Expression and secretion of a biologically active glycoprotein hormone, ovine follicle stimulating hormone, by *Pichia pastoris*

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ABSTRACT

The methylotrophic yeast, *Pichia pastoris*, has been used to co-express recombinant genes formed by fusion of the mating factor- α (MF α) leader and ovine follicle stimulating hormone (oFSH) α and β subunit coding sequences. *Pichia* strains carrying single copies of the two fusion genes secreted recombinant oFSH (roFSH) to concentrations of approximately 51.0 ng/ml and 17.5 ng/ml, measured by RIA or *in vitro* bioassay respectively, whereas a strain with two copies of the α and one copy of the β subunit fusion genes secreted roFSH to concentrations of 61 ng/ml (RIA) and 22 ng/ml (bioassay).

It appears that the *Pichia*-derived roFSH had about one-third the *in vitro* bioactivity of native oFSH or, alternatively, only one-third of the roFSH is bioactive. Measurements of secreted roFSH α and β subunit concentrations indicated less than 10% of α and 25–33% of β subunits were stably dimerized. The receptor binding properties of the roFSH resemble those of native oFSH. In summary this paper reports the production, by *P. pastoris*, of a heterodimeric glycoprotein hormone (roFSH) that has *in vitro* biological activity.

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INTRODUCTION

Follicle stimulating hormone (FSH) secreted by pituitary gonadotrophs promotes ovarian steroidogenesis and follicular growth (Richards 1994). Administration of exogenous FSH can be used to promote the maturation of multiple ovarian follicles (i.e. superovulation), a procedure that has found practical application in both human infertility treatment and animal breeding programmes (Ben-Cherit *et al.* 1996, De Koning *et al.* 1994). At present, the FSH used for the superovulation of domestic animals is purified from animal-derived material; however, the issues of raw material supply and potential pathogen transmission indicate a requirement for 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 three pituitary glycoprotein hormones, FSH, luteinizing hormone (LH) and thyroid stimulating hormone (TSH) (Combarnous 1992, Nagaya & Jameson 1994) – and a hormone-specific β subunit that confers receptor binding specificity (Combarnous 1992). 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 the 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 methylotrophic yeast, *Pichia pastoris*, has been developed as an expression system for high-level production of recombinant proteins (Buckholz & Gleeson 1991, Cregg *et al.* 1993). *Pichia* offers the features of (i) methanol-induced expression of heterologous genes integrated into the genome adjacent to the alcohol oxidase 1 (*AOX1*) gene promoter, (ii) growth to high cell density in inexpensive, chemically defined media, and (iii) the capacity to carry out post-translational modifications resembling those of mammalian cells (Cregg *et al.* 1993). Using either native or heterologous secretion sequences, recombinant proteins can be directed into the yeast cell secretory pathway, wherein disulphide bond formation and glycosylation can occur before secretion into the

growth media (Cregg *et al.* 1993). A wide range of biologically active recombinant proteins have now been produced in *P. pastoris*, including secreted proteins containing intramolecular disulphide bonds (Clare *et al.* 1991, Vedvick *et al.* 1991). Furthermore, co-expression of two different genes in *Pichia* has resulted in the production of biologically active protein heterodimers (Kalandadze *et al.* 1996). The success of these experiments indicated that *Pichia pastoris* might be a suitable expression system for the production of biologically active recombinant ovine FSH (roFSH). This paper reports the co-expression of ovine FSH α and β subunit cDNA sequences in *P. pastoris* to produce biologically active roFSH.

MATERIALS AND METHODS

Choice of yeast expression system

The original cloning procedure was designed with the objective of co-expressing ovine FSH α and β subunit cDNA sequences in the budding yeast, *Saccharomyces cerevisiae*, using the expression vector, pYES2 (Invitrogen, San Diego, CA, USA). To this end, fusion genes were constructed with the *S. cerevisiae* mating factor- α (MF α) leader sequence fused in-phase to sequences encoding the oFSH α and β mature proteins, forming fusion genes MF α oFSH α and MF α oFSH β , respectively, as described in detail below. Note that, with both fusion genes, the FSH subunit mature protein coding sequences were positioned immediately 3' to the two codons of the MF α leader sequence encoding the Lys-Arg dibasic motif recognized by the *kex2* protease (Julius *et al.* 1984). Attempts to produce biologically active roFSH using *S. cerevisiae* were unsuccessful (A Fidler, unpublished results), leading to investigation of the yeast, *Pichia pastoris*, as an alternative yeast expression system.

