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Characterization and analysis of a novel glycoprotein from snake venom using liquid chromatography-electrospray mass spectrometry and Edman degradation

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An N-linked glycosylation in a novel C-lectin protein from snake venom was observed by Edman degradation and liquid chromatography-electrospray mass spectrometry. The peptides obtained by trypsin cleavage were analyzed to confirm the amino acid sequence and Asn5 was found to be the N-glycosylation site. The result was further confirmed by *N*-glycosidase digestion. In addition, the protein and tryptic peptides with and without glycan chain were characterized by mass spectrometry according to the mass difference. The glycopeptide obtained from proteolytic digestion was analyzed and the glycoforms were identified as high-mannose type by tandem MS coupled with α-mannosidase digestion. An oxidized Met residue was detected and located in the protein by mass spectrometry.

Keywords: *Trimeresurus stejnegeri* lectin; glycoprotein; electrospray mass spectrometry; Edman degradation.

Glycosylation is a post-translational processing frequently observed in proteins. The diversity of monosaccharides and the variety of linkages give rise to the complexity of glycoproteins. Glycosylation plays an important role in the biological properties of many proteins, but brings about difficulties in their characterization.

Reverse-phase high-performance liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) has been shown to be a rapid and sensitive means for characterizing the primary structure of proteins and their post-translational modifications including glycosylation [1–7]. To determine the site of glycosylation and site-specific microheterogeneity of glycoprotein, endoproteases are usually employed to obtain the glycopeptides which are separated by HPLC and identified by electrospray mass spectrometry. LC-ESI-MS coupled with protease and glycanase (such as *N*-glycosidase F) digestion can identify the sites of glycosylation, detect whether a glycan is N-linked or O-linked, and even differentiate high-mannose, complex and hybrid forms of N-linked glycan.

C-type lectins [8] are sometimes glycoproteins themselves [9,10]. A novel C-type lectin glycoprotein [11] was isolated from the venom of *Trimeresurus stejnegeri* and designated as TSL. The protein sequence, deduced from cDNA, is KYKPGCHLASFHRLAESLDIAEYISDYHKRQAEVWIGLLDRKKDFSWEWTDRSCTDYLNWDKNQPDHYKDKEFCVELVSLTGYHRWNDQVCESKNSFLCQCKF. The protein is composed of 135 amino acids, with a calculated mass of 16 207.3 Da. The N-terminal 30-amino acid residues except residue 5, a potential N-glycosylation site, were sequenced by Edman degradation. In addition, the molecular mass measured by mass spectrometry was not consistent with the calculated value, possibly as a result of glycosylation. In this paper, by mass spectrometry and Edman degradation, coupled with protease and glycosidase digestion, we report the post-translational modifications of TSL, including the site of N-glycosylation and the structure of glycan chains attached to the protein.

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Abbreviations: LC-ESI-MS, liquid chromatography-electrospray ionization mass spectrometry; MS/MS, tandem mass spectrometry; TIC, total ion current; TSL, *Trimeresurus stejnegeri* lectin; CAM, carboxyamidomethyl.

Enzymes: *N*-glycosidase F (EC 3.5.1.52); Tos-Phe-CH_2Cl-trypsin (EC 3.4.21.4); Glu-C (EC 3.4.21.19); α-mannosidase (EC 3.2.1.24).

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MATERIALS AND METHODS

Dithiothreitol, iodoacetamide, ammonium bicarbonate and α-mannosidase were from Sigma. Trifluoroacetic acid was obtained from Merck. HPLC grade acetonitrile was from BDH. Water from Milli-Q system was used. Endoprotease Glu-C and *N*-glycosidase F were from Boehringer Mannheim. Tos-Phe-CH_2Cl-trypsin was made in our laboratory.

Reduction and S-carboxyamidomethylation of TSL

HPLC-purified TSL dissolved in 30 mM NH_4HCO_3 (pH 7.8) to a final concentration of 1 mg·mL^{-1}, was reduced for 50 min at 40 °C with 10 mM dithiothreitol, and S-carboxyamidomethylated with 25 mM iodoacetamide for 30 min at 37 °C. The reduced and S-carboxyamidomethylated TSL (CAM-TSL) was purified by HPLC to remove dithiothreitol and iodoacetamide.

N-terminal sequencing

N-terminal sequencing of the sample loaded on Prosorb™ was performed on a PE-ABI 491A protein sequencer.

