Amino Acids (2010) 39:859–869
DOI 10.1007/s00726-010-0542-2

ORIGINAL ARTICLE

Purification of recombinant growth hormone by clear native gels for conformational analyses: preservation of conformation and receptor binding

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Received: 19 February 2010 / Accepted: 24 February 2010 / Published online: 19 March 2010
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Abstract Most protein preparations require purification steps prior to biophysical analysis assessing protein stability, secondary structure and degree of folding. It was, therefore, the aim of this study to develop a system to separate and purify a protein from a commercially available medicinal product, recombinant human growth hormone (rhGH) and show preservation of conformation and function following the gel-based procedure. The rhGH was run on clear native (CN) gels and recovered from the gels by electroelution using D-Tube Dialyzer Midi under rigorous cooling. Melting point studies indicated preservation of the structural integrity. This finding was confirmed by synchrotron radiation circular dichroism spectroscopy (SRCD) revealing an identical folding pattern for the sample before and after electrophoretic separation and purification. Synchrotron small-angle X-ray scattering (SAXS) indicated that the sample was folded and monomeric, both before and after separation and purification, and that its shape corresponded well to the known crystal structure of GH. Binding properties of rhGH to a receptor-model system before and after clear native electrophoresis were comparable. This analytical and preparative approach to purify and concentrate a protein preserving conformation and function may be helpful for many applications in analytical, protein and stereochemistry.

Keywords Growth hormone · Electroelution · Conformation · SRCD · Function

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Introduction

In a concise and comprehensive review Seelert and Krause (Seelert and Krause 2008) listed many applications of polyacrylamide gel electrophoresis (PAGE) including elution of bioparticles.

However, a method for separation and purification of native proteins prior to further analytical work is mandatory and represents a major step forward in protein chemistry.

A large series of mild electrophoretic conditions including blue/native gels have been reported, which allowed further studies on proteins (Albertini et al. 2007; Dunahay and Staehelin 1985; Evans and Anderson 1987; Ford 1987; Ford et al. 1987; Heinz and Siefermann-Harms 1981;

Knoetzel et al. 1988; Lancaster et al. 1999; Madhavarao et al. 2003; Poetsch et al. 2000; Schafer et al. 2006, 2007; Seelert et al. 2003; Siefermann-Harms et al. 1982; Singh and Wasserman 1970; Tsiotis et al. 1993) such as crystallization, spectrophotometric assays, enzymatic assays, reconstitution into liposomes and atomic force electron microscopy. An approach for carrying out native gels would represent a valuable analytical tool for further investigation of conformational and functional studies. Native and clear native gels are proposed to fulfill these requirements, because no protein staining/destaining procedure, as necessary for studies from blue or green native gels, would be required (Dunahay and Staehelin 1985; Green et al. 1982; Kuchler et al. 2002; Poetsch et al. 2000; Staehelin et al. 1984). The use of clear native gels CN/PAGE (Krause 2006, 2007; Schagger 2002; Schagger et al. 2004) e.g., allowed studying the assembly of respirasomes.

In the present study we aimed to use a protein, which is in medical use, recombinant human growth hormone (rhGH), to develop methods for protein separation and purification, by clear native gel electrophoresis followed by electroelution, and to show structural integrity by determination of melting point and the evaluation of structural properties by synchrotron radiation circular dichroism spectroscopy (SRCD), and synchrotron small-angle X-ray scattering (SAXS). The determination of the preserved function of rhGH was carried out by an immunofunctional assay representing receptor-binding activity. Moreover, mass spectrometrical characterization of the protein was performed and 100% sequence coverage was obtained.

As this analytical principle showed conformational integrity and preserved function as compared to the non-treated rhGH protein, we propose this simple and non-sophisticated procedure for characterization, separation/purification of proteins for further structural and functional analysis. A large series of applications in protein chemistry for academia and industry are warranted by this procedure.

Materials and methods

Material

The rhGH (Nutropin®, Genentech, Inc., South San Francisco, CA, USA) is a recombinant protein expressed in *E. coli* and was purchased. All the chemicals are from Sigma (Munich, Germany) apart from those noted from other sources.

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis was performed essentially as reported (Chen et al. 2009a, b); 60 µg of rhGH in a volume of 10 µL was mixed with the re-hydration buffer (8 M urea, 4% (w/v) CHAPS, 10 mM 1,4-dithioerythritol, 0.5% IPG carrier ampholytes, 0.008% bromophenol blue) to a final volume of 350 µL and this solution was applied on immobilized pH 3–10 nonlinear gradient strips. Focusing was started at 200 V and the voltage was gradually increased to 8,000 V at 4 V/min and then kept constant for a further 3 h (approximately 150,000 V h totally). After the first dimension, strips (18 cm) were equilibrated for 15 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, strips were loaded on 10–16% gradient sodium dodecylsulfate polyacrylamide gels for second-dimensional separation. The gels (180 × 200 × 1.5 mm) were run at 40 mA per gel. Immediately after the second-dimension run, gels were fixed for 12 h in 50% methanol, containing 10% acetic acid; the gels were stained with Colloidal Coomassie Blue (Invitrogen, Carlsbad, CA, USA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Bio-Rad Laboratories, Hercules, CA, USA) covering the range 10–250 kDa. The p*I* values were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess of dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Amersham Biosciences, Uppsala, Sweden).

