n = 3$ for the larger peptides S and L, and $n = 2$ for the glycopeptides W and K, respectively. (1)–(4) Indicate the following quasi-molecular ions of the glycopeptides S and L: $[M + 2H + Na]^{3+}$, $[M + H + 2Na]^{3+}$, $[M + H + Na + K]^{3+}$, and $[M + 2Na + K]^{3+}$, respectively; used symbols: bi, biantennary glycan; tri, triantennary glycan; F, fucose; SA, sialic acid; MS conditions as specified in Section 2. Symbols: (●) hexose, (■) *N*-acetyl-hexose, (▲) deoxy-hexose, (♦) sialic acid.

3. Results and discussion

3.1. Strategy of analysis

The investigations in this work were carried out on the peptide level after proteolytic digestion of AT by trypsin. When dealing with glycopeptides, the normal mass spectrometric peptide mapping techniques often fail because of distinctive ionization suppression effects regarding the glycopeptides in the presence of non-glycosylated peptides. This effect becomes more important the lower the relative abundance of a certain glycopeptide-isoform is. In our instance, we had to consider minor isoforms below a few percent relative abundance. In order to ensure the significance and reliability of the data, it was thus necessary to pre-separate the glycopeptides prior to mass spectrometry. This was done by means of lectin-affinity chromatography (AFC) using concanavalin A (ConA). ConA selectively retains mannose and glucose containing carbohydrates and is thus appropriate for the enrichment of glycopeptides with N-linked complex-type glycans, particularly with biantennary structure. The eluate from lectin-AFC was eventually applied to the ESI-mass spectrometer without further purification and desalting. The α-D-methylmannopyranoside, employed for the affinity elution of the glycopeptides from the ConA-column and, thus, present in the sample, turned out to not impede the ionization/desorption or the spraying process in ESI. The amino acid sequences of the AT-derived tryptic peptides carrying carbohydrate moieties are given below. These peptides will be referred to by their first amino acid, i.e., L, K, S, and W. The smallest glycopeptide obtained by trypsin digestion was KANK. It contains one theoretical tryptic cleavage site that obviously could not be split, probably due to the adjacent glycosylation.

Table 1
Primary glycan structures attached to the four peptides as determined by various mass spectrometric techniques

Glycopeptide/amino acid sequence	Attached glycan structure		ESI–QIT–MS			Confirmed by	
			Average masses of $[M+H]^+$			$ESI–MS^2$	MALDI-TOF
			Theoretical	Measured	Δm		
LGACNDTLQQLMEVFK		(a)	4074.2	4074.5	0.3	√	√
92–107			3782.9	3784.4	1.5	–	√
KANK		(a)	2666.5	2665.8	−0.7	√	−(c), (d)
133–136			2375.2	2374.8	−0.4	√	−(c)
SLTFNETYQDISELVYGAK		(a)	4385.4	4385.6	0.2	√	√
			4531.5	4531.7	0.2	√	√
			4094.1	4095.8	1.7	–	√
151–169		(b)	5042.0	5041.9	−0.1	√	−(c)
		(b)	4750.7	4750.7	0.0	√	−(c)
WVSNK		(a)	2839.7	2839.0	−0.7	√	−(c), (d)
189–193			2548.4	2548.0	−0.4	–	−(c)

Theoretical as well as measured molecular masses of the mono-protonated glycopeptides. (The mono-protonated masses are calculated from the directly measured masses of the triply and doubly charged ions.) (a) predominant structure, (b) present in very low concentration, (c) insufficient ionization yield by MALDI, (d) confirmation possible after derivatization of the glycopeptides; Symbols: (○) hexose, (■) *N*-acetyl-hexose, (▲) deoxy-hexose, (♦) sialic acid.

3.2. Mass spectrometric structure analysis

The essential part of the analysis was done by means of electrospray multistage mass spectrometry, ESI–QIT–MS^n. This technique comprised low energy collision-induced fragmentation of the glycopeptides and the analysis of the yielded fragments by use of a quadrupole ion trap (QIT) tandem analyzer.

