Production of biologically active tethered tilapia LHβα by the methylotrophic yeast Pichia pastoris

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Abstract

In fish, luteinizing hormone (LH) stimulates processes leading to final oocyte maturation and ovulation in females, and spermiation in males. The hormone is a heterodimeric glycoprotein composed of two non-covalently associated subunits. In this study, we describe the expression of tilapia LH (tLH) as a biologically active, single-chain polypeptide using the methylotrophic yeast Pichia pastoris. The tLHβα and α mature protein-coding sequences were joined to form a fusion gene that encodes a “tethered” polypeptide in which the tLHβα-chain forms the N-terminal part and the α-chain forms the C-terminal part. A “linker” sequence of six amino acids (three Gly-Ser pairs) was placed between the βα- and α-chains to assist in the chimerization of the subunits, and a six-His tail was placed at the end of the βα-subunit, to enable purification of the recombinant protein. Western blot analysis of the pituitary LH resolved by SDS–PAGE yielded a band of 35 kDa, while the recombinant tLHβα had a molecular mass of 45 kDa, and was found to possess only N-linked carbohydrates. Recombinant tLHβα stimulated the release of 11-ketotestosterone from mature testes, whereas its release from immature testes was less pronounced.

Keywords: Recombinant; 11-Ketotestosterone; Gonadotropin; Glycosylation; Tilapia

1. Introduction

Two distinct gonadotropins (GtH I and GtH II) were discovered in salmon (Suzuki et al., 1988a,b,c). Due to their similarity to their vertebrate counterparts, GtH I and II were renamed follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively. As in mammals, both GtHs are heterodimeric glycoproteins, sharing an identical α-subunit, and distinct βα-subunits which confer their biological specificity. cDNA sequences encoding gonadotropin subunits have been isolated and characterized from more than 19 fish species, representing seven teleostean orders (Yaron et al., 2003).

A steroidogenic gonadotropin (taGtH) was isolated from pituitaries of tilapia hybrids by affinity chromatography, followed by anion-exchange chromatography and gel filtration, and a homologous radioimmunoassay (RIA) was developed (Bogomolnaya et al., 1989). The antibodies raised against taGtH immunolabeled the gonadotrophs in the proximal pars distalis, but also stained some isolated cells in the rostral pars distalis and in the pars intermedia (Bogomolnaya et al., 1989). Based on the immunocytochemical evidence, and since taGtH was purified from mature fish, it was assumed that this gonadotropin was tilapia LH (tLH). DNA sequences encoding two gonadotropin βα-subunits have been first described in the pituitary of Mozambique tilapia (Oreochromis mossambicus) (Rosenfeld et al., 1997), indicating the presence of two distinct gonadotropins in the genus. Recently, two gonadotropin βα-subunit cDNAs of
the Nile tilapia (*Oreochromis niloticus*) were also sequenced (Parhar et al., 2003).

Studies based primarily on salmonids suggest that FSH and LH play distinct roles in controlling gonadal development. FSH is generally important for early gonadal development and vitellogenesis, whereas LH was found to be involved in processes leading to final oocyte maturation and ovulation in females and spermiation in males (Yaron et al., 2003). LH was found to be more potent than FSH in stimulating the secretion of the maturation-inducing steroid (17α,20β-dihydroxy-4-pregn-3-one) from post-vitellogenic oocytes (Suzuki et al., 1988c). In tilapia pituitary, cells expressing LHβ mRNA have been detected at all developmental stages. Nevertheless, in sexually mature male tilapia, pituitary FSHβ mRNA levels are highest during early gonadal stages while those of LH are highest during the later stages (Gur et al., 2000).

Oligosaccharide structures of glycoprotein hormones play an essential role in many of the molecule’s functional characteristics (Ulloa-Aguirre et al., 1999). They are important not only for the folding, assembly, conformational maturation and post-translational processing of the subunits but also for heterodimer secretion, metabolic fate, interaction with its cognate receptor, and signal transduction (Boime and Ben-Menahem, 1999; Sairam, 1989; Sairam and Bhargavi, 1985). The metabolic clearance rate and the in vivo biological potency of the gonadotropins are highly dependent on the specific type of terminal residues present in their oligosaccharide structures. Oligosaccharides present in human FSH and chorionic gonadotropin (CG) terminate predominantly in sialic acid; the content of this sugar, and particularly the number of exposed terminal galactose residues, are essential in determining the hormone’s survival in the circulation (Ulloa-Aguirre et al., 1999). The tertiary structure of both α- and β-subunits is constrained by intramolecular disulfide bonds, and is post-translationally modified with N-linked carbohydrate moieties, the structure of which appears to modulate in vivo biological activity (Leanos-Miranda et al., 2003).

