G. Such Sanmartín[1], R. Gutiérrez Gallego[1,2,*], J. Bosch[1], D. Andreu[2] and J. Segura[1,2]

5 kDa and 17 kDa Hydrolysis Products of Human Growth Hormone

[1]Pharmacology Research Unit, Municipal Institute of Medical Research (IMIM), [2]Department of Experimental and Health Sciences, University Pompeu Fabra (UPF), C/ Doctor Aiguader 80, 08003-Barcelona, Spain, * Corresponding author

ABSTRACT

Growth hormone 5 kDa and 17 kDa isoforms are generated through specific proteolysis of the 22 kDa isoform and are believed to reach high concentrations in serum. In the anti-doping field, the interest in these isoforms resides in whether they are generated only from the endogenous 22 kDa isoform or also from the exogenous 22 kDa isoform after administration. The generation of these isoforms has already been reported through DNA-expression. In addition to this, alternative methodologies for their preparation consisting of solid phase synthesis for the 5 kDa isoform and 22 kDa limited proteolysis for the 17 kDa isoform are reported here.

INTRODUCTION

Growth hormone detection

Growth hormone (GH) anti-doping assays commonly work under the basis of the ratio between the distinct GH isoforms existent in blood. Basically two approaches are being used at present: the first focuses on the ratio between the 22 kDa isoform and the 20 kDa isoform, while the second one compares the 22 kDa isoform to all other isoforms [1-3]. Both assays rely on the suppression of the endogenous production of GH after an exogenous administration of the recombinant 22 kDa isoform resulting in altered ratios. However, those GH variants that are not expressed individually but derived from the main 22 kDa isoform (post-translational processes) are expected to be present regardless the origin of the 22 kDa isoform, and therefore could interfere with the mentioned assays.

Especially two of these variants, the 5 kDa and 17 kDa isoforms, were reported at high concentration in blood. Both isoforms of 5 kDa and 17 kDa are believed to originate from a

unique cleavage of the 22 kDa isoform at the position AA43-44, generating the sequences AA1-43 and AA44-191, and as such these circulating isoforms could cross-react with the antibodies used in GH detection assays. In order to address this issue we have developed a method to synthesise the 5 kDa isoform and a fast and simple procedure to generate the 17 and 5 kDa variants through limited proteolysis. Finally, cross-reactivity experiments have been done with selected antibodies against particular isoforms through surface plasmon resonance (SPR) experiments.

EXPERIMENTAL

Materials – Amino acids with side chains protected with specific groups and with N-terminal 9-fluorenylmethoxycarbonyl (Fmoc), 4-(hydroxymethyl) phenoxy methyl (Wang) resin functionalised with serine, N,N-dimethylformamide (DMF), N-hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) , diisopropyl ethyl amine (DIEA), 1,2-ethanedithiol (EDT), and triisopropyl-silane (TIPS) were acquired from Senn chemicals (Dielsdorf, Switzerland). Thermolysin, pepsin, elastase and trypsin were purchased from Sigma Aldrich. Monoclonal anti-22 kDa antibody (clone n^0 A36020047P) was purchased from Biospacific Corporate (Emeryville, CA, USA). Monoclonal anti-20 kDa antibody (D05) was provided by Mitsubishi Kagaku Bioclinical Laboratories (Tokyo, Japan). Monoclonal anti-GH antibody (A 54/9) was purchased from Bioclone (Marrickville, Australia). Monoclonal anti-5 kDa antibody was generated in collaboration with Dr. M. Wu (China Doping Control Center, NRISM). Recombinant growth hormone from E. coli was obtained from Pharmacia & Upjohn (Barcelona, Spain; 22 kDa) and from Mitsubishi Kagaku Bioclinical Laboratories (20 kDa). Recombinant growth hormone (22kDa) from CHO cells was obtained from Serono Laboratories (Barcelona, Spain). Pituitary derived growth hormone standard material (80/505) was purchased from the National Institute of Biological Standards and Controls (NIBSC, United Kingdom). All other chemicals were of the highest grade commercially available.

