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Preparation of biologically active monomeric recombinant zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) recombinant growth hormones

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Abstract Fish growth hormones (GHs) play an important role in regulating growth, metabolism, reproduction, osmoregulation, and immunity and have thus garnered attention for their application in aquaculture. Zebrafish GH (zGH) cDNA or rainbow trout GH (rtGH) cDNA was cloned into the pMon3401 vector, expressed in MON105-competent *Escherichia coli* and purified to homogeneity. Their biological activity was evidenced by their ability to interact with ovine GH receptor extracellular domain and stimulate GH receptor-mediated proliferation in FDC-P1-3B9 cells stably transfected with rabbit GH receptor. The relative affinity of zGH and rtGH, estimated by IC_{50}, was about 38-fold and 512-fold lower, respectively, than ovine GH. This is likely the reason for the low biological activity in cells with rabbit GH receptor, ~ 36-fold lower for zGH and ~ 107-fold lower for rtGH than for human GH. This was not due to improper refolding, as evidenced by circular dichroism analysis. Predicting the activity of fish GHs is problematic as there is no one single optimal in vitro bioassay; heterologous assays may be ambiguous, and only homologous assays are suitable for measuring activity.

Keywords Growth hormone · Fish · Recombinant · Zebra fish · Rainbow trout

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Introduction

Growth hormone/prolactin (GH/PRL) family in teleosts includes GH, PRL, and two distinct somatolactins (SLα and SLβ) (Zhou et al. 2004). GH, PRL, and SL are single polypeptides consisting of 190–200 amino acids with a molecular mass in the range of 22–24 kDa; they have four conserved domains and four α-helix loops. Teleost GHs are produced by somatotroph cells in the anterior pituitary gland of fish and have been shown to be involved not only in promoting growth and development but also in reproduction, immune response, osmoregulatory adaptation, and metabolism (Santos et al. 2003; Canosa et al. 2007; Yada 2007). The actions of GH are initiated by its binding to localized GH receptor (GHR) located on the cell membrane of target tissues (liver, gonads, gill, intestine, kidney, and brain), possibly by forming of a trimeric complex via a homodimeric receptor and a GH molecule (van den Eijnden et al. 2006). Binding of GH to its receptor activates the tyrosine kinases that initiate a cascade of actions resulting in signaling induction through the activation of the signal transducer and activator of transcription protein (STAT) family of transcription factors (Herrington and Carter-Su 2001; Zhu et al. 2001). Given these biological

functions, the teleost GH has attracted attention in the field of aquaculture for development as a growth-promoting agent or immune stimulator. Using recombinant GH as an oral administration, fish growth rate was increased without accumulation of GH in the fish body (Moriyama et al. 1993). In aquaculture applications, fish growth levels were increased after using recombinant GHs (from diverse fish species) produced in various recombinant systems, mainly *Escherichia coli*. Bacterial expression systems are commonly used due to easy manipulation, inexpensive culture, high-level expression, and quick generation of recombinant protein (Chan et al. 2003).

To better characterize the biological properties of zebrafish (*Danio rerio*) GH (zGH) and rainbow trout (*Oncorhynchus mykiss*) GH (rtGH), and to provide a valuable tool for pharmacological homology studies, a procedure for large-scale production of both recombinant GHs was established and the biological activity of the purified protein was tested in vitro in a heterologous system.

Materials and methods

Recombinant hGH, ovine (o) GH and the extracellular domain of oGH receptor (oGHR-ECD), was purchased from Protein Laboratories Rehovot (Rehovot, Israel). FDC-P1-3B9 cells stably transfected with rabbit (rb) GHR were obtained from Dr. Mike Waters. Restriction enzymes used in the molecular biology experiments were from Fermentas (Vilnius, Lithuania); RPMI-1640 medium, nalidixic acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO); fetal calf serum (FCS) was from Biolab Co. (Jerusalem, Israel); and Superdex 75 HR 10/30 column and Q Sepharose were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Antibiotic-antimycotic solution (5×10^4 U/ml penicillin, 50 mg/ml streptomycin, 0.125 mg/ml fungizone), NaCl, and Tris base were purchased from Bio-Lab Ltd. (Jerusalem, Israel). Also used were bacto-tryptone, bacto-yeast extract, glycerol, EDTA, HCl, Triton X-100, urea (ENCO Diagnostics Ltd., Petah-Tikva, Israel), and NaOH (Frutarom Ltd., Haifa, Israel). Molecular markers for SDS gel electrophoresis were purchased from BioRad (Hercules, CA). All other materials were of analytical grade.

