Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 1703–1710

BIOORGANIC & MEDICINAL CHEMISTRY

Determination of a Sugar Chain and Its Linkage Site on a Glycoprotein TIME-EA4 from Silkworm Diapause Eggs by Means of LC-ESI-Q-TOF-MS and MS/MS

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Received 7 November 2001; accepted 19 January 2002

Abstract—The electrospray ionization (ESI)-tandem quadrupole/orthogonal-acceleration time-of-flight (Q-TOF) mass spectrometer combined with the nano-HPLC system was utilized to determine the glycosylation site and the glycan structure in glycoprotein TIME-EA4 (EA4) from *Bombyx* diapause eggs. LC–MS analysis of EA4 and deglycosylated EA4 indicated that the carbohydrate moiety of EA4 has the mass of 730.58 Da. Then, EA4 was digested with trypsin and chymotrypsin to identify the glycosylated peptide. The peptide fragment from G1y21 to Phe25 was found to carry the carbohydrate moiety. LC–MS/MS analysis of this peptide fragment revealed the sequence of the attached oligosaccharide and the glycosylation site at the same time. The present methodology utilizing the combination of the nano-HPLC system and a highly sensitive Q-TOF mass spectrometer is demonstrated to be quite effective for analyses of glycoproteins of relatively low purity and limited availability from natural sources.

Introduction

An ATPase called TIME-EA4 (EA4) is a glycoprotein, which was isolated from diapausing eggs of the silkworm, *Bombyx mori*, as a key to the termination of embryonic diapause. EA4 exhibits one-time transitory ATPase activity during the chilling of eggs to terminate the diapause, probably due to the continuous conformational change.[1] The carbohydrate moiety of EA4 seems to play an important role in regulating this protein conformational change through the interaction with peptides called PIN.[2] Although six PIN peptides consisting of 28–38 amino acid residues have already been characterized,[3] the glycosylation site and the glycan structure in EA4 have remained unclear, preventing detailed discussion on the interaction between EA4 and PIN peptides.

The attachment site and the structure of the oligosaccharide in EA4 was speculated based on the specificity of enzymes and several consensus rules.[2] First, EA4 was subjected to the lectin assay. *Sambucus sieboldiana* aglutinin (SSA), *Datura stramonium* agglutinin (DSA), and concanavalin A (Con A), and to a lesser extent *Aleura aurantia* lectin (AAL) and *Ricinus communis* agglutinin (RCA) 120, were found to react with EA4. Then, EA4 was treated with peptide-*N*-glycosidase F (PNGase F), resulting in an abolished Con A binding and a decrease in molecular mass by approximately 2.5 kDa as deduced from SDS-PAGE. Considering the well-known specificity of PNGase F that hydrolyzes virtually all carbohydrate–asparagine bonds and the well-defined consensus sequence motifs (Asn-Xxx-Ser/Thr/Cys, Xxx≠Pro) where *N*-glycosylation always takes place, the oligosaccharide in EA4 was elucidated to be a *N*-glycoside attached to the asparagine residue at the Asn22-Ile23-Thr24 sequence. In addition to the trimannosyl core structure common to all *N*-glycans, Gal, Fuc, and Sia residues were supposed to be attached to the core structure as deduced from the lectin assay.

Recent advances in mass spectrometry are beginning to enable structural analyses of glycoproteins of limited

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0968-0896/02/$ - see front matter
PII: S0968-0896(02)00040-8

availability.[4] We have introduced the electrospray ionization (ESI)-tandem quadrupole/orthogonal-acceleration time-of-flight (Q-TOF)[5] mass spectrometer combined with the appropriately-adjusted nano-HPLC system equipped with a column (internal diameter 0.3×150 mm) to accurately determine the position and the structure of any modifications in proteins. A Q-TOF-MS/MS features fully resolved product-ion spectra with mass accuracies less than 0.1 Da. The nano-HPLC system, which was carefully constructed to minimize the void volume to the nL level, purifies and concentrates a small amount of a crude sample in a buffered solution to the optimum conditions for the direct introduction into the ESI source. As a methodology to investigate glycoproteins, the following advantages are stressed: (1) A Q-TOF-MS/MS has enabled to obtain clear-cut information on the sequence of peptides, attached glycans, and the glycosylation site at the same time. (2) On-line mass spectrometric analysis has enabled sensitive characterization of minute carbohydrate heterogeneity with considerable reliability when combined with the nano-HPLC chromatographic analysis.

In this paper, we investigated the sugar chain structure on EA4 in detail by means of LC-ESI-Q-TOF-MS, and MS/MS. Utilizing 30–60 pmol (0.5–1.0 μg) of the protein sample, the glycosylation site and the glycan structure in EA4 was unambiguously determined.

Results

Elucidation of the sugar-chain structure by use of the immobilized lectin column

Although we have already reported a lectin blot analysis of EA4, this analytical method cannot exclude the possibility that minor carbohydrate ingredients might react with lectins. To deduce the structure of the main oligosaccharide chain in EA4, lectin binding experiments by use of the immobilized lectin column were carried out.

