Growth Factors, June 2008; 26(3): 152–162

informa
healthcare

Characterisation of the 5 kDa growth hormone isoform

GERARD SUCH-SANMARTÍN[1], JAUME BOSCH[1], JORDI SEGURA[1,2], MOUTIAN WU[3], HONGWU DU[4], GUANGYU CHEN[5], SHAN WANG[3], MIQUEL VILA-PERELLÓ[2,†], DAVID ANDREU[2], & RICARDO GUTIÉRREZ-GALLEGO[1,2‡]

[1]*Pharmacology Research Unit, Municipal Institute for Medical Research (IMIM), Barcelona, Spain,* [2]*Department of Experimental and Health Sciences, Pompeu Fabra University (UPF), Barcelona, Spain,* [3]*China Doping Control Center, National Research Institute of Sports Medicine, Beijing, People's Republic of China,* [4]*Department of Biological Science and Technology, University of Science and Technology, Beijing, People's Republic of China, and* [5]*Laboratory of Molecular Genetics, Beijing Institute of Basic Medical Sciences, Beijing, People's Republic of China*

(Received 6 December 2007; revised form 4 April 2008; accepted 8 April 2008)

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

Abstract

Objectives: The 5 kDa N-terminal fragment of 43 amino acids of human growth hormone (GH) shows a specific and significant *in-vivo* insulin-like activity. This isoform can be easily obtained by solid phase synthesis methods. Our objective in this study is to describe this procedure in detail and to provide structural information of the protein.

Methods: Solid phase synthesis was employed for the synthesis of the 5 kDa GH isoform. Circular dichroism and limited proteolysis have been carried out to provide structural information about the folded state of the protein in solution. Surface plasmon resonance was used to compare the structural equivalence between the synthetic protein and a proteolytic homologue at an antibody binding level. For this purpose, a murine monoclonal antibody specific for the 5 kDa isoform was generated and characterised employing this and several other GH isoforms.

Results: Circular dichroism and proteolysis results suggested that the C-terminal segment of the 5 kDa protein folds in an α-helix. The comparison of the synthetic product to its proteolytic homologue at an antibody binding level suggested structural equivalency. A highly specific antibody against the 5 kDa GH isoform was generated with null cross-reactivity for 17, 20 and 22 kDa isoforms. Kinetic data on the interaction with the synthetic 5 kDa GH was obtained.

Conclusions: The structure of the protein appears to be different in comparison to when it is included within the 22 kDa GH isoform. Finally, a highly specific antibody has been generated. The possible significance of the 5 kDa protein as a potential agent for obesity-related diseases is discussed.

Keywords: *Growth hormone, 5 kDa, GH_{1-43}, solid phase synthesis, limited proteolysis*

Abbreviations: *GH, human growth hormone; T2DM, type 2 diabetes mellitus; SPS, solid phase synthesis; CD, circular dichroism; SPR, surface plasmon resonance; TAT, 1,3,5-triacryloyl-hexahydro-*s*-triazine; TFE, 2,2,2-trifluoroethanol; MALDI-TOF, matrix-assisted laser desorption ionisation-time of flight; Wang resin, 4-(hydroxymethyl) phenoxymethyl; HBTU, 2-1H- benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate; HOBt,* N*-hydrozybenzotriazole; HBS-EP, 10 mM HEPES, 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA) and 0.005% Tween20, pH 7.4); DMF, 20% in* N,N*-dimetilformamide; DIEA,* N,N*-diisopropylethylamine; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; E/S, enzyme–substrate ratio; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RU, resonance units*

Correspondence: R. Gutiérrez-Gallego, Barcelona Biomedical Research Park, C/Doctor Aiguader, 88, E-08003 Barcelona, Spain. Fax: 34 93 316 04 67. E-mail: rgutierrez@imim.es

[†]Present address: Rockefeller University, 1230 York Avenue, New York, NY 10021, USA.

ISSN 0897-7194 print/ISSN 1029-2292 online © 2008 Informa UK Ltd.
DOI: 10.1080/08977190802127952

RIGHTSLINK

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

Introduction

Human growth hormone (GH) is primarily involved in growth and development but also in protein, lipid and carbohydrate metabolism (Ayuk and Sheppard 2006). Human GH does not exist as a unique form, but comprises a family of isoforms mostly produced in the pituitary gland, released to the circulation in a pulsatile manner, and expressing in some cases differentiated biological effects (Baumann 1999; Lewis et al. 2000; Popii and Baumann 2004; De Palo et al. 2005). The main GH isoform is a non-glycosylated 22 kDa sequence of 191 amino acids (AA_{1-191}) structured in four α-helices and several mini-helices. Furthermore, minor constituents of the GH family are generated through alternative splicing (20 and 17.5 kDa), glycosylation (24 kDa), deamidation, homo- and hetero-oligomerisation (Baumann 1999; Lewis et al. 2000; Ryther et al. 2003). The 22 kDa GH isoform is known to be proteolitically processed in different tissues (Wroblewski et al. 1993; Alam et al. 1998; Ge et al. 2007; Komatsu et al. 2007), resulting in a number of isoforms with different biological activity. This fact suggested a pro-hormone role for the 22 kDa isoform (Sinha and Jacobsen 1994; Haro et al. 1996; Rowlinson et al. 1996). A single specific cleavage to generate two contiguous fragments of 5 kDa (AA_{1-43}) and 17 kDa (AA_{44-191}) has been reported to yield significant amounts of these isoforms in circulation (Warner et al. 1993; Lewis et al. 1994, 2000; Sinha and Jacobsen 1994; Lopez-Guajardo et al. 1998). Another cleavage has been reported at the $AA_{134-150}$ segment (Wroblewski et al. 1993; Alam et al. 1998; Ge et al. 2007; Komatsu et al. 2007), although the existence of the resulting fragments has not been confirmed as no specific assay is available. Different biological roles have been reported both for described isoforms and for distinct sequence segments. These include an insulin-like effect for the 5 kDa isoform (Frigeri et al. 1988; Salem 1988; Salem and Wolff 1989) and a diabetogenic effect for the 17 kDa isoform (Lewis et al. 1991; Hettiarachchi et al. 1997), and anti-angiogenic (Struman et al. 1999), lipolytic/anti-lipogenic (Heffernan et al. 2000) and mitogenic (Jeoung et al. 1993) effects for the AA_{1-134}, $GH_{177-191}$ and $GH_{108-129}$ fragments respectively. These activities coincide in some cases with those exerted by the 22 kDa isoform, such as lipolytic/anti-lipogenic or diabetogenic activity (Rosenfalck et al. 2000; Shadid and Jensen 2003), but in other cases represents the opposite such as angiogenic (Struman et al. 1999) or anti-mitogenic (Jeoung et al. 1993) activity. Of interest, anti-diabetogenic/insulin-like activity has also been attributed to GH when the 22 kDa isoform is administered at low doses (Rosenfalck et al. 2000; Yuen et al. 2002, 2005). In the case of the 5 kDa isoform, the insulin-like activity has not been related to concentration or dose, a fact that might be relevant in the treatment of diseases such as type 2 diabetes mellitus (T2DM), where the use of the 22 kDa GH isoform has already been suggested (Ahn et al. 2006).

Studies related to the 5 kDa GH isoform were initiated from the high amount of the protein found in pituitary extracts (Singh et al. 1983). In the past, the 5 kDa protein has been obtained by solid phase synthesis (SPS) methodology (Frigeri et al. 1988; Sinha and Jacobsen 1994; Zhu et al. 2003; Shimizu et al. 2004). Fast access to multi-mg amounts of protein with a high grade of purity are well-known advantages of this methodology, which for relatively small proteins is advantageous over techniques such as purification procedures of human pituitary glands, generation through proteolysis from the 22 kDa GH isoform or recombinant DNA-expression (Rowlinson et al. 1996). However, so far no detailed description of the SPS procedure has been presented or any information of the resulting protein structure has been provided. Considering the significantly different conditions where a protein becomes folded in a SPS environment in comparison to the homologous biological procedure, details of the procedure and resulting structure are not minor issues. Furthermore, the exact determination of the 5 kDa concentration in circulation is still an unresolved issue. Known to be negligible in the pituitary gland (Shimizu et al. 2004), its concentration in circulation has been observed to reach levels comparable to the 22 kDa isoform (Lewis et al. 1994; Lopez-Guajardo et al. 1998).