Methods – Solid phase synthesis was performed on an ABI 433A Peptide Synthesizer from Applied Biosystems (Foster City, CA). The synthesis was initiated at the C-terminus (AA43) with serine-functionalised Wang resin through Fmoc chemistry. Purification of the protein by reversed phase HPLC (5% to 65% of acetonitrile:water system) was performed on a Shimadzu LC-8A HPLC instrument (Kyoto, Japan) which was coupled to a Phenomenex

Luna C8 column (3 µm, 0.46 x 5 cm for analytical HPLC and 10 µm, 2.1 x 25 cm for preparative HPLC), and to an ultra-violet detector measuring at wavelength of 220 nm. Data was acquired with Shimadzu Class-vp software. Circular dichroism measurements were conducted on a Spectro polarimeter Jasco (Ishikawacho Hachioji, Tokyo, Japan) J-810 with thermostated cuvette-device (Neslab RP-100, Bonsai Technologies Group, S.A, Barcelona, Spain), in 5 kDa concentrations of 15 µM in 25 mM sodium phosphate buffer pH 7.4 at a temperature of 5 ºC. Data were analyzed by the spectral deconvolution method JFIT provided by Dr. B. Rupp (Lawrence Livermore National Laboratory, Livermore, CA). Mass spectrometric analyses were carried out on a MALDI-TOF Voyager-DE™ STR Biospectrometry workstation (Applied Biosystems, Foster City, CA, USA), equipped with a N_2 laser (337 nm) of aliquots directly mixed with saturated solutions of sinapinic acid matrix. All recorded data were processed using Data Explorer™ Software (Applied Biosystems, Foster City, CA, USA). Reduction and alkylation of proteins were performed with a prior incubation with 10 mM dithiothreitol / 0.1M ammonium bicarbonate during 30 minutes at 56ºC followed by another incubation with 55 mM iodoacetamide 0.1 M ammonium bicarbonate at room temperature during 30 minutes in the dark. Proteolytic digestions in solution were performed by incubating 1 µg of protein in 50 µl ammonium bicarbonate (100 mM, pH 7.8) with 0.02 µg trypsin for 16 h at 37 ºC in a heater block, or in calcium chloride 10 mM and acetic acid (pH 4), sodium carbonate (pH 7), or ammonium chloride (pH 9) 10 mM buffer adjusted at indicated pH for a limited time with 0.02 µg thermolysin at room temperature. Surface Plasmon Resonance analyses were performed on a BIAcore 3000 instrument (Uppsala, Sweden) using sensor chips of high-density carboxy-methylated dextran layer (CM5). Standard running buffers used in SPR were based on HBS-EP (HEPES 10 mM pH 7.4, NaCl 150 mM, EDTA 1 mM, and 0.005% (v/v) Polysorbate 20 (Tween 20)). All these components were purchased from BIACore (Uppsala, Sweden). SPR analyses were done at 25 ºC at the typical flow rate of 5 µl/min. Immobilisation of antibodies on the surface was performed basically as described by Howell and Johnsson[4;5]. Coupling of the biomolecule of interest was achieved through the primary amine groups employing solutions of 50-60 µg/ml for high level immobilisations in 10mM sodium acetate buffer (coupling buffer, pH 5.0). All sensorgrams were obtained with the Control Software v4.0.1 and processed with the BIAevaluation software v4.0.1 (Biacore, Uppsala, Sweden).

RESULTS AND DISCUSSION

Solid phase synthesis of 5 kDa

The solid phase synthesis of the 5 kDa sequence AA1-43 was successfully accomplished through Fmoc chemistry[6]. The sequence integrity was controlled by MALDI-TOF mass spectrometry at different points of the synthesis and at the end (not shown), and the crude end-product was purified through RP-HPLC. The mass spectrum obtained of the pure protein (figure 1) showed the protonated variant of the correct sequence (5212.62 Da), a series of matrix adducts spaced at 107 Da, and a minor impurity corresponding to a homologues sequence with one amino acid deletion: Ile or Leu by mass. At the end, a total of 110 mg of the 5 kDa protein were synthesised representing an overall yield of 25 % with respect to the initial amount of resin.