Purified EA4 protein dissolved in the binding buffer (20 mM Tris–HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4) was loaded on the immobilized concanavalin A or lentil lectin column. The column was washed with the binding buffer and the effluent was analyzed with SDS-PAGE. Then the elution buffer (0.5 M methyl-α-D-glucopyranoside, 20 mM Tris–HCl, 0.5 M NaCl, pH 7.4) was passed through the column and the effluent was also analyzed with SDS-PAGE. The results are shown in Figure 1. In both cases. EA4 was predominantly detected in the elution buffer, suggesting that both concanavalin A and lentil lectin bound EA4 at high affinity. Binding by other lectins such as wheat germ lectin and peanut lectin were also examined in the same manner, but it was not necessarily clear whether these lectins specifically recognized EA4 or not.

In the case of mammalian glycans, Asn-linked oligosaccharides can be classified into three major subgroups, high-mannose-type, complex-type, and hybrid-type oligosaccharide. Assuming similar structures for the insect glycoprotein. we evaluated the EA4 glycan structure based on the lectin binding experiments.[6] The binding experiment by concanavalin A indicated that the oligosaccharide in EA4 contained the trimannose structure, because strong binding by concanavalin A enough to be retained on the immobilized lectin column requires at least two nonsubstituted or 2-*O*-substituted α-mannopyranosyl residues. Strong binding by lentil lectin, on the other hand, indicated that the oligosaccharide in EA4 had an α1–6-linked fucose at the innermost GlcNAc residue in addition to the trimannose structure. Therefore, we assumed the sugar-chain structure in Figure 2 as the most probable basic skeleton for EA4.

Mass spectrometric analyses of EA4 and deglycosylated EA4

A highly sensitive Q-TOF mass spectrometer combined with the nano-HPLC system has enabled to analyze an extremely limited amount of a sample. Utilizing this system, we carefully analyzed the glycoprotein EA4. In order to measure the molecular mass of EA4 and deglycosylated EA4 as accurately as possible, we utilized a sequencially-homologous Cu,Zn-SOD protein as a calibration standard. Figure 3 shows the electrospray

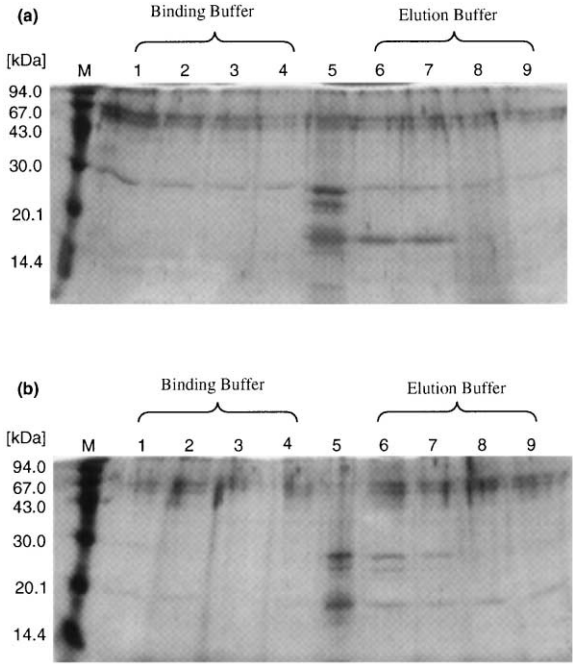

Figure 1. SDS-PAGE of the lectin column effluent on a 12.5% gel: (a) the effluent from an immobilized concanavalin A column, (b) the effluent from an immobilized lentil lectin column. M, molecular mass standards; lanes 1–4, fractions 1–4 of the binding buffer effluent; lane 5, the purified EA4 sample loaded on the lectin column; lanes 6–9, fractions 1–4 of the elution buffer effluent.

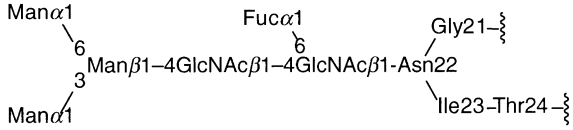

Figure 2. The oligosaccharide structure estimated from the lectin binding experiments by use of the immobilized lectin column.

mass spectrum of EA4. Treatment of EA4 with PNGase F resulted in a decrease in molecular mass from 17,336.79 to 16,607.19 (Fig. 4). The calculated average mass of the amino acid sequence of EA4 deduced from cDNA[7] was 16,606.28. Considering that PNGase F converts the glycosylated Asn residue to the Asp upon hydrolysis of the glycoside, the observed molecular mass of 16,607.19 for PNGase F-treated EA4 was in complete agreement with the calculated value (16,607.27) for EA4 having the -Gly21-Asp22-Ile23- sequence. The observed value for the sugar-chain moiety was then 730.58 Da, corresponding to two mannose and two *N*-acetylglucosamine residues (calculated value 730.26 Da).

Enzymatic digestion of EA4 for the localization of the glycosylation site

EA4 was digested with trypsin and the obtained tryptic peptides were analyzed with LC-ESI-Q-TOF-MS to find the glycosylated peptide fragment. Then, the tryptic EA4 in phosphate buffer was directly analyzed with Develosil ODS-HG-5 column without any off-line purification operation and the column effluent was introduced into the mass spectrometer without splitting. Among the 14 tryptic peptides, the T3 peptide from Gly2l to Lys32 (1306.65 Da) shown in Figure 5 was suggested to accompany the carbohydrate moiety having the mass of 730.58 Da. Indeed, the glycosylated T3 peptide was eluted around 22 min as a doubly-charged ion (*m*/*z* 1019.51) as shown in Figure 6. Thus, the observed difference in molecular mass was 730.37 Da, which was in good agreement with the calculated value of 730.26 Da for two mannoses and two *N*-acetylglucosamines.

