Production of biologically active tethered ovine FSHβα by the methylotrophic yeast *Pichia pastoris*

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Abstract

The pituitary-derived glycoprotein hormone FSH plays a central role in controlling vertebrate gonadal function. In female mammals the maturation of ovarian follicles is critically dependent upon stimulation by FSH. Moreover, injection of exogenous FSH is used extensively to stimulate increased numbers of follicles to ovulate. Structurally FSH is a heterodimeric glycoprotein composed of two non-covalently associated polypeptide subunits. The tertiary structures of both the α- and β-subunits are constrained by intramolecular disulphide bonds and are post-translationally modified with two N-linked carbohydrate moieties, the structure of which appears to modulate *in vivo* biological activity.

Here we report the expression of ovine FSH (oFSH) as a biologically active single-chain polypeptide using the methylotrophic yeast *Pichia pastoris*. Sequences encoding the mature oFSH α- and β-proteins were fused to form a gene encoding a fusion protein with the C-terminus of the β-chain joined to the N-terminus of the α-chain, with the chains separated by a two amino acid linker sequence. This fusion gene was itself fused to two alternative *Pichia* leader sequences (mating factor alpha and acid phosphatase) and transformed into the *Pichia* strains GS115 and SMD1168. The recombinant fusion protein (oFSHβα) was expressed at approximately 0·1 µg/ml in 'shake-flask' cultures. The *Pichia*-expressed tethered protein was biologically active in an *in vitro* bioassay, had a molecular mass of 28 kDa, as determined by SDS-PAGE, and bound the bovine FSH receptor with a binding profile similar to that of native oFSH.

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Introduction

Follicle-stimulating hormone (FSH) secreted by pituitary gonadotrophs promotes ovarian follicular growth and maturation (Richards 1994, Kumar *et al.* 1997). The administration of exogenous FSH can be used to stimulate increased numbers of ovarian follicles to ovulate, a procedure that has practical applications in human infertility and animal breeding management (Devroey *et al.* 1993, de Koning *et al.* 1994, Anderiesz *et al.* 2000). Presently, the FSH used for the superovulation of domestic animals is purified from animal-derived material. To minimise the potential risk of pathogen transmission, future requirements of hormone for superovulation purposes may require a serum-free source of recombinant FSH (de Koning *et al.* 1994).

Structurally, FSH is a heterodimer formed by the non-covalent association of an α-subunit, which is common to the three pituitary glycoproteins hormones, FSH, luteinising hormone (LH) and thyroid-stimulating hormone (TSH) (Combarnous 1992, Nagaya & Jameson 1994), and a hormone-specific β-subunit, which confers receptor-binding specificity. The secondary structures of the α- and β-subunits are constrained by intramolecular disulphide bonds, five in the α-subunit and six in the β, and both subunits carry two N-linked carbohydrate moieties (Combarnous 1992). Variation in these carbohydrate structures results in both pituitary and serum FSH comprising a heterogeneous population of molecules varying in parameters such as receptor-binding affinity and metabolic clearance rate (Ulloa-Aguirre *et al.* 1995).
The methylothrophic yeast *Pichia pastoris* has been developed as an expression system for high-level production of recombinant proteins (Sudbery 1996, Hollenberg & Gellisen 1997). *P. pastoris* offers the features of: (i) methanol-induced expression of heterologous genes integrated into the yeast genome downstream of the alcohol oxidase 1 (*AOX1*) gene promoter; (ii) growth to high cell density in an inexpensive, serum-free, chemically defined medium; and (iii) the capacity to carry out post-translational modifications resembling those of mammalian cells. Using either native or heterologous secretion sequences, recombinant proteins can be directed into the yeast's secretory pathway wherein disulphide bond formation and glycosylation can occur before secretion into the growth medium (Grinja & Tschopp 1989, Sudbery 1996, Hollenberg & Gellisen 1997).

In a previous paper we reported the production of biological active ovine FSH (oFSH) using transformed *Pichia* strains coexpressing oFSH α- and β-subunit cDNA sequences fused to nucleotide sequences encoding the mating factor alpha (MFα) leader sequence (Fidler et al. 1998). Such *Pichia* strains expressed biologically active oFSH, albeit at low levels. However, most of the subunits that were secreted had not dimerised and furthermore it appeared that only one-third of the dimers produced were biologically active (Fidler et al. 1998). Therefore the primary aim of this study was to test whether the issue of the low level of interchain dimerisation could be addressed by using *P. pastoris* to express recombinant tethered proteins formed by joining the C-terminus of the oFSH β-chain to the N-terminus of the α-chain, with a two amino acid linker sequence. Secondary aims of this study were to test a variety of alternative leader sequences and two alternative *Pichia* strains as well as altered codon usage within the oFSHβ-coding region in attempts to increase the production of biologically active recombinant oFSH.