3.2.1. MS^1 mode of operation

The electrospray ionization yielded predominantly triply charged quasi-molecular ions $[M + 3H]^{3+}$, for the larger glycopeptides, S and L, and doubly charged quasi-molecular ions $[M + 2H]^{2+}$, for the smaller ones, W and K. Besides these protonated molecular ions several sodium and potassium adducts were present, i.e., $[M + 2H + Na]^{3+}$, $[M + H + 2Na]^{3+}$, $[M + H + Na + K]^{3+}$, $[M + H + Na]^{2+}$, $[M + 2Na]^{2+}$, etc. The MS^1 spectrum of the glycopeptide mixture is shown in Fig. 1, and the according $[M + H]^+$ values (average masses), evaluated from the *m/z* values of the measured multiply charged ions, are listed in Table 1.

On the basis of the agreement of these measured molecular masses with the corresponding theoretically calculated values, the glycopeptide structures listed in Table 1, could be deduced. This result was obtained using the MS^1 mode of operation (i.e., no CID). It will be shown below that all these structures could be confirmed by MS^2 experiments.

The predominantly present glycans had the canonical (primary) structure published long time ago by Franzén et al. [6] and Turk et al. [7]. With much lower abundances (in the order of a few percent) several other glycan structures were found. These structures are essentially slight variations of the canonical structure, and, the presence of these variations turned out as being site-specific.

Per accident, the peaks of the low abundant monosialylated glycoform of S interfered with the $[M + 2H + Na]^{3+}$ peak of the higher abundant bi-sialylated glycoform of L. The presence of this glycopeptide was thus additionally confirmed by means of MALDI-TOF-MS using a linear TOF analyzer. By the MALDI-TOF technique the glycopeptides were detected predominately as singly charged intact molecular ions, either as $[M + H]^+$ in the positive ion mode or as $[M - H]^-$ in the negative ion mode, giving simple spectra which were not affected by the mentioned interference. (With this ionization technique most of the glycopeptides of S and L could be confirmed, whereas the two smaller glycopeptides, K and W, were not detected. This is not unexpected as with these glycopeptides the molecular mass of the glycan is more than three-fold as large as that of the peptide backbone and ion suppression is observed in such instances [22].)

3.2.2. MS^2 and MS^3 mode of operation

MS^2 experiments were performed for all glycopeptides listed in Table 1 in order to confirm the primary structures (monosaccharide sequence) concluded on the base of the MS^1 data. The low energy CID-based fragment analysis started from the protonated quasi-molecular cations $[M + H]^+$. For the selection of this precursor ion a mass spectrometric resolution of about 200 was used. Due to the soft ionization and low energy CID conditions in the ESI–QIT–MS instrument, exclusively fragmentation of the carbohydrate chain took place in the MS^2 mode giving very clear and reliable spectra. As an example, the MS^2 spectra of two glycopeptides are shown in Fig. 2; glycopeptide W with the biantennary, disialylated glycan, and glycopeptide S with the fucosylated, biantennary, disialylated glycan.