Nano-LC-ESI-CID/ETD-MS/MS analysis

Nano-LC-ESI-CID/ETD-MS/MS analysis was performed essentially as reported (Chen et al. 2009a). Gel pieces of interest were cut and put into a 1.5-mL tube. They were washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate repeatedly. Addition of 100% acetonitrile resulted in gel shrinking and the shrunk gel plugs were then SpeedVac-dried in a SpeedVac Concentrator 5301 (Eppendorf, Hamburg, Germany). The dried gel pieces were re-swollen and in-gel digested with 40 ng/mL trypsin (Promega, Madison, WI, USA) in digestion buffer (consisting of 5 mM octyl b-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated overnight at 37°C. Chymotrypsin digestion was performed by addition of 25 mM ammonium bicarbonate containing 25 ng/mL chymotrypsin (sequencing grade; Roche Diagnostic, Mannheim, Germany) and incubated for 30 min at 30°C. Peptide extraction was performed with 15 mL of 1% formic acid (FA) in 5 mM OGP for 30 min, 15 mL of 0.1% FA for 30 min, and 15 mL of 0.1% TFA in 20% acetonitrile for mass spectrometric analysis for 30 min. The extracted peptides were pooled for nano-LC-ESI-CID/ETD-MS/MS analysis.

The HPLC used was an Ultimate 3000 system (Dionex Corporation, Sunnyvale, CA, USA) equipped with a PepMap100 C-18 trap column (300 μm × 5 mm) and PepMap100 C-18 analytic column (75 μm × 150 mm). The gradient was ($A = 0.1\%$ formic acid in water, $B = 0.08\%$ formic acid in acetonitrile) 4–30% B from 0 to 105 min, 80% B from 105 to 110 min, 4% B from 110 to 125 min. A HCT Ultra ETD II (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of m/z 350–1,500, and MS/MS spectra in information-dependent data acquisition over the mass range of m/z 100–2,800. Repeatedly, MS spectra were recorded followed by three data-dependent CID MS/MS spectra and three ETD MS/MS spectra generated from three highest intensity precursor ions. An active exclusion of 0.4 min after two spectra was used to detect low abundant peptides. The voltage between ion spray tip and spray shield was set to 1,500 V. Drying nitrogen gas was heated to 150°C and the flow rate was 10 L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). Searches were done by using the Mascot 2.2.06 (Matrix Science, London, UK) against the latest UniprotKB database for protein identification. Searching parameters were set as follows: enzyme selected as trypsin or chymotrypsin with two maximum missing cleavage sites, species limited to human, a mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of methionine oxidation and phosphorylation (Tyr, Thr, and Ser). Positive protein identifications were based on a significant MOWSE score.

Gel-free ESI–MS analysis of rhGH

Growth hormone 20 μL (about 100 μg) was added to 1 mL cold acetone and kept at −20°C overnight to precipitate the protein. Centrifugation was done at 14,000×g at 4°C for 15 min. Pellets were washed again with 1 mL cold acetone, centrifuged at 14,000×g at 4°C for 15 min, dried at 30°C for 30 min, dissolved in 500 μL of 1% formic acid and 50% acetonitrile. Samples were injected to Bruker HCT Ultra ETD II at a flow rate of 240 μL/h. The MS spectrum was recorded over the mass range of m/z 1,200–1,800. The voltage between ion spray tip and spray shield was set to 4,000 V. Drying nitrogen gas was heated to 300°C and the flow rate was 10 L/min. The MS spectrum was deconvoluted in Bruker Data Analysis 4.0 software, and shifted spectra were also generated.

Native PAGE

All steps except the polymerization procedure were and had to be carried out in a cold room at 4°C from the beginning of the experiment.

For preparation of the 10% separation polyacrylamide gel (4.5 mL volume) a mixture of 1.5 mL 30% acrylamide/piperazine diacrylamide (29.25 g/0.75 g per 100 mL), 1.125 mL 1.5 M Tris–HCl pH 8.8, 1.875 mL H_2O, 2.7 μL TEMED and 27 μL of 10%(w/v) ammonium persulfate, was used for gel casting into Mini-Protean 3 plates (Biorad, Hercules, CA, USA). 1 mL of 50% 2-propanol (v/v) was added to the gel surface and polymerization was done at 25°C for 10 min. The gel surface was cleaned with distilled water and the sample loading comb was inserted.

For preparation of the 4.5% stacking gel a mixture of 0.13 mL 30% acrylamide/piperazine diacrylamide as given above, 0.25 mL 0.5 M Tris–HCl pH 6.8, 0.62 mL H_2O, 1.4 μL TEMED, 10 μL 10%(w/v) ammonium persulfate, was used and polymerized at 25°C for 10 min. The Mini-Protean R 3 electrophoresis cell (Bio-Rad, Hercules, CA, USA) was set up and 900 mL running buffer (25 mM Tris, 192 mM glycine, pH 8.3) was added to the electrophoresis chamber. A prerun, in order to remove unpolymerized acrylamide and other constituents, was done at 100 V for 1 h. A solution of 240 μL containing 1,400 μg rhGH was mixed with 50 μL loading buffer (125 mM Tris–HCl, 20% glycerol, and 0.2% (w/v) bromophenol blue, pH 6.8.

Eight aliquots of this solution was subsequently loaded into each well. Electrophoresis was performed at a constant voltage of 50 V in the cold room for 30 min, and then at 100 V for 2 h using a running buffer (25 mM Tris and 192 mM glycine, pH 8.3). Native protein markers (Invitrogen, Carlsbad, CA, USA) were loaded to one well.

Electroelution and dialysis

Two longitudinal strips, i.e., one lane containing markers and one lane containing rhGH, were cut out and stained with E-Zinc staining kit (Pierce, Rockford, IL, USA) while keeping the rest of the gel on a glass plate on ice. The stained strips were lined up along the edges of the unstained gel and used as a guide to cut out bands of interest from the unstained gel. The excised bands were placed into D-Tube Midi (3.5 kDa cutoff, Novagen, Germany), 800 μL native page running buffer (25 mM Tris, 192 mM Glycine, pH 8.3) were added, and tubes were fixed on a horizontal electrophoresis chamber, Sub-Cell R GT cell (Biorad, Hercules, CA, USA). The chamber was filled with native PAGE running buffer and electroelution in D-Tube Midi was performed at 100 V for 5 h in the cold room. Reverse electrophoresis was done in D-Tube Midi to recover protein attached to the tubes' membrane for 2 min.

