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Probing for Characteristics of Growth Hormone by LC-ESI-MS/MS

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Introduction

Growth hormone (GH, Figure 1) was identified as the growth-promoting principle of the human pituitary gland in 1956 [1], and with its isolation and preparation from hypophysis its structural heterogeneity became evident. Detailed investigations elucidated the actual reasons for numerous GH variants, which were found to originate from an alternative splice site at the mRNA level [2, 3], from post-translational modifications such as *N*-terminal acylation or deamidation [4], as well as from oligomerization [5-7]. In addition, artificial products such as scissions within the primary structure supposedly resulting from harsh extraction conditions or long-term storage have been identified [8, 9]. Several assays enabling the determination of GH levels as well as the potential misuse of recombinant preparations have been published primarily utilizing sophisticated immunoassays [6, 10-17]. In order to optimize immunoassays in terms of cross reactivity as well as specificity, the characterization of antigens and their natural as well as artificial variants is of paramount importance and has been subject of considerable research in the past. Objects of investigation have been natural monomers (e.g. 22 kDa and 20 kDa variants) [18, 19], covalent as well as non-covalent oligomers [6, 7, 20-24], and chemical, proteolytic or metabolic degradation products of growth hormones [9, 25].
In the present study, mass spectrometric investigations regarding quality and composition of recombinant as well as pituitary growth hormone preparations are described, providing unambiguous data on degradation after long-term storage and post-translational modification of GH samples.

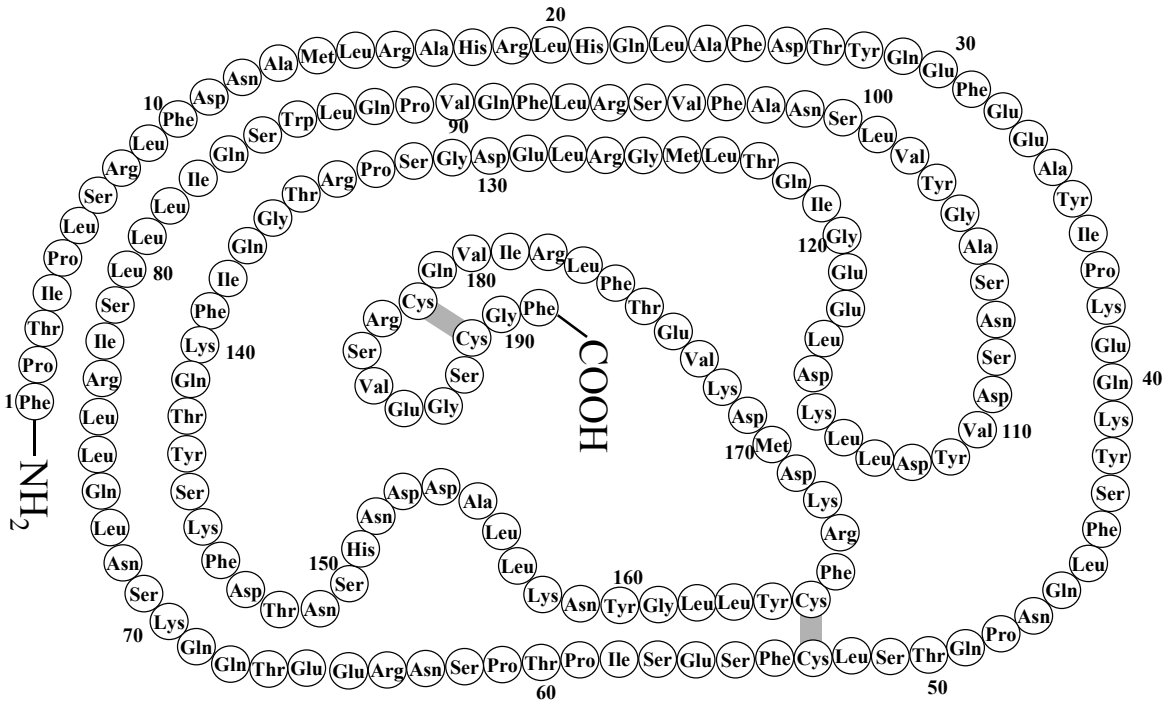


Figure 1: Primary structure of the 22 kDa isoform of human growth hormone consisting of 191 amino acids and two disulfide bonds between the cysteine residues 53 and 165 as well as 182 and 189.

