ENVIRONMENTAL BIOTECHNOLOGY 7 (2) 2011, 53-64

A simple, rapid method for the production of soluble recombinant proteins from the duplicated growth hormone genes of the tetraploid common carp

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Keywords: common carp, gene/genome duplication, growth hormone

ABSTRACT

The simple protein production method developed in the present study enables the production of large quantities of active recombinant common-carp growth hormone proteins in a few brief steps. With this method, the recombinant protein is obtained in a soluble form without the need for refolding. This method can be utilised for determining if the two common-carp growth hormones have similar functions *in vitro* and *in vivo* and what role each plays in growth regulation. If differences in activity exist, this method can also be used to produce intermediate forms of the two common carp growth hormones rapidly in order to determine which of the six amino acids that differ between the two proteins are responsible for the altered activity. Common carp has the same tetraploid ancestor as goldfish, and the products of the growth hormone paralogues in goldfish also differ. It would be interesting to understand how the same duplicated orthologues have evolved, and the methods elaborated in this study could be used for such comparative investigations.

INTRODUCTION

Polyploidy is common in teleosts, such as catostomids, cyprinids, poeciliids and salmonids. The study of duplicated genes is important for investigating the evolutionary processes that follow the tetraploidisation event (Bailey et al. 1978; Futami et al. 2001, 2005; Hughes and Hughes 1993; Larhammar et al. 2002; McLysaght et al. 2002; Nadeau and Sankoff 1997; Ohno 1970). The common carp, *Cyprinus carpio*, is a tetraploid fish species from the family Cyprinidae that arose about 20-50 Myr ago. This species represents one of the most recent genome duplications. Today, common carp is among the most important species in freshwater fish culture, as it is mainly raised for human consumption (FAO Fishery Statistics 2008). A rapid growth rate and high vitality are economically the most important traits of this species. When studying the growth mechanisms in common carp, it is necessary to remember that many genes important for growth are duplicated in this fish.

Growth hormone (GH), which is produced in the pituitary gland, is a cardinal factor for growth regulation in vertebrates. GH is a protein of approximately 22 kDa that contains two disulphide bridges (Charrier and Martal 1988). The two common carp growth hormones differ by six amino acids. With the assistance of evolution tests, such as those of Tajima (1993) and the SCR3 programme (Hughes et al. 1990), Murakaeva (2008) demonstrated that these two genes have different evolution rates. The relative rate test of Tajima (1993) revealed a statistically significant increase in the evolution rate of the common carp *GH I* gene. The SCR3 programme demonstrated that non-synonymous mutations occur in such a way that certain amino acid properties of interest (in this case polarity) change to a greater extent than would be expected by random substitution (neutral evolution). This finding established that one of the two common carp growth hormones has relaxed functional constraints regarding polarity. Therefore, the activity of the two proteins could differ. To verify this hypothesis, the activity of both common carp growth hormones should be tested *in vitro* or *in vivo*.

Fine et al. (1993) have produced recombinant common-carp growth hormone, but the *E. coli* expressed rcGH was found only in insoluble refractile bodies (inclusion bodies), not in the supernatant of the cell extract. Subsequent refolding and purification yielded a bioactive rcGH fraction of only 10-15%. The refolding of inclusion bodies is not

straightforward and is an extremely time consuming process, and in the end, much of the recombinant protein is lost because it is not possible to properly refold 100% of the protein. In order to obtain sufficient quantities of recombinant protein for bioactivity tests from insoluble refractile bodies in *E. coli*, one would need large volumes of bacterial growth media to culture a sufficient number of *E. coli* cells and large volumes of buffers to solubilise the inclusion bodies. To test the hypothesis that the activities of the two common carp growth hormones and their intermediate forms are different, it is necessary to obtain soluble recombinant protein in its native form without refolding from small amounts of *E. coli* in order to save time, operations and laboratory equipment. The elaboration of a new facile method for the production of active recombinant cGH proteins at a high yield is necessary.

The *E. coli* expression system is generally used for the production of recombinant proteins. One disadvantage of expressing heterologous proteins in *E. coli* is that these proteins are frequently expressed as insoluble aggregated intermediates.

In most cases, solubility is not an all-or-nothing phenomenon; combinations of the vector, host, and culture conditions can change the proportion of soluble and insoluble forms of protein obtained. The choice of vector and expression host can significantly increase the activity and amount of target protein in the soluble fraction (Aslund et al. 1999; Bessette et al. 1999; Collins-Racie et al. 1995; LaVallie et al. 1993; Nygren et al. 1994; Prinz et al. 1997; Stewart et al. 1998; Zheng et al. 2003). Many proteins require the formation of stable disulphide bonds to fold properly into a native conformation. Without disulphide bonds, these proteins may be degraded or accumulate in inclusion bodies. The appropriate vector can enhance solubility and/or folding in one of the following ways: first, by enabling the fusion of the protein to a highly soluble polypeptide (e.g. glutathione-S-transferase (GST), thioredoxin (Trx), or N utilisation substance A (NusA)) (LaVallie et al. 1993; Nygren et al. 1994; Zheng et al. 2003), and second, by enabling the fusion of the protein to an enzyme that catalyses disulphide bond formation (e.g. Trx, DsbA, or DsbC) (Collins-Racie et al. 1995; Stewart et al. 1998). Recombinant protein folding can be also improved by using hosts that allow for cytoplasmic disulphide bond formation. The high reducing potential of the cytoplasmic compartment of *E. coli* limits the production of properly folded proteins. Bacterial strains with mutations in glutathione reductase (gor) and/or thioredoxin reductase (trxB) overcome this limitation and enhance the formation of disulphide bonds in the *E. coli* cytoplasm (Aslund et al. 1999; Bessette et al. 1999; Prinz et al. 1997). Another approach to improve the production of recombinant proteins in their native form is to optimise the cultivation conditions. Under optimal growth conditions in complex *E. coli* medium at 37°C, the overexpression of recombinant proteins led to the formation of inclusion bodies, but at 24°C, the proteins were folded properly (Kopetzki et al. 1989). By lowering the temperature or the gene expression rate by using weaker promoters or partial induction, the formation of inclusion bodies can be reduced or avoided completely (Bowden and Georgiou 1990; Chalmers et al. 1990; Kopetzki et al. 1989; Schein 1989; Shatzman 1990; Strandberg and Enfors 1991).

