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Selective glycopeptide mapping of erythropoietin by on-line high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

Selective glycopeptide mapping of recombinant human erythropoietin (rhEPO) used as a model glycoprotein was successfully carried out by on-line high-performance liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) using a Vydac C_{18} column eluted in acetonitrile–1 m*M* ammonium acetate, pH 6.8. rhEPO expressed in a Chinese hamster ovary clone was exhaustively digested into four glycopeptides and nine peptides with endoproteinase Glu-C. Both glycopeptides and peptides were eluted with trifluoroacetic acid as the eluent, whereas only glycopeptides were eluted selectively with ammonium acetate in the following order: *N*38, *N*24, *O*126, and *N*83. Furthermore, many glycoforms included in each glycopeptide were found to be separated by differences in the numbers of sialic acid and *N*-acetyllactosaminyl repeats. Twenty, 16 and 22 different *N*-linked oligosaccharides were determined at Asn24, 38, and 83, respectively, and two different *O*-linked oligosaccharides were observed at Ser126. Our method is simple, rapid, and useful for determining the carbohydrate structures at each glycosylation site and for elucidating the site-specific carbohydrate heterogeneity.

Keywords: Erythropoietin; Glycoproteins; Glycopeptides; Peptides; Proteins

1. Introduction

It is well known that the carbohydrate moieties of glycoproteins play an important role in biological activities and biological functions, such as secretion, biological half-life, antigenicity, and cell–cell interactions [1]. Many structural analyses of the carbohydrates in the glycoproteins have been performed by using carbohydrates released from glycoproteins chemically or enzymatically [2–5]. Glycoproteins are digested into peptides and glycopeptides, and the carbohydrates released from the glycopeptides are used for structural analysis of the carbohydrates at each glycosylation site [6,7]. Since fractionation of glycopeptides from the peptide and glycopeptide pools is time-consuming and complicated, simple and rapid methods are desirable to elucidate the carbohydrate structures at each glycosylation site.

Electrospray ionization mass spectrometry (ESI-MS) is a powerful technique for the structural analysis of proteins [8,9] and carbohydrates [7,10–14], and the carbohydrate structures at each glycosylation site in glycoproteins have recently

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been analyzed by peptide and glycopeptide mapping using high-performance liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) [7,11–14]. However, both the chromatograms and mass spectra of peptide and glycopeptide maps are complicated owing to the carbohydrate heterogeneity, which makes structural assignments more difficult. Selective glycopeptide mapping, which enables the detection of only glycopeptides and separation of glycoforms should be useful for the structural analysis of the carbohydrates at each glycosylation site.

Erythropoietin (EPO) is a sialoglycoprotein composed of about 40% (w/w) carbohydrates and possesses three *N*-linked glycosylation sites (Asn24, 38, and 83) and one *O*-linked glycosylation site (Ser126) [4,6,15–18]. In this study, we used recombinant human EPO (rhEPO) as a model glycoprotein and demonstrated glycopeptide mapping in which only four glycopeptides are detected and separated by the difference in glycoforms. Our method is useful for elucidating the detailed site-specific carbohydrate heterogeneity of glycoproteins.

2. Experimental

2.1. Materials

rhEPO expressed by Chinese hamster ovary cells was a kind gift from Chugai (Tokyo, Japan). Endoproteinase Glu-C (Glu-C) was obtained from Boehringer-Mannheim. All other chemicals used were of the highest purity available.

2.2. Proteolytic digestion of EPO

rhEPO (1 mg) was dissolved in 1 ml of 100 m*M* ammonium acetate, pH 8.0, and was incubated with 0.25 mg of Glu-C dissolved in 62 μl of 100 m*M* ammonium acetate, pH 5.6, at 37°C for 20 h. The digest was stored at −20°C before use.

2.3. LC–MS analysis

2.3.1. Peptide and glycopeptide mapping

The high-performance liquid chromatography (HPLC) apparatus used was a Finnigan MAT spectra system equipped with a p4000 pump and a UV2000 instrument. HPLC was performed with a Vydac C_{18} column (Vydac 218TP52, particle size 5 μm, 250× 2.1 mm I.D.) at a flow-rate of 0.25 ml/min at 40°C. The effluent was monitored at 206 nm. An eluent program was performed with a linear gradient from 1% to 35% acetonitrile containing 0.05% trifluoroacetic acid (TFA) in 130 min. An aliquot of 20 μl of the sample was injected into the HPLC apparatus.

A Finnigan-MAT TSQ 7000 triple-stage quadruple mass spectrometer equipped with an ESI source (Finnigan-MAT, San Jose, CA, USA) was used. The mass spectrometer was operated in the negative ion mode and scanned in the m/z region from 500 to 2400 with a scan duration of 4 s. The ESI voltage, capillary temperature, sheath gas pressure and auxiliary gas were set at 4500 V, 225°C, 70 p.s.i., and 10 units, respectively (1 p.s.i.=6894.76 Pa).

