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Expression of bovine follicle-stimulating hormone subunits in a *Hansenula polymorpha* expression system increases the secretion and bioactivity *in vivo*

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ABSTRACT

Bovine follicle-stimulating hormone (bFSH), a pituitary gonadotropin, is a heterodimer hormone that consists of a common α-subunit non-covalently associated with the hormone-specific β-subunit. Unfortunately, expression levels of recombinant bFSH or its subunits are invariably low. We report here the secretory expression of biologically active bFSHα and bFSHβ subunit in the methylotrophic yeast *Hansenula polymorpha*. A slightly higher level of expression of recombinant bFSH subunits was achieved by using the *Sacchromyces cerevisiae*-derived calnexin (ScCne1) as a chaperone in engineered *H. polymorpha* strains. The preliminary data also suggested that bFSH subunits expressed in *H. polymorpha* appeared to be less-glycosylated. This isoform had been shown to be 80% increase in *in vivo* bioactivity compared with the hyperglycosylated *Pichia pastoris*-derived recombinant bFSHα/β. More sophisticated applications of bFSH would profit from the assembled less-glycosylated heterodimer.

Bovine follicle-stimulating hormone (bFSH)[2], which belongs to the family of glycoprotein hormones produced either in the pituitary or in the placenta, is a heterodimer composed of two non-covalently associated α- and β-subunits. There are five intrachain disulfide bridges in α-subunit and six in β-subunit, each containing two N-linked carbohydrate moieties [1]. Purified FSH has been used to improve reproductive efficiency in both human and economically important animals, but the results with the hormone or with pregnant mare serum gonadotropin, which contains intrinsic FSH and luteinizing hormone activity, have been variable. The use of recombinant bovine FSH (bFSH), which is homologous to the species in which it is applied most frequently, may improve superovulation results. Nevertheless, bFSH has been difficult to purify and to obtain in sufficient quantities from bovine pituitaries [2]. Therefore, a standardized source of recombinant bFSH would provide an attractive alternative to produce large quantities of highly purified bFSH protein or its subunits to allow their use in many fertility related applications. Recombinant bFSH or its subunit has been expressed in the milk of transgenic mice [2], in insect cells [3], in plants [4], in the milk of transgenic rabbits [5], and in *Pichia pastoris* [6,7]. However, secretory expression of recombinant bFSH is invariably low, which could be attributed to inefficient gene expression due to a hairpin structure at the 5′ end of mature FSHβ mRNA [8] and poor protein folding capacity due to the complex arrangement of disulfide bonds of FSH subunits [6]. In eukaryotes, protein folding and disulfide bond formation are well-known bottlenecks in the secreted heterologous proteins [9]. The specific folding of these proteins occurs in the endoplasmic reticulum (ER), where several proteins are involved in this process. Notable members of this group are the lectin chaperone calnexin and the Hsp70-like chaperone BiP [10,11]. Calnexin is involved in the folding and quality control of nascent glycoproteins [9]. Cne1, a homologue of calnexin in *Saccharomyces cerevisiae*, exhibits chaperone activities in a manner similar to mammalian calnexin, and participates in protein folding, assembly, and post-translational modification through an oligosaccharide moiety, $Glc_1Man_9GlcNAc_2$ [10]. Modulation of these chaperones has been successfully used to improve heterologous protein folding and secretion [12,13]. However, the effect of heterologous chaperone gene on the secretion of heterologous protein has not been investigated in *Hansenula polymorpha*.

The N-linked carbohydrate chains of natural pituitary FSH exhibit considerable variation in both size and structure [14–16]. Moreover, oligosaccharides modulated glycoprotein hormone efficacy through an influence on hormone conformation [17]. Biologically active recombinant bFSH has been expressed in different systems [2–7], which appeared to be hyperglycosylated. However, to date, there is no report about expression of less-glycosylated FSH protein or its subunits and investigation of the effect this isoform on its

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[2] *Abbreviations used*: bFSH, bovine follicle-stimulating hormone; Cne1, calnexin; ScCne1, *Saccharomyces cerevisiae* calnexin; BiP, binding protein; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; hCG, human Choriogonadotropin.

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biological activity. For the above reasons, high production at low cost and less-glycosylation are essential when choosing engineered organisms to generate and assemble the recombinant bFSH subunits. Consequently, we chose *H. polymorpha* expression system for expression of bFSH as it offers some unique advantages over other yeast expression systems. *Hansenula polymorpha* is a methylotrophic yeast that has been used as a host organism for industrial scale production of eukaryotic proteins with clinical and therapeutic values, such as hirudin, hepatitis B surface antigen, aprotinin, and human urokinase [18], because of its capability of faster growth in simple defined media and the low antigenicity of proteins produced in *H. polymorpha* [19]. In addition, the prevention of hyperglycosylation in the *H. polymorpha* system constitutes an additional advantage over other yeast expression systems such as *S. cerevisiae* and *P. pastoris* for recombinant glycosylated proteins production [20].

In this study, we report a slightly higher expression level of recombinant bFSH subunits by codon usage optimization scheme and co-expression with *S. cerevisiae*-derived calnexin (ScCne1) in *H. polymorpha*. Thus, it adds another genetic tool to the growing toolbox for efficient secretion of heterologous proteins in *H. polymorpha*. Furthermore, the preliminary data suggested that bFSHα and bFSHβ expressed in *H. polymorpha* existed as a low molecular weight isoform, which appeared to be less-glycosylated. This isoform exhibited 80% increase in *in vivo* bioactivity compared with recombinant *Pichia*-derived hyperglycosylated bFSHα/β. This information can further be exploited for the structural-based functional study of glycoprotein hormone.