Tryptic digestion

The HPLC-purified CAM-TSL dissolved in 30 mM NH_4HCO_3 (pH 7.8) to a concentration of 1 mg·mL^{-1} was mixed with Tos-Phe-CH_2Cl-trypsin to give an enzyme to substrate ratio of

1 : 50 (w/w). The digestion proceeded at 37 °C for 4 h, followed by addition of another equal aliquot of enzyme. After 24 h, the digestion was stopped by freezing to −20 °C.

N-Glycosidase F deglycosylation and reduction of native TSL

It was performed as reported with some modification [12]. *N*-Glycosidase F (0.5 unit in 2 μL of water) was added to the native TSL (in 100 mM NH_4HCO_3 to a concentration of 1 mg·mL^{-1}, pH 8.3). The mixture was incubated at 37 °C for 2 h. Reduction with dithiothreitol was followed as aforementioned in this article. The reaction was stopped by injection onto the HPLC.

N-Glycosidase F deglycosylation of CAM-TSL

N-Glycosidase F (0.5 unit in 2 μL of water) was added to the CAM-TSL (in 100 mM NH_4HCO_3 to a concentration of 1 mg·mL^{-1}, pH 8.3). The mixture was incubated at 37 °C for 2 h. The reaction was stopped by injection onto the HPLC.

N-Glycosidase F deglycosylation of tryptic digest

It was performed as reported with some modification [12]. *N*-glycosidase F (0.5 unit in 2 μL of water) was added to the tryptic digest solution (pH 7.8) and the mixture was incubated at 37 °C for 2 h. The reaction was terminated by freezing to −20 °C.

Glu-C digestion

The HPLC-purified CAM-TSL dissolved in 100 mM NH_4Ac (pH 4.0) to a concentration of 1 mg·mL^{-1} was mixed with Glu-C to give an enzyme to substrate ratio of 1 : 50 (w/w). The digestion proceeded at 37 °C for 4 h, followed by addition of another equal aliquot of enzyme. After 24 h, the digestion was stopped by freezing to −20 °C.

α-Mannosidase cleavage of Glu-C digest

The reaction was performed as described [13] with some modification. Glu-C digest was boiled and cooled to −20 °C to inactivate the Glu-C. α-Mannosidase (0.5 unit in 2 μL of buffer: 3.0 M $(NH_4)_2SO_4$, 0.1 mM zinc acetate, pH 7.5) was added to the thawed digest. The mixture was incubated at 37 °C for 5 h and 22 h. The reaction was stopped by freezing to −20 °C.

Chromatography

The on-line HPLC separation was performed on HP1100 at a flow rate of 0.2 mL·min^{-1}. Solvent A was 0.02% trifluoroacetic acid in water, solvent B was 0.02% trifluoroacetic acid in 98% acetonitrile/2% water. For protein, 2.1 × 30 mm C8 reverse-phase column (ABI-RP-300) was used, the gradient was 0–2 min 0% B, 2–15 min 0–100% B. A 2.1 × 150 mm C18 reverse-phase column (HP) was used for proteolytic digest separation. For Glu-C digest, the gradient was 0–2 min 0% B, 2–90 min 0–60% B. For tryptic digest, the concentration of trifluoroacetic acid in the solvent was increased to 0.05%; the gradient was 0–2 min, 0% B, 2–90 min 0–50% B.

Mass spectrometry

The mass spectra were obtained on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with an electrospray ionization source; spray voltage was 4.25–5 kV. The heated capillary was maintained at 180–200 °C; capillary voltage was 15 V. Ion collection included three microscans with a maximum ion injection time of 100 ms. The instrument was scanned from 50 to 2000 *m/z*. The mass spectrometer conditions were optimized with Ultramark provided by the manufacturer. The ZoomScan and tandem MS (MS/MS) was performed in data dependent mode, consisting of a full scan, followed by a high resolution ZoomScan and MS/MS experiment of the ion selected in the ZoomScan. The MS/MS collision energy value was 45–47%.