Fusing tags to recombinant proteins can facilitate the detection and purification of a target protein. Smith et al. (1987) found that small peptides containing several histidine and tryptophan residues bind strongly to transition metal ions immobilised on IDA-Sephadex G 25. Hochuli et al. (1988) showed for the first time that the use of IMAC to separate an expressed recombinant protein fused to a hexahistidine peptide tag yielded highly pure protein in a single chromatographic step under both native and denaturing conditions.

The activity of recombinant common carp GH in the present study was tested for its ability to stimulate IGF-I mRNA expression in primary cultured hepatocytes from common carp. IGF-I plays a major role in endocrine growth regulation, and GH acts via IGF-I. The dependence of IGF-I mRNA expression on recombinant tilapia growth hormone has been demonstrated in cultures of primary liver cells from tilapia (Schmid et al. 2000). A method for preparing primary liver cell cultures from teleosts was developed by Schmid et al. (1999).

MATERIAL AND METHODS

1. RNA preparation and first-strand cDNA synthesis

All common carp pituitary gland and primary liver cell samples were placed in TRIzol reagent (Invitrogen), frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated from the fish tissues using TRIzol reagent (Invitrogen). Samples were homogenised with Ultra-Turrax (IKA Labortechnik). Chloroform was added, and the mixture was vortexed vigorously, incubated for 5min at room temperature and centrifuged at 4°C for 15min at 12,000·g. RNA was extracted from the aqueous phase with isopropanol and precipitated by centrifugation (15min, 12,000·g, 4°C). The pellet was washed with ethanol (75%, v/v), centrifuged (10min, 12,000·g, 4°C), dried and resuspended in diethylpyrocarbonate (DEPC)-treated water.

Prior to cDNA synthesis, all samples were treated with RNase-free deoxyribonuclease I (Fermentas) and following phenol extraction and ethanol precipitation was applied (Sambrook et al. 1989). Subsequently, RNA (1μg) was reverse transcribed in a 20μl reaction for 5min at 37°C, 60min at 42°C and 10min at 70°C. The reaction mixture included 5pmol oligo dT primer and 200U RevertAidTM H Minus M-MuLV Reverse Transcriptase (Fermentas) in 1·reverse transcriptase buffer, 10mM dNTPs and 10U RNase inhibitor (Fermentas).

The assessment of RNA was carried out on the Biophotometer (Eppendorf) at the following wavelengths:

240nm (background absorption and possible contamination), 260nm (specific for nucleic acid) and 280nm (specific for protein). The optical density (OD) 260 measured the quantity, the ratio of OD 260/280 gave the quality and the OD 260/240 estimated the purity and the extraction performance. An OD 260/280 ratio greater than 1.8 indicated good RNA quality.

2. PCR conditions and PCR product purifications

The primer pairs for the production of GH I and GH II cDNA to be used for cloning are listed in Table 1. Each PCR reaction (total volume of 25μl) contained 1·PCR buffer (20mM Tris-HCl, 10mM $(NH_4)_2SO_4$, 10mM KCl, 0.1% Triton X-100, pH 8.8), 4mM $MgCl_2$, 0.8mM dNTPs, 1.0μM of each primer, 5μl cDNA and 1.25 units of *Pfu* DNA polymerase (MBI-Fermentas). The amplification conditions were identical for both primer pairs: 95°C for 3min followed by 35 cycles of 95°C for 30sec, 55°C for 30sec and 72°C for 1min.

The four sticky-end PCR primers (A-D) were constructed, and the sequences are listed in Table 1. These primers were used to clone the full-length coding sequences of the two growth hormones, including the stop codons, into the Nco I (CCATGG) and Bam HI (GGATCC) sites of the expression vectors pET-30 and pET-32. The locations of the restriction sites in the sticky-end PCR primers are marked by bold italic letters (Table 1). Primers A and B contain the coding sequence for the first seven amino-acid residues in GH I and II. Primers C and D contain the complement of both the stop codon (underlined) and the coding sequence of the last seven amino-acid residues of GH I and II. Primers A and D were used in the PCR reaction AD with the pGEM®-T Easy Vector and either the GH I or II insert. The primers B and C were used in the PCR reaction BC with the pGEM®-T Easy Vector and either the GH I or II insert. The proofreading DNA polymerase *Pfu* (Fermentas) was used for PCR under the following conditions: 94°C for 3min followed by 35 cycles of 94°C for 1min, 54°C for 1min, and 72°C for 2min.

Table 1. Primer pairs used in present study.

Description of the primer pairs	Primer sequences and designation
Primer pair for GH I cDNA, PCR product should be 656 bp long	**K-F:** CTG AGC GAA ATG GCT AGA GT **K-R:** TAC ACC GGT GCC ATC TAC AG
Primer pair for GH II cDNA, PCR product should be 612 bp long	**CH-F_2:** ACC AGG GGA GAG CAT CAG AT **CH-R_2:** TGC AGG CAC TGA CTA GCA ATA
Nucleotide sequences of the four sticky-end PCR primers	**A:** ***CATGGG*** A TCA GAC AAC CAG CGG CTC TT **B:** ***G*** A TCA GAC AAC CAG CGG CTC TT **C:** ***GA TCC*** TTA CTA CAG GGT GCA GTT GGA ATC **D:** ***C*** TTA CTA CAG GGT GCA GTT GGA ATC
Primer pair for IGF-I cDNA, PCR product should be 549 bp long	**Fas:** ACA GTC CCA GGA CAC CAA AG **Ras:** CAA GGG TTC CAA ACG GTC TA
Primer pair for ß-actin cDNA, PCR product should be 484 bp long	**Fß-ac:** AAG GCC AAC AGG GAA AAG AT **Rß-ac:** TAC CGC AAG ACT CCA TAC CC

To analyse the ability of rcGH to stimulate the IGF-I mRNA expression in common carp primary cultured hepatocytes, primer pairs for the IGF-I (GenBank accession number D83271) and ß-actin (M24113) cDNA sequences were constructed (Table 1). The actin signal served as an internal standard. The amplification conditions were optimised to reach a linear PCR signal.