Preparation of expression plasmids encoding zGH and rtGH

Synthetic cDNA encoding the sequences of rtGH (NCBI gene database accession number NM_001124689) and zGH (NCBI gene database accession number NM_001020492) was ordered from GENEWIZ (South Plainfield, NJ). The cDNA was modified to ensure better codon usage and expression in *E. coli*. The cDNA in corresponding pUC57-Amp plasmids was digested with NcoI and HindIII, extracted, and ligated into linearized pMon3401 expression vector. *E. coli* MON105-competent cells were transformed with the new expression plasmid and plated on Luria-Bertani (LB) agar plates containing 75 μg/ml spectinomycin for plasmid selection. Four *E. coli* colonies were isolated and confirmed to contain the respective cDNA by digestion with NcoI/HindIII restriction enzymes. All colonies were positive and one of them was sequenced.

Expression, refolding, and purification of zGH and rtGH

E. coli (strain MON105) cells transformed with the respective plasmids were grown in 2.5-l flasks at 37 °C, 200 rpm, in 500 ml Terrific Broth (TB) medium to an absorbance of 0.9, at 600 nm after which nalidixic acid (2.5 ml of 10 mg/ml in 0.1 N NaOH) was added. The cells were grown for an additional 4 h, pelleted for 10 min at 7000 × *g*, and stored at − 20 °C. Inclusion bodies (IBs) for subsequent refolding of zGH and rtGH were prepared as described previously (Raver et al. 2002) and frozen. The IBs containing rtGH were solubilized in a total volume of 100 ml containing 4.5 M urea buffered with 40 mM Tris base. Cysteine was added to a final concentration of 1 mM. The pH was increased to 11.0 with NaOH, and the clear solution was gently stirred at 4 °C for 1.5 h. The solution was then diluted with three 300 ml volumes of 0.667 M cold arginine, stirred at 4 °C for 2 h, and then dialyzed against 5 × 10 l of 10 mM Tris–HCl pH 10 for 48 h. The solution was next loaded at 120 ml/h onto a Q Sepharose column pre-equilibrated with 10 mM Tris–HCl pH 10 at 4 °C. Elution was carried out using a discontinuous NaCl gradient in the same buffer, and 40-ml fractions were collected.

IBs from bacteria expressing zGH, obtained from 2.5 l fermentation culture, were suspended in 100 ml cold DDW double distilled water and sonicated. IB

suspension (30 ml) was used for refolding in a total volume of 300 ml containing 4.5 M urea buffered with 40 mM Tris base. Cysteine was added to a final concentration of 0.1 mM. The pH was increased to 11.0 with NaOH, and the clear solution was gently stirred at 4 °C for 1.5 h. The solution was then diluted with two volumes (600 ml) of cold DDW, stirred at 4 °C for 2 h, and then dialyzed against 5 × 10 l of 10 mM Tris–HCl pH 8 for 48 h. Then NaCl was added to a final 150 mM, and the solution was loaded onto a Q Sepharose column (2 × 6 cm) pre-equilibrated with 10 mM Tris pH 8.0 containing 0.15 M NaCl. The flow-through was collected, concentrated to 20 ml, and loaded onto a preparative G-75 column (2.6 × 60 cm) pre-equilibrated with 25 mM Tris–HCl buffer pH 8.0 containing 300 mM NaCl. In both cases, the protein concentration was determined by absorbance at 280 nm, and monomer content was determined by gel-filtration chromatography on a Superdex 75 (HR 10/30) column. Fractions containing the monomeric protein were pooled, dialyzed against $NaHCO_3$ (4:1 protein-to-salt ratio), and lyophilized.

