JOURNAL OF MASS SPECTROMETRY, VOL. 32, 948–958 (1997)

Glycopeptide Profiling of Human Urinary Erythropoietin by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry

Henrik Rahbek-Nielsen,[1] Peter Roepstorff,[1]* Heinz Reischl,[2] Manfred Wozny,[2] Hans Koll[2] and Anton Haselbeck[2]

[1] Department of Molecular Biology, Odense University, Campusvej 55, DK-5230 Odense, Denmark
[2] Boehringer Mannheim, Penzberg, Germany

The site-specific glycan heterogeneity of human urinary erythropoietin was investigated by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Owing to the small amount of protein available, a strategy combining optimal sensitivity and specificity was used. Erythropoietin was reduced, *S*-alkylated and digested with endoproteinase Lys C. The peptides were separated by reversed-phase high-performance liquid chromatography and the molecular masses of the peptides determined by MALDI-MS. The peptides were identified by comparing the experimental masses with the masses predicted from the cDNA derived amino acid sequence. Glycopeptides were identified from the mass spectra based on the peak pattern caused by the glycan heterogeneity. They were further characterized after treatment with neuraminidase and endoproteases. All *N*-glycosylation sites exhibited fucose-containing complex-type glycans. The *N*-glycosylation sites at Asn_{38} and Asn_{83} are mainly occupied by tetraantennary glycans, whereas Asn_{24} is occupied by a mixture of bi-, tri- and tetraantennary glycans. A molecular mass glycoprofile for each glycosylation site was established based on the relative peak intensities observed in the MALDI mass spectra of the desialylated glycopeptides. © **1997 by John Wiley & Sons, Ltd.**

J. Mass Spectrom. **32**, 948–958 (1997)
No. of Figs: 6 No. of Tables: 5 No. of Refs: 25

KEYWORDS: erythropoietin; glycosylation; neuraminidase; matrix-assisted laser desorption/ionization mass spectrometry

INTRODUCTION

Erythropoietin (Epo) is a glycoprotein hormone involved in the regulation of the erythrocyte level by stimulating differentiation of erythroid progenitor cells to mature erythrocytes. In adults the hormone is produced in the kidney and brought to the target cells in the bone marrow via blood circulation. Since Epo increases the number of peripheral red blood cells, there has been a considerable interest in the therapeutic use of Epo for the treatment of severe anemia.

Human Epo is a protein of 165 amino acid residues. It was first purified from the urine of aplastic anemia patients[1] and has been made available as a recombinant protein.[2,3] Approximately 40% of the mass has been assigned to carbohydrate structures attached at Ser_{126} and the three *N*-glycosylation sites at Asn_{24}, Asn_{38} and Asn_{83}.[4] Early studies of the biological activity indicated that the glycosylation is essential for the function of human Epo. Deglycosylation of Epo results in aggregation and removal of the *in vivo* activity. However, *in vitro* interaction with bone marrow target cells was retained to some degree.[5] In addition, desialylation alone eliminates the *in vivo* activity but increases the *in vivo* activity.[6] Variation of the biological activity of recombinant human Epo expressed in different host cells has been proposed to be caused by differences in their glycan structure.[7] Glycans liberated from urinary human Epo have been characterized and compared with those liberated from recombinant Epo using a variety of chromatographic techniques[8] combined with fast atom bombardment mass spectrometry (FAB-MS)[9] and NMR spectroscopy.[10] Investigation of the isolated recombinant Epo glycans by NMR spectroscopy[10] and FAB-MS[9] and a combination of the two techniques[11] demonstrated that Epo contains complex-type glycans with the predominant fraction having fucose attached to the Asn-linked *N*-acetylglucosamine residue. The site-specific glycosylation pattern of the four glycosylation sites at Ser_{126}, Asn_{24}, Asn_{38} and Asn_{83} has been estimated for recombinant Epo by FAB-MS,[12] NMR and FAB-MS,[13] NMR and matrix-assisted laser desorption/ionization (MALDI)-MS[14] and electrospray ionization MS.[15,16] These studies revealed significant differences in the antennarity and heterogeneity of the complex type glycans linked to the three *N*-glycosylation sites. A minor fraction of the glycan on Asn_{24} was found to be a high-manose structure.[14] Depending on the techniques actually used, the limited amount of human urinary Epo available makes it difficult to obtain sufficient material for a full characterization of the site-specific glycosylation. MALDI-MS allows the mass analysis of peptides and glycopeptides in the low-picomole to

* Correspondence to: P. Roepstorff, Department of Molecular Biology, Odense University, Campusvej 55, DK-5230 Odense, Denmark.

Contract grant sponsor: Danish Biotechnology Program.

CCC 1076–5174/97/090948–11 $17.50

Received 1 April 1997
Accepted 29 May 1997

femtomole range[17] and is an excellent method for the analysis of mixtures generated by proteolytic digestion of proteins or by heterogeneous glycosylation. This technique is rapidly developing to be an alternative or complementary method for glycopeptide analysis.[18–21] In the present work we examined the site-specific glycosylation of human urinary Epo by MALDI-MS of the endoproteinase Lys C fragments derived from the protein.

EXPERIMENTAL

Materials

Human urinary Epo was obtained from StemCell Technologies (Vancouver, Canada). Neuraminidase from *Clostridium perfringens* (EC 3.4.1.18), sequencing grade trypsin (EC 3.4.21.4), endoproteinase Glu-C (EC 3.4.21.19) and endoproteinase Lys-C (EC 3.4.99.30) were purchased from Boehringer Mannheim (Mannheim, Germany). High-performance liquid chromatographic (HPLC)-grade acetonitrile (MeCN) and trifluoroacetic acid (TFA) were purchased from Ratburn Chemicals (Walkerburn, UK), α-cyano-4-hydroxycinnamic acid (4HCCA), 4-vinylpyridine and ammonium hydrogencarbonate from Sigma Chemical (St Louis, MO, USA) and 2,5-dihydroxybenzoic acid (DHB) solution from Hewlett–Packard (Palo Alto, CA, USA). Microsep microconcentrators (molecular mass cut-off 10 kDa) were supplied by Filtron (Northborough, MA, USA). Water obtained using an Elgastat water purification unit (Elga, High Wycombe, UK) was used throughout.

Reduction and *S*-alkylation of human urinary Epo

Human urinary Epo was desalted on a Vydac C_4 HPLC column with a linear gradient from 0.1% trifluoroacetic acid (TFA) in water to 80% MeCN, 0.09% TFA in water. The Epo-containing fraction was dried in a Speedvac concentrator, resuspended in 100 μl of 5 mM dithioerythritol, 6 M guadinium·HCl, 12 mM EDTA, 0.1 M Tris–HCl (pH 8.5) and incubated for 90 min at 37 °C. Subsequently, 4-vinylpyridine was added to the solution to a final concentration of 15 mM and the solution was incubated for 30 min at 37 °C.

Proteolytic digestion with endoproteinase Lys C

A Filtron microconcentrator unit was used to transfer 10 μg of pyridylethylated Epo into 80 μl of 25 mM Tris, 7 M urea (pH 8.5) buffer. The solution was diluted by the addition of 320 μl of Tris–HCl (pH 8.5) buffer solution. After the addition of 0.7 μg of endoproteinase Lys C the protein was digested for 16 h at 32 °C.

