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Glycoproteomics of cerebrospinal fluid in neurodegenerative disease

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Abstract

Cerebrospinal fluid (CSF) from individual patients with Alzheimer's disease (AD) was separated by narrow range two-dimensional (2D) gel electrophoresis and analyzed by electrospray FT-ICR MS in this glycoproteomic study. Because several altered proteins in the comparison between AD patients and healthy controls individuals are isoforms of glycoproteins, it is important to determine if the modifying glycans are also altered. FT-ICR MS and fragmentation of glycopeptides with infrared multiphoton dissociation (IRMPD) offers abundant fragment ions through breakage at the glycosidic linkages with excellent mass accuracy, which facilitates the structural determination of the site-specific N-linked glycosylation. We present results from a structural comparison of proteins from three AD patients and three control individuals of different glycosylated isomers of α-1-antitrypsin, β-trace and apolipoprotein J.

Keywords: Cerebrospinal fluid; Glycoprotein; Ion cyclotron resonance; MS/MS; Fourier transform; FT-ICR MS; FTMS; IRMPD

1. Introduction

The causes and mechanisms of the neurodegenerative disorder, Alzheimer's disease (AD), are still unknown and the only way to confirm the diagnosis is the postmortem identification of senile plaques and neurofibrillary tangles in the brain. Elevated cerebrospinal fluid (CSF) levels of β-amyloid-42, the major component of senile plaques, combined with analysis of hyperphosphorylated tau-protein, found in neurofibrillary tangles, is predictive of AD [1,2]. These biochemical changes can be determined in the CSF due to its direct contact with the brain.

In order to diagnose AD earlier, more accurately, and to discriminate AD from other primary dementia disorders, efforts have been made to identify peripheral biochemical markers. Proteomic studies with two-dimensional (2D) gel electrophoresis and mass spectrometry of CSF have recently revealed new potential biomarkers for AD [3]. Several of the altered proteins in the comparison between AD patients and controls were isoforms of glycoproteins such as α-1-antitrypsin, β-trace, α-1β-glycoprotein, apolipoprotein J, and apolipoprotein E. Therefore, potential changes in the glycosylation patterns of these proteins and their role in AD may prove important in the etiology of AD.

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) provides the high mass accuracy [4] needed to improve the specificity for protein database search results and prediction of glycoforms. Previous reports demonstrate sub femtomole sensitivities with FT-ICR MS [5,6] permitting analysis of 2D gel separated complex human protein mixtures in the femtomole range. Fragmentation of large multiply charged ions, such as peptides, can be performed with infrared multiphoton dissociation (IRMPD) [7]. The glycosidic bonds of N-linked glycans, (amino acid consensus sequence N–X–S/T/C, in which X cannot be P) are selectively dissociated by IRMPD [8]. In this study, we apply FT-ICR MS and IRMPD to structurally determine the glycosylation states of altered isoforms of CSF proteins from individual AD patients compared to controls.

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2. Experimental

2.1. CSF proteins

Cerebrospinal fluid samples were obtained from the Clinical Neurochemical Laboratory, Sahlgrenska University Hospital/Mölndal, Sweden. The AD group consisted of eight patients aged 77 ± 6 years, three men and five women, all of whom underwent a comprehensive clinical examination and were diagnosed with AD according to NINCDS ADRDA criteria [9].

The control group consisted of eight individuals aged 67 ± 5 years, three men and five women, with no history, symptoms, or signs of psychiatric or neurological disease. Lumbar puncture was performed in the L4–L5 vertebral interspace. The first 12 ml of CSF was collected and gently mixed to avoid possible gradient effects. The CSF samples were centrifuged at 2000 × *g* (4 °C) for 10 min to eliminate cells and other insoluble material, and stored at −80 °C. The study was approved by the Ethical Committee of Göteborg.

2.2. Gel electrophoresis

The 2D gel electrophoresis method has previously been described in detail [3]. Briefly, 300 μl of CSF was mixed with 900 μl of ice-cold acetone and stored for at least 2 h at −20 °C. The mixture was centrifuged at 10 000 × *g* (4 °C) for 10 min. The pellet was air-dried and mixed with 10 μl of a buffer containing 0.35 M sodium dodecyl sulfate (SDS) and 150 mM dithiothreitol (DTT) and boiled for 2–3 min. The sample was diluted with 70 μl of isobuffer (9 M urea, 4% 3-[(cholamidopropyl) dimethylammonio] propanosulfonate hydrate (CHAPS), 35 mM Tris, 65 mM DTT, bromphenol blue) and 70 μl of rehydration buffer (9 M urea, 4% immobilized pH gradient (IPG) buffer, 18 mM DTT, bromphenol blue). Isoelectric focusing was performed with IPG strips, pH 4.7–5.9, 7 cm (BioRad, Hercules, CA, USA), in the Protean IEF Cell (BioRad). Active rehydration was performed at 50 V. The focusing step was completed at 20,000 V h, following a program of 250 V for 15 min and 4 kV for 2 h at 20 °C. After equilibration of the IPG strips in buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, bromphenol blue) containing 1% DTT for 15 min and 2.5% iodoacetamide for a further 15 min, the second dimension separation was carried out by use of the Nu-PAGE gel system (NOVEX, San Diego, CA, USA) combined with MOPS running buffer [1 M 3-(*N*-morpholino) propane sulfonic acid (MOPS), 1 M Tris, 69 mM SDS, 20 mM EDTA] for about 45 min.