2.3.2. Selective glycopeptide mapping

HPLC was performed with a Vydac C_{18} column at a flow-rate of 0.2 ml/min at 40°C, and the effluent was monitored at 206 nm. The elution program from solvent A (1 m*M* ammonium acetate, pH 6.8) to solvent B (80% acetonitrile containing 1 m*M* ammonium acetate) was as follows: time 0–60 min, gradient from 1% to 6% solvent B; 60–140 min, gradient from 6% to 36% solvent B. An aliquot of 15 μl of the sample was injected into the HPLC apparatus. The mass spectrometer was operated in the negative ion mode and the positive ion mode. The mass range acquired was m/z 1000–2400 with a scan duration of 3 s. The ESI voltage, capillary temperature, sheath gas pressure, and auxiliary gas were set at 4500 V, 225°C, 70 p.s.i., and 10 units, respectively.

3. Results

3.1. Peptide and glycopeptide mapping of rhEPO

To determine the carbohydrate structures at each glycosylation site, rhEPO was digested into four glycopeptides and nine peptides by Glu-C, which hydrolyzes at Glu-X and Asp-X [19]. Fig. 1A shows the total ion current (TIC) chromatograms of the rhEPO digest by LC–MS in the negative ion mode.

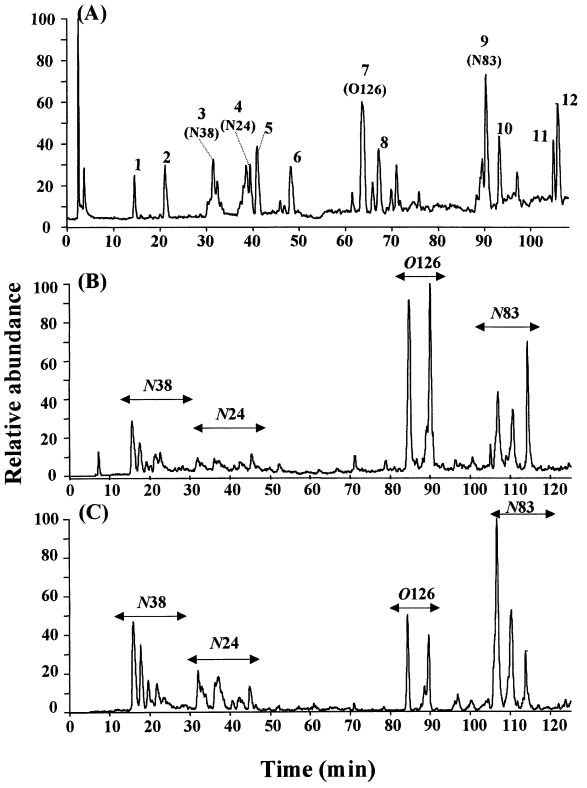

Fig. 1. TIC chromatogram of rhEPO digested with Glu-C by on-line LC–MS.

The peptide and glycopeptide map of rhEPO was obtained with a Vydac C_{18} column and with TFA as the eluent. Peptides were separated as single peaks. The amino acid residues of the individual peaks were determined from the mass spectra (Table 1). Peaks 3, 4, 7, and 9 were eluted as multiple peaks due to the carbohydrate heterogeneity and were determined as *N*38, *N*24, *O*126, and *N*83 glycopeptides, respectively. The carbohydrate structures at each glycosylation site were characterized by the same way as described in our previous paper in which a Pegasil ODS column was used [14,20]. The elution profile obtained with the Vydac C_{18} column was the same as obtained with Pegasil ODS column.

3.2. Selective glycopeptide mapping of rhEPO

Generally, hydrophobic and large peptides could not be eluted with a low concentration of salt as an eluent. An eluent containing a low concentration of ammonium acetate was examined instead of 0.05% TFA in order to elute only glycopeptides in the rhEPO digest selectively. Although similar elution profiles were obtained by both 1 m*M* and 5 m*M* ammonium acetate, mass spectra obtained by 1 m*M* ammonium acetate provided higher intensity of pseudomolecular ions of glycopeptides than that obtained by the 5 m*M* one. Four glycopeptides in the

Table 1
Peak number, amino acid residues and theoretical and observed *m*/*z* of the peptide in the rhEPO digest

Peak No.	Residue	Charge state	Theoretical *m*/*z*		Observed *m*/*z*
			Monoisotopic	Average	
1	56–62	1−	728.4	728.8	727.7
2	9–13	1−	601.3	601.7	600.7
3*	38–43 (*N*38)				
4*	22–37 (*N*24)				
5	(1–8) S–S (160–165)	1−	1501.7	1502.7	1501.9
6	14–18	1−	691.4	691.8	691.0
7*	118–136 (*O*126)				
8	44–55	1−	1570.8	1571.9	1570.6
9*	73–96 (*N*83)				
10	63–72	1−	1113.6	1114.3	1113.0
11	137–159	2−	1416.8	1417.7	1416.7
12	97–117	1−	2210.3	2211.6	2210.6
		2−	1104.6	1105.3	1104.0

*, Glycopeptides. The peptide containing amino acid residues from 19 to 21 was not detected.

EPO digest were eluted in the same elution order by 5 m*M* ammonium acetate at both pH 4.0 and 6.8, however separation of many glycoforms included in each glycopeptide at pH 6.8 was better than at pH 4.0. Separation of many glycoforms with a Vydac C_{18} column was better than with a Pegasil ODS column. Therefore, we chose 1 m*M* ammonium acetate, pH 6.8 as an eluent and a Vydac C_{18} column for selective glycopeptide mapping of EPO.