Each PCR reaction mix (total volume of 25μl) was composed of 1·PCR buffer (10mM Tris-HCl, 50mM KCl, pH 8.3), 2.5mM $MgCl_2$, 0.1mM dNTPs, 0.2μM of each primer, 5μl cDNA and 0.25 units of *Taq* DNA polymerase (MBI-Fermentas). The amplification conditions were as follows for ß-actin cDNA: 95°C for 3min, then 30 cycles consisting of 95°C for 30sec, 54°C for 30sec, 72°C for 1min. The amplification conditions for IGF-I cDNA were the same, only more cycles were carried out (35 cycles) for amplification.

The PCR products were either cleaned directly from the PCR reaction mix using the E.Z.N.A. Cycle-Pure Kit (Peqlab Biotechnologie) or fragments were gel-purified using the E.Z.N.A. Gel Extraction Kit (Peqlab Biotechnologie).

All PCR products were visualised on 1.7% agarose gels using ethidium bromide staining and UV light with the GeneRulerTM 100bp DNA Ladder Plus (100-3000bp) (Fermentas) as a molecular weight marker.

PCR products obtained with the constructed primer pairs were sequenced for verification purposes. Automated sequencing was carried out on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) or on a Beckman Coulter CEQ 8000 using the CEQ-DTCS Quick Start Kit (Beckman Coulter). The forward and reverse strands were aligned and assembled using SeqEd v.1.0.3 (Applied Biosystems) or the Beckman Coulter CEQ 8000 Genetic Analysis System.

3. The cloning of the GH I and GH II coding sequences into expression vectors and the expression of recombinant GH proteins

Purified PCR products corresponding to GH I and GH II cDNA were cloned using the pGEM®-T Easy Vector system (Promega) according to the manufacturer's recommendations.

The procedure developed by Shih et al. (2002) was used for the parallel cloning of genes into expression vectors without needing to restriction digest the PCR products. This method utilises sticky-end PCR and directional cloning, thereby allowing for the production of inserts with sticky ends that correspond to restriction enzyme sites that are present within the coding sequence. For the two common carp growth hormone proteins, this method enabled both coding sequences to be maintained in the same way and to be inserted into different expression vectors. Two PCR products from one template should be obtained with two primer pairs (Figure 1).

The PCR products AD and BC for the GH I coding sequence were adjusted to equal concentrations and mixed. The same was done with the PCR products AD and BC for the GH II coding sequence. The PCR products AD and BC were denatured for 5min at 95°C, annealed for 5min at 65°C and cooled to room temperature.

The expression vectors were ligated with sticky-end PCR products in 1·T4 DNA ligase buffer (40mM Tris-HCl (pH 7.8 at 25°C), 10mM $MgCl_2$, 10mM DTT, 0.5mM ATP) with 5 units T4 DNA Ligase (Fermentas) in a total volume of 10μl.

The automated sequencing of the GH-I and GH-II coding inserts in the cloning and expression vectors was carried out as described above.

Four *E. coli* expression hosts (BL21, BLR, HMS174, and Rosetta-gami (Novagen)) were transformed with the expression vector pET-30 or pET-32 (Novagen) containing the coding region of GH I or GH II (Table 2 and Table 3). All four expression hosts are lysogens of bacteriophage DE3, a

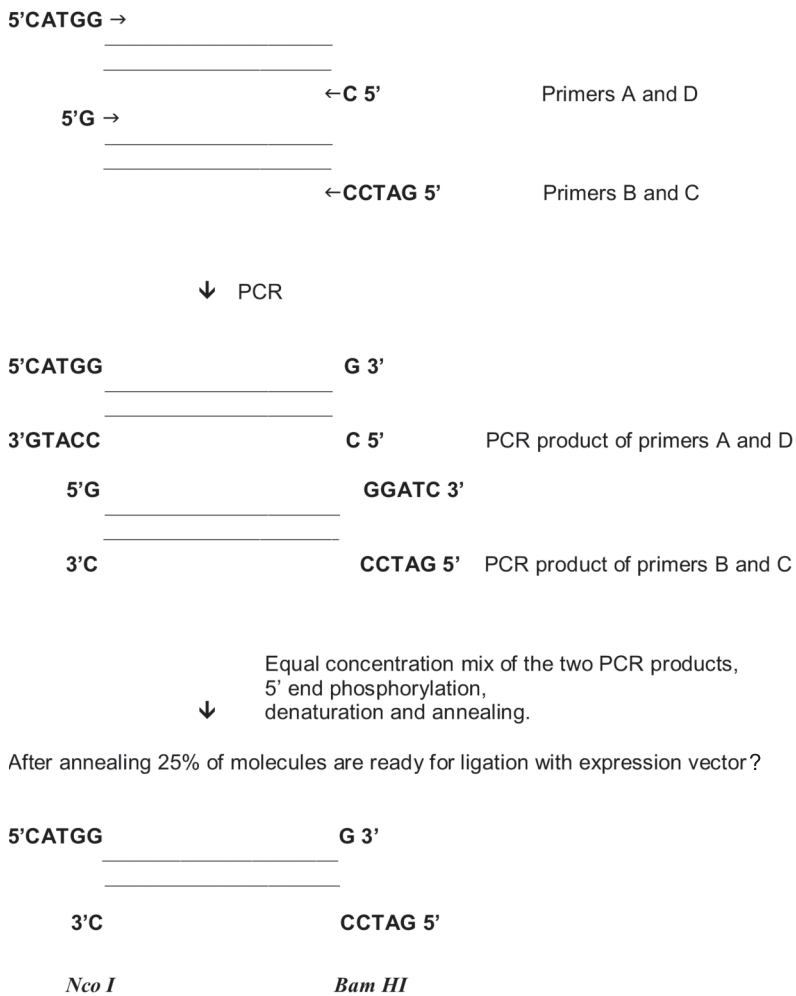

Figure 1. Molecular cloning strategy: Four PCR primers and two PCRs are used to create double-stranded DNA inserts with cohesive ends that are ready for ligation with expression vectors. *Nco* I - C↓CATGG; *Bam HI* - G↓GATCC restriction sites used to insert *GH I* and *GH II* coding sequences in expression vectors.

Table 2. *E. coli* expression strains used in present study.

