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Muscular hypertrophy and growth-promoting effects in juvenile pejerrey (*Odontesthes bonariensis*) after oral administration of recombinant homologous growth hormone obtained by a highly efficient refolding process

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Abstract

Growth hormone (GH) can be orally administrated to fish in order to increase growth rates. Fish growth is characterized by the hyperplasia and hypertrophy of muscle fibre throughout adult life. In this respect, GH could affect directly and indirectly (by growth and metabolic factors) the development and growth of muscle fibres. Recombinant pejerrey GH (r-pjGH) was expressed in *Escherichia coli* and refolded in a highly efficient batch dilution system, obtaining 0.1 g L^{-1} of hormone without protein precipitation during the refolding procedure. Orally administered hormone to pejerrey produced a 30% increase in mean weight and stimulated liver insulin-like growth factor type I (IGF-I) mRNA expression after 1 month of treatment. Histological analyses showed that muscle growth was generated mainly by hypertrophy of the fibres. A higher r-pjGH dose increased muscle fibre hypertrophy but somatic growth was negatively affected probably due to a reduced capacity of generating new fibres.

Keywords: pejerrey, growth hormone, refolding, IGF-I, muscle, hypertrophy

Introduction

Pejerrey (*Odontesthes bonariensis*, *Atheriniformes*) is an inland water native fish from South America. The high qualities of its flesh and its commercial and social importance have made pejerrey a good candidate for aquaculture. Even though efforts are being made to optimize pejerrey intensive and semi-intensive culture, adequate growth rates for commercial purposes have not been reached yet (Somoza, Miranda, Berasain, Colautti, Remes Lenicov & Strüssmann 2008). Regarding pejerrey aquaculture requirements, reproduction of this species has already been optimized in order to obtain high-quality eggs and seedlings (Miranda, Berasain, Velasco, Shirojo & Somoza 2006; Miranda & Somoza 2009). Then, advances in pejerrey growth endocrinology knowledge become essential for future improvements of pejerrey culture.

Pejerrey growth hormone (pjGH) and insulin-like growth factor type I (pjIGF-I) were sequenced and characterized (Sciara, Rubiolo, Somoza & Arranz 2006; Sciara, Somoza & Arranz 2008). It has also been reported that intraperitoneal injections of recombinant pjGH (r-pjGH) induced pjIGF-I mRNA expression in pejerrey liver (Sciara *et al.* 2008). Although potential biotechnological applications arise from these works, several improvements must be carried out to effectively use growth hormone (GH) as a growth enhancer in pejerrey. First, r-pjGH should be efficiently refolded by using simple methods and low volumes of refolding solution; second, an adequate method for r-pjGH delivery to fish

should be used; and finally, the fact that r-pjGH promotes growth has to be demonstrated.

It has been reported that GH transgenic expression could produce dramatic growth enhancement when low-expression promoters were used (Sin 1997). A 40-fold increase in growth rate was achieved when β-metallothionein promoter was used to direct homologous GH expression in salmon *Oncorhynchus kisutch* (Devlin, Yesaki, Biagi, Donaldson, Swanson & Chan 1994). Also and in order to obtain rapid growth promotion in aquaculture, several key species were transfected with GH constructions with success; for example, tilapia *Oreochromis niloticus* (Martinez, Estrada, Berlanga, Guillén, Hernandez, Cabrera, Pimentel, Morales, Herrera, Morales, Pina, Abad, Sanchez, Melamed, Lleonart & De La Fuente 1996; Rahman, Mak, Ayad, Smith & Maclean 1998), carp *Cyprinus carpio* (Chen, Kight, Lin, Powers, Hayat, Chatakondi, Ramboux, Duncan & Dunham 1992) and channel catfish *Ictalarus punctatus* (Dunham, Ramboux, Duncan, Hayat, Chen, Lin, Gonzalez-Villasenor & Owers 1992). The use of GH has other associated advantages, mainly the increase of food conversion, for example transgenic tilapia expressing GH increased food conversion up to 290% compared with non-transgenic fish (Martinez, Juncal, Zaldivar, Arenal, Guillen, Morera, Carrillo, Estrada, Morales & Estrada 2000). Genetically modified fish have considerable potential to further increase aquaculture yields but have prompted serious concerns about the possible environmental impact on wild species (Muir 2004), and also market acceptance. To overcome these drawbacks, the external administration of GH has been considered as an alternative (Habibi, Ewing, Bajwa & Walker 2003). This technique is usually performed by intraperitoneal injection (Tsai, Lin, Kuo & Chen 1995; Guillen, Lleonart, Agramonte, Morales, Morales, Hernandez, Vazquez, Diaz, Herrera, Alvarez-Lajonchere, Hernandez & De La 1998; Promdonkoy, Warit & Panyim 2004) or oral administration (Moriyama, Yamamoto, Sugimoto, Abe, Hirano & Kawauchi 1993; Ben Atia, Fine, Tandler, Funkenstein, Maurice, Cavari & Gertler 1999). Owing to its simplicity, oral administration is normally the method of choice for aquaculture peptide or drug dispensing. The effect of oral GH administration in fish has been associated with growth promotion in several species such as coho salmon (Moriyama, Duguay, Conlon, Duan, Dickhoff & Plisetskaya 1993), flounder *Paralichtys olivaceus* (Jeh, Kim, Lee & Han 1998), perch *Perca fiuviatilis* (Jentoft, Aastveit & Andersen 2004) and giant catfish *Pangasianodon gigas* (Promdonkoy *et al.* 2004). This strategy could be used in teleost fish because their intestine epithelium is capable of absorbing intact high-molecular-weight proteins (McLean and Donaldson 1990; Habibi *et al.* 2003).

Muscle is the tissue that concerns the main interest in fish farming and it compromises $> 50\%$ of whole body weight. Therefore, it is important to understand the effects of different treatments in muscle fibre formation. Growth hormone axis is known to affect muscle fibre differentiation and hypertrophy. An increase in thymidine uptake above the basal levels is caused by IGF-I in primary salmon myoblasts culture, which was not observed when they were incubated with insulin (Castillo, Codina, Martinez, Navarro & Gutierrez 2004). In the muscular tissue of GH-transformed char *Salvelinus alpinus* L., the enhanced growth was clearly associated with the proliferation of muscle cells (hyperplasia), whereas in heart tissue both cell proliferation and increase in cell size (hypertrophy) were enhanced (Pitkanen, Krasnov, Teerijoki & Molsa 1999). Nevertheless, the effects of GH and IGFs on muscle growth and differentiation are barely known and should be analysed independently for each species.