The electroelution tubes were dialyzed in 3 L of 10 mM phosphate buffer pH 7.0 overnight with gentle stirring in the cold room and this procedure was repeated once. All steps were carried out in the cold room at 4°C.

Protein concentration and Bradford assay

The electroeluted and dialyzed sample was transferred to 1.5 mL 3,000 Da cutoff tubes (Millipore, Bedford, MA, USA), centrifuged at 13,000×*g* at 12°C for about 2 h to reduce the volume to about 100 µL. Concentration of the sample at 4°C would triple the time for this step; 1 µL of the sample was used to measure protein concentration by the Bradford assay (Bradford 1976).

Differential scanning fluorimetry

Differential scanning fluorimetry (DSF) analysis is employed to screen an optimal buffer for protein stability (Ericsson et al. 2006). In this work, it was used to check recovery of the protein structure before and after electroelution assays. The temperature of protein unfolding is monitored via the rise in the dye SYPRO Orange (Invitrogen, Carlsbad, CA, USA) fluorescence signal while binding to hydrophobic patches of the protein that become accessible during the heating process (20–95°C in 1 h). The experiments were carried out in a real-time PCR instrument with filters calibrated to absorb signals from the SYPRO Orange dye: iQ5 multicolor Real-Time PCR Detection System (Biorad, Hercules, CA, USA). For rhGH samples, a final concentration of ~ 4.5 mg/mL and 100× dye were used.

Synchrotron radiation CD spectroscopy

SRCD spectra were collected essentially as previously described (Majava et al. 2010). The samples of rhGH before electrophoresis and after recovery were diluted with phosphate buffer (as given above) if necessary, and phosphate buffer was also used as the baseline in the measurement. Both samples were measured at approximately 5 mg/mL; 1 µL of sample was loaded onto the center of a circular calcium fluoride cuvette with a path length of 5 µm.

The cuvette was mounted on the BESSY II SRCD beamline 3 m-NIM-C. SRCD spectra were measured between 260 and 170 nm, with 3 s at each wavelength step of 1 nm. Three spectra were collected and averaged. For background correction, similar spectra were collected for the cuvette alone filled with phosphate buffer. The circular cuvette was always in the same orientation with respect to the incoming beam. After subtracting the background, spectra were normalized by dividing with the peptide absorption peak at 190 nm for direct comparison. The data were also analyzed for secondary structure content at the Dichroweb server (Lobley et al. 2002).

As an additional experiment, dried films of the GH samples were prepared directly on the cuvette. GH was pipetted directly onto the CaF2 cuvette and allowed to dry, first in a hood, and then in vacuum for a few minutes. Subsequently, SRCD spectra were measured down to a wavelength of 130 nm.

Small-angle X-ray scattering

For SAXS, the samples were dialyzed into 10 mM HEPES, pH 7.5. SAXS data were collected on beamline I711 at MAX-Lab, Lund, Sweden (Cerenius et al. 2000) and processed using the in-house BliI711 software. Further data processing and analyses were done with programs of the ATSAS package (Konarev et al. 2006). For comparisons, the crystal structure of human growth hormone was used (PDB entry 1HWG) (Sundstrom et al. 1996).

Immunofunctional assay

An immunofunctional assay (IFA) was performed according to a previous publication (Strasburger et al. 1996). Anti-hGH mAb 7B11, which binds to an epitope largely overlapping with binding site 2 of the hGH molecule, was adsorbed to 96-well flat bottom microtiter plates (Nunc, Roskilde, Denmark) by incubation of 500 ng mAb 7B11 in 200 µL 50 mmol/L sodium phosphate buffer, pH 9.6. The plates were sealed with a self-adhesive cover film and stored at 4°C for 12 h. After aspiration of the coating solution, the plates were washed three times with washing solution. Growth hormone samples were diluted 100,000 times and also 500,000 times. Twenty-five microliters of standard or sample was pipetted into the wells, followed by 175 µL assay buffer. The plates were incubated at ambient temperature for 3 h on a horizontal shaker. After a threefold wash step, 50 rigs/well biotinylated rhGHBP were added in 200 µL assay buffer, and the plates were sealed and incubated overnight (12–16 h) at 4°C. After a threefold wash step, the plates were incubated with streptavidin-europium, and after 5–15 min of agitation on a horizontal shaker, the plate was placed into a Delfia 1232 time-resolved fluorometer to read the signal.

Concentrations of hGH were measured using the automated Immulite 2000 chemiluminescent assay system (Siemens, Bad Nauheim, Germany). This sandwich-type immunoassay involves a monoclonal mouse-anti-hGH capture- and a polyclonal rabbit-anti-hGH detection-antibody. Within assay coefficient of variance (CV) was 3.2, 2.3 and 2.3% at concentrations of 0.2, 2.1 and 27.1 ng/mL, respectively. Between-assay variability at the same

concentrations was 3.0, 7.4 and 2.3%, respectively. The lower limit of quantification was 0.05 µg/L, the linear working range was 0.05–40 µg/L.

Results

In two-dimensional gel electrophoresis (Fig. 1a) rhGH showed an apparent molecular weight of 19 kDa (theoretical molecular weight is 22 kDa), p*I* from 6 to 6.5 along with some minor spots at different molecular weights, that were not taken for further analysis. The protein was characterized by nano-LC–ESI–MS/MS and identified unambiguously with complete sequence coverage (100%) as residues 27–217 of human growth hormone (SOMA Human). Details of identification are listed in Table 1.

There were two bands, a very strong band and a minor band, in the native PAGE of rhGH (Fig. 1b). Both bands were picked and identified as residues 27–217 of human growth hormone (Table 1).

Determination of the molecular mass of the entire protein revealed 22,125.49 Da (Fig. 2); the calculated molecular weight of 27–217 of SOMA Human was 22,129.00 Da that would show integrity of rhGH and probably indicate absence of heavy modification.