The methylotrophic yeast *Pichia pastoris* has been developed as an expression system for the high-level production of recombinant proteins (Hollenberg and Gellissen, 1997). *P. pastoris* offers the features of: (i) methanol-induced expression of heterologous genes integrated into the yeast genome downstream of the alcohol oxidase I (AOXI) gene promoter; (ii) growth to high cell density in the yeast genome downstream of the alcohol oxidase I induced expression of heterologous genes integrated into sen, 1997). This expression system has already been used for the production of human CG (Sen Gupta and Dighe, 1999), ovine, bovine, and porcine FSH (Fidler et al., 1998; Richard et al., 1998; Samaddar et al., 1997; respectively), and more recently, eel FSH (Kamei et al., 2003).

The first objective of the present study was to express glycosylated, properly folded and biologically active LH using an expression system capable of producing large quantities of the recombinant protein. Such a system could be used as a model system for investigating structure–function relationships and folding of the subunits during biosynthesis. For this purpose, we chose the *P. pastoris* yeast expression system, which fulfills all the prerequisites for hyperexpression of glycoprotein hormones. The second objective was to test the in vitro stereo-drogenic responsiveness to recombinant tilapia LH in male tilapia at different stages of its reproductive style.

### 2. Materials and methods

#### 2.1. Fish

Approximately 600 14-day-old *O. niloticus* fry were obtained from the Institut fur Tierzucht und Haustiergenetik, Georg-August Universität, Göttingen, Germany. The fish were originally from different families of a population originating from Lake Manzala (Egypt). The fish were kept and bred in the fish facility unit at the Hebrew University, in 500-l tanks, at 26 °C, under natural photoperiod. Fish were fed every morning ad libitum with commercial pellets and flakes, containing 50% protein, 6% fat, 5.6% ash, and 26% cellulose (Zemach Feed Mills, Zemach, Israel). Fish were anesthetized using 0.01% tricane methanesulfonate (3-aminobenzoic acid ethyl ester; Sigma Chemical, St. Louis, MO). All experimental procedures were in compliance with the Animal Care and Use Guidelines at the Hebrew University and approved by the local Administrative Panel on Laboratory Animal Care Committee.

#### 2.2. Amplification of the α- and β-subunit sequences of tLH

Total RNA was extracted from the pituitaries of four mature males *O. niloticus* [53 ± 5.5 g BW; GSI (gonado-somatic index, i.e., gonadal weight percentage of body weight) = 2.78 ± 0.23%] by TRIzol reagent (Gibco-BRL, Life Technologies, Paisley, UK) and was reverse-transcribed at 37 °C using MMLV reverse transcriptase (Promega, Madison, WI) and oligo(dT) primer (Promega), according to the manufacturer’s protocols. Two recombinant plasmids containing either tLHβ or tilapia glyco-protein hormone α (tGPα) subunits were constructed (Products A and B, respectively; Fig. 1). Primers were designed for the amplification of tGPα and tLHβ.
sequences, to form fusion genes to be subcloned into the
*Pichia* expression vector. The oligonucleotide primers
used to clone the subunit DNAs from the pituitary of
*O. niloticus* were designed according to the nucleotide
sequences of *O. mossambicus* tGP<sub>afii9825</sub>/afii9825 (Gur et al., 2001) and
tLH<sub>afii9826</sub>/afii9826 (Rosenfeld et al., 1997). The tLH<sub>afii9826</sub>
forward primer (P1, Table 1) contained an
*Eco*<sub>R</sub> site (italics) and 15
nucleotides encoding the
*W*<sub>ve</sub> N-terminal amino acid res-
sidues of the mature tGP<sub>afii9826</sub>-sub-
unit. The reverse primer (P2, Table 1) contained a
*Bam*<sub>H</sub> site (italics), a sequence
encoding six histidine residues (underlined), and 21
nucleotides encoding the seven C-terminal amino acid
residues of the mature tGP<sub>afii9826</sub>-sub-
unit. The tGP<sub>afii9825</sub>
forward
primer (P3, Table 1) contained a
*Bam*<sub>H</sub> site (italics), which is also a Gly-Ser string, another two Gly-Ser motif
(underlined) and 24 nucleotides encoding the eight N-
terminal amino acid residues of the mature tGP α-sub-
unit. The reverse primer (P4, Table 1) contained a
*Not*<sub>I</sub> site, the stop codon (TAA), and 23 nucleotides encoding the C-terminal amino acid residues of the mature tGP α-
subunit. The cDNA portions encoding the mature
secreted forms of the tGP<sub>afii9825</sub>- and tLH<sub>afii9826</sub>-subunits were
amplified by PCR using the aforedescribed primers and
subcloned into pGEM-T easy (Promega) to give rise to
pGEM-T-tLH<sub>afii9826</sub> (Product A, Fig. 1) and pGEM-T-tGP<sub>afii9825</sub> (Product B, Fig. 1). To amplify the tLHβ for subcloning
into the *Pichia* expression vector, we used the tLHβ for-
ward primer (P1, Table 1) and the reverse primer (P5,
Table 1) containing a *Not*<sub>I</sub> site, a sequence encoded for
six histidine residues (underlined), and 21 nucleotides

Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Position/subunit</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1 (β)</td>
<td>3′<em>GAATTC</em>AGCTGCGCCTGGTG5′</td>
</tr>
<tr>
<td>P2</td>
<td>436 (β)</td>
<td>3′<em>GGATCC</em>ATGATGATGATGATGATGATGATGATGATGAGAAGGGGATGTCA5′</td>
</tr>
<tr>
<td>P3</td>
<td>87-110 (α)</td>
<td>3′*GAATCCGGTTTCTGTTTCTATCCCAACATTGATTATCAAAC5′</td>
</tr>
<tr>
<td>P4</td>
<td>363-346 (α)</td>
<td>3′*GGGCGCGCTTATATCTTTATGAAAGTAACAGGTG5′</td>
</tr>
<tr>
<td>P5</td>
<td>436-456 (β)</td>
<td>3′*GGGCGCGCTTATATCTTTATGAAAGTAACAGGTG5′</td>
</tr>
<tr>
<td>P6</td>
<td>1(α)</td>
<td>3′*GAATCTTATCCCAACATTGATTTATCAAAC5′</td>
</tr>
</tbody>
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Fig. 1. Construction of the expression vector for tLHβ<sup>His</sup>, tGPα, and tLHβ<sup>His</sup>. The expression vector pPIC9K is an *Escherichia coli–Pichia pastoris* shuttle vector with sequences required for selection in each host. It has the 5′ promoter and 3′ transcription termination sequences of the alcohol (methanol) oxidase gene (AOX1) flanking the cloning site into which the tLHβ, tGPα or tLHβ were introduced. The vector has an α yeast mating factor (αMF) signal peptide downstream of the AOX1 promoter to which tLHβ<sup>His</sup>, tGPα or tLHβ<sup>His</sup> were fused.
encoding the seven C-terminal amino acid residues of the mature β-subunit. The cDNA portion encoding the mature secreted form of the tLHβ-subunit was subcloned into pGEM-T easy (Promega), giving rise to pGEM-T-tLHβ (Product D, Fig. 1). To amplify tGPα for subcloning into the Pichia expression vector, we used the tGPα forward primer (P6, Table 1) containing an EcoRI site (italics) and 24 nucleotides encoding the eight N-terminal amino acid residues of the mature α-subunit, and the reverse primer (P4, Table 1). The cDNA portion encoding the mature secreted form of the tGPα-subunit was subcloned into pGEM-T easy (Promega) giving rise to pGEM-T-tGPα (Product F, Fig. 1).

2.3. Subcloning of fusion genes into Pichia expression vectors

The tLHβα fusion gene was formed by three-piece ligation between: (i) the pGEM-T-tGPα (Product B, Fig. 1) BamHI–NotI fragment, (ii) the pGEM-T-tLHβ (Product A, Fig. 1) EcoRI–BamHI fragment and (iii) a pPIC9K (Invitrogen, USA) vector linearized by double digestion with EcoRI and NotI, to obtain pPIC9KtLHβαHis (Product C, Fig. 1). The tLHβ fusion gene was formed by ligation between pGEM-T-tLHβ (Product D, Fig. 1) and the pPIC9K (Invitrogen) vector linearized by double digestion with EcoRI and NotI, giving rise to pPIC9KtLHβHis (Product E, Fig. 1). The GPα fusion gene was formed by ligation between pGEM-T-tGPα (Product F, Fig. 1) and the pPIC9K (Invitrogen) vector linearized by double digestion with EcoRI and NotI, giving rise to pPIC9KtGPα (Product G, Fig. 1). The ligation reactions were transformed into DH5α cells, and plasmids with EcoRI–NotI inserts of the desired size (approximately 674, 376, or 296 bp for pPIC9KtLHβαHis, pPIC9KtLHβHis, or pPIC9KtGPα, respectively) were identified by restriction-enzyme digestion. All the genes were cloned into the vector pPIC9K that has yeast α mating factor (αMF) signal peptide downstream of the AOX1 promoter (Fig. 1). The structure of the fusion genes was confirmed by DNA sequencing (Sequencing Unit, Weizmann Institute, Israel).

2.4. Expression of recombinant tLH (rtLH)

Before yeast transformation, pPIC9KtLHβHis, pPIC9KtGPα, and pPIC9KtLHβαHis were linearized with BglII to get Mut3 (slow methanol utilization) transformants. The BglII-linearized expression cassette was then used to transform the host strain GS115 (his4; Invitrogen) by electroporation (GenePulser, Bio-Rad, Richmond, CA). The transformants were selected on His-deficient medium, and 300 clones from each construct were selected for higher resistance to antibiotic (1 mg/ml G418; Gibco-BRL). Twenty His7/Mut3 clones from each construct were cultivated with shaking for 1 day (growth phase) in 1.5 ml of BMG (1.34%, w/v, yeast nitrogen base with ammonium sulfate and without amino acids, 1%, v/v, glycerol, 0.00004%, biotin, and 100 mM potassium phosphate buffer, pH 6) at 28°C. Cells were harvested (1500g, 5 min), resuspended and cultivated for 3 days (induction phase) in 1 ml of BMM (same as BMG but supplemented with 0.5% methanol every 24h). The mature proteins coding sequences were positioned immediately 3’ to the codons of the αMF leader sequence enabling the proteolytical cleavage of the recombinant protein.