Experimental

Chemicals and reagents. Recombinant human GH (Genotropin) was purchased from Pfizer (Karlsruhe, Germany). Pituitary GH was obtained from the National Institute for Biological Standards and Control (NIBSC 80/505, South Mimms, UK). NuPage mini gels (12% Bis-Tris, 1.0 mm x 10 well), NuPage LDS sample buffer, NuPage reducing agent and NuPage MOPS SDS running buffer were bought from Invitrogen (Carlsbad, CA). Tris(carboxyethyl)phospine (TCEP) and dithiothreitol (DTT) were purchased from Sigma (Steinheim, Germany). GelCode Blue stain reagent was obtained from Pierce (Rockford, IL).

Gel electrophoresis. 1D-gel electrophoresis was conducted on a Hoefer SE 260 gel electrophoresis using Invitrogen NuPage Bis-Tris (12%) pre-cast gels and MOPS SDS running buffer. Aliquots of 26 µL of aqueous samples containing 50 pmol of analyte were mixed with 10 µL of LDS running buffer and, in case of reducing conditions, with 4 µL of reducing agent and incubated at 70°C for 10 minutes. After cooling to ambient temperature, 20 µL were placed in each mini gel well, and gels were run at 200 V (constant) for 50 minutes. Staining was accomplished by gently shaking the gel in Coomassie GelCode blue staining reagent for two hours. Subsequently, gels were washed with deionized water for 1 hour to enhance the intensity of visualized bands.

Reduction of intact proteins. Aqueous solutions of GH were treated either with 10 mM TCEP or DTT for 10 minutes at 60°C.

Liquid chromatography-mass spectrometry. Liquid chromatography-mass spectrometry was performed on an Agilent 1100 Series capillary LC equipped with a Zorbax SB300 C18 column (0.3mm x 50mm, particle size 3 µm, pore size 300 Å). Solvents used were A: 0.1% acetic acid containing 0.01% TFA, and B: acetonitrile-water (80% acetonitrile/20% water) containing 0.1% acetic acid and 0.01% TFA. After loading of 5µL of sample onto a trapping conlumn (Zorbax SB300 C8, 1.0mm x 17mm) for 3 minutes, analytes were transferred onto the analytical column at a flow rate of 10 µL/min using a linear gradient from 90% A to 0% A in 15 minutes. The mass spectrometer was an Applied Biosystems Qtrap 4000 analyzer operated either in the full scan or product ion scan mode using the linear ion trap at a scan rate of 1000 u/s. The declustering potential was set to 50V and collision offset voltages varied according to the stability of selected precursor ions. Positive ionization was used for all analyses at a spray voltage of 5000V.

Results

In Figure 2a, the ESI mass spectrum of recombinant GH is shown consisting of a charge-envelope from $(M+12H)^{12+}$ to $(M+23H)^{23+}$. In comparison, Figure 2b represents the ESI spectrum generated from GH extracted from pituitaries. Here, two charge-envelopes are visible demonstrating the presence of the two most abundant GH variants at 22 kDa and 20 kDa as shown by means of deconvolution in the inset.

a)

b)