**Materials and methods**

**Amplification of the oFSH α- and β-subunit sequences**

Primers were designed for the amplification of the oFSH β and α cDNA sequences for formation of fusion genes that could be subcloned into two *Pichia* expression vectors, namely pPIC9, encoding the MFα leader sequence and pHILS1, encoding the acid phosphatase (*PHO1*) signal sequence (Invitrogen, Carlsbad, CA, USA). As the 5′ region of the oFSHβ mature protein-coding sequence was to be fused to three different leader sequences, three different forward primers were required for amplification of the oFSHβ mature protein-coding sequence: PrimerβA 5′-CCCTCGAGAAAA GAAGCTGCGAGCTGACCAATC-3′, with primer-encoded vector sequence including the ‘minimal’ *KEX2* dibasic proteolytic cleavage site (-Lys-Arg-); PrimerβB 5′-CCCTCGAGAAAAAG AGGCGTGAAGCTAGCTGCGAGCTGACCAATC-3′, with primer-encoded vector sequence including the ‘extended’ *KEX2* proteolytic cleavage site consisting of the dibasic motif (-Lys-Arg-) followed by the tetrameric sequence -Glu-Ala-Glu-Ala-; and PrimerβC 5′-CCCTCGAGATGTAGCTGCAGCTGACCAATC-3′, which encodes the *PHO1* signal peptide sequence cleavage site. In all three primers the vector sequences are underlined, the oFSHβ mature protein-coding sequence (nucleotides 113–133 of Mountford et al. (1989)) are shown in bold type and the XhoI site is double-underlined. Primers βA and βB were used for subsequent subcloning into the vector pPIC9 (Invitrogen) and PrimerβC for subcloning into pHILS1 (Invitrogen). The oFSHβ reverse primer, PrimerβD, was 5′-CCGGATCTTCTTCTCTGATGTCACTGAAGAGGA-3′, with the oFSHβ-coding sequence (nucleotides 422–442 of Mountford et al. (1989)) shown in bold type and the BamHI site double-underlined. The oFSHα forward primer (PrimerαA) was 5′-CCGGATCTTCTTCTCTGATGTGAAGTTCACA-3′, with the oFSHα-coding sequence (nucleotides 143–163 of Bello et al. (1989)) shown in bold type and the BamHI site double-underlined. The oFSHα reverse primer (PrimerαB) was 5′-CCGGATCTGGAATCTTCTAAAGATTTGTGATAATAACAAACATC3′, with the oFSHα-coding sequence (nucleotides 413–433 of Bello et al. (1989)) shown in bold type and the SacI and EcoRI sites double-underlined. First-strand cDNA was synthesised from 5 µg ovine pituitary total RNA using Superscript reverse transcriptase (Gibco BRL, Bethesda, MA, USA). The mature protein-coding sequences of the oFSH α- and β-subunits were amplified by Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany)-catalysed PCR using the following reaction conditions: 94 °C,
sequencing. of the fusion genes was confirmed by DNA by restriction enzyme digestion. The structure of the size wanted (approximately 0·6 kb) identified cells and plasmids with XhoI-EcoRI inserts of ligation reactions were transformed into DH5α.

Construction of the oFSHβα fusion genes

The oFSHβα fusion genes were formed by three-way ligations between (i) the oFSHβ-coding regions (isolated as approximately 0·3 kb XhoI-BamHI fragments from a 1% (w/v) LMP agarose gel (QiaQuick), (ii) the oFSHα-coding region (isolated as an approximately 0·3 kb BamHI-SacI fragment from a 2% (w/v) LMP agarose gel and (iii) vector pGem7Z (Promega) linearised by double-digestion with XhoI and SacI and isolated from a 1% (w/v) LMP agarose gel. As there were three different oFSHβ-coding sequences, corresponding to the three different oFSHβ forward primers (Primers Α, Β and ΒC, above) three different oFSHβα fusion genes were produced. The ligation reactions were transformed into DH5α cells and plasmids with XhoI-EcoRI inserts of the size wanted (approximately 0·6 kb) identified by restriction enzyme digestion. The structure of the fusion genes was confirmed by DNA sequencing.

Subcloning of fusion genes into Pichia expression vectors

The oFSHβα fusion genes (cloned in pGem7Z) were subcloned into the P. pastoris expression vectors pPIC9 or pHILS1 (Invitrogen). Selection of the appropriate vector was determined by the oFSHβ forward primer used in the initial PCR amplification of the oFSHβ sequence: PrimerβA and βB products were cloned into pPIC9 and PrimerβC products into pHILS1. Vectors pPIC9 and pHILS1 were linearised by double-digestion with XhoI and EcoRI and isolated from 1% (w/v) LMP agarose gels. Similarly, inserts encoding the oFSHβα fusion genes were isolated from 1% (w/v) LMP agarose gels after XhoI and EcoRI double-digestion of corresponding pGem7Z-based constructs. Inserts and vectors were ligated and the desired recombinant plasmids were first identified by restriction enzyme analysis and the sequences confirmed by DNA sequencing. The three plasmid constructs were denoted pPIC9 FSHβα, pPIC9 EAFSHβα and pHIL FSHβα.