Determination of purity and monomer content

SDS-PAGE was carried out according to Laemmli (1970) in a 15% polyacrylamide gel under reducing conditions. The gel was stained with Coomassie Brilliant Blue R. Size exclusion chromatography (SEC) was performed on a Superdex 75 HR 10/30 column for determination of monomeric content of zGH and rtGH. The column was pre-equilibrated and developed using TN buffer (25 mM Tris–HCl, 300 mM NaCl, pH 8) at room temperature. The columns were calibrated with leptin (16 kDa), hGH (21.5 kDa), and bovine serum albumin (66 kDa).

Determination of CD spectra

Circular dichroism (CD) spectra were recorded using a J-810 spectropolarimeter (JASCO) in a 0.1-cm quartz cuvette for far-ultraviolet CD spectroscopy. Far-ultraviolet CD spectra were collected over 190–260 nm at 25 °C. Lyophilized GHs were dissolved in water, dialyzed against 50 mM phosphate buffer pH 7.5 for 20 h, and adjusted to 50 μM concentrations. The CD measurements were performed at 25.0 °C as controlled by thermoelectric Peltier elements to an accuracy of 0.1 °C. The CD spectra were measured in five repetitions to obtain an average spectrum for each protein. Standard deviation of the averaged CD signal at 222 nm was in the 5% range. For secondary-structure determination, CD data were expressed in degree square centimeter per decimole per mean residue, based on respective molecular mass. For secondary-structure analysis, the DICHROWEB program was used, and the α-alpha helical content was calculated using the analysis program SELCON3 (Sreerama and Woody 2000; Whitmore and Wallace 2004).

Detection of rtGH and zGH complex formation with oGHR-ECD and complex stability determination by SEC

To evaluate the binding stoichiometry of rtGH and zGH with oGHR-ECD, the latter was mixed with each GH separately at different molar ratios, incubated for 10 min at 4 °C, and then separated under nondenaturing conditions by SEC using an analytical Superdex 75 HR 10/30 column equilibrated with TN buffer (25 mM Tris–HCl, 300 mM NaCl, pH 10) at room temperature, as described previously (Niv-Spector et al. 2005). To estimate the stability of each complex, the complex solution with 10 μM of each component was subjected to consecutive dilutions and the retention time (RT) of each complex was determined by SEC.

Receptor-binding assay

In the competitive binding experiment, immobilized oGHR-ECD was used as a receptor, and biotinylated oGH served as the ligand that could be competed off by zGH or rtGH. Polystyrene 96-well microtiter plates were coated overnight at 4 °C with 100 μl of 42 μM oGHR-ECD in phosphate-buffered saline (PBS), pH 7.4. Wells were then washed once with PBST (PBS containing 0.05% *w*/*v* Tween 80) and blocked with PBS containing 3% (*w*/*v*) skim milk for 2 h at room temperature. All further incubations were carried out at room temperature. Wells were washed again once with PBST and incubated with different concentrations of unlabeled zGH or rtGH (50 μl/well) for 30 min, and then 50 μl of 72 pM biotinylated oGH was added to each well for another 2 h. The wells were then washed three times with PBST and incubated with 1:10,000 diluted streptavidin/HRP for 1 h. Wells were washed three times with PBST and then developed using 100 μl/well 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (KPL, Zotal, Israel) diluted 1:4 and incubated

for 30 min. The reaction was stopped with 50 μl of 2 N H_2SO_4, and the absorbance was read at 450 nm by ELISA MicroPlate Reader ELx808 (Bio-Tek Instrument Inc., Winooski, VT).

