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Tandem Mass Spectrometric Analysis of Glycopeptides Derived from the Tryptic Digestion of Human Chorionic Gonadotropin (hCG)

Lay-Harn Gam [1, *] **and Aishah Latiff** [2]
[1] School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.
* layharn@usm.my. Telephone (+ 604) 6533888 extention 2208, Facsimile (+ 604) 6570017.
[2] Doping Control Center, University Sains Malaysia, 11800 Penang, Malaysia.

ABSTRACT Analysis of hCG glycopeptides by ion trap mass spectrometer is difficult due to ion suppression effect attributed to the sugar components of glycopeptides. In addition, collision induced dissociation of glycopeptides also could not provide useful product ion spectrum. In our present study, determination of amino acid sequences of hCG glycopeptides was accomplished by enzymatic deglycosylation prior to tandem mass spectrometric analysis. Enzymatic deglycosylation was found more effective when performed on short stranded tryptic fragments compared to its intact molecule. Deglycosylated-peptides display greater resistance to collision induced dissociation compared to peptides, which revealed higher collision energy required for fragmentation.

ABSTRAK Penganalisaan glikopeptida daripada hCG dengan menggunakan spectrometer jisim perangkap ion adalah sukar disebabkan kesan penurunan ion oleh komponen gula bagi glikopeptida. Tambahan lagi, penceraian langgaran teraruh bagi glikopeptida juga tidak dapat memberikan spektrum ion produk yang berguna. Berdasarkan kajian terbaru, penghitungan turutan asid amino bagi glikopeptida hCG boleh dijayakan dengan pendeglikosilan secara berenzim dilakukan sebelum analisis tandem spectrometer jisim. Pendeglikosilan secara berenzim keatas potongan triptik yang pendek didapati lebih berkesan daripada pendeglykosilan keatas molekul sempurna. Peptida terdeglikosilan menunjukan rintangan yang lebih terhadap penceraian langgaran teraruh berbanding peptida, yang mana tenaga langgaran yang lebih tinggi diperlukan untuk frakmentasinya.

(Glycopeptide, Deglycosylated-peptide, Tandem Mass Spectrometry, Human Chorionic Gonadotropin).

Subtitle: Mass spectrometric analysis of hCG glycopeptides.

Nonstandard Abrreviation: hCG, Human chorionic gonadotropin; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Parent ion, molecular ion; TFA, triflouroacetic acid; ACN, acetonitrile.

INTRODUCTION

Human chorionic gonadotropin is composed of two dissimilar subunits, α- and -β-. The approximate molecular weights of the subunits are 14.5 kDa and 22.2 kDa, respectively [1]. Carbohydrate consists of 30% of the total weight of hCG, which comprises of 10 - 11% of neutral sugar, 10 - 11% amino sugar and 8 - 9% of sialic acids [2]. There are mainly two types of carbohydrate linkages on hCG, namely N-linked oligosaccharide [3] and O-linked oligosaccharide [4]. Tsuguo and Akira [5] reported that different types of O-linked and N-linked sugar chains are present in hCG. The sugar content of hCG was accounted for the heterogeneous property of hCG [7].

The N-linked oligosaccharide involves the well-established 'Asn-X-Thr' pattern, where X represents Val, His, Ala or Thr. This pattern is apparently recognized by the carbohydrate transferase that adds acetyglucosamine to asparagine residues. These large carbohydrate moieties are attached to the 52^{th} and 78^{th} amino acid residues of the α-subunit as well as the 13^{th} and 30^{th} amino acid residues of the β-subunit [6]. The O-linked oligosaccharide is made up of a less

complex carbohydrate side chain that is attached to the hydroxyl group of the serine residues. These carbohydrate moieties are found at residues 121, 127, 132 and 138 of the C-terminus of hCG β-subunit [6].

