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## **Mass spectrometric identification of human pituitary growth hormone variants**

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### **Introduction**

Growth hormone (GH) is one of the most important growth promoting substance in the human body. It acts directly on lipolysis and has indirect anabolic effects by activating the production of IGF-1. Effects and side effects are mainly known from people with growth hormone deficiency or acromegaly [1,2]. Although it is presumed that GH is abused by athletes, studies on the effect of recombinant growth hormone in healthy man are controversial and do not clearly show performance enhancing effects [3-6].

Human growth hormone is synthesized in the pituitary gland and consists of several isoforms and fragments. The predominant form of human pituitary growth hormone has 191 amino acids and a molecular weight of 22 kDa. The heterogeneity of human growth hormone [7,8] results from splice variants, proteolytic cleavage, oligomerization or posttranslational modification. The main splice variant has 20 kDa and exhibits amino acids 32-46 [9], proteolytic cleavage is described to result in fragments of 5 and 17 kDa [10], and posttranslational modifications were shown to include phosphorylation [11], glycosylation [12], deamidation [13] and N-acylation. Furthermore, oligomerization was observed that includes is mainly the formation of dimers, which can be homo- or heterodimers with covalent or non-covalent linkage [14,15].

In contrast, recombinant growth hormone consists only of the unmodified 22 kDa form of GH.

The different isoforms of human pituitary growth hormone have different metabolic actions [16,17] as well as different clearance times from the circulation, which is connected to different affinities to binding proteins.

Detailed analysis and characterization of growth hormone in blood and pituitary shall provide structural information on different variants and may demonstrate connections between the appearance of isoforms and special metabolic states or conditions. The identification of additional fragments may also complement the knowledge about differences between recombinant and endogenous human growth hormone and provide targets for future drug testing assays.

Hence, in the present work human pituitary growth hormone was analyzed by two dimensional gel electrophoresis and orbitrap mass spectrometry after trypsin digestion of Coomassie blue stained spots.

### **Material and Methods**

Pituitaries were homogenized in 2 mL of multichaotropic sample solution (MCSS, 7.7 M urea, 2.2 M thiourea, 4.4 % CHAPS, 44 mM tris) and sonicated for 30 min. After centrifugation (5 min, 9000 g) the supernatant was transferred to a new tube and frozen at -20 °C until analysis. Details on the origin of the samples are listed in Table 1. Each sample was prepared and analyzed at least three times to confirm results and exclude sample preparation as reason for differences and conspicuities.

Table 1 Analyzed pituitaries

	gender	age [years]	height [cm]	weight [kg]	autolysis [days]	cause of death
<b>1</b>	male	20	174	64	1	craniocerebral injury
<b>2</b>	male	42	170	97	3	acute heart failure
<b>3</b>	male	56	174	70	3	brain mass bleeding
<b>4</b>	male	81	unknown		5	traumatic brain bleeding
<b>5</b>	female	43	165	65	5	unknown
<b>6</b>	female	56	156	47	3	diabetes mellitus, pancreatitis
<b>7</b>	female	80	171	56	3	chronic pneumonia

Separation of pituitary proteins was performed using two-dimensional polyacrylamide gel electrophoresis (PAGE). Prior to isoelectric focussing, 50 µL of pituitary extract were added to 140 µL of MCSS, and proteins were reduced with 10 µL of 1 M dithiothreitol (DTT). Reduced cysteine residues were derivatized with 30 µL of 1 M acrylamide, and the excess of