Fig. 1B and C show glycopeptide maps measured in the positive ion mode and the negative ion mode, respectively. Only four glycopeptides in the rhEPO digest were eluted in the following order: *N*38, *N*24, *O*126, and *N*83. The sensitivity for *N*-linked oligosaccharide-containing glycopeptides measured in the negative ion mode was higher than measured in the positive ion mode. Fig. 2A–D show an expanded view of Fig. 1C. Individual glycopeptides were further separated by differences in the structure of the oligosaccharides attached to each glycosylation site.

3.2.1. Carbohydrate structures of N38 glycopeptides

As shown in Fig. 2A, the *N*38 glycopeptides of rhEPO were separated into 11 sharp peaks A1–A11. The mass spectra of peaks A1–A11 are shown in Fig. 3A1–A11, respectively. Glycopeptides were detected as the 3− and 4− charge state ions. Ion peaks were assigned on the basis of the theoretical *m*/*z* of peptides containing amino acid residues from Asn38 to Asp43 and oligosaccharides reported in our previous paper [14]. Table 2 indicates the oligosaccharide structures of individual ion peaks in Fig.

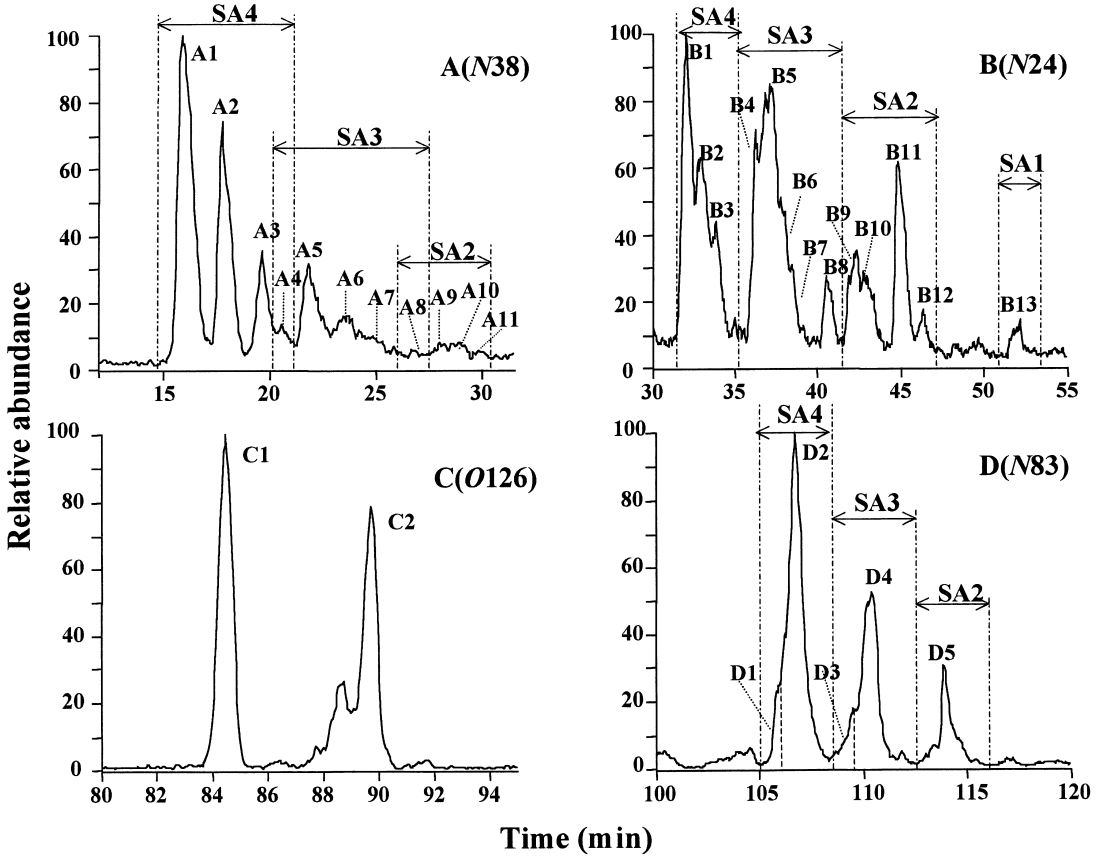

Fig. 2. Expanded view of the glycopeptides at *N*38 (A), *N*24 (B), *O*126 (C) and *N*83 (D) in Fig. 1C. SA, Sialic acid (*N*-acetyl neuraminic acid).