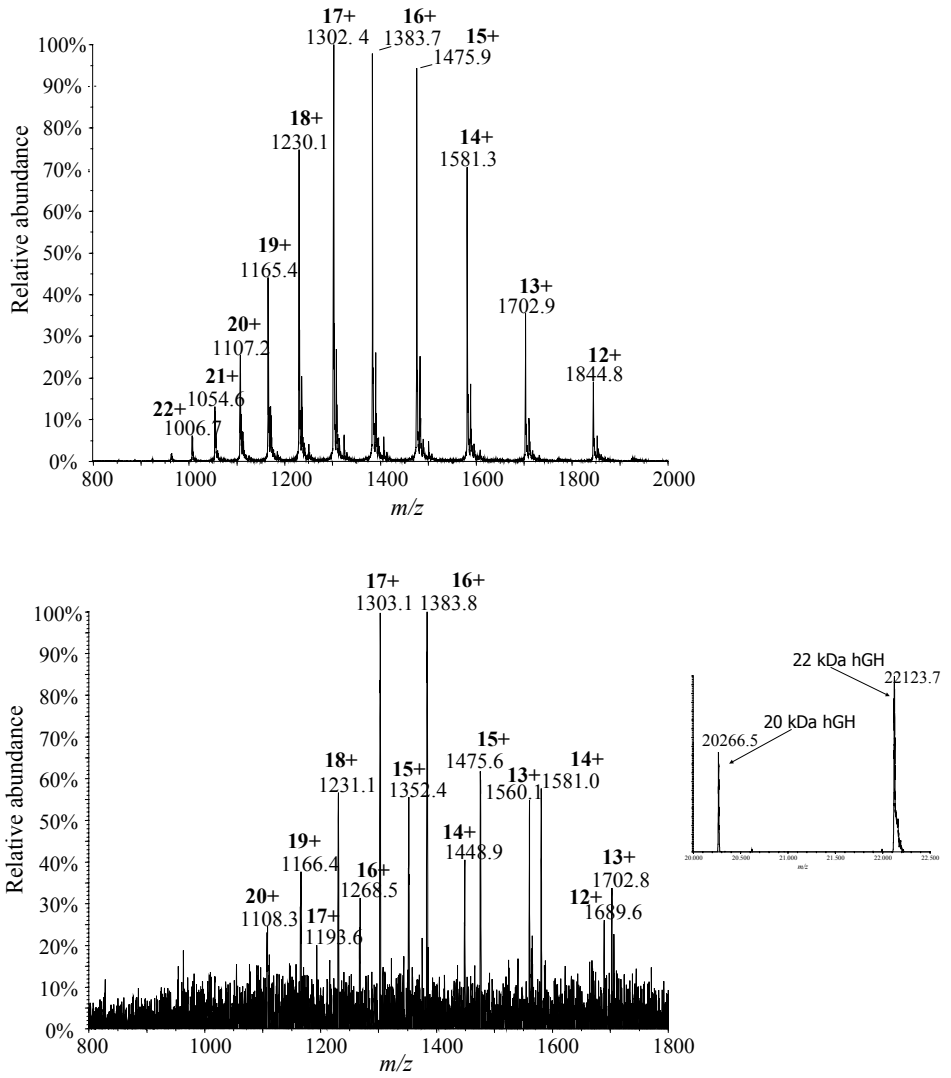


Figure 2: ESI spectra of a) recombinant GH (Genotropin) and b) GH from pituitary extracts recorded on an Applied Biosystems Qtrap 4000.

The long-term storage of hGH in solution at +4°C causes a considerable degradation as described in the literature. In Figure 3, a collage of 1D-gel electrophoreses is depicted prepared from fresh recombinant GH and pituitary GH after storage for 4 months in a refrigerator in aqueous solution.

The lanes A-B contain recombinant GH without cleavage of disulfide bonds while lanes C-D were prepared with disulfide reduction. In both cases only one band at 22 kDa was observed demonstrating that a freshly prepared solution of GH does not degrade under reducing conditions. Here, DTT as well as TCEP were employed generating identical results (data not shown). In contrast to recombinant GH, GH samples from hypophysis gave rise to several bands upon 1D-gel electrophoresis. Lanes E-F of Figure 3 were prepared without disulfide

reduction and numerous bands were assigned, the most abundant one of which is the 22 kDa variant of GH. The 20 kDa isoform was separated and visualized below the 22 kDa band, and above an intense but artificial “24 kDa” variant was observed. In addition, dimeric structures are shown at 45 kDa. The naturally occurring 24 kDa variant obtained by glycosylation of GH is usually low abundant, hence an artificial origin was assumed for the intense band shown in Figure 3 (E-F). The disulfide reduction of pituitary GH followed by gel electrophoresis gave rise to the lanes G-H that did not contain the “24 kDa” GH band but a new band at approximately 16 kDa. The phenomenon of a “24 kDa” GH has been described in the literature severalfold, and a sequence cleavage between phenylalanine 139 and lysine 140 of the 22 kDa GH isoform was assumed causing altered migration properties of the protein under 1D-gel electrophoresis conditions due to additional C- and N-termini. The presence of a disulfide bond between the cysteine residues 53 and 165 prevents a loss of the C-terminal peptide, but reduction of the protein results in an elimination of the liberated peptide.