Materials and methods

Strains, plasmids and reagents

Hansenula polymorpha NCYC495 (leu 1.1) was a gift from Dr. Jan A.K.W. Kiel, University of Groningen, the Netherlands, and served as the standard host for protein expression. pHMOXG-alpha-A, a G418-selectable plasmid, contains a methanol oxidase (*MOX*) promoter from *H. polymorph*a fused to the α-mating factor from *S. cerevisiae* for directing the protein to the secretory pathway. pHFMDHZ-A, a zeocin-selectable plasmid, contains a formate dehydrogenase (*FMD*) promoter from *H. polymorph*a. The two plasmids were constructed in our laboratory [21] and used for cloning and expression in *H. polymorph*a. Methanol oxidase (*MOX*) and formate dehydrogenase (*FMD*) promoters derived from key genes of the methanol utilization pathway are strong inducible promoters [19]. Strong induction does not exclusively depend on the presence of toxic methanol but can be elicited by limited addition of glycerol or glucose [19]. *Saccharomyces cerevisiae* strain AH109 was purchased from Clontech. *Escherichia coli* DH5α and BL21 (DE3) (Novagen), kept in our laboratory, were used as a cloning strain and an expression strain, respectively. Taq DNA polymerase and endonucleases were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). All other chemicals were of analytical grade and obtained from the local commercial resources.

Polyclonal antibody production

The polyclonal antisera against calnexin were obtained by injecting rabbits with GST-fused Cne1p fusion proteins expressed in *E. coli*, according to the standard immunization protocol. The chaperone ScCne1 fragment was amplified with primers F1 (5′-GAATCCATGAAGTTTTCTGCGTATTTA-3′, the EcoRI site is underlined) and R1 (5′-GCGGCCGCCTATGTAAATACTACACA-3′, the NotII site is underlined) and cloned between the EcoRI and NotI sites of pGEX-6P-1. Expression and purification of GST-fused Cne1p proteins were performed as described by Xu et al. [22].

Construction of the vectors

The native and codon-optimized genes of bFSH α- and β-subunits without their cognate signal peptide sequences were assembled from synthetic oligonucleotides by overlap-extension PCR, respectively. A 6× His-tag was added at the C-terminus of each codon-optimized gene for further purification. As shown in Supplementary Fig. S1, the PCR amplified codon-optimized products are 315 nucleotides for bFSHα and 363 nucleotides for bFSHβ. The synthetic genes bFSHα and bFSHβ were assembled, respectively, from oligonucleotides. Briefly, the oligonucleotides were synthesized on a 50 nmol scale with no purification and dissolved in water to a final concentration of 100 μM each. To assemble the oligonucleotides, PCR reactions were performed as described [23] with minor modifications. To construct the specific pHMOXG-alpha-bFSHα and pHMOXG-alpha-bFSHβ, the synthesized bFSHα and bFSHβ genes were cloned into pHMOXG-alpha-A using the restriction sites XhoI and NotI, respectively. Then, the pHMOXG-alpha-bFSHα vector was digested with BglII and BamHI, and ligated into BglII-digested pHMOXG-alpha-bFSHβ. The resulting vector contains bFSH subunits α and β, and was designated pHMOXG-alpha-bFSHα-bFSHβ. To construct the specific pHFMDZ-ScCne1, the chaperone ScCne1 fragment was amplified from genomic DNA of *S. cerevisiae* with primers F2 (5′-CCATGGTGAAGTTTTCTGCGTATTTA-3′, the NcoI site is underlined) and R2 (5′-TCTAGACTATGTAAATACTACACA-3′, the XbaI site is underlined). The resulting ScCne1 fragment was digested by appropriate restriction enzymes and subcloned into similarly digested pHFMDZ-A, yielding pHFMDZ-ScCne1.

Yeast transformation and selection of recombinant clones

To express separately the bFSHα and bFSHβ genes, *H. polymorpha* competent cells were transformed with the DraI-linearized pHMOXG-alpha-bFSHα and pHMOXG-alpha-bFSHβ by electroporation [24], respectively, and screened at 37 °C on YPD selective plates (1% (w/v) difco yeast extract, 2% (w/v) bacto-tryptone and 2% (w/v) dextrose) supplemented with 200 μg/ml G418. Similarly, to co-express the bFSHα and bFSHβ genes, *H. polymorpha* competent cells were transformed with the BglII-linearied pHMOXG-alpha-bFSHα-bFSHβ by electroporation. Selection of transformants containing a single copy of bFSHα or bFSHβ gene was carried out by PCR amplification of the promoter fragment described by Brito et al. [25]. The positive transformants were designated *H. polymorpha*/bFSHα, *H. polymorpha*/bFSHβ and *H. polymorpha*/bFSHα/β, respectively. To co-express ScCne1 and either bFSH α- or β-subunit, *H. polymorpha*/bFSHα and *H. polymorpha*/bFSHβ competent cells were transformed, respectively, with the ApaI-linearized pHFMDZ-ScCne1 by electroporation and screened at 37 °C on YPD selective plates containing 100 μg/ml zeocin. The selected co-expression recombinants were validated by PCR. The resulting positive transformants were designated *H. polymorpha*/bFSHα/ScCne1 and *H. polymorpha*/bFSHβ/ScCne1, accordingly.

Expression of recombinant bFSHα and bFSHβ in shaking flask

Pre-culture from a single colony was grown with shaking at 37 °C for 12 h in 3 ml of YPD selective medium in a test tube supplemented with 100 μg/ml zeocin. The first pre-culture was collected by centrifugation (2000*g*, 15 min) and resuspended in 30 ml of YPD medium in a 500 ml flask and inoculated at 37 °C for 24 h (to OD_{600} of 2–6). The cells were harvested and resuspended in 300 ml of YPD medium in a 2-L flask to an initial OD_{600} of approximately 1.0. For induction, methanol (100%) was