Cloning of the yeast MF α leader sequence

Nucleotide sequences encoding the yeast MF α leader sequence were amplified using forward primer 5'-CCAAGCTTATGAGATTTCTTCAATTTTAA-3' (HindIII site underlined; remaining primer sequence corresponds to nucleotides 1–22 of Kurjan & Herskowitz (1982)) and reverse primer 5'-GGGAATTCAGGCCTTTTATCCAAAGATACC-3' (EcoRI and StuI restriction sites underlined; remainder is the complement of nucleotides 255–237 of Kurjan & Herskowitz (1982)). After HindIII and EcoRI (Boehringer Mannheim, Mannheim, Germany) digestion, the amplification products were electrophoresed through 1% (w/v) low melting point agarose gels (FMC BioProducts,

Rockland, ME, USA), in-gel ligated into pUC18 using T4 DNA ligase (Boehringer Mannheim) and transformed into competent DH5 α cells. Plasmids having inserts were identified by restriction enzyme digestion and T7 Sequenase catalysed DNA sequencing (Amersham Life Science Ltd, Amersham, Bucks, UK). The resulting construct was termed pUC18MF α .

Construction of fusion genes MF α oFSH α and MF α oFSH β

First-strand cDNA was synthesized from 5 μ g 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 (Boehringer Mannheim)-catalysed PCR: FSH α subunit forward primer 5'-TTTCTGATGAGAGTTTACAATGCAGGGT-3' (nucleotides 143–172 of Bello *et al.* (1989)), reverse primer 5'-CCGAATTCAAATATTTAAGATTTGTGATAA-3' (EcoRI site underlined; remainder corresponding to nucleotides 439–418 of Bello *et al.* (1989)); FSH β subunit forward primer 5'-AGCTGCGAGCTGACCAACATCACCATCAC-3' (corresponding to nucleotides 113–141 of Mountford *et al.* (1989)), reverse primer 5'-CCGAATTCCTCTTTATTCTCTGATGTCACT-3' (EcoRI site underlined; remaining sequence the complement of nucleotides 449–430 of Mountford *et al.* (1989)). Reaction conditions were: 94 °C for 3 min, 60 °C for 3 min, 72 °C for 5 min, one cycle; 94 °C for 30 s, 62 °C for 1 min, 72 °C for 2 min, 35 cycles; 72 °C for 5 min, one cycle. The PCR amplification products were incubated with T4 DNA polymerase (Boehringer Mannheim), to remove 3'A overhangs, digested with EcoRI, and ligated with StuI/EcoRI double-digested pUC18MF α . The sequences of the resulting HindIII–EcoRI fragments, encoding fusion genes either MF α oFSH α or MF α oFSH β , were confirmed by DNA sequencing (Amersham) as being free of errors introduced by *Taq* polymerase.

Subcloning of fusion genes MF α oFSH α and MF α oFSH β into pAO815 to form plasmids pAOM α and pAOM β

As originally constructed, the MF α oFSH α and MF α oFSH β fusion genes were encoded on HindIII–EcoRI fragments (HindIII at 5' end, EcoRI at 3' end) to facilitate cloning into the *S. cerevisiae* expression vector, pYES2 (Invitrogen). To allow subcloning into the single EcoRI site of the *P. pastoris* expression vector, pAO815 (Invitrogen), EcoRI sites were required at both the

5' and 3' ends of the fusion gene sequence (Cregg *et al.* 1993). Such EcoRI sites were introduced by co-ligating the HindIII–EcoRI fragments encoding the MF α oFSH α and MF α oFSH β fusion genes into the EcoRI site of pUC18, along with linker oligomers 5'-AATTCGGTACCA-3' and 5'-AGCTTGGTA CCG-3' thereby introducing an EcoRI site into the 5' end of each fusion gene. The resulting EcoRI fragments, encoding the two fusion genes, MF α oFSH α and MF α oFSH β , were ligated into EcoRI digested, dephosphorylated pAO815, forming plasmids pAOM α and pAOM β respectively. The orientation of the inserts with respect to the *AOX1* promoter was confirmed by restriction enzyme analysis and DNA sequencing.