RESULTS

Molecular mass determination of TSL chain

In the previous report, nonreducing SDS/polyacrylamide gel electrophoresis of TSL revealed four bands of 20 000, 40 000, 60 000 and 80 000 Da, while the reducing SDS/polyacrylamide gel electrophoresis only showed one band of 20 000 Da [11]. The molecular mass of TSL chain measured by mass spectrometry was 17 924.1 Da (Fig. 1) and 18 494.9 Da for reduced TSL and CAM-TSL, respectively. The increase of 570.7 Da was consistent with the ten Cys residues in TSL. However, the

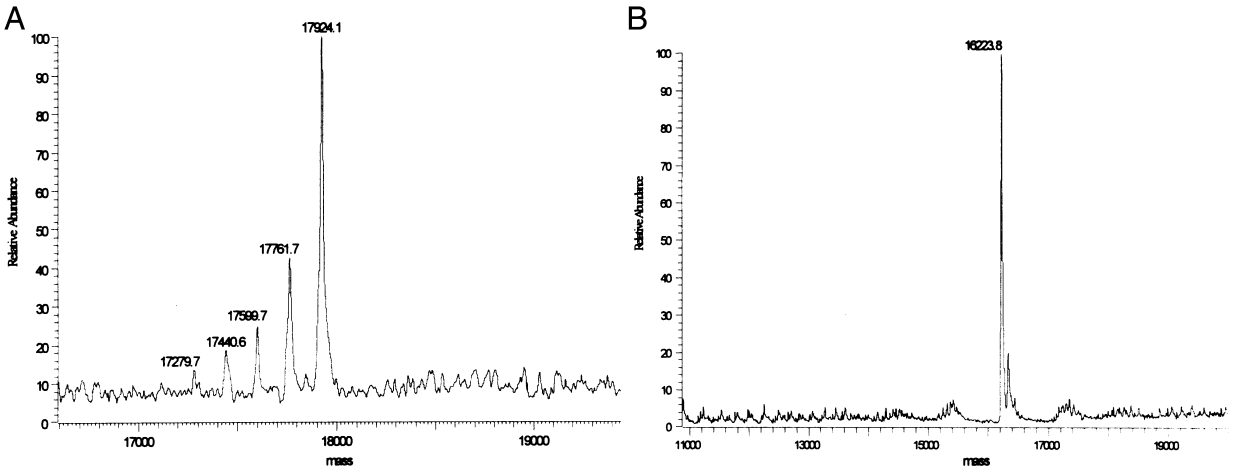

Fig. 1. Mass spectra of intact TSL chain. (A) The observed mass of reduced TSL is revealed. Heterogeneity resulted from the different length of glycan linked to the protein. (B) The measured mass of reduced TSL removal of glycan is indicated: the mass deceased by 1700.3 Da, compatible with the mass of glycan on the protein.