The gels were stained with Pro-Q Emerald 300 Glycoprotein stain [10] (Molecular Probes, Leiden, The Netherlands) scanned in a Fluor-S Multi-Imager (BioRad) then stained with Sypro Ruby Protein Stain (Molecular Probes) according to the supplier's protocols and scanned again in order to obtain an image of the protein pattern. The optical density of protein spots is proportional to protein concentration. The spots were detected, quantified and matched by use of the PD-Quest 2D-gel analysis software. Only statistically significant results (Mann–Whitney *U*-test <0.05) were considered.

Duplicate gels for individual CSF samples (500 μl) were stained with a Colloidal Blue staining kit (Invitrogen, Carlsbad, CA, USA). Gel spots containing isoforms of glycoproteins were removed from three colloidal blue-stained AD patient gels and three control individual gels, respectively.

2.3. In-gel digestion of glycoproteins

The method described by Shevchenko et al. [11] was applied with minor modifications. Briefly, the gel pieces were washed twice in 200 μl of H_2O/CH_3CN (1:1 v/v) for 15 min and destained with 200 μl of acetonitrile. The gel pieces were dried in a vacuum centrifuge, rehydrated in 10–20 μl of digestion buffer (25 mM NH_4HCO_3) containing 12.5 ng/μl sequencing grade trypsin (Promega, Madison, WI, USA)], and incubated at 37 °C overnight. Any remaining digestion was stopped with 20 μl 2% formic acid (FA) in water. The supernatant was collected, and the peptides were extracted twice with 30 μl of 2% FA in water/CH_3CN (1:1). The combined supernatants were evaporated to dryness in a vacuum centrifuge.

2.4. Accurate mass measurements of tryptic digests

Tryptic digests were reconstituted in 20 μl 0.1% formic acid (Sigma, St. Louis, MO, USA) in water and desalted with C_{18} ZipTips (Millipore, Bedford, MA, USA). The peptides were then directly eluted for 2× in 5 μl 1:1 (v/v) CH_3CN/H_2O with 0.1% FA. Eluted samples were diluted to a final volume of 20 μl with a stock solution of electrospray solvent containing 1:1 methanol (J.T. Baker, Philipsburg, NJ, USA) and water with 2.5% acetic acid (Sigma), resulting in estimated tryptic peptide concentrations of approximately 100 fmol/μl, or lower.

The mass spectrometry analysis has previously been described in detail and was applied with minor modifications [12]. A custom-built, passively shielded, microelectrospray ionization (ESI) [13] 9.4 T FT-ICR mass spectrometer [14] equipped with a quadrupole mass filter, allowing mass-selective [15] external ion accumulation [16] and a middle octopole, modified to allow improved ejection of ions along the *z*-axis [17] was employed for mass mapping of the tryptic peptides. Dual, switchable, 50 μm i.d. fused-silica ESI emitters [18] were used to infuse the peptides and a calibration solution (Agilent Technologies, Wilmington, DE, USA), without mixing the two solutions [19]. The flow rate was 300 nl/min in both cases, and the switching interval for the emitter containing the calibration solution was 400 ms. The emitters were operated in positive-ion mode. Following external accumulation for 20 s or longer, glycopeptide ions were transferred through an octopole ion guide and captured by gated trapping in an open cylindrical ICR cell.

Peptide ions were subjected to chirp excitation (72–480 kHz at 150 Hz/μs) and direct-mode broadband detection (512 Kword data points). Hanning apodization and one zero fill was applied prior to fast Fourier transform followed by magnitude calculation. The experimental event sequence was controlled by a modular ICR data acquisition system (MIDAS) [20,21]. Internal calibration was performed from ions of two mass-to-charge (*m*/*z*) ratios, 922.00979 and 1521.97146. The spectra were generated from sums of 5–10 time-domain transients, depending on the duration of the external ion accumulation.