In vitro biological activity

FDC-P1-3B9 cells transfected with rbGHR were grown as suspension cultures in 75-cm^2 tissue-culture flasks (Nunc, Kamstrup, Denmark). For maximal growth and routine maintenance, cells were cultured in RPMI-1640 medium containing 5% (*v*/v) FCS supplemented with antibiotic-antimycotic cocktail solution and 100 ng/ml recombinant hGH was added to each flask to promote growth. The cells were incubated under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Before seeding, the cells were washed three times with PBS and centrifuged at 1000×*g* for 4 min. The cells were then resuspended in RPMI-1640 medium containing 5% (*v*/*v*) horse serum. The experiment was performed using 96-well plates (Nunc) in which 0.1-ml suspensions containing 2.0×10^4 cell/well were seeded. Aliquots (10 μl) of increasing concentrations of zGH or rtGH were added (from 0.23 to 1500 ng/ml), and GH-dependent cell proliferation was determined by MTT assay after 48 h as described previously (Solomon et al. 2006). Briefly, 20 μl of the MTT solution (0.625% in PBS) was added to each well and the plates were left at 37 °C. Two hours later, 100 μl of the solubilization/stop solution (containing 20 g of SDS in 100 ml of 50% dimethylformamide and 2.5 ml of 80% acetic acid) was added, and the plates were left overnight at 37 °C. Cell proliferation was determined by monitoring the absorbance at 595 nm, using an ELISA plate reader (ELx800, Bio-Tek Instruments, Inc., Winooski, VT). The average absorbance in wells without GH (negative control) was used as a blank value and subtracted from other absorbance values to yield the corrected absorbance values. The growth curves were drawn using the Prizm (4.0) nonlinear regression sigmoidal dose-response curve and, the EC_{50} values were calculated.

Results

Purification and chemical characterization of recombinant zGH and rtGH

E. coli MON105 cells transformed with the expression plasmids containing rtGH cDNA were induced with nalidixic acid (50 mg/flask). In a preliminary experiment performed with four clones, the induced cells expressed rtGH (~21 kDa) as a main cell protein and one clone was chosen for large-scale preparation. Over 95% of the rtGH was found in the insoluble IBs. The refolded rtGH was purified on a Q Sepharose column by stepwise elution with increasing concentrations of NaCl. Fractions eluted with 0.15 M NaCl that contained monomeric rtGH were pooled, dialyzed against $NaHCO_3$ (at a 4:1 protein-to-salt ratio), and lyophilized. Similarly, the zGH was purified by two-step chromatography, first on the Q Sepharose column and then by preparative SEC. The purity of these fractions was further confirmed by SDS-PAGE analysis in the presence of reducing agents (Fig. 1) and by SEC analysis on a Superdex 75 HR 10/30 analytical column (Fig. 2a, c). As shown, both GHs consisted of > 90% monomers eluted at RTs of 14.80 min (rtGH) and 14.54 min (zGH). The respective yield of pure monomeric rtGH and zGH obtained from 5 l of *E. coli* culture varied between 12 and 18 mg of pure monomeric protein. The specific absorbance at 280 nm for a 0.1 mg/ml solution of zGH and rtGH as calculated from amino acid composition using DNAman program was, respectively, 0.42 and 0.62.

To characterize the folding propensity of our recombinant proteins, we performed CD analysis. Figure 3a shows the CD spectra of zebrafish and rainbow trout GHs at neutral pH and Fig. 3b that of hGH. A high

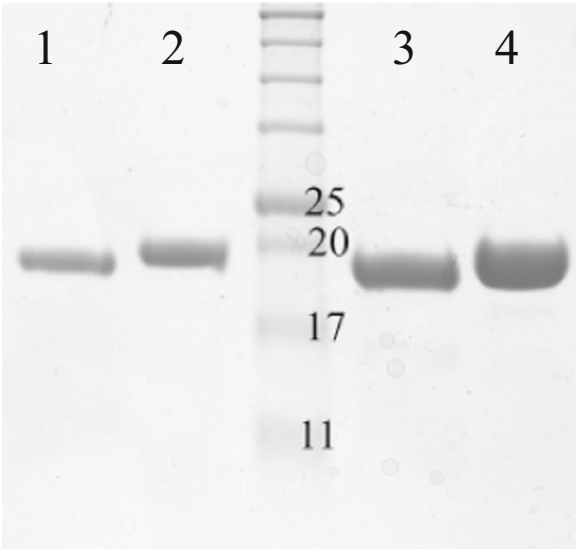

Fig. 1 SDS-PAGE analysis (15%) of recombinant rainbow trout and zebrafish GHs. Lane 1, 5 μg zGH; lane 2, 5 μg rtGH; lane 3, 10 μg rtGH; lane 4, 10 μg rtGH