Strains	Genotype and/or description	Source
BL21	*F - ompT hsdS$_B$ (r$_B$ - m$_B$) gal dcm (DE3)* Expression host with protease deficiency	Novagen
BLR	*F - ompT hsdS$_B$ (r$_B$ - m$_B$-) gal dcm Δ(srl-recA)306::Tn10(Tc^R) (DE3) recA-* Expression host with protease deficiency, recommended for use with tandem repeats	Novagen
HMS 174	*F- recA hsdR (rK$_{12}$ - mK$_{12}^+$) Rif^R (DE3) recA - K12*	Novagen
Rossetta-gammi	*Δara-leu7697 lacX74 ΔphoAPvuII ΔphoR araD139 ahpC galE galK prsL F'[lac+(lacIq)pro] gor522::Tn10(TcR) trxB::kan (DE3) pRARE (CmR)* Expression host with two mutations in cytoplasmic disulfide reduction pathway enhance disulfide bond formation in *E. coli* cytoplasm, provides rare codon tRNAs	Novagen

lambda derivative that carries the immunity region of phage 21 and a DNA fragment containing the lacI gene, the lacUV5 promoter, and the gene for T7 RNA polymerase (Studier and Moffatt 1986). Once a DE3 lysogen is formed, the only promoter known to direct transcription of T7 RNA polymerase is the lacUV5 promoter, which is inducible by isopropyl-ß-D-thiogalactopyranoside (IPTG). The addition of IPTG to a growing culture of the lysogen induces T7 RNA polymerase production, which, in turn, transcribes the target DNA in the plasmid. BL21 and BLR strains are deficient in the Lon protease and lack the ompT outer membrane protease that can degrade proteins during purification (Grodberg and Dunn 1988). The Rosetta-gami strain carries the *trxB* and the *gor* mutations, which enhance the potential for disulphide bond formation and ultimately increase the solubility and activity of recombinant proteins (Aslund et al. 1999; Bessette et al. 1999; Prinz et al. 1997). In addition, the Rosetta-gami strain is specially designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* (Brinkmann et al. 1989; Kane 1995; Kurland and Gallant 1996; Seidel et al. 1992).

In the pET-30 and pET-32 plasmids, target genes are expressed by strong bacteriophage T7 transcription and, optionally, translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. Additionally, pET-32 can enhance the solubility and/or folding of recombinant proteins by fusing the recombinant protein to Trx, which is highly soluble (Table 3).

To transform the expression vectors into competent cells, 0.2ng of plasmid was added to 20μl of competent cells. The cells were incubated on ice for 5min, heat-shocked at 42°C for 30sec, and cooled on ice. Afterward, 80μl SOC was added, and the reaction mixture was incubated for 60min at 37°C. The bacteria were plated on agar plates containing the appropriate antibiotic.

Antibiotics were filter-sterilised and added aseptically to all media, such as the agar plates and the shake flasks. The final antibiotic concentrations were as follows: carbenicillin, 50μg·ml^{-1}; chloramphenicol, 34μg·ml^{-1}; and tetracycline, 25μg·ml^{-1}.

The cells were grown at 37°C in LB medium (Table 4). The culture was cooled to 20°C before inducing the production of recombinant protein with IPTG.

Table 3. Tested expression vectors (Novagen).

Vector	Selection	Promoter	Fusion tags	Protease site
pET-30	Kan TA 1535	T7lac	His.Tag, S.Tag	Thrombin, Enterokinase
pET-32	Amp	T7lac	Trx.Tag^{TM}, His.Tag, S.Tag	Thrombin, Enterokinase

4. Recombinant protein analysis and purification

Polyacrylamide gel electrophoresis of proteins under denaturising conditions was performed in a Mini-PROTEAN 3 Cell (Bio-Rad). A resolving gel of Tris/HCl 10% with SDS was prepared according to Laemmli (1970). The samples were run on the gels at 200V for approximately 45-50min in running buffer (Table 4). The Protein Molecular Weight Marker (Fermentas), with molecular weights ranging from 14.4 to 116kDa, served as a standard.

Table 4. Media and buffers used in present study for recombinant protein production, analysis and purification.

Name of medium or buffer	Composition
Luria-Bertani (LB) medium	Bacto trypton 10g·l^{-1}; yeast extract 5g·l^{-1}; NaCl 5g·l^{-1}
Agar plates	Bacto trypton 10g·l^{-1}; yeast extract 5g·l^{-1}; NaCl 5g·l^{-1}; Agar 15g·l^{-1}
Lysis buffer	50mM potassium phosphate pH 7.8, 400mM NaCl, 100mM KCl, 0.5% Triton X 100, 10% glycerol, 5mM imidazole. 30mg·ml^{-1} lysozyme (Sigma)
2·sample loading buffer	150mM Tris/HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 4% (v/v) 2 mercaptoethanol, 0.2% (w/v) bromphenolblue
Running buffer	25mM Tris, 192mM glycine, 0.1% (w/v) SDS
Binding buffer	20mM sodium phosphate, 0.5M NaCl, 20mM imidazole, pH 7.4
Elution buffer	20mM sodium phosphate, 0.5M NaCl, 500mM imidazole, pH 7.4

To prepare the soluble protein fraction and the insoluble cell pellet from total cell lysates for polyacrylamide gel electrophoresis, the cells were harvested by centrifugation at 4500·g for 5min at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 500µl lysis buffer (Table 4). The tube was shaken for 2-3 hours at 225rpm. The lysate was centrifuged at 14000·g for 20min at 4°C. The supernatant (100µl) or some amount of the pellet was mixed with 100µl of 2·SDS sample buffer. The other samples for polyacrylamide gel electrophoresis were mixed with 2·SDS sample buffer at a ratio of 1:1. The tubes were incubated immediately at 95°C for 5min.

Coomassie blue staining and silver staining were used to visualise the proteins on the polyacrylamide gels. Coomassie blue staining was used for qualitative analysis of the proteins, whereas silver staining was used to analyse the purity of the recombinant proteins.

Protein concentrations were estimated with Coomassie Plus-The Better Bradford™ Assay Kit (PIERCE); this procedure was carried out according to the manufacturer's recommendations. Bovine serum albumin (BSA) (PIERCE) was used as a standard. The absorption at 595nm was measured on a Biophotometer (Eppendorf).