2.5. Purification of pPIC9KtLHβHis and pPIC9KtLHβαHis

The supernatant of the induced culture obtained using pPIC9KLHβHis or pPIC9KLHβαHis (Products E and C, respectively, Fig. 1) were purified using one-step nickel batch purification according to manufacturer’s instructions. Briefly, the pH of the supernatant was adjusted to 8.0 using a few drops of 5 N sodium hydroxide. Then the beads (QIAexpressionist; Qiagen, Alameda, CA) were added to the medium and vortexed for 18 h at 4°C. The beads were collected in a column previously washed with 50 to 60 bed volumes of PBS pH 8.0, containing 10 mM Tris–HCl. The bound protein was then eluted with PBS pH 4.5, containing 250 mM imidazole. The eluted protein was dialyzed against PBS pH 7.5. The purified protein, tagged with a six-His tail, was detected on a Western blot by QIAexpress anti-His antibodies (Qiagen).

2.6. SDS–PAGE and Western blot analysis

From culture supernatants, reduced and non-reduced samples were electrophoresed on 10% SDS–polyacrylamide running gels, with a 5% stacking gel. After blocking with 5% low-fat milk, the gels were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). rtLHβαHis, rtGPα, and rtLHβHis were visualized with three different antibodies, one raised against the whole native LH purified from tilapia pituitaries and used previously for homologous RIA (Bogomolnaya et al., 1989) and the second against tLHβ (Melamed et al., 1999). The third antibody (QIAexpress anti-His antibodies; Qiagen) was used to detect the six-His tail. The membranes were incubated in PBS plus 1% non-fat milk with the antibodies (1:10,000 dilution for all antibodies used) for 1 h at room temperature, then with goat anti-rabbit horseradish peroxidase conjugate (1:5000; Jackson, ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. After washing, the membranes were treated with enhanced chemiluminescence reagent (ECL; Biological Industries, Beit Ha’emek, Israel) to reveal immunoreactive bands.
2.7. Glycosylation analysis of rtLHβx or rtLHβ

Based on supplier recommendations (Roche Applied Science, Mannheim, Germany), 100 ng of reduced and denatured rtLHβx or rtLHβ were incubated for 2 h at 37°C in the presence or absence of N-glycosidase F (PNGase F) which hydrolyzes all types of N-glycans chains. Deglycosylated proteins were analyzed by Western blot analysis.

2.8. Enzyme immunoassay for tLH using biotin

A direct and simple homologous enzyme immunoassay (EIA) for tLH using biotin–streptavidin amplification was developed according to Prakash et al., 2002, 1999. Briefly, 40 μg of tLH previously purified from tilapia pituitaries (Bogomolnaya et al., 1989) was dissolved in 100 μl PBS; 12 μl of biotinamidocaproate–N-hydroxysuccinimideester (biotin; Sigma) dissolved in 1 mg/ml dimethyl sulfoxide (DMSO; Sigma) was added, and the mixture was incubated for a further 3 h at room temperature. The coupling reaction was stopped by the addition of 20 μl NH4Cl (1 M). The first coating was performed by adding 1 μg of sheep anti-rabbit IgG dissolved in 1 mg/ml sodium carbonate (pH 9.6) per well of the microtiter plate (Nunc, Roskilde, Denmark). After blocking, 50-μl duplicates of unknown samples or tLH standards ranging from 6.25 to 1600 pg/50 μl were added into the respective wells along with 100 μl of LH antibody (Bogomolnaya et al., 1989) diluted 1:1000 in assay buffer (50 mM NaPO4, 0.15 M NaCl, pH 7.4). Plates were incubated overnight before adding 100 μl biotinyl-tLH conjugate diluted 1:200 in assay buffer. Then 20 ng streptavidin-peroxidase (Sigma) were added to all wells and the plates were incubated for a further 1 h. The wells were washed and incubated for 1 h after adding 150 μl substrate solution/well (substrate buffer: 0.05 M citric acid, 0.11 M Na2HPO4, pH 4.0 with HCl; substrate solution: 17 ml substrate buffer plus 340 μl 3,3',5,5'-tetramethyl benzidine, 12.5 mg/ml DMSO, Sigma). The reaction was stopped by the addition of 50 μl of 4 N H2SO4 and the color measured at 450 nm.