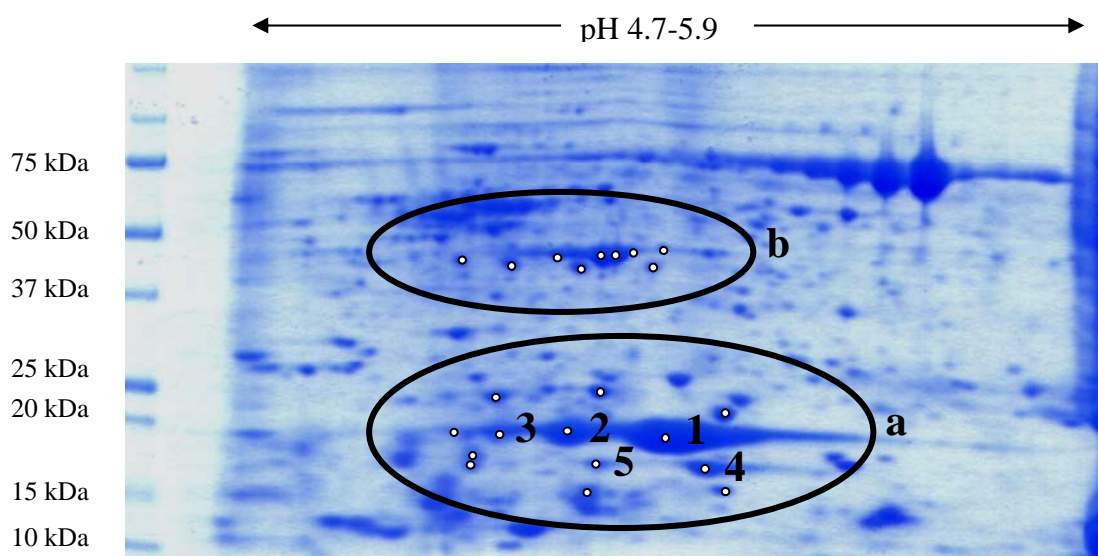
acrylamide was reduced by further 20  $\mu$ L of DTT. Isoelectric focussing was performed on 11 cm IPG strips with a pH gradient of 4.7-5.9 (Biorad, Munich, Germany). Equilibration of IPG strips was performed using an equilibration solution that contained 6 M urea, 125 mM tris, 2% (w/v) sodium dodecyl sulfate (SDS) and 0.01% Bromo Phenol Blue. The voltage gradient for IEF was as follows: 100-300 volts in 2 h, 300 to 10000 volts in 6 h and 10000 volts until 80000 vhs were reached. The current limit was 50  $\mu$ A per strip. For SDS-PAGE 8-16% gradient tris gels (Proteome Systems, Massachusetts, USA) were used, and the SDS running buffer contained 250 mM of tris, 1.92 M of glycine and 1 % (w/v) of SDS. SDS-PAGE was performed with 30 mA per gel. Gels were stained with Coomassie blue (Perbio, Bonn, Germany) and selected spots were digested with trypsin (Promega, Mannheim, Germany) at 37 °C over night. For the extraction of the resulting peptides, 1% trifluoroacetic acid in 50% acetonitrile and 50% deionized water (Water Lab System, Millipore, Eschborn, Germany) was used. Peptides were separated by capillary liquid chromatography and analyzed by electrospray ionization orbitrap mass spectrometry and tandem mass spectrometry (Table 2). All reagents and solutions were bought from Sigma (Munich, Germany). All buffer ingredients were of electrophoresis grade and reagents for trypsin digestion were of analytical grade.

Table 2 LC-MS conditions

<b>Capillary Liquid Chromatography</b>	Agilent 1100
Trap column	Zorbax 300SB-C18, 0.3 x 5 mm
Analytical Column	Zorbax 300SB-C18, 0.3 x 50 mm
Solvents	A: 0.1% acetic acid, 0.01% TFA B: 80% acetonitril, 0.1% acetic acid, 0.01% TFA
Solvent gradient	0-2 min 95% A, 2-20 min 60% A, 20-34 min 3% A, 34-44 min 3% A, 95% A, 15 min hold
<b>Mass Spectrometry</b>	Thermo LTQ Orbitrap
Ionization voltage	3.5 kV
Resolution	30000
Normalized Collision Energy in automated MS/MS experiments	35%

## Results and Discussion

Analysis of the pituitary extracts resulted in 22 spots, which contained growth hormone as proven with a sequence coverage of 35-80% (spots with white dots in Figure 1).



*Figure 1 Pituitary extract from a 42 year old male (white dots mark spots containing growth hormone)*

The spots in circle **a** fit the molecular weight of monomeric forms of growth hormone. While spots 1-3 represent the predominant 22 kDa variant, spots 4 and 5 were expected to contain the 20 kDa form. Spots with a slightly higher molecular weight may be glycosylated [12].