Table 2
Peak number, glycoforms, and theoretical and observed m/z of the *N*38 glycopeptides in the rhEPO digest[a]

Peak No.	Glycoform	Charge state	Theoretical m/z		Observed m/z
			Monoisotopic	Average	
A1	Tetra-SA4 (a)	3−	1439.2	1440.0	1439.4
		4−	1079.2	1079.8	1079.1*
A2	Tetra-Lac1-SA4 (b)	3−	1560.9	1561.8	1560.5
		4−	1170.4	1171.1	1170.3*
A3	Tetra-Lac2-SA4 (c)	3−	1682.6	1683.6	1682.7
		4−	1261.7	1262.4	1261.6*
A4	Tri-SA3 (d)	3−	1220.5	1221.2	1220.8*
	Tetra-Lac3-SA4 (e)	4−	1353.0	1353.8	1352.8
		5−	1082.2	1082.8	1082.2
	Tetra-SA4-Ac1 (f)	3−	1453.2	1454.0	1453.1
		4−	1089.7	1090.3	1089.8
	Tetra-Lac2-SA4 (c)	3−	1682.6	1683.6	1682.6
		4−	1261.7	1262.4	1261.9
A5	Tetra-SA3 (or Tri-Lac1-SA3) (g)	3−	1342.2	1342.9	1341.5*
		4−	1006.4	1006.9	1006.7
A6	Tetra-Lac1-SA3 (or Tri-Lac2-SA3) (h)	3−	1463.9	1464.7	1464.1
		4−	1097.7	1098.3	1097.4*
A7	Tetra-Lac2-SA3 (i)	3−	1585.6	1586.5	1585.4
		4−	1188.9	1189.6	1189.1*
A8	Tri-SA2 (j)	2−	1685.6	1686.6	1685.4
		3−	1123.4	1124.1	1123.4
	Tetra-SA3-Ac1 (or Tri-Lac1-SA3-Ac1) (k)	3−	1356.2	1356.9	1356.3*
		4−	1016.9	1017.5	1017.2
	Tetra-Lac1-SA4-Ac1 (l)	3−	1574.9	1575.8	1574.9
		4−	1180.9	1181.6	1181.0
	Tetra-Lac3-SA3 (m)	3−	1707.3	1708.3	1707.9
		4−	1280.2	1281.0	1280.1
A9	Tetra-SA2 (or Tri-Lac1-SA2) (n)	2−	1868.2	1869.3	1867.5
		3−	1245.1	1245.8	1245.4*
A10	Tetra-SA2 (or Tri-Lac1-SA2) (n)	3−	1245.1	1245.8	1245.4*
	Tetra-Lac1-SA2 (or Tri-Lac2-SA2) (o)	3−	1366.9	1367.6	1366.5
A11	Tetra-Lac2-SA2 (p)	3−	1488.6	1489.4	1489.4*
		4−	1116.2	1116.8	1116.1
	Tetra-Lac1-SA2 (or Tri-Lac2-SA2) (o)	3−	1366.9	1367.6	1367.6

[a] All carbohydrates contain a fucosylated core. *, Base peak in the mass spectrum of each HPLC peak in Fig. 2A; Ac, Acetyl; (a) to (p) shown in Fig. 3.

3, their theoretical m/z of monoisotopic values and average values, and the observed m/z values. Peaks A1–4, A4–8, and A8–11 in Fig. 2A were assigned to tetra-, tri-, and disialylated glycopeptides, respectively. Tetrasialylated glycopeptides were further separated by the difference in the number of *N*-