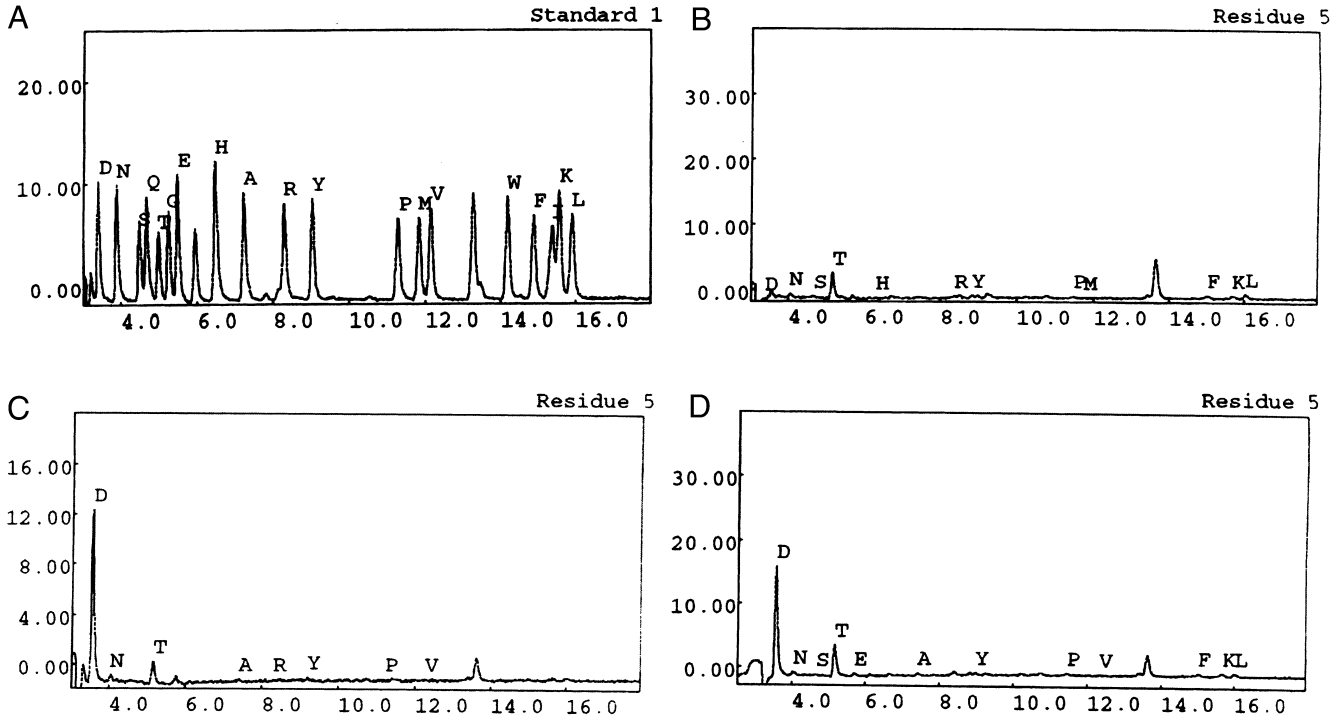

Fig. 2. N-terminal sequencing of the fifth residue of TSL. (A) The N-terminal sequencing standard. (B) The result of native TSL: the fifth residue could not be obtained as a result of glycosylation. (C) and (D) The fifth residue of CAM-TSL and native TSL, respectively, after removal of the glycan. Asn-glycan was converted to Asp by deglycosylation.

observed mass was not consistent with the value calculated from the protein sequence. Furthermore, the observed mass of reduced TSL had obvious heterogeneity, there were several substances with mass less than that of the main TSL peak, and the two neighboring peaks differed by about 162 Da from each other. The molecular mass of TSL was reduced to 16 223.8 Da after *N*-glycosidase F digestion, with a difference of 1700.3 Da, consistent with the predicted mass of an oligosaccharide chain with five hexose residues attached to the core oligosaccharide structure of $(GlcNAc)_2$-$(Man)_3$. The heterogeneity resulted

Table 1. Tryptic peptides observed in the LC/MS analysis of CAM-TSL.

Tryptic peptide	Amino acid	Expected mass (Da)	Observed mass (Da)	Retention time (min)	Peptide sequence
T1[a]	1–16	3641.5	3642.0	36.81–38.69	SC^{c}C^{c}TN^{a}DSLPMNGMC^{c}YK
T1[a,b]	1–16	3657.5	3658.0	32.82	SC^{c}C^{c}TDN^{a}SLPM^{b}NGMC^{c}YK
T2	17–22	747.8	747.4	29.77	IFDEPK
T3	23–30	1028.1	1027.3	50.23	TWEDAEMF
T4	23–33	1344.3	1344.3	46.36	TWEDAEMFC^{c}R
T5	34–45	1472.6	1472.0	34.03	YKPGC^{c}HLASFHR
T6	46–55	1123.2	1122.8	47.89	AESLDIAEYI
T7	46–62	2023.2	2022.6	55.65	LAESLDIAEYISDYHKR
T8	63–73	1299.4	1299.2	58.45	QAEVWIGLLDR
T9	75–84	1369.5	1369.2	48.53	KDFSWEWTDR
T10	76–84	1241.3	1240.8	51.75	DFSWEWTDR
T11	85–94	1301.3	1301.7	43.45	SC^{c}TDYLNWDK
T12	85–101	2184.3	2183.6	42.37	SC^{c}TDYLNWDKNQPDHYK
T13	85–106	4119.4	4118.8	57.22	SC^{c}TDYLNWDKNQPDHYKDKEFC^{c}VELVSLTGYHR
T14	85–103	2427.5	2427.3	40.83	SC^{c}TDYLNWDKNQPDHYKDK
T15	95–117	2836.1	2836.4	53.13	NQPDHYKDKEFC^{c}VELVSLTGYHR
T16	102–117	1953.1	1952.8	54.69	DKEFC^{c}VELVSLTGYHR
T17	104–117	1709.9	1709.4	56.12	EFC^{c}VELVSLTGYHR
T18	118–126	1165.2	1164.6	28.40	WNDQVC^{c}ESK
T19	127–134	1056.1	1055.8	33.11	NSFLC^{c}QC^{c}K
T20	127–135	1203.3	1203.0	45.75	NSFLC^{c}QC^{c}KF

[a] Indicates the glycopeptides; [b] shows the peptides with oxidized Met; [c] marks the carboxyamidomethylated Cys.