Protein 1.6 mg was loaded onto native PAGE. Following electroelution and dialysis in phosphate buffer, about 0.82 mg rhGH remained in 6.4 mL (about 51% recovery). After concentration in 3 kDa cutoff tubes to about 100 µL the remaining protein amount was about 0.520 mg. The final recovery ratio was about 32.5%.

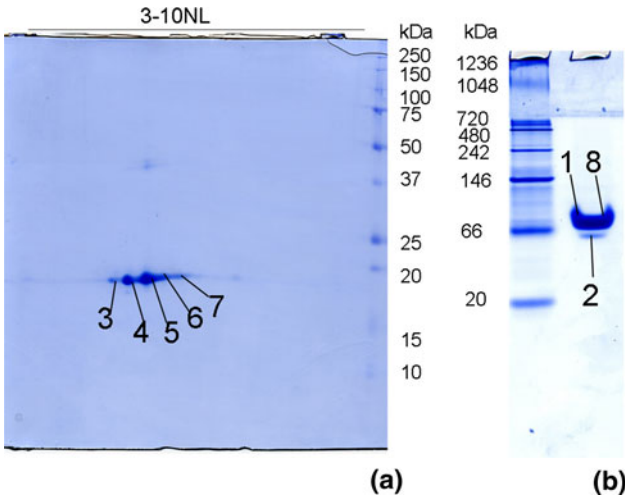

Fig. 1 **a** Two-dimensional gel electrophoresis of rhGH. 60 µg rhGH was separated on 3–10NL IPG strip and then 10–16% SDS-PAGE. **b** Native PAGE analysis of rhGH. Native markers and 30 µg rhGH were loaded on the gel

Results from DSF analysis of rhGH before electrophoresis and after recovery are shown in Fig. 3. No significant difference in the shape of the curves could be observed. Similar melting points at 74°C (before) and 71.5°C (after) were observed. This result suggests that the recovered protein is still folded and shows similar folding to the protein before electrophoresis.

The folding of the original and recovered rhGH samples was further studied using SRCD. The absorbance at 190 nm was between 0.55 and 0.65 for the measured samples. SRCD spectra could be measured reliably down to a wavelength of 170 nm using a 5 µm CaF_2 cuvette. The concentration-corrected spectra for the two samples are basically identical, unambiguously proving efficient recovery of correctly folded rhGH (Fig. 4a). Since the vacuum ultraviolet (VUV) region of the SRCD spectrum is very sensitive to even minor changes in conformation and protein interactions, the result is a reliable proof of essentially identical structure between the two samples. The spectra visually looked like those obtained for purely alpha helical proteins. This was confirmed by analyses on the Dichroweb server using different data sets of proteins available. The protein was calculated to be purely alpha helical, with the fraction of helical secondary structure ranging between 70 and 90%, depending on the reference data set used.

SRCD spectra were also measured from dried rhGH films down to a wavelength of 130 nm (Fig. 4b), thus avoiding the absorption by water, which normally hampers attempts to measure CD data below 168 nm. Both spectra were similar also in the low-wavelength region, having a minimum at 160 nm and a maximum at 140 nm. The result corresponds well to recent results obtained by SRCD from films of proteins with helical conformation (Nesgaard et al. 2008).

The solution structure of rhGH before and after electrophoresis and purification was also probed by SAXS (Fig. 4c; Table 2). The scattering curves of both samples were similar and corresponded well to scattering expected from monomeric GH. Ab initio model building based on the SAXS data also resulted in a shape that could well accommodate the structure observed in the crystal structure (Fig. 4d, e). All in all, the SAXS data indicated that the rhGH, both before and after electrophoretic separation and purification, was a monomeric folded protein with a folded 3D structure corresponding to that observed in the crystal structure. The dimensions of the molecule were slightly larger than of those observed in the corresponding crystal structure, but this is exactly as expected, when analyzing molecules in solution versus the crystal state.

The immunofunctional assay of growth hormone shows that the ratio between IFA and Immulite is in the same