HPLC separation of the endoproteinase Lys C digest

Peptides were separated on an Aquapore RP300 C_8 column (220 × 2.1 mm i.d.) at a flow rate of 0.2 ml min^{-1}. The eluting solvents were 0.1% TFA in water (solvent A) and 70% MeCN, 0.09% TFA in water (solvent B). The column was equilibrated for 15 min with solvent A and the separation was performed by gradient elution (2% solvent B per minute). The peptides were collected manually and dried in a Speedvac concentrator.

Neuraminidase digestion of glycopeptides

Amounts of 100–200 pmol of the dried peptides were dissolved in 50 mM NH_4HCO_3 (pH 8.5) (final peptide concentration 10 pmol μl^{-1}) and incubated with 3 mU of neuraminidase overnight at 37 °C.

Proteolytic subdigestion of glycopeptides

Aliquots of 0.7 μl were withdrawn from the neuraminidase digests for analysis by MALDI-MS. The remaining sample was further digested with appropriate proteases. The peptides K4 and K6 were incubated at 37 °C for 1 h with ~2% (m/m) trypsin. Glycopeptide K2 was incubated at 37 °C for 4 h with 5% (m/m) endoproteinase Glu C. The digests of K2 and K4 were purified by HPLC as described above.

Mass spectrometry

MALDI mass spectra were acquired with a prototype linear time-of-flight (TOF) mass spectrometer (Applied Biosystems, Uppsala, Sweden) equipped with a 0.7 m flight tube, a 337 nm nitrogen laser and a 300 MHz digitizer. Delayed extraction MALDI mass spectra were obtained on a Voyager Elite instrument (PerSeptive Biosystems, Framingham, MA, USA) using a 20 kV acceleration voltage, 95% grid voltage, 0.2% guide wire voltage and 150 ns delay time. The 4HCCA matrix was dissolved in 70% acetonitrile at 15 g l^{-1}. If not stated otherwise, the dried peptides were dissolved in 50% MeOH, 1% acetic acid in water prior to analysis by MALDI-MS. 4HCCA sample preparations were performed in two steps: the probe tip was precoated with matrix by addition of 0.5 μl of 4HCCA solution and drying at ambient temperature. Subsequently, 0.7 μl of sample solution and 0.5 μl of 4HCCA solution were applied to the probe tip, mixed gently and dried at ambient temperature. For sample preparation with a DHB matrix, equal volumes of sample and DHB solution were mixed in a test-tube and the mixture was applied to the target and left to dry at ambient temperature. Spectra were accumulated from 20–60 laser shots. The MALDI mass spectra were externally calibrated; the calibration constants were derived from spectra of human insulin. In general, the mass accuracy obtained with the prototype instrument was better than 0.2%, which is acceptable for this instrument when using external calibration. The accuracy of mass differences derived from spectra containing multiple peaks and for spectra recorded with the PerSeptive instrument was better than 0.1%. Theoretical peptide masses were calculated from the human erythropoietin protein

sequence[2] (residues 28–193 of human proerythropoietin) using the software GPMAW (Lighthouse Data, Odense, Denmark).

RESULTS

Digestion of Epo with endoproteinase Lys C

Epo was reduced, alkylated and digested with endoprotease Lys C (Lys C). The peptides were separated by reversed-phase (RP)-HPLC (Fig. 1) and the fractions corresponding to the major peaks (for numbering, see Fig. 1) were collected manually. The number of peptides found exceeds the number of peptides predicted from a complete digest of the protein. All the fractions collected were examined by MALDI-MS and assigned to the Epo sequence according to the masses determined (Table 1). The peptides to be expected from a complete digest of Epo are designated K1–K9 according to their positions relative to the *N*-terminus (Fig. 2). Based on the masses determined, fractions 8, 5, 9 and 7 were found to contain the peptides K1, K3, K5 and K7, respectively. The expected dipeptide K8 was only found as part of peptides resulting from incomplete cleavage present in fraction 11 (K5–K6–K7–K8 and K5–K6–K7–K8–K9). In agreement with previous reports,[22] the *C*-terminal peptide K9 was found as a truncated version lacking the *C*-terminal arginine. A few minor peaks present in the chromatogram (fractions 1, 4 and 10) could not be assigned to the Epo sequence and are probably due to unknown impurities. After further characterization as described below, the glycopeptides K2 and K6 were assigned to fractions 3 and 6, respectively. Finally, the most hydrophobic fraction 12 was found to contain glycopeptide K4 and several other components (oxidized K4 and small amounts of the incompletely cleaved peptides K4–K5 and K3–K4). In addition, a distribution of peaks separated by 44 Da around *m/z* 1000 was observed in fraction 12. These signals may originate from a detergent containing glycol esters that was used during the purification of Epo.

Analysis of the *O*-glycosylated peptide K6

The MALDI mass spectrum of the presumed *O*-glycosylated peptide K6 (fraction 6 in Fig. 1) contains three peaks separated by 290.4 and 290.2 Da [Fig. 3(A)]. Within the limits of error of mass determination, this pattern is characteristic of sialylated glycopeptides. As expected, neuraminidase treatment of K6 resulted in a single peak at *m/z* 2867.5 [Fig. 3(B)] corresponding to the signal with lowest *m/z* before neuraminidase treatment. The mass of this peptide is in good agreement

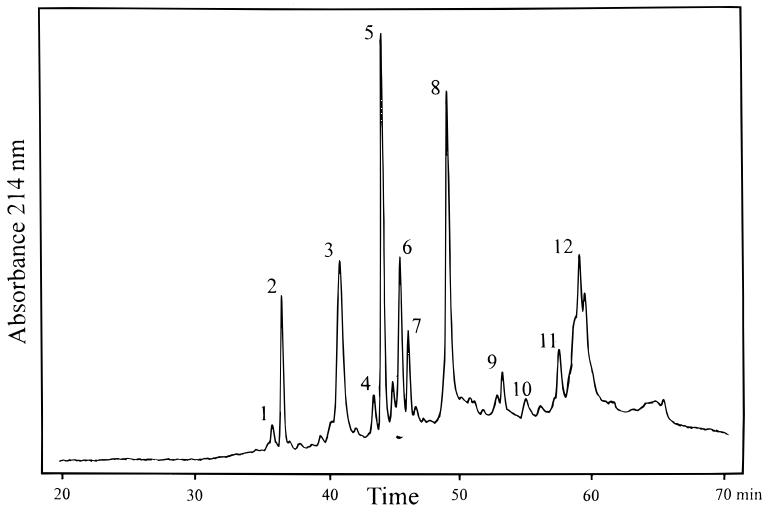


Figure 1. RP-HPLC separation of an endoprotease Lys C digest of human urinary Epo.