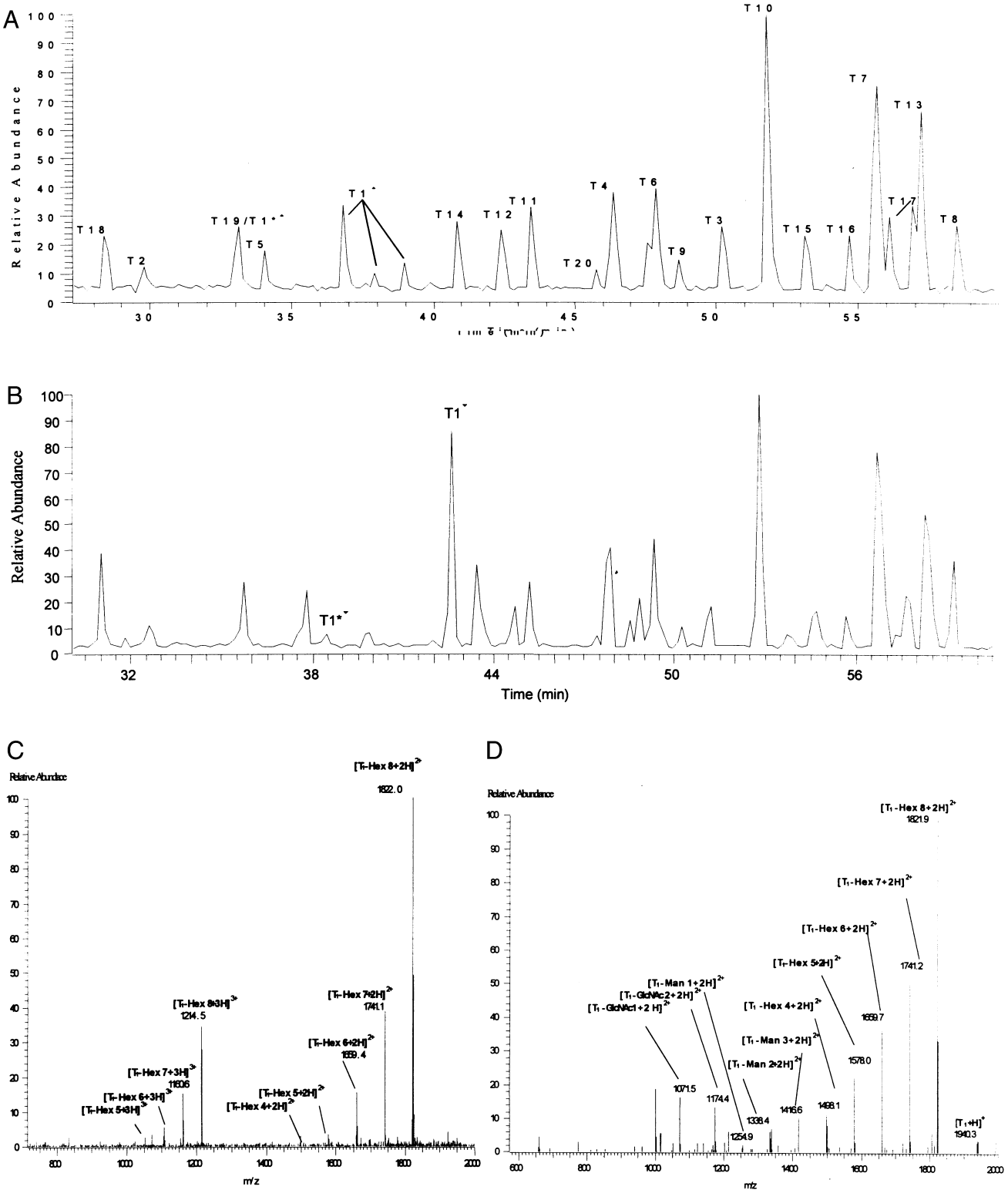

Fig. 3. TIC and mass spectra of tryptic digest of CAM-TSL before and after *N*-glycosidase F treatment. (A) and (B) TIC maps of tryptic digest of CAM-TSL before and after deglycosylation, respectively. Peaks marked with (▲) are the glycopeptides, and peaks marked with (▼) are the peptides removal of glycan. The peptides with oxidized Met are marked with (*); (C) and (E) show the mass spectra of glycopeptides and nonglycosylated peptides; (D) and (F) show the MS/MS spectra of the 2+ charged ions of glycopeptide (*m/z* 1822.0) and nonglycosylated peptide (*m/z* 970.9), respectively; (G) and (H) show the MS and MS/MS spectra of peptide with oxidized Met and without glycan; (*) indicates the peptide and fragments with 16 Da mass addition resulting from oxidized-Met.