The spots in circle **b** were supposed to contain dimeric forms due to their molecular weight of approximately 45 kDa.

Comparison of the extracts from the different pituitaries showed one noticeable difference being the appearance of four dimeric spots in only three of the seven analyzed pituitaries as shown by comparison of Figure 1 and Figure 2 (see arrow in Figure 2). These spots were found in pituitaries 2, 4 and 7 (Table 1). Comparison of the data listed in Table 1 did not give a clear reason or advice for the appearance of these spots. Age and gender of the people the pituitaries were taken from as well as the cause of death and autolysis time did not fit systematically with the appearance of these spots.

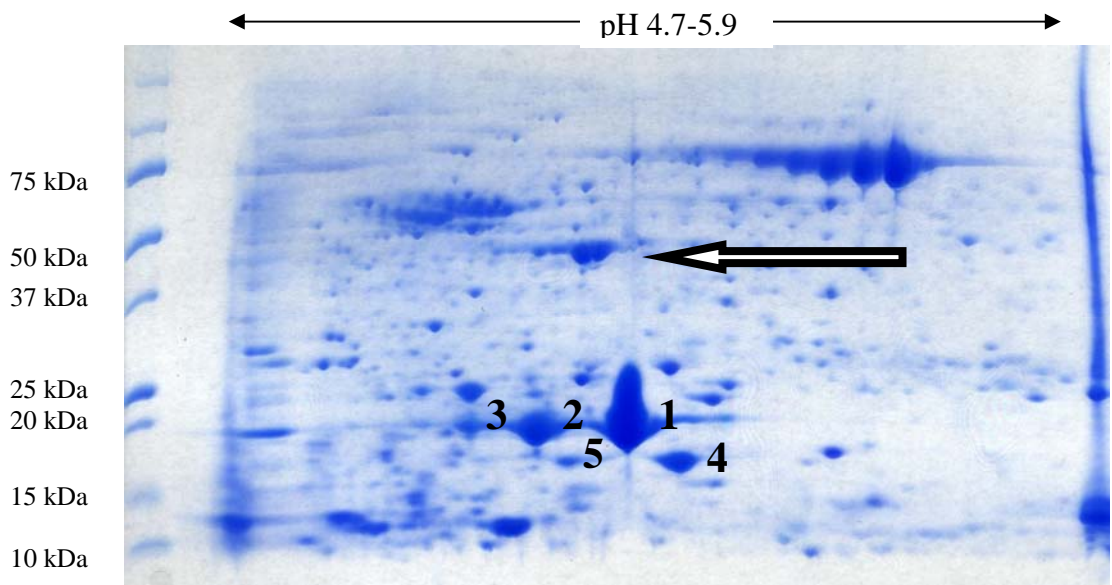


Figure 2 Pituitary extract from a 20 year old male missing the dimeric spots.

Spots 4 and 5 were shown to contain the 20 kDa splice variant by MS/MS experiments of the “new” peptide resulting from the missing amino acids 32-46 and therefore consisting of amino acids 20-31 and 47-64 (Figure 3). The precursor ion is triply charged with an  $m/z$  value of 1181.2 and the MS/MS spectrum shows expected most abundant ions for a proline directed dissociation ( $y_6$  and  $b_{24}$ , Figure 3).