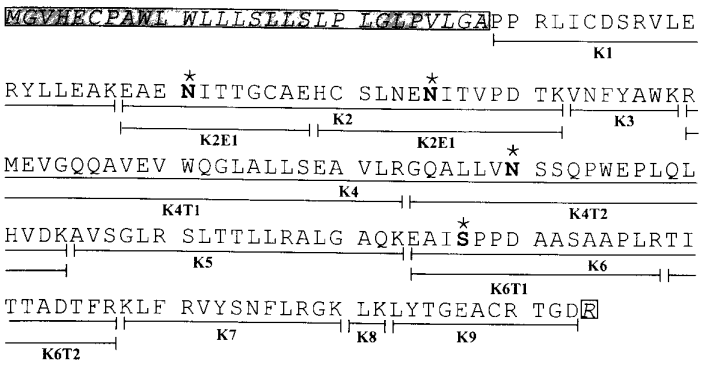


Figure 2. Primary amino acid sequence of human Epo prepeptide obtained by conceptual translation of the cDNA sequence. Hatched sequences correspond to *N*- and *C*-terminal sequences which were not found to be present in human urinary Epo. Peptides generated by endoproteinase Lys C and endoproteinase Glu C are indicated by underlining. An asterisk indicates glycosylated amino acid residues.

Table 1. Identification of the peptides derived from endoproteinase Lys C digestion of human urinary Epo based on MALDI-MS data

HPLC fraction in Fig. 1	*m/z* observed	Calculated mass (Da)	Peptide identification
1	1545.6		Not identified
2	1291.5	1290.4	K9[c]
3	Distribution around 8104.6	2900.2[b]	K2[d]
4	1392.4		
	1525.2		Not identified
	2419.8		
5	928.3	927.0	K3
6	2868.2	2499.8[b]	K6 linked to HexNAc–Hex[d]
	3158.6		K6 linked to HexNAc–Hex–SA
	3448.8		K6 linked to HexNAc–Hex–2SA
7	1503.1	1499.8	K7
8	2447.0	2448.9	K1
9	1955.9	1955.3	K5
10	5553.0		
	6032.0		Not identified
	6136.0		
11	6525.9	6160.2[b]	K5–K8 linked to HexNAc, Hex
	6816.6		K5–K8 linked to HexNAc, Hex–SA
	7107.8		K5–K8 linked to HexNAc, Hex–2SA
	7797.2	7432.6[b]	
	8088.3		K5–K9[c] linked to HexNAc–Hex
	8381.4		K5–K9[c] linked to HexNAc–Hex–SA
			K5–K9[c] linked to HexNAc–Hex–2SA
12[a]	7542.3	5024.8[b]	K4[d]
	7832.9		
	8121.1		

[a] Only the major peaks of the fraction are listed.
[b] Mass of peptide without glycosylation.
[c] The Epo *C*-terminal fragment K9 is only identified in a processed version without the *C*-terminal arginine.
[d] Glycopeptide, identified after further subdigestion and subsequent MALDI-MS analysis.

with the molecular mass of 2865.1 Da calculated for peptide K6 containing an *O*-linked GalNAc–Gal (GalNAc = *N*-acetylgalactosamine; Gal = galactose) structure. In order to verify this assignment further the asialo peptide was further digested with trypsin.

The MALDI mass spectrum of the resulting mixture showed two peaks with masses corresponding to Thr_{132}–Lys_{140} and to Glu_{117}–Arg_{131} linked to HexNAc–Hex (HexNAc = *N*-acetylhexosamine; Hex = hexose) (Table 2). This locates the *O*-glycosylation to either Ser_{120} or Ser_{126}, supporting previous reports assigning the *O*-linked glycosylation to Ser_{126}.[4]

Analysis of peptide K2 containing Asn_{24} and Asn_{38}

Peptide K2 contains two of the consensus sequences for *N*-glycosylation, namely Asn_{24} and Asn_{38}. As expected

Table 2. Analysis of the glycosylated K6 peptides (fraction 6 of Lys C peptide map in Fig. 1)[a]

Sample	*m/z* observed	Calculated mass (Da)	Interpretation
Fraction 6 in Fig. 1	2868.2	2865.14	K6 linked to HexNAc–Hex
(Lys 6)	3157.6	3156.39	K6 linked to HexNAc–Hex–SA
	3448.8	3447.65	K6 linked to HexNAc–Hex–2SA
Neuraminidase-treated Lys 6	2867.5	2865.14	K6 linked to HexNAc–Hex
Neuraminidase- and	1835.6	1830.96	Glu_{117}–Arg_{131} linked to HexNAc–Hex
trypsin-treated Lys 6	1055.8	1052.20	Thr_{132}–Lys_{140}

[a] The peptides were successively digested with neuraminidase and trypsin. Aliquots were analyzed by MALDI-MS prior to addition of each of the enzymes. The observed masses are compared to the calculated masses of putative glycopeptide structures of the K6 and the K6 derived peptides.