Pichia strain transformation and screening of transformants

Pichia host strains GS115 (his4) and SMD1168 (his4, pep4) (Invitrogen) were transformed with plasmids using the method of Dohmen et al. (1991) (Pichia EasyComp; Invitrogen). His+ transformants were selected for and then screened for both normal and slow growth phenotypes on medium containing methanol as the sole carbon source, thereby identifying transformants with both the His+Mut+ and His+Mut8 phenotypes (Sreekrishna & Kropp 1996).

Alteration of fusion gene codons by in vitro mutagenesis

Three codons within the oFSHβ-coding region of the oFSHβα fusion gene were altered by site-directed mutagenesis using the overlap-extension technique (Horton et al. 1990). Complementary oligomers were designed to the region of the oFSHβ gene sequence that encoded the three codons that were to be changed: CGC (Arg88), CGA (Arg96) and GGG (Gly99) being altered to CGT, CGT and GGT respectively; forward codon change primer (FCCP): 5′-ACCGTGACAGCA CTGACTGCACCGTGCGTGGCCTGGGTCC A−A−3′ (nucleotides 372–413, Mountford et al. 1989) and reverse codon change primer (RCCP): 5′-TGGGACCCAGGCCACGCAC GGTGCTGACAGCA CTGACTGCACCGTGCGTGGCCTGGGTCC A−A−3′ (complementary to
nucleotides 413–372, Mountford et al. (1989)). The procedure for changing the codons was as follows. Plasmids pPIC9 FSHβα and pPIC9 EAFSHβα were digested with BglII to provide linearised templates. In the first round of PCR amplification, the pPIC9 FSHβα- and pPIC9 EAFSHβα-encoded oFSHβα genes were amplified with two pairs of primers thereby amplifying the codon-altered oFSHβα fusion genes in two sections. The two primer pairs were: (i) FCCP paired with the oFSH reverse primer (PrimerαB) and (ii) RCCP paired with either the oFSHβ forward primer PrimerβA (used with the pPIC9 FSHβα template) or PrimerβB (used with the pPIC9 EAFβα template). The amplification reaction used Taq DNA polymerase and the following reaction conditions: 94 °C, 2 min, one cycle; 94 °C, 30 s, 60 °C, 30 s, 72 °C, 1 min, ten cycles; 94 °C, 30 s, 62 °C, 30 s, 72 °C, beginning 1 min and increasing 20 s per cycle, 30 cycles; 72 °C, 10 min, one cycle. Amplification products were isolated after agarose gel electrophoresis and pooled, heated in a boiling water bath and then cooled slowly to room temperature before being placed on ice. The annealed mixtures were then used as templates for a second round of amplification using one of two primer pairs, either the oFSHβ forward primer PrimerβA paired with the oFSHα reverse primer PrimerαB or the oFSHβ forward primer PrimerβB paired with PrimerαB. The amplification conditions were: 94 °C, 2 min, one cycle; 94 °C, 30 s, 60 °C, 30 s, 72 °C, 1 min, ten cycles; 94 °C, 30 s, 60 °C, 30 s, 72 °C, 1 min, 30 cycles; 72 °C, 10 min, one cycle using Taq DNA polymerase. The amplification products were electrophoresed through a 1% (w/v) LMP agarose gel, products of the anticipated size (approximately 0·6 kb) purified and cloned into pGemT-easy (Promega). Plasmids with inserts were identified by restriction enzyme digestion and the inserts sequenced to confirm the structure of the fusion genes, the presence of the desired codon changes and the absence of any other (random) mutations introduced by Taq DNA polymerase. Inserts encoding the altered FSHβα gene (denoted ochFSHβα for ovine codon changed FSHβα) sequence were ligated into XhoI/EcoRI double-digested pPIC9 forming two plasmids that were denoted pPIC9 EAcchFSHβα and pPIC9 cchFSHβα. The structure of these plasmids was confirmed by DNA sequencing. Plasmids pPIC9 EAcchFSHβα and pPIC9 cchFSHβα were linearised by BglII digestion before transformation of Pichia host strains.

Induction of recombinant protein production

Pichia culturing and induction followed ‘shake-tube’ and ‘shake-flask’ procedures described previously (Barr et al. 1992, Sreekrishna & Kropp 1996). Briefly, Pichia cultures were grown for 2 days, reaching high cell density in medium with a glycerol carbon source (BMGY medium: 1·0% (w/v) yeast extract, 2·0% (w/v) bacto-peptone, 1·0% (v/v) glycerol, 0·1 M potassium phosphate buffer pH 7·0, 1·34% (w/v) bacto-yeast nitrogen base without amino acids, 0·4 µg/ml biotin). Growth conditions included vigorous aeration and were at 30 °C. The cells were then resuspended in a fifth of the volume of medium containing methanol as the sole carbon source (BMMY medium: made up as for BMGY medium, but lacking glycerol, and supplemented to 0·5% (v/v) with methanol). After 2 days of growth in BMMY medium the growth medium was clarified by centrifugation and dialysed extensively against 1× PBS at 4 °C. Samples were stored at −20 °C before analysis by radioimmunoassay (RIA), radioreceptor assay (RRA) and in vitro bioassay.