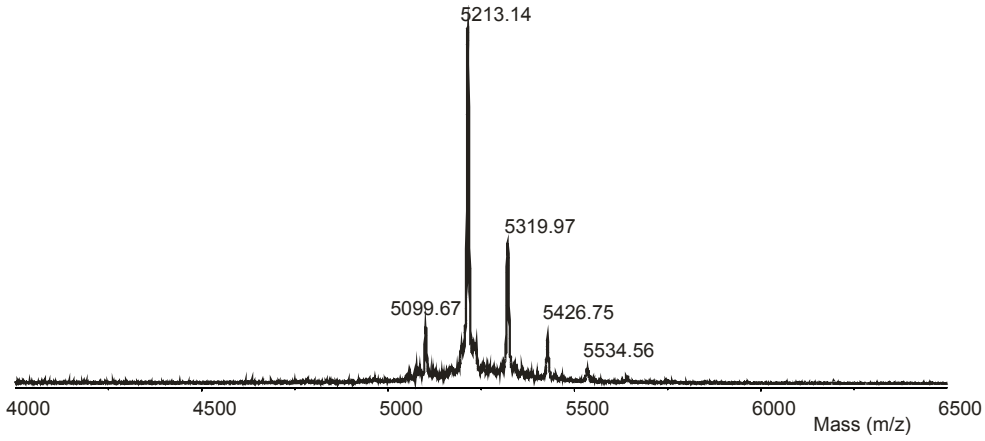

Figure 1. MALDI-TOF spectrum of the 5 kDa protein purified by HPLC.

An additional verification of the protein sequence was done by trypsinolysis followed by MS (not shown), covering the totality of the sequence (fragments obtained: AA9-16/MSO16, AA1-8, AA9-16, and AA17-43). Based on relative MALDI intensities between peaks of the correct and the shorter sequence, a qualitative approach of the percentage of the erroneous sequence was established at *ca.* 6 %. The identification of the AA deletion to Ile4 or Leu6 was based on the observation of the analogue of the AA1-8 peptide with a 113.47 Da mass difference.

Circular dichroism.

The circular dichroism (CD) spectra of the 5 kDa isoform were obtained at 5 ºC in phosphate buffer (pH 7) with 0 %, 20 % and 50 % of the structuring agent TFE[7] (figure 2, right-hand side corresponds to a normalised plot). The analysis of CD data is based in the theoretical

deconvolution into the curves corresponding to pure structures, typically α-helix, β-sheet and random coil (unfolded state)[8;9]. Deconvolution of CD data using JFIT software yielded a 40 %, 78 % and 98 % of α-helix content in measurements with TFE values of 0 %, 20 % and 50 % respectively, verifying that the protein is partially folded in solution.

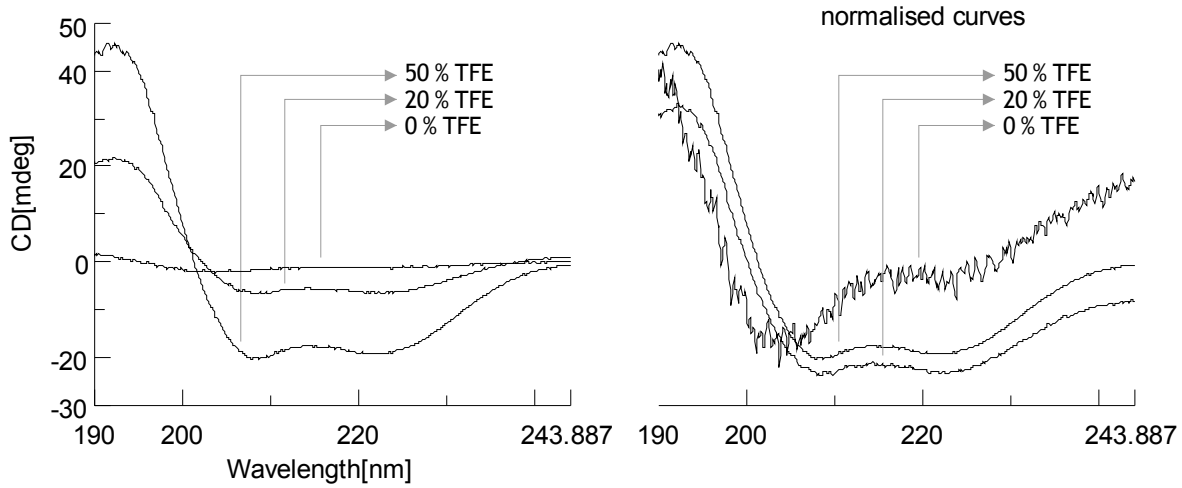

Figure 2. CD spectrum of the synthesised protein dissolved at neutral pH in phosphate buffer, at 5ºC, mixed with 0 %, 20 % and 50 % of TFE. Right-hand side graph represents the normalised view of the curves.

Limited proteolysis of 5 kDa.