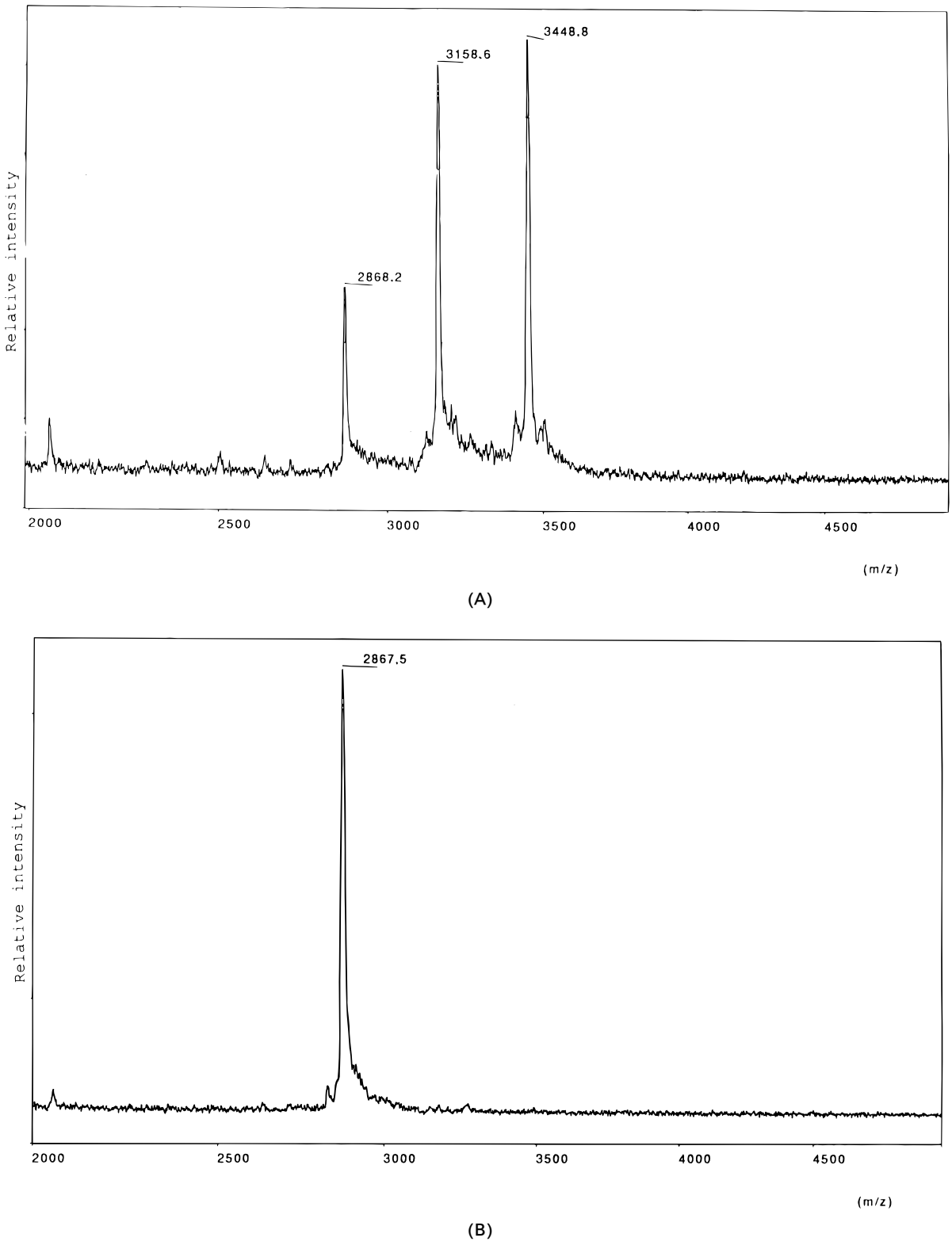


Figure 3. MALDI mass spectra used for the characterization of the peptides contained in fraction 6 in Fig. 1 (glycopeptide K6). The spectra were acquired in the linear mode using 4HCCA as matrix. (A) Intact glycopeptide, (B) neuraminidase-digested glycopeptide.

for a high degree of heterogeneity of glycosylation, the MALDI mass spectrum of fraction 3 in Fig. 1 containing K2 [Fig. 4(A)] shows a broad, unresolved peak around m/z 8100. Upon neuraminidase treatment a distinct but still complex pattern was observed [Fig. 4(B)]. To separate the two *N*-linked glycosylation sites, the peptides of fraction 3 were further cleaved with endoproteinase Glu-C. The resulting peptides (K2E1, K2E2, see Fig. 2) were separated by RP-HPLC and analyzed by MALDI-MS.

The analysis of peptide K2E1 containing Asn_{24} was only successful with delayed extraction (DE) MALDI-MS and DHB as a matrix [Fig. 4(C)]. The major peaks observed can putatively be assigned to bi-, tri- and