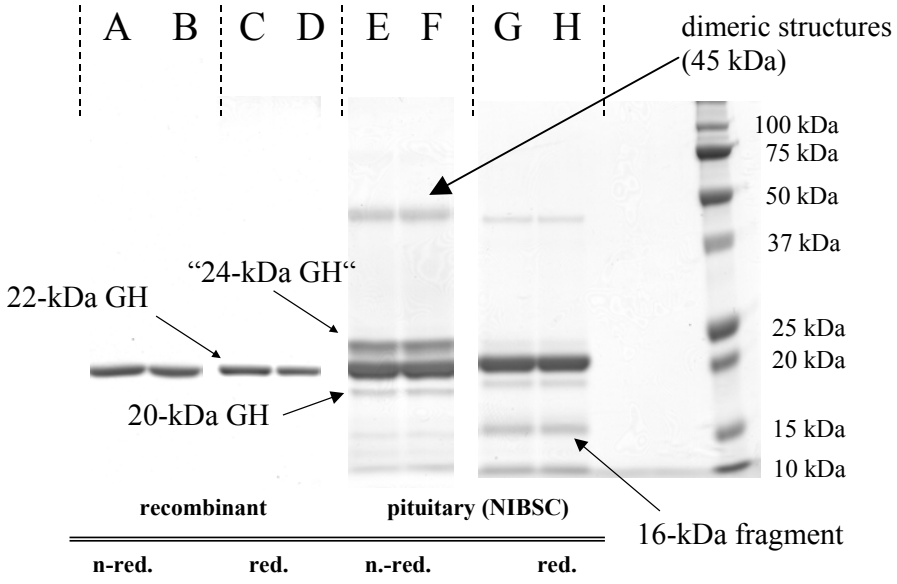
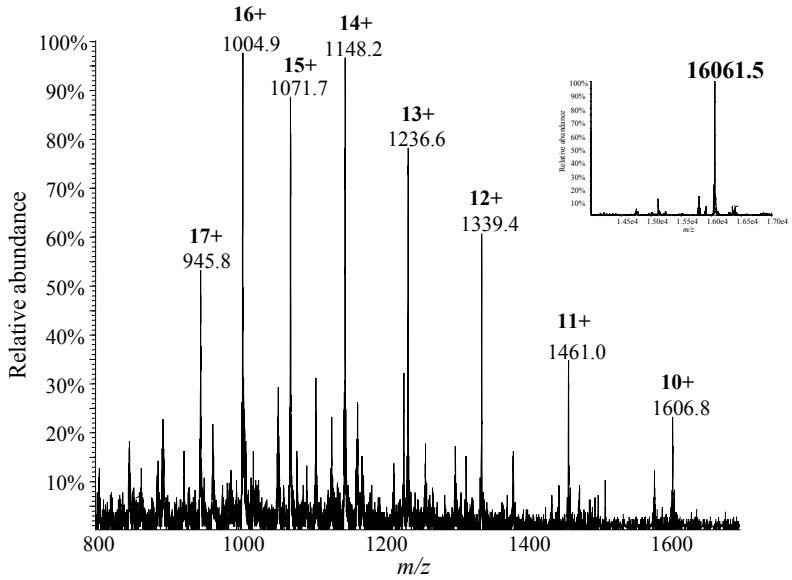


Figure 3: 1D-gel electrophoreses of recombinant and pituitary GH without (n-red.) and with (red.) disulfide reduction.