Consequently, this manuscript describes the synthesis and purification of the 5 kDa GH isoform of AA_{1-43} sequence by SPS methodology, and studies the structure of the protein in solution through circular dichroism (CD) and limited proteolysis experiments. Concerning the existence of endogenous 5 kDa isoform in blood circulation, a murine monoclonal antibody was generated using the synthetic 5 kDa protein, which constitutes a first step in the development of a specific immunoassay for this isoform. The antibody was characterised against 5 kDa and other GH isoforms and kinetic analyses were carried out by means of surface plasmon resonance (SPR). Furthermore, the antibody was assayed against a proteolytic 5 kDa isoform generated through specific cleavage of the 22 kDa isoform. The synthetic protein was expected to show close structure homology to the endogenous protein.

Materials and methods

Materials

Recombinant 22 kDa isoform (genotropin) was a generous gift from Pfizer Laboratories (Barcelona, Spain). Recombinant 20 kDa isoform was kindly provided by Mitsui & Co. LTD (Tokyo, Japan).

RIGHTSLINK

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

Proteolytic 5 kDa (purified sequences AA_{1-43} and AA_{1-44}) and 17 kDa (a mixture of sequences AA_{44-191} and AA_{45-191}) isoforms were obtained by limited cleavage of recombinant 22 kDa isoform in a comparable process to the described by Spolaore et al. (2004), which is to be published in a separate manuscript. Thermolysin, trypsin, 1,3,5-triacryloylhexahydro-*s*-triazine (TAT) and 2,2,2-trifluoroethanol (TFE) were purchased from Sigma-Aldrich (Barcelona, Spain). POROS R2 resin (20 μm) and matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) Sequazyme Peptide Mass Standards Kit were purchased from Applied Biosystems (Barcelona, Spain). Fmoc-protected amino acids were purchased from Senn Chemicals (Dielsdorf, Switzerland). Side chain protecting groups were *t*-butyloxycarbonyl (Lys, Trp), *t*-butyl (Asp, Glu, Ser, Thr, Tyr), 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg) and trityl (Asn, Gln, His). 4-(Hydroxymethyl) phenoxymethyl (Wang) resin loaded with the C-terminal Fmoc-Ser(*t*Bu) was from Bachem (Bubendorf, Switzerland). 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*-hydrozybenzotriazole (HOBt) were from Albatross Chem. (Montreal, Canada). CM5 sensor chips and HBS-EP buffer saline (10 mM HEPES, 150 mM NaCl, 3.4 mM ethylenediaminetetraacetic acid (EDTA) and 0.005% Tween20, pH 7.4) were purchased from Biacore (Izasa, Barcelona, Spain).

Protein synthesis

Solid phase synthesis was performed on an ABI 433A synthesizer (Applied Biosystems, Foster City, CA, USA) running Fmoc/tBu chemistry programs. The synthesis was initiated at the C-terminus (AA_{43}) with serine-functionalised Wang resin (182 mg equivalent to 0.1 mmol of amino acid): N-terminal Fmoc groups were removed with piperidine (20% in *N*,*N*-Dimetilformamide (DMF)), the resin was washed with DMF and the next Fmoc-protected amino acid, activated with HBTU/HOBt in the presence of N,N-diisopropylethylamine (DIEA); amino acid:HBTU:HOBt:DIEA (ratio 1:1:1:2) was coupled in an excess of 10 equivalents within the 20–40 min range adjusted to the nature of each residue, followed by extensive washes with DMF. Coupling of predicted difficult linkage between Glu_{33} and Glu_{32} (Peptide Companion software, CoshiSoft, San Diego, CA, USA) was performed manually and repeated until a negative Kaiser ninhydrin test (Kaiser et al. 1970) was obtained. Manual synthesis included prolonged deprotection times (20% piperidine in DMF, 2 × 5 min) to ensure quantitative Fmoc removal, as well as longer couplings (30 min or more). Side chains deprotection and resin cleavage of the full-length protein sequence was done by treatment with trifluoroacetic acid (TFA) in the presence of scavengers (TFA:ethanedithiol:water:triisopropylsilane 94:2.5:2.5:1) for 90 min. The protein material was precipitated from the cleavage filtrate by addition of chilled methyl *t*-butyl ether and centrifugation (4000 rpm, 5°C, 10 min). The crude protein pellet was solubilised in a minimal volume of acetic acid (10% v/v), freeze-dried and subsequently purified by preparative reversed phase high-performance liquid chromatography (HPLC).

Reversed phase HPLC

The synthesised protein resuspended in H_2O mQ was purified by preparative reversed phase HPLC (Shimadzu LC-8A) on a Phenomenex Luna C8 column (10 μm, 2.1 × 25 cm) eluted with a 5–65% acetonitrile gradient (60 min, 25 ml/min, 220 nm UV detection). Fractions estimated to be of sufficient purity by analytical HPLC were pooled, freeze-dried and further characterised by MALDI-TOF mass spectrometry.

Generation of anti-5 kDa monoclonal antibody

Two six weeks old female Balb/c mice were immunised three times by subcutaneous injection. The first immunisations were with 50 μg of synthetic AA_{1-43} in Freund's complete adjuvant, followed by a second and third immunisations with 50 μg of synthetic AA_{1-43} in Freund's incomplete adjuvant every two weeks, respectively. One month following the last immunisation, serum was obtained from each mouse and assayed for the presence of antibodies specific to synthetic AA_{1-43}, with the screening enzyme-linked immunosorbent assay (ELISA) procedure, described below. The mouse with the highest antibody titre received a boost of 50 μg of synthetic AA_{1-43} in Freund's incomplete adjuvant three days prior to removal of the spleen. The splenocytes were fused to SP2/0 myeloma cells using the procedure described by Kennett (1979). Hybridomas were selected in hypoxanthine–aminopterin–hymidine medium. Hybridomas producing anti-synthetic AA_{1-43} were screened through the antibody capture ELISA. A limited number of hybridomas corresponding to a single clone were re-assayed for anti-5 kDa activity through the supernatants, selecting the clone of highest sensitivity. *ELISA procedure*: High binding ELISA plates (Corning 3922, Corning, New York, USA) were coated with 100 μl/well of a 1 μg/ml synthetic AA_{1-43} solution and incubated overnight at 4°C. The wells were blocked with 200 μl/well of 1% bovine serum albumin (BSA)-PBS (8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride, 1% w/v BSA, pH 7.4) for 1 h at 37°C. A total of 100 μl/well of hybridoma culture supernatant or two-fold serial

RIGHTSLINK

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

dilutions of monoclonal antibody in 1% BSA-PBS were added to the plate for 1 h at 37°C. The wells were washed three times with PBS-Tween 20 (8 mM sodium phosphate, 2 mM potassium phosphate, 140 mM sodium chloride, 10 mM potassium chloride, 0.1% v/v Tween 20, pH 7.4). Hundred microlitre of peroxidase-coupled goat anti-mouse IgG (Immuno-Hunt, Beijing, China) at a dilution of 1:10,000 were then added to the wells and the binding reaction was carried out for 1 h at 37°C. The wells were then washed five times with PBS-Tween 20. A total of 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate solution was then added to each well. After 15 min incubation, the reaction was stopped with the addition of 50 μl/well of 2 M H_2SO_4. The optical density (450 nm) of each well was subsequently measured with a Tecan Sunrise Elisa plate reader. Hybridomas of interest were further subcloned by limiting dilution. Supernatants from wells containing a single clone were re-screened to verify the binding to synthetic AA_{1-43}.