2.5. Bioinformatics tools

Monoisotopic peak lists were generated by the THRASH algorithm [22]. Measured multiple charged mass values for peptides that could not be matched to the identified protein sequence were examined for the presence of glycosylation by use of the GlycoMod tool (http://us.expasy.org/tools/glycomod) [23]. The SwissProt accession number corresponding to the protein identity and unmatched monoisotopic masses was entered, and a mass deviation of 10 ppm was tolerated. The number of possible hexoses (Hex) was set to 3–10, *N*-acetylhexosamines (HexNAc) 2–10, deoxyhexoses 0–3, and *N*-acetylneuraminic acids (NeuAc) 0–6.

2.6. Infrared multiphoton dissociation

Infrared multiphoton dissociation was performed with the 9.4 T FT-ICR instrument described above. The instrument is equipped with a 40 W, 10.6 μm CO_2 laser (Synrad, Mukilteo, WA, USA). An off-axis geometry with a 2.5× beam expander was used [19]. Precursor glycopeptide ions were isolated by a quadrupole mass filter and by stored waveform inverse Fourier transform (SWIFT) ejection [24]. Photon irradiation period and laser power were optimized to produce the greatest number and abundance of product ions, typically in the range of 100 ms at 20% laser power. The spectra represent averages of 5–10 time-domain transients. The external ion accumulation was typically increased to more than 60 s to attain a maximum number of possible precursor ions.

3. Results and discussion

Isoelectric focusing of proteins with micro-narrow strips (pH 4.7–5.9) improves the separation of different glycoprotein isomers compared to separation over a large pH range. Trains of spots, containing five isoforms of α-1β glycoprotein, eight isoforms of α-1-antitrypsin, two isoforms of β-trace and eight isoforms of apolipoprotein J and apolipoprotein E were separated as shown in Fig. 1. Statistical comparison of eight AD patients versus eight control individuals highlighted several spots as significantly changed ($P < 0.05$, Mann–Whitney), each marked with a circle on the gel. In a previous study of Sypro Ruby total protein stained 2D gels, 18 protein spots were found to be up or down regulated [25] and, consistent with this study, nine of the altered spots were glycoproteins. The glycoprotein specific Pro-Q Emerald 300 stain was used to verify that the different protein isoforms were glycosylated. The image of

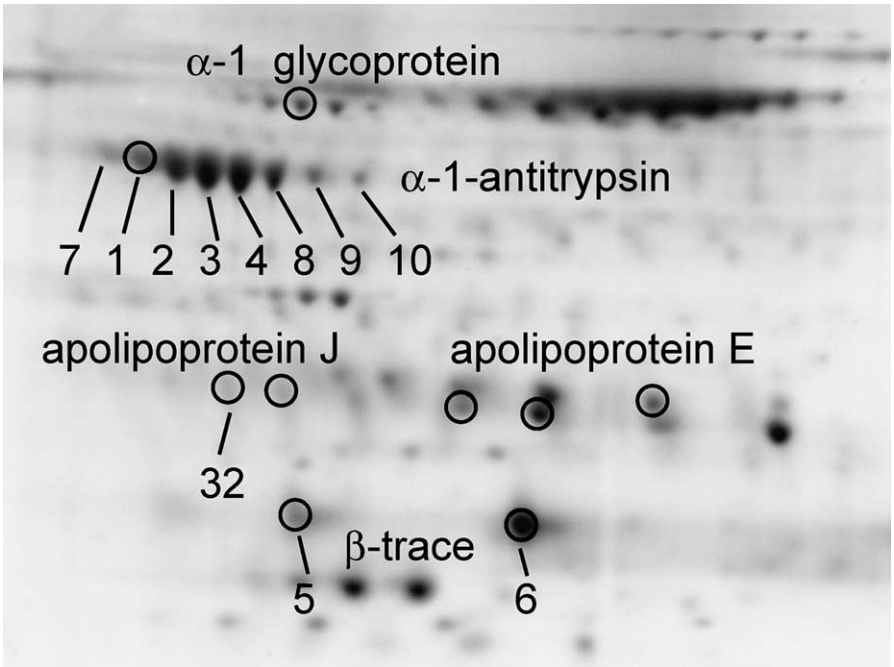

Fig. 1. Two-dimensional gel electrophoresis, pH 4.7–5.9, of 300 μl human cerebrospinal fluid. This gel is an example from one AD patient and is stained with Sypro Ruby. Circles denote the altered proteins compared to control individuals analyzed with PD-Quest. Numbered spots were excised from duplicate gels (500 μl CSF) stained with colloidal blue for analysis by FT-ICR MS.