Mass spectrometric evidence for this proposal was obtained by analyzing the degraded and disulfide-reduced sample by LC-MS. In Figure 4, two mass spectra are shown representing a 16 kDa (a) as well as 6 kDa (b) fragment of GH, the measured masses of which are shown in respective insets obtained by deconvolution. Considering an additional water molecule due to the hydrolyzed peptide bond, the molecular masses are exactly matching a cleavage of the GH

primary structure between Phe139 and Lys140, substantiating the proposed degradation process. In addition to the determination of protein fragment masses, top-down as well as bottom-up sequencing provided unambiguous information on the origin of the investigated signals.

a)



b)

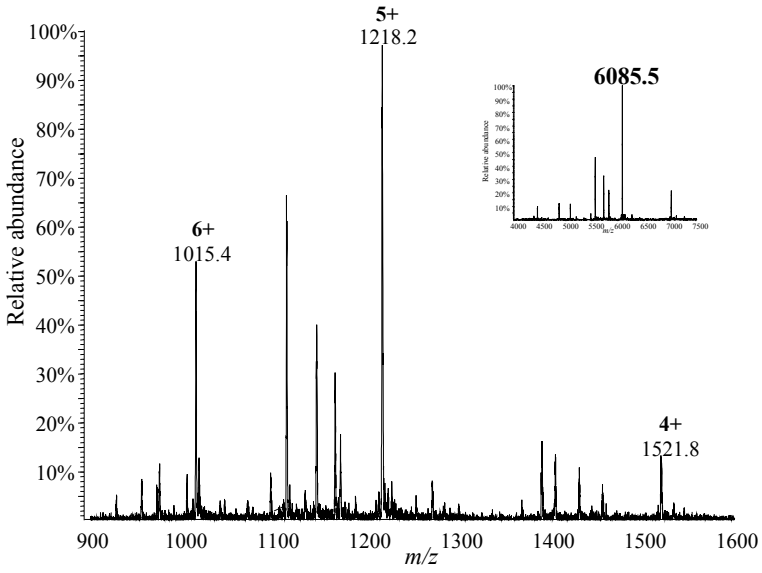


Figure 4: ESI-mass spectra of a 16 kDa fragment (a) and a 6 kDa fragment (b) obtained from degraded and disulfide-reduced pituitary GH. Masses of analytes were obtained by deconvolution and represent the first 138 or the last 53 amino acid residues of GH.

Top-down sequencing of the 6 kDa peptide gave rise to the product ion spectrum shown in Figure 5a allowing the assignment of a sequence tag (NDDALLKN) unambiguously demonstrating its origin from human GH. In addition, the 16 kDa fragment allowed the identification of a sequence tag of EAYIPKEQK that represents the N-terminal region of GH from residue 33 to 41. Moreover, the 16 kDa band was excised from the 1D-gel (Figure 3), hydrolyzed by means of trypsin, and resulting peptides were measured by MALDI-MS allowing a peptide mass fingerprint of GH with a sequence coverage of 87%.

In addition to the identification of degradation products of GH generated by long-term storage, post-translational modifications such as inter-molecular disulfide bonds and phosphorylation/sulfonation were observed. In Figure 6a a product ion mass spectrum is shown that was recorded from a doubly charged precursor ion at *m/z* 784.3 derived from tryptically digested pituitary GH. The amino acid sequence tag obtained by CID enabled the assumption of a homodimeric peptide composed by SVEGSCGF, the C-terminus of hGH. The presence of this homodimer was postulated in the literature earlier but was not proven by mass spectrometry. In order to substantiate the finding, the peptide SVEGSCGF was synthesized, dimerized in solution and analyzed under identical conditions as used for the trypsin-digested hGH. The resulting product ion spectrum is shown in Figure 6b proving identity with the peptide isolated from pituitary GH.

Finally, a peptide presumably composed by the amino acid residues VETFLR was detected as shown in Figure 7. However, its molecular mass was incremented by 80 u accounting either for a phosphorylation or sulfonation. At present, the identification of the modification has not been finished.