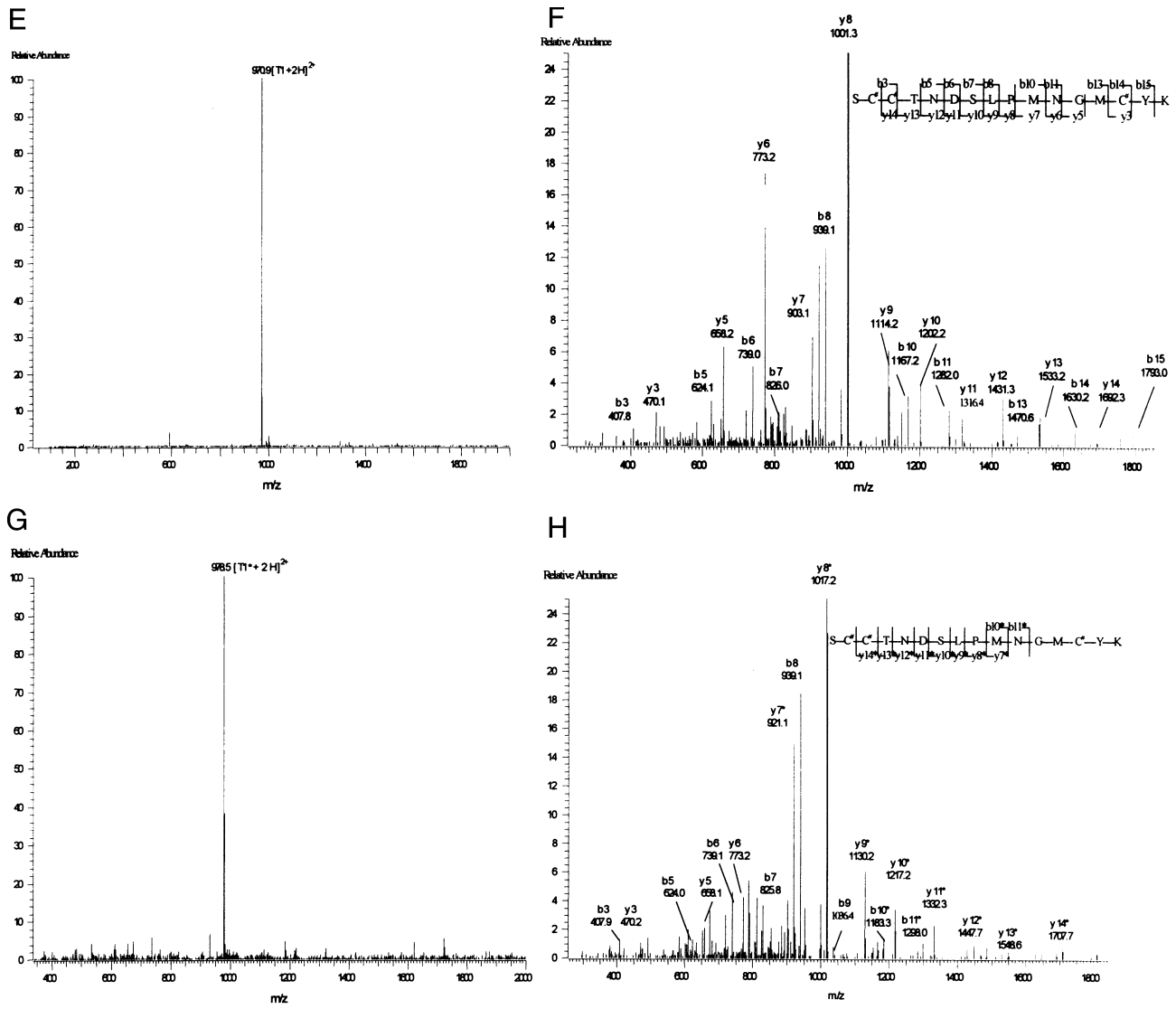

Fig. 3. continued.

from the addition of one to five hexose residues to the core structure, corresponding to the five peaks in the mass of reduced TSL.