Liu and Bowers (1996) have reported that detection of intact or subunits of hCG using MS analysis was not possible [7]. Therefore, in this study, we have subjected the hCG to digestion with trypsin prior to MS analysis. Seven glycopeptides were expected to form from the digestion. However, only two of the glycopeptides were detected in the LC/MS/MS analysis. The failure to detect other glycopeptides may be explained by the ion suppression effect caused by the sugar moieties of the glycopeptides. In order to detect these glycopeptides, the sugar components of the glycopeptides were removed enzymatically, the removal of the sugar component resulted in the successful detection of the deglycosylated peptides. Furthermore, we were able to confirm the amino acid sequences of the deglycosylated peptides via tandem mass spectrometric method. In this study, we reported the identification and confirmation of the amino acid sequence of 6 glycopeptides of hCG using ESI-ion trap mass spectrometer.

MATERIALS AND METHODS

Chemicals

Human chorionic gonadotropin (Lot CG 10) prepared from pooled pregnancy urine; guanidine HCl; Tris base and TPCK treated bovine pancreas trypsin were purchased from Sigma (St Louise, MO, USA), dithiotreitol; iodoacetic acid; trifluoroacetic acid, acetic acid and ammonium bicarbonate were obtained from Fluka (Buchs, Switzerland), sodium hydroxide from J.T. Baker (Phillipsburg, NJ, USA), Deglycosylation enzymatic kit was purchased from Bio-Rad (Hercules, CA, USA). HPLC grade acetonitrile and methanol were purchased from Fisher Chemical (Pittsburgh, PA, USA). Ultra pure H_20 collected from ELGA deioniser (Bucks, England, UK) at 18.2MΩ was used throughout the experiment.

Equipments

Ion trap mass spectrometer, LCQ Classic, Finnigan MAT (San Jose, CA, USA); HPLC, binary pump and degasser, series 1100, Agilent Technology (Waldbronn, Germany); incubation oven, UBS, Memmert (Schwabach, Germany); freeze dryer, Christ LDC-2, B. Braun (Osterode am Harz, Germany), syringe pump, A-9606, Harvard Apparatus (Halliston, MA, Canada),

Preparation of deglycosylated hCG

Five microgram of hCG was subjected to trypsin digestion according to method described in Gam *et al.* (2003) [8]. The digested tryptic-fragments were dried and dissolved in 12 µl of distilled water. Four microliters of reaction buffer (250 mM sodium phosphate, pH 6) was added, followed by 2 µl of NANase II and 2 µl of O-glycosidase. The mixture was then incubated at 37°C for 1 hour, after which 10 µl of distilled water and 10 µl of the pH-adjustment buffer (0.5 M sodium phosphate dibasic) were added. Finally, 2 µl of PNGase F was added to the reaction mixture. The deglycosylation reaction was allowed to take place at 37°C for 24 hours. The deglycosylated glycopeptides were then lyophilized and stored at -20°C.

HPLC separation

The deglycosylated-peptides mixture was reconstituted in 50 µl of deionized distilled H_2O and 2 µl was injected to an RPC-column (C_{18} 300 Å, 5 µm, 1 mm X 150 mm, Vydac) connected to a Hewlett Packard HPLC (series 1100). The flow rate was set at 1 ml/min and then split by a high pressure splitter to 20 µl/min through-column flow rate. The gradient used was 10 - 60% B for 60 minutes and held constant at 60% B for 5 minutes. Mobile phase A was 0.05% TFA in H_2O (Maxima ultra pure water, ELGA) and mobile phase B was 0.05% TFA in ACN. The HPLC was interfaced to an ion trap mass spectrometer (ThermoQuest).

Mass Spectrometry

The parameters for enquiring MS data was as follows; heated capillary temperature was 200°C, sheath gas flow rate was 60 arb, spray voltage was 4 kV, tube lens offset was -60 V and the capillary voltage was 38 V. Data dependent MS/MS scans were created for the analysis of tryptic glycopeptides of hCG. Predicted precursor ions were programmed to the precursor ion list. The parameters set for data dependent scan were: default charge state was 2, minimum

signal acquired was 5 x 10^{-4} counts, and the isolation width was 2 m/z.