Circular dichroism

CD spectra were acquired on a Jasco J-810 spectropolarimeter (Jasco Corporation, Tokyo, Japan) purged with nitrogen (25 ml/min) and using a quartz 0.1 cm path length cell fitted to a Neslab RP-100 thermostated device (Bonsai Technologies, Barcelona, Spain) set at 5°C, at protein concentration of 15 μM in 25 mM sodium phosphate buffer, pH 7.4. Alpha-helical secondary structure formation was induced by solvent titration with 10–50% (v/v) TFE. For each spectrum, two 190–260-nm scans were accumulated, at 10 nm/min, with 0.2 nm resolution, 2 s response, and 1.0 nm band width. Data were analyzed by the spectral deconvolution JFIT software (Rupp 1996).

Mass spectrometry

Mass spectra were recorded on a Voyager-DE STR Biospectrometry workstation (Applied Biosystems, Barcelona, Spain) using a pulsed nitrogen gas laser (337 nm) in positive linear mode, with accelerating voltage of 20 kV. Spectra were generated from the accumulation of 500 laser pulses. Sample aliquots (1 μl volume) were mixed with either α-cyano-4-hydroxycinnamic or sinapinic acid matrices diluted in acetonitrile:trifluoroacetic acid (50:0.1% v/v) saturated aqueous solution in 1:1 (v/v) proportion. Calibration was performed with the Sequazyme Peptide Mass Standards kit and data analysed with the Data Explorer software version 4.5 (Applied Biosystems). Peptide mass fingerprint analyses used 200 nmols of protein digested with trypsin at an enzyme–substrate (E/S) ratio of 1/50 (w/w) for 16 h at 37°C in 10 mM calcium chloride solution (pH 7.0), purified through Geloader tips containing Poros R2 resin (Gobom et al. 1999). Limited proteolysis was carried out using 0.05 g/l protein concentrations digested with thermolysin at an E/S ratio of 1/50 (w/w) in 10 mM calcium chloride solutions (pH 4.0/7.0). The progress of the reaction was monitored at different time points measuring aliquots directly mixed with the matrix and crystallised onto the plate.

Cross-linking experiments

Intra-molecular cross-linking experiments of the 5 kDa isoform used 0.4 g/l protein concentrations in 10 mM calcium chloride solutions, pH 7.0 (minimum incubation of 60 min of the protein in solution at room temperature) with 1 μl of TAT (aqueous saturated solution). The final mixture was incubated at 37°C during 1 h and subsequently analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Mini-Protean 3 cell electrophoresis system (Bio-Rad, Barcelona, Spain), 150 V, 15% acrylamide, silver staining procedure (Gorg et al. 2000).

Surface plasmon resonance experiments

SPR experiments were carried out on a BIAcore™ 3000 (25.0°C). Immobilisation of the antibodies onto a CM5 sensor chip was done through standard amine coupling (HBS-EP 5 μl/min, 800 μg/ml 65 μl anti-5 kDa antibody in 10 mM pH 5.0 sodium acetate). The reference surface was generated by simple activation and deactivation steps, following the same coupling procedure. Sensorgrams were recorded at a flow rate of 5–10 μl/min and analyte (typically a sample volume for a 3-min association phase) was allowed to interact with the surface-bound material. Typically samples were allowed to dissociate from the surface upon reaching baseline levels, prior to the following sample injection. Kinetic constants were obtained from a series of injections of the 5 kDa protein. Regenerations, when required, were achieved through injections with formic acid solutions (1%). Acquired data were evaluated with the simultaneous estimation of association and dissociation constants, applying the 1:1 Langmuir binding model including the mass transport effects using the BIAcore Control Software v4.0.1.

Results

Synthesis of 5 kDa protein

The solid phase synthesis of the 43 amino acid protein corresponding to the 5 kDa GH isoform was successfully accomplished through Fmoc/*t*-butyl chemistry (Fields and Noble 1990). The main peak from the reversed-phase HPLC purification of the synthetic material gave a MALDI-TOF mass spectra (Figure 1) with a main singly charged ion at *m*/*z* 5216.93, consistent with the expected average molecular mass

RIGHTSLINK

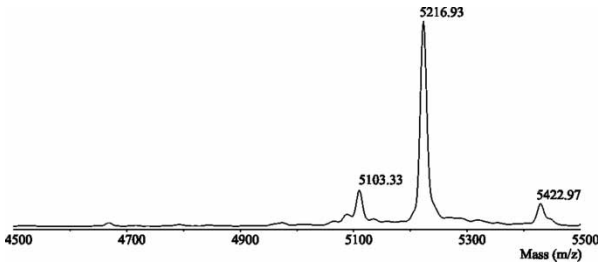

Figure 1. MALDI-TOF spectrum of the synthesised 5 kDa protein after purification by HPLC. An aliquot (1 μl) of the HPLC collected peak (5–65% acetonitrile gradient) corresponding to the pure protein was directly crystallised with sinapinic matrix (1:1 v/v).

of 5215.92 Da, a peak 206 Da above the previous one, corresponding to a sinapinic acid photo-adduct (Beavis and Chait 1990), and a third peak at *m/z* 5103.33 Da, i.e. 113.60 Da below the molecular ion, attributed to deletion of either Ile or Leu (113.16 Da of theoretical difference, average mass). This low-abundance deletion product could not be purified-out by further HPLC steps. Concerning the specific linkage between amino acids 33 and 32 that was manually performed, no deletion was detected. A total of 110 mg of the protein was obtained, representing an overall yield of 25% with respect to the initial amount of resin.

Peptide mass fingerprint

Verification of the protein sequence was achieved by peptide mass fingerprinting following trypsin digestion. The resulting mass spectrum (Figure 2) showed a number of peaks in the mass range between 700 and 3500 Da that can be assigned to the 5 kDa isoform (sequence Phe-Pro-Thr-Ile-Pro-Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala-Met-Leu-Arg-Ala-His-Arg-Leu-His-Gln-Leu-Ala-Phe-Asp-Thr-Tyr-Gln-Glu-Phe-Glu-Glu-Ala-Tyr-Ile-Pro-Lys-Glu-Gln-Lys-Tyr-Ser). Peaks at isotopic *m/z* 930.53, 979.50, 1890.97, 2706.25, 3091.47 and 3341.61 corresponded to the sequences AA_{1-8}, AA_{9-16}, AA_{1-16}, AA_{17-38}, AA_{17-41} and AA_{17-43}, respectively. An additional peak at *m/z* 1734.88 was attributed to the sequence AA_{1-15}, an unexpected cleavage product. These peaks covered the total sequence of the 5 kDa verifying the correct amino acid sequence of the product (Table I). Furthermore, an additional peak was observed at *m/z* 817.45. This peak showed a mass difference of 113.08 Da with respect to the AA_{1-8} sequence and was consistent with the Ile/Leu deletion observed in the main HPLC fraction, detected here with isotopic resolution. While the mass difference clearly suggested the absence of either Ile_4 or Leu_6, the precise position of the deletion could not be established, as electrospray ionisation tandem mass spectrometry experiments yielded poor fragmentation, probably due to the adjacent Pro residue. On the basis of MALDI-TOF spectra peak intensities, this deletion-analogue was estimated to be less than 5% of the total amount of protein.