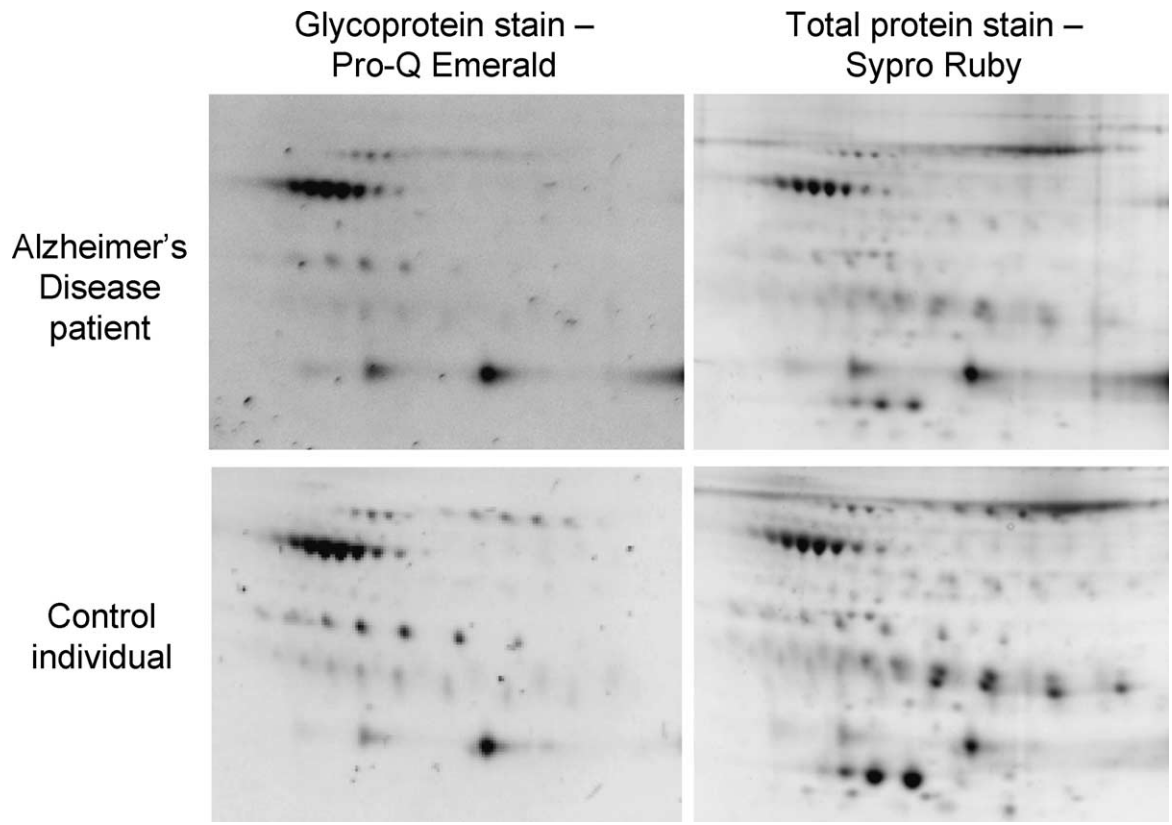

Fig. 2. Images with differential staining demonstrate the glycoproteins in the left column and the total protein content in the right column. Two-dimensional gels of 300 μl human CSF were first stained with Pro-Q Emerald, scanned, followed by total protein staining and a second scanning. Images were used to verify the presence of glycosylation to compare AD patients with control individuals.

the glycoprotein specific gel was used to target glycoprotein spots for further analysis. Typical gels from this study, demonstrating the difference between glycoprotein and protein images, are illustrated in Fig. 2. Spots were excised from colloidal blue stained duplicate gels from three individual AD patients and three individual control persons. In total, 108 samples from three proteins from six gels were analyzed with microelectrospray FT-ICR MS. α-1β Glycoprotein was not analyzed due to a larger gel-to-gel variation than the other four proteins. Results for apolipoprotein E were poor, and therefore, not reported here. For a more complete picture of the glycosylation pattern in apolipoprotein J, further analysis is needed. In this paper we show an example of characterization of apolipoprotein J to demonstrate the analytical capacity of FT-ICR MS for glycoproteomic investigations.

Accurate mass assignments of tryptic peptides were submitted in database queries to provide protein identification. Unmatched peptide masses, derived from multiply charged ions, were submitted to the Glyco-Mod software tool in order to obtain suggested assignments of glycan composition.

The analysis of the isoforms of α-1-antitrypsin and of β-trace are summarized in Tables 1 and 2. Because this study is the first glycoproteomic characterization of CSF from individual AD patients, comparative mapping of all of the eight glycosylated isoforms of α-1-antitrypsin (Table 1) is of interest.