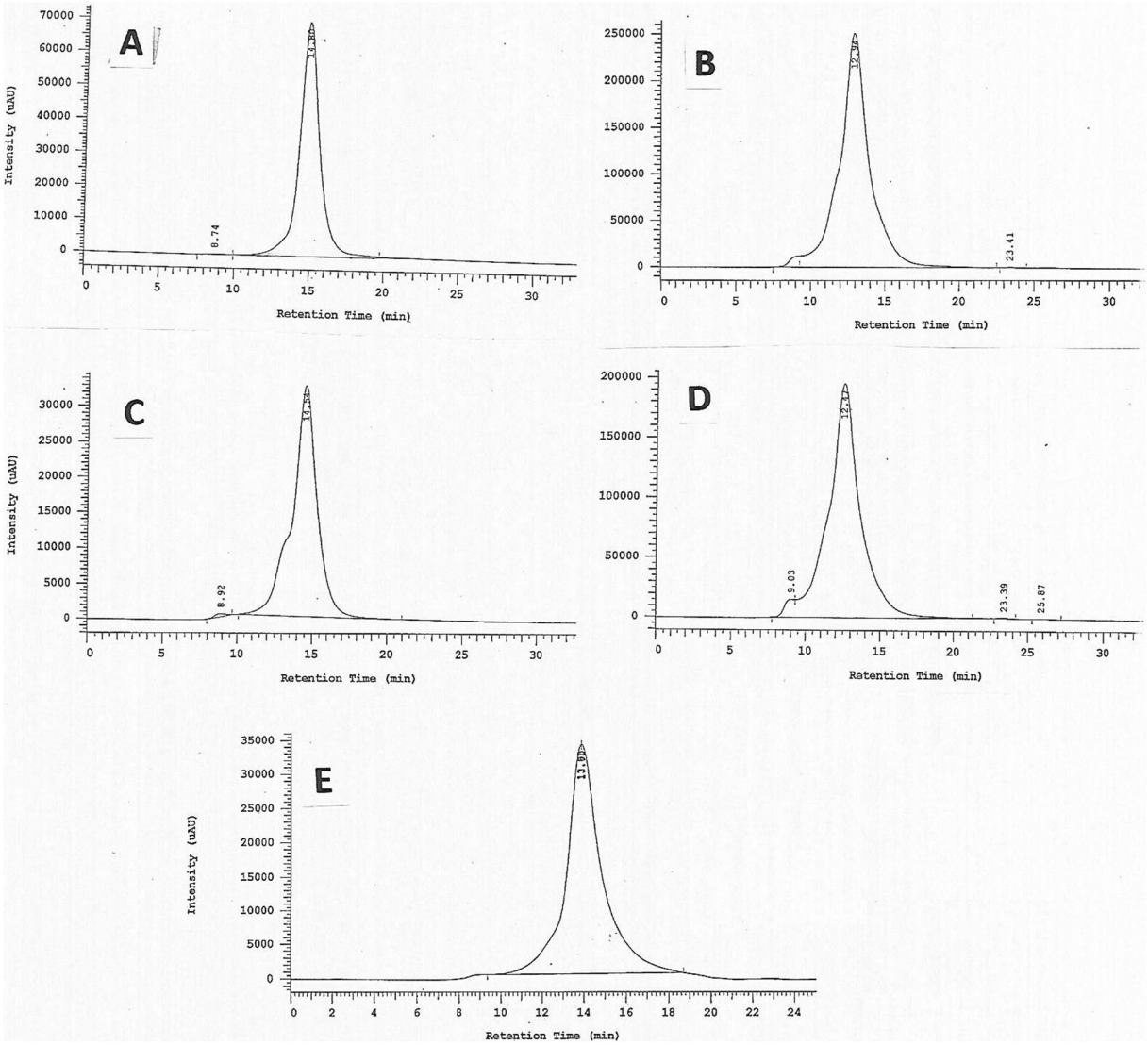

Fig. 2 Size exclusion chromatography (SEC) of purified proteins and their complexes with oGHR-ECD. **a** rtGH, **b** rtGH-oGHR-ECD complex, **c** zGH, **d** zGH-oGHR-ECD complex, and **e** oGHR-ECD. SEC was performed on a Superdex 75 HR 10/30 column. The column was pre-equilibrated and developed using TN buffer (25 mM Tris–HCl, 300 mM NaCl, pH 8) 0.8 ml/min at room temperature. The columns were calibrated with leptin (16 kDa), hGH (21.5 kDa), and bovine serum albumin (66 kDa)

content of α-helix for both rtGH, zGH, and hGH indicated proper refolding.