Construction of plasmids pAOM α M β and pAO(M α)₂M β

Plasmid pAOM β was double-digested with restriction enzymes BglII and BamHI and a 1.9-kb fragment, encoding the *AOX1* promoter, MF α oFSH β fusion gene and 3' *AOX1* transcription termination sequences, was ligated into BamHI-digested, dephosphorylated pAOM α , producing plasmid pAOM α M β . The relative orientation of the two fusion genes in pAOM α M β was determined by both restriction enzyme analysis and DNA sequencing. Plasmid pAOM α M β was digested with BglII and BamHI and an approximately 3.8-kb fragment, encoding the two fusion genes, MF α oFSH α and MF α oFSH β , with their transcription control sequences, was ligated into BamHI-digested, dephosphorylated pAOM α , producing plasmid pAO(M α)₂M β . Insert orientation was determined by restriction enzyme analysis and DNA sequencing.

GS115 transformation and screening of transformants

The *Pichia* host strain, GS115 (his4) (Invitrogen), was transformed with BglII-linearized plasmids using the lithium chloride method (Ausubel *et al.* 1994). His⁺/Mut^S transformants were isolated by selection for His⁺ auxotrophy and screening for slow growth on media containing methanol as the sole carbon source (Mut^S) (Sreekrishna & Kropp 1996). The presence of oFSH α and β subunit sequences in His⁺/Mut^S transformants was confirmed by PCR from genomic DNA templates (Linder *et al.* 1996).

Induction of recombinant protein production

Pichia culturing and induction followed 'shake tube' and 'shake flask' procedures in which cultures were

grown to high density in rich media with a glycerol carbon source and then resuspended in media containing methanol as the sole carbon source (Barr *et al.* 1992, Sreekrishna & Kropp 1996). After 2 days of induction, the culture medium was clarified by centrifugation, a protease inhibitor cocktail (Complete, Boehringer Mannheim) was added and the mixture dialysed extensively against 1 × phosphate buffer saline (PBS) at 4 °C. Samples were stored at –20 °C before analysis by radioimmunoassay and bioassay.

Radioimmunoassays

Immunoreactive FSH dimer and oFSH α were measured using RIA kits supplied by the NIH National Hormone and Pituitary Program (NIH, Bethesda, MD, USA). Samples were assayed for FSH dimer using United States Department of Agriculture (USDA)-oFSH-19-selective immunoaffinity purified (SIAFP)-I-2 for iodination, USDA-oFSH-19-SIAFP-RP-2 (94 × NIH-oFSH-S1; biological potency=2351 IU/mg) for reference preparation and National Institute of Diabetes and Digestive and Kidney Diseases-anti oFSH-1 antiserum. For the assay of oFSH α subunit, WRR-1-Alpha was used for iodination and reference preparation, and the antiserum was National Institute of Arthritis, Metabolism and Digestive Diseases-anti-oLH Alpha-1. For the assay of oFSH β subunit, oFSH β (Bioscan Continental Inc., Quebec, Canada) was used for iodination and reference preparation, and the antiserum was anti-porcine FSH β (Biogenesis Ltd, Poole, Dorset, UK). Standards were iodinated using the Chloramine T method and purified by gel chromatography (FSH α and β subunits) or ion exchange chromatography (FSH dimer) (Moore *et al.* 1997). Assays were performed by incubating 100 μ l sample or standard, 100 μ l primary antibody and 50 μ l tracer overnight at room temperature. Separation of free from bound hormone was by the second antibody method, using sheep anti-rabbit IgG, followed by 1 ml 6% polyethylene glycol 8000 (Carbowax Union Carbide Co., Danbury, CT, USA). Sensitivities of the assays for FSH α , FSH β and FSH dimer (90% of zero binding) were 0.1, 0.5 and 0.2 ng/ml respectively. Intra- and inter-assay coefficients of variation for the above assays were 9% and 18.8%, 10.9% and 15.4%, and 7.9% and 10.8% respectively.