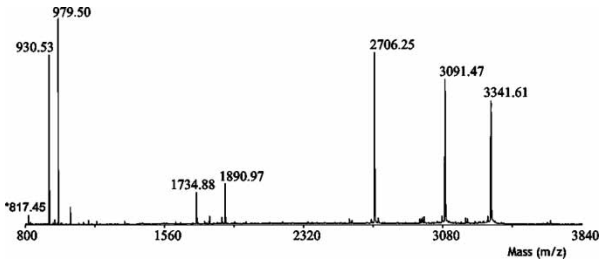

Figure 2. MALDI-TOF spectrum of a tryptic digest of the 5 kDa protein. Identification of the observed masses is included in Table I. Approximately, 200 nmols of protein were digested at an enzyme–substrate ratio of 1/50 (w/w) for 16 h at 37°C in 10 mM pH 7.0 calcium chloride solution. The digest was purified through Poros R2 resin, eluting directly with α-cyano-4-hydroxycinnamic matrix in acetonitrile:trifluoroacetic acid (50:0.1%) aqueous solution.

Circular dichroism

Information about the secondary structure of the 5 kDa synthetic protein was assessed by far-UV circular dichroism. The protein was measured in buffer and after addition of TFE, where a reinforcement of the secondary structure and a disruption of the tertiary structure is expected (Buck 1998). Figure 3 shows overlaid spectra corresponding to 0, 20 and 50% (v/v) of TFE (right-hand side graph corresponds to a normalised plot). Spectra were deconvoluted by means of the Yang equation (Yang et al. 1986) to give 40, 78 and 98% of α-helical content for 0, 20 and 50% TFE concentrations, respectively.

Limited proteolysis

Limited proteolysis of the synthetic 5 kDa isoform provided detailed information about local structure in relation to the amino acids sequence. Digestions

Table I. Mass identification of generated monoisotopic ions from trypsin proteolysis of the 5 kDa protein.

Fragment sequence	Theoretical mass ion	Mass ion
AA_{1-8}*	817.37	817.45
AA_{1-8}	930.53	930.53
AA_{9-16}	979.49	979.50
AA_{1-15}	1734.91	1734.88
AA_{1-16}	1891.02	1890.97
AA_{17-38}	2706.32	2706.25
AA_{17-41}	3091.52	3091.47
AA_{17-43}	3341.61	3341.61

*Ion corresponding to a shorter sequence of the synthesis, probably by deletion of Ile4 or Leu6.

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

RIGHTSLINK

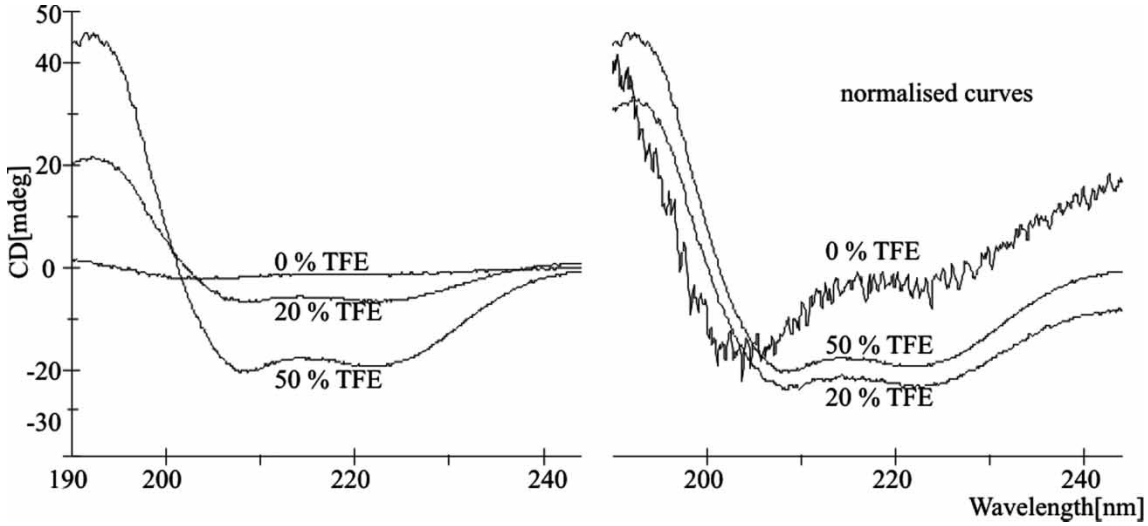

Figure 3. Circular dichroism spectrum of the synthesised protein (15 μM protein concentration in 25 mM sodium phosphate buffer (pH 7.4)) mixed with 0, 20 and 50% of 2,2,2-trifluoroethanol. On the right the normalised view of the curves is represented.

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

were carried out with thermolysin at pH 7.0, yielding a cleavage pattern shown in Figure 4(A), and upon acidification and thus denaturation of the protein (Fink et al. 1994), yielding a cleavage pattern shown in Figure 4(B). Obtaining a particular fragment pattern different from the theoretical cleavage (depicted in Figure 5) is directly related to how exposed and flexible the structure of the protein is (Hubbard 1998). Fragment assignment of peaks observed in neutral and acidic pH are showed in Table II. At acidic pH thermolysin was strongly degraded and longer times of reactions were required to obtain the first peaks of the digestion. Modifications of thermolysin specificity in function of media pH were discarded with equal patterns observed in acidic and neutral digestions with other proteins (not shown).

5 kDa cross-linking

Actual state of the 5 kDa protein oligomerisation was determined through covalent intra-molecular cross-linking reactions of the oligomers in solution and posterior analysis with SDS-PAGE electrophoresis. Whereas covalent oligomers could be directly viewed in the gel, non-covalent oligomers required previous covalently fixing by TAT reagent (Dienys et al. 1998). The existence of covalent dimers were not observed in protein analysis without TAT reagent (Figure 6, lane 1), but multiple non-covalent oligomers were showed under the same buffer conditions used in limited proteolysis (Figure 6, lane 3). This non-covalent oligomerisation state was disrupted with the addition of urea (8 M) as expected (Bennion and Daggett 2003), observing exclusively the monomer in such conditions (Figure 6, lane 4).

Characterisation of the anti-5 kDa antibody

Murine monoclonal antibody from the final selected clone specific against the synthetic 5 kDa protein was characterised by means of SPR. The antibody was immobilised on the SPR CM5 surface through standard amine chemistry (Johnsson et al. 1991) obtaining a high density surface [11,500 resonance units (RU)]. Saturation of the antibody at the surface was obtained at a concentration of 1 μM of 5 kDa protein. This yielded a signal of 600 RU. Compared to the theoretical maximum response (R_{max}, calculated using the formula provided by the manufacturer), the 600 RU empirical value represented a 75% of the theoretical R_{max} value, demonstrating particularly good surface properties of the antibody. The sensitivity of the surface was approached by a series of injections within the nanomolar range (2–26 nM) of the protein in buffer matrix (Figure 7(A)). The lowest concentration detected was situated at the 2–6 nM 5 kDa value. From equivalent sensorgrams obtained after successive injections of 5 kDa protein at equivalent concentrations within the same nanomolar range, a mathematical model was adjusted to calculate the kinetic constants (Table III), obtaining an equilibrium dissociation constant (Kd) of 55.4 nM. Crossreactivity information was obtained after injection of recombinant 20 and 22 kDa GH isoforms and proteolytic 5 and 17 kDa GH isoforms over the surface. Sensorgrams obtained did not show any affinity of the antibody for the 17, 20 and 22 kDa GH isoforms at concentrations as high as 50 nM (Figure 7(B)), concentrations between 100 and 1000 times higher than what would be expected to be found in blood. In the case of the proteolytic 5 kDa isoform, the affinity for the antibody was equivalent to that of the synthetic 5 kDa isoform, producing both a signal around 140 RU at the same 50 nM concentration (Figure 7(B)).