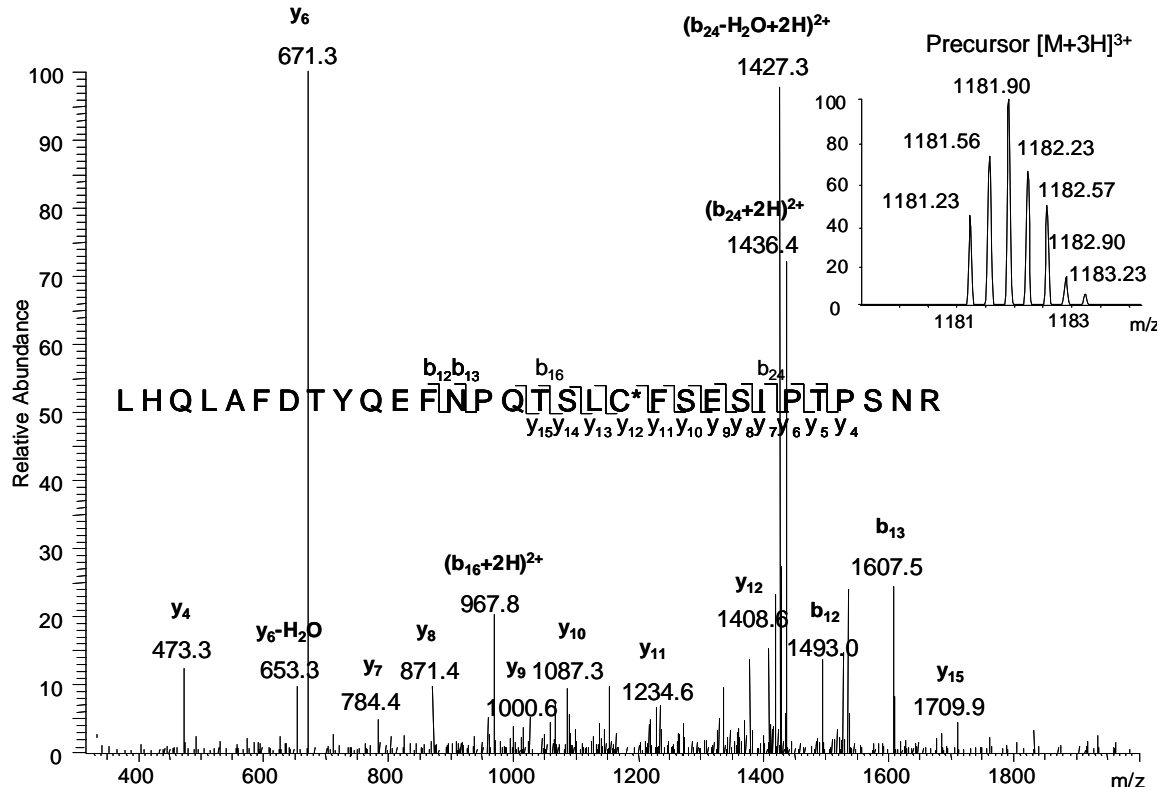


Figure 3 MS/MS spectrum of the 20 kDa peptide resulting from the missing amino acids 32-46

## Phosphorylation

Searching for the reason for pI shift of the 20 and 22 kDa spots, phosphorylations were found at serine 106 and 150 by MS/MS experiments (Figure 4 and Figure 5).

The peptides comprising the amino acids 146-158 and 100-150 were found to be modified at serine 150 and serine 106, respectively, by an additional 80 u as shown in product ion spectra depicted in Figure 4 and Figure 5. Phosphorylation was reasoned by the loss of 98 u in the MS/MS spectra representing the loss of phosphoric acid. A sulfonation which also leads to a mass shift of 80 u usually shows a loss of 80 instead of 98 u. Phosphorylation at serine 150 was further verified by high resolution, high accuracy orbitrap mass spectrometry which additionally differentiated the phosphorylation from a sulfonation by comparison of experimental and calculated average  $m/z$  values:

<b>Experimental average <math>m/z</math>:</b>	<b>785.779</b>
<b>Calculated average <math>m/z</math> for a phosphorylation of peptide aa 146-158:</b>	<b>785.779</b>
<b>Calculated average <math>m/z</math> for a sulfonation of peptide aa 146-158:</b>	<b>785.820</b>

Phosphorylation at serine 150 was detected in spots 2, 3 and 5, whereas phosphorylation at serine 106 was only found in spot 3 (Figure 1).