The purification of polyhistidine-containing soluble recombinant protein was carried out in the following way. Cells were harvested by centrifugation at 10000·g for 10min, and the supernatant was discarded. The pelleted cells were resuspended in lysis buffer (Table 4), and this solution was shaken for 2-3 hours at 225rpm followed by centrifugation at 15000·g for 20min. The supernatant was transferred to a fresh flask. Ammonium sulphate was added to the supernatant at a final concentration of 1.36M, and the resulting protein precipitate was collected by centrifugation at 15000·g for 20min. The precipitate was redissolved in binding buffer (Table 4) for purification by immobilised metal-ion affinity chromatography. The prepacked HiTrap affinity columns contained precharged Ni-Sepharose™ (Amersham Biosciences). The purification was carried out on the AKTA FPLC system (Amersham Biosciences) using UNICORN 5.01 software. The columns were equilibrated with binding buffer (Table 4). After protein loading, the column was washed at first with binding buffer and then was subjected to a gradient wash of 20 to 500mM imidazole (Table 4). The absorbance at 280nm was monitored to identify the peaks.

The Recombinant Enterokinase Kit (Novagen) was used to remove Trx and the His-Tag from the recombinant GH proteins. The cleavage reaction was carried out according to the manufacturer's recommendations. After cleavage, the protein mixture was loaded on the pre-equilibrated HiTrap affinity column, as described above.

Monomer content was determined by HPLC gel-filtration chromatography on a Nucleosil 125-5 GFC column (Goehler) in a Gilson apparatus. The column was pre-equilibrated with 0.05M NaH_2PO_4 and 0.25M NaCl at pH 7 and subsequently run in the same buffer at a rate of 1.0ml·min^{-1} at room temperature. Protein content was monitored by absorbance at 280nm. The retention times of several known proteins, such as bovine serum albumin, hGH and ß-lactoglobulin, were determined to calibrate the column.

MALDI-TOF mass spectrometry of the rcGHs was performed on a Voyager-DE Pro Biospectrometry Workstation (Applied Biosystems). Measurements were taken in linear positive ion mode with an accelerating voltage of 25kV, a grid voltage of 92%, and a delay time of 600nsec. The matrix solution consisted of 10mg·ml^{-1} sinapinic acid in acetonitrile:water:formic acid (50:50:0.1). Spectra were accumulated in delayed extraction mode. For MALDI-TOF analysis, the recombinant proteins were digested with trypsin (Roche Diagnostics GmbH) at a 1:50 ratio of trypsin to rcGH. The digest was performed overnight at 37°C in 100mM Tris-HCl buffer (pH 8.5).

5. Recombinant common-carp GH activity test

The livers of common carp weighing approximately 300-500g were perfused *in situ* to isolate hepatocytes according to the protocol by Schmid et al. (1999). The animals were injected

Table 5. Media used for perfusion and culture of common carp hepatocytes.

Component	CMF	CMC	MEM
NaCl (mM)	142	142	142
KCl (mM)	5.4	5.4	5.4
CaCl (mM)	0.0	2.4	2.4
$MgSO_4$ $7H_2O$ (mM)	0.00	0.00	0.81
Na_2HPO_4 (mM)	0.42	0.42	0.42
KH_2PO_4 (mM)	0.44	0.44	0.44
$NaHCO_3$ (mM)	0.43	0	0.43
HEPES (mM)	15	15	20
Amino acids (50·)	0	0	1:50
Vitamins (100·)	0	0	1:50
NaEDTA (mM)	5	0	0
Glutamine (mM)	0.01	0.00	0.00

with a single dose of 3000U heparin (Roth) dissolved in 0.5ml dH_2O and anaesthetised by immersion in water containing 0.1% MS 222 (Sigma). A catheter (1mm diameter) was introduced into the bulbus arteriosus, and the liver was retrogradely perfused with 100ml calcium- and magnesium-free (CMF) medium (Table 5) to wash out the blood. The tissue was digested with 50ml calcium- and magnesium-containing (CMC) medium (Table 5) supplemented with collagenase D (0.5mg·ml^{-1}) (Roche). The liver was exposed to collagenase D for 20min after which it was perfused with 50ml CMF medium, excised, transferred into ice-cold CMF medium and minced. The cells were selected by filtration through nylon gauze with 250, 100 or 50μm mesh. Dispersed cells were washed and collected by two centrifugation steps (5min at 700rpm and 5min at 500rpm) using ice-cold CMF medium. The supernatant was discarded each time, and the final cell pellet was resuspended in minimal essential medium (MEM) (Table 5). Cells were counted and subsequently adjusted to approximately $1.5 \cdot 10^6 \cdot ml^{-1}$ in MEM. Aliquots (1.5ml) of this cell suspension were seeded as a monolayer on sterile plastic Petri dishes (35mm diameter) (Primaria, Falcon). The cultures were incubated at 20°C in high humidity with normal air in an incubator (Heraeus).

All media were adjusted to pH 7.5, and after sterile filtration, each 100ml of media was supplemented with 10000U penicillin and 10mg streptomycin immediately prior to use. All chemicals were obtained from Sigma, except for amino acids, vitamins and glutamine, which were purchased from Serva.

In order to test the activity of the purified rcGH, the IGF-I mRNA level was measured in common-carp hepatocytes exposed to the recombinant growth hormone. The hepatocytes were exposed to 10 or 25nM rcGH for 10.5 hours. As a control, the IGF-I mRNA level was measured in primary cultured hepatocytes growing in MEM without any treatment. At 0 (control dishes) and 10.5 (control and treated dishes) hours, the medium was removed, and the cells were harvested for expression analysis of IGF-I and ß-actin.

To analyse the dependence of the IGF-I mRNA expression on rcGH in primary cultured hepatocytes by RT-PCR, the cDNA sequence of common carp IGF-I (GenBank accession number D83271) was used to construct specific primers. This cDNA sequence is from one of the two *IGF-I* genes found in common carp. The constructed primers were specific for the transcript of only one of common carp duplicated *IGF-I* genes. There are no data on whether these duplicated common-carp *IGF-I* genes have similar expression patterns. Therefore, the mRNA level of only one *IGF-I* gene was ascertained to minimise complications that could arise from potentially distinct regulation of the duplicated common-carp *IGF-I* genes. The ß-actin signal served as an internal standard. Only one sequence for ß-actin (M24113) in common carp was available in GenBank.

PCR fragments were separated on 1.7% agarose gels and were stained with ethidium bromide. The optical density of the IGF-I bands was normalised to that of the ß-actin bands.

RESULTS AND DISCUSSION

From eight combinations of expression hosts and vectors, only the combination of Rosetta-gami cells with the pET-32 expression vector containing the GH I or GH II coding region allowed for the abundant production of soluble recombinant proteins. The other combinations of expression hosts and vectors produced recombinant proteins only in inclusion bodies. For all combinations, protein production was carried out at 20°C.