The masses of the deglycosylated glycopeptides and product ions were calculated using PAWS program (version 8.5, freeware edition).

RESULTS AND DISCUSSION

Mass spectrometric analysis of the glycopeptides

Each hCG molecule comprises of 8 glycosylation sites; two at the α-subunit whilst 6 at the β-subunit. Digestion of hCG with trypsin will result in the formation of 7 glycopeptides fragments. Six of the glycopeptides contained single carbohydrate attachment site and one contained two glycosylation sites. The hCG glycopeptides were designated as αT6, αT9, βT3, βT4, βT13, βT14 and βT15, where βT14 contained two glycosylation sites. Table 1 shows the amino acid sequences of the hCG glycopeptides.

Figure 1 shows the mass spectrometric analysis of the hCG glycopeptides, two of the glycopeptides (βT3 and βT4) were detected,. Both the glycopeptides were co-eluted in one chromatographic peak. The multiple m/z precursor ions of βT3 glycopeptide revealed the multiple glycoforms of this glycopeptide. However, a single m/z precursor ion of βT4 showed its single glycoform. The detection of the multiple glycoforms is common in glycopeptides due to their oligosaccharide heterogeneity. Besides, the difference in sialic acid contents [3], [4], the oxidation of methionine amino acid residues [9] and the different carbohydrate moieties that attached to the common glycosylation sites [10] are other factors that contribute to the heterogeneity of hCG.

The multiple glycoforms of βT3 observed were βT3N1, βT3N3, βT3N4 and βT3N5, where N1 to N5 refer to the different N-linked oligosaccharides [10]. In addition, βT3N6 and βT3N7 glycoforms were also detected in a separate experiment (data not shown). The βT4 glycoform detected was identified as βT4N2. The glycopeptides were ionized mainly to triply-charged ions whilst the doubly-charged ions of some of the glycoforms were also detected but at lower ion intensity.

The precursor ions of the two glycopeptides (βT3 and βT4) were subjected to MS/MS scan. The product ions of the glycopeptides existed as irregular spiked signals, which are not useful for indicating of sequence. Therefore, interpretable MS/MS spectra cannot be obtained. Neither increasing nor reducing collision energy brought improvement to the spectra.

Table 1. Predicted glycopeptides derived from the tryptic digestion of hCG

GLYCOPEPTIDE	AMINO ACID RESIDUES	AMINO ACID SEQUENCE	$[M+H]^+$ AFTER SUGAR WAS REMOVED
αT6	α-subunit; 52-63	[a]NgVTSESTCCVAK	1357.6
αT9	α-subunit; 76-91	[a]VENgHTACHCSTCYYHK	2069.7
βT3	β-subunit; 9-20	[a]CPINgATLAVEK	1372.8
βT4	β-subunit; 21-43	[a]EGCPVCITVNgTTICAGYCPTMTR	2665.3
βT13	β-subunit; 115-122	[b]FQDSSSSgK	885.4
βT14	β-subunit; 123-133	[b]APPPSgLPSPSgR	1105.6
βT15	β-subunit; 134-145	[b]LPGPSgDTPILPQ	1234.7

[a] shows the N-link glycopeptide
[b] shows the O-link glycopeptide
g represents the sugars' attachment sites either at Arginine (N) or Serine (S) residues.