Table 1 Mass spectrometry result of rhGH spots

Spot	Sequence coverage (%)	Identified peptide (enzyme/ion score/mass error)
1	72.25	27A.FPTIPLSR.L 34 (Try/27/−0.0822)
		35R.LFDNAM*LR.A Oxidation (M) 42 (Try/45/−0.0479)
		35R.LFDNAMLR.A 42 (Try/51/−0.0696)
		46R.LHQLAFDTYQEFEEAYIPK.E 64 (Try/138/−0.1072)
		68 K.YSFLQNPQTSLCFSESIPTPSNR.E 90 (Try/69/−0.129)
		97K.SNLELLR.I 103 (Try/57/−0.0517)
		121R.SVFANSLVYGASDSNVYDLLK.D 141 (Try/126/−0.1807)
		142K.DLEEGIQTLM*GR.L Oxidation (M) 153 (Try/91/−0.0981)
		142K.DLEEGIQTLMGR.L 153 (Try/86/0.0056)
		172K.FDTNSHNDDALLK.N 184 (Try/76/−0.0416)
		185K.NYGLLYCFR.K 193 (Try/55/−0.1221)
		194R.KDM*DKVETFLR.I Oxidation (M) 204 (Try/46/−0.1348)
		194R.KDMDKVETFLR.I 204 (Try/65/−0.1911)
		195K.DM*DKVETFLR.I Oxidation (M) 204 (Try/44/−0.007)
		195K.DMDKVETFLR.I 204 (Try/56/−0.0872)
		199K.VETFLR.I 204 (Try/41/−0.0116)
		210R.SVEGSCGF.- 217 (Try/38/−0.1696)
2	56.54	27A.FPTIPLSR.L 34 (Try/28/−0.0616)
		35R.LFDNAM*LR.A Oxidation (M) 42 (Try/42/−0.0931)
		35R.LFDNAMLR.A 42 (Try/48/−0.0856)
		46R.LHQLAFDTYQEFEEAYIPK.E 64 (Try/126/−0.149)
		97K.SNLELLR.I 103 (Try/47/−0.0455)
		121R.SVFANSLVYGASDSNVYDLLK.D 141 (Try/127/−0.1037)
		142K.DLEEGIQTLM*GR.L Oxidation (M) 153 (Try/95/−0.1185)
		142K.DLEEGIQTLMGR.L 153 (Try/77/−0.0732)
		172K.FDTNSHNDDALLK.N 184 (Try/96/−0.1051)
		185K.NYGLLYCFR.K 193 (Try/59/−0.0341)
		194R.KDM*DKVETFLR.I Oxidation (M) 204 (Try/62/−0.0199)
		194R.KDMDKVETFLR.I 204 (Try/79/−0.0675)
		195K.DM*DKVETFLR.I Oxidation (M) 204 (Try/46/−0.0754)
		195K.DMDKVETFLR.I 204 (Try/64/−0.029)
3	71.20	27A.FPTIPLSR.L 34 (Try/25/−0.0644)
		35R.LFDNAM*LR.A Oxidation (M) 42 (Try/39/−0.0715)
		35R.LFDNAMLR.A 42 (Try/45/−0.0764)
		46R.LHQLAFDTYQEFEEAYIPK.E 64 (Try/92/−0.1717)
		68K.YSFLQNPQTSLCFSESIPTPSNREETQQK.S 96 (Try/51/−0.1869)
		97K.SNLELLR.I 103 (Try/70/−0.0669)
		121R.SVFANSLVYGASDSNVYDLLK.D 141 (Try/133/−0.1151)
		142K.DLEEGIQTLM*GR.L Oxidation (M) 153 (Try/100/−0.1151)
		142K.DLEEGIQTLMGR.L 153 (Try/97/−0.1094)
		172K.FDTNSHNDDALLK.N 184 (Try/85/−0.1609)
		185K.NYGLLYCFR.K 193 (Try/55/−0.0427)
		194R.KDM*DKVETFLR.I Oxidation (M) 204 (Try/73/−0.0742)
		194R.KDMDKVETFLR.I 204 (Try/74/−0.1263)
		195K.DM*DKVETFLR.I Oxidation (M) 204 (Try/42/−0.066)
		195K.DMDKVETFLR.I 204 (Try/58/−0.0679)

Table 1 continued

Spot	Sequence coverage (%)	Identified peptide (enzyme/ion score/mass error)
4	65.45	27A.FPTIPLSR.L 34 (Try/35/−0.0744)
		35R.LFDNAM*LR.A Oxidation (M) 42 (Try/45/−0.0907)
		35R.LFDNAMLR.A 42 (Try/50/−0.0792)
		46R.LHQLAFDTYQEFEEAYIPK.E 64 (Try/130/−0.1114)
		97K.SNLELLR.I 103 (Try/64/−0.0913)
		121R.SVFANSLVYGASDSNVYDLLK.D 141 (Try/111/−0.1839)
		142K.DLEEGIQTLM*GR.L Oxidation (M) 153 (Try/96/−0.1013)
		142K.DLEEGIQTLMGR.L 153 (Try/100/−0.0696)
		161R.TGQIFKQTYSK.F 171 (Try/58/−0.1518)
		172K.FDTNSHNDDALLK.N 184 (Try/90/−0.1585)
		185K.NYGLLYCFR.K 193 (Try/53/−0.1131)
		194R.KDM*DKVETFLR.I Oxidation (M) 204 (Try/74/−0.1297)
		194R.KDMDKVETFLR.I 204 (Try/73/−0.1089)
		195K.DM*DKVETFLR.I Oxidation (M) 204 (Try/49/−0.025)
		195K.DMDKVETFLR.I 204 (Try/64/−0.1394)
		199K.VETFLR.I 204 (Try/49/0.0156)
		210R.SVEGSCGF.- 217 (Try/44/−0.0636)
5	86.91	27A.FPTIPLSRLFDNAMLR.A 42 (Try/66/−0.1547)
		35R.LFDNAM*LR.A Oxidation (M) 42 (Try/45/−0.0435)
		35R.LFDNAMLR.A 42 (Try/57/−0.1134)
		46R.LHQLAFDTYQEFEEAYIPK.E 64 (Try/162/−0.1834)
		68K.YSFLQNPQTSLCFSESIPTPSNR.E 90 (Try/92/−0.193)
		97K.SNLELLR.I 103 (Try/62/−0.0725)
		104R.ISLLLIQSWLEPVQFLR.S 120 (Try/48/−0.1506)
		121R.SVFANSLVYGASDSNVYDLLK.D 141 (Try/141/−0.0827)
		142K.DLEEGIQTLM*GR.L Oxidation (M) 153 (Try/95/−0.1315)
		142K.DLEEGIQTLMGR.L 153 (Try/90/−0.0586)
		161R.TGQIFKQTYSK.F 171 (Try/49/−0.1017)
		172K.FDTNSHNDDALLK.N 184 (Try/89/−0.103)
		185K.NYGLLYCFR.K 193 (Try/53/−0.0739)
		194R.KDM*DKVETFLR.I Oxidation (M) 204 (Try/73/−0.1645)
		194R.KDMDKVETFLR.I 204 (Try/89/−0.1105)
		195K.DM*DKVETFLR.I Oxidation (M) 204 (Try/54/−0.0862)
		195K.DMDKVETFLR.I 204 (Try/68/−0.098)
		199K.VETFLR.I 204 (Try/38/−0.071)
		210R.SVEGSCGF.- 217 (Try/52/−0.0832)
6	65.44	27A.FPTIPLSR.L 34 (Try/27/−0.0568)
		35R.LFDNAM*LR.A Oxidation (M) 42 (Try/35/−0.0923)
		35R.LFDNAMLR.A 42 (Try/45/−0.101)
		46R.LHQLAFDTYQEFEEAYIPK.E 64 (Try/118/−0.1549)
		97K.SNLELLR.I 103 (Try/52/−0.0785)
		121R.SVFANSLVYGASDSNVYDLLK.D 141 (Try/110/−0.1733)
		142K.DLEEGIQTLM*GR.L Oxidation (M) 153 (Try/95/−0.1305)
		142K.DLEEGIQTLMGR.L 153 (Try/91/−0.0952)
		161R.TGQIFKQTYSK.F 171 (Try/43/−0.1004)
		172K.FDTNSHNDDALLK.N 184 (Try/90/−0.1087)
		185K.NYGLLYCFR.K 193 (Try/60/−0.1113)
		194R.KDM*DKVETFLR.I Oxidation (M) 204 (Try/54/−0.127)
		194R.KDMDKVETFLR.I 204 (Try/76/−0.1404)
		195K.DM*DKVETFLR.I Oxidation (M) 204 (Try/55/−0.0942)
		195K.DMDKVETFLR.I 204 (Try/59/−0.0637)
		199K.VETFLR.I 204 (Try/53/−0.121)
		210R.SVEGSCGF.- 217 (Try/33/−0.0378)