RIGHTSLINK

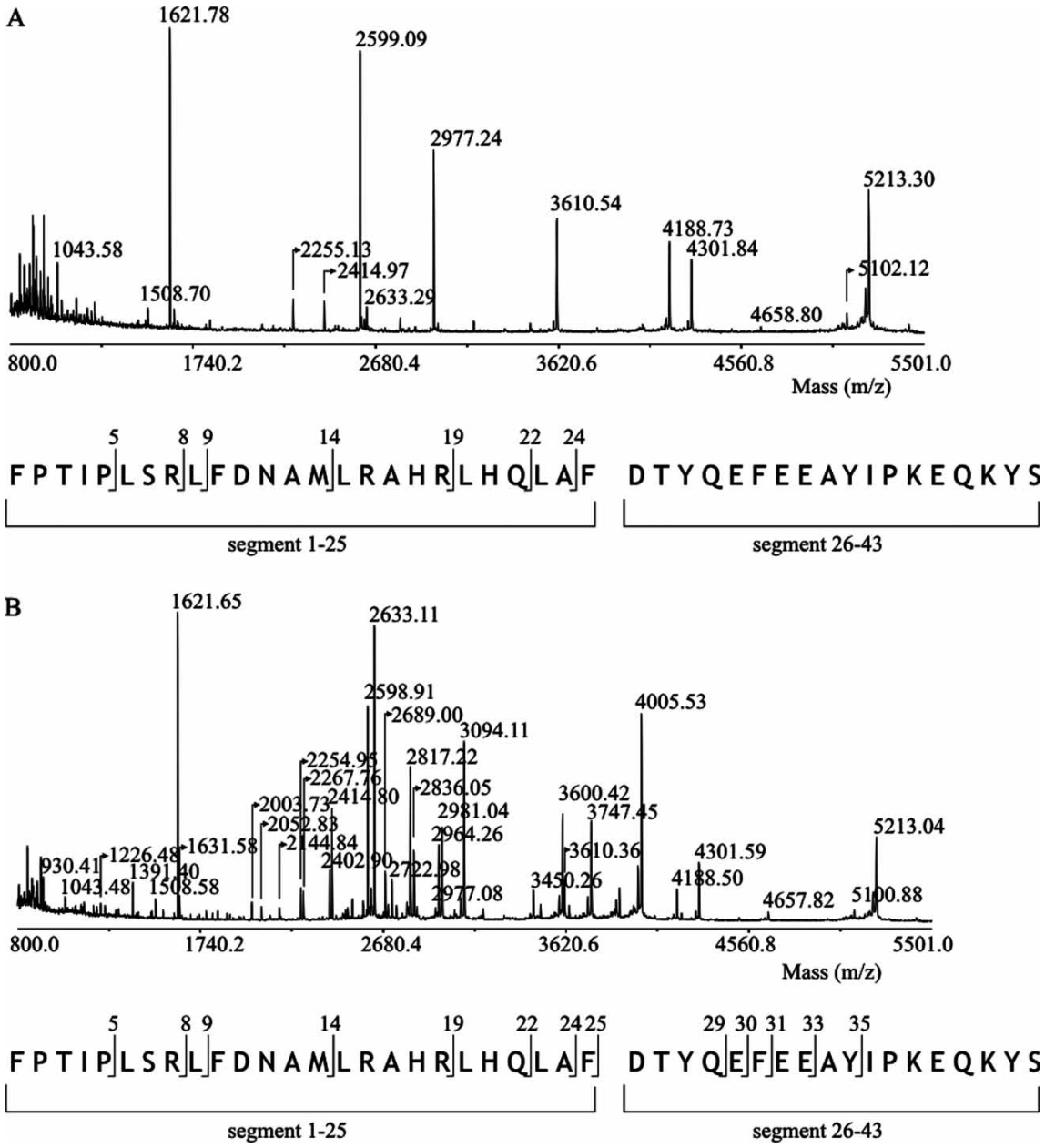

Figure 4. MALDI-TOF spectrum of 5 kDa fragments generated from a limited thermolysin proteolysis carried out at (A) pH 7, minute 5, and (B) pH 4, minute 240. Identification of the observed ions is included in Table II. Five kiloDalton protein was diluted to a 0.05 g/l concentration and digested with thermolysin at an enzyme–substrate ratio of 1/50 (w/w), at room temperature.

Within the generation process of the proteolytic 5 kDa of sequence AA_{1-43}, deriving from the 22 kDa sequence AA_{1-191}, the sequence AA_{1-44} was also generated, separated and purified. This variant was also assayed against the antibody observing a comparable affinity to that showed by both the proteolytic and the synthetic 5 kDa, although obtaining a slightly lower value around 110 RU (Figure 7(B)), indicative of a lower affinity.

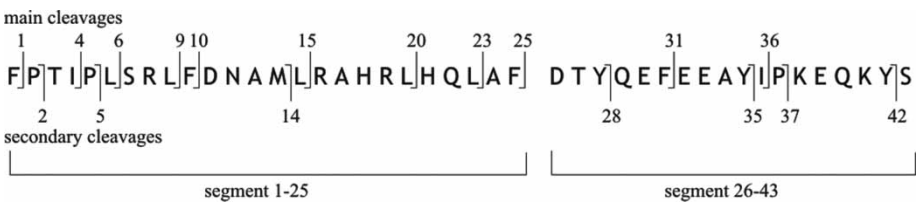

Figure 5. Scheme of the amide bonds cleaved by thermolysin of the 5 kDa sequence in a totally exposed structure. Segments AA_{1-25} and AA_{26-43} are indicated to illustrate the differences of enzyme activity in both regions.

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

RIGHTSLINK

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

Table II. Table of fragments (monoisotopic mass) of the 5 kDa after proteolysis with thermolysin. Fragments are grouped by segments of amino acids (separated by dotted line) sharing the same C-terminus of the sequence.

Identified fragment	Theoretical mass ion	Mass ion (pH 7)	Mass ion (pH 4)
AA_{1-43}	5213.64	5213.30	5213.04
AA_{1-43}*	5100.55	5102.12	5100.88
AA_{6-43}	4658.33	4658.80	4657.82
AA_{9-43}	4302.11	4301.84	4301.59
AA_{10-43}	4189.03	4188.73	4188.50
AA_{15-43}	3610.81	3610.54	3610.36
AA_{20-43}	2977.43	2977.24	2977.08
AA_{23-43}	2599.23	2599.09	2598.91
AA_{25-43}	2415.11	2414.97	2414.80
AA_{26-43}	2268.04	–	2267.76
AA_{31-43}	1631.80	–	1631.58
AA_{34-43}	1226.65	–	1226.48
AA_{20-35}	2003.91	–	2003.73
AA_{1-33}	4006.01	–	4005.53
AA_{6-33}	3450.71	–	3450.26
AA_{9-33}	3094.49	–	3094.11
AA_{10-33}	2981.40	–	2981.04
AA_{15-33}	2403.19	–	2402.90
AA_{23-33}	1391.60	–	1391.40
AA_{1-31}	3747.93	–	3747.45
AA_{9-31}	2836.40	–	2836.05
AA_{10-31}	2723.32	–	2722.98
AA_{15-31}	2145.10	–	2144.84
AA_{1-30}	3600.86	–	3600.42
AA_{9-30}	2689.34	–	2689.00
AA_{1-25}	2964.62	–	2964.26
AA_{9-25}	2053.10	–	2052.83
AA_{1-24}	2817.55	–	2817.22
AA_{1-22}	2633.43	2633.29	2633.11
AA_{1-19}	2255.23	2255.13	2254.95
AA_{1-14}	1621.85	1621.78	1621.65
AA_{1-14}*	1508.76	1508.70	1508.58
AA_{1-9}	1043.63	1043.58	1043.48
AA_{1-8}	930.55	–	930.41

*Ions corresponding to a shorter sequence of the synthesis, probably by deletion of Ile4 or Leu6.