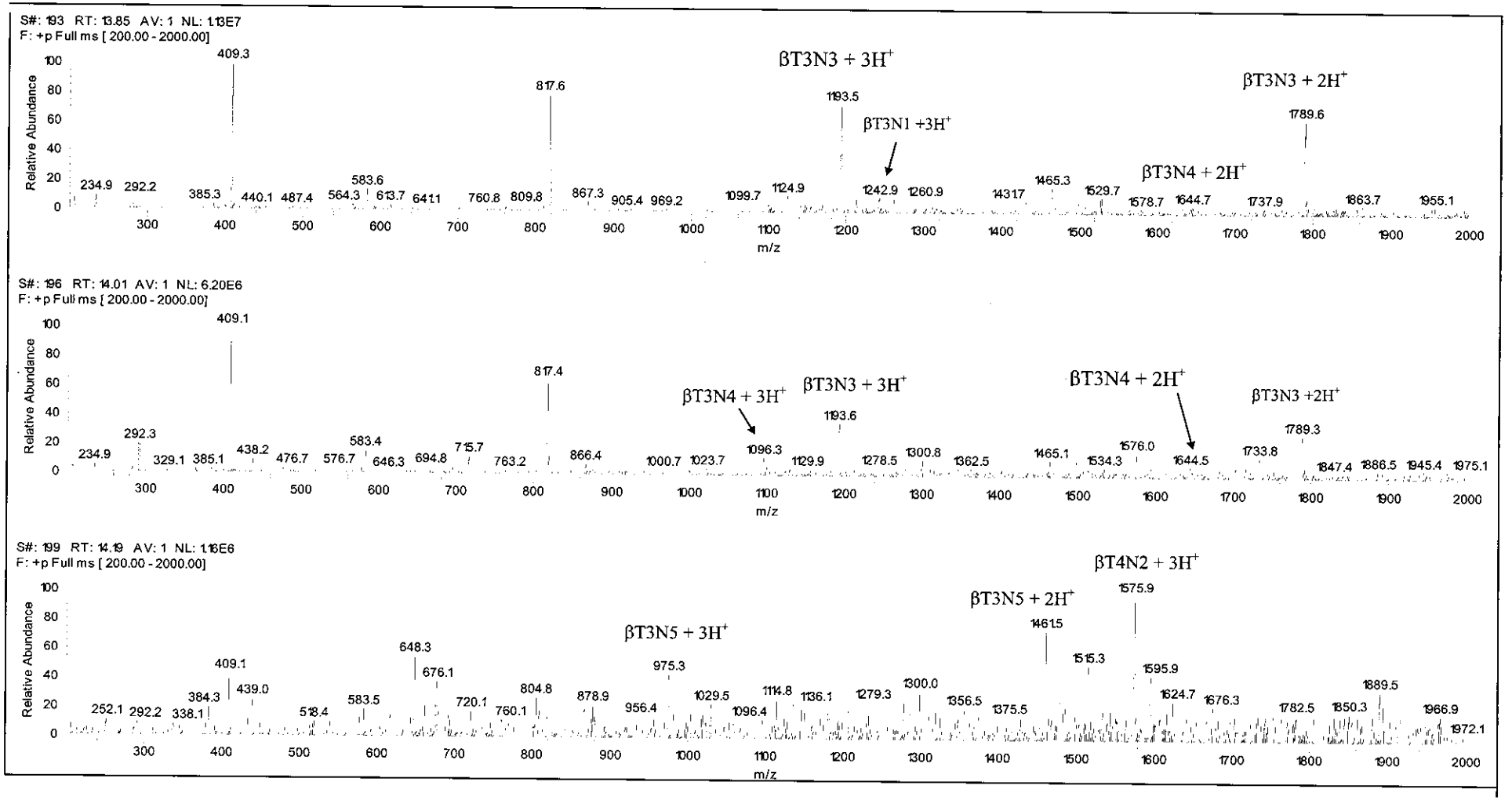

Figure 1. Mass spectrum of glycopeptides detected: Doubly and triply charged parent ion of βT3N3, 1789.6 and 1193.5 respectively. Doubly and triply charged ion of βT3N4, 1644.5 and 1096.2 respectively. Doubly and triply charged ion of βT3N5, 1461.5 and 975.3 respectively. Triply charged ion of βT3N1, 1242.9. Triply charged ion of βT4N2, 1575.8. (note: 409.3 and 817.4 are the parent ion for αT5 peptide, which was co-eluted with the glycopeptide).