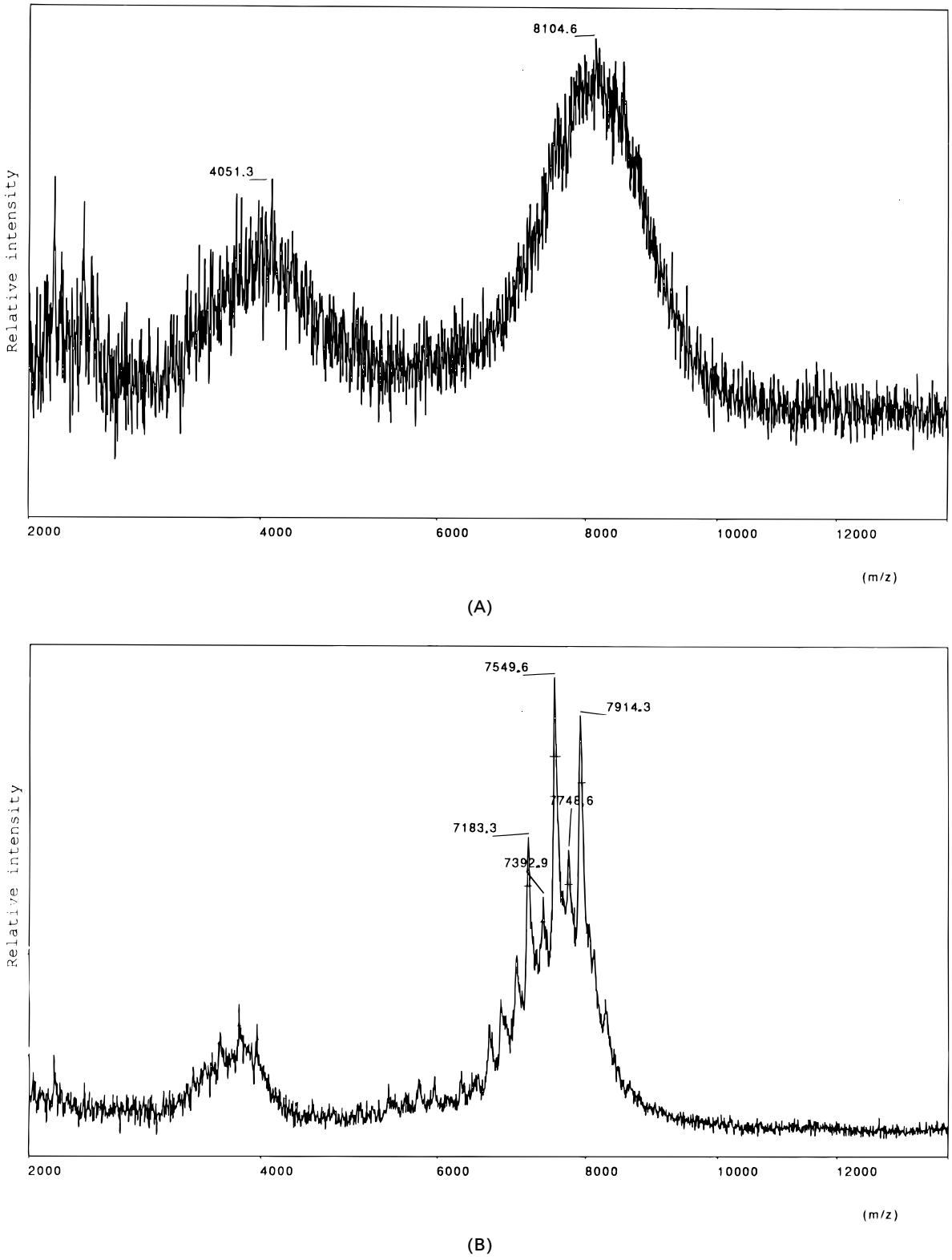
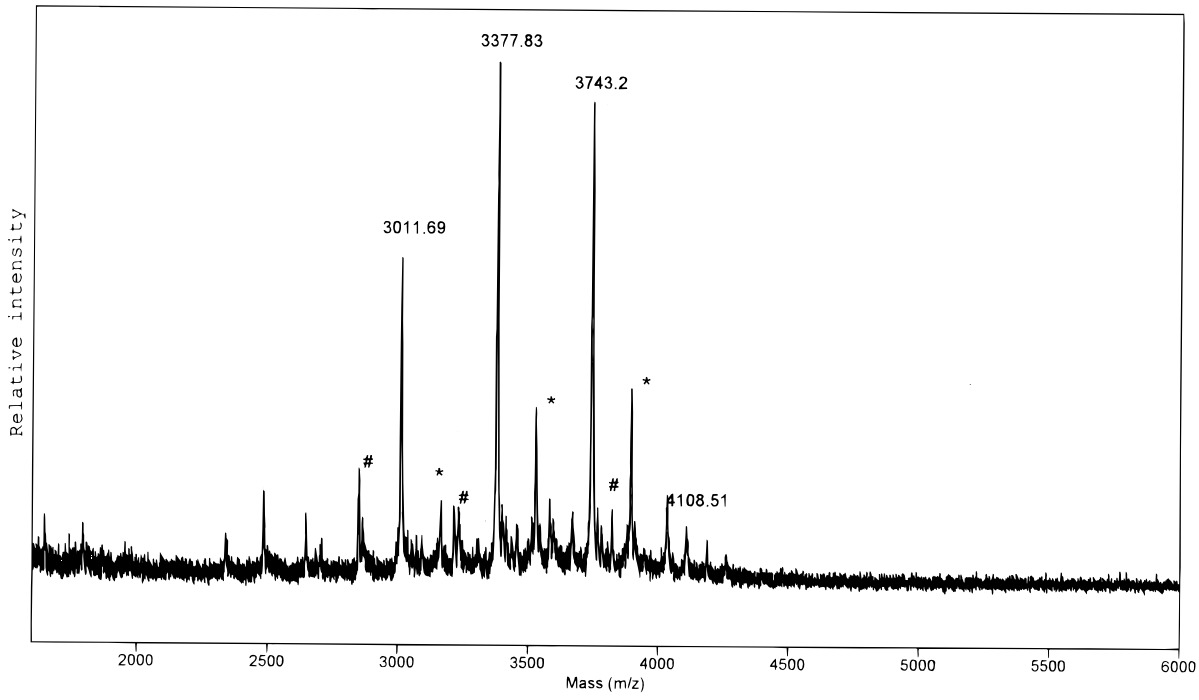
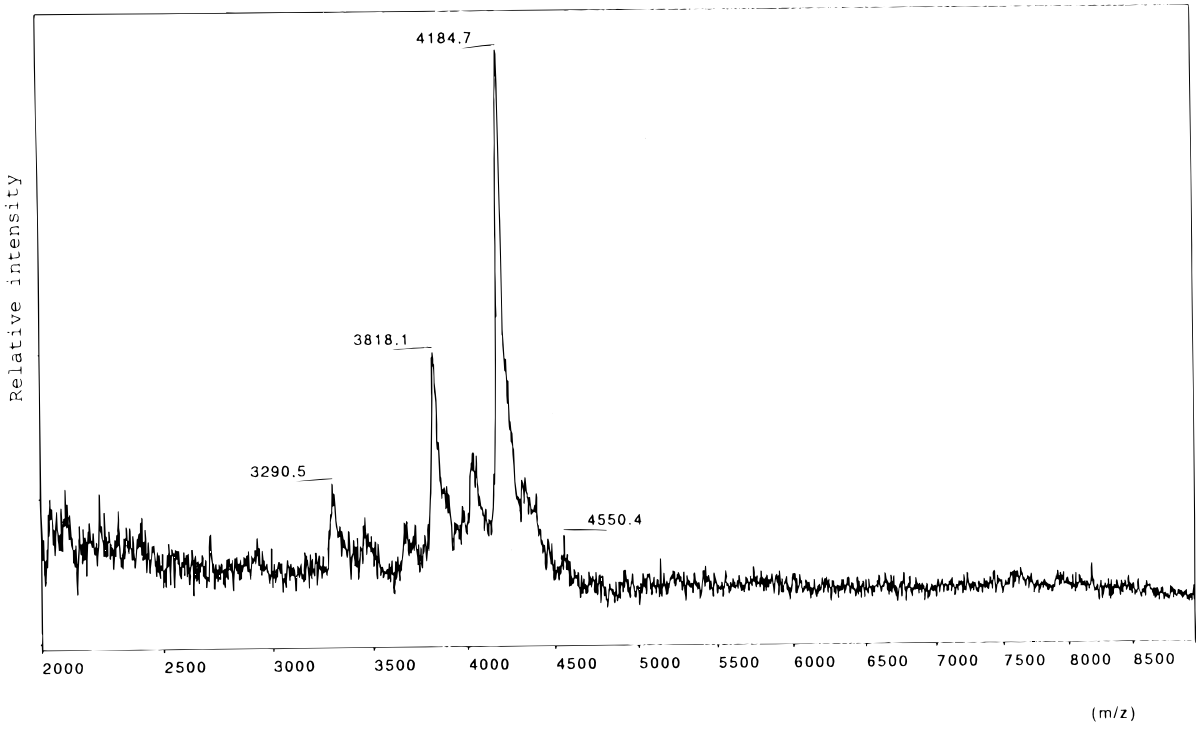


Figure 4. MALDI mass spectra used for the characterization of the peptides contained in fraction 3 in Fig. 1 (glycopeptide K2 containing Asn_{24} and Asn_{38}). (A) Intact glycopeptide; (B) neuraminidase-digested glycopeptide; (C) glycopeptide K2E1 containing Asn_{24}; (D) glycopeptide K2E2 containing Asn_{38}. The peptides K2E1 and K2E2 were produced by endoproteinase Glu-C digestion of the peptides of fraction 3 and subsequent RP-HPLC purification. The MALDI mass spectra in (A), (B) and (D) were obtained using 4HCCA as matrix and that in (C) was obtained with delayed extraction and DHB as matrix. An asterisk indicates DHB adducts. Peaks marked with # contain glycans without fucose.

tetraantennary fucosylated complex-type oligosaccharides. In addition, the data indicate the presence of a small amount of a fucosylated tetraantennary oligosaccharide with a single *N*-acetyllactosaminyl repeat (see Table 3). A series of peaks observed 152 Da above the major peaks are most likely photochemically induced DHB matrix adducts [indicated by asterisks in Fig. 4(C)]. Furthermore, minor peaks corresponding to



(C)



(D)

Figure 4. *Continued*

complex-type glycans without fucose are present. These complex-type glycans without fucose correspond to ~10% of the total peak intensity and are equally distributed between the different glycoforms.

Peptide K2E2 containing Asn_{38} was analyzed with 4HCCA as a matrix. The spectra indicate glycosylation with triantennary and tetraantennary fucosylated complex-type oligosaccharide (see Fig. 4(D) and Table 3).