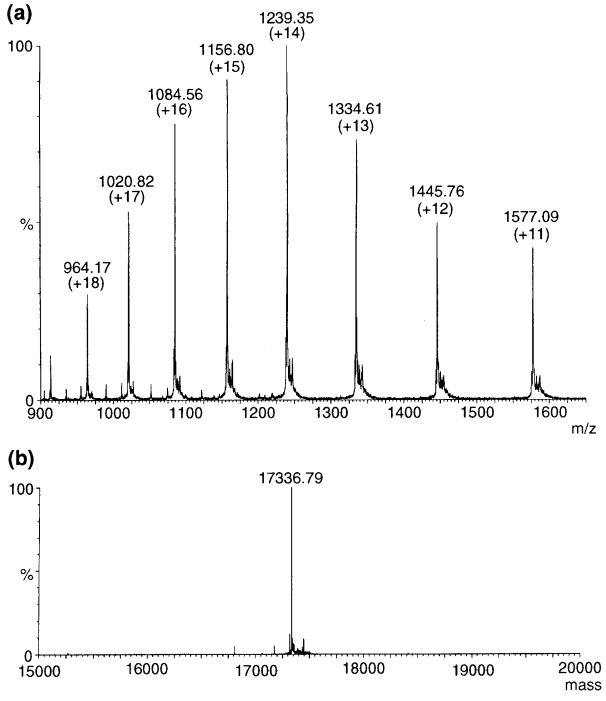

Figure 3. (a) The raw electrospray data of EA4; (b) the mass spectrum produced by MaxEnti processing[8] of the raw electrospray data above. The cone voltage was 40 V.

The glycosylated T3 peptide was analyzed with LC-ESI-Q-TOF-MS/MS, but the glycosylation site could not be determined due to the facile fragmentation of the carbohydrate moiety in the collision cell. Higher collision energy is generally required to fragment longer peptide chains for MS/MS measurements. In order to suppress the fragmentation of the fragile saccharide chain in the mass spectrometer. the glycosylated T3 peptide was further digested by chymotrypsin into two smaller peptides T3Y1 and T3Y2 at the C-terminal side of Phe25 before LC-ESI-Q-TOF-MS/MS analysis (Fig. 5). To avoid operational complexity and minimize a sample loss, the glycosylated T3 peptide was digested without isolation. Both peptide fragments were successfully detected as shown in Figure 7. It should be noted that exactly the same amount of the starting EA4 protein was required for analyses of T3Y1 and T3Y2. The T3Y2 peptide was found unmodified as a doubly-charged ion (*m*/*z* 388.20) and the amino acid sequence of T3Y2 was confirmed with LC-ESI-Q-TOF-MS/MS. The T3Y1 peptide, on

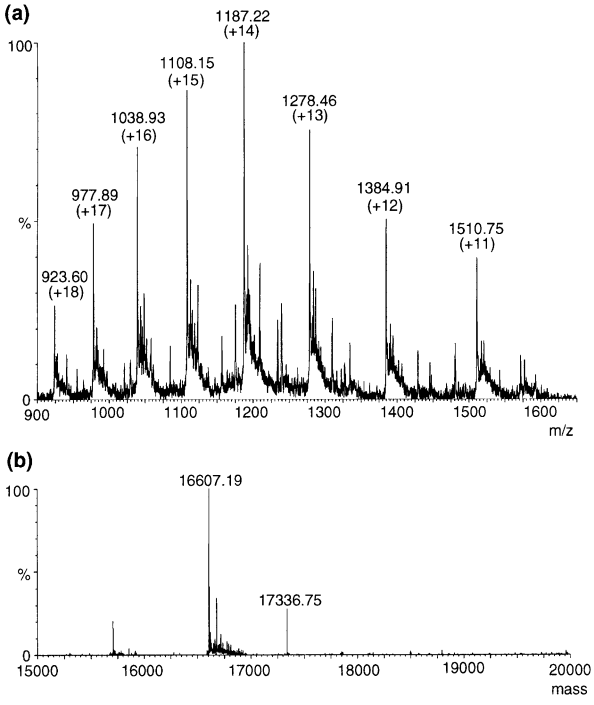

Figure 4. (a) The raw electrospray data of PNGase F-treated EA4; (b) the mass spectrum produced by MaxEnt 1 processing[8] of the raw electrospray data above. The cone voltage was 40 V.

T3

Gly^{21}-Asn^{22}-Ile^{23}-Thr^{24}-Phe^{25}-Thr^{26}-Gln^{27}-Val^{28}-Gln^{29}-Asp^{30}-Gly^{31}-Lys^{32}

Monoisotopic Mass = 1306.65

T3Y1

Gly^{21}-Asn^{22}-Ile^{23}-Thr^{24}-Phe^{25}

Monoisotopic Mass = 550.28

T3Y2

Thr^{26}-Gln^{27}-Val^{28}-Gln^{29}-Asp^{30}-Gly^{31}-Lys^{32}

Monoisotopic Mass = 774.39

Figure 5. Amino acid sequence and the monoisotopic mass of the T3, T3Y1, and T3Y2 peptides.

the other hand, was found to accompany the carbohydrate moiety (m/z 641.31, in the doubly charged state) as shown in Figure 7. The observed value of 730.34 Da for the carbohydrate moiety attached to the T3Y1 peptide agrees very well with the theoretical value of 730.26 Da for two mannoses and two *N*-acetylglucosamines, too.