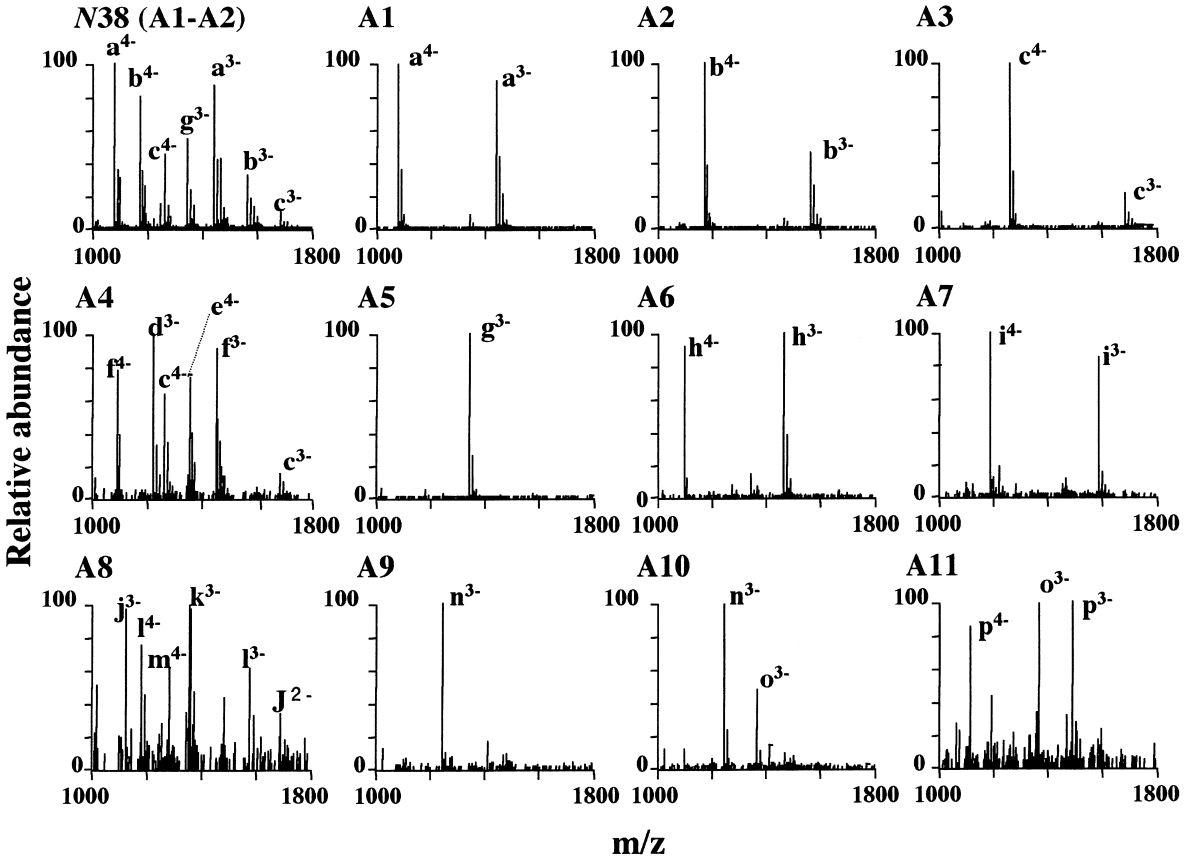

Fig. 3. Mass spectra of *N*38 glycopeptides (peaks A1–A11) and peaks A1–A11 in Fig. 2A. The carbohydrate structures of ions a–p are indicated in Table 2.

acetyllactosaminyl repeats. Peaks A1, A2, A3, and A4 were assigned to fucosylated tetrasialylated tetraantennary oligosaccharides (Tetra-SA4), fucosylated tetrasialylated mono-*N*-acetyllactosamine tetraantennary oligosaccharides (Tetra-Lac1-SA4), Tetra-Lac2-SA4, and Tetra-Lac3-SA4, respectively. Peaks A4–A8 were characterized as Tri-SA3, Tetra-SA3 or Tri-Lac1-SA3, Tetra-Lac1-SA3 or Tri-Lac2-SA3, Tetra-Lac2-SA3, and Tetra-Lac3-SA3, respectively. Minor peaks A8–A11 were determined as shown in Table 2. Our results indicate that the major oligosaccharides at Asn38 are fucosylated tetrasialylated tetraantennary oligosaccharides containing 0–2 *N*-acetyllactosamines.

Under the HPLC conditions presented here, the order of *N*38 glycopeptide elution was: tetra-, tri-, and disialylated glycopeptides, and the glycopeptides in each group were further separated by differences in the number of *N*-acetyllactosaminyl repeats. Our method provides for a excellent separation of glycopeptides based on differences in glycoforms.

3.2.2. Carbohydrate structures of N24 glycopeptides

As shown in Fig. 2B, the *N*24 glycopeptides of rhEPO were separated into 13 peaks, B1–B13. The structural assignments of peaks B1–B13, and their theoretical and observed *m*/*z* values are summarized in Table 3. Under these HPLC conditions, many glycoforms of *N*24 glycopeptides were separated in the order of group SA4 (peaks B1–B3), SA3 (peaks B4–B8), SA2 (peaks B9–B12), and SA1 (peak

Table 3
Peak number glycoforms and theoretical and observed m/z of the *N*24 glycopeptides in the rhEPO digest[a]

Peak No.	Glycoform	Charge state	Theoretical m/z		Observed m/z
			Monoisotopic	Average	
B1	Tetra-SA4	4−	1337.0	1337.8	1337.4*
		5−	1069.4	1070.0	1069.5
B2	Tetra-Lac1-SA4	4−	1428.3	1429.1	1428.5*
		5−	1142.4	1143.1	1142.2
B3	Tetra-Lac2-SA4	4−	1529.6	1520.5	1519.8*
		5−	1215.4	1216.2	1215.5
	Tetra-Lac3-SA4	4−	1610.9	1612=1.8	1611.6
		5−	1288.5	1289.2	1288.6
B4	Tri-SA3	3−	1564.2	1565.2	1564.4
		4−	1172.9	1173.6	1173.1*
B5	Tetra-SA3 (or Tri-Lac1-SA3)	3−	1686.0	1687.0	1686.2
		4−	1264.2	1265.0	1264.3*
	Tetra-Lac1-SA3 (or Tri-Lac2-SA3)	4−	1355.5	1356.3	1356.1
B6	Tetra-Lac1-SA3 (or Tri-Lac2-SA3)	3−	1807.7	1808.7	1808.4
		4−	1355.5	1356.3	1355.8*
	Tetra-Lac2-SA3	4−	1446.8	1447.6	1446.5
B7	Tetra-Lac2-SA3	3−	1929.4	1930.5	1929.2
		4−	1446.8	1447.6	1446.4*
	Tetra-Lac3-SA3	4−	1538.1	1539.0	1538.2
B8	Tri-SA3	3−	1564.2	1565.2	1564.7
		4−	1172.9	1173.6	1173.0*
	Tetra-SA3-Ac1 (or Tri-Lac1-SA3-Ac1)	3−	1700.0	1701.0	1699.2
		4−	1274.7	1275.5	1274.8
	Tetra-Lac1-SA4-Ac1	3−	1821.7	1822.7	1821.7
		4−	1365.8	1366.8	1365.9
B9	Tri-SA2	3−	1467.2	1468.1	1467.3*
		4−	1100.2	1100.8	1100.1
	Tetra-SA2 (or Tri-Lac1-SA2)	3−	1588.9	1589.9	1589.4
		4−	1191.4	1192.2	1191.2
B10	Tetra-SA2 (or Tri-Lac1-SA2)	3−	1588.9	1589.9	1589.1
		4−	1191.4	1192.2	1191.3*
	Tetra-Lac1-SA2 (or Tri-Lac2-SA2)	3−	1710.6	1711.7	1711.2
		4−	1282.7	1283.5	1282.8
	Tetra-Lac2-SA2	3−	1832.3	1833.4	1831.7
		4−	1374.0	1374.8	1374.2
	Tetra-Lac3-SA2	3−	1954.1	1955.2	1954.8
		4−	1465.3	1466.2	1465.8
B11	Bi-SA2	3−	1345.5	1346.3	1345.8*
		4−	1008.9	1009.5	1009.3
B12	Tri-SA2	3−	1467.2	1468.1	1467.1
		4−	1100.2	1100.8	1100.0*
B13	Bi-SA1	2−	1873.2	1874.3	1873.5
		3−	1248.5	1249.2	1248.3*