RIA

Immunoreactive FSH was measured using RIA kits supplied by the NIH National Hormone and Pituitary Program (NIH, Bethesda, USA). Samples were assayed for FSH dimer using USDA-oFSH-19-SIAFP-I-2 for iodination, USDA-oFSH-19-SIAFP-RP-2 (94 × NIH-oFSH-S1; biological potency=2351 IU/mg) for reference preparation and NIDDK-anti-oFSH-1 antisera. Standards were iodinated using the chloramine T method and purified by ion exchange chromatography for FSH dimer (Moore et al. 2000). Assays were performed by incubating 100 µl sample or standard, 100 µl primary antibody and 50 µl tracer overnight at room temperature. Separation of free hormone from bound was by the second antibody method using sheep anti-rabbit IgG, followed by 1·0 ml 6% (w/v) PEG8000 (Carbowax; Union Carbide Co., Danbury, CT, USA). Sensitivities of the assays for FSH (90% of zero binding) was 0·2 ng/ml. Intra- and inter-assay coefficients of
variation for the above assays were 7·9 and 10·8% respectively.

RRA

Clarified supernatants from methanol-induced shake-flask cultures of *Pichia* strains were filtered (0·22 µm), concentrated 10-fold by ultrafiltration (Amicon, Beverly, MA, USA) and dialysed extensively against 10 mM MgCl₂, 50 mM Tris–HCl pH 7·3 at 4 °C. FSH concentrations measured by RRA were quantified using membrane fractions from bovine testes as previously reported (Moore et al. 1997) using ¹²⁵I-labelled USDA-oFSH-19-SIAFP-I-2 as tracer and USDA-oFSH-19-SIAFP-RP-2 for reference preparation. Samples from methanol-induced *Pichia* cultures were assayed at supernatant protein concentrations of less than 50 µg/ml to avoid non-specific binding interference that was detected when the supernatants were assayed at higher concentrations. Total protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA).

*In vitro* FSH bioassay

The FSH bioassay, using a Chinese hamster ovary (CHO) cell line expressing the human FSH receptor, was essentially as described by Albanese et al. (1994). However, the assay end-point was cAMP production, as measured using the RIA methodology described previously (McNatty et al. 1989), rather than luciferase reporter gene expression. Native oFSH (USDA-oFSH-A-SIAFP-RP-2; NIH) was used as a standard. The sensitivity of the assay was 1 ng/ml and the intra- and inter-assay coefficients of variation were 8 and 14% respectively.

Preparation of E. coli-expressed recombinant oFSHα antigen

A cDNA sequence encoding the oFSHα mature protein was obtained by BamHI and EcoRI double-digestion of the pMOS oFSHα construct (described above), which was then sub-cloned into the expression vector pET28a (+) (Novagen, Madison, WI, USA) so that the resulting recombinant oFSHα protein would include an N-terminal histidine tag. The pET28 oFSHα construct was transformed into the host strain BL21(DE3) (Novagen), recombinant oFSHα protein production induced and the recombinant protein purified by affinity chromatography using Ni-NTA agarose (Qiagen). The procedures for bacterial culture, gene expression induction and protein purification followed the manufacturer’s instructions (Novagen). The identity of the purified recombinant oFSHα antigen was confirmed by Western blotting using antibody raised to the α-subunit of oFSH (National Hormone and Pituitary Program of USA, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), #AFP5999189).

Preparation of antiserum against recombinant oFSHα

New Zealand white rabbits were immunised by s.c. injections of 200 µg purified *E. coli*-expressed recombinant oFSHα emulsified in complete Freund’s adjuvant (Sigma, St Louis, MO, USA). The animals were boosted twice at three-weekly intervals using 100 µg of the same antigen. Two weeks after the last injection, the rabbits were bled and the antisera examined for binding to an in-house, highly purified (i.e. >90% pure by HPLC), native ovine pituitary standard purified in our laboratory (oFSH Wal; 1·4 × USDA-oFSH-19-SIAFP-RP-2) using Western blotting. Details regarding the purification of this in-house oFSH standard are published elsewhere (Moore et al. 1997).