2.9. In vitro bioassay

The bioassay procedure was generally according to that described by Bogomolnaya and Yaron (1984) for tilapia ovaries. Immature (16.1 ± 0.97 g BW; GSI = 0.65 ± 0.04%) or maturing (19.75 ± 0.62 g BW; GSI = 3.28 ± 0.10%) testes were dissected from the fish and divided into uniformly sized fragments (about 20 mg/piece). The fragments were pre-incubated in a 96-well culture plate at 26°C in the presence of 0.1 ml/well of BME containing NaHCO3 (4 mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and nystatin (1.251U/ml) (Biological Industries) and 0.3% BSA (Sigma). The medium was replaced after 3 h by the same medium containing 0.5 mM isobutylmethylxantine (IBMX; Sigma) with or without the gonadotropins being tested. Stimulation with rtLHβ, rtLHβx, or hCG (CG100; Sigma), at graded doses, was continued for another 18 h. These incubations were performed in triplicate wells per treatment. The incubation medium was collected and stored at −20°C until assay for 11-ketotestosterone (11-KT) concentration by ELISA.

2.10. ELISA for 11-KT

The androgen 11-KT was determined by ELISA according to Cuisset et al. (1994) and Nash et al. (2000) using acetylcholinesterase as a label. The anti-11KT was donated by Prof. D.E. Kime (Sheffield, UK) and is described in Cuisset et al. (1994). All samples were analyzed in duplicate, and for each ELISA plate, a separate standard curve was run. The lower limit of detection was 0.93 pg/ml. The intra- and inter-assay coefficients of variance were less than 7 and 11%, respectively. Steroid levels in the medium determined by ELISA were validated by verifying that serial dilutions were parallel to the standard curve.

2.11. Statistical analysis

Data are presented as mean ± SEM. The significance of the differences between group means of hormone levels was determined by one-way analysis of variance (ANOVA) followed by Newman–Keuls test using the GraphPad Prism software (GraphPad, San Diego, CA). To test for parallelism between different regression lines, we used the analysis of covariance at: http://www.ubmail.ubalt.edu/~harsham/Business-stat/otherapplets/ANOCOV.htm.

3. Results

3.1. Western-blot characterization of the Pichia-expressed rtLHβHis and rtLHβ2His proteins

Fig. 2 shows a Western blot of supernatant from untransfected GS115 or GS115 cells transformed with pPIC9KtLHβHis, pPIC9KtGPx or pPIC9KtLHβxHis, together with tilapia pituitary extract. These proteins were immunoreacted with antibodies raised against tLH purified from pituitaries (Bogomolnaya et al., 1989). This antibody recognized both α- and β-subunits (Fig. 2A), while the antisera generated against tLHβ alone (Melamed et al., 1999) recognized only the β-subunit (Fig. 2B). Under non-reducing conditions, in tilapia pituitary extract which was immunoreacted with anti-LH (Fig. 2A, lane 1), α- and β-subunits were revealed as bands of...
12 and 14 kDa, respectively, and a band at 14 kDa represented the β-subunit after immunoreaction with anti-LHβ (Fig. 2B, lane 1). Both the pituitary and recombinant LH, immunoreacted with both antibodies (anti-LH and anti-LHβ), yielded bands of 35 and 45 kDa, respectively. As expected, the anti-LH recognized the rtGPα and yielded a band of 45 kDa (Fig. 2A, lane 3), whereas rtGPα immunoreacted with anti-LHβ did not yield any band (Fig. 2B, lane 3). rtLHβ was recognized by both antibodies and yielded bands at 25 and 35 kDa (Fig. 2A and B, lane 4). Additional bands present in both pituitary and recombinant gonadotropin probably represent highly glycosylated mature protein, a dimer and some degradation of the proteins, or incomplete processing of the αMF leader sequence (Fig. 2). A large smear ranging from 45-120 kDa is recognized by both antibodies immunoreacted with rtLHβα (Fig. 2A, lanes 2 and 3; Fig. 2B, lane 2). Part of this smear may be due to high degree of glycosylation of the proteins. However, high bands are also shown in the tilapia pituitary (Figs. 2A and B, lane 1). Western blot analysis of supernatant from untransfected GS115 did not show any immunoreacted bands, verifying that the bands detected by the Western blot are specific to the strains transfected with the vectors encoding recombinant proteins (Figs. 2A and B, lane 5).