[a] All carbohydrates contain a fucosylated core. *, Base peak in the mass spectrum of each HPLC peak in Fig. 2B. Ac, Acetyl.

B13). Each group was further separated in the order of zero-, mono-, di-, and tri-*N*-acetyllactosamines, the same as the *N*38 glycopeptides. The structures of the main oligosaccharide were fucosylated bi-, tri-, and tetraantennary oligosaccharides containing 2–4 sialic acids and 0–2 *N*-acetyllactosamines. The glycosylation pattern at Asn24 was different from that at Asn38, which consisted of fucosylated tetrasialylated tetraantennary oligosaccharides mainly. It was particularly noteworthy that the biantennary oligosaccharides Bi-SA2 (peak B11) and Bi-SA1 (peak B13) were observed only in *N*24 glycopeptides.

The predominant ions of peaks B4 and B8 were observed at m/z 1173.1 and 1173.0, respectively, which are consistent with the theoretical m/z of the $[M-4H]^{4-}$ ion of Tri-SA3. This means that peaks B4 and B8 are isomers caused by differences in branching [5]. Similarly, peaks B9 (m/z 1100.1) and B12 (m/z 1100.0) may be isomers of Tri-SA2. These isomers could not be distinguished by MS, but were successfully separated from each other by HPLC. Thus, on-line LC–MS made it possible to distinguish the isomers caused by the differences in branching.

3.2.3. Carbohydrate structures of N83 glycopeptides

Fig. 2D shows an expanded view of the *N*83 glycopeptides of rhEPO. Peaks D1–2, D3–4, and D5 were assigned to tetra-, tri-, and disialylated glycopeptides, respectively. The oligosaccharide structures of D1 to D5, which were determined by the mass spectra, are summarized in Table 4. The carbohydrate in the largest peak, peak D2, was assigned to fucosylated tetrasialylated tetraantennary oligosaccharides containing 0–2 *N*-acetyllactosamines. A shoulder peak, D1, was assigned to Tetra-Lac1-SA4, Tetra-Lac2-SA4, Tetra-Lac3-SA4, and Tetra-Lac4-SA4. A few Tetra-Lac4-SA4 and Tetra-Lac4-SA3, which were not combined at Asn38, were detected in shoulder peaks D1 and D3, respectively. To the best of our knowledge, there have been no reports on the existence of Tetra-Lac4-SA4 and Tetra-Lac4-SA3 in rhEPO. Our results demonstrate that the oligosaccaride structures at Asn83 are similar to those at Asn38, except for tetraantennary oligosaccharides containing four *N*-acetyllactosaminyl repeats.

3.2.4. Carbohydrate structures of O126 glycopeptides

As shown in Fig. 2C, the *O*126 glycopeptides of rhEPO were separated into two peaks, C1 and C2. The structural assignments of peaks C1–C2, and their theoretical and observed m/z values are summarized in Table 5. The difference between the m/z values of the two peaks (m/z 145.1) is equal to that of a sialic acid residue in the two charge ion state. Peaks C1 and C2 were assigned to disialylated Gal-GlcNAc and monosialylated Gal-GlcNAc, respectively. The two peaks were completely separated under the HPLC conditions we used, but they were not separated in the previous paper [7,17].

4. Discussion

The carbohydrate moieties of glycoproteins play an important role in their biological activities and biological functions, and carbohydrate heterogeneity at each glycosylation site may mediate their different functions [1]. Therefore, analysis of the detailed site-specific carbohydrate heterogeneity of glycoproteins is necessary to understand their biological function, and MS is one of the most useful methods of doing so. The site-specific carbohydrate heterogeneity of rhEPO has been investigated by ESI-MS [7,13], fast atom bombardment mass spectrometry (FAB-MS) [6,18], and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [21]. Glycopeptides have been mapped, isolated, and analyzed by ESI-MS, FAB-MS and MALDI-MS.