Western blotting procedure

Clarified supernatants from methanol-induced *Pichia* cultures were filtered (0·22 µm) and concentrated 20-fold by ultrafiltration using a stirred cell with a 10 kDa cut-off filter (Amicon). Thereafter, the yeast concentrates and the ovine pituitary oFSH Wal standard were separated by SDS-PAGE through a 13·5% acrylamide gel (11·7% (w/v) acrylamide, 0·3% (w/v) N,N’-bis-methylene acrylamide) and electrophoretically transferred to a mixed ester nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech) in alkaline transfer buffer (2% (v/v) methanol, 25 mM Tris, 190 mM glycine) at 4 °C. Following transfer, the filters were stained in Ponceau S solution (0·5% (w/v) Ponceau S, 1·0% (v/v) acetic acid) and the positions of molecular mass standards (Bio-Rad Laboratories, Madison, WI, USA) were located using a Bio-Rad UV transilluminator.
Hercules, CA, USA) marked with pencil. After pre-incubation in blocking solution (20 mM Tris–HCl pH 7·5, 0·5 M NaCl, 0·1% (v/v) Tween-20, 5·0% (w/v) non-fat dried milk) for 1 h at room temperature the filters were washed in TBS buffer (20 mM Tris–HCl pH 7·5, 0·5 M NaCl, 0·1% (v/v) Tween-20) and then incubated overnight at room temperature with the rabbit antiserum raised against either native oFSHβ/afii9826 (R4; supplied by Dr K Henderson, Wallaceville Animal Research Centre, NZ) or the recombinant oFSHβ/afii9825 generated for this study (see above) diluted 1/500 in blocking solution. Unbound primary antibody was removed by extensive washing in TBS buffer and the filter incubated for 4–6 h at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody (HRP-goat anti-rabbit IgG) (Sigma) diluted 1/2000 in blocking solution. The unbound secondary antibody was removed by extensive washing in TBS buffer and labelled bands were visualised by incubating the filter with HRP substrate (0·22 mg/ml 3,3’-diaminobenzidine tetrahydrochloride dihydrate (Bio-Rad), 0·67 mg/ml 4-chloro-1-naphthol (Bio-Rad), 6·7 x 10⁻³ (v/v) H₂O₂ dissolved in 0·1% (v/v) methanol/1 × PBS). The visualisation reaction was terminated by washing in distilled water and the image was photographed on the same day.

**Results**

**Construction of fusion genes encoding tethered oFSHβα proteins**

Three different oFSH fusion gene constructs were produced for expression in *P. pastoris* (Fig. 1A–C). In all three constructs the oFSH β and α mature protein-coding sequences were separated by a six-nucleotide BamHI site encoding the amino acids glycine (G) and serine (S), which act as linker sequence. Transcription of the recombinant genes is from the vector-encoded, methanol-inducible AOX1 promoter.

**Figure 1** Schematic diagram of the oFSH fusion gene (oFSHβα) constructs in *P. pastoris* expression vectors. (A) pPIC FSHβα: the oFSHβα fusion gene attached to the MFα leader sequence and positioned immediately adjacent to sequences encoding the KEX2 protease cleavage site (KR) encoded in the vector pPIC9. (B) pPIC9 EAFSHβα: the oFSHβα fusion gene attached to the MFα leader sequence and positioned adjacent to the KEX2 protease dibasic cleavage site (in vector pPIC9) and with inclusion of the EAEA (Glu-Ala-Glu-Ala) sequence. (C) pHIL FSHβα: the oFSHβα fusion gene cloned adjacent to the acid phosphatase leader sequence (PHO1) encoded by vector pHLS1. In all three plasmid constructs the nucleotides sequences encoding the oFSH β and α mature proteins are separated by a six-nucleotide BamHI site encoding the amino acids glycine (G) and serine (S), which act as linker sequence. Transcription of the recombinant genes is from the vector-encoded, methanol-inducible AOX1 promoter.
encoding a ‘linker’ sequence of the two amino acids (glycine and serine), was placed between the β- and α-chains to facilitate the cloning procedure. The three fusion gene constructed differed with respect to the leader sequence to which they were fused. In two, pPIC9 FSHβα and pPIC9 EAFSHβα (Fig. 1A and B), the fusion gene includes the MFα-coding sequence so that, in the corresponding protein, the oFSHβ mature protein N-terminal sequence (SCEL) is placed adjacent to the dibasic (Lys-Arg/KR) KEX2 protease cleavage site (Fig. 1A and B). Construct pPIC9 EAFSHβα is essentially the same as pPIC9 FSHβα but includes, following the KR motif, the additional EEA (Glu-Ala-Glu-Ala) tetrameric sequence, the presence of which may enhance the efficiency of proteolytic cleavage by the KEX2 protease (Fig. 1B). The third construct, pHIL FSHβα, encodes a oFSHβα fusion gene with the FSHβ chain N-terminus joined to the PHO1 leader sequence (Fig. 1C). Transcription of all three fusion genes is from the vector-encoded, methanol-inducible AOX1 promoter.

Production of biologically active oFSHβα tethered protein

The three plasmid constructs (pPIC9 FSHβα, pPIC9 EAFSHβα and pHIL FSHβα) were transformed into two different Pichia host strains (GS115 (PEP4; hisα) and SMD1168 (pep4; his4)) (Invitrogen) and transformants selected for by requiring a His+ phenotype for growth. The His+ transformants were then screened for either the Mut+ phenotype (i.e. slow growth on methanol as a sole source of carbon), a phenotype indicating homologous recombination of the construct into the genomic AOX1 gene, or the Mut+ phenotype (i.e. normal growth on methanol), a phenotype indicating integration of the construct into the Pichia genome by non-homologous recombination. Following this procedure four strains could potentially be produced for each plasmid construct.