Analysis of peptide K4 containing Asn_{83}

The largest and most hydrophobic peptide K4 eluted in fraction 12 from the reversed-phase column. The MALDI spectrum of K4 is complex [Fig. 5(A)]. Although the spectrum is not fully resolved, three significant peaks could be assigned to represent putatively

Table 3. Analysis of the glycosylated K2 peptides (fraction 3 of Lys C peptide map in Fig. 1)[a]

Sample	*m/z* observed	Calculated mass (Da)	Interpretation[b]
Fraction 3 in Fig. 1 (Lys 3)	Broad distribution around 8100		
Neuraminidase-treated Lys 3	—	6441.49	K2 + biant. + biant. + 2 Fuc
	—	6806.82	K2 + biant. + triant. + 2 Fuc
	7183.3	7172.16	K2 + biant. + tetraant. + 2 Fuc or K2 + triant. + triant. + 2 Fuc
	7392.9	7391.34	K2 + triant. + tetraant. + Fuc
	7549.6	7537.49	K2 + triant. + tetraant. + 2 Fuc
	7748.6	7756.68	K2 + tetraant. + tetraant. + Fuc
	7914.3	7902.83	K2 + tetraant. + tetraant. + 2 Fuc
Fraction 1 of V8 digest of neuraminidase-treated Lys 3	3011.7	3012.97	K2E1 + biant. + Fuc
	3377.8	3378.30	K2E1 + triant. + Fuc
	3743.2	3743.64	K2E1 + tetraant. + Fuc
	4108.5	4108.98	K2E1 + tetraant. repeat + Fuc
Fraction 2 of V8 digest of neuraminidase-treated Lys 3	3818.1	3811.85	K2E2 + triant. + Fuc
	4184.7	4177.19	K2E2 + tetraant. + Fuc

[a] The peptide was examined by MALDI-MS prior to and after neuraminidase treatment and then sub-digested with endoproteinase Glu C. The endoproteinase Glu C fragments were separated by RP-HPLC and collected manually. The observed masses are compared with calculated masses of putative glycopeptide structures of the K2 and K2-derived peptides. K2, Glu_{21}–Lys_{45}; K2E1, Glu_{21}–Glu_{31}; K2E2, His_{31}–Lys_{45}.
[b] Fuc = fucose.

sialylated forms (see Table 4). Neuraminidase digestion of the sample resulted in a simpler MALDI mass spectrum but also in a poor signal-to-noise ratio and reduced resolution and mass accuracy, most likely due to interaction with buffer.

To confirm the identification of the peptide and to obtain improved mass accuracy of the peaks corresponding to the different glycoforms, neuraminidase-treated K4 was cleaved after Arg_{76} by trypsin and the resulting peptides were purified by RP-HPLC. Although K4 possesses only one cleavage site for trypsin, three peptides were obtained from the digest. One of the peptides exhibited the mass expected for K4T1, whereas a second peptide showed a mass 16 Da above the theoretical mass of K4T1. This peptide is most likely oxidized at Met_{54} (Table 4). From the chromatogram of the tryptic digest of fraction 12 (data not shown) the extent of oxidation of peptide K4T1 is estimated to be 50%. The third fraction was analyzed by linear MALDI-MS using 4HCCA as a matrix [Fig. 5(B)]. It was demonstrated to contain the glycosylated K4T2 peptide. The two major peaks at *m/z* 4492.6 and 4859.1 indicate the presence of both the tri- and tetra-antennary fucosylated complex-type glycans; the former, being a minor component, is masked in the more complex spectrum of the intact K4 glycopeptide.

Table 4. Analysis of the glycosylated K peptides (fraction 12 of Lys C peptide map in Fig. 1)[a]

Sample	*m/z* observed	Calculated mass (Da)	Interpretation
Fraction 12 in Fig. 1 (Lys 12)	7542.3[b]	7525.31	K4 + tetraant. glycan
	7832.9[b]	7816.33	K4 + tetraant. glycan + SA
	8121.1[b]	8107.59	K4 + tetraant. glycan + 2SA
	8409.0[b]	8398.84	K4 + tetraant. glycan + 3SA
Tryptic digest of neuraminidase-treated Lys 12:			
RP-HPLC fraction 3	2684.0	2683.12	K4T1
RP-HPLC fraction 2	2699.0	2699.12	K4T1 oxidized
RP-HPLC fraction 1	4492.6	4494.63	K4T2 + triant. glycan
	4859.1	4859.97	K4T2 + tetraant. glycan

[a] The peptides were successively digested by neuraminidase and trypsin. The tryptic digest of the neuraminidase-treated glycopeptides was fractionated by RP-HPLC (chromatogram not shown). The observed masses are compared with calculated masses of putative glycopeptide structures of the K4 and K4-derived peptides. K4, Arg_{53}–Lys_{97}; K4T1, Arg_{53}–Arg_{76}; K4T2, Gly_{77}–Lys_{97}.
[b] The measured masses are between 10 and 18 Da higher than the calculated masses owing to the putative oxidation of Met_{54}.