Gel-filtration (SEC) experiments

To characterize the binding stoichiometry between rainbow trout or zebrafish GH and oGH-ECD, the respective ligands and oGH-ECD were mixed in different molar ratios and separated by SEC using an analytical Superdex 75 (HR 10/30) column to determine the molecular mass of the binding complex under nondenaturing conditions. The experiments were performed using a constant 70 μM of the respective ligand and oGHR-ECD. Both zGH and rtGH proteins complexed at a 1:1 M ratio with oGHR-ECD (Fig. 2d, b, respectively), as evidenced by the appearance of a single main peak for the complex with shorter RT (12.47–12.94 min), as compared to the higher retention times of oGHR-ECD (13.90 min; Fig. 2e) and the GHs (14.54–14.80 min; Fig. 2a, c). This main peak appeared when the components were mixed at a 1:1 M ratio, whereas at a 2:1 M ratio of ECD to either zGH of rtGH, excess ECD was observed (not shown). This

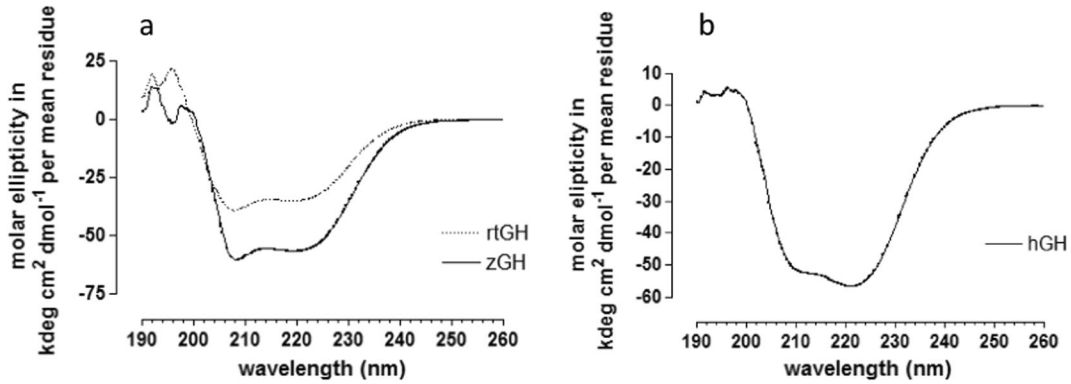

Fig. 3 Comparing the 2D structure of zGH and rtGH (**a**) shows CD of zGH and rtGH, while (**b**) shows the CD of hGH. The CD spectra of purified recombinant zGH and rtGH were collected over 190–260 nm at 25 °C. For secondary-structure analysis, the DICHROWEB program was used. CD spectra were recorded using a J-810 spectropolarimeter (JASCO) in a 0.1-cm quartz cuvette for far-ultraviolet CD spectroscopy. Far-ultraviolet CD spectra were collected over 190–260 nm at 25 °C. Lyophilized GHs were dissolved in water, dialyzed against 50 mM phosphate buffer pH 7.5 for 20 h, and adjusted to 50 μM concentrations. The CD measurements were performed at 25.0 °C as controlled by thermoelectric Peltier elements to an accuracy of 0.1 °C. For experimental details, see text

indicated that at that concentration, only 1:1 M complex is formed. The complex's calculated molecular mass, based on the peak's RT, was 41 kDa in both cases, close to the predicted value of 49.5 kDa. Tenfold dilution of the complexes to 7 μM followed by gel filtration indicated partial dissociation of the complex (not shown).

Binding activity

The binding activity of rtGH and zGH was compared to that of oGH in a competitive nonradioactive binding assay using biotinylated oGH as the ligand and oGHR-ECD as the receptor. Both rtGH and zGH displaced the biotinylated oGH with respective EC_{50} values of 1.75×10^{-5} M and 2.21×10^{-6} M, which were statistically significantly different (Fig. 4).