The transformed Pichia strains were grown in shake-tube cultures, induced to express the FSHβα fusion gene and the culture supernatants assayed for the presence of oFSH by three different methods: RIA, RRA and an in vitro bioassay (Fig. 2). The culture supernatants of the negative control strains, either non-transformed strains GS115 and SMD1168 (Fig. 2, data sets 1 and 10 respectively) or transformed with the vector pPIC9 alone (Fig. 2, data sets 2, 3, 11 and 12), had no oFSH detected using any of the three FSH assays. Screening of between 10 and 36 His+ transformants of strains transformed with each of the three oFSHβα fusion gene constructs (pPIC9 FSHβα, pPIC9 EAFSHβα, pHIL FSHβα, Fig. 1) detected both Mut+ and Mut+ transformants that secreted oFSH (GS115 transformants: Fig. 2 data sets 4–9, SMD1168 transformants: Fig. 2 data sets 13–18). Generally, the concentrations of FSH measured using the three assay methods were in agreement with all strains secreting oFSHβα in the range of approximately 60–140 ng/ml with the actual values varying between independent inductions.

Western blot characterisation of the Pichia-expressed oFSHβα protein

All the strains of GS115 and SMD1168 transformed with constructs pPIC9 FSHβα and pPIC9 EAFSHβα were characterised by Western blotting. Figure 3 shows a Western blot of a supernatant from strain GS115 transformed with pPIC9 FSHβα together with oFSH Wal using, as primary antibodies, rabbit antisera raised against either oFSHα or oFSHβ (Fig. 3). The oFSH Wal preparation contained labelled products of 22 kDa and 23–26 kDa respectively. The supernatants of GS115 transformed with the oFSHβα fusion gene constructs contained a labelled product of estimated molecular mass approximately 28 kDa. GS115 transformed with the vector alone (i.e. GS115/pPIC9 (Mut+)) (Fig. 3A and B, lane 4) acted as the negative control. In addition to a 28 kDa band, the GS115/pPIC9 FSHβα supernatant contained additional labelled bands of >45 kDa and <17 kDa.

Receptor-binding characteristics of the oFSHαβ fusion protein

RRAs were used to compare the receptor-binding characteristics of the recombinant oFSHαβ fusion protein expressed by a range of Pichia transformants with those of native oFSH (Fig. 4). Supernatants from methanol-induced Pichia cultures were concentrated 10-fold and dialysed against binding buffer and then used to competitively displace radiolabelled native oFSH from the bovine FSH receptor. Sigmoidal displacement curves have been transformed into linear plots using the logit-log
transformation (Fig. 4). The regression lines for hormone displacement of the transformed *Pichia* strains had the following slopes: GS115/0 (i.e. untransformed GS115), \(-0.530\); GS115/pPIC9 Mut\(^s\), \(-0.342\); GS115/pPIC9 FSH\(\beta\alpha\) Mut\(^s\), \(-0.868\); GS115/pPIC9 EAFSH\(\beta\alpha\) Mut\(^s\), \(-0.861\); and GS115/pAOM\(\beta\alpha\) Mut\(^s\), \(-0.884\), while the regression line slope for native oFSH was \(-0.814\). The results of the negative control supernatants (GS115/0 and GS115/pPIC9 Mut\(^s\)) indicate non-parallelism with the reference standard (RP2) (Fig. 4). Supernatants from two strains expressing tethered oFSH\(\beta\alpha\) protein (GS115/pPIC9 FSH\(\beta\alpha\), Mut\(^s\); and GS115/pPIC9 EAFSH\(\beta\alpha\), Mut\(^s\)) competitively displaced radiolabelled oFSH in a similar manner to the reference oFSH standard (RP2) as shown by the slopes of their displacement regression lines being similar (\(-0.868\) and \(-0.861\) respectively) to that of the native oFSH standard RP2 (\(-0.814\)). Furthermore the supernatant from a previously described strain which expresses the oFSH\(\alpha\)- and \(\beta\)-chains separately (GS115/pAOM\(\alpha\)M\(\alpha\) Mut\(^s\), Fidler et al. (1998)) produced a displacement curve parallel to that of the strains expressing the tethered oFSH\(\beta\alpha\) protein.

Supernatants from the transformed *Pichia* strain SMD1168/pPIC9 FSH\(\beta\alpha\) Mut\(^s\) were also tested for linearity by RIA, bioassay and RRA with respect to the reference pituitary FSH standard USDA-oFSH-19-SIAFP-RP-2. As with the aforementioned studies, displacement curves were transformed using the log-log transformation. In the RIA, the regression lines for the aforementioned recombinant FSH\(\beta\alpha\) protein and reference FSH standard (RP-2) were \(-0.9137\) and \(-0.9269\).
respectively; these were not significantly different from one another. In the bioassay, the regression lines for the recombinant FSHα protein and RP-2 standard were $-0.7197$ and $-0.7052$, and these were not significantly different. In the RRA the regression lines for the recombinant FSHβα protein and RP-2 standard were $-0.9740$ and $-0.9032$, and these were not significantly different from one another.