Mass spectrometric analysis of deglycosylated-peptides

Sequential enzymatic cleavage of carbohydrates was carried out to remove the sugar moieties from the intact molecules of hCG prior to digestion with trypsin. The resulting fragments were then subjected to tandem mass spectrometric analysis. Only two of the deglycosylated-peptides, βT3 and αT6 were detected and their identities were confirmed by their respective product ion spectrum. We have increased the concentration of deglycosylation enzymes in order to improve deglycosylation of the glycopeptides, nevertheless, the number of the deglycosylated-peptides detected remain the same. This observation helps us to deduce the possibility of complex folding of the glycoprotein that hinders the accessibility of the enzymes to the sugar attachment sites.

An alternative experiment was conducted to improve the deglycosylation of glycopeptides. Whereby the enzymatic deglycosylation was performed on the short stranded tryptic-digested fragments of hCG instead of its intact molecules. This alternate experiment increased the number of the deglycosylated-peptides detected from 2 to 6. The deglycosylated-peptides were found to fragment at different collision energy. At 25 collision energy, only αT6 and βT3 were fragment in the predictable manner to generate interpretable MS/MS spectra. Whilst the MS/MS spectra for other deglycosylated-peptides were appeared as spiking signals and therefore cannot be interpreted. When the collision energy was increased to 35, interpretable MS/MS spectra for βT4 and βT13 were obtained. Further increases in collision energy to 40 resulted in the generating of interpretable MS/MS spectra for βT14 and αT9 deglycosylated-peptides. Using this approach, we were able to confirm the deglycosylated-peptides sequences.

The data of our experiments indicate that fragmentation of deglycosylated-peptides were harder to perform compared to the hCG peptides, which were fragment at collision energy 25. With regards to their chemical composition, there is no obvious chemical rationale for the difference in the fragmentation energy required for peptides and deglycosylated-peptides. The fragment ions profiles for the 6 deglycosylated-peptides reveal that the asparagine's and serine's peptide bonds were able to fragment freely as in the peptides. Asparagine and serine are the residues that bind to sugars. Moreover, the full scan MS spectra for deglycosylated-peptides shows that they were ionized mainly to doubly-charged ions, a phenomenon that is similar to peptides. Thus, the rationale for the higher collision energy needed to fragment deglycosylated-peptides is not clear. Figures 2, 3 and 4 show the examples of MS/MS spectra of three deglycosylated-peptides that were fragment at different collision energy.

The attempt to detect βT15 was not successful although collision energy was periodically increased from 40 to 50, no MS/MS spectrum that match βT15 sequence was observed. βT15 is derived from the C-terminus end of β-subunit, where the sequence was not clearly defined; hence this may lead to the discrepancy in βT15 precursor ion mass [11].