The altered spot 1 did not show any difference in glycosylation patterns between AD and control individuals; therefore, the observed difference in the protein image may be a result of difference in the protein amount. In the overall structural determination of the glycosylation pattern, there is a large similarity to the glycoforms reported from the analysis of the pooled control material [12]. From the total number detected, the AD samples contained more glycans than the controls. In total, 58 glycans were found in the eight isoforms from the three AD patients and 32 glycans were found in the three control gels.

The two isoforms of β-trace were both altered when compared from the protein stained 2D-gel. In Table 2, there is a trend toward greater microheterogeneity in isoform spot 6 from the control individuals, whereas there is about the same amount in the two isoforms from the AD patients. One possible explanation for this finding may be a more acidic β-trace in AD patients due to an increase in sialic acid content.

The levels of the apolipoproteins were the most altered in previous comparison of CSF in AD patients [3]. Apolipoprotein J (ApoJ/clusterin), is a multifunctional glycoprotein capable of interacting with a broad spectrum of molecules. In pathological conditions, ApoJ is an amyloid-associated protein, co-localizing with the cerebrovascular deposits found in AD. ApoJ expression is up-regulated in a wide variety of conditions and may represent a defense response against lo-

Table 1
Summary of the different glycoforms found in the eight isoforms of α-1-antitrypsin (SwissProt accession no P01009), labeled in Fig. 1

Glycoform		Composition					Gel spot							
Mass	Charge	Sequence	Hex	HexNAc	Fuc	NeuAc	7	1	2	3	4	8	9	10
1091.71	5+	244–274	5	4	0	1			*		*			
1149.93	5+	244–274	5	4	0	2		#**	##	*	*			
1223.87	3+	244–259	5	4	0	1	*	##**	#***	#**	#**	#*	*	*
1278.59	4+	40–69 (met-ox)	5	4	0	1			*	*				
1320.90	3+	244–259	5	4	0	2	*	#*	##***	###***	##**	#**	**	**
1351.36	4+	40–69 (met-ox)	5	4	0	2			#**	#**	*	*		
1387.89	4+	40–69 (met-ox)	5	4	1	2			*		*			
1437.17	4+	244–274	5	4	0	2		##**	##*	##*		#		
1474.92	4+	70–101	5	4	0	2		##**	##***	#**	#*	#		
1566.20	4+	70–101	6	5	0	2			*	*	*			

The presence of a specific glycoform is visualized with symbols in the three AD patients (marked *) and the three control individuals (marked #) analyzed by FT-ICR MS.

Table 2
Summary of the different glycoforms found in the two isoforms of β-trace (SwissProt accession no P41222), labeled in Fig. 1

Glycoform		Composition					Gel spot	
Mass	Charge	Sequence	Hex	HexNAc	Fuc	NeuAc	5	6
1214.52	3+	21–34	5	4	1	1		###*
1282.21	3+	21–34	5	5	1	1		###**
1311.54	3+	21–34	5	4	1	2	##*	###*
1326.93	3+	45–63	5	4	1	1	#**	##**
1344.58	3+	21–34	4	6	2	1		#*
1379.25	3+	21–34	5	5	1	1	#**	###*
1389.30	3+	45–63	4	5	2		*	#*
1394.62	3+	45–63	5	5	1		#**	###**
1423.96	3+	45–63	5	4	1		##**	###***
1466.67	3+	45–64	5	4	1		#**	##
1491.66	3+	45–63	5	5	1		#**	###***
1534.36	3+	45–64	5	5	1		*	#

The presence of a specific glycoform is visualized with symbols in the three AD patients (marked *) and the three control individuals (marked #) analyzed by FT-ICR MS.

cal damage to neurons, including binding to hydrophobic regions of partially unfolded proteins, and therefore, ApoJ may play a protective role against aggregation in a chaperone-like manner [26]. The 26 kDa β-subunit of apolipoprotein J, which possesses three possible potential N-linked glycosylation sites, was identified in spot 32. In the FT-ICR MS spectra of spot 32 from one AD patient (Fig. 3), 10 unmodified peptide masses identified ApoJ and 4 unmatched peptide masses could be assigned to glycopeptides (Table 3). The mass accuracy (better than 10 ppm) provided by FT-ICR MS reduces the number of possible glycoforms per peptide and simplifies the interpretation of the IRMPD data (Table 4).