In order to achieve these goals, the present work describes a highly efficient method for pjGH refolding by solubilization of inclusion bodies in high urea concentration and batch dilution. Also, the effect of orally supplemented r-pjGH on the liver IGF-I mRNA expression and growth promotion was tested. Additionally, we analysed the effect of pjGH supplementation on muscle fibre growth and differentiation.

Materials and methods

Hormone solubilization

Recombinant pejerrey GH was obtained in *Escherichia coli* as already described in Sciara *et al.* (2006). In order to determine the effect of urea concentration on r-pjGH intermolecular interactions, recovered inclusion bodies were solubilized in different concentrations of urea and 100 mM Tris-HCl pH 10.5 for 3 h. Samples were analysed in a 12% polyacrylamide and 8 M urea gel electrophoresis with 0.25 M Tris and 1.9 M glycine pH 8.5 buffer. The effect of the solubilization time on the r-pjGH disulphide formation was also evaluated. Inclusion bodies were solubilized in 8 M urea, 100 mM Tris-HCl pH 10.5 using different incubation times. Disulphide formation was evaluated by the migration rate of r-pjGH in a 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) without reducing agents. The gels were stained with 0.1% Coomassie brilliant blue R 250.

Hormone refolding by dilution and dialysis

Inclusion bodies were solubilized in 8 M urea and 100 mM Tris-HCl pH 10.5. This solution was incubated for 1 h under mild agitation and was centrifuged at 10 000 *g* for 10 min. Hormone concentration was adjusted to 1 mg pjGH mL^{-1} before the beginning of the folding process. The effect of different folding solutions was evaluated. One volume of r-pjGH solution was slowly added to nine volumes of the folding solution with a peristaltic pump at 10 °C. Both solubilization and folding processes were set to last 6 h. After centrifugation at 20 000 *g* for 20 min, the solution was dialysed three times in 20 mM Tris-HCl, 0.5 mM EDTA pH 10.5; 20 mM Tris-HCl, 0.5 mM EDTA pH 9.5 and $NaHCO_3$ 0.05% for 12 h each. Final r-pjGH concentration was estimated using 280 nm absorption (estimated absorption coefficient is 1.39 mg mL^{-1} cm^{-1}). The purity of the refolded r-pjGH was analysed by HPLC using a Zorbax Bio Series GF-450 gel filtration column (9.4 mm ID × 250 mm, Rockland Technologies, Mount Vernon, NY, USA). Elution was performed isocratically with Tris-HCl 20 mM pH 9 buffer. Purity was determined by integration of the peaks.

pjGH structure determination

Circular dichroism spectrum of soluble r-pjGH was obtained at 25 °C in the wavelength range of 200–250 nm using a JASCO J810 spectropolarimeter (Jasco, Easton, MD, USA). The sample was scanned 10 times for data accumulation and the average spectrum was plotted. Primary structure hydrophobic plots were obtained using a Java-based Internet facility (http://www.vivo.colos tate.edu/molkit/hydropathy). Secondary structure estimation using circular dichroism spectra was achieved using DICROPROT (V 2.6) program (Deleage & Geourjon 1993). Secondary structure prediction using protein sequence was made using SYMPRED program (http://bio-cluster.iis.sinica.edu.tw/bioapp/hyprosp2/index.html; Simossis & Heringa 2004). Tertiary structure prediction was achieved using homology modelling PHYRE Internet facility (http://www.sbg.bio.ic.ac.uk; Nett-Lovsey, Herbert, Sternberg & Kelley 2008) and with these predictions, pjGH 3D image was plotted using the PYMOL MOLECULAR GRAPHIC SYSTEM v 0.99.

Western blot analysis

A homologous and specific antiserum against pjGH was used in order to detect pjGH using western blot. Preparation and characteristics of this antiserum were described by Sciara *et al.* (2006). Total proteins from pituitary extracts and r-pjGH were separated on 15% SDS-PAGE with Tris-glycine buffer (Laemmli 1970) and electroblotted on nitrocellulose membrane. The strips were equilibrated in phosphate buffer saline (PBS) pH 7.2, and blocked for 2 h in blotto (5% non-fat dry milk in PBS pH 7.2) at room temperature. The membrane was then incubated in 1/5000 dilution of the antiserum for 2 h in blotto, fully washed and allowed to react for 1 h with horseradish-peroxidase-labelled goat anti-rabbit IgG (1/10 000 dilution in blotto). After washing in blotto (3 × 15 min), the strips were rinsed briefly in PBS and finally developed by incubation for 15–20 min in a freshly prepared substrate solution of 20 mL of 100 mM Tris-HCl pH 7.2 containing 2 mg of 3,3-diaminobenzidine, 8 mg $NiCl_2$ and 7.5 µL of ice cold 30% hydrogen peroxide. Colour development was stopped by washing the strips in water.

Fish

Pejerrey fish were obtained from IIB-INTECH aquatic facilities (Chascomús, Buenos Aires, Argentina). Juvenile fish (30 days after hatching) were maintained in 100 L open circulation tanks. The temperature was maintained at 19 ± 1 °C and water salinity at 4 ppm. The fish were exposed to a constant photoperiod (14 L:10 D). The fish were fed to satiation twice a day with a commercial food powder (Shulet, Gral, Las Heras, Bs As, Argentina) and twice a day with *Artemia sp.* nauplii. All animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Santa Fe, Argentina) and the IIB-INTECH local regulations.

Growth-promoting effects

Biological activity of the purified r-pjGH was evaluated by measuring the growth-promoting effect on pejerrey by oral administration. From preliminary experiments with various oral doses of r-pjGH, the amount of r-fGH for oral delivery was determined as one dose every week at a dosage of 2 and 20 µg r-pjGH per gram of body weight (gbw) of pejerrey. A total of 300 juvenile fish were randomly distributed in three 100 L tanks. Artificial food containing two different doses of r-pjGH or carrier was provided once a week for 6 weeks providing a ration of 2% of the estimated body weight. Fish were starved the day before the administration of r-pjGH-containing food.