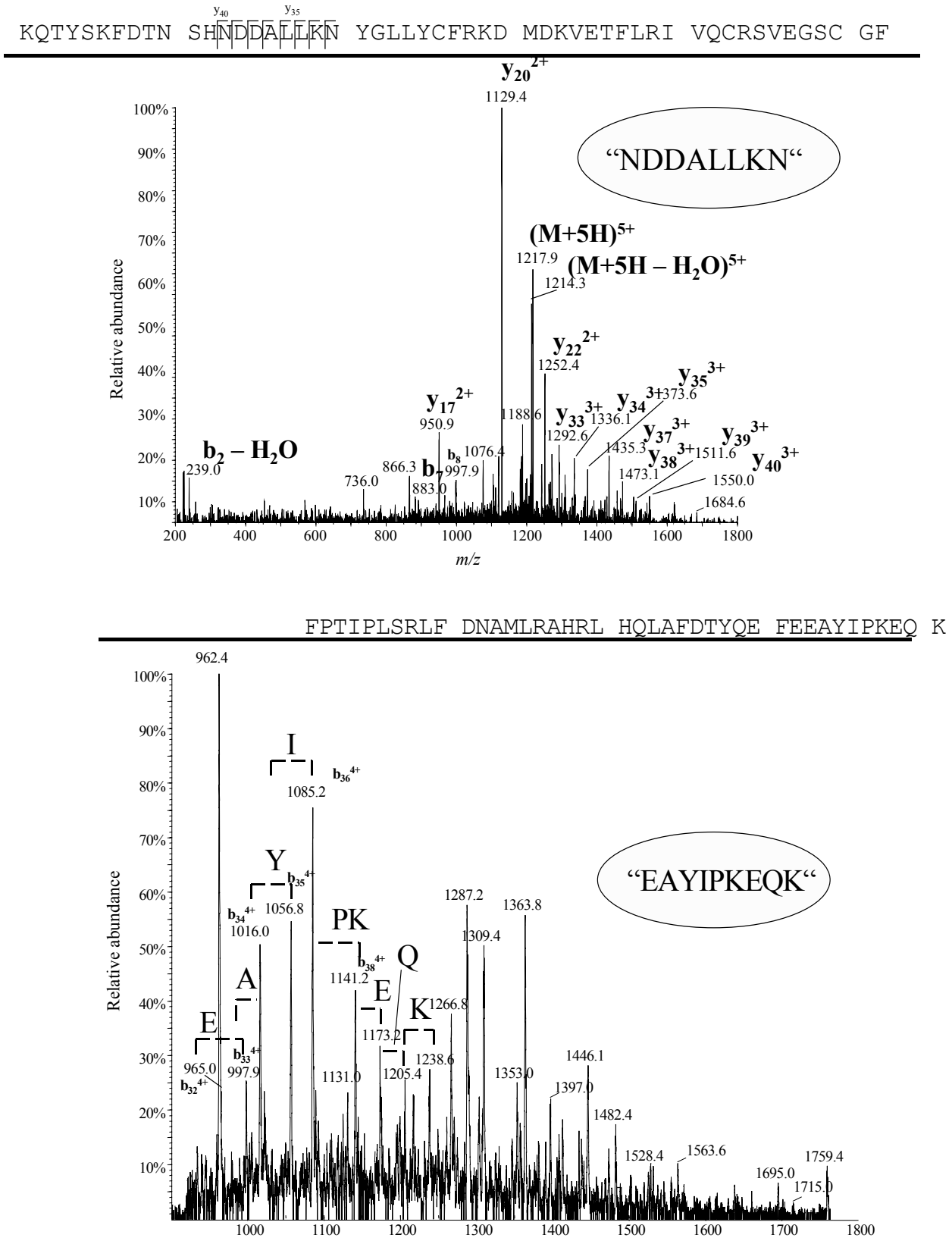
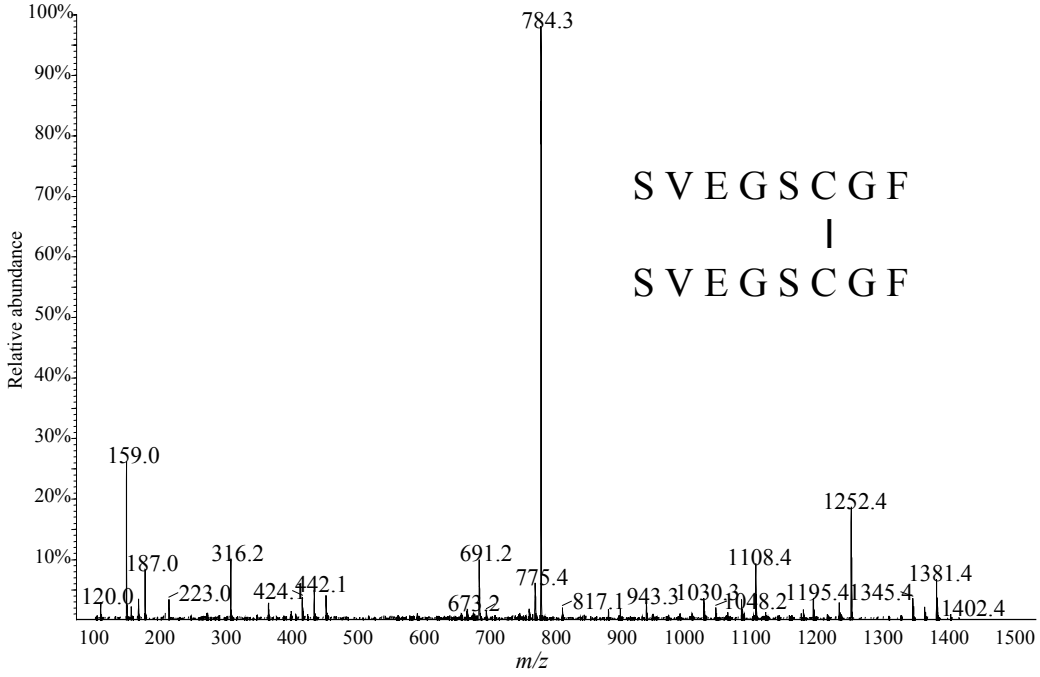