LC-ESI-Q-TOF-MS/MS analysis of the trypsin–chymotrypsin digest of EA4

The glycosylated T3Y1 peptide was analyzed with LC-ESI-Q-TOF-MS/MS to investigate the glycosylation site and the glycan structure. In Figure 8 is shown the MaxEnt3-processed[8] data of the raw MS/MS spectrum of the glycosylated T3Y1 peptide. The glycan structure having the mass of 730.34 Da was clarified by the facile fragmentation of the carbohydrate moiety. Three intense molecular ion peaks (m/z 1119.68, 957.59 and 754.50) were observed between the parent glycopeptide ion peak (m/z 1281.76) and the fragmentation ion peak of the peptide moiety (m/z 551.38). The mass difference of 162.05 and 203.08 corresponds to the fragmentation of a mannose (Man) and an *N*-acetyiglucosamine (GlcNAc) residue, respectively. Therefore, the oligosaccharide structure of EA4 was characterized as -GlcNAc-GlcNAc-Man-Man in order from the reducing end. In addition, the peptide structure was also confirmed as shown in Figure 8.

The glycosidic linkage is fragmented much more readily than the peptide backbone in the collision cell. So it is generally impossible to determine the glycosylation site with tandem mass spectrometry of glycopeptides. However, we found that the final monosaccharide residue attached to the peptide exhibits some resistance to fragmentation, when the peptide backbone to be analyzed is small enough to be fragmented with relatively low collision energy. Compared in Figure 9 is the MS/MS spectrum of the glycosylated T3Y1 [Fig. 9(a)] with that of the peptide moiety of T3Y1 generated in the mass spectrometer by fragmentation with the high cone voltage [Fig. 9(b)]. As shown in the MS/MS spectrum in Figure 9(a), the b_2 (m/z 172.10), b_3 (m/z 285.20), and b_4 (m/z 386.26) ions accompanied the fragment ions shifted by 203.08 Da (m/z 375.21, 488.31, and 589.37, respectively). On the other hand, the observed y_1'' (m/z 166.11), y_2'' (m/z 267.18) and y_3'' (m/z 380.28) ions did not show any sign of the glycoside attachment. Therefore, the glycosylation site of EA4 from *Bombyx* diapause eggs was unambiguously determined to be Asn22. It should be noted that the comparison of the MS/MS spectra of the glycosylated and the deglycosylated T3Y1 peptide was absolutely necessary, because the fragment ion at m/z 369.24 in Fig. 9(a) seemingly agreed with the $y_1'' + 203.08$ ion. This possibility, however, was ruled out by the presence of the fragment ion at m/z 369.26 in the MS/MS spectrum of the deglycosylated T3Y1 in Fig. 9(b). The other point to be noted is that deglycosylation of the glycopeptide T3Y1 should be accomplished through the in-source fragmentation with the high cone voltage, because PNGase-F-deglycosylation inevitably accompanies conversion of the Asn to the Asp residue.

LC-ESI-Q-TOF-MS analysis of minute carbohydrate heterogeneity in EA4

Although the major carbohydrate structure in EA4 was successfully determined as -GlcNAc-GIcNAc-Man-Man with LC-ESI-Q-TOF-MS, MS/MS, the result obtained in this study was partly contradictory to the previous lectin binding assay[2] suggesting the usual trimannosyl core structure carrying Gal, Fuc and Sia residues. Then, we considered the possibility that a small amount of minor oligosaccharide chains in EA4 was present and might react with lectins.

Electrospray mass spectrometry of EA4 in Figure 3 indicated that the sample contained the protein having

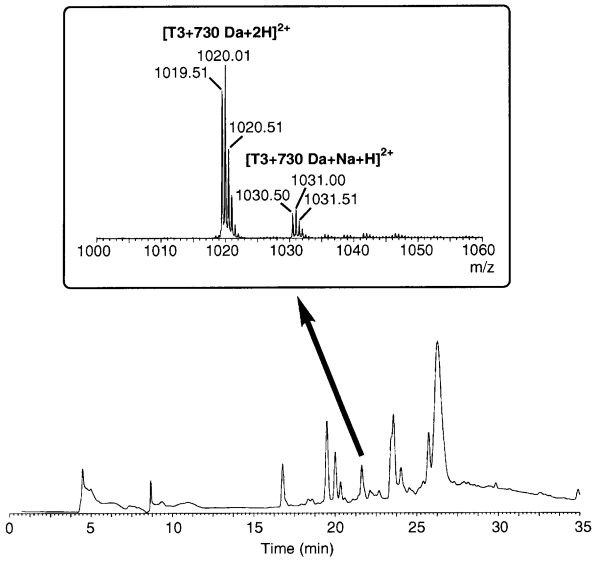

Figure 6. Develosil ODS-HG-5 chromatographic analysis of the peptide mixture from the tryptic EA4. The column effluent was monitored at 210 nm. Inset: The on-line ESI-Q-TOF-MS spectrum of the column effluent around 22 min. The cone voltage was 30 V.