Discussion

Synthesis and structure of 5 kDa GH isoform

The 5 kDa isoform was successfully synthesised, obtaining more than 100 mg of protein within a short period of time. The final structure of the protein is derived from the inherent tendency of the sequence once deprotected and released from the resin. This folded state could differ from the endogenous structure of the isoform, which is originated from the initial structure of the AA_{1-43} sequence when included in the main 22 kDa isoform. As such, structural characterisation of the synthetic isoform was approached through CD and limited proteolysis experiments. CD provided a global insight of the total secondary structure of the protein and showed a partial helicoidal structure of the 5 kDa isoform in agreement with the conformation shown on the AA_{1-43} sequence within the 22 kDa structure

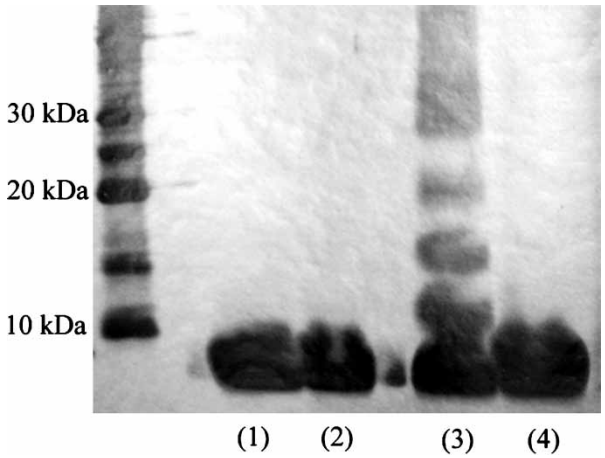

Figure 6. SDS polyacrylamide (15%) gel of 5 kDa synthetic protein (0.4 g/l) in 10 mM calcium chloride solutions, pH 7.0, after 1 h of incubation at 37°C. Left lane: molecular weight markers. Lane 1: 5 kDa protein. Lane 2: 5 kDa protein with urea (8 M). Lane 3: 5 kDa protein with 1,3,5-triacryloyl-hexahydro-*s*-triazine reagent (TAT). Lane 4: 5 kDa protein with urea (8 M) and TAT.

(Kasimova et al. 2002). The addition of TFE to the media resulted in a fast increment of the α-helix content revealing a low resistance of the rest of the protein to adopt a folded state, synonym of a notable grade of flexibility within the chain. Limited proteolysis allowed to detail which segments of the sequence were folded and which were not. Limited proteolysis experiments are based on the premises that the activity of broad-specificity proteases (e.g. thermolysin) (Park and Marqusee 2004), over amino acid chains is guided by the folded state of the sequence (Hubbard 1998). If extensive proteolysis is done all cleavage positions become available yielding a full cleavage, but in a time-controlled reaction those regions more accessible and less structured are preferentially cleaved. Proteolysis of the 5 kDa isoform with thermolysin in a neutral pH environment showed a major activity over the AA_{1-25} N-terminal part of the sequence (Table II), thus suggesting that under those conditions the Cterminal segment of the protein sequence (approximately AA_{26-43}) existed in a folded state. To corroborate this findings an additional proteolysis was performed under acidic conditions where enzyme activity was verified to be fully preserved while the protein was completely denatured. Under such conditions the protein became unfolded and the enzyme extensively cleaved the whole sequence (Table II).

Furthermore a possible influence of protein oligomerisation towards the enzyme activity was studied. Oligomers of the 5 kDa protein were covalently fixed through intra-molecular cross-linking of protein nucleophilic functional groups at neutral pH. Subsequently these were assayed through SDS-PAGE (Figure 6). The oligomeric organisation of the protein could stabilise a different structure leading to a thermolysin cleavage pattern not indicative of the real

RIGHTSLINK

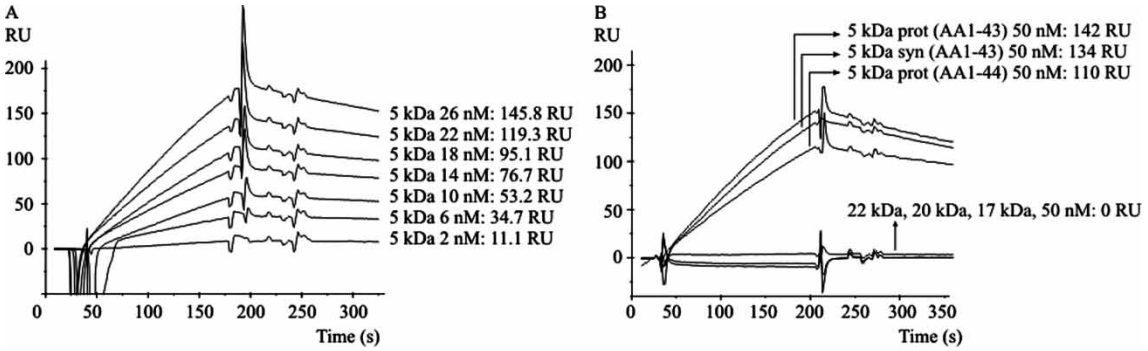

Figure 7. SPR sensorgrams of interaction between immobilised monoclonal antibody and (A) synthetic 5 kDa isoforms at 2 to 26 nM concentrations and (B) synthetic 5 kDa isoform, proteolytic 5 kDa isoform, AA_{1-44} proteolytic 5 kDa protein, 17 kDa isoform, 20 kDa isoform and 22 kDa isoform at 50 nM concentrations, employing 5 μl/min flow rate and 15 μl of injection volume. First part of the sensorgrams (positive slope) corresponds to the association phase of the interaction where the antigen is binding to the antibody. The second part of the sensorgrams (negative slope) corresponds to the dissociation phase of the interaction while flowing buffer across.

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

structure of the monomer, although representative of the solution state of the protein. Oligomers of the 5 kDa protein formed at neutral pH, which were disrupted with the addition of 8 M urea. However, limited proteolysis of the 5 kDa protein with and without 8 M urea (not shown) did not reveal any difference in the obtained cleavage pattern, indicative of a lack of relation between the activity of thermolysin and the existence of 5 kDa oligomers in solution.

In conclusion, the structure of the synthetic 5 kDa isoform is shown to have the N-terminal part of the protein (AA_{1-25}) apparently unfolded and the C-terminal part (AA_{26-43}) firmly structured, in physiological-like pH conditions. This constitutes a modification in the structure of the AA_{1-43} sequence in comparison when it is included into the AA_{1-191} 22 kDa sequence. Within the latter, the 5 kDa fragment conforms the first helix of the 22 kDa protein (Kasimova et al. 2002), having therefore the central part of the 5 kDa sequence structured in an helicoidal state. The C-terminal part of the 5 kDa sequence is known to constitute part of the first mini-helix of the 22 kDa isoform, but it has been observed that such mini-helix might not be present in the 22 kDa free isoform, i.e. when is not bound to the GH binding protein (Kasimova et al. 2002).

Monoclonal antibody against 5 kDa GH isoform

The generation of a monoclonal antibody employing the synthesised protein was followed by a characterisation by means of SPR experiments. Cross-reactivity with other GH isoforms was assayed in sight of the large segments of identical sequences shared between them. The group of included isoforms consisted of the main 22 kDa isoform of AA_{1-191} sequence, the 5 and 17 kDa isoforms of AA_{1-43} and AA_{44-191} sequences, and the 20 kDa isoform of $A_{(1-31)-(47-191)}$ sequence (the AA_{1-191} sequence lacking the segment AA_{32-46}) reported to represent a 5–10% of the circulating GH (Hashimoto et al. 1998). These proteins could constitute the most populated GH isoforms in circulation. SPR results showed the highest specificity for the synthetic 5 kDa isoform not showing any interaction towards the other isoforms in concentrations as high as 50 nM (where endogenous values could lie within the picomolar range). The null interaction of the antibody towards the 22 kDa isoform, in spite of sharing the AA_{1-43} sequence, could be explained by the different structure of the 5 kDa sequence when it constitutes a unique fragment. Interestingly, this lack of cross-reactivity towards the 22 kDa isoform was also reported in two other specific antibodies against the 5 kDa isoform (Lopez-Guajardo et al. 1998; Shimizu et al. 2004). In this case, the stated different structure could actually be caused by the synthetic origin of the protein. This hypothesis was discarded at an antibody binding level through the use of an alternative 5 kDa protein of the same AA_{1-43} sequence originating from a specific cleavage onto the 22 kDa sequence, in a similar procedure to that described elsewhere (Spolaore et al. 2004). The structure of this fragment derives from the structure of the main 22 kDa isoform and consequently, it is expected to maintain the maximum homology with the native 5 kDa isoform, also generated by proteolysis. The equivalent signals measured by SPR with both 5 kDa proteins obtaining totally superimposable curves suggests an equivalent structure for both 5 kDa isoforms.