From gel spot 32, labeled in Fig. 1, a glycopeptide candidate was fragmented by IRMPD to yield the specific cleavage of glycosidic bonds for the determination of the glycan structure in this isoform. By prediction there is often more than one possibility for the composition of an attached glycan based only on accurate mass measurement. Ideally, several candidates should be fragmented but the low sample volume of (20 μl, in this case) restricts the number of possible precursors to fragment. As an example of IRMPD frag-

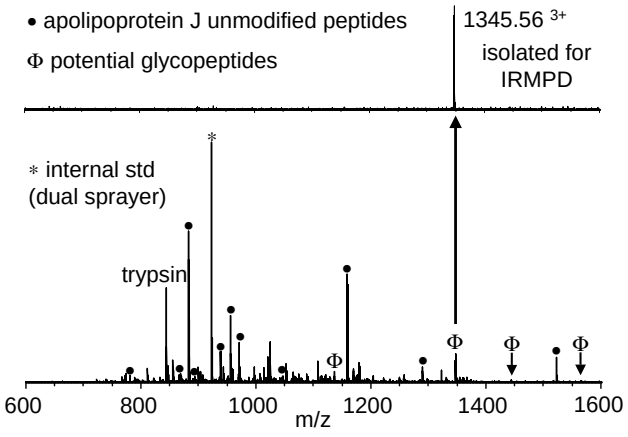

Fig. 3. Mass spectrum of unfractionated desalted tryptic peptides from apolipoprotein J gel spot 32, obtained by microelectrospray ionization 9.4 T FT-ICR MS. The upper part shows a peak selected for IRMPD by use of the external quadrupole mass filter and SWIFT internal ion ejection. The lower spectrum is a sum of seven scans and was obtained following 20 s external accumulation at a flow rate of 300 nl/min. Masses and peak assignment are found in Tables 3 and 4.

Table 3
Glycopeptides identified by GlycoMod for the tryptic digests of apolipoprotein J (SwissProt accession no P10909) gel spot 32 from one AD patient

Theoretical peptide mass (M)	Mass difference (ppm)	Glycoform composition[a]				Peptide sequence		Missed cleavage
		Hex	HexNAc	Deoxyhexose	NeuAc			
Search results for *m/z* 1134.791, charge +3, mono mass 3401.352								
3401.350	0.7	4	1	2	1	81–89	KEDALNETR	1
3401.362	−3.1	0	2	0	2	82–94	EDALNETRESETK	1
3401.392	−11.7	2	3	0	1	287–297	EIRHNSTGCLR	1
3401.392	−11.9	2	2	1	1	287–297	EIRHNSTGCLR	1
3401.307	13.2	5	4	0	0	290–297	HNSTGCLR	0
3401.306	13.4	5	3	1	0	290–297	HNSTGCLR	0
3401.375	−6.8	5	0	0	1	290–299	HNSTGCLRMK	1
Search results for *m/z* 1345.560, charge +3, mono mass 4033.659								
4033.609	12.5	5	3	1	1	287–297	EIRHNSTGCLR	1
4033.608	12.7	5	2	2	1	287–297	EIRHNSTGCLR	1
4033.590	17.1	3	6	0	1	290–299	HNSTGCLRMK	1
4033.591	17.0	3	5	1	1	290–299	HNSTGCLRMK	1
4033.656	**0.7**	**2**	**2**	**1**	**2**	**372–385**	**LANLTQGEDQYYKR**	**0**
Search results for *m/z* 1442.566, charge +3, mono mass 4321.676								
4324.662	3.3	7	1	3	2	81–89	KEDALNETR	1
4324.674	0.3	3	2	1	3	82–94	EDALNETRESETK	1
4324.704	−6.5	5	3	1	2	287–297	EIRHNSTGCLR	1
4324.704	−6.6	5	2	2	2	287–297	EIRHNSTGCLR	1
4324.656	4.4	1	5	3	3	290–297	HNSTGCLR	0
4324.686	−2.5	3	6	0	2	290–299	HNSTGCLRMK	1
4324.686	−2.4	3	5	1	2	290–299	HNSTGCLRMK	1
4324.752	−17.8	2	2	1	3	372–385	LANLTQGEDQYYLR	0
Search results for *m/z* 1564.297, charge +3, mono mass 4689.869								
4689.806	13.3	4	3	1	3	82–94	EDALNETRESETK	1
4689.928	−12.7	0	4	0	1	97–118	VCNETMMALWEECK	0
4689.836	7.0	6	4	1	2	287–297	EIRHNSTGCLR	1
4689.836	6.9	6	3	2	2	287–297	EIRHNSTGCLR	1
4689.789	17.0	2	7	2	3	290–297	HNSTGCLR	0
4689.788	17.1	2	6	3	3	290–297	HNSTGCLR	0
4689.818	10.7	4	7	0	2	290–299	HNSTGCLRMK	1
4689.819	10.6	4	6	1	2	290–299	HNSTGCLRMK	1
4689.884	−3.4	3	3	1	3	372–385	LANLTQGEDQYYLR	0

The glycoform at *m/z* 1345.560 $^{3+}$ was verified by IRMPD, marked in gray. The search results demonstrate the importance of high mass accuracy to decrease the number of possible glycoforms per peptide mass submitted.