Expression of oFSHβα fusion genes with altered codon usage

Using in vitro mutagenesis, three codons within the oFSHβ portion of the oFSHβα fusion gene were changed. Specifically two arginine codons, CGC, encoding Arg$_{98}$, and CGA, encoding Arg$_{96}$, were both altered to CGT and a nearby glycine codon, encoding Gly$_{99}$, was changed to GGT. The altered oFSHβα genes were cloned into pPIC9 to produce the plasmids pPIC9 cchFSHβα, with the MFα leader sequence and minimal KEX2 proteolytic cleavage site, and pPIC9 EAcchFSHβα, with the extended KEX2 site. Plasmids pPIC9 cchFSHβα and pPIC9 EAcchFSHβα were transformed into Pichia strains GS115 and SMD1168. After selection for the His$^+$ phenotype, and screening for the Mut$^+$ and Mut$^+$ phenotypes, 10–30 transformants in each group were assayed by RIA, RRA and in vitro bioassay for production of oFSHβα fusion protein following methanol induction in shake-tube cultures. Irrespective of whether the various
transfectants were assayed by RIA, RRA or bioassay, all strains secreted oFSHβα within the range 60–140 ng/ml (data not shown). No consistent differences in the level of oFSHβα production were apparent between the GS115 and SMD1168 transformants or between transformants sharing a common plasmid–host combination but differing in their Mut*/Mut* phenotypes (data not shown).

**Discussion**

The results presented here show that recombinant tethered oFSHβα fusion proteins produced by *P. pastoris* were biologically active as assessed by an *in vitro* bioassay. Moreover the results show that a variety of di-*Pichia* transformant strains secreted the oFSHβα proteins at concentrations in the range 60–140 ng/ml. The finding that the oFSHβα fusion protein was biologically active *in vitro* and bound the FSH receptor with a binding profile similar to that of native oFSH indicates that the fusion protein is able to adopt a conformation resembling that of native oFSH.

Although not required for FSH receptor binding, N-linked glycosylation influences FSH bioactivity both *in vitro* and *in vivo* (Ulloa-Aguirre et al. 1995). In the bioassay used in the present study, oFSHβα stimulated the production of cAMP, suggesting that suitable glycosylation structures have been added to the recombinant protein within the *Pichia* secretory pathway (Albanese et al. 1994). Western blotting studies, using antibodies directed against both the α- and β-portions of the oFSHβα protein, labelled a product of approximately 28 kDa. As the predicted size of the secreted 208 amino acid portion of the oFSHβα fusion protein is 23.4 kDa, the additional size detected is likely to be due to glycosylation of the fusion protein. The Western blotting results also showed that in addition to a 28 kDa product for the oFSHβα protein there were additional products of <17 kDa and >45 kDa. We suggest that the <17 kDa product is likely to be a degraded form of oFSHβα whereas the >45 kDa products may well be multimers (Ben-Menahen et al. 1999).

The present results represent a significant improvement on previous results where the oFSH α- and β-chain were expressed separately and the levels of recombinant FSH measured by bioassay were only a third of those measured by RIA (Fidler et al. 1998). However, the amounts of oFSHβα secreted by the *Pichia* transformants in this work (approximately 0.1 μg/ml) are still modest relative to what *Pichia* has been reported to achieve (Sudbery 1996). In a report from another group examining *Pichia* for the production of biologically active recombinant porcine FSH, protein values in the 1–10 μg/ml range were reported (Richard et al. 1998). In part the differences between our yields and those for porcine FSH may relate to the ‘potencies’ of the reference standards used to determine the yields. For example, in the present study, the reference preparation was the ovine standard USDA-oFSH-19-SAEP-RP-2, which is 94 times more potent than the original reference standard NIH-oFSH-S1. In the Richard et al. (1998) report, the yields were related to a porcine standard CY1844II, which is 29 times more potent than the NIH-FSH-P1 standard. From a comparison of S1 and P1 standards (Reichert & Wilhelmi 1978) using a receptor assay it can be calculated that the yields generated by Richard et al. (1998) are approximately 15-fold greater than those achieved in the present study.

To test whether higher levels of oFSHβα could be obtained we examined a number of other factors that might influence yield. To this end the oFSHβα fusion gene was attached to three different leader/signal sequences, i.e. MFα (with the ‘minimal’ KEX2 protease cleavage sequence), MFα (with the ‘extended’ KEX2 cleavage sequence) and PHO1. Directing the protein into the secretory pathway is required to ensure the protein is glycosylated, to promote correct disulphide bond formation and, obviously, to lead to secretion of the protein which can greatly assist its subsequent purification (Sudbery 1996, Hollenberg & Gellisen 1997). The selection of these leader sequences was determined largely by their being commercially available in the *Pichia* expression plasmids pPIC9 and pHILS1 (Invitrogen). Our results showed that varying these leader sequences did not significantly affect the level of oFSHβα production in any of the constructs tested.

In addition to varying the leader sequence to alter protein expression we examined the consequences of transforming all three constructs into different *Pichia* strains, namely GS115 (his4, PEP4) and SMD1168 (his4, pep4). The PEP4 gene encodes the protease A enzyme, so strain SMD1168, being protease A-deficient, may have lower levels of
proteolytic degradation of expressed recombinant proteins (Gleeson et al. 1998). However, in this work, the strain genotype did not influence the levels of oFSHβα protein in the culture supernatants. The other genotypic difference investigated was between the strains with the recombinant gene integrated by homologous recombination into the AOX1 gene (identified from the Mut<sup>a</sup> phenotype) and those strains with the recombinant gene integrated by non-homologous recombination (Mut<sup>b</sup> phenotype). In this work the Mut phenotype did not appear to influence the level of recombinant protein produced. Finally the methanol/glycerol ratios in the induction medium of the Pichia cultures were altered with no observed increase in oFSHβα protein levels above about 0·1 µg/ml (data not shown).