Radioreceptor assay (RRA)

Clarified supernatants from methanol-induced 'shake flask' cultures of strains GSpAO815,

GSpAOM α M β and GSpAO(M α)₂M β were dialysed extensively against 10 mM MgCl₂, 50 mM Tris-HCl pH 7.3 before being concentrated tenfold by ultrafiltration (Amicon, Beverly, MA, USA). FSH concentrations measured by RRA were quantified using membrane fractions from bovine testes as previously reported (Moore *et al.* 1997) using USDA-¹²⁵I 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 30 µg per tube (300 µg/ml) to avoid the detection of non-specific binding interference 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 previously (Albanese *et al.* 1994). However, the assay end-point was cAMP production, as measured using RIA rather than luciferase reporter gene expression. Native oFSH (NIH-FSH-RP2) (NIH) was used as the standard.

RESULTS

Plasmid pAO815-based constructions and transformation into *Pichia* host strain GS115

Four plasmid constructs were produced: (i) pAOM α , encoding the MF α oFSH α fusion gene alone, (ii) pAOM β , encoding the MF α oFSH β fusion gene alone, (iii) pAOM α M β , encoding both the MF α oFSH α and MF α oFSH β fusion genes in tandem, and (iv) pAO(M α)₂M β , encoding two copies of the MF α oFSH α gene and a single copy of the MF α oFSH β gene (Fig. 1A). In those plasmids with multiple fusion genes, each gene is transcribed from its own *AOX1* promoter and is separated from adjacent genes by the 3' *AOX1* transcription termination sequence. As it has been found that the level of recombinant protein production is frequently positively correlated with the number of copies of the corresponding gene (Sreekrishna & Kropp 1996), plasmid pAO(M α)₂M β was constructed to mimic more closely the stoichiometry of the α / β subunit dimerization reaction in gonadotrophs, in which the α subunit is present in excess of the β subunit. Plasmids were linearized by BglII digestion and transformed into the strain GS115 (his4) (Invitrogen) and *Mut*^S/*His*⁺ transformant

strains identified. For each plasmid construct, six to ten *Mut*^S/*His*⁺ transformants were screened for secreted recombinant protein production, and transformed strains that consistently produced the greatest amount of protein were selected for further characterization. The presence of oFSH α or oFSH β cDNA sequences, or both, in the genomes of the transformed strains was confirmed by PCR using primers specific for the oFSH α and β subunit sequences (Fig. 1B,C).

Measurement of the concentrations of secreted recombinant oFSH α and β subunits in the growth media of GS115 transformants

RIA was used to determine the concentrations of secreted oFSH α and β subunits present in the growth media of methanol-induced 'shake tube' cultures of GS115 transformants (Fig. 2). No immunoreactivity to either the anti-oFSH α or the anti-oFSH β antisera was detected in the media of either untransformed GS115 (GS0) or GS115 transformed with the vector pAO815 (GSpAO815). In a representative triplicate induction, the growth media of strain GSpAOM α contained a mean $\pm$ S.E.M. of 752 $\pm$ 27 ng/ml immunoreactive oFSH α and no detectable oFSH β , whereas the reverse was found with strain GSpAOM β , with which immunoreactive oFSH β was detected in the supernatant at a mean $\pm$ S.E.M. concentration of 209 $\pm$ 15 ng/ml, with no detectable oFSH α . These findings are consistent with the presence and absence of corresponding genes as determined by PCR (Fig. 1B,C) and confirm that there is no significant cross reaction between the two recombinant α and β subunits in the RIA. Co-expression of the α and β fusion genes, in strain GSpAOM α M β , resulted in the secretion of recombinant oFSH α and β subunits into the growth media to mean $\pm$ S.E.M. concentrations of 547 $\pm$ 6 and 196 $\pm$ 9 ng/ml respectively. The growth media of strain GSpAO(M α)₂M β contained α subunit at a mean $\pm$ S.E.M. concentration of 1386 $\pm$ 174 ng/ml and oFSH β subunit at a mean $\pm$ S.E.M. concentration of 192 $\pm$ 18 ng/ml.