[a] N-linked core structure with 3 Man (Hex) and 2 GlcNAc (HexNAc) not included.

Table 4
Mass assignment and sequence coverage for the β-subunit of ApoJ as analyzed with FT-ICR MS corresponding to the spectra in Fig. 3

Observed mass	Charge	Matched mass	Error	Start–end[a]	Sequence
779.403	+1	779.405	−2.7	438–443	(K)ALQEYR(K)
865.923	+2	1730.842	−2.4	430–443	(K)FMoxETVAEKALQEYR(K)
881.918	+2	1762.828	−0.2	307–322	(R)EILSVDCSTNNPSQAK(L)
907.519	+1	907.525	−6.7	340–346	(R)KYNELLK(S)
937.498	+2	1873.991	−1.5	409–425	(K)LFDSDPITVTVPVEVSR(K)
954.462	+1	954.462	1.6	430–437	(K)FMETVAEK(A)
970.456	+1	970.456	0.4	430–437	(K)FMoxETVAEK(A)
1039.989	+2	2078.960	5.6	305–322	(K)CREILSVDCSTNNPSQAK(L)
1157.599	+2	2314.178	5.5	386–408	(R)VTTVASHTSDSDVPSGVTEVVVK(L)
1288.646	+1	1288.639	5.7	326–336	(R)ELDESLQVAER(L)
1345.560	+3	4033.659	0.7	372–385	(R)LAN(*glycan*)LTQGEDQYYLR(V)

Amino acids, 228–270 SLMPFSPYEPLNFHAMFQPFLEMIHEAQQAMDIHFHSPAFQHP; 271–310 PTEFIREGDDDRTVCREIRH**N**STGCLRMKDQC-DK*CREILS*; 311–350 *VDCSTNNPSQAK*LRRELDESLQVAERLTR*KYNELLK*SYQW; 351–390 KML**N**TSSLLEQLNEQFNWVSR*LAN**LTQGEDQYYLRV-TTVA*; 391–430 *SHTSDSDVPSGVTEVVVKLFDSDPITVTVPVEVSR*KNPKF; 431–449 *METVAEKALQEYR*KKHREE.

[a] Amino acid position corresponds to the β-subunit of ApoJ (see above, with the MS data coverage shown with italic letter), which is the second half of the precursor protein (acc no P10909).

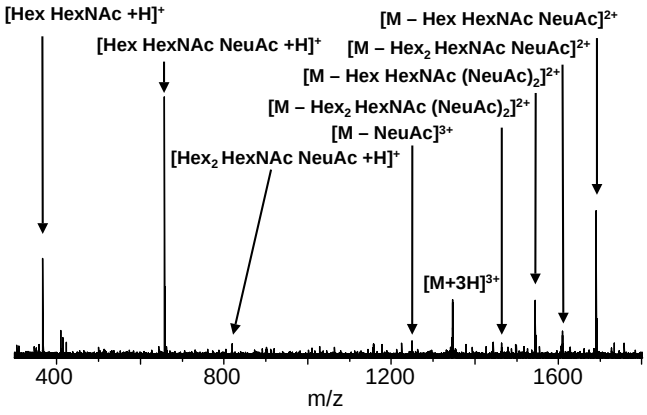

Fig. 4. IRMPD product ion spectrum of apolipoprotein J glycopeptide, at *m/z* 1345.560 3+, from gel spot 32, confirming the glycoform $(Hex)_2(HexNAc)_2(DeoxyHexose)_1(NeuAc)_2(Hex)_3(HexNAc)_2$ predicted by GlycoMod. The product ions show different levels of losses of glycan residues as well as the parent ion $[M + 3H]^{3+}$. For example the most abundant product ion corresponds to loss of one antenna from the core in the glycan structure. Following *m/z* were observed and their assignments are displayed in this IRMPD spectrum; 366.156 $^{1+}$, 657.261^{1+}, 819.311^{1+}, 1248.518 $^{3+}$, 1345.560 $^{3+}$, 1463.156 $^{2+}$, 1544.166 $^{2+}$, 1608.673 $^{2+}$ and 1689.712 $^{2+}$.