An unsuitable codon-usage profile in a gene is often cited as a possible constraint on recombinant protein production in a heterologous system (Batard et al. 2000). Therefore the coding sequences of the oFSH α and β genes were compared with their porcine and bovine homologues to determine if differences in codon usage could provide an explanation for constraints on production. Codons possibly unsuitable for the Pichia translation processes were determined from a codon-usage table derived from the analysis of codon usage in five highly expressed Pichia genes (Sreekrishna & Kropp 1996). Within this table are six codons not present in any of the five highly expressed genes, specifically GGG (Gly<sup>99</sup>), ATA (Ile<sup>99</sup>), CGA (Arg<sup>96</sup>), CGC (Arg<sup>98</sup>), CCG (Pro<sup>99</sup>) and TGA (stop). For the purposes of this discussion these codons will be referred to as ‘undesirable’ codons. Some of these ‘undesirable’ codons were present in the oFSHα and β mature protein-coding regions, in oFSHα, GGG (Gly<sup>98</sup>) (Bello et al. 1989) and in oFSHβ, ATA (Ile<sup>20</sup>), CGA (Arg<sup>80</sup>), CGC (Arg<sup>86</sup>) and GGG (Gly<sup>99</sup>) (Mountford et al. 1989). None of the amino acid-coding ‘undesirable’ codons was detected in the porcine FSHα mature protein-coding sequence (Hirai et al. 1989) while the porcine FSHβ sequences had two such codons, ATA (Ile<sup>19</sup>) and GGG (Gly<sup>99</sup>) (Kato 1988). Furthermore ATA and GGG codons were also present at homologous positions in the bovine FSHβ gene, which had been expressed, using Pichia, at levels as high as 4 µg/ml (Esch et al. 1986, Samaddar et al. 1997). Thus it appeared that neither the ATA nor the GGG ‘undesirable’ codons greatly constrained synthesis of the porcine and bovine FSHβ proteins by Pichia.

Thus, by a process of elimination, it appeared that, if codon usage were indeed a limitation on oFSH production, the more likely candidates are those ‘undesirable’ codons present in the oFSH sequences, but which were absent from the porcine and bovine homologues, specifically the oFSHβ codons CGC (encoding Arg<sup>86</sup>) and CGA (encoding Arg<sup>96</sup>) (Mountford et al. 1989). The nearby codon GGG (encoding Gly<sup>99</sup>) was also altered during in vitro mutagenesis because alteration of all three codons could be effected using a single oligonucleotide pair. The three codons were altered to codons encoding the same amino acids but, based on the Pichia codon-usage table, possibly more suitable for expression in Pichia. However, these gene sequence changes did not result in significant increases in oFSHβα production by the corresponding Pichia transformants. Thus, from these results, it does not appear that the codon-usage profile of the oFSH α and β genes is indeed a significant constraint on the levels of protein produced. In support of this conclusion is the observation that the human chorionic gonadotrophin (hCG) β gene-coding sequence has a large number of codons which would be ‘undesirable’ as defined by the criteria used here (i.e. one CGA (Arg) codon, eight CGC (Arg) codons and five CCG (Pro) codons) and yet the corresponding protein can be secreted by Pichia strains at levels as high as 3 µg/ml (Sen Gupta & Dighe 1999). Thus it does not appear that inappropriate codon-usage profiles in the oFSH α and/or β genes provide an explanation for the relatively modest levels of recombinant hormone production in this, and previous, work (Fidler et al. 1998).

In a previous study, Sen Gupta & Dighe (2000) reported that a Pichia-expressed fusion protein formed by joining the two subunits of hCG in the arrangement α-subunit C-terminus–β-subunit N-terminus was biologically active. Interestingly, although the levels of production of recombinant hCG by Pichia were high (12–16 mg/l), when the hCG α- and β-chains were synthesised separately, and when the same coding sequences were joined, the production of the corresponding fusion protein was reduced to a level (i.e. 50–100 µg/l) similar to the amounts reported in this work for FSHβα (Sen Gupta & Dighe 1999, 2000). In contrast, in creating an oFSH tethered protein, a modest increase in levels of production of biologically active oFSH, from around 20 ng/ml to as much
as 120 ng/ml, has been achieved (Fidler et al. 1998).

In summary, this paper reports the expression, by P. pastoris, of tethered oFSH (oFSHβα) that is biologically active in vitro. The level of production was in the range 60–140 ng/ml and a number of parameters (i.e. protein leader sequence, host strain PEP4 genotype, transformed strain Mut phenotype and codon usage) were varied to determine if a particular combination produced higher levels of oFSHβα; however, all showed a similar level of production.

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