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Characterisation of modified pharmaceutical proteins: the somatropin case

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INTRODUCTION

Somatropin, a recombinant protein containing 191 amino acids, is derived from the endogenous human growth hormone, somatotropin [1]. This protein is clinically used in children and adults with inadequate endogenous growth hormone to stimulate a normal bone and muscle growth. In addition, somatropin is currently being investigated for the diagnosis and radiotherapy of certain hormonal cancers. The modification of the protein with a chelating agent like NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) allows the inclusion of metals coupled to the protein. The NOTA unit is selectively introduced on a lysine side chain. This yields 9 possible labelling sites for somatropin: 38, 41, 70, 115, 140, 145, 158, 168 and 172.

As site-specific labelling is necessary to avoid active region interactions, characterisation of the chelate-modified somatropin is indispensable. Therefore, we have applied an enzymatic digestion procedure using trypsin, chymotrypsin and Staphylococcus aureus V-8 proteases. The resulting peptides were then monitored using HPLC-MS^n, allowing the investigation of the exact position of amino acid modifications.

EXPERIMENTAL

The cysteine residues of somatropin were first reduced and alkylated using DL-dithiothreitol (DTT) and iodoacetamide, respectively. The solution was then desalted, using a PD-10 desalting column. Subsequently, an aliquot of the solution was incubated with trypsin (4 hours, 37°C), chymotrypsin (24 hours, 37°C) and *Staphylococcus aureus* V-8 (18 hours, 37°C) proteases.

After NOTA-labelling of somatropin [2], as well as additional metal complexation, the same procedure for protein digestion was performed. In addition, a mixture of trypsin and chymotrypsin was added to the protein and incubated for 24 hours at 37°C.

The resulting peptides were separated using a Vydac Everest C_{18} column (250 mm x 2.1 mm i.d., 5 µm particle size, 300 Å) in an oven set at 45°C, with a mobile phase consisting of A) 0.1% w/v formic acid in water, and B) 0.1% w/v formic acid in acetonitrile. A linear gradient was employed and the flow rate was set at 0.2 mL/min.

RESULTS and DISCUSSION

Bioinformatics

Bioinformatics determined the active regions of somatropin as 1-16, 41-68, 103-120, 167-175. Based upon the calculated pKa values of the different lysine amino acids, one lysine amino acid was found to be a good candidate for chelate modification using the proprietary process [2]. This specific lysine amino acid is indicated in yellow in Figure 1.

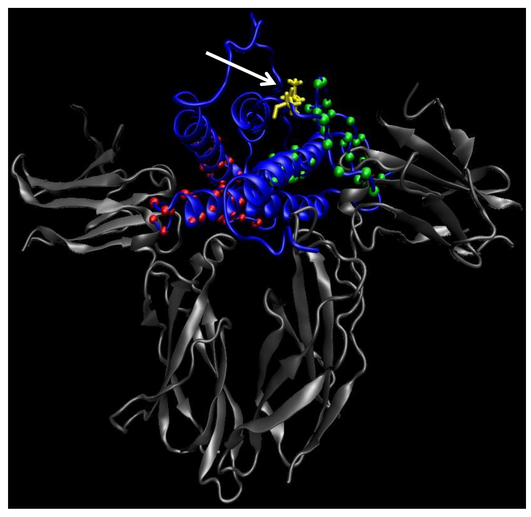

Figure 1: Somatropin bound to the hGHR

Characterisation of unmodified somatropin

Enzyme	General lysine coverage	Specific lysine amino acid
Trypsin	100 %	7 %
Chymotrypsin	89 %	18 %
Staphylococcus aureus V-8 protease	22 %	Not recovered

Staphylococcus aureus V-8 protease digestion of somatropin is unsuitable for localisation of modified lysine amino acids, as the lysine coverage is inadequate. Chymotrypsin and trypsin digestion are thus the most appropriate methods for characterising the lysine amino acids.

Characterisation of NOTA-modified somatropin

Enzyme	General lysine coverage	Specific lysine amino acid
Trypsin	100 %	11 %
Chymotrypsin	67 %	18 %
Trypsin and chymotrypsin combination	89 %	17 %

The use of an enzyme mixture of trypsin and chymotrypsin is suitable for localisation of modified lysine amino acids. In that way, an enhanced information efficiency is obtained.

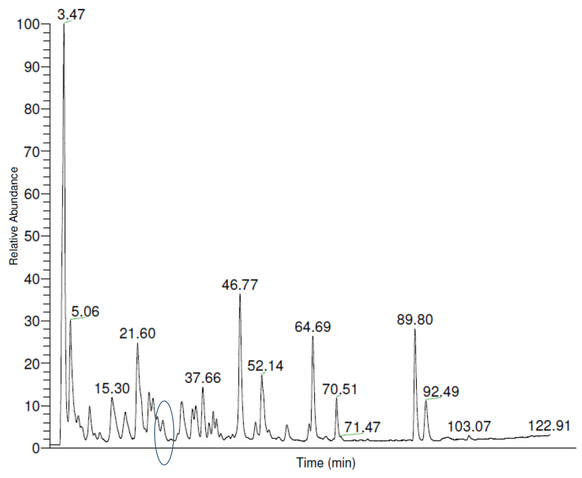

Figure 2: Typical HPLC-MS of the digest

Characterisation of metal-labelled NOTA-somatropin

The use of trypsin and chymotrypsin is suitable to investigate the incorporation of a metal ion into the NOTA-protein conjugate. Figure 3 gives the mass spectrum of a metal-chelate modified peptide fragment, encircled in Figure 2. This fragment elutes after 27 minutes and has a mono-isotopic mass of 2434.77, corresponding to SESIPTPSNREETQQK(-NOTA-Ga)S.

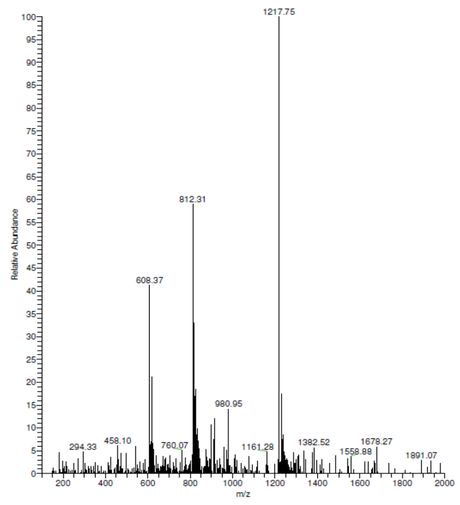

Figure 3: Mass spectrum

CONCLUSIONS

The use of a mixture of trypsin and chymotrypsin is most suitable for the peptide-mapping characterisation of chelate modified somatropin. The use of this enzyme combination gives an enhanced information efficiency.

REFERENCES

[1] European Pharmacopoeia 6.0, 01/2008:0951.
[2] Patent application GB1006285.9