Random samples of 20 fish were taken from each tank 1 day after the hormone treatment every 2 weeks in order to measure weight and standard length of each fish. Feed pellets containing the oral formulation were prepared as follows: 1 g of artificial food was thoroughly mixed with 1.5 mL of distilled water, 1 mL of soluble r-pjGH (0, 0.1 or 1 mg mL^{-1}) in 0.05% $NaHCO_3$ and 0.5 mL of gelatinized 4% starch. In order to obtain a 1 mg mL^{-1} solution, the 0.1 refolded r-pjGH solution was freeze-dried and dissolved in a proper volume of 0.05% $NaHCO_3$. The food preparation was freeze-dried and processed to obtain a powder of 0.5–1.5 mm in diameter. Final concentrations of r-pjGH in the preparations were 0, 0.1 and 1 mg g^{-1}. Thereafter, doses of 0, 2 and 20 μg r-pjGH gbw^{-1} were provided to each group. Mortality during the experiment was 15% average with no significant difference between treatments. In addition, all fish were necropsied in order to visually determine the amount of adipose tissue in the coelomic cavity.

Muscle fibre measurements

Ten healthy specimens of *O. bonariensis* were randomly selected from each treatment at day 28. Fish were sacrificed by anaesthetic overdose using benzocaine solution (100 ppm). Muscle samples were obtained by sectioning the caudal peduncle immediately after the caudal border of the first dorsal fin. All samples were fixed in 10% buffered formalin, and embedded in paraffin wax. Sections of 5 μm thickness were collected on slides and allowed to dry overnight, after which the sections were dewaxed, hydrated, stained with haematoxylin and eosin, dehydrated and cover slipped. Cross-sections of each caudal peduncle were photographed at × 100 magnification. For image analysis, four areas of white muscle (2.5×10^5 μm^2) were randomly chosen for the assessment of mean fibre area (MFA) and the percentage of fibres with an area of < 500 μm^2 ($F < 500$) in order to evaluate the effect of r-pjGH administration in muscle fibre growth and differentiation. All measurements were performed on digitized images using IMAGEJ v 1.36b in the public domain available from the National Institutes of Health, USA.

Hepatic IGF-I mRNA relative quantification after pjGH oral administration

In order to evaluate the effect of r-pjGH oral administration on liver IGF-I mRNA expression, 72 pejerrey of 14.5 ± 4.5 g weight were distributed in three 100 L tanks. Fish were acclimatized for 1 week before the beginning of the treatment and were fed three times a day with a commercially available food. Two different doses of r-pjGH- or carrier-containing food were administered to each tank at an amount of 2% of the estimated body weight at weekly intervals for a total of 4 weeks. Fish were starved 1 day after the oral administration of r-pjGH-containing food (0 and 2 μg r-pjGH gbw^{-1} doses). In the first sampling, six fish (two fish from each tank) were randomly chosen 1 day before the first r-pjGH administration (day 0). In the second and third sampling, six fish were taken from each tank 2 days after the third and the fifth r-pjGH administration (days 17 and 31). Fish were sacrificed by anaesthetic overdose using benzocaine solution (100 ppm) and liver samples were collected rapidly and stored in RNAlater solution (Qiagen, Crawley, UK) at − 20 °C for further analysis. Total RNA was extracted from 50 to 100 mg liver explants of each fish using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Then, RNA concentration was estimated by measuring the absorbance at 260 nm, its purity was assessed by the $A_{260/280\,nm}$ ratio and the integrity on an agarose gel. The remaining DNA was removed by DNAse digestion. First-strand cDNA was reverse transcribed from 1 mg of total RNA using an oligonucleotide annealing to the poly-A tail (oligo(dT)16mer) during 1-h incubation at 42 °C using MMLV reverse transcriptase (100 U, Promega, Madison, WI, USA), 10 mM dNTPs and 1 × buffer in a final volume of 10 μL. For the real-time PCR, 2 μL of cDNA at 10-fold dilutions was subjected to triplicates using a master mix containing deoxynucleotides (200 mM each), 12 pmol of each oligonucleotide primer and 0.5 U Platinum Taq DNA Polymerase (Invitrogen), 1 Platinum Taq DNA Polymerase Buffer and 1 × SYBR green fluorophore (Qiagen) in 20 μL of the final incubation volume. The amplification programme was 40 cycles of 94 °C for 40 s, 60 °C for 40 s and 72 °C for 30 s. Target gene primers (IGX5: AACTGCGGCGCCTGG AAATG and IGY6: GTCTTGTCTGGCTGCTGTGCTG TC) and reference gene primers (Actin-fw: CTCTG GTCGTACCACTGGTATCG and Actin-rv: GCAGAGCG TAGCCTTCATAGATG) were displayed in separate real-time PCR tubes (Axygen, Union City, CA, USA). Control tubes with no template were used to confirm that the reagents were not contaminated. Reverse transcriptase was omitted from RT reactions to test for interference from residual genomic DNA in RNA preparations.

Statistical analysis

For growth assay, time and treatment analysis was achieved using the Variance Analysis Model I and the LSD multiple comparison test. Single factor analyses were performed using Kruskal–Wallis tests with the Dunn's multiple comparison test.

Results

Optimization of r-pjGH solubilization and oxidation of cysteines

Pejerrey growth hormone was expressed in an *E. coli* highly efficient laboratory-scale system, which produced 0.5 g L^{-1} of inclusion bodies that contained 87% pure protein (Sciara *et al.* 2006). To evaluate the optimal conditions for hormone solubilization, inclusion bodies were solubilized in 0.5–8 M urea solution and analysed in 12% polyacrylamide gels with 8 M urea. Intermolecular interactions between polypeptides, which formed high-molecular-weight aggregates, disappeared only when inclusion bodies were solubilized with 8 M urea (Fig. 1). Although pjGH has four cysteine residues, we found that the interactions observed were not caused by intermolecular disulphide bonds because SDS-PAGE analysis of the same samples under non-reducing conditions showed only the presence of the monomeric polypeptide (data not shown). The appearance of multiple conformations of the monomeric r-pjGH when 8 M urea was used in the solubilization media suggests that different three-dimensional structures of the polypeptide are present even when high concentrations of chaotropic agent were used.

The effect of solubilization time in the formation of disulphide bonds was analysed. Non-reducing SDS-PAGE showed a significant increase in the fast migration r-pjGH conformation when the sample was incubated previously for 2 h (Fig. 2). Increasing the incubation time did not produce an increment in the r-pjGH oxidized/reduced rate and long incubation times produced new r-pjGH conformations. These findings show that the optimal duration of the solubilization process should last between 2 and 8 h.