Figure 5: ESI-product ion spectra of fragments derived from degraded pituitary GH after disulfide reduction. a) 6 kDa fragment; precursor at *m/z* 1218 $(M+5H)^{5+}$, and b) 16 kDa fragment; precursor at *m/z* 1339 $(M+12H)^{12+}$.

a)



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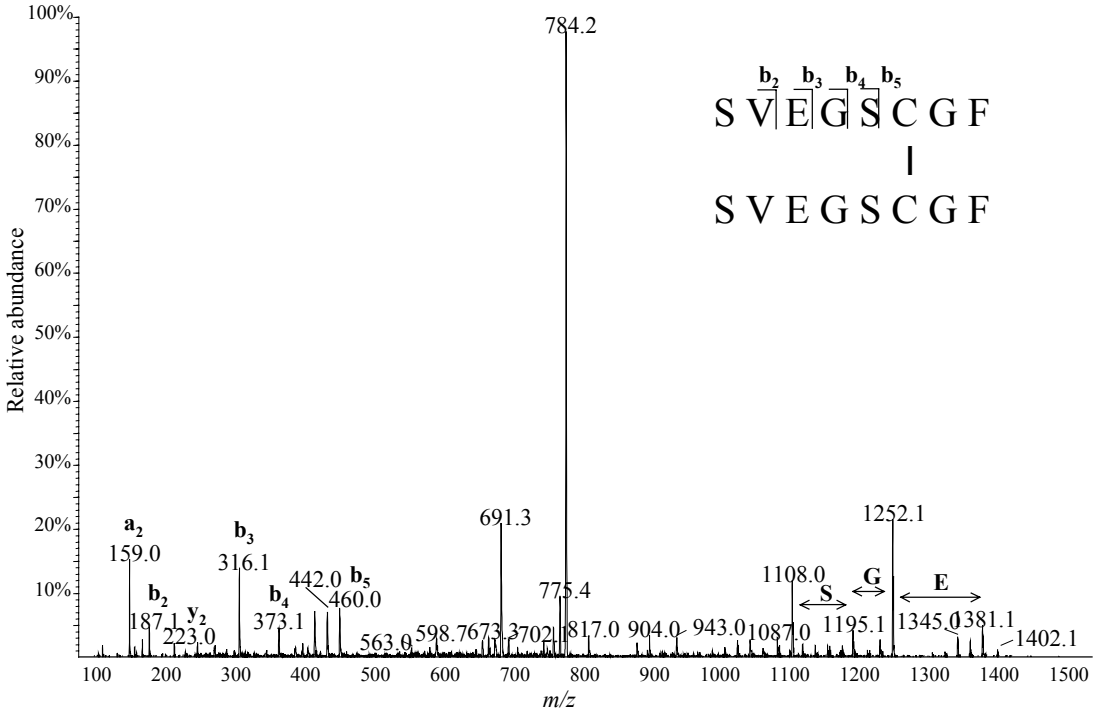