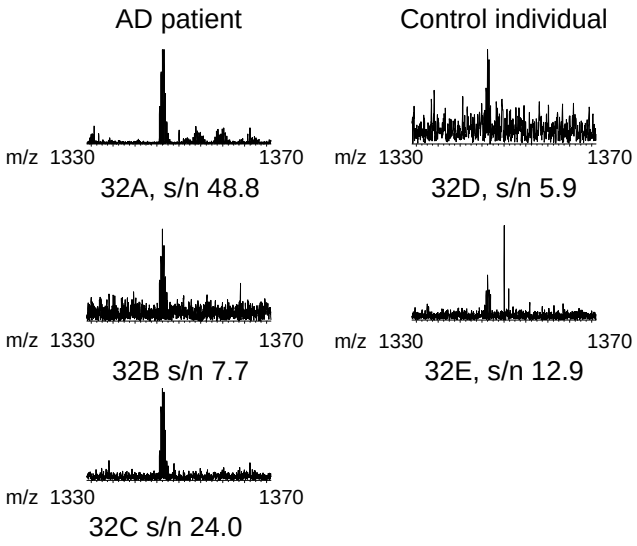

Fig. 5. Comparison of CSF samples from Alzheimer's Disease patients vs. control based on MS from gel spot 32. Glycopeptide at *m/z* 1345.560^{3+} shows qualitative indications that are interesting for further analysis, The measurement is not quantitative. Data for the third control sample is missing.

mentation of a glycopeptide candidate and the dissociation of the glycosidic bonds, the spectrum of the triply charged precursor at *m/z* 1345.560 is shown in Fig. 4. The assignment of the glycoform and peptide sequence could be confirmed from the IRMPD data. For the confirmed glycan in ApoJ (Fig. 4), the non-glycosylated peptide was not observed. The MS parameters were optimized to detect as many high mass multiple charged (typically glycopeptides) as possible, and therefore, many unmodified peptides of the remaining amino acid sequence would not have been detected.

The dissociation of the glycans is observed as fragment ions, probably produced through a charge-remote mechanism [8]. The mass of the fragment ions correspond to the mass of the entire glycopeptide minus the loss of various mono-/oligosaccharide fragments. In the low molecular mass region, the characteristic ions of the positively charged HexHexNAc at 366.140 and HexHexNAcNeuAc at 657.235 are annotated as antennae. The suggested structures are based on compositions derived from the determined molecular masses of the oligosaccharides and by analogy to what is known about N-linked carbohydrates produced in mammalian cells. The glycan core structure attached to the asparagine consists of two *N*-acetyl-D-glucosamines (HexNAc) and three mannoses (Hex) and the antennae are linked to the mannoses. The doubly charged ion at *m/z* 1463.156 represents the largest loss of oligosaccharide ($Hex_2HexNAc\ (NeuAc)_2$) from the glycopeptide and suffices to determine that this glycoform is a bisialoantennary structure, e.g., two antennae with sialic acids (NeuAc) at the ends. Because no fucose loss was observed, it is most likely attached directly to the core.

As a first screening method for possible AD specific glycoforms or alterations in the presence of a glycoform, the MS spectra of the samples for the altered protein in spot 32 were compared. Such a comparison is illustrated in Fig. 5, in which the signal-to-noise ratio in combination with the low mass error for this glycopeptide yielded a possible trend for a difference in the CSF between AD patients and healthy control individuals. Trends found in the comparison are not quantitative in this analysis but the MS spectra show qualitative indications that are interesting for further analysis. The main aim is to compare any structural differences in the glycoforms from any altered protein. Due to the low sample number, it will be necessary to analyze more samples from single patients to have a statistically relevant data, and then evaluate the finding in relation to the biochemistry.

4. Conclusion

The ability to characterize the glycan structures in glycoproteins derived from the very scarce samples available from cerebrospinal fluid from individual patients is very important in the search of new biomarkers. In many cases, altered protein profiles of tissues or cells are the result of altered protein post-translational modifications rather than altered gene expression.

FT-ICR MS and IRMPD can provide detailed structural information regarding N-linked glycosylation from individual AD patient CSF samples. This method requires no enzymatic deglycosylation and no extra sample manipulation of the in-gel digested glycoprotein, and therefore, leaves the localization information of the glycoform intact. IRMPD provides valuable structural information of the glycan with very limited dissociation of the peptide backbone.

Characterization of glycoproteins, such as apolipoproteins in CSF, can reveal altered glycosylation patterns for development of new biomarkers.

The full data sets for the more abundant eight isoforms of α-1-antitrypsin and the two isoforms of β-trace have been analyzed and the presence of different glycoforms in three single AD patients has been compared for three control individuals, demonstrating the analytical capacity of FT-ICR MS for glycoproteomic investigations. Further analysis of clinical CSF samples will increase our understanding of the role of glycosylation in neurodegenerative disorders.

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