High-yield r-pjGH refolding

Once the optimal solubilization conditions for r-pjGH were achieved, different refolding solutions were tested. The presence of the oxidized monomer of r-pjGH in a western blot was used as an indication of correctly folded r-pjGH. Results showed that the Tris solution appears to be more appropriate than PBS; and that when arginine is present in the refolding solution reduced r-pjGH (misfolded form) is diminished (Fig. 3). The optimal arginine concentration in the refolding solution was 50 mM because a higher concentration of the amino acid did not increase the oxidized form of r-pjGH (data not shown). Hence, Tris 40 mM NaOH and arginine 50 mM pH 10.5 were selected as the buffer composition for the renaturalization process. The optimal r-pjGH concentration in the solubilization solution was 1 mg mL^{-1} because higher concentration of pjGH generates an increase of the reduced (misfolded) r-pjGH conformation (data not shown). Globally, the refolding process did not produce any r-pjGH precipitation after centrifugation and almost a 90% of the protein was found in a properly oxidized form (Fig. 4A). The other 10% of r-pjGH was found forming dimers and high-molecular-

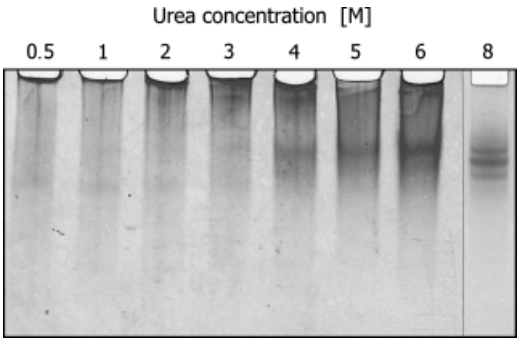

Figure 1 Molecular aggregation of r-pjGH in different urea concentrations. Inclusion bodies were incubated 3 h in 0.5–8 M urea solution with 100 mM Tris-HCl pH 10.5. The solubilized r-pjGH (4 μg) was analysed in a 12% polyacrylamide gel with 8 M urea. High-molecular-weight r-pjGH aggregates disappeared when 8 M urea was added to the solubilization solution.

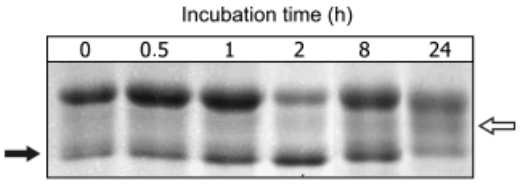

Figure 2 Effect of solubilization time on the reduction state of growth hormone. Inclusion bodies were dissolved in 8 M urea, 100 mM Tris-HCl pH 10.5 at different times and 4 μg of r-pjGH was analysed in a 15% non-reducing SDS-PAGE. The black arrow indicates the completely reduced protein. The white arrow indicates the appearance of a partially reduced conformation.

weight aggregates. In addition, the purity of monomeric r-pjGH determined by HPLC was 85% (Fig. 4B).

We bore out the refolded r-pjGH structure using circular dichroism analysis. The CD spectrum showed that refolded r-pjGH has a typical α-helix conformation, characteristic of GHs (Fig. 4C). Moreover, secondary structure prediction determined that 56% of the amino acids are involved in α-helix structures.

The *in silico* analysis of pjGH showed a primary structure hydrophobic plot, which differs from that in human GH mainly in the presence of two higher hydrophobic peaks in the first α-helix (indicated by the first two arrows in Fig. 5) and a new hydrophobic peak in the second α-helix (indicated by the third arrow in Fig. 5). The tertiary structure prediction submitted seven highly probable conformations ($E > 5.7 \times 10^{-19}$) and the one with the highest score ($E = 5.3 \times 10^{-23}$) was plotted (supporting information Figure S1). Six tyrosine and four phenylalanine amino acid residues (aromatic) that were found to be oriented towards the protein surface are shown.

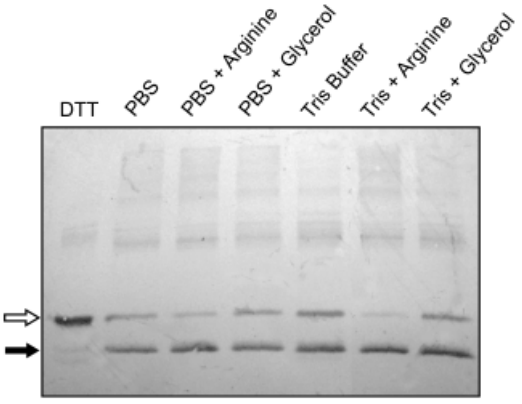

Figure 3 Effect of different buffer systems and low-molecular-weight solutes in the r-pjGH refolding process. The effect of different buffer systems on the refolding process was analysed by western blot. Recombinant growth hormone (0.5 μg) was run in a non-reducing 15% SDS-PAGE, transferred to a nitrocellulose membrane and detected with 1/5000 pjGH anti-serum dilution. The white arrow shows the position of fully denatured (reduced) pjGH. The black arrow indicates the position of native-like, disulphide-containing (oxidized) pjGH. DTT, 5 mM dithiothreitol-treated sample.

Growth-promoting effect of r-pjGH

The effect of purified r-pjGH on growth of juvenile pejerrey is shown in Fig. 6. After 4 weeks (day 28), fish fed with 2 μg r-pjGH gbw^{-1} showed an increase of 30% in mean weight ($P = 0.002$) and a 12% increase in mean length ($P = 0.006$) compared with the control group. After 6 weeks (day 42), the mean length of the treated group with 2 μg pjGH gbw^{-1} was also higher than the control group ($P = 0.04$). Nevertheless, although the mean weight of the lower r-pjGH dose at day 42 of the experiment was 15% higher than the control group, this difference was not statis-

