Expression of a Novel Follicle-Stimulating Hormone Glycoform Relating to Fertility

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1. Abstract

Gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), play significant roles in follicular development and maintenance of the estrous cycle. FSH specifically functions to stimulate follicle growth, estrogen synthesis and serve as a selection factor for dominant follicles, which are essential to maintain fertility. FSH exists in two glycoforms: diglycosylated FSH (DiGlycFSH) and tetraglycosylated FSH (TetGlycFSH). The DiGlycFSH contains carbohydrates on the α subunit only, while TetGlycFSH has carbohydrates on both α and β subunits. Pituitary extraction of FSH shows that in young reproductive age women, the DiGlycFSH is more abundant than TetGlycFSH, whereas post menopausal women have more pituitary TetGlycFSH. Bioassay of DiGlycFSH shows that it has greater biological activity than TetGlycFSH. Due to limited availability of DiGlycFSH, bacterial expression of recombinant human (h)FSH (rec hFSH) is needed to provide sufficient glycoform for structural and biological studies. We report our efforts towards synthesis of DiGlycFSH, which involves expression of rec hFSHβ, separation, purification from soluble and insoluble fractions, folding, and reassociation with human chorionic gonadotropin (hCGα). Protein function will be characterized by receptor binding and steroidogenesis assays.

2. Introduction

FSH is a member of the glycoprotein hormone family, which also includes thyroid stimulating hormone (TSH), luteinizing hormone (LH), and chorionic gonadotropin (CG) [1]. Of these members, FSH, LH, and CG are gonadotropins, while TSH is a metabolic hormone. Glycoprotein hormones are heterodimers produced by the anterior pituitary gland and placenta. They are composed of α and β subunits of which the α subunit is common to all glycoprotein hormones [1]. The β subunit is different for each member of the glycoprotein hormones, and it determines the function of each hormone.

FSH has four N-linked oligosaccharides, two are attached to each α and β subunits. The purposes of N-glycosylation are to facilitate folding, stabilize the heterodimer, extend the half-life of the glycoprotein in the serum, as well as activate the receptor. The α subunit glycosylation sites are conserved in all mammalian species at Asn^{56} and Asn^{82}, except in humans, in which the α subunit is glycosylated at Asn^{52} and Asn^{78} due to the absence of four N-terminal amino acid residues. $Asn^{52/56}$ oligosaccharide is important for both LH and FSH biological activity [2]. The glycans at Asn^{78} function in subunit stabilization and aid in proper folding of the α subunit [3]. Asn^{78} glycans are also involved in FSH signal transduction [4,5]. Due to N-terminal heterogeneity involving loss of residues 1 and 2, hFSHβ glycosylation sites are located at either Asn^{5} and Asn^{22} or at Asn^{7} and Asn^{24}.

Western blot analysis of FSH using monoclonal antibodies, such as hFSHβ-specific RFSH20 or hFSHβ peptide-specific PO3, reveals two gel bands, one at 21 kDa and another at 24 kDa [6,7]. Mass spectrophotometry analysis of 21kDa hFSHβ revealed two peaks one at *m/z* 12,566 and the other at *m/z* 12,361 representing the 1-111 and 3-111 residue isoform peptide masses, respectively. The 24 kDa hFSHβ preparation provided a series of ions ranging from 13,450-13,900, consistent with the presence of at least one N-glycan. Amino acid sequence of each band confirmed that 21 kDa form had no carbohydrates attached while the 24 kDa form had both carbohydrates attached. Human FSH samples collected from the pituitaries of two sets of females, one group of three 21-24 years of age and a second group of three aged 71-81 years, showed that DiGlychFSH was more abundant than TetGlychFSH in all the younger females of reproductive age while TetGlychFSH was more abundant in the older, postmenopausal females (Unpublished data). Biological activity analysis of purified DiGlychFSH and TetGlychFSH glycoforms indicates that DiGlychFSH has greater receptor binding affinity and biological activity than TetGlychFSH.