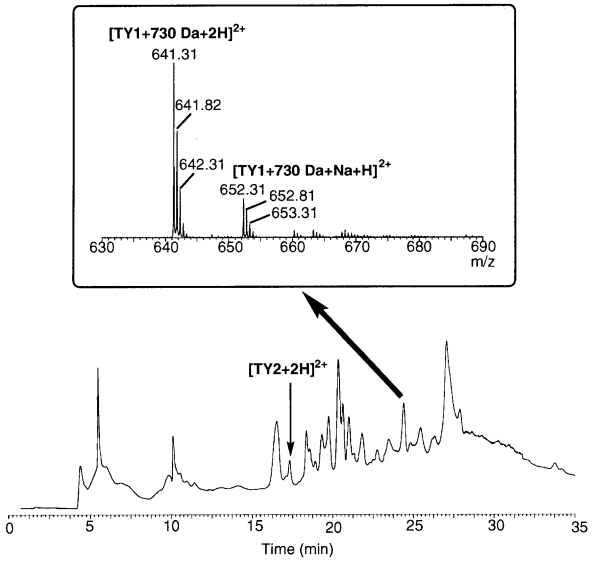

Figure 7. Develosil C30-UG-5 chromatographic analysis of the peptide mixture from the trypsin–chymotrypsin digest of EA4. The column effluent was monitored at 210 nm. Inset: The on-line ESI-Q-TOF-MS spectrum of the column effluent around 24.5 min. The cone voltage was 30 V.

the mass of 17,336.79 Da exclusively. The intensity of possible minor species with different glycan structures were too weak to observe well above the noise level. Considering the poor ionization efficiency and the broadened signals of the intact EA4 protein, investigation of such minor species as large glycoproteins seemed undesirable. Instead, the tryptic EA4 was investigated with LC-ESI-Q-TOF-MS to find out other glycans attached to the T3 peptide, utilizing the combination of the accurately-determined molecular mass and the chromatographic behavior of minor species in the sample. Shown in Figure 10 is the ESI-Q-TOF-MS spectrum of

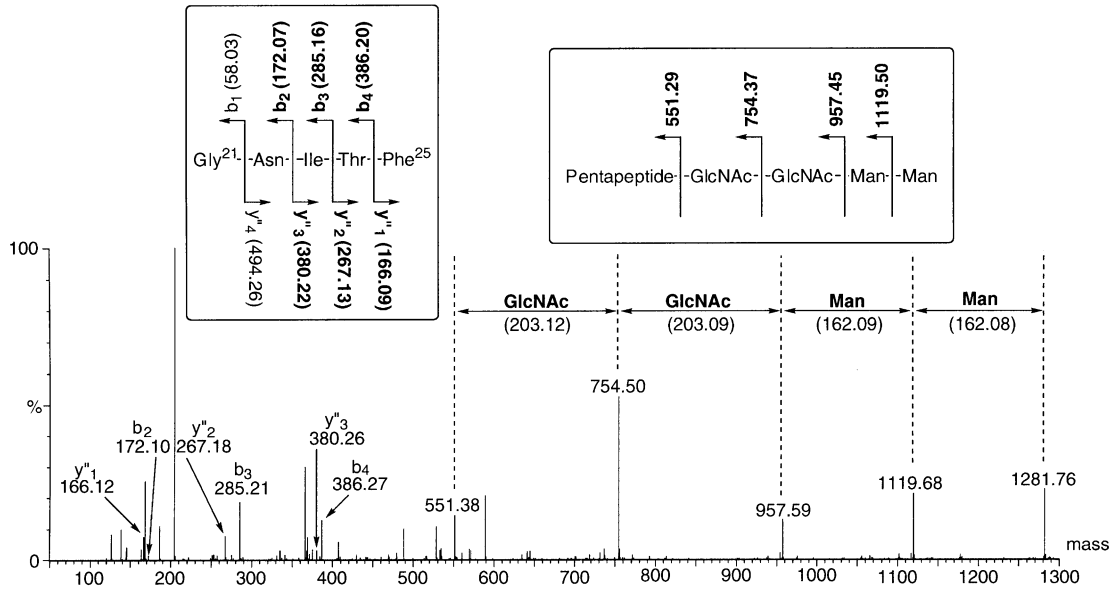

Figure 8. The MaxEnt3 processed[8] data of the raw MS/MS spectrum of the glycosylated T3Y1 peptide (m/z 641.31, in the doubly charged state). Observed b- and y″-fragments of the peptide moiety are shown in the spectrum. The fragmentation patterns of the oligosaccharide and the peptide are shown above. In the peptide sequence are shown the theoretical values of b- and y″-fragments. Observed ions are shown in bold. The MS/MS spectrum was obtained at cone voltage of 30 V and valiable collision energy between 15 and 26 V.