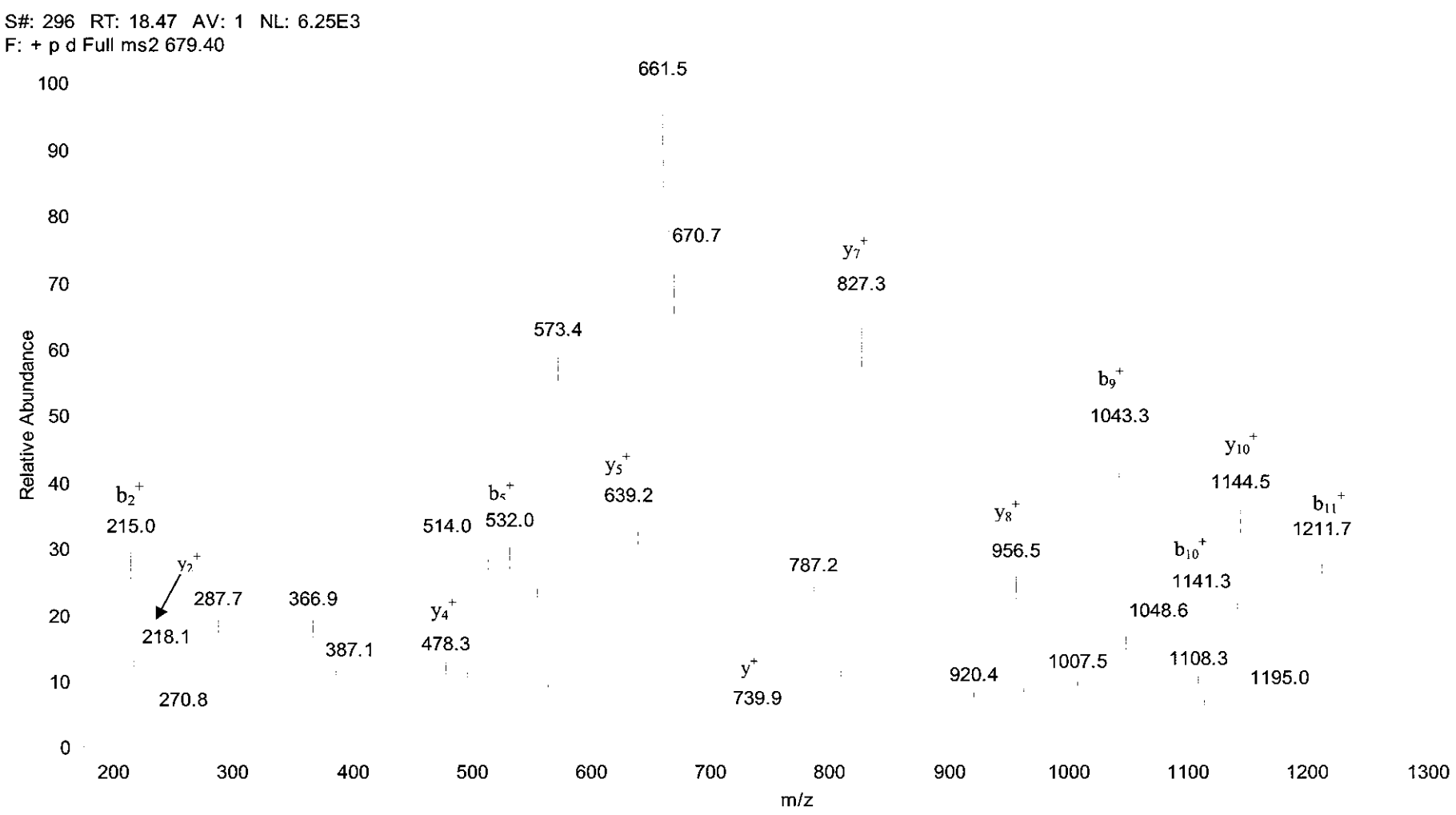

Figure 2. The Full scan MS/MS spectrum for the doubly charged ion of deglycosylated-peptide αT6. Fragmentation occurred at collision energy 25.

Figure 2. The Full scan MS/MS spectrum for the doubly charged ion of [illegible]