RIA and bioassay measurements of the concentration of secreted recombinant oFSH dimer in the growth media of GS115 transformants

As FSH is biologically active as an α / β -subunit heterodimer, both RIA and an *in vitro* bioassay were used to determine the concentration of roFSH dimer present in the growth media of the transformed *Pichia* strains. Neither FSH immunoreactivity nor bioactivity was detected either in

FIGURE 1. (A) Arrangement of fusion genes MF α FSH α and MF α FSH β in plasmids pAOM α , pAOM β , pAOM α M β and pAO(M α)₂M β . The cloning strategy is outlined in Materials and Methods. Plasmid pAOM α encodes the fusion gene MF α FSH α , which consists of the *S. cerevisiae* MF α leader sequence fused in phase to the ovine FSH α mature protein coding region, flanked by the *AOX1* promoter (5'AOX1) and transcription termination (TT) sequences. Plasmid pAOM β resembles pAOM α , except that it encodes the fusion gene MF α FSH β . Plasmid pAOM α M β encodes both fusion genes MF α FSH α and MF α FSH β , each flanked by 5'AOX1 and the TT sequences and orientated with respect to each other as shown. Plasmid pAO(M α)₂M β encodes two copies of the MF α FSH α fusion gene and a single copy of the MF α FSH β gene, with each fusion gene being flanked by the *AOX1* transcription control sequences. All pAO815-derived plasmid constructs were linearized by BglII digestion before being transformed into *Pichia* strain GS115. Transformants in which the linearized plasmids had incorporated into the *AOX1* gene by homologous recombination were identified by sequentially selecting for the *His*⁺ phenotype and then screening for the *Mut*^S phenotype. The resulting *His*⁺/*Mut*^S strains were designated GSpAOM α , GSpAOM β , GSpAOM α M β and GSpAO(M α)₂M β . (B,C) Detection of oFSH α and β subunit cDNA sequences in the genomes of transformed *Pichia* strains. Genomic DNAs from strains GS115 (host strain untransformed) (lane 1), GSpAO815 (host strain transformed with plasmid pAO815) (lane 2), GSpAOM α (lane 3), GSpAOM β (lane 4), GSpAOM α M β (lane 5) and GSpAO(M α)₂M β (lane 6) were used as templates for PCR with primers specific to the ovine FSH α (B) and ovine FSH β (C) cDNA sequences. The PCR primers were those used to amplify the oFSH α and β subunit coding regions for construction of the fusion genes and amplified products of 296 bp (FSH α) and 336 bp (FSH β). The amplification products were electrophoresed through 2% (w/v) agarose gels, ethidium bromide-stained and photographed under u.v. illumination.

the growth media of control strains, GS0 and GSpAO815, or the media of strains transformed with only one of the two fusion genes, strains GSpAOM α and GSpAOM β (Fig. 3). In contrast, the growth media of *Pichia* strains carrying at least one copy of the α and β subunit fusion genes (i.e. strains GSpAOM α M β and GSpAO(M α)₂M β) secreted roFSH dimer into their growth media: GSpAOM α M β (RIA mean $\pm$ S.E.M. concentration 50.9 ± 1.4 ng/ml; bioassay mean $\pm$ S.E.M. concentration 17.5 ± 2.7 ng/ml); GSpAO(M α)₂M β (RIA mean $\pm$ S.E.M. concentration 61.2 ± 5.6 ng/ml; bioassay mean $\pm$ S.E.M. concentration 21.8 ± 2.4 ng/ml) (Fig. 3).