Table III. Kinetic values obtained from SPR measurements of the antibody anti-5 kDa interacting with the synthetic 5 kDa GH isoform.

ka (1/Ms)	kd (1/s)	Ka (1/M)	Kd (M)	χ^2
4.55×10^4	2.52×10^{-3}	1.8×10^7	5.54×10^{-8}	4.93

ka, kd: rate constants. Ka, Kd: equilibrium constants

The kinetic characterisation of the antibody against the 5 kDa protein revealed an equilibrium dissociation constant (Kd) of 50 nM. The surface properties in

RIGHTSLINK

terms of sensitivity of the antibody in relation to the chemistry used in the SPR immobilisation procedure were significant, obtaining an approximated 75% well-oriented antibodies. Considering the random-oriented amine chemistry used (Bonroy et al. 2006), this percentage is clearly superior to typical obtained values in our laboratory lying within a 10–30% range. As a result, with this antibody it was possible to detect 5 kDa in SPR down to the low nanomolar range of 5 kDa in non-biological samples. Further work on the detection of 5 kDa isoform in complex biological matrix is ongoing.

Possible biological relevance of the 5 kDa GH isoform

The possible role of 5 kDa isoform as a native insulin mediator could represent a new possible therapeutic target for insulin-related and obesity-related diseases such as diabetes (Lewis et al. 2000). How the 5 kDa isoform stimulates tissue by promoting the insulin action is uncertain. The action of the 5 kDa isoform is known not to be specific for the adipose tissue (Frigeri et al. 1988). Different segments of the 5 kDa isoform comprising mostly of its sequence were studied (Frigeri et al. 1988; Ohkura and Hori 2000) and separate activity for different sequences on different tissues was suggested (Frigeri et al. 1988). The accentuated insulin-like effects of the 5 kDa isoform in comparison with those shown by the 22 kDa isoform could be related to the different structure adopted by the 5 kDa fragment once cleaved from the 22 kDa isoform. Of special interest, the 5 kDa protein lacks any diabetogenic or anti-insulin effects, which on the contrary has been shown in the 22 kDa isoform (Rosenfalck et al. 2000; Shadid and Jensen 2003). Indeed, although the 22 kDa protein is currently being evaluated as a potential therapeutic agent for its insulin-like effects (Ahn et al. 2006), the 5 kDa protein could constitute a better candidate. Furthermore, the recent identification of the 5 kDa protein as an *in-vitro* dipeptidyl peptidase IV (DP-IV) substrate (Zhu et al. 2003) might promote the study of possible relations with the use of DP-IV inhibitors or protein mimetics in analogy to incretin mimetics (Boyle and Freeman 2007) in treatments for T2DM.

Acknowledgements

We thank Drs M.L. Valero for assistance with the peptide synthesis and B.Ó. Mathúna for careful reading of the manuscript. This project has been carried out with financial support of WADA and the Ministry of Science and Technology of China (contract number: 2003DA904B05).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Ahn CW, Kim CS, Nam JH, Kim HJ, Nam JS, Park JS, Kang ES, Cha BS, Lim SK, Kim KR, Lee HC, Huh KB. 2006. Effects of growth hormone on insulin resistance and atherosclerotic risk factors in obese type 2 diabetic patients with poor glycaemic control. Clin Endocrinol (Oxf) 64:444–449.

Alam KS, Morimoto M, Yoshizato H, Fujikawa T, Furukawa K, Tanaka M, Nakashima K. 1998. Expression and purification of a mutant human growth hormone that is resistant to proteolytic cleavage by thrombin, plasmin and human plasma in vitro. J Biotechnol 65:183–190.

Ayuk J, Sheppard MC. 2006. Growth hormone and its disorders. Postgrad Med J 82:24–30.

Baumann G. 1999. Growth hormone heterogeneity in human pituitary and plasma. Horm Res 51(Suppl 1):2–6.

Beavis RC, Chait BT. 1990. Rapid, sensitive analysis of protein mixtures by mass spectrometry. Proc Natl Acad Sci USA 87:6873–6877.

Bennion BJ, Daggett V. 2003. The molecular basis for the chemical denaturation of proteins by urea. Proc Natl Acad Sci USA 100:5142–5147.

Bonroy K, Frederix F, Reekmans G, Dewolf E, De Palma R, Borghs G, Declerck P, Goddeeris B. 2006. Comparison of random and oriented immobilisation of antibody fragments on mixed self-assembled monolayers. J Immunol Methods 312:167–181.

Boyle PJ, Freeman JS. 2007. Application of incretin mimetics and dipeptidyl peptidase IV inhibitors in managing type 2 diabetes mellitus. J Am Osteopath Assoc 107(Suppl):S10–S16.

Buck M. 1998. Trifluoroethanol and colleagues: cosolvents come of age. Recent studies with peptides and proteins. Q Rev Biophys 31:297–355.

De Palo EF, De FV, Gatti R, Spinella P. 2005. Growth hormone isoforms and segments/fragments: molecular structure and laboratory measurement. Clin Chim Acta.

Dienys G, Sereikaite J, Gavenas G, Kvederas R, Bumelis VA. 1998. Cross-linking of protein subunits by 1,3,5-triacryloyl-hexahydro-s-triazine. Bioconjug Chem 9:744–748.

Fields GB, Noble RL. 1990. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int J Pept Protein Res 35:161–214.

Fink AL, Calciano LJ, Goto Y, Kurotsu T, Palleros DR. 1994. Classification of acid denaturation of proteins: intermediates and unfolded states. Biochemistry 33:12504–12511.

Frigeri LG, Teguh C, Ling N, Wolff GL, Lewis UJ. 1988. Increased sensitivity of adipose tissue to insulin after in vivo treatment of yellow Avy/A obese mice with amino-terminal peptides of human growh hormone. Endocrinology 122:2940–2945.

Ge G, Fernandez CA, Moses MA, Greenspan DS. 2007. Bone morphogenetic protein 1 processes prolactin to a 17-kDa antiangiogenic factor. Proc Natl Acad Sci USA 104:10010–10015.

Gobom J, Nordhoff E, Mirgorodskaya E, Ekman R, Roepstorff P. 1999. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. J Mass Spectrom 34:105–116.

Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, Weiss W. 2000. The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21:1037–1053.

Haro LS, Singh RN, Lewis UJ, Martinez AO, Galosy SS, Staten NR, Krivi GG. 1996. Human growth hormone deletion mutant (hGH44-191) binds with high affinity to lactogenic receptors but not to somatogenic receptors. Biochem Biophys Res Commun 222:421–426.

Hashimoto Y, Ikeda I, Ikeda M, Takahashi Y, Hosaka M, Uchida H, Kono N, Fukui H, Makino T, Honjo M. 1998. Construction of a

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

RIGHTSLINK

specific and sensitive sandwich enzyme immunoassay for 20 kDa human growth hormone. J Immunol Methods 221:77–85.

Heffernan MA, Jiang WJ, Thorburn AW, Ng FM. 2000. Effects of oral administration of a synthetic fragment of human growth hormone on lipid metabolism. Am J Physiol Endocrinol Metab 279:E501–E507.