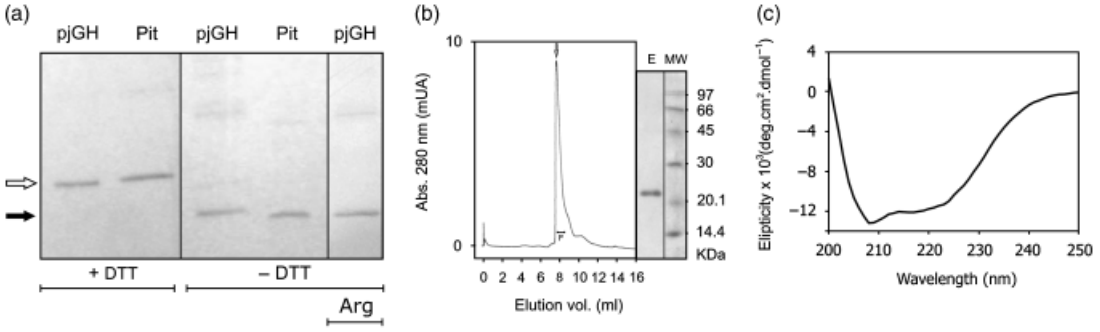

Figure 4 Western blot analysis and CD spectrum of refolded r-pjGH. Purified inclusion bodies were solubilized in 100 mM Tris-HCl pH 10.5 and refolded with 40 mM Tris-HCl/20 mM arginine pH 10.5. Folded pjGH was dialysed in 20 mM Tris-HCl pH 10.5, 9.5 and 9, 12 h each one, and in 0.05% $NaHCO_3$ for 12 h. (A) Western blot of folded r-pjGH (without arginine in the folding buffer) and pejerrey pituitary homogenate was achieved using specific pjGH antibodies (1/5000 anti-serum dilution). Reducing (left panel) and non-reducing (centre and right panels) electrophoresis are indicated by +DTT and −DTT. Right panel shows refolded r-pjGH using arginine in the refolding buffer. White arrow shows the position of fully denatured (reduced) pjGH. Black arrow indicates the position of native-like, disulphide-containing (oxidized) pjGH. (B) Gel filtration HPLC analysis of refolded pjGHr. Arrow indicates the peak of r-pjGH. The elution fraction analysed by western blot is shown (E). (C) Near-UV range spectrum (200–250 nm) of 0.05 mg mL^{-1} folded pjGH denotes a typical α-helix structure, as found in all growth hormone structures. F, the collected elution fraction; MW, molecular weight markers stained with Ponceau red.

tically significant. Mean weight and length of the high-dose group did not show any difference with control group at day 28, while at day 42 both parameters were significantly lower in high-dose group than control and lower dose group ($P = 0.003$ and 0.04 respectively).

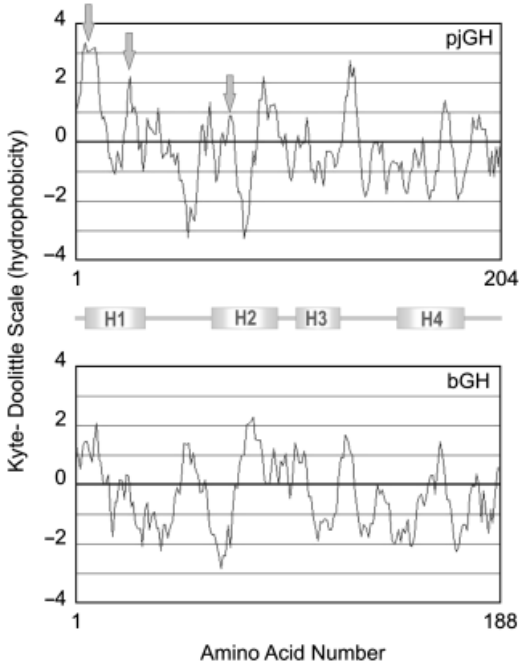

Figure 5 Hydrophobicity plot of growth hormone primary structure. Pejerrey (pjGH) and bovine (bGH) sequences excluding signal peptide were used. Graphic was made with a seven amino acid window. Predicted secondary structure of pjGH sequence is shown in the graphic between panels. H1, H2, H3 and H4 indicate the pjGH α-helixes predicted by the SYMPRED program.

In order to further evaluate the effect of r-pjGH treatment, we analysed the weight distribution of treated and control fish. Figure 7 shows that weights frequencies between r-pjGH-treated (2 μg gbw^{-1}) and control groups were shifted towards higher weights, being the shape of the plots almost identical between each other. Nevertheless, a narrower and higher curve could be observed in the r-pjGH-treated group.

The macroscopic examination of adipose tissue in the coelomic cavity during the necropsies of fish revealed a complete depletion of these reserves in both treated groups.

Effect of pjGH on myotome differentiation and muscle growth

The results from the microscopy studies are summarized in Fig. 8. The 20 μg r-pjGH gbw^{-1}-treated fish presented an increase in MFA ($P < 0.05$) in compari-

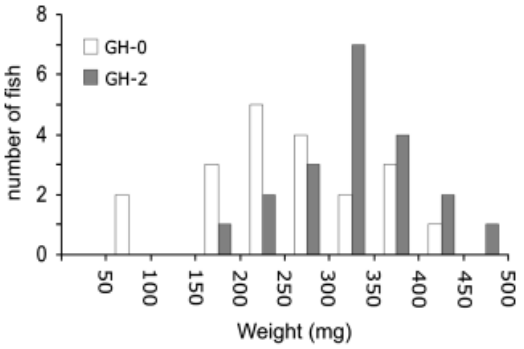

Figure 7 Weight distribution frequencies of 2 μg r-pjGH gbw^{-1}-treated and control fish at day 28 of treatment. The number of fish that lies within a weight range was plotted.

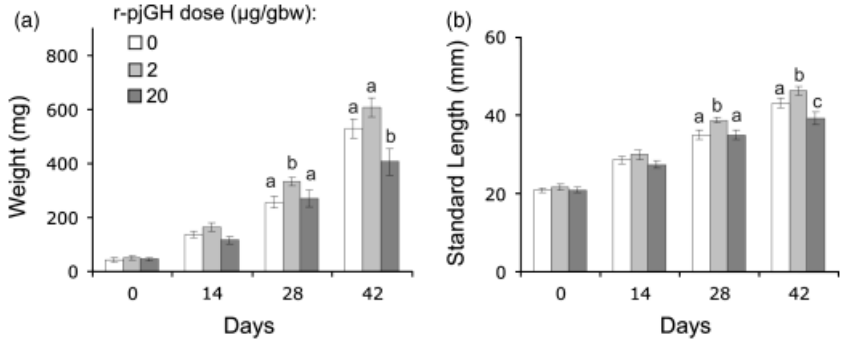

Figure 6 Growth of pejerrey juveniles after weekly pjGH oral administration. Fish were fed weekly with an artificial food containing 0, 0.1 or 1 mg r-pjGH g^{-1} (0, 2 or 20 μg gbw^{-1} doses). Weight (A) and standard length (B) of 20 randomly selected fish were measured every 2 weeks. Statistical differences among groups are indicated with a different letter. *P* value for each day and measurement: $P = 0.002$ (weight, day 28), $P = 0.006$ (length, day 28), $P = 0.003$ (weight, day 42), $P = 0.04$ (length, day 42).