The triplicate induction results indicated that duplication of the α subunit fusion gene, strain GSpAO(M α)₂M β , resulted in an increase in production of roFSH dimer when compared with strain GSpAOM α M β , which carries a single copy of the α subunit fusion gene. To confirm this, ten 'shake-tube' cultures of strains GSpAOM α M β and GSpAO(M α)₂M β were methanol-induced and the concentration of roFSH in the growth medium measured by both RIA and bioassay. The results showed a greater mean concentration of roFSH in the growth media of strain GSpAO(M α)₂M β (RIA mean $\pm$ S.E.M. concentration 63.1 ± 2.8 ng/ml; bioassay mean $\pm$ S.E.M. concentration 22.6 ± 1.8 ng/ml) compared with strain GSpAOM α M β (RIA mean $\pm$ S.E.M. concentration 45.0 ± 1.6 ng/ml; bioassay mean $\pm$ S.E.M. concentration 15.3 ± 1.2 ng/ml), with the differences from both assays being statistically significant at the 1% level.

FSH receptor binding characteristics of the secreted recombinant oFSH

RRAs were used to compare the receptor binding characteristics of the recombinant oFSH with that of native oFSH. Radiolabelled native oFSH was competitively displaced from the bovine FSH receptor by increasing amounts of unlabelled native oFSH or increasing amounts of dialysed and concentrated culture media from methanol-induced cultures of strains GSpAOM α M β and GSpAO(M α)₂M β (Fig. 4). The sigmoidal displacement curves have been transformed into linear plots using the logit-log transformation. The regression lines for hormone displacement by the culture media of strains GSpAOM α M β and GSpAO(M α)₂M β paralleled each other, with slopes of -0.876 and -0.811 respectively, and that of native oFSH standard, slope -0.811 , indicating that the binding properties of the roFSH from the two strains resemble each other and that of the native oFSH. Control RRAs using the dialysed culture media from induced

FIGURE 2. RIA measurements of the concentration of FSH α and β subunits secreted into the growth media of transformed *P. pastoris* strains. *Pichia* strains GSO, GSpAO815, GSpAOM α , GSpAOM β , GSpAOM α M β and GSpAO(M α)₂M β were grown for 2 days before heterologous gene expression was methanol-induced for a further 2 days. The cells were pelleted by centrifugation and the clarified supernatant dialysed extensively against 1 $\times$ PBS before the FSH α (■) and β (□) subunit concentrations (ng/ml) were assayed by RIA. Values shown are means of three inductions, with s.e.m. bars indicated.

cultures of negative control strain GSpAO815 indicated that non-specific interference with native oFSH binding occurred at culture media protein concentrations greater than 300 μ g/ml (30 ng/tube) (Fig. 4). Using ED₅₀ values from the RRA, the concentrations of roFSH in the tenfold concentrated culture media were calculated as 230 ng/ml for strain GSpAOM α M β and 342 ng/ml for GSpAO(M α)₂M β .

DISCUSSION

Biologically active recombinant FSH has been produced previously using transformed mammalian and insect cell lines and transgenic mice (Keene *et al.* 1989, Greenberg *et al.* 1991, Mountford *et al.* 1994, Arey *et al.* 1997, Hakola *et al.* 1997). The CHO cell-derived recombinant human FSH has been used successfully in infertility treatment (Devroey *et al.* 1993). Furthermore, it was recently reported that *Pichia*-expressed bovine FSH β subunit could be combined with native bovine α subunit to produce biologically active FSH (Samaddar *et al.* 1997). Extending these approaches, we have now reported that co-expression of

recombinant genes encoding the yeast MF α leader sequence fused to the ovine FSH α and β subunit mature protein coding regions results in the secretion of biologically active recombinant ovine FSH (roFSH).