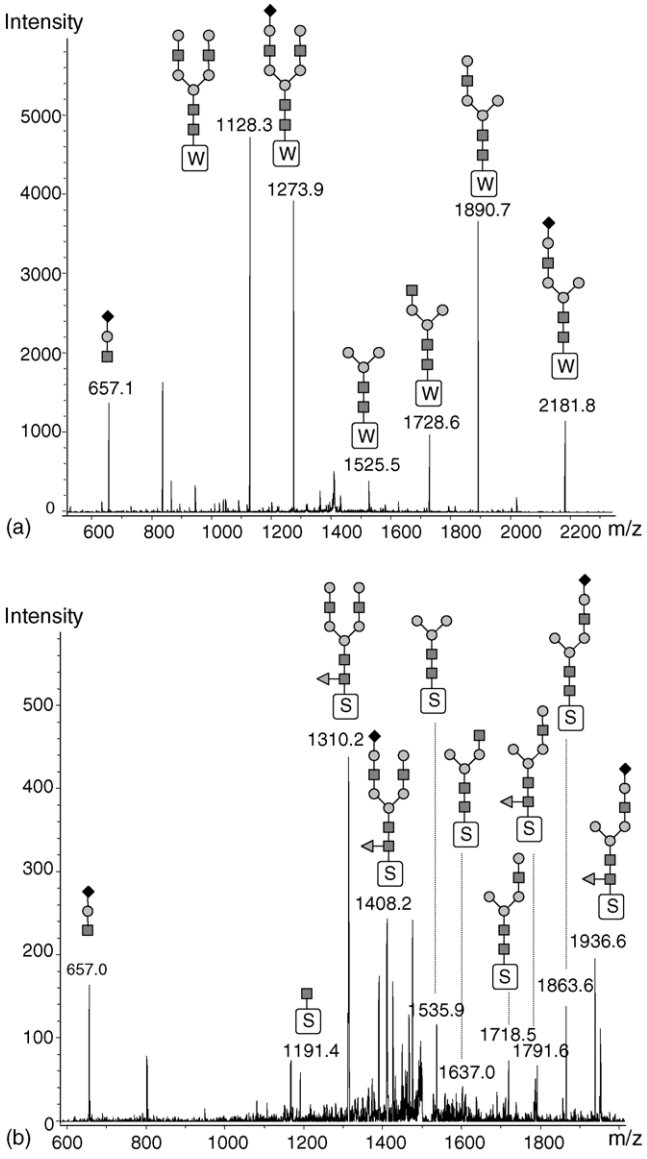


Fig. 2. Positive ion ESI–QIT–MS^2 spectra involving low energy CID of two glycopeptides. (a) glycopeptide W with biantennary, disialylated glycan, monoisotopic molecular mass 2836 Da (selected precursor ion: $[M + 2H]^{2+}$, *m/z* 1420), (b) glycopeptide S with biantennary, disialylated, core fucosylated glycan, monoisotopic molecular mass 4528 Da (selected precursor ion: $[M + 3H]^{3+}$, *m/z* 1510). Symbols as in Fig. 1.

The amino acid sequence in the peptide backbone could be confirmed by subsequent MS^3 experiments, selecting in the ion trap the respective peptide with only one core-GlucNAc attached as precursor ion for the MS^3 stage. The amino acid sequence of the glycopeptides L and S could be confirmed by the detection of a large number of fragment ions belonging to the respective b and y series.

4. Conclusion

Considering the site-specific variation of the glycan structures shown above, the four glycopeptides clearly did not exhibit the same variability. Glycopeptide S (containing N^{155}) showed the highest variability. Apart from the main glycoform four other forms were present at this peptide, all with significant lower abundances: one was fucosylated at the core-GlucNAc, one was deficient of one of the terminal SAs, one had a triantennary-trisialo- and, finally, one a triantennary-disialo structure. The shortest glycopeptide, K (containing N^{135} and lacking glycosylation in case of β-AT) occurred also in the triantennary-trisialo and the biantennary-monosialo glycoform. Glycopeptides L (containing N^{96}) and W (containing N^{192}) had only one additional glycoform each, i.e., those deficient in one of the terminal SAs. There was no fucosylation observed apart from glycopeptide S. Together, 7 minor populated glycoforms could be determined.

From our CZE-MS experiments reported previously [5], which established the accurate molecular mass of the intact glycoprotein isoforms, the occurrence of only one single fucosylated glycan was concluded, all other glycans being of canonical structure. Analogously, the presence of either one triantennary glycopeptide or one glycopeptide deficient in one SA was concluded. All these isoforms were measured with relative abundances in the order of a few percent. The results of the present investigation shown in Table 1 confirm these structures postulated before [5]. They show that core-fucosylation in only found in glycopeptide S. This is interesting as human plasma AT was believed to be completely non-fucosylated [18]. Opposite to this, the glycans in recombinant AT obtained from baby hamster kidney cells were reported as fully fucosylated at all four sites [9].

In a cumulative overview on the results obtained so far, one can conclude that the types of variation in the glycans of AT are rather limited. The variability in the carbohydrate structures was found to differ site-specifically. Most isoforms exhibit only one variation at the time (except the loss of a terminal SA which is observed also in the triantennary structure). Isoforms with cumulated variations are expected to occur in trace amounts far below 1% relative abundance, they were not detected and characterized so far. No AT was found lacking a complete glycan at other positions than N^{135}.

All data refer to AT gained from a plasma pool of about 1500 L plasma. They are thus representative and can be seen as reference values when comparing with recombinant samples. Together with our previous data given in [5], we could give a quite complete qualitative and quantitative image of the glycopeptides present in AT with abundances higher than 1%. It will be the topic of our further research to establish the individual scatter in AT glycosylation in a healthy population and to compare the AT phenotype between healthy and diseased persons by means of proteomic methodology. On the base of these data, it should be possible to find out whether there exists a correlation between glycosylation pattern and certain diseased states.

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