N-terminal sequencing of TSL before and after *N*-glycosidase F treatment

The N-terminal sequence of CAM-TSL was obtained, except for the fifth residue, by Edman degradation. The fifth residue as shown by the cDNA sequence was Asn, indicating that the TSL contained a consensus sequence of N-linked glycosylation: Asn-X-Ser [14,15]. After being treated by *N*-glycosidase F [16] the fifth residue was determined as Asp.

Figure 2 shows the N-terminal sequences before and after *N*-glycosidase F treatment. The N-terminal sequencing revealed that both native TSL and CAM-TSL could be deglycosylated by *N*-glycosidase F, indicating that native TSL was sensitive to the enzyme. The region of TSL with glycan might be exposed and easily accessible to the enzyme.

Table 2. Relative abundance of different glycosylated isoforms in glycopeptides of CAM-TSL.

Glycopeptide	Relative abundance (%)
T1-Hex8	61.30
T1-Hex7	25.97
T1-Hex6	10.43
T1-Hex5	1.55
T1-Hex4	0.74

MS determination of tryptic digest of CAM-TSL before and after *N*-glycanase F treatment

Tryptic digestion of CAM-TSL was carried out to obtain more detailed information on the molecule. Figure 3A shows the reconstructed total ion current (TIC) map of the tryptic digest separated by HPLC. The MS of tryptic digest confirmed the protein sequence deduced from cDNA except that the N-terminal peptide from residues 1–16 (T1) contained an N-linked glycosylation site. In Fig. 3A, three peaks marked with (▲) could not be interpreted by the amino acid sequence. On the other hand, in the TIC map of the tryptic

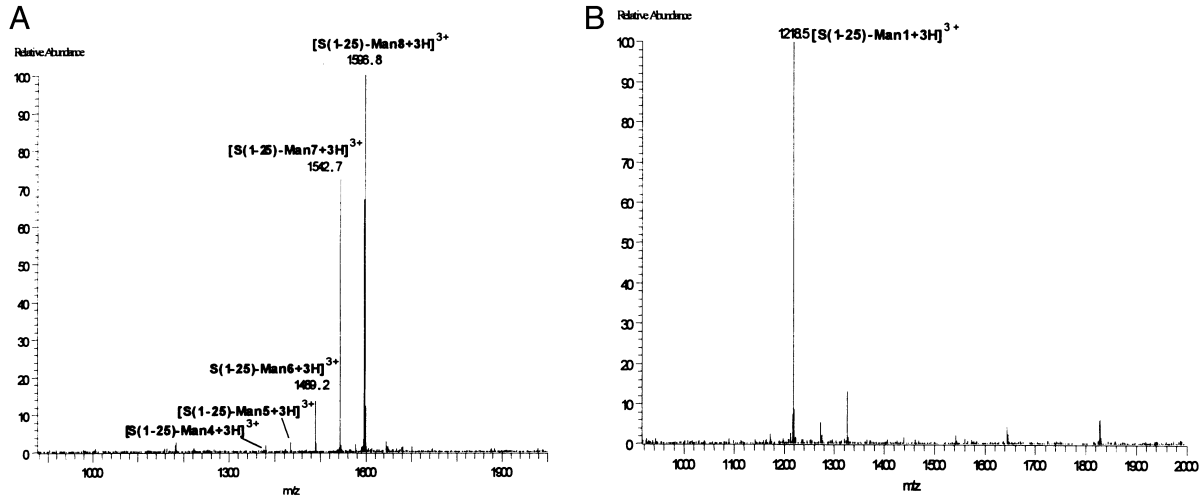

Fig. 4. Mass spectra of Glu-C glycopeptides with or without treatment of α-mannosidase. Mass spectra of glycopeptides: (A) with glycan Man_8-Man_4; (B) treated with α-mannosidase for 22 h, resulting in removal of all the mannose except the β-mannose linked to the GlcNAc in the core.