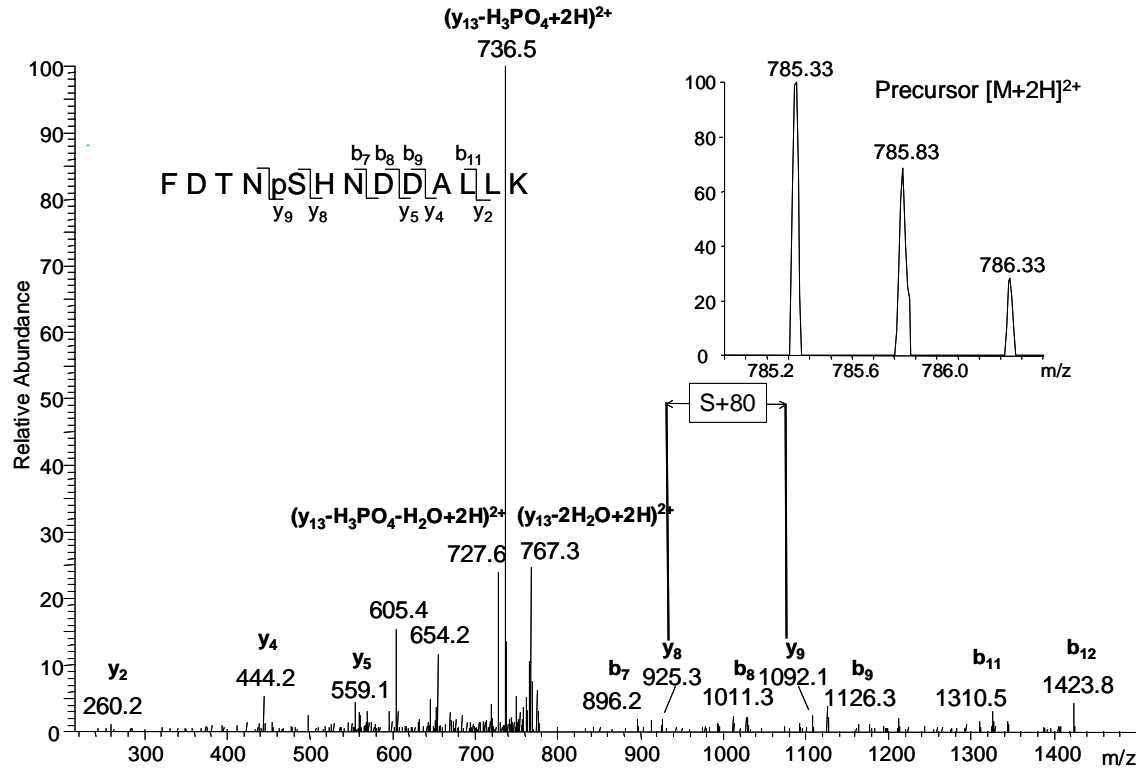


Figure 4 MS/MS spectrum of peptide 146-158 phosphorylated at serine 150

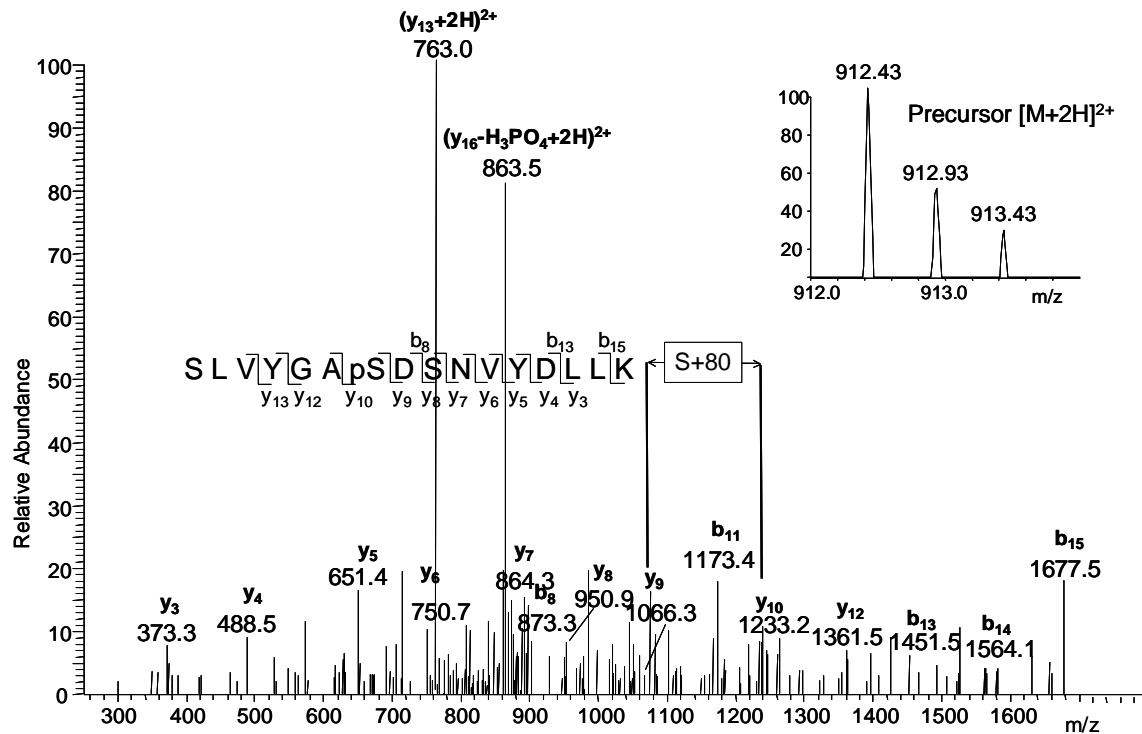


Figure 5 MS/MS spectrum of peptide 100-115 phosphorylated at serine 106

## Tryptophan oxidation

Another modification detected in human growth hormone was tryptophan oxidation. Growth hormone contains one tryptophan in position 86.

Tryptophan oxidation was detected earlier for enzymes and proteins being involved in reduction and oxidation reactions in mitochondria [18,19] or being exposed to oxygen radicals or light like in the eye lens [20] and therefore the oxidation pathway was shown earlier [18] (Figure 6).

In Figure 7 the MS/MS spectrum of the peptide 78-94 with the tryptophan residue oxidated to *N*-Formyl-Kynurenine is presented. The calculated *m/z* value for two additional oxygens (elemental composition C<sub>99</sub>H<sub>161</sub>O<sub>26</sub>N<sub>23</sub>, *m/z* 1044.0986) differs from the measured accurate monoisotopic mass (*m/z* 1044.0976) by only 0.952 ppm, which substantiates the assumed modification. Beside *N*-Formyl-Kynurenine, other related modifications from Figure 6 were also identified by MS/MS experiments.