Two proteolyses were done corresponding to the folded protein and to the protein partially unstructured by means of a thermic shock applied before the digestion[10;11] (figure 3a and 3b respectively). Comparison of the resulting mass spectra showed the disappearance of peaks AA1-14, AA9-43, AA10-43 and AA20-43 and the significant increase of the peak AA25-43 (figure 3b), suggesting an alteration of the protein structure due to the thermic shock (95 ºC during 1 minute) disfavouring the activity of the enzyme towards the segment AA1-25.

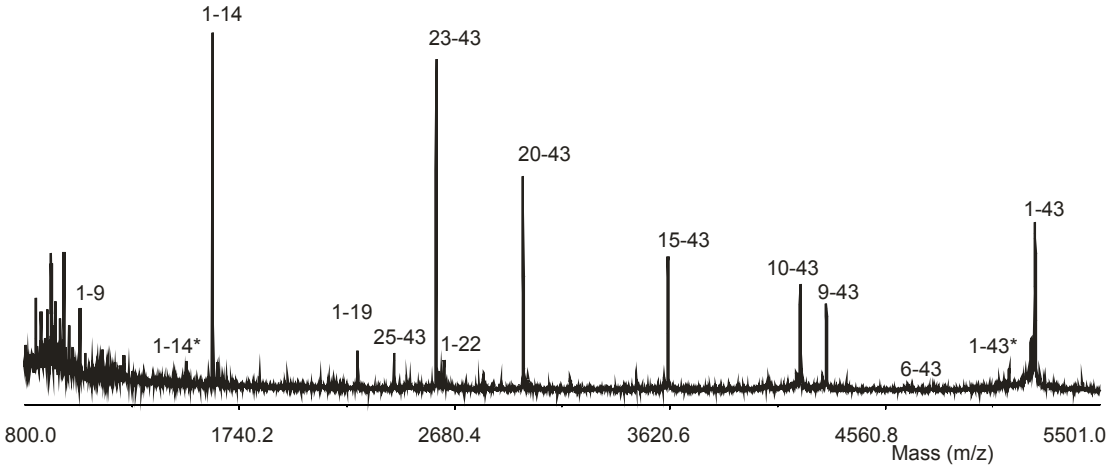

Figure 3a. MALDI-TOF spectrum of thermolysin digestion of 5 kDa at pH 9.

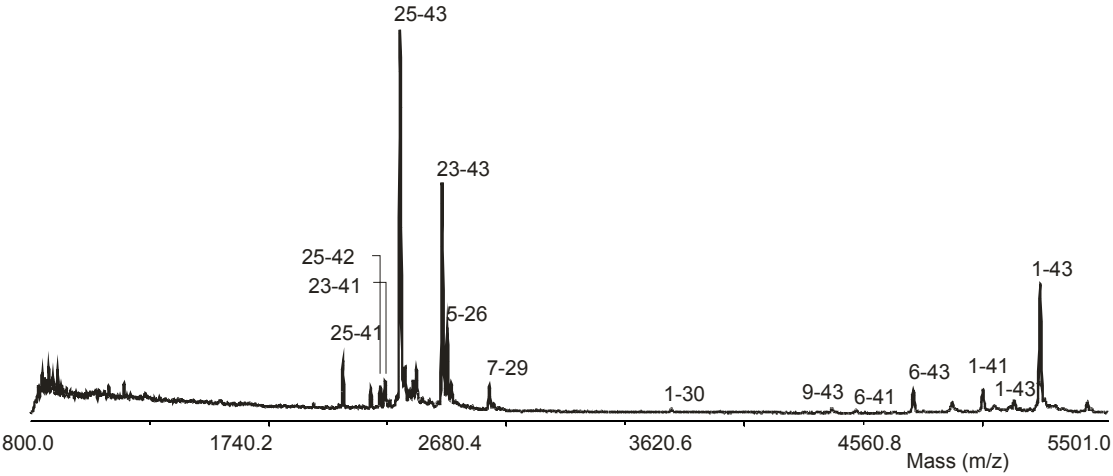

Figure 3b. MALDI-TOF spectrum of thermolysin digestion of 5 kDa at pH 9 with a previous thermic shock of 95 ºC during 1 minute.