Figure 6: Product ion mass spectra of $(M+2H)^{2+}$ at *m/z* 784.3 from a) tryptically hydrolyzed pituitary GH, and b) synthesized homodimeric peptide.

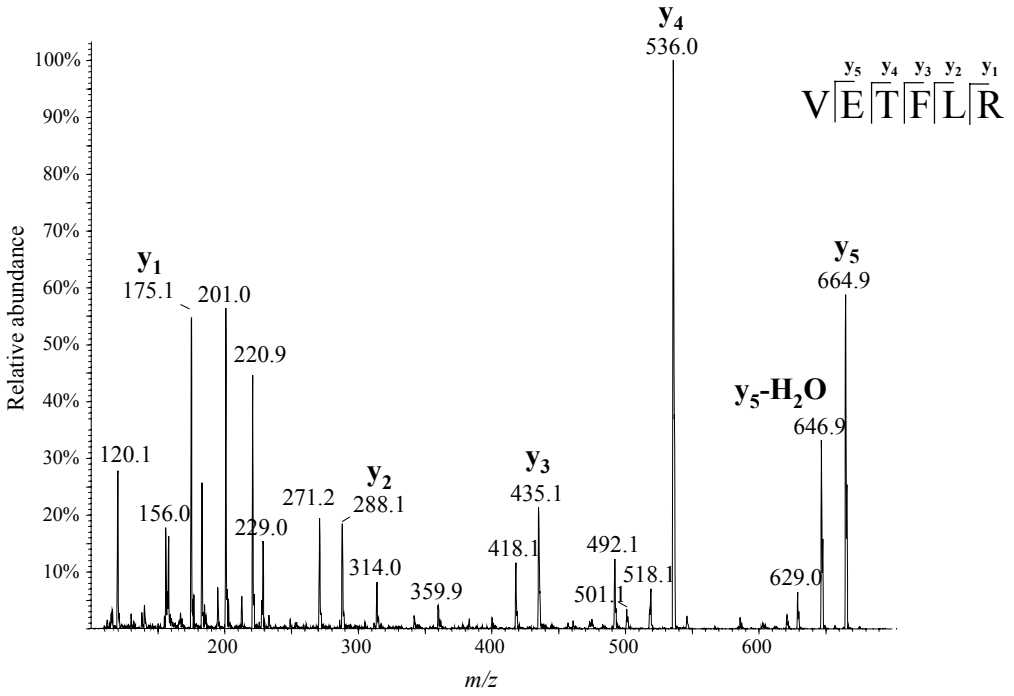

Figure 7: Product ion mass spectrum of $(M+2H)^{2+}$ at *m/z* 422.5 from tryptically hydrolyzed pituitary GH.

Conclusion

Mass spectrometric studies enable a valuable insight into target analytes and natural reference material of doping controls. In particular when using peptides and proteins frequent quality control analyses are recommended owing to the liability to degradation, and mass spectrometry supports the identification of degradation products that may possibly interfere with antibody assays. Moreover, the identification of specific characteristics of target compounds may support the preparation of complementary or more selective and robust assays employing antibody-antigen strategies.

Acknowledgments

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