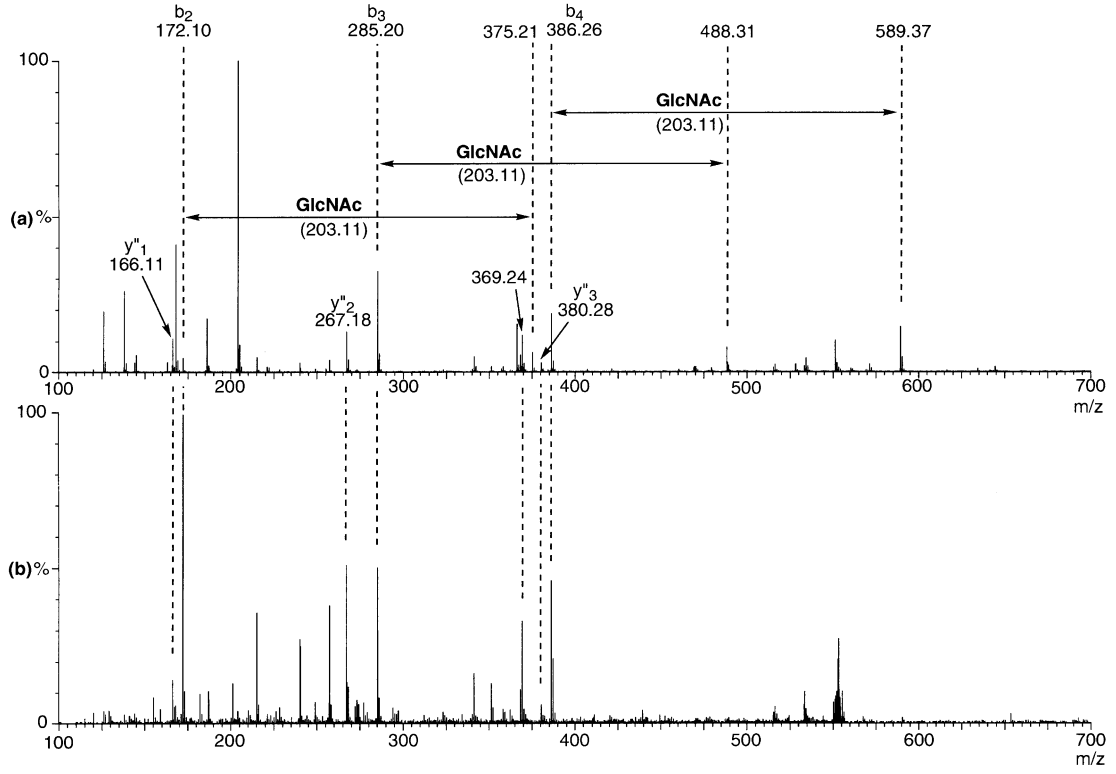

Figure 9. ESI-Q-TOF-MS/MS spectra of (a) the glycosylated T3Y1 peptide (m/z 641.31, in the doubly charged state) and (b) the T3Y1 peptide (m/z 551.40, in the singly charged state). The glycosylated T3Y1 peptide was ionized at cone voltage of 30 V. At higher cone voltage of 50 V, the deglycosylated T3Y1 peptide was generated in the mass spectrometer by fragmentation. Both MS/MS spectra were obtained at valiable collision energy between 20 and 32 V.

the column effluent from 20 to 24 min in Figure 6. Other than the T3 + 730 Da peptide, additional mannose or fucose (Fuc) attachment was found out, although the intensity of these minor species was less than 1% of that of the T3 + 730 Da peptide. As seen in the ion chromatograms in Figure 11, these minor species exhibited the exactly identical reversed-phase chromatographic behavior with the T3 + 730 Da glycopeptide. Thus, these minor species, for which MS/MS measurement seemed practically impossible, could be characterized with considerable reliability.

The T3Y1 peptide was investigated likewise to further confirm this result. Shown in Figure 12 is the ESI-Q-TOF-MS spectrum of the column effluent from 22.5 to 26.5 min in Figure 7. The mass values corresponding to the minor species having additional mannose or fucose were detected in the same manner. In addition, these minor species were eluted from Develosil C30-UG-5 column at exactly the same retention time with the T3Y1 + 730 Da peptide (Fig. 13). A series of evidence suggested that EA4 had minor oligosaccharide chains ($GlcNAc_2Man_3$ and $GlcNAc_2Man_2Fuc$) attached to the same amino acid with the major one.

Discussion

The mass spectrometric result described in this paper was partly contradictory to the previous estimation of the glycan structure in EA4.[2] First of all, the mass of the glycan measured with a Q-TOF mass spectrometer was 730.58 Da, which was considerably smaller than the value estimated from SDS-PAGE (2.5 kDa). We considered the possibility of an unexpected in-source fragmentation of the sugar chain of EA4 in the mass spectrometer and examined three pyridylaminated sugar chains as model compounds under similar measurement conditions. Parent ion peaks were clearly observed for three model sugar chains having five or 10 sugar units.[9] Thus, the molecular mass of the glycan in EA4 was determined to be 730.58 Da when EA4 was isolated and purified as described in the Experimental. It was already reported that by employing high-percentage (12.5–15%) acrylamide gels, a glycan-free polypeptide chain produced by PNGase F migrates slightly faster on SDS-PAGE than the same polypeptide chain with one GlcNAc residue produced by Endo F.[10] Although there seems to be no systematic investigation on the mobility of intact and deglycosylated glycoproteins on SDS-PAGE, the value of 2.5 kDa for the glycan in EA4 might probably be overestimated. Another point to be

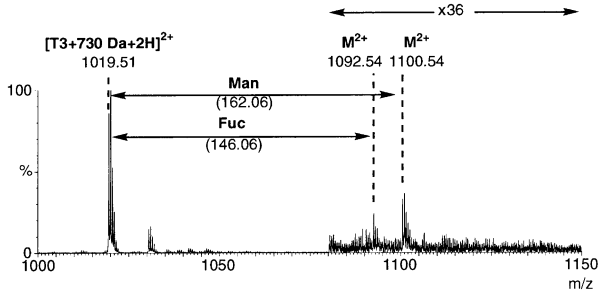

Figure 10. The on-line ESI-Q-TOF-MS spectrum of the column effluent from 20 to 24 min where the T3 peptide eluted. HPLC chromatogram at 210 nm is shown in Figure 6. The cone voltage was 30 V.