Antibody specific against 5 kDa

A specific antibody against the 5 kDa isoform was generated with the synthesised protein in collaboration with Dr. M. Wu (China Doping Control Center, NRISM). The characterisation of this antibody was done through SPR experiments, consisting in the immobilisation of the antibody onto a surface and a posterior injection of 5, 20 and 22 kDa isoforms solutions at a concentration of 400-500 nM. The unique interaction observed corresponded to the 5 kDa isoform. Analysis of the thermodynamic parameters is ongoing.

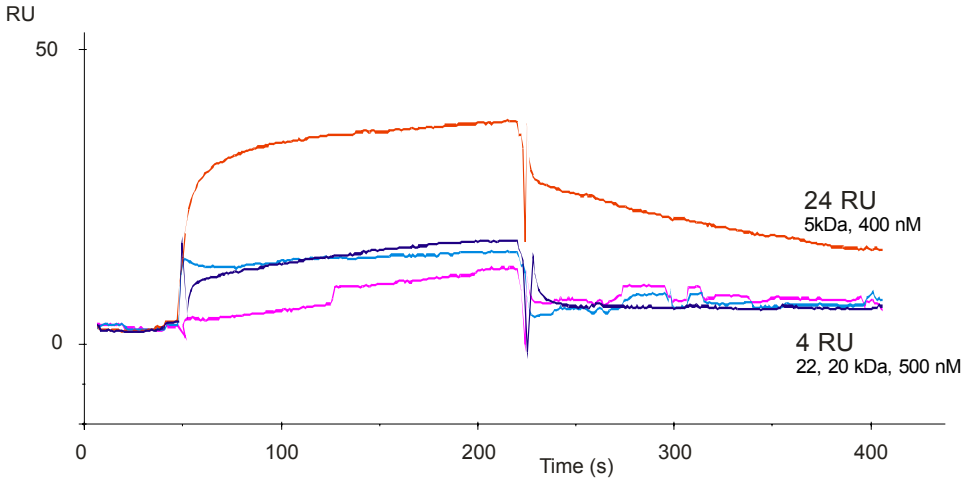

Figure 4. SPR sensorgram of the interaction of the antibody anti-5 kDa immobilised onto a surface and the 5, 20 and 22 kDa isoforms.

17 kDa isoform

The generation of the 17 kDa fragment corresponding to the GH isoform through limited proteolysis of the 22 kDa isoform was evaluated with the enzymes pepsin, elastase and thermolysin. The latter yielded promising proteolysis, cleaving at the desired bond AA43-44 and to a lesser extent (depending on the reaction conditions) also at the bond AA44-45 (figure 5). Proteolysis conditions were evaluated extensively and included various buffers, solvents, times, temperature and E/S ratios. Optimum conditions were selected for further experiments.

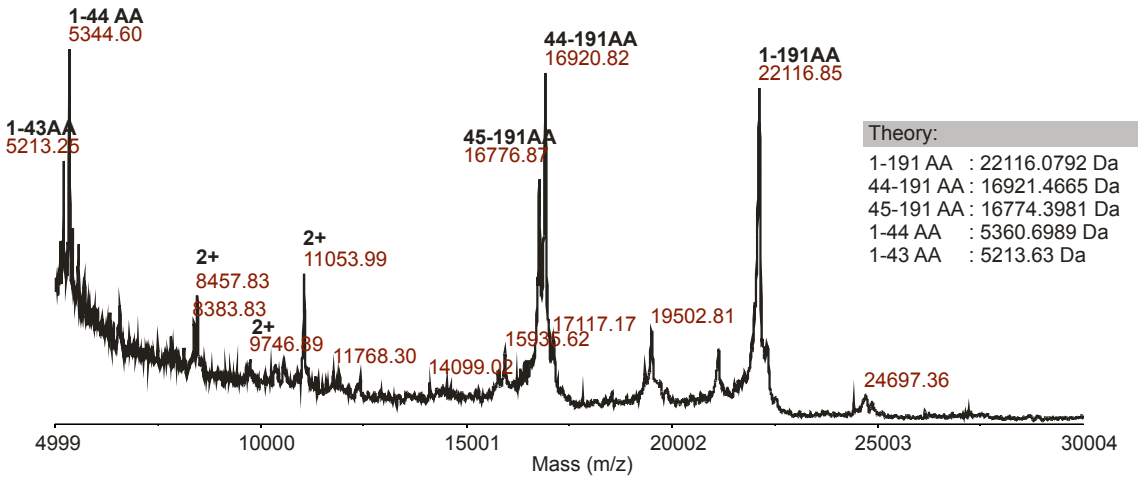

Figure 5. MALDI-TOF spectrum of thermolysin digestion of 22 kDa at pH 7 measured at 1 hour of digestion**.**