The overall hypothesis in our lab is that DiGlychFSH and TetGlychFSH are the two functionally significant hFSH glycoforms in the human pituitary. Both glycoforms vary in abundance during the menstrual cycle in women and DiGlychFSH declines in abundance at menopause. Because DiGlychFSH is cleared from circulation significantly faster than TetGlychFSH, the loss of the more rapidly cleared glycoform may

contribute to the significant postmenopausal rise in circulating hFSH.

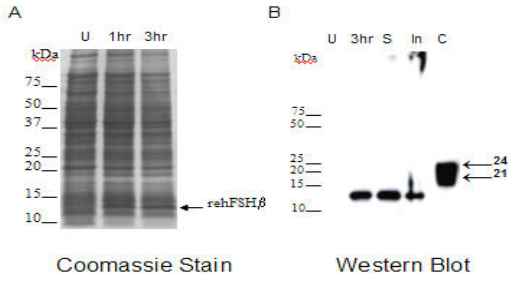
Historically, hFSH has been primarily isolated from pooled pituitary tissue and postmenopausal urine. The amount of DiGlychFSH recovered from these sources is limited because it only represents 15-20% of the total hFSH and because it is difficult to purify. Therefore, we want to develop a system in which we can synthesize sufficient DiGlychFSH for characterization in biological studies. Expressing rec DiGlychFSH using a bacterial system has several advantages. First, prokaryotes, such as *E. coli*, lack the ability to perform N-glycosylation. Second, the expression system is low in cost and mg quantities of protein can be synthesized in 3 hr, while in 48 hr, only µg quantities can be obtained from mammalian cell cultures. Moreover, bacterial expression can be scaled up more rapidly than when using a eukaryotic cell line.

2. Experimental Results and Discussion

The plasmid, pKR8, that contains the cDNA sequence for hFSHβ was amplified using PCR primers based on coding region of hFSHβ. The forward primer was 5'gaattcgatgaagacactccagtttttc3' and the reverse primer was 5'gtcgacttctttcatttcaccaaagga3'. The hFSHβ gene sequence was cloned and sequenced to confirm that no mutation in the sequence occurred during amplification
before cloning into the expression vector PET-33b(+).

Figure 1. Detection of expression of rec hFSHβ

A. SDS-PAGE gel of expressed protein. Samples collected from 5mL of bacterial cells induced with 1mM IPTG for 3 hours. The soluble fraction is



diluted to 1:5 and uninduced, inclusion bodies and total cells are diluted 1:100. The arrow indicates the expected size of rec hFSHβ. B. Western blot of rec hFSHβ. The primary antibody was anti- hFSHβ peptide monoclonal PO3. The control used was 1 µg pituitary hFSH and the arrow indicates the two different molecular weight bands that correspond to di- and tetra-glycosylated hFSH.

The resulting expression vector was then transfected into an *E. coli* strain called *Origami*. The bacterial cells were cultured in 200 mL LB at 37°C until OD_{600} reached 0.8-1 and then induced with 1 mM IPTG for 3 hours at 30°C. A small sample of the culture was used for Western blot analysis to detect rec hFSHβ using monoclonal antibody PO3 (Fig. 1). The remaining culture was centrifuged to pellet the cells. The cell pellet was incubated with 1 mg/mL lysozyme and sonicated to lyse the cells. The soluble and insoluble protein fractions were separated by centrifugation. The inclusion body was further purified by washing in 50 mM ammonium acetate, pH 5.0, containing 1% Triton-X100. Reverse phase HPLC detection of reduced and alkylated sample of inclusion body protein indicated that it achieved > 90% purity. The soluble fraction was purified by using Affi-Gel Blue affinity chromatography, which can bind the parallel Cys knot loops 1 & 3. The bound fraction was desalted by Superdex 75 gel filtration.

3. Conclusions

We have successfully expressed rec hFSHβ protein in the *E. coli Origami* strain. The inclusion body hFSHβ preparation achieved >90% purity. For soluble fraction, we are facing a challenge purifying the protein. Our next step is to fold rec hFSHβ and combine it with hCGα to form a functional hormone. We will then test the biological activity of rec hFSH hormone with receptor assays and steriodogenesis assay.

4. Acknowledgment

I would like to thank Dr. V. Butnev for showing me how to do Western blots and receptor-binding assays.

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