3.2. Purification of rtLHβαHis or rtLHβαβHis by one-step nickel batch purification

To facilitate purification of the recombinant protein from the culture medium, a six-His tag was introduced at the C-terminal end of the LHβ cDNA by incorporating the appropriate sequence into the 3’ PCR primer (P2 and P5, Table 1) and cloning the amplified DNA into pPIC9K to obtain the constructs pPIC9KLHβαHis and pPIC9KLHβαβHis (Products C and E, respectively, Fig. 1). The yeast transformants were selected and screened as already described. rtLHβαHis was purified using one-step nickel batch purification (Fig. 3). In a typical preparation, 100 ml of the induced culture supernatant containing rtLHβαHis (Fig. 3A, lane 1) was passed through a 1-ml bed volume of Ni-NTA agarose. The unabsorbed
fraction contained very small amounts of rtLHβ<sup>His</sup> (Fig. 3, lane 2); while the eluted fraction (Fig. 3, lane 3) contained large amounts of the recombinant subunit. rtLH-β<sup>His</sup> was immunoreacted with anti-His antibodies to yield bands of 20 and 45 kDa, probably representing the nascent and glycosylated forms of the protein. An additional band of <10 kDa appeared after purification of rtLHβ<sup>His</sup>, probably due to degradation. Hundred ml of such a culture yielded around 400 µg (n = 2). Similar results were obtained when rtLHβ<sup>α</sup>β<sup>His</sup> was purified by one-step batch purification (Fig. 3B), only that the culture supernatant contained such a small amount of the protein that it was unable to be visualized in the western blot. Indeed, 100 ml of the culture medium, grew in shake flask yielded only 8 ± 1.2 µg (n = 3) of rtLHβ<sup>α</sup>β<sup>His</sup>, as measured by EIA.

Deglycosylation of rtLHβ<sup>α</sup> resulted in a shift from a 55- or 60-kDa band to 45 kDa (Fig. 4A). An additional band at 20 kDa appeared after the deglycosylation of rtLHβ<sup>α</sup>, probably due to degradation (Fig. 4A). Treatment of rtLHβ with PNGase F resulted in a shift from 40 to 20 kDa, representing the nascent β-subunit (Fig. 4B).

3.3. Parallelism of rtLHβ<sup>α</sup>β<sup>His</sup>, tilapia pituitary extract, or standard LH

The curves yielded by serial dilutions of tLH, tilapia pituitary extract (TPE), or rtLHβ<sup>α</sup>β<sup>His</sup> are shown in Fig. 5. Each point is the mean of triplicate determinations in a single sample. The dilution curve of rtLHβ<sup>α</sup> was found to parallel both that of LH standard, and that of TPE, indicating that rtLHβ<sup>α</sup> is immunologically similar to the native LH. The LOGIT-transformed linearized standard curves of tilapia pituitary and standard LH were highly correlated with those of rtLHβ<sup>α</sup>β<sup>His</sup> (r<sup>2</sup> = 0.9976 ± 0.007, r<sup>2</sup> = 0.9965 ± 0.010 and r<sup>2</sup> = 0.9997 ± 0.003, respectively; p ≤ 0.0001) (Fig. 5). The slope of the displacement curve obtained with rtLHβ<sup>α</sup> (slope ± SEM: −0.1941 ± 0.0015) was similar to that obtained with the LH standard, (−0.2160 ± 0.0015), and to that of the pituitary extract (−0.1768 ± 0.0038) suggesting that the recombinant rtLHβ<sup>α</sup> is immunologically similar to tLH (Fig. 5). However, the slope of the EIA displacement curve obtained with rtLHβ (−0.3544 ± 0.034) differed from that obtained with the authentic LH (data not shown). The culture medium of the host cells did not show any displacement in the assay (data not shown), thus excluding the possibility of non-specific interactions from the culture medium. Since the rtLHβ<sup>α</sup> used in the parallelism test also contained the six-His tail, the parallelism between rtLHβ<sup>α</sup>β<sup>His</sup> and authentic LH (Fig. 5) suggests that the addition of six His residues at the C-terminus do not affect the folding of the molecule.

3.4. In vitro biological activity of rtLHβ<sup>α</sup>β<sup>His</sup> and rtLHβ<sup>α</sup>β<sup>His</sup>

The release of 11-KT from the testes of tilapia was used as a bioassay for rtLHβ<sup>α</sup>. hCG, which is broadly used for spawning induction in various fish (Zohar and Mylonas, 2001), and which belongs to the LH subfamily of gonadotropins, was used as a positive control. The amount of rtLHβ<sup>α</sup> used for the stimulation was calculated by EIA. The hCG IU were translated into units of mass for the sake of comparison (100 IU of the hCG utilized = 1 mg). Tilapia testes stimulated with culture supernatant of cells transformed with the expression vector not containing rtLHβ<sup>α</sup> cDNA did not stimulate 11-KT production at all (Fig. 6, control). However, incubation of testicular tissue from maturing tilapia (GSI > 0.3, Fig. 6B) with increasing concentrations of rtLHβ<sup>α</sup> resulted in significant increases in the concentration of 11-KT in the incubation media. A significant, albeit less pronounced increase was noticed after stimulating the
4. Discussion