Biological activity in vitro

To measure the somatogenic activity of the GHs, a cell line stably transfected with rbGHR (FDC-P1-3B9) was employed. Results of a typical experiment (out of three)

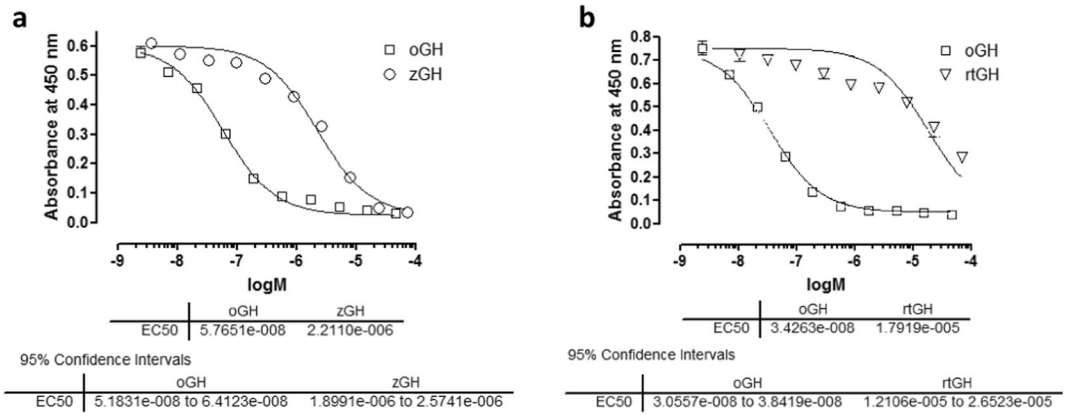

Fig. 4 Competitive displacement of biotinylated oGH bound to plated oGHR-ECD by zGH (**a**) or rtGH (**b**). In the competitive binding experiment, immobilized oGHR-ECD was used as a receptor and biotinylated oGH served as the ligand that could be competed off by zGH or rtGH. Polystyrene 96-well microtiter plates were coated overnight at 4 °C with 100 μl of 42 μM oGHR-ECD in phosphate-buffered saline (PBS), pH 7.4. Wells were then washed once with PBST (PBS containing 0.05% *w*/*v* Tween 80) and blocked with PBS containing 3% (*w*/*v*) skim milk for 2 h at room temperature. All further incubations were carried out at room temperature. Wells were washed again once with PBST and incubated with different concentrations of unlabeled zGH or rtGH (50 μl/well) for 30 min, and then 50 μl of 72 pM biotinylated oGH was added to each well for another 2 h