It is of interest to note that the concentration of roFSH as measured by *in vitro* bioassay was only about one-third of that determined by RIA, indicating either that the roFSH has one-third the biopotency of native oFSH or, alternatively, that only about one-third of the immunoreactive roFSH is biologically active. The difference in the measurements of the two assays cannot be attributed to non-specific factors within the growth media interfering with the bioassay, as native oFSH standards diluted in strain GSpAO815 growth media were accurately measured using the bioassay (data not shown). It should be borne in mind that the two assays used (RIA and bioassay) differ with respect to the end-points of measurements, with the RIA requiring interaction of the roFSH with a polyclonal antisera, whereas the bioassay requires roFSH binding to a G-protein-coupled membrane receptor, followed by cAMP synthesis (Albanese *et al.* 1994). The RRA measurements gave roFSH concentration estimates between those of the RIA

FIGURE 3. RIA and bioassay measurements of the concentration of recombinant oFSH heterodimer secreted into the growth media of transformed *P. pastoris* strains. Clarified culture supernatants from methanol-induced strains GS0, GSpAO815, GSpAOMα, GSpAOMβ, GSpAOMαMβ and GSpAO(Mα)₂Mβ were dialysed against 1 × PBS and assayed for roFSH heterodimer by RIA (■) or *in vitro* bioassay (□). RIA values are the means of three inductions, the bioassay values the means of six inductions. S.E.M. bars are shown.

and the bioassay, suggesting the existence of three classes of secreted roFSH: (i) receptor-binding, biologically active, (ii) receptor-binding, biologically inactive, and (iii) non-receptor-binding. The possible lack of biological activity of two-thirds of the roFSH may result from the recombinant molecules adopting tertiary structures inappropriate for receptor binding, activation, or both. In particular, *N*-linked carbohydrate moieties significantly influence the biological activity of gonadotropins (Thotakura & Blithe 1995, Ulloa-Aguirre *et al.* 1995, Arey *et al.* 1997), as they are required for activation of intracellular signalling pathways in addition to being determinants of *in vivo* bioactivity through their influence on the metabolic clearance rate (MCR) of the hormones (Ulloa-Aguirre *et al.* 1995, Arey *et al.* 1997). As the glycosylation moieties synthesized by *P. pastoris* differ from those of mammalian cells, being of the high-mannose type characteristic of yeast (Grinna & Tschopp 1989), an important future goal will be to determine the glycosylation patterns and MCR of *Pichia*-derived roFSH. Comparison of the mean concentrations of the secreted roFSH α and β subunits with those of the roFSH heterodimer

indicates that only approximately 9% and 4% of the α subunits, 26 and 32% of the β subunits, secreted by strains GSpAOMαMβ and GSpAO(Mα)₂Mβ respectively, are present in the culture media as dimers. Thus it would appear that dimerization of the roFSH α and β subunits occurs inefficiently or the roFSH heterodimers formed are unstable, dissociating into their subunits after secretion. At the molecular level, there are a number of explanations for the low level of stable roFSH dimer formation: the 85-amino acid MFα leader sequence may sterically hinder dimerization (Kurjan & Herskowitz 1982, Julius *et al.* 1984), *N*- and *O*-linked carbohydrate moieties may block dimerization, as has been reported for the native α subunit (Blithe 1990, Thotakura & Blithe 1995), or a β subunit intramolecular disulphide bond that forms a 'seat-belt' structure believed to be important in stabilizing gonadotrophin dimers (Lapthorn *et al.* 1994) may not be formed efficiently in *Pichia* cells.

Comparison of the α and β subunit concentrations in the culture media of GS115 transformants in which the fusion genes were expressed alone, GSpAOMα and GSpAOMβ, with those of strain

FIGURE 4. Comparative displacement of ^{125}I -labelled oFSH from bovine FSH receptors by native oFSH and the dialysed culture media of transformed *Pichia* strains GSpAO815, GSpAOM α M β , and GSpAO(M α) $_2$ M β . Radio-labelled (^{125}I) oFSH (USDA-oFSH-19-SIAFP-I-2) was specifically displaced from bovine FSH receptors by increasing concentrations of oFSH standards (USDA-oFSH-19-SIAFP-RP-2) (●) and tenfold concentrated dialysed culture media from methanol-induced cultures of strains GSpAOM α M β (▼) and GSpAO(M α) $_2$ M β (▽). At supernatant concentrations of protein greater than 30 $\mu\text{g}/\text{tube}$, non-specific displacement of the ^{125}I -oFSH was observed using the culture media from negative control strain GSpAO815 (○). Concentrations of native oFSH standards (●) are Ln (ng oFSH/tube) while concentrations of culture media (GSpAO815 (○), GSpAOM α M β (▼), GSpAO(M α) $_2$ M β (▽)) are Ln (ng total protein/tube). Logit B/Bo, $\log_e (b/100 - b)$ where b is the proportion of tracer bound expressed as a percentage of that in the zero standard. Values shown are the means of duplicate assays.