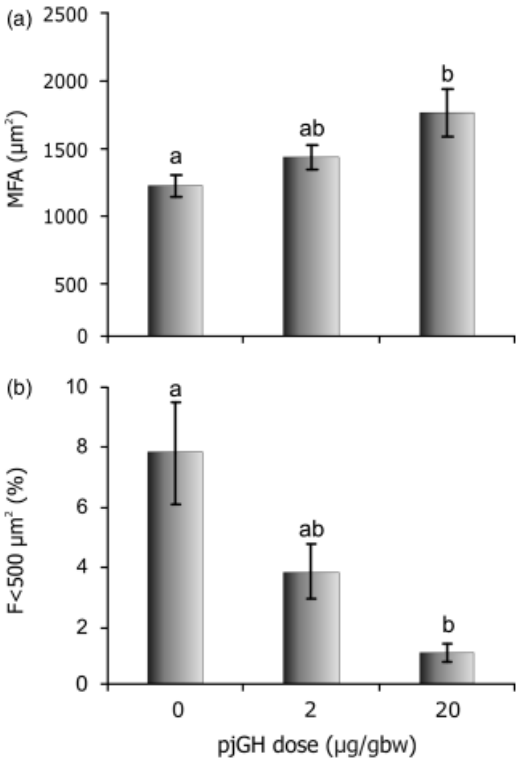

Figure 8 Effect of pjGH oral administration on muscle fibre area and the number of small muscular fibres. (A) MFA determined in caudal peduncle of control and r-pjGH-treated fish ($P = 0.023$, $n = 10$). (B) Percentage of fibres of $< 500\ \mu m^2$ ($F < 500$) of control and r-pjGH-treated fish ($P = 0.001$, $n = 10$). Different letters indicates significant differences.

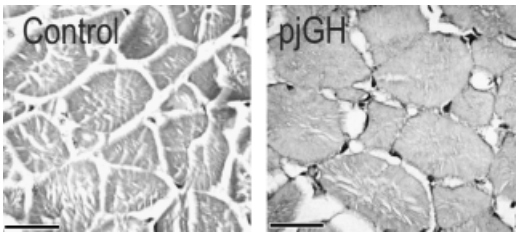

Figure 9 Typical result found in a muscle transversal section of control and 20 µg r-pjGH gbw^{-1}-treated group. Bar: 40 µm, × 100 magnification.

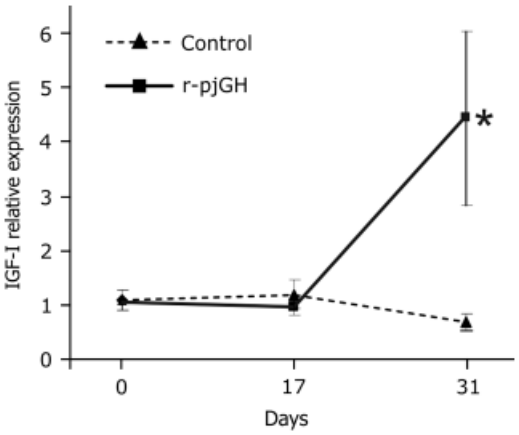

Figure 10 Liver IGF-I mRNA expression after GH administration. Liver relative levels of IGF-I transcript following GH oral administration were measured by real-time PCR. Full line: r-pjGH (2 µg gbw^{-1})-treated group. Dashed line: control group. All means are shown as the relative change ± SE. *significant difference ($P < 0.001$, $n = 6$).

son with the control group (Fig. 8A). Lower dose r-pjGH treatment did not present MFA statistical difference with the control and the higher dose treated fish. The effect of r-pjGH treatment on the percentage of small fibres ($F < 500$) was dose dependent. A higher dose produced a decrease of the $F < 500$ in relation to the control group (Fig. 8B). In addition, the low-dose group also showed a lower $F < 500$ than the control group. Figure 9 photomicrographs clearly illustrate the increase in muscle fibre diameter typically observed in the slides.

Hepatic IGF-I mRNA expression

To examine whether and when oral GH administration induces IGF-I expression, a weekly oral administration of pjGH to 1-year pejerrey fish was conducted during 4 weeks. We used the 2 µg r-pjGH gbw^{-1} doses because it was the only one that produced a significantly increase in growth. Liver IGF-I mRNA significantly increased (Fig. 10) in GH-treated fish compared with controls after four doses of treatment (day 31; $P = 0.0007$) but no effect was observed at day 17 (two doses of hormone). Interestingly, the dispersion of the values obtained in samples from hormone-treated fish was greater than that obtained from controls, suggesting that individual genetic variations could condition IGF-I level upon stimulation.

Discussion

Several fish recombinant GH refolding protocols, which imply simple techniques of dilution and dialysis, are found in the bibliography (Moriyama, Yamamoto *et al.* 1993; Cheng, Lin, Shamblott, Gonzalez-Villasenor, Powers, Woods & Chen 1995; Tsai *et al.* 1995; Guillen *et al.* 1998). It is also well