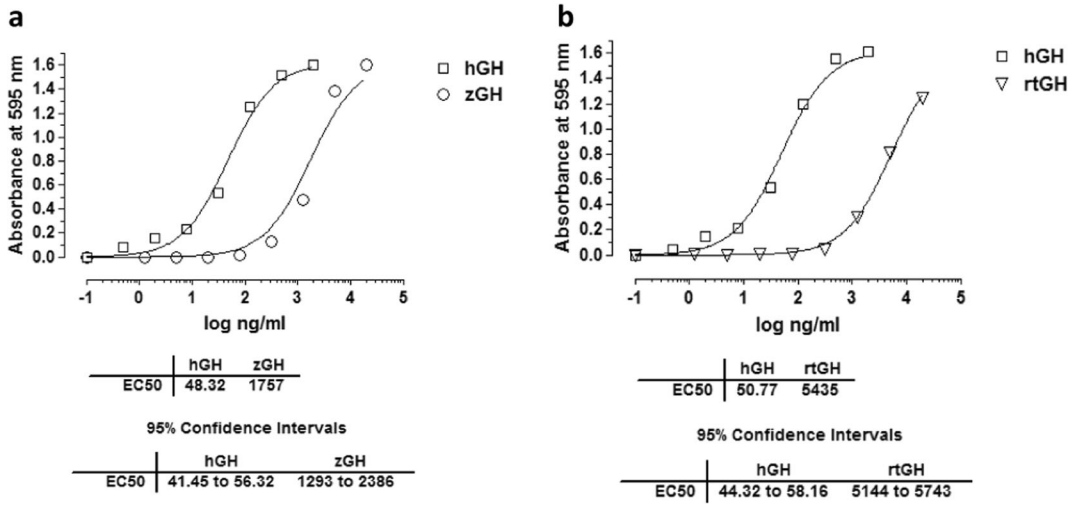

Fig. 5 Comparison of biological activity of zGH (**a**) and rtGH (**b**) in FDC-P1-3B9 cells stably transfected with rbGHR. The experiment was performed using 96-well plates (Nunc) in which 0.1-ml suspensions containing 2.0×10^4 cells/well were seeded. Aliquots (10 μl) of increasing concentrations of zGH or rtGH were added (from 0.23 to 1500 ng/ml), and GH-dependent cell proliferation was determined by MTT assay after 48 h as described previously (Solomon et al. 2006)

are presented in Fig. 5. The biological activity of both GHs was compared with that of hGH. The results were analyzed by PRISM software, according to a nonlinear regression sigmoidal dose-response curve. The mitogenic activity of zGH was ~ 36-fold lower, whereas that of rtGH was ~ 107-fold lower than that of hGH.

Discussion

Recombinant rtGH and zGH were purified to homogeneity, as documented by SDS-PAGE, and contained more than 95% monomeric protein, as evidenced by gel-filtration analyses. The secondary structure of zGH was similar to that of hGH, whereas the α-helix content of rtGH indicated a slightly different, though still similar, secondary structure, thus indicating proper folding. The biological activity of the purified proteins was assessed by analyzing their ability to bind to oGHR-ECD and by in vitro heterologous bioassays. The binding stoichiometry clearly showed that though both zGH and rtGH have the capacity to interact with oGHR-ECD, they form a 1:1 M complex in contrast to mammalian GHs which show a 2:1 oGHR-ECD ratio. This indicated not only reduced affinity to GHR site one, as shown by the binding experiments (see Fig. 2), but even lower affinity to site two. The binding activity of zGH and rtGH was compared to that of oGH (serving as a positive control) in a nonradioactive binding assay. Both GHs competed with biotinylated oGH for binding to immobilized oGHR-ECD, and their relative affinity, as judged by IC_{50} value, was about 38-fold (zGH) and 512-fold (rtGH) lower than that of oGH. This lower affinity is likely the reason for the low biological activity in cells possessing rbGHR (Fig. 5), ~ 36-fold lower for zGH and ~ 107-fold lower for rtGH as compared to hGH in FDC-P1-3B9 cells stably transfected with the rbGH receptor. These findings raised the question of whether the lower affinity of rtGH and zGH results from improper refolding or is an intrinsic property. A previous report showed that purified dolphin fish (*Coryphaena hippurus*) GH exhibits growth-stimulating activity in FDC-P1-3B9 cells, even though its bioactivity is 300-fold lower than that of hGH (Paduel et al. 1999). Interestingly, common carp GH activity in FDC-P1-3B9 was only 20-fold lower than that of hGH, whereas the gilthead sea bream GH was not active at all. It seems that rather minor differences in primary structure account for different species specificities (Paduel et al. 1999). Most studies investigating the growth-stimulating activity of recombinant fish GHs have been performed in vivo by intraperitoneal injection or oral administration to fish (Poen and Pornbanlualap 2013); in vitro growth-stimulating function has rarely been confirmed in fish cell lines. Chung et al. (2015) evaluated the biological activity of recombinant giant grouper (*Epinephelus lanceolatus*) GH (ggGH) by determining its growth-promoting activity on zebrafish liver cells.

Cells in starvation media were treated with different concentrations of purified ggGH, hGH, and BSA and then examined using a WST-1 (water-soluble tetrazolium salt of (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate)) assay. The ggGH significantly stimulated the proliferation of zebrafish liver cells compared to the control group, and this proliferation occurred in a dose-dependent manner for concentrations ranging from 1 to 10 ng/ml, indicating that species specificity plays a major role.

In conclusion, using a relatively simple protocol for the purification and refolding of zGH and rtGH into a biologically active form, we obtained pure recombinant proteins, thus providing a research tool for future in vitro and in vivo investigations. Based on our results, we suggest that there is no one single optimal in vitro bioassay for teleost GHs; the heterologous assays may be ambiguous, and only the homologous assays are suitable for measuring activity.

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