Table 1 continued

Spot	Sequence coverage (%)	Identified peptide (enzyme/ion score/mass error)
7	71.20	27A.FPTIPLSR.L 34 (Try/26/0.0068)
		35R.LFDNAM*LR.A Oxidation (M) 42 (Try/49/−0.0837)
		35R.LFDNAMLR.A 42 (Try/62/−0.0758)
		46R.LHQLAFDTYQEFEEAYIPK.E 64 (Try/151/−0.177)
		68K.YSFLQNPQTSLCFSESIPTPSNREETQQK.S 96 (Try/58/−0.0018)
		97K.SNLELLR.I 103 (Try/65/−0.0631)
		121R.SVFANSLVYGASDSNVYDLLK.D 141 (Try/101/−0.1625)
		142K.DLEEGIQTLM*GR.L Oxidation (M) 153 (Try/93/−0.1405)
		142K.DLEEGIQTLMGR.L 153 (Try/90/−0.0934)
		172K.FDTNSHNDDALLK.N 184 (Try/65/−0.0946)
		185K.NYGLLYCFR.K 193 (Try/54/−0.0549)
		194R.KDM*DKVETFLR.I Oxidation (M) 204 (Try/73/−0.1135)
		194R.KDMDKVETFLR.I 204 (Try/78/−0.1119)
		195K.DM*DKVETFLR.I Oxidation (M) 204 (Try/47/−0.136)
		195K.DMDKVETFLR.I 204 (Try/59/−0.0343)
		199K.VETFLR.I 204 (Try/49/−0.061)
8	78.01	27A.FPTIPLSRLF.D 36 (Chy/36/0.0521)
		27A.FPTIPLSRLFDNAM*L.R Oxidation (M) 41 (Chy/45/−0.1791)
		33L.SRLFDNAM*L.R Oxidation (M) 41 (Chy/41/−0.099)
		33L.SRLFDNAML.R 41 (Chy/44/−0.067)
		42L.RAHRLHQLAFDTYQEFEEAY.I 61 (Chy/65/−0.1711)
		47L.HQLAFDTY.Q 54 (Chy/42/−0.0291)
		47L.HQLAFDTYQEFEEAYIPKEQKY.S 68 (Chy/52/−0.1346)
		52F.DTYQEFEEAYIPKEQKY.S 68 (Chy/63/−0.0806)
		55Y.QEFEEAYIPKEQKY.S 68 (Chy/90/−0.1204)
		69Y.SFLQNPQTSL.C 78 (Chy/56/−0.041)
		69Y.SFLQNPQTSLCF.S 80 (Chy/88/−0.0437)
		71F.LQNPQTSLCF.S 80 (Chy/58/0.0141)
		100L.ELLRISL.L 106 (Chy/40/−0.1133)
		103L.RISLLLIQSW.L 112 (Chy/45/0.0389)
		108L.LIQSWLEPVQF.L 118 (Chy/66/−0.1046)
		124F.ANSLVYGASDSNVY.D 137 (Chy/64/−0.0618)
		151L.M*GRLEDGSPRTGQIF.K Oxidation (M) 165 (Chy/60/−0.1611)
		151L.MGRLEDGSPRTGQIF.K 165 (Chy/56/−0.1397)
		152M.GRLEDGSPRTGQIF.K 165 (Chy/74/−0.0903)
		170Y.SKFDTNSHNDDALL.K 183 (Chy/66/−0.1371)
		170Y.SKFDTNSHNDDALLKNY.G 186 (Chy/78/−0.1328)
		170Y.SKFDTNSHNDDALLKNYGLL.Y 189 (Chy/76/−0.048)
		173F.DTNSHNDDALL.K 183 (Chy/44/−0.0673)
		173F.DTNSHNDDALLKNY.G 186 (Chy/79/−0.1322)
		191Y.CFRKDM*DKVETF.L Oxidation (M) 202 (Chy/48/−0.1086)
		191Y.CFRKDMDKVETF.L 202 (Chy/51/−0.1109)
		193F.RKDM*DKVETF.L Oxidation (M) 202 (Chy/47/−0.0259)
		193F.RKDMDKVETF.L 202 (Chy/51/−0.0567)
		203F.LRIVQCRSVEGSCGF.- 217 (Chy/58/−0.1441)
		204L.RIVQCRSVEGSCGF.- 217 (Chy/43/−0.1118)

All spots are identified as growth hormone (27–217, P01241, SOMA Human) by trypsin or chymotrypsin. Combining all trypsin results, the total sequence coverage was 90.58%. Combining all chymotrypsin results, the sequence coverage was 78.01%. Combining trypsin and chymotrypsin results, the sequence coverage was 100%

order of magnitude (approx 1.8–2.1) for all rhGH preparations (Table 3). This means that at least there is no dramatic change in the binding sites of the rhGH interacting with the receptor, and therefore before electrophoresis and after recovery seem to be equally bioactive.