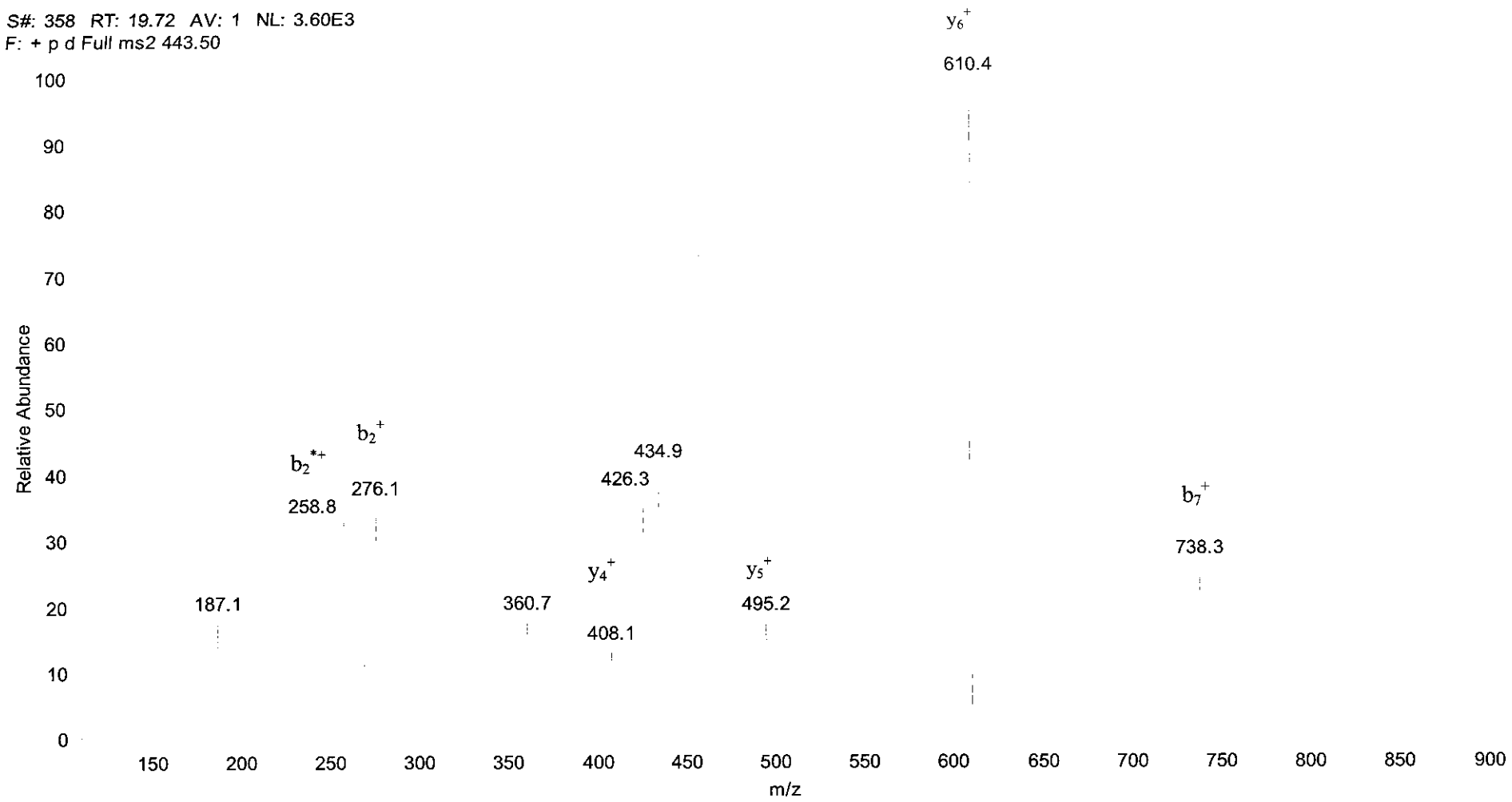

Figure 3. The Full scan MS/MS spectrum for the doubly charged ion of deglycosylated-peptide βT13. Fragmentation occurred at collision energy 35.