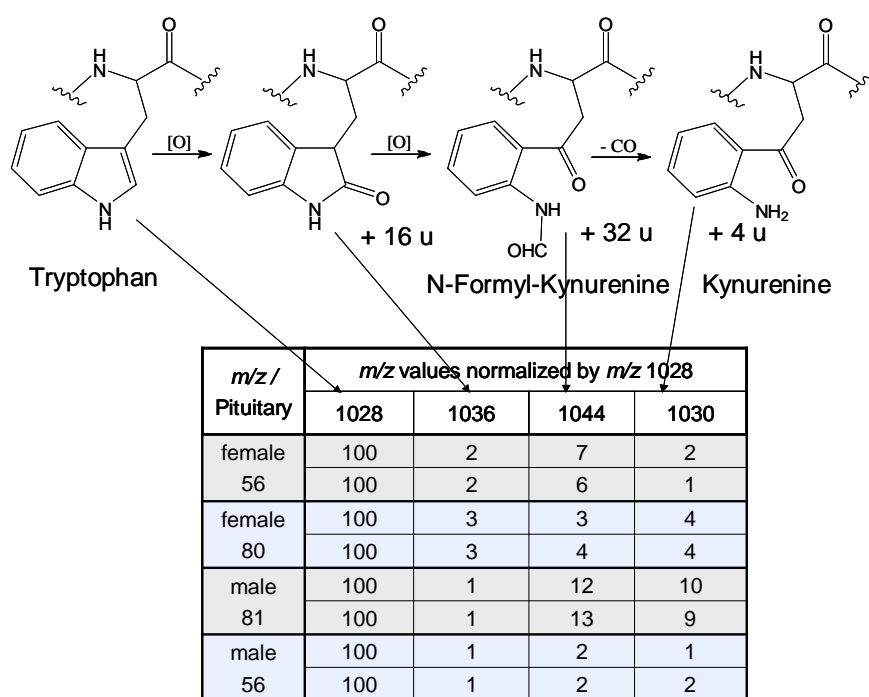
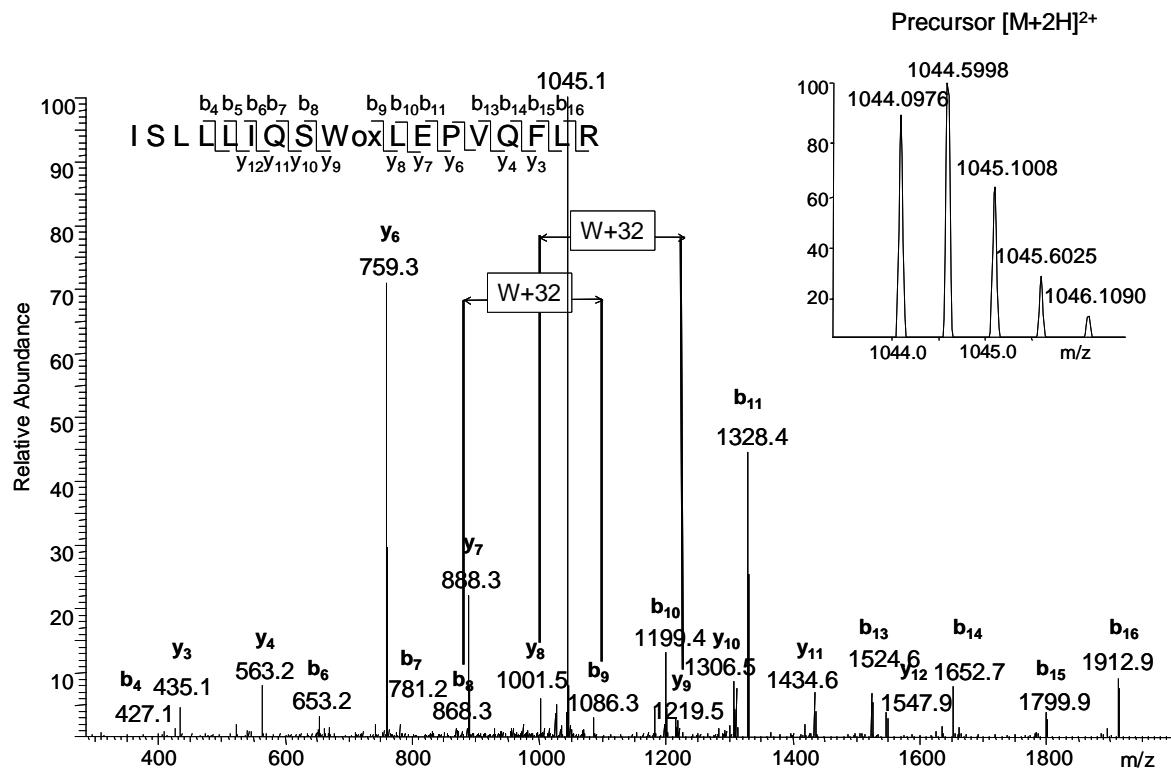


Figure 6 Tryptophan oxidation and interindividual differences

All tryptophan modifications were found in spots 1-5 from Figure 1. Analyzing spot 1 from different pituitaries showed that the ratio of the different oxidation forms differs interindividually (see Table in Figure 6). Repeated sample preparation and analysis yielded the same values which excludes sample preparation as reason for the oxidated tryptophan forms.





## Conclusion

The variants of human pituitary growth hormone detected here show only a small part of the heterogeneity, and single spots may contain a far more complex set of modifications.

In this work phosphorylations were found to be responsible for pI shift to more acidic pH. Tryptophan residues were found to be oxidized in several growth hormone variants and oxidation states were interindividually different. Furthermore, dimeric forms of human growth hormone were only found in some of the analyzed pituitaries.

Isoforms that appear interindividually different should be studied in detail because changes in the three dimensional structure may influence the binding properties in antibody based assays.

For anti-doping purposes, it will be essential to compare the variants found in the pituitary extracts and plasma samples.

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