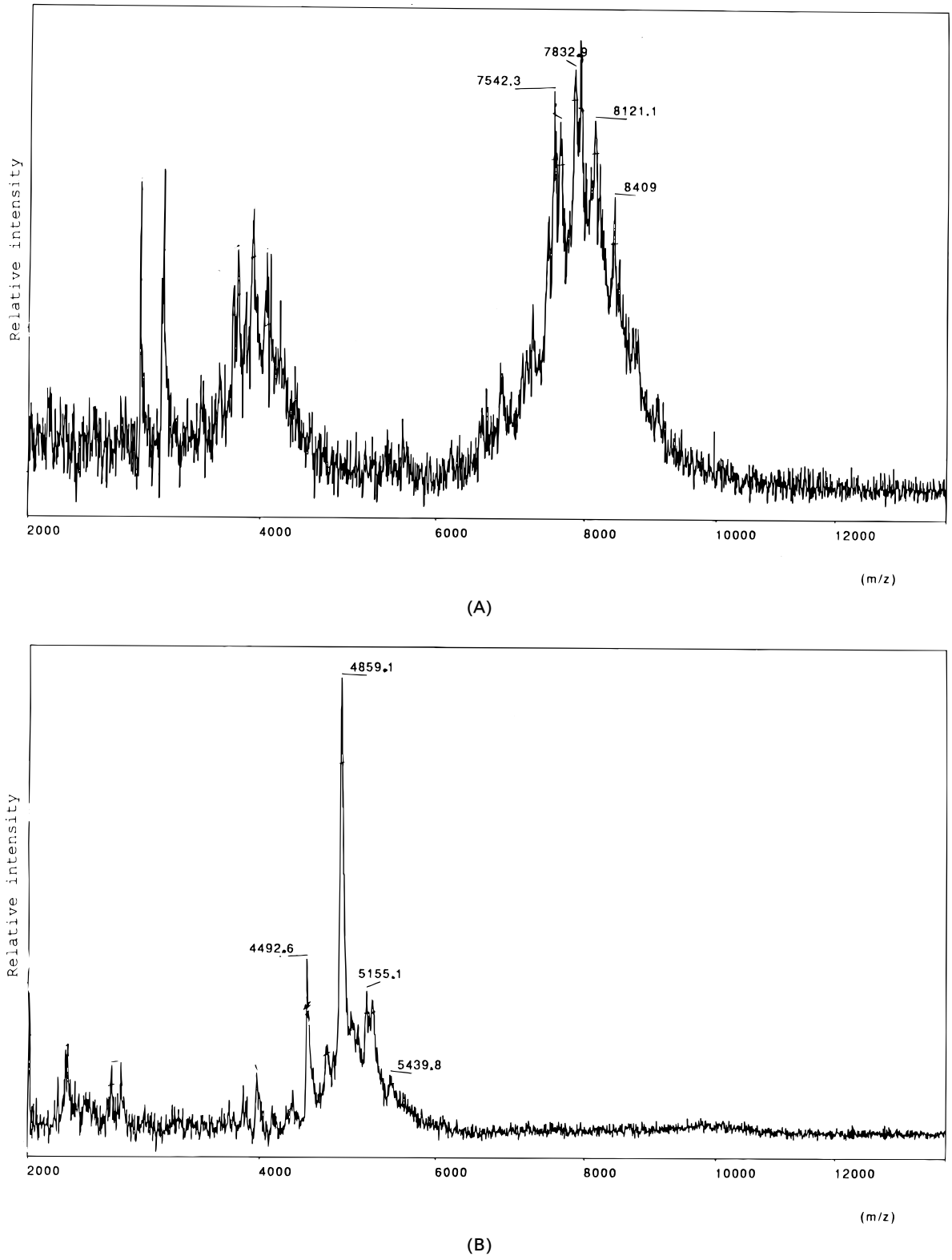


Figure 5. MALDI mass spectra used for the characterization of the peptides contained in fraction 12 in Fig. 1 (glycopeptide K4 containing Asn_{83}). (A) Intact glycopeptide K4; (B) HPLC-purified glycopeptide K4T2 obtained after digestion with neuraminidase and trypsin. The MALDI mass spectra were obtained using 4HCCA as matrix.

Establishing a molecular mass glycoprofile of Epo glycans

In principle, the mass spectra of the different glycopeptides constitute a site-specific fingerprint or glycoprofile of a glycoprotein. A site-specific glycoprofile can be determined on two levels, a qualitative profile showing which types of glycans are present and a quantitative profile showing the relative abundancies of the different forms.

It is well known that the presence of sialic acid resi-

Table 5. Comparison of the present MALDI molecular mass glycoprofile with published glycoprofiles transformed into a molecular mass glycoprofile (values in %)

	MALDI-MS	Takeuchi *et al.*[8]	Tsuda *et al.*[10] (A)	Tsuda *et al.*[10] (B)
Biantennary	8	9.0	48.4	13.4
Triantennary	30	23.6	26.0	24.6
Tetraantennary	55	59.9	25.1	62.0
Tetraantennary + Lac	7	6.9	ND	ND
Tetraantennary + 2 Lac	ND	0.6	ND	ND

dues cannot be unambiguously determined from MALDI mass spectra, since sialylated glycans tend to undergo both prompt and metastable fragmentation.[20] For this reason, the spectra of the sialylated peptides can only be used for an estimate of the qualitative glycan pattern and are not suitable for the estimation of the quantitative glycan pattern. However, for neutral oligosaccharides[23] and glycopeptides with neutral oligosaccharides,[18,24] it has been demonstrated that the mass spectrum reflects the true quantitative glycan heterogeneity. Therefore, the MALDI mass spectra of the desialylated peptides shown are expected to represent the true quantitative site-specific glycoprofiles.

In order to test whether the mass spectra of the desialylated glycopeptides can be used for establishing a quantitative glycoprofile, the peak areas of the different glycoforms were evaluated. In the case of glycopeptide K2E1 [Fig. 4(C)], 25, 37, 36 and 2% of the total intensity can be assigned to bi-, tri- and tetraantennary fucosylated complex-type glycans and to tetraantennary complex-type glycans with a single *N*-acetyllactosaminyl repeat, respectively. Similar analyses were performed for the other *N*-glycosylation sites. An overview of the results is presented in Fig. 6. Table 5 shows a comparison of the overall MALDI-derived glycoprofile with glycoprofiles reported in the literature.

DISCUSSION

In agreement with a previous report stating that Ser_{126} is glycosylated,[4] an *O*-linked glycan consisting of a Hex–HexNAc core and a variable number of sialic acids residues was found to be located on either Ser_{120} or Ser_{126}. The previously reported *O*-linked GalNAc with or without sialic acid[25] could not be confirmed by our data.

Furthermore, the data demonstrate that all of the three potential *N*-glycosylation sites of human urinary Epo are glycosylated. The dominating *N*-linked glycans found were of the complex type containing one fucose residue and a variable number of sialic acids. Minor peaks corresponding to structures without fucose were also present just above the noise level of the spectra.

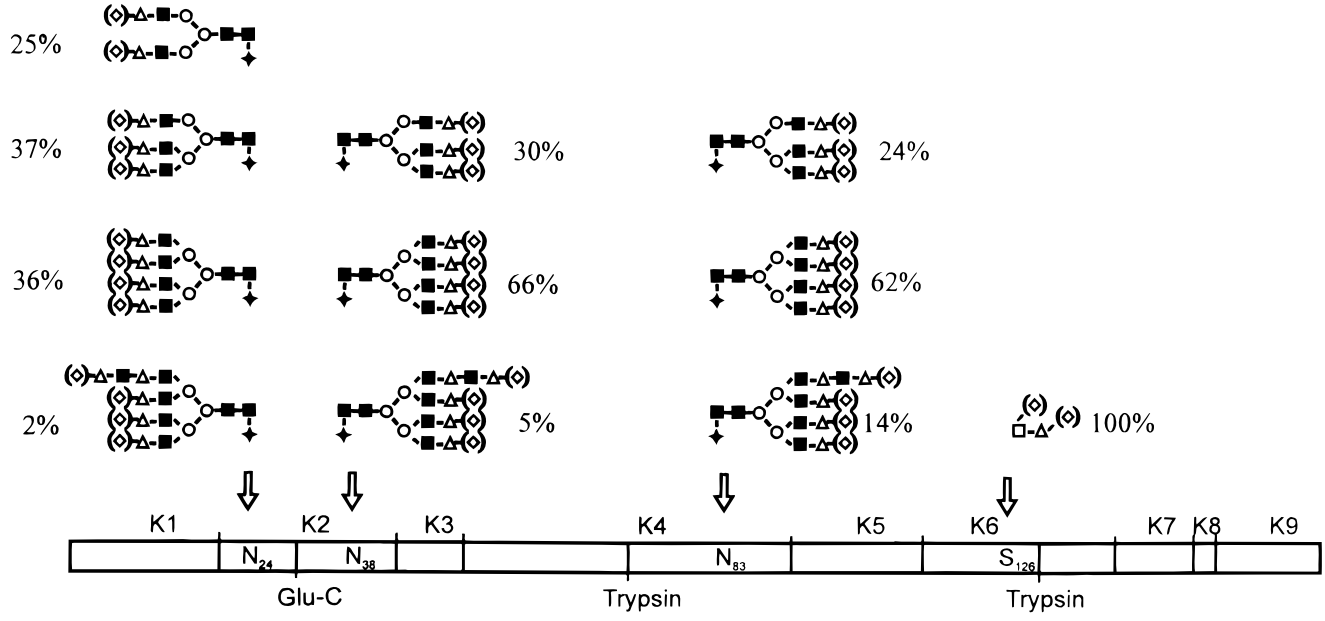


Figure 6. MALDI molecular mass glycoprofile of human urinary Epo as derived from the masses and peak intensities of the MALDI mass spectra of the desialylated glycopeptides. The endoproteinase Lys C generated fragments are indicated by the numbers used in Fig. 1; the cleavage sites for endoproteinase Glu C in K2 and for trypsin in K4 and K6 are also indicated. The pictograms basically represent the monosaccharide composition of the glycans. Symbols: ■ and □, *N*-acetylhexoseamines, most likely *N*-acetylglycosamine and *N*-acetylgalactosamine, respectively; ◆, deoxyhexose, most likely fucose; ○ and △, hexoses, most likely mannose and galactose, respectively; and ◇, sialic acid.