documented that recombinant GHs expressed in bacteria are always obtained as inclusion bodies. These protein aggregates are easily purified by centrifugation allowing them to be used as the starting material for refolding. Nevertheless, the major counter backs for this method are the low final yield due to an extensive precipitation of the polypeptide and the low final concentration of the soluble refolded hormone (Tsumoto, Ejima, Kumagai & Arakawa 2003). In order to obtain good yields of active hormone, all the steps of the refolding protocol must be optimized. Accordingly, solubilization of the protein contained in the inclusion bodies is essential to achieve an efficient folding process (Vallejo & Rinas 2004). In human and bovine, GHs refolding methods were established and involved dissolution of the hormone containing inclusion bodies in low urea concentrations (2–3 M) solutions (Khan, Rao, Eshwari, Totey & Panda 1998; Patra, Mukhopadhyay, Mukhija, Krishnan, Garg & Panda 2000). Under these conditions, the protein would retain the native secondary structure present in the inclusion bodies allowing higher folding yields. In order to efficiently refold r-pjGH, similar solubilization and refolding protocols were tried but low urea dissolution of r-pjGH inclusion bodies produced extremely low yields of soluble protein after direct dilution (data not shown). In a folding process, protein precipitation could be caused by interactions of the folding intermediates mainly by association between hydrophobic regions. In this respect, differences between bovine, human and pejerrey GH folding using low urea protocols could be caused by differential hydrophobic regions, which are observed in pjGH and absent in the bovine GH (bGH) primary structure hydrophobic plot. Interestingly, in bGH, the modification of α-helix number 3 (found between amino acid 107 and 128) by a single mutation (Lys to Leu) promotes intermolecular interactions due to an increased affinity between hydrophobic surfaces (Brems, Plaisted, Havel & Tomich 1988). Moreover, the nine hydrophobic amino acid residues that are found facing towards the solution in the predicted tertiary structure of pjGH could have been involved in protein–protein interaction. Consequently, these residues could favour polypeptide aggregation and precipitation. In a similar experimental approach, refolding of *S. aurata* GH was achieved by dissolving the inclusion bodies in 4.5 M urea pH 11.3, a twofold dilution water and serial dialysis in solutions of progressively lower pHs. Preliminary studies with r-pjGH using this method did not produce the expected results (data not shown).

In order to obtain a highly efficient refolding process, r-pjGH disulphide bond formation was optimized without the addition of oxidized/reduced compounds as glutathione or cysteine. Oxidation process by solubilized oxygen was driven mainly by high pH a condition that accelerates disulphide formation because thiolate ion produces the nucleophilic attack to form new bonds or disrupts the existing ones. We also evaluated the adequate disulphide formation during the solubilization of the inclusion bodies in order to maximize the number of polypeptides with a partially folded tertiary structure at the beginning of the folding process. Arginine was added to the refolding solution, and prevented r-pjGH precipitation during this critical step. Finally, in order to avoid the presence of divalent cations, which could catalyse the exchange between the recently formed disulphide bonds, EDTA was added to the dialysis buffers. The refolded hormone was 100% soluble, has native-like disulphide bonds and a high percentage of α-helix secondary structure (56%), as expected for a GH structure. Moreover, secondary structure predicted using a bioinformatics resource yielded 59% of α-helix. After refolding, the reduced form of the hormone was not observed in a western blot, but a 10% average of the r-pjGH was found forming dimers and soluble high-molecular-weight aggregates as estimated by densitometry of western blot bands (data not shown). The formation of these aggregates could not be avoided by changes in the refolding protocol.

A batch dilution refolding method was earlier developed for *Oncorhynchus mykiss* GH (Cheng *et al.* 1995) that was stabilized using lactose and mannitol (0.2% each) and a pH 8 buffer. Unfortunately, the efficiency of the refolding method was not provided in that manuscript. A similar protocol was used with tilapia GH and showed a 67% efficiency caused by precipitation and the presence of high-molecular-weight aggregates (Guillen *et al.* 1998). Lower efficient methods (1–2% of the initial protein) were obtained when *Acanthopagrus latus* GH inclusion bodies were dissolved in 6 M guanidine–HCl pH 10 and refolding was achieved by dialysis in NH_4HCO_3 pH 10 (Tsai *et al.* 1995). In comparison, pjGH refolding method provided a better yield (100% of soluble protein that contained 85% of r-pjGH monomer) along with a high protein concentration solution (0.1 mg mL^{-1}). Refolded pjGH maintained in 0.05% $NaHCO_3$ pH 8.8 is stable at 4 °C for several days. The pjGH stability decreases with time and also when the pH of the protein solution drops below 8.5, probably because lower pH diminishes the superficial charge of soluble GH

and concomitantly promotes interaction between the polypeptides. Solutes such as sucrose, mannitol, glycerol or arginine could be added to the solution so as to maintain monomeric pjGH soluble for longer periods of time. The effect of these molecules on pjGH aggregation must still be evaluated. In summary, a simple and efficient protocol for r-pjGH solubilization and refolding was established. As r-pjGH was expressed in *E. coli*, this method could be easily escalated from the laboratory to fed-batch production.

In order to test the growth-promoting effect of pjGH incorporated to fish food, a growth assay was performed using doses of 2 and 20 μg r-pjGH gbw^{-1} once a week. A significant 30% difference was observed in juvenile pejerrey at day 28 in the lower dose group compared with the control group. Similarly, Promdonkoy *et al.* (2004) used lower GH doses (0.1 or 1 μg soluble homologous GH gbw^{-1} week^{-1}) and produced a 25% increase of body weight in 3 g giant catfish. In 6 g flounder, 40 μg GH gbw^{-1} was orally delivered weekly, obtaining a 24% non-significant increase in body weight compared with control group (Jeh *et al.* 1998). In the same way, 8.2 g rainbow trout were weekly dispensed with 5 μg gbw^{-1} of polymer-protected GH producing a significant increase in weight after 14 days and a significant increase in length after 35 days (Moriyama, Yamamoto *et al.* 1993). Statistical significance was maintained until week 6 of treatment.

The reduction in mean values of weight and length differences of the 20 μg r-pjGH group compared with the control group after 42 days of treatment is an unexpected event that affects future applications of this procedure. Interestingly, when 0.5 μg GH gbw^{-1} were orally delivered to tilapia, growth promotion was observed after 9 weeks, but this effect disappeared when a 2.5 μg tilapia GH gbw^{-1} dose was used. In addition to this, adipose tissue was hardly found in GH-treated pejerrey peritoneum indicating a limitation in energy food supplies that could affect growth when lipid reserves became exhausted. Moreover, GH is a lipolytic hormone and nutritional factors could affect lipid utilization in adipocytes (Albalat, Gomez-Requeni, Rojas, Medale, Kaushik, Vianen, Van Den, Gutierrez, Perez-Sanchez & Navarro 2005). Consequently, lower GH concentrations should be tested in pejerrey in order to achieve a continuous effect in growth promotion.