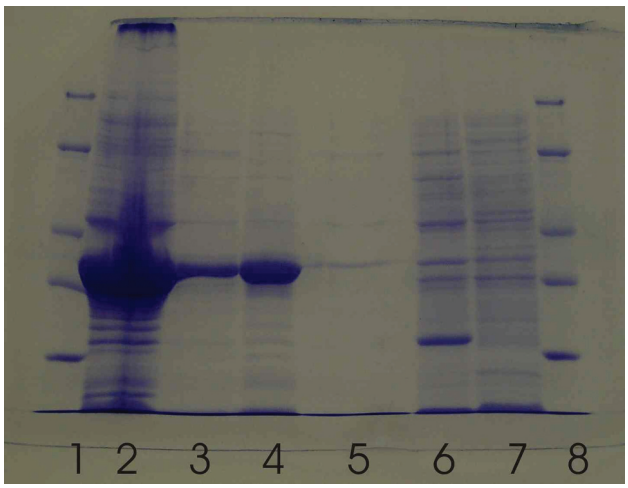

Figure 2. Expression of recombinant protein in Rossetta-gammi cells transformed with pET-32. Electrophoretic analysis on a 10% SDS-PAGE under denaturing reducing conditions. Gel was stained with Commassie brilliant blue. Induction was performed by IPTG. Lanes 1 and 8, Protein Molecular Weight Markers (Fermentas) (from top to bottom in kDa): 116, 66, 45, 35, 25; Lane 2, nonsoluble pellet after cell lysis; Lane 3, soluble protein fraction in the supernatant after cell lysis; Lane 4, precipitate of proteins after concentration from supernatant (Lane 3) with ammonium sulfate; Lane 5, proteins remained in supernatant after concentration from supernatant (Lane 3) with ammonium sulfate; Lane 6, Rossetta-gammi transformed with expression vector pET-32 without induction by IPTG; Lane 7, Rossetta-gammi without expression vector induced by IPTG.

The analyse of inclusion bodies and soluble fractions from Rosetta-gami expression host with the pET-32 expression vector containing the GH I or GH II coding region on SDS-polyacrylamide gel with Coomassie blue staining detected the recombinant protein in both fractions after IPTG induction of protein expression (Figure 2, lanes 2 and 3). The recombinant protein constituted approximately 43% of the total protein present in the soluble fraction after cell lysis, as determined by densitometric scanning (Figure 2, lane 3). In addition, two negative controls were included on the gel: the first control was whole protein extract from Rosetta-gami cells carrying the pET-32 vector but without IPTG induction (Figure 2, lane 6), and the second control was whole protein extract from induced by IPTG Rosetta-gami cells but not carrying the pET-32 vector (Figure 2, lane 7). The common carp growth hormone protein is approximately 22kD. With the fusion of growth hormone to Trx and a His-Tag in the pET-32 expression vector, the theoretical molecular weight of the resulting target protein is approximately 39kD. In lanes 2, 3 and 4 of Figure 2, a notably intense band at this molecular weight was present. In the control lanes 6 and 7, this band was not present with great intensity.

The successful production of abundant and soluble recombinant common-carp growth hormone by using a combination of Rosetta-gami cells and the pET-32 expression vector can be explained by a combination of positive attributes. The pET-32 expression vector enables the fusion of growth hormone to Trx, which is a highly soluble enzyme that catalyses disulphide bond formation. Among the other bacterial hosts utilised, only Rosetta-gami cells have two mutations in the cytoplasmic disulphide reduction pathway. These two mutations in glutathione reductase (gor) and thioredoxin reductase (trxB) enhance disulphide bond formation in the cytoplasm of *E. coli.* Additionally, the Rosetta-gami strain enables the productive expression of eukaryotic proteins that contain codons rarely used in *E. coli*.

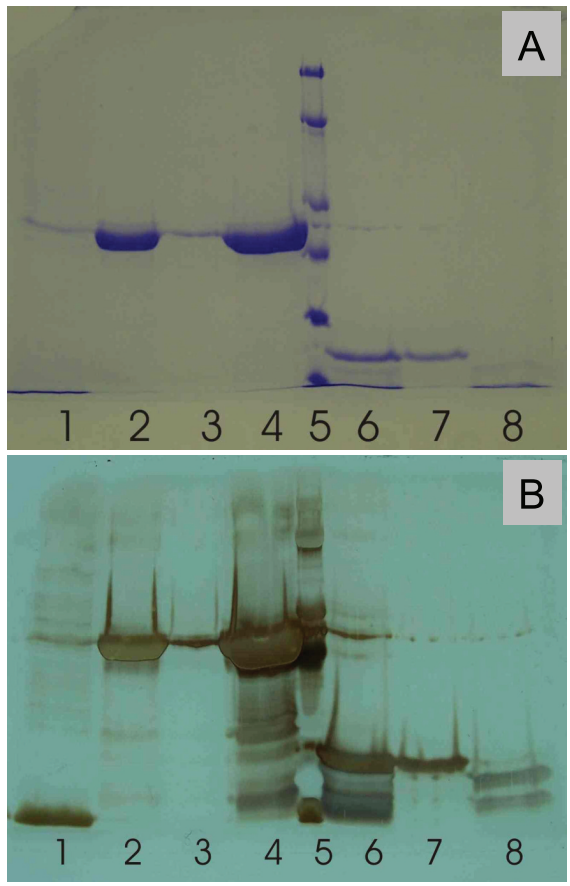

Figure 3. Different stages of purification. Electrophoretic analysis on a 10% SDS-PAGE under denaturing reducing conditions. (A) Gel was stained with Commassie brilliant blue. (B) Gel was stained with very sensitive for proteins silver staining. Lanes 1, 2, 3, 4, first separation on the HiTrap affinity column with Ni-Sepharose (Figure 4A); Lanes 1 and 3, the first peak containing untagged, contaminating proteins; Lanes 2 and 4, the second peak containing fusion of growth hormone protein with highly soluble protein thioredoxin (Trx) and HisTag; Lane 5, Protein Molecular Weight Marker (Fermentas) (from top to bottom in kDa): 116, 66, 45, 35, 25, 18; Lane 6, protein mixture after cleavage of the fusion protein with Enterokinase; Lane 7, 8, second separation of protein mixture on the HiTrap affinity column with Ni-Sepharose after cleavage with Enterokinase (Figure 4B); Lane 7, the first peak containing recombinant growth hormone; Lane 8, the second peak containing fusion of thioredoxin, HisTag and contaminating proteins.