GSpAOM α M β indicates that β subunit production is unaffected by co-expression of the α subunit gene, whereas the secreted α subunit concentrations are significantly reduced when co-expressed with the β subunit gene ($P < 0.001$). In contrast, α subunit production is increased by duplicating the MF α oFSH α gene, the mean concentration of α subunit secreted by strain GSpAO(M α) $_2$ M β (1386 ng/ml) being approximately double that produced by strain GSpAOM α (752 ng/ml) (a difference that is statistically significant at the 5% level). Increased α subunit secretion with doubling of gene copy number is more dramatic when comparing strains GSpAOM α M β and GSpAO(M α) $_2$ M β . The more than twofold differ-

ence in α subunit secretion between these strains may indicate that increased α subunit production overcomes the apparent inhibitory effect of β subunit co-expression. Although the fraction of α subunit secreted by strain GSpAO(M α) $_2$ M β that is dimerized with β subunit is low at 4% (compared with 9% with strain GSpAOM α M β), duplicating the MF α oFSH α gene did result in a 40–50% increase in roFSH dimer production as measured by RIA, bioassay and RRA.

As noted above, at 20–60 $\mu\text{g}/\text{l}$, the amounts of roFSH secreted by the transformed *Pichia* strains are modest when compared with the mg/l to g/l values reported for some *Pichia*-produced heterologous proteins (Sreekrishna & Kropp 1996). However, the amounts are comparable to the values of 300–400 $\mu\text{g}/\text{l}$ previously reported for recombinant heterodimer secretion by *Pichia* (Kalandadze *et al.* 1996). Although the low level of roFSH production may, in part, reflect the low efficiency of roFSH dimer formation, concentrations of secreted α and β subunit themselves are at the lower end of the range of reported recombinant protein production by *Pichia* (Sreekrishna & Kropp 1996). roFSH subunit synthesis is probably influenced by the conditions of yeast growth and induction, and by the sequences of the recombinant genes. The growth and induction procedures used in this work were standard 'shake tube' or 'shake flask' procedures, both of which produced similar concentrations of secreted recombinant proteins. However, these growth and induction conditions are by no means optimal, and the use of bioreactors frequently results in a substantial increase in heterologous protein production (Sreekrishna & Kropp 1996). Both the nucleotide composition and codon usage of heterologous genes influence their level of expression in *Pichia* (Sreekrishna & Kropp 1996). Although the A+T content of the FSH α and β subunit coding sequences, 54% and 44% respectively, is within the 30–55% range considered compatible with high level expression, both the α and β subunit coding sequences include one or more codons believed to be unfavourable for translation in *Pichia*: in the α subunit, GGG encoding Gly $_{34}$, and in the β subunit, GGG (Gly $_{99}$), ATA (Ile $_{20}$), CGC (Arg $_{88}$) and CGA (Arg $_{96}$) (Bello *et al.* 1989, Mountford *et al.* 1989, Sreekrishna & Kropp 1996). Furthermore, it is possible that heterodimer formation and stability could be enhanced using fusion gene constructs encoding secretory signals other than the MF α leader sequence. In addition, heterodimer formation and stability might be enhanced by cloning of the β and α subunit coding regions in tandem, to produce a biologically active single polypeptide, as has been reported for human FSH

expressed by mammalian cells (Sugahara *et al.* 1996a,b).

In summary, the key finding of this paper is that biologically active recombinant ovine follicle stimulating hormone has been produced by *Pichia pastoris*. To our knowledge, this is the first report of the production of a biologically active glycoprotein hormone using this particular yeast. Further studies will be needed to evaluate whether yields of *P. pastoris*-derived roFSH can be increased, and whether the roFSH is biologically active *in vivo*.

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