Here we show the expression, secretion, and purification of tLH using P. pastoris as the expression system. Very little information is available in fish on the structure-function relationships of the gonadotropins. To perform such studies, the ability to express a heterodimer as a single, biologically active chain would likely avoid mutagenesis-induced defects and secretion of individual subunits. Furthermore, since subunit dissociation inactivates the in vivo activity of a heterodimer, a single-chain analog could have a longer biological half-life. The C-terminal region of the α-subunit and the N-terminal region of the β-subunit of the gonadotropins have been shown to be important for receptor binding (Chen et al., 1992; Huang et al., 1993). In contrast, the N-terminal end of the β-subunit can be modified with no significant effects on receptor binding or signal transduction (Fares et al., 1992). Therefore, in the first construct (Product C, Fig. 1), the tLHβ and rGPα mature protein-coding sequences were joined to form a fusion gene that encodes a “tethered” polypeptide consisting of the tLHβ-chain forming the N-terminal part and the α-chain forming the C-terminal part. A “linker” sequence of six amino acids (three Gly-Ser pairs) was placed between the β- and α-chains to facilitate tethered protein folding thereby allowing interaction between the fused β- and α-chains. Most of the gonadotropins that have been expressed in P. pastoris in a tethered form have been human chorionic gonadotropin (Sen Gupta and Dighe, 1999, 2000). In hCG, the last 30 amino acids of the β-subunit contain a C-terminal peptide (CTP) that plays a pivotal role as a linker (Sugahara et al., 1995). Only when the CTP is placed on either the C-terminal end of the FSHβ-subunit or in the N-terminal region of the α-subunit, assembly, secretion, and signal transduction of the dimers are comparable to the wild-type hormones (Fares et al., 1992; Furuhashi et al., 1995; Sugahara et al., 1995). Since the tLH lacks a CTP or any similar structure at its C-terminus, the fusion between the two subunits was bridged by a flexible Gly-Ser linker. The Gly-Ser linker sequence is minimally hydrophobic or charged, and can therefore maximize the opportunity for chimerized subunits folding into their native conformational structures for independent interaction with their receptors. This six-amino-acid Gly-Ser spacer provides some distance between the α- and β-subunits. A similar strategy was used in fusing IL-3 and GM-CSF (Curtis et al., 1991), idiotype antibody and GM-CSF (Tao and Levy, 1993), and HBV surface antigen and the immune stimulatory region of human IL-1 (Rao and Nayak, 1990).

The slight differences in the apparent molecular masses between the pituitary and recombinant LH (35 versus 45 kDa) and between the pituitary and recombinant β-subunits (14 versus 25 kDa) resulted in part from the additions (the six-His tail and the linker) to the recombinant proteins. Different degrees of glycosylation may also have contributed to these differences. The molecular masses of the bands representing the α (12 kDa) and β (14 kDa) subunits are in agreement with those of native LH purified from other teleosts (Kamei et al., 2003; Shimizu and Yamashita, 2002). The Western blotting results also showed the existence of other products, in addition to the tLHβx protein. We suggest that the smaller product is a degraded form of tLHβx whereas the larger product may be either a highly glycosylated form of tLHβx or multimers, as reported in Ben-Menahem et al. (1999). Another possibility for the presence of bands of different sizes is an incomplete processing of the MF α leader sequence. tLH has one putative N-glycosylation sequence (Asn-X-Ser/Thr) at amino acid 40 of the β-subunit (Rosenfeld et al., 1997), and two
sites at amino acid 78 and 103 of the α-subunit (Gur et al., 2001). This may explain the different degrees of glycosylation by Pichia between the α- and β-subunits of the recombinant LH. The rtLHβ appeared as a distinct band in Western blot analysis, probably due to the smaller degree of glycosylation relative to rtGPα, which appeared as a smear. The mobility of the latter in SDS–PAGE was very similar to that of rtLHβ. Although not required for LH-receptor binding, N-linked glycosylation influences LH bioactivity both in vitro and in vivo, especially in the mechanisms of receptor coupling to adenylate cyclase (Arey et al., 1997). We performed deglycosylation of rtLHβHis and rtLHβHis with PNGase F, which hydrolyzes all types of N-glycans chains. PNGase F reduced the glycosylated forms to the nascent translated protein (45 and 15 kDa, respectively). Moreover, these results implied, for the first time in fish, that the carbohydrate modifications on the mature protein occur exclusively through N-linked, and not at all through O-linked glycosylation.

One of the objectives of this work was to develop a protocol for the production of recombinant gonadotropins for use in studying fish gonadotropins for which antibodies are unavailable. This is why it was crucial to add a His tail to the gonadotropin gene that would enable affinity purification of the recombinant protein. As seen from the slopes of the EIA curves, the addition of six His residues at the C-terminus of rtLHβ did not have any noticeable effect on the overall folding of the molecule. Moreover, the recombinant protein rtLHβ-His was capable of binding to its receptor and stimulating 11-KT secretion from tilapia testes. The biological potency of the recombinant LH suggests that the protein was folded properly and that appropriate glycosylation structures were added to the recombinant protein within the Pichia secretory pathway. It may be concluded that rtLHβHis is very similar to its pituitary-derived counterpart with respect to its immunological identity and biological activity.