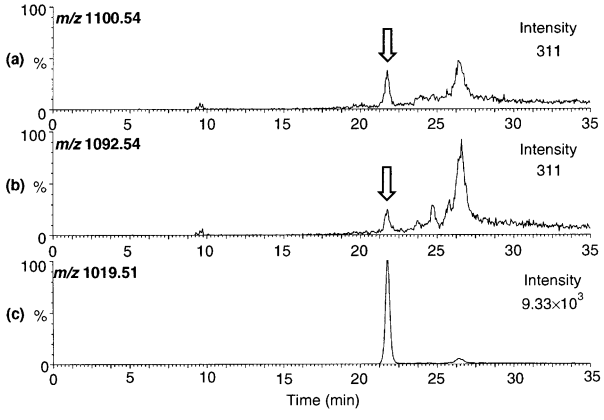

Figure 11. Ion chromatograms for molecular ions observed at (a) *m/z* 1100.54, (b) *m/z* 1092.54, and (c) *m/z* 1019.51.

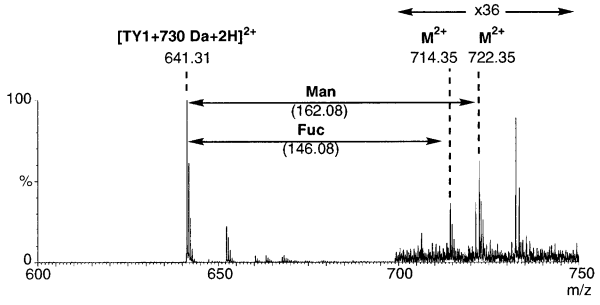

Figure 12. The on-line ESI-Q-TOF-MS spectrum of the column effluent from 22.5 to 26.5 min where the T3Y1 peptide eluted. HPLC chromatogram at 210 nm is shown in Figure 7. The cone voltage was 30 V.

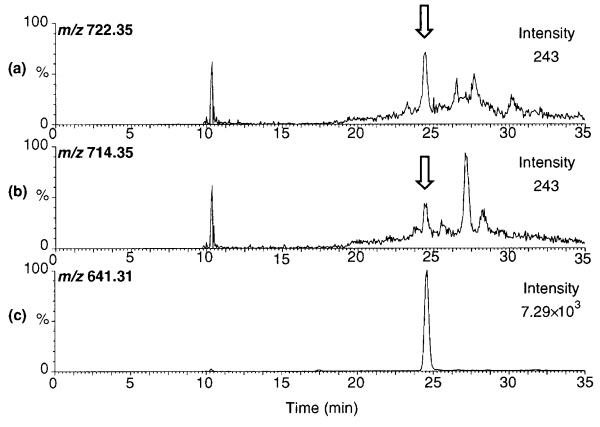

Figure 13. Ion chromatograms for molecular ions observed at (a) *m/z*

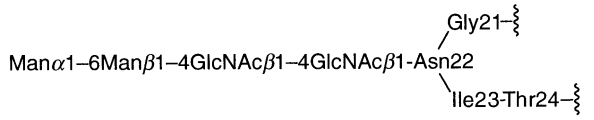

Figure 14. Proposed structure for the carbohydrate moiety attached to Asn22 in EA4.

mentioned is the discrepancy between the estimated structure based on the lectin assay and the present mass spectrometric structure. We examined the presence of a small amount of minor oligosaccharide heterogeneity in EA4 that might react with lectins. As a preliminary investigation, two minute oligosaccharide chains were identified with considerable reliability utilizing the combination of the nano-HPLC and the Q-TOF mass spectrometer. Although it is not clear at present whether these minor species might react with lectins to result in the overall positive lectin reactions, the present mass spectrometric method provides complementary information for the lectin binding assay.

Finally, we would like to propose the oligosaccharide structure in Figure 14 as the most probale one for EA4. Exactly the same structure was already reported for membrane glycoproteins of the three insect cell lines *Maniestra brassicae* (Mb-0503), *Bombyx mon* (Bm-N), and *Spodoptera frugiperda* (Sf-2l) by means of two-dimensional HPLC.[11] Unexpectedly, this straight sugar chain seemed to be bound at high affinity by concanavalin A and lentil lectin, in the absence of the trimannose core structure and the fucose residue.

Conclusions

We described herein a detailed analysis of glycoprotein EA4, which was found to have the truncated oligosaccharide with two mannoses and two *N*-acetylglucosamines attached to Asn22 as a major component. The Q-TOF mass spectrometer combined with the nano-HPLC system was successfully utilized to analyze the glycosylation site and the glycan structure in EA4. As demonstrated in this paper, nano-HPLC purification and concentration before on-line mass spectrometric analysis has enabled to obtain clear-cut MS and MS/MS spectra for samples of low purity and limited availability without any off-line purification operation. In addition, it is advantageous to carefully examine mass spectrometric results in combination with reversed-phase chromatographic analysis, because samples from natural sources sometimes contain misleading protein impurities. This advantage was highlighted in the investigation of minute carbohydrate heterogeneity in EA4. The methodology presented here could be applicable to analyses of any glycoproteins from natural sources in the same manner.