Based on the observed mass differences, putative glycan structures can be suggested by analogy with known structure types. As a result, the relative distribution between different numbers of antennae can be estimated. However, it must be borne in mind that these estimates are based on mass differences which only reflect the monosaccharide composition of each form.

The estimated distribution of bi-, tri- and tetra-antennary glycans varies significantly between the three *N*-glycosylation sites (see Fig. 6). The glycans at Asn_{24} are the most heterogenous, and Asn_{24} is the only glycosylation site containing biantennary glycans. The glycans at Asn_{38} and Asn_{83} are mainly tetraantennary and triantennary. All the *N*-linked sites were found to contain small amounts ($<10\%$) of tetraantennary glycans with *N*-acetyllactosaminyl repeats. The types of glycan structures observed for human urinary Epo are the same as those reported at recombinant Epo.[12–16]

To compare the molecular mass glycoprofiles obtained by MALDI-MS with the glycoprofiles of human urinary Epo determined by traditional methods, the site-specific MALDI glycoprofiles were used to construct an overall mass glycoprofile. Furthermore, the literature glycoprofiles were recalculated to mass glycoprofiles by calculating the theoretical masses of the glycoforms and adding the amounts of glycoforms with identical theoretical masses. Sialic acids were disregarded in this calculation, since the mass profiles were intended to be compared with the data for desialylated glycans. The comparison of the different sets of data is presented in Table 5. It is evident that a good correlation exists between the glycoprofile derived from MALDI-MS and between the glycoprofile reported by Takeuchi *et al.*[8] and also between one of the two glycoprofiles reported by Tsuda *et al.*[10] The other glycoprofile reported by Tsuda *et al.* differs considerably with regard to the amount of biantennary glycans. However, it must be taken into consideration that the glycoprofiles can be influenced by different sources of urinary Epo and by the purification procedures employed.

In summary, we have demonstrated that MALDI-MS is a sensitive method for the investigation of the glycoprofiles of individual glycosylation sites of human urinary Epo. In addition, the overall molecular mass glycoprofile derived from the mass spectra correlates well with independently determined glycoprofiles of human urinary Epo reported in the literature. This indicates that MALDI-MS may constitute a reliable, fast and very sensitive method for the determination of both qualitative and quantitative glycoprofiles.

Acknowledgement

This study was performed in the frame of the Protein Engineering Research Center financed by the Danish Biotechnology Program.

REFERENCES

1. T. Miyake, C. K. H. Kung and E. Goldwasser, *J. Biol. Chem.* **252**, 5558 (1977).
2. K. Jacobs, C. Shoemaker, R. Rudersdorf, S. D. Neill, R. J. Kaufman, A. Mufson, J. Seehra, S. S. Jones, R. Hewick, E. F. Fritsch, M. Kawakita, T. Shimizu and T. Miyake, *Nature* (*London*) **313**, 806 (1985).
3. F. Lin, S. Suggs, C. Lin, J. K. Browne, R. Smalling, J. C. Egrie, K. K. Chen, G. M. Fox, F. Martin, Z. Strabinsky, S. M. Badrawi, P. Lai and E. Goldwasser, *Proc. Natl. Acad. Sci. USA* **82**, 7580 (1985).
4. P. Lai, R. Everett, F. Wang, T. Arakawa and E. Goldwasser, *J. Biol. Chem.* **261**, 3116 (1986).
5. M. S. Doredale, F. Wang and E. Goldwasser, *Endocrinology*, **116**, 2293 (1985).
6. E. Tsuda, G. Kawanishi, M. Jeda, S. Masuda and R. Sasaki, *Eur. J. Biochem.* **188**, 405 (1990).
7. M. Goto, K. Akai, A. Murakami, C. Hashimoto, E. Tuda, M. Ueda, G. Kawanishi, N. Takahashi, A. Ishimoto, H. Chiba and R. Sasaki, *Biotechnology* **6**, 67 (1988).
8. M. Takeuchi, S. Takasaki, H. Miyazaki, K. Takashi, S. Hoshi, N. Kochibe and A. Kobata, *J. Biol. Chem.* **263**, 3657 (1988).
9. H. Sasaki, B. Bothner, A. Dell and M. Fukuda, *J. Biol. Chem.* **262**, 12059 (1987).
10. E. Tsuda, M. Goto, A. Murakami, K. Akai, M. Ueda and G. Kawanishi, *Biochemistry* **27**, 5646 (1988).
11. E. Watson, A. Bhide and H. Van Halbeek, *Glycobiology* **4**, 227 (1994).
12. H. Sasaki, N. Ochi, A. Dell and M. Fukuda, *Biochemistry* **27**, 8618 (1988).
13. M. Nimtz, W. Martin, V. Wray, K. D. Kloppel, J. Augustin and H. S. Conradt, *Eur. J. Biochem.* **213**, 39 (1993).
14. M. Nimtz, V. Wray, A. Rüdiger and H. S. Conradt, *FEBS Lett.* **365**, 203 (1995).
15. K. B. Linsley, S. Y. Chan, S. Chan, B. B. Reinhold, P. J. Lisi and V. N. Reinhold, *Anal. Biochem.* **219**, 207 (1994).
16. R. S. Rush, P. L. Derby, D. M. Smith, C. Merry, M. F. R. Rogers and V. Katta, *Anal. Chem.* **67**, 1442 (1995).
17. O. Vorm, P. Roepstorff and M. Mann, *Anal. Chem.* **66**, 3281 (1994).
18. C. W. Sutton, J. A. O'Neill and J. S. Cottrell, *Anal. Biochem.* **218**, 34 (1994).
19. M. J. Treuheit, C. E. Costello and B. Hasall, *Biochem. J.* **283**, 105 (1992).
20. M. C. Huberty, J. E. Vath, W. Yu and S. A. Martin, *Anal. Chem.* **65**, 2791 (1993).
21. A. Tsarbopoulos, M. Karas, K. Stupart, B. N. Pramanik, T. L. Nagabhushan and F. Hillenkamp, *Anal. Chem.* **66**, 2062 (1994).
22. M. A. Recny, H. A. Scobel and Y. Kim, *J. Biol. Chem.* **262**, 17156 (1987).
23. T. J. P. Naven and D. J. Harvey, *Rapid Commun. Mass Spectrom.* **10**, 1361 (1996).
24. E. Mørtz, T. Sareneva, I. Julkunen and P. Roepstorff, *J. Mass Spectrom.* **31**, 1109 (1996).
25. N. Inoue, M. Takeuchi, K. Asano and R. Shimizu, *Arch. Biochem. Biophys.* **301**, 375 (1993).