An approach to selective detection of glycopeptides by LC–ESI-MS–MS has been reported [22,23], and that method only detects glycopeptides by measuring carbohydrate-selective marker ions, such as at m/z 204 ($HexNAc^+$) and 366 (Lac^+). It would be useful to know the location of glycopeptides in the peptide map, but individual glycopeptides cannot be expected to be separated based on differences in the structure of various oligosaccharides at each glycosylation site. However, the mass spectra of glycopeptides are very complicated because of their carbohydrate heterogeneity, structural assignment of their carbohydrates is difficult, and many glycoforms need to be separated from each other to elucidate the carbohydrate structures of glycopeptides easily.

Table 4
Peak number, glycoforms, and theoretical and observed m/z of the *N*83 glycopeptides in the rhEPO digest[a]

Peak No.	Glycoform	Charge state	Theoretical m/z		Observed m/z
			Monoisotopic	Average	
D1	Tetra-Lac2-SA4	4−	1764.7	1765.8	1764.8*
		5−	1411.6	1412.4	1411.5
	Tetra-Lac1-SA4	4−	1673.5	1674.4	1673.6
		5−	1337.9	1339.3	1338.6
	Tetra-Lac3-SA4	4−	1856.0	1857.1	1856.5
		5−	1484.6	1485.5	1484.4
	Tetra-Lac4-SA4	4−	1947.3	1948.4	1947.7
		5−	1557.6	1558.5	1558.0
	Tetra-Lac2-SA4-Ac1	4−	1775.2	1776.3	1775.3
	Tetra-Lac2-SA4-Gly1	4−	1768.7	1769.8	1769.7
D2	Tetra-SA4	4−	1582.2	1583.1	1582.6*
		5−	1265.5	1266.3	1266.0
	Tetra-Lac1-SA4	4−	1673.5	1674.4	1674.1
		5−	1338.6	1339.3	1338.7
	Tetra-SA4-Ac1	4−	1592.7	1593.6	1593.5
	Tetra-SA4-Gly1	4−	1586.2	1587.1	1586.9
	Tetra-Lac1-SA4-Ac1	4−	1684.0	1684.9	1684.6
D3	Tetra-Lac1-SA3	4−	1600.7	1601.6	1601.2*
		5−	1280.3	1281.1	1280.7
	Tetra-Lac2-SA3	4−	1692.0	1692.9	1692.0*
		5−	1353.4	1354.2	1353.7
	Tetra-Lac3-SA3	4−	1783.2	1784.3	1784.4
		5−	1426.4	1427.2	1427.2
	Tetra-Lac4-SA3	4−	1874.5	1875.6	1875.1
		5−	1499.4	1500.3	1499.5
	Tetra-Lac1-SA3-Ac1	4−	1611.2	1612.1	1612.1
	Tetra-Lac2-SA3-Ac1	4−	1702.5	1703.5	1703.0
D4	Tetra-SA3 (or Tri-Lac1-SA3)	3−	2012.9	2014.0	2013.5
		4−	1509.4	1510.3	1509.9*
	Tetra-Lac1-SA3 (or Tri-Lac2-SA3)	3−	2134.6	2135.8	2135.1
		4−	1600.7	1601.6	1601.7
	Tri-SA3	3−	1891.2	1892.3	1891.6
		4−	1418.0	1418.9	1418.8
	Tetra-SA3-Ac1 (or Tri-Lac1-SA3-Ac1)	4−	1519.9	1520.8	1520.1
	Tetra-Lac1-SA3-Ac1 (or Tri-Lac2-SA3-Ac1)	4−	1611.2	1612.1	1611.7
D5	Tetra-SA2 (or Tri-Lac1-SA2)	3−	1915.8	1917.0	1916.7
		4−	1436.6	1437.5	1437.0*
	Tri-SA3	3−	1794.2	1795.2	1794.5
		4−	1345.4	1346.1	1345.4
	Tetra-Lac1-SA2 (or Tri-Lac2-SA2)	3−	2037.5	2038.7	2037.6
		4−	1527.9	1528.8	1527.8
	Tetra-Lac2-SA2	3−	2159.3	2160.5	2161.0
		4−	1619.2	1620.1	1620.5
	Tetra-Lac3-SA2	3−	2281.0	2282.3	2282.5
		4−	1710.5	1711.5	1710.9

[a] All carbohydrates contain a fucosylated core. *, Base peak in the mass spectrum of each HPLC peak in Fig. 2D. Ac, Acetyl; Gly, NeuGc (*N*-glycolyl neuramic acid).

Table 5
Peak number, glycoforms, and theoretical and observed m/z of the O126 glycopeptides in the rhEPO digest

Peak No.	Glycoform	Charge state	Theoretical m/z		Observed m/z
			Monoisotopic	Average	
C1	SA2-Gal-GalNAc	2−	1391.1	1391.9	1391.1
C2	SA-Gal-GalNAc	2−	1245.6	1246.3	1246.0

We succeeded in separating the glycopeptides based on differences in the structure of various oligosaccharides at each glycosylation site. Our method provides the following advantages: (1) only glycopeptides in the glycoprotein digest are separated and analyzed selectively, (2) many glycopeptides containing different carbohydrates at each glycosylation site are further separated based on differences in the numbers of sialic acid and *N*-acetyllactosaminyl repeats, (3) a few glycoforms can be easily detected by on-line LC–MS, (4) isomers due to differences in branching are separated and detected from each other, and (5) it is found to be simple, rapid, and useful for determining oligosaccharide structures at each glycosylation site and for elucidating site-specific carbohydrate heterogeneity.

