Luteinizing Hormone Glycopeptide Preparation for Mass Spectrometry

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1. Introduction

Luteinizing hormone (LH) is a member of the heterodimeric glycoprotein hormone family. As these hormones are members of the cystine knot growth factor superfamily, each subunit is composed of a pair of protease-resistant hairpin loops, designated L1 and L3, on one side of the cystine knot and a protease-sensitive loop, L2, on the other side. N-linked oligosaccharides are attached to L2 and L3 in the alpha subunit common to all glycoprotein hormones and one N-linked oligosaccharide is linked to L1 in the LH beta subunit. A second N-glycosylation site is found in beta L1 of other hormones. When associated with the LH beta subunit, the common alpha subunit is glycosylated in a LH-specific manner. The Asn-linked oligosaccharides are essential for biological activity and variations in their structures somehow modulate LH activity. Total deglycosylation of LH increases receptor binding affinity, but virtually eliminates biological activity. In the alpha subunit L2 oligosaccharide plays a critical role in conferring biological activity, but this is modulated by beta L1 oligosaccharide. Accordingly, it is essential to characterize the oligosaccharides at both alpha L2 and beta L1 in order to predict the biological activity of LH. The present study is part of an effort to simultaneously characterize LH N-glycosylation sites using mass spectrometry. Site identification relies on the mass differences in the glycosylation signal sequences, or sequons, which differ in the second residue of the Asn-Xaa-Thr sequon ($N^{\alpha 56}$IT, $N^{\alpha 82}$HT, and $N^{\beta 13}$AT for oLH and $N^{\alpha 52}$VT, $N^{\alpha 78}$HT, and $N^{\beta 30}$TT for hLH).

2. Material and Methods

Preparation of Glycopeptides:

Samples containing 5.0 mg reduced, carboxymethylated oLH and hLH were incubated with a 500µg proteinase-K at 37^{o}C for 24 hours. The samples were dried by evaporation in a Savant Speed Vac. Each sample was then resuspended in 1ml of milli-Q water.

Determination of Monosaccharide Compositions:

A 10µl aliquot of each sample was dried by Savant Speed Vac and then hydrolyzed with 200µl 4N TFA at 100^{o}C for 4 hours. Each aliquot was then dried and resuspended in 200µl water containing deoxyglucose for monosaccharide composition analysis using a Dionex carbohydrate analyzer. A 200µl portion of each digest was fractionated by Superdex gel filtration chromatography.

Monosaccharide-Screening of the collected Fractions:

Samples of each fraction were subjected to carbohydrate and protein sequencing analysis. Protease-resistant peptides were treated with pronase, subtilisin or peptide-N-Glycanase and the products analyzed by gel filtration using a Superdex 75 column.

3. Results

Human and sheep LH preparations were reduced and alkylated and subjected to proteinase K digestion. Initially, glycopeptides and protease were separated by centrifugal ultrafiltration over 10,000MW cutoff membranes. However, the glycopeptide yields were low and carbohydrate analysis indicated significant glycopeptide retention. Fractionation of the retained fraction by Superdex 75 gel filtration revealed two glycopeptide fractions. Protein sequencing demonstrated that glycopeptides were the major components of carbohydrate-rich fractions. The high MW fractions consisted of either a 13-residue oLH beta glycopeptide (Fig.1, fraction 1, calculated mass 1,450.72 Da) or a 30-residue (Fig.1, fractions A and B calculated mass 3,345.94 Da) hLH beta glycopeptide the low MW fractions consisted of tri- and tetra-peptides derived from both alpha subunit L2 and L3 glycosylation sites (Fig.1, Fraction 2, NIT theorical mass 346.38 Da, NHT 370.37 Da and fraction C, KNVT 460.53 Da, NHT 370.37).The masses calculated for beta subunit glycopeptides combined with known LH oligosaccharide masses (m/z 771.3 – 2,095.6) are too large to be detected by electrospray mass spectroscopy which has an upper limit of 2,000 Da for accurate mass determination. Pronase, subtilisin, and peptide N-glycanase (PNGase) digestion of the sheep LHαL1 glycopeptide revealed that while it was protease insensitive, the oligosaccharide could be released with PNGase. Fractionation of the hLH and oLH proteinase K digest without previous ultrafiltration on a Superdex peptide gel filtration column produced multiple fractions, of which 3-4 possessed carbohydrate, while the others consisted of peptides (Fig.2). Rechromatography of each glycopeptide fraction on the Superdex 75 column

revealed complex mixtures in some fractions and relatively pure peptides in others. Peptide components in each fraction were identified by protein sequencing.