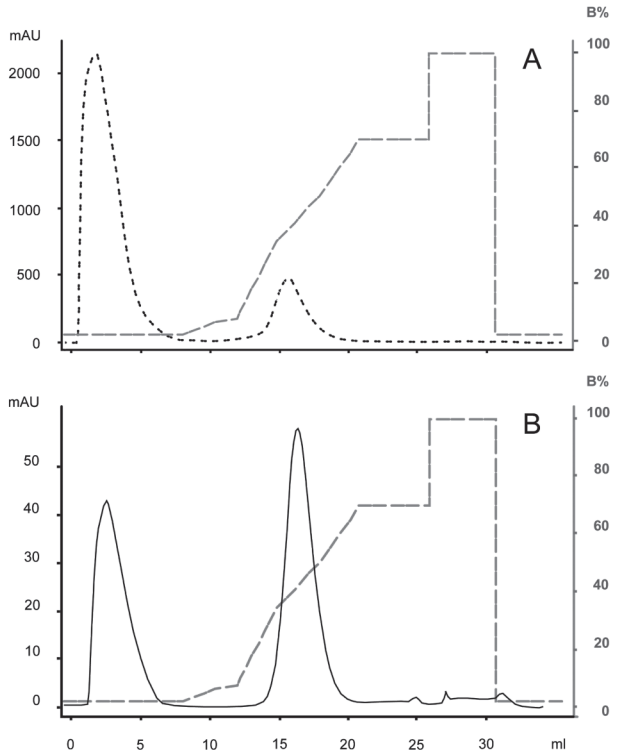

Figure 4. The separation on the HiTrap affinity column with Ni-Sepharose. Chromatogram is from AKTA FPLC system (Amersham Biosciences). The column was equilibrated with 20mM sodium phosphate buffer with 20mM imidazole, pH 7.4, and washed with the same buffer. Then, elution was carried out in the same buffer using a gradient with imidazole from 20mM to 500mM. (A) The separation of growth hormone fusion with thioredoxin (Trx) and HisTag from contaminating proteins. The precipitate of proteins (after ammonium sulfate concentration) was solubilized in 20mM sodium phosphate buffer with 20mM imidazole and applied to the column. Untagged, contaminating proteins were removed at 20mM imidazole (first peak). The fusion of growth hormone with thioredoxin (Trx) and HisTag was eluted at 60-260mM of imidazole (second peak). (B) The separation of recombinant growth hormone from thioredoxin with HisTag. The separation was done after cleavage of fusion protein with Enterokinase. The recombinant growth hormone was eluted at 20mM imidazole (first peak) and thioredoxin connected with HisTag was eluted at 60-260mM imidazole (second peak).

The proteins in the soluble fraction were concentrated by ammonium sulphate precipitation (Figure 2, lanes 4 and 5). The precipitate was dissolved in 20mM sodium phosphate binding buffer and loaded onto a HiTrap affinity column with Ni-Sepharose. The separation of proteins was carried out in the AKTA FPLC system (Amersham Biosciences). Using gradient washes of 20 to 500mM imidazole, most of contaminating proteins were eluted at 20mM imidazole (Figure 3A and 3B, lanes 1 and 3), and thus the purity of growth hormone fused to a His-Tag and thioredoxin was significantly increased (Figure 3A and 3B, lanes 2 and 4). During the first separation on the HiTrap affinity column with Ni-Sepharose, the fusion protein eluted between 60 and 260mM imidazole in wash buffer (Figure 4A). The affinity of the fusion protein for Ni-Sepharose is high because of the six adjacent histidine residues in the His-Tag (Hochuli et al. 1987, 1988; Smith and Pidgeon 1986; Smith et al. 1987). Compared with other proteins, higher concentrations of imidazole are needed to remove a recombinant fusion protein from the column, which helps isolate the recombinant protein with high purity.

After the first separation on the HiTrap affinity column, the fusion protein was cleaved to remove Trx and the His-Tag. Figure 3A and 3B (lane 6) show the protein mixture after fusion protein cleavage with enterokinase. After cleavage, enterokinase was captured with EKapture Agarose (Recombinant Enterokinase Kit, Novagen). Next, the protein mixture was separated again on a HiTrap affinity column using the same buffer and wash conditions as in the first separation. The recombinant growth hormone was eluted at 20mM imidazole (Figure 3A and 3B, lane 7, and Figure 4B). The fusion of thioredoxin with the His-Tag eluted between 60 and 260mM imidazole (Figure 3A and 3B, lane 8, and Figure 4B). Without the His-Tag, the recombinant growth hormone had a notably weak affinity for Ni-Sepharose and consequently was easily separated from the Trx-His-Tag fusion protein and any other remaining contaminating proteins with high affinity for Ni-Sepharose.

In this study, the yield of rcGH varied between 5 and 8mg per 6g of precipitated *E. coli* cells, which is approximately 7 times more protein per 1g of precipitated *E. coli* cells than was obtained in earlier studies (Fine et al. 1993).

The purity of rGH was notably high after all purification steps (Figure 3B, lane 7). HPLC on a Nucleosil 125-5 GFC column demonstrated that common-carp rGH consisted of 95% monomeric GH with a retention time equal to that of recombinant hGH.

MALDI confirmed that the purified recombinant proteins were common-carp growth hormones.

In summary, the two-step purification of recombinant growth hormone, achieved first on a HiTrap affinity column with Ni-Sepharose as a fusion to His-Tag and Trx and second without His-Tag and Trx, enabled the production of highly pure recombinant GH protein. In the first step, all contaminating proteins with low affinity for Ni-Sepharose were removed, and in the second step, all contaminating proteins with high affinity for Ni-Sepharose were eliminated. The scheme of the production and purification steps for recombinant cGH is shown in Figure 5.