digest after removal of glycan (Fig. 3B), these three peaks disappeared or weakened and a new peak marked with (▼) was detected. Mass spectra of the new peak show only one signal of a 2+ charged ion at *m/z* 970.9 (Fig. 3E) with a mass of 1939.8 Da, consistent with the calculated mass of T1 after conversion of glyco-Asn5 to Asp5 by *N*-glycosidase F. MS/MS of the 2+ charged ion (Fig. 3F) further confirmed the amino acid sequence. Therefore, the three peaks in the tryptic digest were recognized as glycopeptides derived from N-terminal peptide T1. Mass spectra of the glycopeptides demonstrated two series of heterogeneous signals. Figure 3C shows two clusters of ions, corresponding to the observed mass of the 2+ and 3+ charge state. Each cluster consisted of five peaks with *m/z* values of neighboring peaks that differed by about 81 Da (from 1497.9 to 1822.0 Da) and 54 Da (from 1023.5 to 1214.5 Da) in the clusters of 2+ and 3+ charged ions, respectively. This indicated that microheterogeneity may be due to the number of hexose residues ($2 \times 81 = 162$, $3 \times 54 = 162$, residue weight for hexose) in the N-linked carbohydrate of T1. Because only hexose was found outside the core structure, the glycoform was assumed to be high-mannose type, instead of complex or hybrid type. The ion at *m/z* 1822.0 Da was determined as a 2+ charge state by ZoomScan, with a mass of 3641.9 Da, 1702.9 Da higher than that of the nonglycosylated peptide, indicating a $GlcNAc_2$-Man_8 glycoform. The heterogeneity of the glycopeptide was attributed to the different number of hexose residues attached to the same peptide ranging from $GlcNAc_2$-Man_4 to $GlcNAc_2$-Man_8. The MS/MS of ion *m/z* 1822.0 and the two charged ions of peptide with $GlcNAc_2$-Man_8, represented the glycan chain fragments linked to the peptide (Fig. 3D). The tryptic peptides of TSL detected by LC/MS are listed in Table 1. The relative amounts of oligosaccharide structures were estimated from the ion intensity of the glycopeptides as reported [17] (Table 2).

However, the mass of reduced nonglycosylated protein measured by MS was 16 223.8 Da (Fig. 1B), being 16.5 Da higher than expected (16 207.3 Da). It was assumed that one of the three Met might be oxidized as reported previously [18]. In the tryptic digest, an additional peptide with an increase of 16 Da of the glycopeptide was detected (Fig. 3G). Removal of glycan afforded the peptide with an oxidized Met. MS and MS/MS revealed that Met10 was oxidized (Fig. 3H). The fragments containing only Met10 and those containing both Met10 and Met 14 showed an increase in mass of 16 Da, compared to the Met-unoxidized peptide, while there was no such increase in the fragments only containing Met14. The oxidation was partial as peptides with and without oxidized Met were detected. Further work is being carried out to elucidate the function of the oxidized Met.

MS determination of Glu-C digest of CAM-TSL before and after α-mannosidase treatment

To further demonstrate the high-mannose type glycochain, α-mannosidase was used to cleave the glycan chain. Therefore, if the glycan chain is of high-mannose type, all the mannose can be removed by α-mannosidase except the β-mannose linked to GlcNAc in the core. Because the optimum pH for trypsin digestion is 7.5–8.5, which is not suitable for α-mannosidase (usually used at pH 4.0–5.0), we used Glu-C to digest CAM-TSL at the C-terminal of Glu at pH 4.0 before α-mannosidase treatment. The glycopeptides 1–25 of Glu-C digest had observed masses of 4787.4 Da to 4138.2 Da, indicating peptides with glycan chains of Man_8-Man_4 (Fig. 4A). After being treated by α-mannosidase for 22 h, the observed mass of the glycopeptide was 3652.2 Da (Fig. 4B), in accordance with a calculated mass (3652.3 Da) of glycopeptide with two GlcNAc and one mannose (Man_1) attached. This result confirms that the glycochains of TSL belong to the high-mannose type.

DISCUSSION

On-line HPLC-ESI-MS allows the identification of the glycopeptides eluted from the HPLC. Therefore the glycopeptide could be identified by comparing the ion current of MS of the protease digest and the proteolytic digest before and after deglycosylation. In general, the retention time of nonglycopeptides in reverse-phase HPLC would be longer than that of the glycopepides because the peptides become more hydrophobic when the glycan is removed. The selected ion monitor reported elsewhere was not used to find the glycopeptides as it may give false glycopeptide-positive signals [18,19].

Previous reports usually isolated the glycopeptides resulting from proteolytic digestion [20,21], before the glycan was cleaved with glycanase. In our method, no isolation was needed

before deglycosylation, if compatible pH conditions were used to perform protein and glycan digestions.

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