17 kDa semi-preparative proteolysis

A series of 22 kDa proteolyses at high concentrations were done with thermolysin and subsequently purified through liquid chromatography. A good separation was obtained between 5, 22 and 17 kDa mixtures. The two 17 kDa variants corresponding to the isoform AA44-191 and the similar fragment of sequence AA45-191 could not be separated. The overall yield of each digestion was established to a *ca.*18 %, although the original 22 kDa peak (*ca.* 55 %) could be reused in further procedures. The collected 17 kDa fragment was identified through MS after trypsin proteolysis. Through the observation of the exclusive fragments AA44-64 and AA45-64 and a satisfactory coverage of the sequence (not shown) the identity of the purified fragments were determined.

Cross-reactivity values against 5 kDa and 17 kDa

Three antibodies specific against 22 kDa, 20 kDa and one non-specific (no preference towards 22 and 20 kDa) were immobilised onto a SPR surface and evaluated with the 5 kDa synthetic isoform and the 17 kDa fragment isoform. For the 5 kDa isoform none of the antibodies showed specific affinity towards the isoform (not shown). For the 17 kDa isoform, both the anti-22 kDa and the unspecific antibody showed affinity towards the protein (figure 6). The epitope discussion of these antibodies derives therefore to its location for the anti-22 kDa antibody around the segment AA43-46, for the non-specific antibody around the segment AA44-191, and for the anti-20 kDa antibody to the exclusive union between amino acids AA32 and AA46. Thus it is verified the interference of the 17 kDa isoform with this selected batch of antibodies.

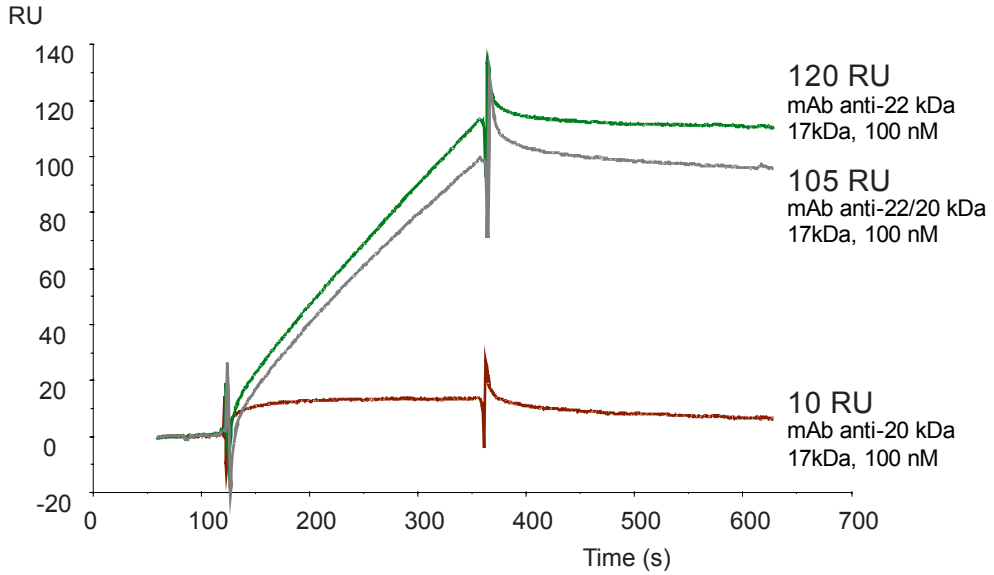

Figure 6. SPR sensorgram of cross-reactivity experiments of the anti-22 kDa, anti-20 kDa and unspecific antibodies towards the 17 kDa isoform.

CONCLUSIONS

Within this paper are described methodologies, other than through recombinant gene-expression, to produce substantial quantities of the 5 kDa and 17 kDa GH isoforms. These have been used for a generation of a specific antibody against the 5 kDa isoform and also to evaluate the affinity towards a selection of monoclonal antibodies. The absolute knowledge of what the antibodies truly recognise in the current GH assays, as well as the developing of specific antibodies towards these isoforms must contribute to the definition of the definitive assay for the detection of exogenous administration of GH.

Acknowledgments

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