A qualitative activity test was performed on one of the two produced common-carp recombinant growth hormones. The activity of rcGH II was tested on common-carp primary cultured hepatocytes. The hepatocytes were incubated for 10.5 hours in medium containing 10nM or 25nM common-carp recombinant growth hormone. The signal of IGF-I was higher at 25nM rcGH II than in control samples or in samples treated with 10nM rcGH II (Figure 6), thereby demonstrating the biological activity of the rcGH II. The activity of lyophilised rcGH II was reduced.

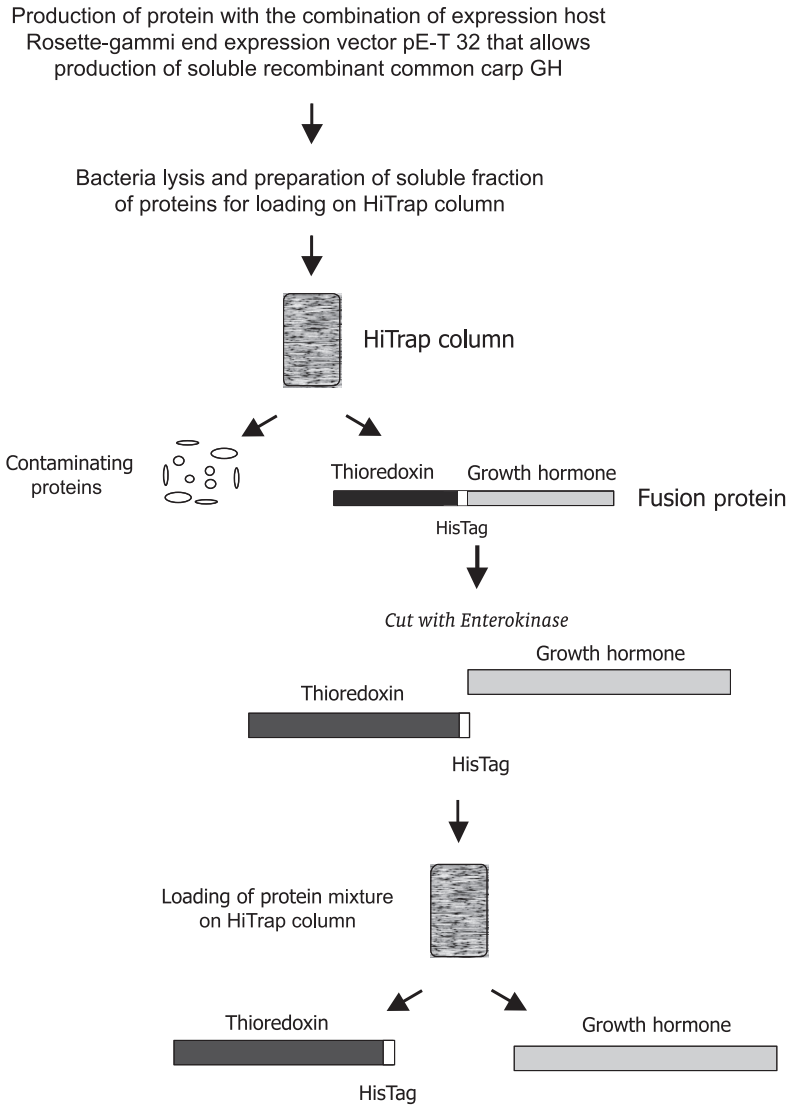

Figure 5. Scheme of recombinant cGH production and purification.

The simple protein production method developed in the present study could be used to rapidly produce abundant active recombinant common-carp growth hormone proteins in a minimal number of steps. The developed method could be employed to determine whether the two growth hormones of common-carp have similar activity *in vitro* and *in vivo*. If differences in activity are identified, the method could be used to produce quickly intermediate forms of the two common carp growth hormones in order to determine which and how many of the six different amino acids are responsible for the altered activity. In addition, common carp has a cognate fish species, namely, the goldfish, and both species share a common tetraploid ancestor. The goldfish growth hormone paralogues also differ. The study of the biological activity of the duplicated *GH* orthologues in these two species could lead to a better understanding of duplicated gene evolution after a tetraploidisation event. The method elaborated in this study could be used for such comparative investigations.

ACKNOWLEDGEMENTS

We thank Prof. Arenz for generously granting permission to use equipment in his laboratory for protein purification. We thank Prof. Linscheid for generously granting permission to use equipment in his laboratory for MALDI-TOF MS, and we thank Dr. Pieper for supporting these analyses. This work was supported by a grant from the “Berliner Programm zur Förderung der Chancengleichheit für Frauen in Forschung und Lehre”.

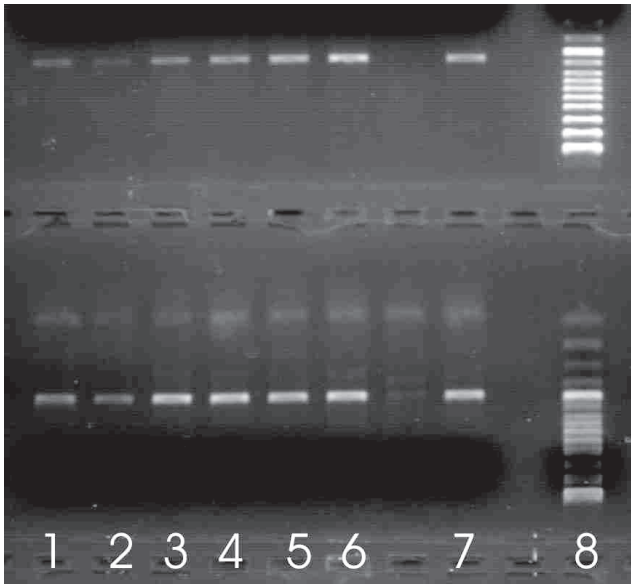

Figure 6. Analysis of dependency of the IGF-I signal (top part of gel) on the rcGH concentration in the culture medium of primary hepatocytes using RT-PCR. The ß-actin signal served as an internal standard in experiment (bottom part of gel).
Lanes 1 and 2, control at 0 hours without rcGH II
Lanes 3 and 4, control at 10.5 hours without rcGH II
Lane 5, hepatocytes exposed to 10nM of rcGH II for 10.5 hours
Lane 6, hepatocytes exposed to 25nM of rcGH II for 10.5 hours
Lane 7, hepatocytes exposed to 25nM of lyophilised rcGH II for 10.5 hours
Lane 8, molecular weight marker Gene Ruler 100bp DNA Ladder Plus (MBI-Fermentas)

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