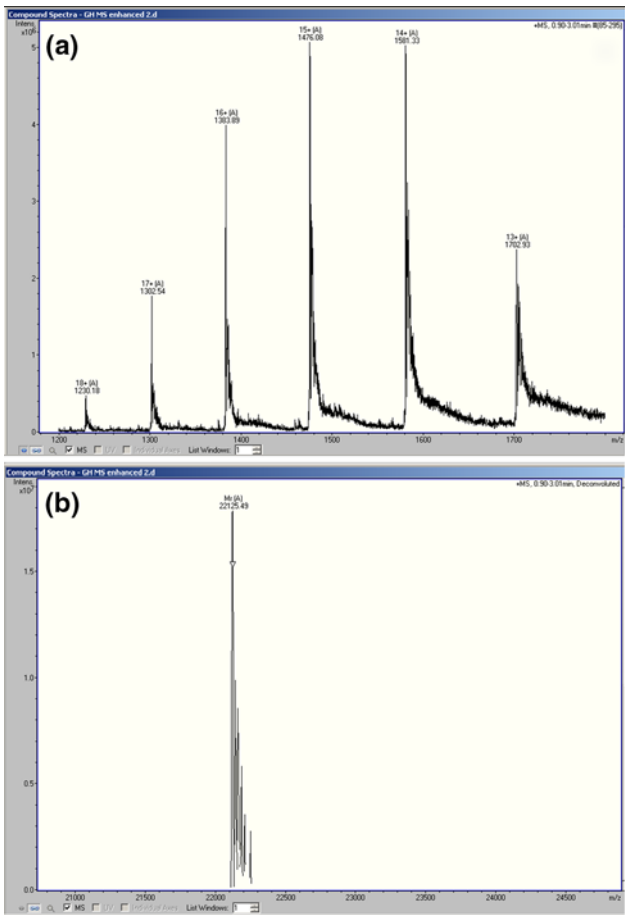

Fig. 2 **a** The raw MS spectrum of rhGH. 13, 14, 15, 16, 17 and 18 charged rhGH peaks are labeled. **b** Deconvoluted MS spectrum of rhGH. The molecular weight of rhGH was 22,125.49

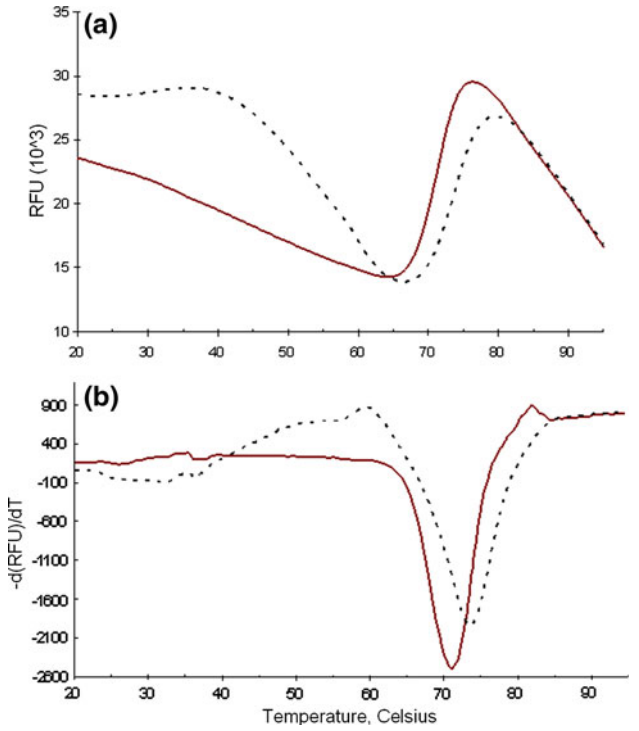

Fig. 3 DSF of rhGH before electrophoresis (*solid line*) and after recovery (*dash line*) from native PAGE. **a** The melting curve of rhGH before electrophoresis and after recovery. **b** The melting peak of rhGH before electrophoresis and after recovery. The minima corresponds to the inflection point (melting temperature) of melting curve in (**a**)

Discussion

Based upon the findings presented above, we propose that the analytical system will be applied as a tool for the characterization, separation/purification, conformational, and functional analysis of proteins.

The rhGH from the native gel was unambiguously identified by in-gel digestion with trypsin and chymotrypsin followed by mass spectrometry using an ion trap with 100% sequence coverage. As rhGH was produced in *E. coli* no posttranslational modifications (PTMs) were studied, but of course this tool would reliably allow characterization of PTMs.

Electroelution was used to obtain the protein from the gel. Several publications have been recommending the use of electroelution (Ihara et al. 1987; McDonald et al. 1986; Seelert and Krause 2008; Shoji et al. 1995). A major prerequisite is that all steps have to be run in a cold room with pre-cooled buffers and other chemicals. Running a gel at room temperature, or simple cooling by standard cooling conditions to run electrophoresis, results in loss of protein conformation, most probably due to the heating effects of the relatively high electrical power needed to recover the protein.

The incomplete recovery of rhGH from the gel by this method may depend on protein and gel properties or limitations of the electroelution method.

The determination of melting temperature is a non-sophisticated and reliable method to obtain conformational information on structural stability and integrity, and indeed, pre/electrophoretic and electroeluted rhGH showed comparable melting points, suggesting that the three-dimensional structure of the protein has been recovered after electroelution under specific conditions.