Hettiarachchi M, Watkinson A, Leung KC, Sinha YN, Ho KK, Kraegen EW. 1997. Human growth hormone fragment (hGH44-91) produces insulin resistance and hyperinsulinemia but is less potent than 22kDa hGH in the rat. Endocrine 6:47–52.

Hubbard SJ. 1998. The structural aspects of limited proteolysis of native proteins. Biochim Biophys Acta 1382:191–206.

Jeoung DI, Allen DL, Guller S, Yen V, Sonenberg M. 1993. Mitogenic and receptor activities of human growth hormone 108-129. J Biol Chem 268:22520–22524.

Johnsson B, Lofas S, Lindquist G. 1991. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Anal Biochem 198:268–277.

Kaiser E, Colescott RL, Bossinger CD, Cook PI. 1970. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. Anal Biochem 34:595–598.

Kasimova MR, Kristensen SM, Howe PW, Christensen T, Matthiesen F, Petersen J, Sorensen HH, Led JJ. 2002. NMR studies of the backbone flexibility and structure of human growth hormone: a comparison of high and low pH conformations. J Mol Biol 318:679–695.

Kennett RG. 1979. Monoclonal antibodies. Hybrid myelomas–a revolution in serology and immunogenetics. Am J Hum Genet 31:539–547.

Komatsu N, Saijoh K, Otsuki N, Kishi T, Micheal IP, Obiezu CV, Borgono CA, Takehara K, Jayakumar A, Wu HK, Clayman GL, Diamandis EP. 2007. Proteolytic processing of human growth hormone by multiple tissue kallikreins and regulation by the serine protease inhibitor Kazal-Type5 (SPINK5) protein. Clin Chim Acta 377:228–236.

Lewis UJ, Lewis LJ, Salem MA, Staten NR, Galosy SS, Krivi GG. 1991. A recombinant-DNA-derived modification of human growth hormone (hGH44-191) with enhanced diabetogenic activity. Mol Cell Endocrinol 78:45–54.

Lewis UJ, Sinha YN, Haro LS. 1994. Variant forms and fragments of human growth hormone in serum. Acta Paediatr (Suppl 399):29–31.

Lewis UJ, Sinha YN, Lewis GP. 2000. Structure and properties of members of the hGH family: a review. Endocr J 47(Suppl):S1–S8.

Lopez-Guajardo CC, Armstrong LS, Jordan L, Staten NR, Krivi GG, Martinez AO, Haro LS. 1998. Generation, characterization and utilization of anti-human growth hormone 1-43, (hGH1-43), monoclonal antibodies in an ELISA. J Immunol Methods 215:179–185.

Ohkura K, Hori H. 2000. Analyses of insulin-potentiating fragments of human growth hormone by computative simulation: essential unit for insulin-involved biological responses. Bioorg Med Chem 8:1733–1740.

Park C, Marqusee S. 2004. Probing the high energy states in proteins by proteolysis. J Mol Biol 343:1467–1476.

Popii V, Baumann G. 2004. Laboratory measurement of growth hormone. Clin Chim Acta 350:1–16.

Rosenfalck AM, Maghsoudi S, Fisker S, Jorgensen JO, Christiansen JS, Hilsted J, Volund AA, Madsbad S. 2000. The effect of 30 months of low-dose replacement therapy with recombinant human growth hormone (rhGH) on insulin and C-peptide kinetics, insulin secretion, insulin sensitivity, glucose effectiveness, and body composition in GH-deficient adults. J Clin Endocrinol Metab 85:4173–4181.

Rowlinson SW, Waters MJ, Lewis UJ, Barnard R. 1996. Human growth hormone fragments 1-43 and 44-191: in vitro somatogenic activity and receptor binding characteristics in human and nonprimate systems. Endocrinology 137:90–95.

Rupp B. http://www.ruppweb.org/cd/cdtutorial.htm 1996.

Ryther RC, McGuinness LM, Phillips JA, III, Moseley CT, Magoulas CB, Robinson IC, Patton JG. 2003. Disruption of exon definition produces a dominant-negative growth hormone isoform that causes somatotroph death and IGHD II. Hum Genet 113:140–148.

Salem MA. 1988. Effects of the amino-terminal portion of human growth hormone on glucose clearance and metabolism in normal, diabetic, hypophysectomized, and diabetic-hypophysectomized rats. Endocrinology 123:1565–1576.

Salem MA, Wolff GL. 1989. Potentiation of response to insulin and anti-insulin action by two human pituitary peptides in lean agouti A/a, obese yellow Avy/A, and C57BL/6J-ob/ob mice. Proc Soc Exp Biol Med 191:113–123.

Shadid S, Jensen MD. 2003. Effects of growth hormone administration in human obesity. Obes Res 11:170–175.

Shimizu T, Murakami Y, Nishiki M, Koshimura K, Oki Y, Kato Y. 2004. Contents and release of human growth hormone1-43 (hGH1-43) in human pituitary glands and hGH-producing pituitary adenoma cells. Biomed Res 25:141–146.

Singh RNP, Seavey BK, Lewis LJ, Lewis UJ. 1983. Human growth hormone peptide 1-43: isolation from pituitary glands. J Protein Chem 2:425–436.

Sinha YN, Jacobsen BP. 1994. Human growth hormone (hGH)-(44-191), a reportedly diabetogenic fragment of hGH, circulates in human blood: measurement by radioimmunoassay. J Clin Endocrinol Metab 78:1411–1418.

Spolaore B, Polverino DL, Zambonin M, Fontana A. 2004. Limited proteolysis of human growth hormone at low pH: isolation, characterization, and complementation of the two biologically relevant fragments 1-44 and 45-191. Biochemistry 43:6576–6586.

Struman I, Bentzien F, Lee H, Mainfroid V, D'Angelo G, Goffin V, Weiner RI, Martial JA. 1999. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. Proc Natl Acad Sci USA 96:1246–1251.

Warner MD, Sinha YN, Peabody CA. 1993. Growth hormone and prolactin variants in normal subjects. Relative proportions in morning and afternoon samples. Horm Metab Res 25:425–429.

Wroblewski VJ, Kaiser RE, Becker GW. 1993. Proteolysis of human growth hormone by rat thyroid gland in vitro: application of electrospray mass spectrometry and N-terminal sequencing to elucidate a metabolic pathway. Pharm Res 10:1106–1114.

Yang JT, Wu CS, Martinez HM. 1986. Calculation of protein conformation from circular dichroism. Methods Enzymol 130:208–269.

Yuen K, Ong K, Husbands S, Chatelain P, Fryklund L, Gluckman P, Ranke M, Cook D, Rosenfeld R, Wass J, Dunger D. 2002. The effects of short-term administration of two low doses versus the standard GH replacement dose on insulin sensitivity and fasting glucose levels in young healthy adults. J Clin Endocrinol Metab 87:1989–1995.

Yuen KC, Frystyk J, White DK, Twickler TB, Koppeschaar HP, Harris PE, Fryklund L, Murgatroyd PR, Dunger DB. 2005. Improvement in insulin sensitivity without concomitant changes in body composition and cardiovascular risk markers following fixed administration of a very low growth hormone (GH) dose in adults with severe GH deficiency. Clin Endocrinol (Oxf) 63:428–436.

Zhu L, Tamvakopoulos C, Xie D, Dragovic J, Shen X, Fenyk-Melody JE, Schmidt K, Bagchi A, Griffin PR, Thornberry NA, Sinha RR. 2003. The role of dipeptidyl peptidase IV in the cleavage of glucagon family peptides: in vivo metabolism of pituitary adenylate cyclase activating polypeptide-(1-38). J Biol Chem 278:22418–22423.

Growth Factors Downloaded from informahealthcare.com by Universitat Pompeu Fabra on 06/27/11
For personal use only.

RIGHTSLINK