Experimental

Instrumentation

MS and MS/MS spectra were measured utilizing a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray type ESI source. All experiments were performed in the positive ion mode. Data were acquired and processed using MassLynx version 3.4. All samples were desalted and separated by the appropriately-adjusted nano-HPLC system (JASCO, Tokyo, Japan) before on-line ESI-MS and MS/MS analysis. Columns used were Develosil C30-UG-5 (Nomura, Seto, Japan, 15 cm×0.3 mm ID) and Develosil ODS-HG-5 (Nomura, 15 cm×0.3 mm ID). The columns were equilibrated with 260 μL of water containing 0.025% trifluoroacetic acid at a flow rate of 10 μL/min and then developed using a linear gradient from 0 to 100% of acetonitrile containing 0.025% trifluoroacetic acid for 40 mm at a flow rate of 5 μL/min. The column effluent was monitored at 210 nm and then introduced into the electrospray nebulizer without splitting.

Materials

Acetonitrile (HPLC grade) and trifluoroacetic acid were purchased from Nacalai tesque (Kyoto, Japan). PNGase F (recombinant) and trypsin, chymotrypsin (sequence grade) were purchased from Roche Diagnostics (Mannheim, Germany).

Purification of EA4

EA4 was extracted from C108 *Bombyx* silkworm diapause eggs according to the procedure reported by Tani et al.[2] Instead of a Sephadex G–25 column reported in the literature procedure, a HiTrap Chelating column (Amersham Pharmacia Biotech) was utilized to purify the crude EA4 precipitate with 80% saturated ammonium sulfate. Purification was performed according to the operation manual supplied from Amersham Pharmacia Biotech. A HiTrap Chelating column (5 mL) was utilized for purification of the crude EA4 precipitate from diapause eggs (40 g), with Zn^{2+} as a chelated metal ion and imidazole as a displacing agent. EA4 in the elution buffer was then passed through HiTrap Desalting columns (Amersham Pharmacia Biotech, 5 mL×3) twice to exchange the buffer to the phosphate buffer (20 mM, pH 7.2). Concentration was achieved by ultrafiltering the sample solution through Centricon YM-10 (Millipore). The amount of the obtained EA4 protein was estimated with the peak area in the reversed-phase HPLC profile, using sequencially-homologous Cu,Zn-SOD as a standard. EA4 (297 μg, 17 nmol) was obtained from C108 *Bombyx* silkworm diapause eggs (40 g). For the determination of the molecular mass of EA4, the solution of EA4 (38 pmol) in phosphate buffer (1 μL) was analyzed with LC-ESI-Q-TOF-MS.

Analysis of EA4 by use of the immobilized lectin column

The immobilized lectin columns (HiTrap Con A, HiTrap Lentil Lectin, Hilrap Wheat Germ Lectin, HiTrap Peanut Lectin) were purchased from Amersham Pharmacia Biotech. According to the operation manual, purified EA4 in the designated binding buffer (1 mL) was loaded on the pre-equilibrated column at the flow rate of 0.1 mL/min for 30 mm. Then, the designated elution buffer was passed through the column at a flow rate of 1 mL/min. Each fraction of the effluent was analyzed with SDS-PAGE (silver-staining).

PNGase F deglycosylation of EA4

To the solution of EA4 (9.9 μg, 567 pmol) in phosphate buffer (26 μL, 62 mM, pH 7.2) was added the solution of

EDTA (2 μL, 160 mM), 2-mercaptoethanol (2 μL, 160 mM), and finally PNGase F (2 μL, 2 units). The solution was incubated at 37 °C for 20 h. 4 μL of the obtained sample was then analyzed with LC-ESI-Q-TOF-MS without any off-line purification operation.

Trypsin–chymotrypsin digestion of EA4

The solution of EA4 (8 μg, 458 pmol) in phosphate buffer (17 μL, 59 mM, pH 7.2) was heated at around 90 °C for 5 min to denature the protein. After cooled in an ice water bath, the solution of trypsin (0.4 μg, 17 pmol) in water (3 μL) was added. The solution was incubated at 37 °C for 18 h. The enzyme was deactivated by heating the solution at about 90 °C for 5 min. A 10 μL of the obtained sample was set aside for LC-ESI-Q-TOF-MS analysis. The remaining tryptic EA4 solution (10 μL) was further digested with chymotrypsin. After addition of chymotrypsin (0.2 μg, 8 pmol) in water (2 μL), the solution was incubated at 37 °C for 18 h. The enzyme was deactivated by heating the solution at about 90 °C for 5 min. A 1 μL of the obtained sample was usually subjected to LC-ESI-Q-TOF-MS analysis of the peptide mixture. In the case of the analysis of minute carbohydrate heterogeneity, 8 μL of the obtained sample was used. For LC-ESI-Q-TOF-MS/MS analysis of the T3Y1 peptide, a 2 μL of the sample mixture was injected.

Acknowledgements

This work was supported by JSPS-RFTF 96L00504. The authors acknowledge Japan Society for the Promotion of Science for financial support to the post-doctoral work for T.K., S.S., and T.F. The authors thank JASCO, Inc. for technical support for the nano-HPLC system.

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