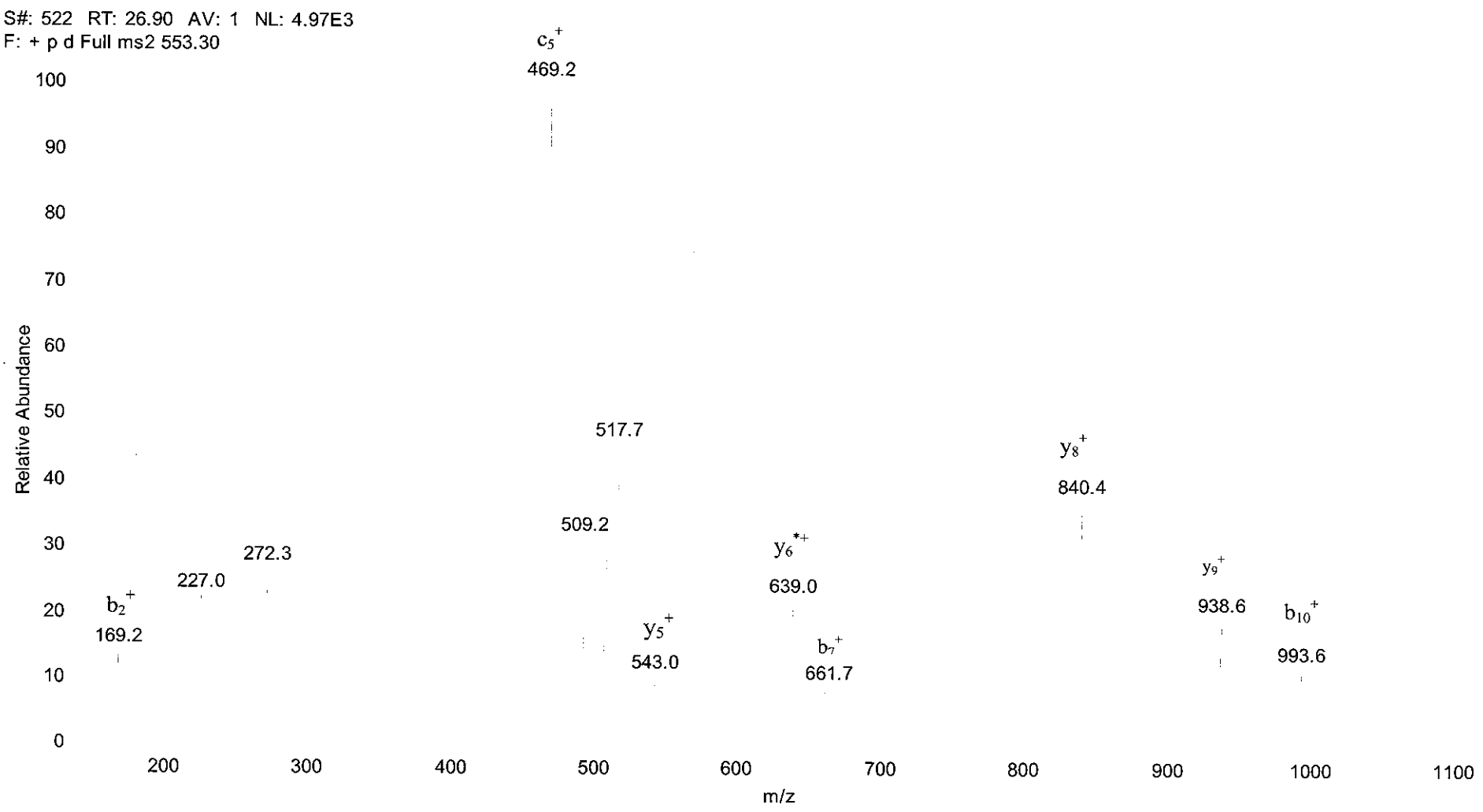

Figure 4. The Full scan MS/MS spectrum for the doubly charged ion of deglycosylated-peptide βT14. Fragmentation occurred at collision energy 40

CONCLUSION

The collision energy required to fragment most of the deglycosylated-peptides were found to be higher than those for peptides of hCG. Furthermore, the intensity of the collision energy required to fragment varies amongst the hCG deglycosylated-peptides. The rational for the phenomenon observed is not fully understood although it seems related to the glycosylation of the peptides.

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SERIES B

PHYSICAL SCIENCES