The use of SRCD showed the presence of a fully alpha helical protein in both samples using only 5 μg of protein for each test. The fact that the spectra were essentially identical in all wavelength regions indicates both, that there were no large differences in secondary structure content, and that also finer details and assembly of the protein structure in the purified sample were indistinguishable from the original sample. Further proof for identical structures of the two samples comes from SAXS experiments, indicating both that the two samples are highly similar in structure and that they also closely correspond to the crystal structure of GH.

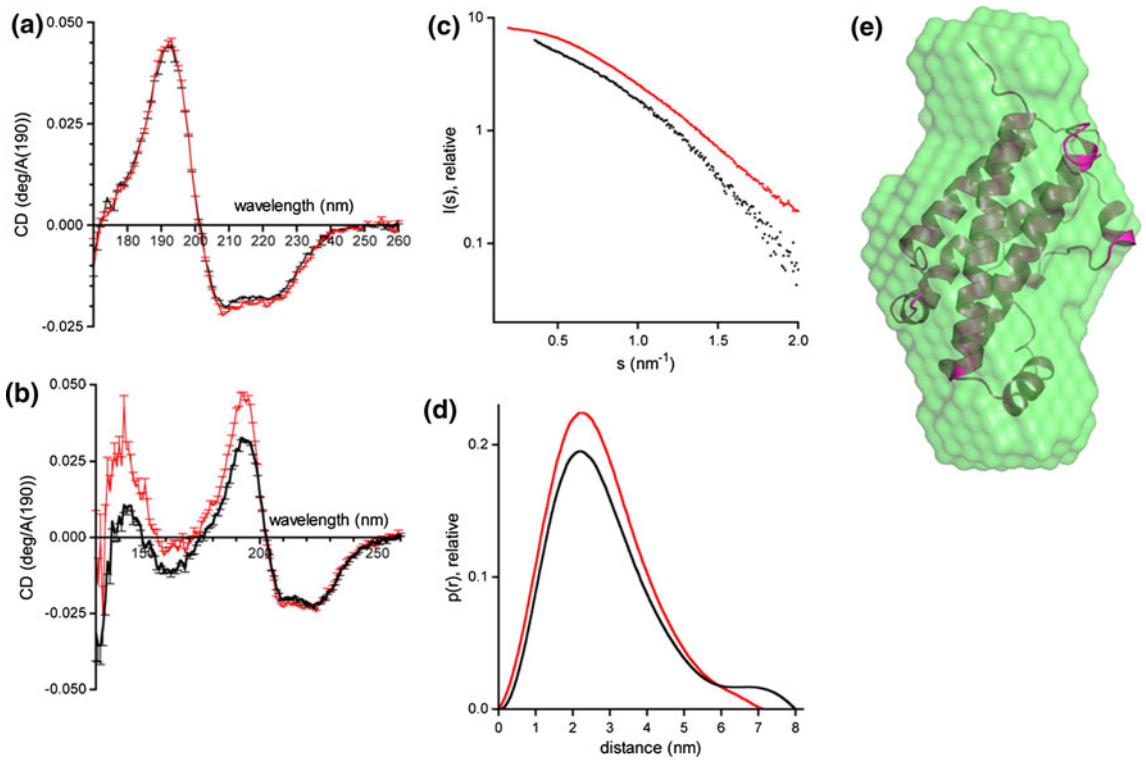

Fig. 4 **a** SRCD analysis of the folding of GH before electrophoresis (*red*) and after recovery (*black*). The spectra were corrected for concentration based on absorbance at 190 nm. *Error bars* (*red*, *upward*; *black*, *downward*) are shown for each point. **b** SRCD spectra of the GH samples after drying as a film onto the cuvette, coloring as in **a**. The spectra were normalized to A(190). **c** SAXS scattering curves for GH, coloring as in **a**. **d** Distance distribution functions for the GH samples, coloring as in **a**. **e** Superposition of the ab initio model of the GH solution structure (*green*) and the crystal structure of GH (*magenta*)

Table 2 Parameters from SAXS data

Sample	R_g (nm)	Volume (nm^3)	D_{max} (nm)	I(0)	MW (kDa)
GH	2.12	44.3	7.2	8.62	28.8
Recovered GH	2.28	43.8	8.0	7.55	25.3
GH crystal	1.80	29.1	6.5	–	22
SAXS control (CaM)	2.06	23.4	7.0	5.08	17

CaM was used as a standard for MW determination

Table 3 Immunofunctional assay result of rhGH before electrophoresis and after recovery

Dilution	Before electrophoresis			After recovery		
	IFA	Immulite	Ratio	IFA	Immulite	Ratio
1:100.000	45.5	24.3	1.9	37.5	18	2.1
1:500.000	8.3	4.5	1.8	8.1	4.4	1.8

IFA means Immunofunctional assay indicator of bioactvity because it involves binding to the (recombinant) extracellular domain of the GH receptor

Immulite is the routine assay which measures "total" GH molecules (as most sandwich type immunoassays the results represents "immunoreactivity" rather than biological acitvity)

Activity of the rhGH could be revealed by the use of an immunofunctional assay. This bioactivity assay reflects binding of GH to the receptor that in turn would require patent conformation, at least of the binding sites.

This approach for a rapid, non-sophisticated biophysical characterization of a protein in terms of primary and higher protein secondary and tertiary structure, and function is herein proposed as a useful tool for a series of applications in academia and industry. Although a series of enzyme activities have been already shown to be recovered by extraction from SDS/gels by so-called renaturation solutions (Afjehi-Sadat and Lubec 2007). The use of this model may even open ways for further analysis of the recovered native protein as e.g., for electron microscopy, diverse spectroscopic, binding and protein interaction studies or further functional assays including enzyme activity studies.

Acknowledgments We wish to thank the excellent support on the synchrotron beamlines involved in this study. In addition, data collection at synchrotrons has been supported by the EU, (European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 226716). We acknowledge the contribution by the Verein zur Durchführung der wissenschaftlichen Forschung auf dem Gebiet der Neonatologie und Kinderintensivmedizin "Unser Kind".

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