Our method for determining the site-specific carbohydrate heterogeneity of rhEPO characterized the structures of the main oligosaccharides of rhEPO at both Asn38 and 83 as fucosylated tetrasialylated tetraantennary oligosaccharides containing 0–2 *N*-acetyllactosamines, and those at Asn24 as fucosylated bi-, tri- and tetraantennary oligosaccharides containing 2–4 sialic acids and 0–2 *N*-acetyllactosamines. These results coincide with the findings reported in the previous paper [6,7,13,14].

In conclusion, using ammonium acetate as an eluent, we have demonstrated that on-line LC–MS equipped with a Vydac C_{18} column is a simple and powerful method for identificaton of the detailed site-specific carbohydrate heterogeneity of four glycosylation sites in rhEPO. In addition, Tetra-Lac4-SA4 and Tetra-Lac4-SA3 are newly detected in *N*83 glycopeptides.

Further research is underway to use this method for the site-specific carbohydrate heterogeneity of other glycoproteins such as thrombomodurin and to make comparisons of the site-specific carbohydrate heterogeneity of rhEPO products from different sources.

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References

[1] A. Varki, Glycobiology 3 (1993) 97.
[2] Y.C. Lee, Anal. Biochem. 189 (1990) 151.
[3] R. Kumarasamy, J. Chromatogr. 512 (1990) 149.
[4] E. Tsuda, M. Goto, A. Murakami, K. Akai, M. Ueda, G. Kawanishi, N. Takahashi, R. Sasaki, H. Chiba, H. Ishihara, M. Mori, S. Tejima, S. Endo, Y. Arata, Biochemistry 27 (1988) 5646.
[5] K.G. Rice, N. Takahashi, Y. Namiki, A.D. Tran, P.J. Lisi, Y.C. Lee, Anal. Biochem. 206 (1992) 278.
[6] H. Sasaki, N. Ochi, A. Dell, M. Fukuda, Biochemistry 27 (1988) 8618.
[7] K.B. Linsley, S.-Y. Chan, S. Chan, B.B. Reinhold, P.J. Lisi, V.N. Reinhold, Anal. Biochem. 219 (1994) 207.
[8] C.G. Edmonds, R.D. Smith, Methods Enzymol. 193 (1990) 412.
[9] S. Akashi, K. Noguchi, R. Yuji, U. Tagami, K. Hirayama, K. Kato, H.-H. Kim, K. Tokioka, I. Shimada, Y. Arata, J. Am. Soc. Mass Spctrom. 7 (1996) 707.
[10] N. Kawasaki, M. Ohta, S. Hyuga, O. Hashimoto, T. Hayakawa, Anal. Biochem. 269 (1999) 297.
[11] K. Hirayama, R. Yuji, N. Yamada, K. Kato, Y. Arata, I. Shimada, Anal. Chem. 70 (1998) 2718.
[12] A.W. Guzzetta, L.J. Basa, W.S. Hancock, B.A. Keyt, W.F. Bennett, Anal. Chem. 65 (1993) 2953.
[13] R.S. Rush, P.L. Derby, D.M. Smith, C. Merry, G. Rogers, M.F. Rohde, V. Katta, Anal. Chem. 67 (1995) 1442.
[14] N. Kawasaki, M. Ohta, S. Hyuga, M. Hyuga, T. Hayakawa, Anal. Biochem. 285 (2000) 82.

[15] E. Tsuda, G. Kawanishi, M. Ueda, S. Masuda, R. Sasaki, Eur. J. Biochem. 188 (1990) 405.
[16] P.-H. Lai, R. Everett, F.-F. Wang, T. Arakawa, E. Goldwasser, J. Biol. Chem. 261 (1986) 3116.
[17] H. Sasaki, B. Bothner, A. Dell, M. Fukuda, J. Biol. Chem. 262 (1987) 12059.
[18] M. Nimtz, W. Martin, V. Wray, K.D. Kloppel, J. Augustin, H.S. Conradt, Eur. J. Biochem. 213 (1993) 39.
[19] G.R. Drapeau, Methods Enzymol. 47 (1977) 189.
[20] T. Hayakawa, M. Ohta, N. Kawasaki, in: Proceedings Biologicals Beyond 2000, Pharmeuropa Special Issue, 2000, p. 87.
[21] H. Rahbek-Nielsen, P. Roestorff, H. Reischl, M. Wozny, H. Koll, A. Haselbeck, J. Mass Spectrom. 32 (1997) 948.
[22] M.J. Huddleston, M.F. Bean, S.A. Carr, Anal. Chem. 65 (1993) 877.
[23] M.F. Bean, R.S. Annan, M.E. Hemling, M. Mentzer, M.J. Huddleston, S.A. Carr, in: J.W. Crabb (Ed.), Techniques in Protein Chemistry VI, Academic Press, London, 1995, p. 107.