The amounts of rtLHβHis secreted by the Pichia transformants in this work (approximately 80 ng/ml) are still modest compared to what Pichia has been reported to achieve (Hollenberg and Gellissen, 1997). Such low levels of recombinant protein in the growing phase are hardly detectable by Coomassie-Blue staining. Similar amounts (50–100 ng/ml) of recombinant protein have been found when a single-chain, biologically active recombinant hCG protein was expressed in Pichia (Sen Gupta and Dighe, 2000). Further work is still required to scale-up these amounts using fermentation to produce sufficient amounts of protein. Interestingly, when the tLHβ- and GPα-subunits were synthesized separately, protein production was higher than when the subunits were joined to form a fusion protein. This may be due to the simplicity of expressing an isolated subunit as compared to expressing a heterodimer. A similar phenomenon has been reported for hCG (Sen Gupta and Dighe, 2000). A relatively low level of glycoprotein hormone expression, even using expression systems known to produce heterologous protein at exceptionally high levels, highlights the constraints on expressing these hormones. Such low expression of recombinant LH is not only characteristic of the Pichia expression system; it also seems to be a crucial obstacle in other expression systems for the glycoprotein hormone family. For example, sea bream gonadotropins that were expressed using baculovirus yielded low levels of gonadotropins (Meiri et al., 2000); expression of FSH using the rat β-casein promoter construct yielded extremely low levels of the hormone in transgenic animals (Greenberg et al., 1991), and expression of eel FSH in P. pastoris also yielded low levels of the recombinant protein (Kamei et al., 2003). However, the expression of catfish gonadotropins in Drosophila cells yielded very high levels (Zmora et al., 2003). Catfish recombinant LH that was produced in the soil amoeba, Dictyostelium discoideum, yielded 700–1000 ng/ml (Vischer et al., 2003).

The displacement curve obtained with rtLHβHis appears to be parallel to that obtained with the native pituitary extract and standard LH, indicating that the fusion protein is able to adopt a conformation similar to that of the native tLH. Moreover, our results show that the α- and β-subunits of tLH encoded as a single chain can fold into an appropriate conformation, and that non-covalent linkage of the subunits is not absolutely required for tLH’s biological activity. It should be mentioned in this context that we can not excluded the possibility that the tethered construct behaves differently from the native tLH.

Comparison of the biological potency of rtLH and hCG showed a similar response to microgram amounts of the heterologous gonadotropin in immature and mature males. The homologous recombinant gonadotropin was efficient already in the nanogram range. However, the levels of 11-KT secreted from the testes of mature males were significantly higher than those secreted from the testes of early maturing males. Testes from mature tilapia, with high GSI, contained running spermatozoa (Gur et al., 2000). Our results corroborate previous ones showing that in male tilapia, circulating levels of 11-KT are correlated with increasing GSI, and that LHβ mRNA levels are high compared with those in immature tilapia (Melamed et al., 1997).

Our results corroborate the findings in salmon whereby LH stimulated 11-KT production in vitro during spermatogenesis (Planas and Swanson, 1995). The gonadotropin’s potency at stimulating the production of 11-KT was dependent on the stage of spermatogenesis and it increased as spermatogenesis progressed. The enhanced potency of LH coincides with the appearance of post-meiotic germ cells and LH receptor in the testes, increases in plasma LH and spermatiation (Swanson et al.,
Although the duality of gonadotropins in fish is well established, the exact role of each gonadotropin in non-salmonid fish, especially multi-spawning fish with asynchronous gonads, such as tilapia, is unknown. Our results, showing that recombinant LH elicits higher secretion levels of 11-KT in mature versus immature males, may indicate a differential function for LH during testicular development where, like in the salmonids, LH is predominant in the mature male tilapia.

Our results show that rtLHß increases the release of 11-KT from mature and immature tilapia testes, while hCG stimulated the release of similar levels at the two stages. The discrepancy in the response mode between the homologous and heterologous gonadotropins suggests that hCG and rtLHß do not necessarily bind to the same receptor at the same potencies. Previous studies have indicated the presence of two types of receptor for GtH in different teleosts, including tilapia (Oba et al., 2001). It was also shown that FSH receptor interacts with both of the homologous LH and FSH, whereas LH receptor interacts specifically with LH in catfish (Vischer et al., 2003) and salmon (Miwa et al., 1994). Since hCG is broadly used for spawning induction in many fish species, it would be important to characterize the types of GtH receptors to which rtLHß is binding as compared to hCG.

In conclusion, we report the expression, secretion, and characterization of a biologically active, single-chain tLH in the methyloptroph yeast P. pastoris. This system can be employed for large-scale production and purification of recombinant fish gonadotropins, and as such can be applied as a model system to study the structure–function relationships of these important hormones.

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