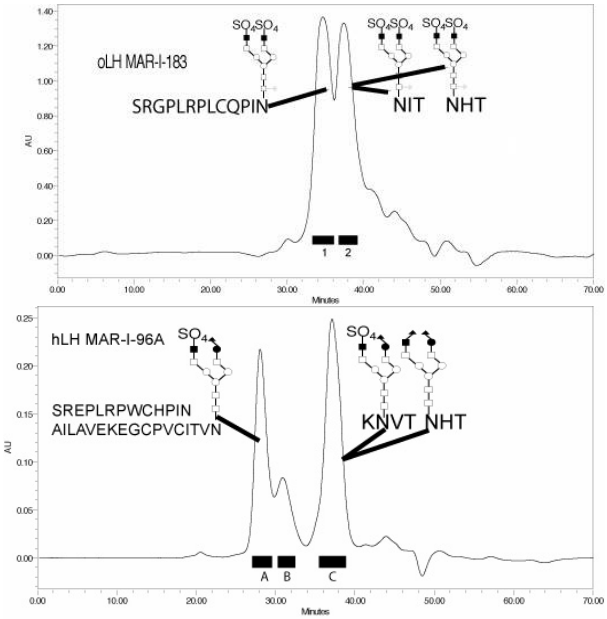
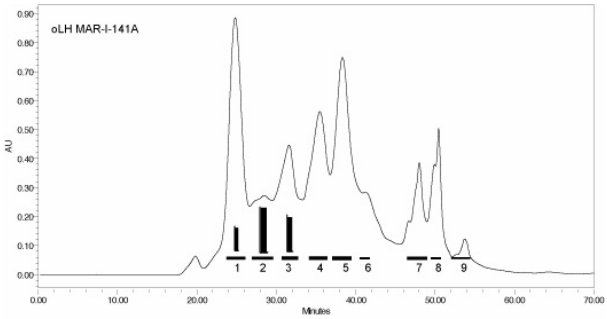


Fig.1. Fractionation of the retained fraction by Superdex 75 gel filtration revealed two glycopeptide fractions. oLH protein sequencing and . hLH Protein sequencing demonstrated that fractions 1, A and B were composed of a 13-or-30 residue LH betaL1 glycopeptide and fractions 2 and C consisted of tri- and tetra-peptides derived from both alpha subunit L2 and L3 glycosylation sites.



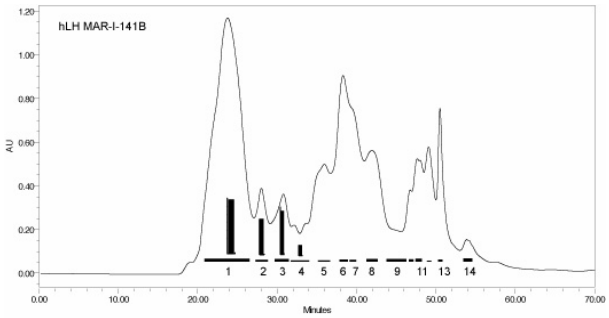


Fig.2. Fractionation of the hLH and oLH proteinase K digest without previous ultrafiltration on a Superdex peptide gel filtration column (0.4ml/min, 0.2M ammonium bicarbonate, Absorbance 230nm) produced multiple fractions, represented by lines, of which 3-4 possesed carbohydrate, while the others consisted of peptides. The relative yields of carbohydrate are represented by vertical bars.

4. Conclusion

The relatively low peptide background and size suggests that the alpha subunit glycopeptides should be good subjects for mass spectrometry. Beta subunit glycopeptides are too big for analysis by electrospray mass spectrometry.

5. Acknowledgements

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