Bichell, Kikuchi and Rotwein (1992) demonstrated that GH administration by oral intubation in rainbow trout induces a plasmatic IGF-I increase after 48 h. Recently, it was determined that short-term GH treatment induces GH receptors and IGF-I expression (Gahr, Vallejo, Weber, Shepherd, Silverstein & Rexroad III 2008). No reports have been found to describe the endocrine effect of oral GH administration in fish. In order to determine the biological activity of orally supplemented r-pjGH in pejerrey, IGF-I mRNA hepatic expression was measured in juvenile fish. Using the growth-promoting effective dose of 2 μg gbw^{-1} week^{-1}, a significant increase in IGF-I expression was detected after 1 month (day 31) of treatment but not at day 17. Gastric administration of 0.1 μg GH gbw^{-1} to rainbow trout raised plasmatic IGF-I after 48 h (Moriyama 1995). Gahr *et al.* (2008) found that intraperitoneal injection of GH significantly increases IGF-I and IGF-II in salmon after 3 days of treatment. In pejerrey, intraperitoneal injection of pjGH caused an increase of the hepatic IGF-I transcript 9 h after the injection (Sciara *et al.* 2008). A similar quick response of the IGF-I transcription and degradation was described in carp (Vong, Chan & Cheng 2003), rainbow trout (Shamblott, Cheng, Bolt & Chen 1995) and tilapia (Chen, Li, Chang, Hua, Gong, Lin, Chen & Wua 2007). Interestingly, metabolic hormones such as insulin, triiodothyronine (T3) or dexamethasone could modulate IGF-I response to GH. Schmid, Lutz, Kloas and Reinecke (2003) found a modest (1.5-fold) stimulation of basal IGF-I mRNA by T3 (10^{-7} M) in tilapia hepatocytes but no effect of T3 (10^{-7} M) was found on basal or GH-stimulated IGF-I mRNA expression in salmon hepatocytes (Pierce, Fukada & Dickhoff 2005), suggesting that differences between species exist with regard to this response. Moreover, T3 strongly potentiated the response to GH in a rat hepatocyte culture (Tollet, Enberg & Mode 1990). Hence, it is possible that, during the first 17 days of pejerrey oral administration with r-pjGH, a transitory effect by GH occurs but this effect could not be detected when IGF-I levels are tested 2 days after the hormone delivery. Instead, after 1 month of treatment, metabolic changes in treated fish could cause a more stable or intense increase in IGF-I expression. It is important to note that no polymer enteric matrix was used in GH-supplemented food ration preparation to protect the hormone from gastric acid proteases degradation. Polymers like HP-55 were used in food preparation of gastric fish like salmon (Moriyama, Yamamoto *et al.* 1993) but could be avoided in stomachless fish like pejerrey. These results show that orally administered r-pjGH is active and could cause changes in hepatic gene expression and suggest that the increase in IGF-I expression level at day 31 could be ascribed to a better growth performance.

It is important to highlight the temporal coincidence between growth promotion in fish larvae and IGF-I over expression in juveniles fish that were orally stimulated with GH. Hepatic IGF-I mRNA expression has been positively associated with growth rates in salmonids (Duan, Plisetskaya & Dickhoff 1995; Pierce, Beckman, Shearer, Larsen & Dickhoff 2001), tilapia (Vera Cruz, Brown, Luckenbach, Picha, Bolivar & Borski 2006) and Japanese eel (Moriyama, Ayson & Kawauchi 2000). Several efforts are being made to establish growth markers in fish cultures and these results in pejerrey endorse the use of IGF-I expression to evaluate growth conditions.

Muscle growth in fish differs from that in mammals in that muscle recruitment continues throughout the life cycle. In mammals, post-natal muscle growth only involves the hypertrophy of the fibres formed before birth. In fish, contribution of hypertrophy (incorporation of myoblasts into pre-existent fibres) and hyperplasia (recruitment of myoblasts into new fibres) is variable and dependent on the species and growth conditions (reviewed in Johnston 1999). Because mature red and white muscle fibres rarely exceed 50 and 200 μm in diameter, respectively, growth to a large body size can only occur through the recruitment of new muscle fibres. For example, in Atlantic salmon *Salmo salar* L., the number of white muscle fibres per myotome is around 5000 at hatching, 180 000 at smoltification and over 1 000 000 in two sea winter fish of 4 kg body mass. In contrast, fish species that only reach a modest ultimate size stop fibre recruitment soon after hatching or at birth in the case of viviparous species (Weatherley, Gill & Lobo 1988). The regulation of fibre mass is thought to be controlled by signalling pathways involving IGF-I and IGF-II (Johnston 2006). Unfortunately, there is no available information regarding pejerrey muscle development and plasticity. In order to evaluate muscle response to pjGH stimulation, we measured muscle fibre diameter (MFA) and the percentage of small fibres ($F < 500$). Results showed that MFA grew and $F < 500$ was reduced when pjGH concentration rose. This effect was not observed in the total length and weight measurements of fish, suggesting that in this particular case, muscle hypertrophy is not the main factor involved in total mass increase. The reduction in small fibres could indicate that in high-dose r-pjGH-treated fish myoblasts are being incorporated to pre-existing fibres and probably the recruitment of new fibres is being negatively affected by treatment. This contradictory effect could be caused by a deficiency in the energy balance when stored lipids are exhausted by GH-mediated lipolysis as described earlier. To summarize, when 2 μg r-pjGH gbw^{-1} dose is used, an enhancement of growth was achieved mainly by hypertrophy of small fibres. On the other side, when a 20 μg r-pjGH gbw^{-1} doses was used, the recruitment of new fibres was severely affected and growth promotion diminished even though the treatment increased fibre area. This complex effect on muscle could be caused by IGF-I or IGF-II endocrine and paracrine effect but direct effect of GH in muscle could not be ruled out. An energetic increase in food composition should be attained in order to balance the metabolism of r-pjGH-treated fish towards a greater muscle mass increment.

Taken together, the present results show that feeding pejerrey with recombinant refolded r-pjGH supplemented food could become an important tool to increase growth rates and overcome culture limitations associated with this highly valuable species.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Computer representation of the mature pjGH folded polypeptide. Protein backbone and the four predicted α-helix structures are shown in green. Panel A shows a view in which four α-helix are exposed. The amino terminus is located at the lower right-hand corner, whereas the carboxyl terminus is hidden in this view. Panel B shows a lateral view

towards the position pointed by the arrow in panel A. R groups which are exposed to the external surface of the molecule are shown in blue (tyrosine) and purple (phenylalanine). The two disulfide bonds in the molecule are shown in red.