

EXPRESSION OF A NOVEL FOLLICLE STIMULATING HORMONE GLYCOFORM

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in biological sciences.

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DEDICATION

To my parents, my sisters and my brothers

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ABSTRACT

Heterodimeric pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), play significant roles in follicular development and maintenance of the female reproductive cycle. FSH functions in the ovary include: stimulation of follicle growth, estradiol synthesis and selection of dominant follicles. FSH exists as two glycoforms, designated di- and tetra-glycosylated FSH. Di-glycosylated FSH contains 2 carbohydrates located only on the α subunit, while tetra-glycosylated FSH has 4 carbohydrates, 2 each on the α and β subunits. Individual pituitary FSH analysis shows that in young, reproductive-age women, di-glycosylated FSH is more abundant than tetra-glycosylated FSH, whereas in postmenopausal women there is more pituitary tetra-glycosylated FSH. In vitro bioassay of di-glycosylated FSH shows that it has significantly greater biological activity than tetra-glycosylated FSH. As the availability of di-glycosylated human FSH from natural or mammalian-generated recombinant sources is limited, bacterial expression of recombinant human FSH β (rec hFSH β) was explored as an avenue to provide sufficient di-glycosylated hFSH for structural and biological function studies. We chose the *E.coli Origami* expression system to make non-glycosylated FSH β , because it lacks the ability to N-glycosylate the wild-type protein sequence. The wild-type hFSH β sequence is desired because mutations to eliminate N-glycosylation are known to reduce expression efficiency. Moreover, mutations to silence the Asn²⁴ glycosylation site, appear to affect protein folding and stability. Bacterially expressed hFSH β was extracted and separated into soluble and insoluble fractions. We obtained a relatively pure (>90%) hFSH β preparation derived from the insoluble fraction, which had to be refolded. We also recovered a small quantity of apparently partially folded hFSH β in the soluble fraction after Affi-Gel Blue purification. The attempts to

fold denatured insoluble fraction hFSH β were unsuccessful, as we could not get the folding reaction product to associate with α subunit and constitute a functional hormone. Furthermore, the soluble fraction, assumed to be folded, because it bound to Affi-Gel Blue via dye intercalation between cystine knot loops 1 and 3, also failed to associate with α subunit. Overall, we could not use a bacterial expression system to make a functional hFSH β to combine with the α subunit to make a functional intact hormone. Moreover, the electrophoretic mobility of the 12.5 kDa bacterially expressed hFSH β reflected the formula weight of the primary sequence more closely than that of the 21 kDa non-glycosylated pituitary hFSH β , suggesting unknown posttranslational modification of the 21 kDa form. However, upon further investigation, the 12.5 kDa band appeared to be a fragment, rather than a full-length subunit. This result rationalizes the failure to fold, as fragments do not fold.

TABLE OF CONTENTS

Chapter	Page
1. Introduction	01
2. Purpose of Study	10
3. Material and Methods	
<i>3.1 Construction of Expression Vectors</i>	10
<i>3.2 Expression of recombinant hFSHβ</i>	
<i>3.2.1 Small Scale Expression of rec hFSHβ</i>	13
<i>3.2.2 Western Blot analysis</i>	13
<i>3.2.3 Large Scale Expression of recombinant hFSHβ</i>	14
<i>3.3 Purification of rec hFSHβ</i>	
<i>3.3.1 Purification of insoluble rec hFSHβ fraction</i>	15
<i>3.3.2 Purification of soluble rec hFSHβ fraction</i>	16
<i>3.4 Recombinant hFSHβ folding and assembly with N⁵² dg-hCGα</i>	17
<i>3.5 FSH Dimer-Specific Radioimmunoassay</i>	17
<i>3.6 FSH Radioligand Receptor Assay</i>	18
<i>3.7 Protein sequence determination</i>	19
<i>3.8 Digestion of Di- and Tetra- glycosylated hFSH with λ Protein Phosphatase</i>	19
4. Result	
<i>4.1 Construction of Expression Vectors</i>	
<i>4.1.1 hFSHβ</i>	20
<i>4.2 Purification of rec hFSHβ</i>	

TABLE OF CONTENTS (cont.)

Chapter	Page
<i>4.2.1 Purification of soluble rec hFSHβ fraction</i>	20
<i>4.2.2 Purification of insoluble rec hFSHβ fraction</i>	21
<i>4.3 Recombinant hFSHβ folding and assembly with N⁵²dg-hCGα</i>	
<i>4.4 FSH Radioligand Receptor Assay</i>	23
<i>4.5 FSH Dimer-Specific Radioimmunoassay</i>	24
<i>4.6 Protein sequence determination</i>	24
<i>4.7 λ Protein Phosphatase Digestion of Di- and Tetra- glycosylated hFSH</i>	25
4. Discussion	25
List of References	34
Appendices	41

ABBREVIATIONS

¹²⁵ I	Iodine-125
Amp	Ampicillin
Asn (N)	Asparagine
BSA	Bovine serum albumin
CK2	Creatinin Kinase 2
COX-2	Cyclooxygenase 2
Cys	Cystine
Dg	Deglycosylated
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMBL	European Molecular Biology Laboratory
ER	Endoplasmic reticulum
formyl-Met	Formyl-methionine
FSH10	FSH dimer specific Antibody
GnRH	Gonadotropin-releasing hormone
CG	Chorionic gonadotropin
FSH	Follicle stimulating hormone
LH	Luteinizing hormone
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IPTG	Isopropyl β -D-1-thiogalactopyranoside

ABBREVIATIONS (cont.)

Kan	Kanamycine
kDa	KiloDalton
Lys	Lysine
<i>m/z</i>	mass-to-charge ratio
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization-Time of flight-mass spectrophotometry
NGF	Nerve growth factor
OD	Optical density
PBS	phosphate buffer saline
PDGF	Platelet-derived growth factor
pI	Isoelectric point
PKC	Protein kinase C
PM	Post Menopausal
PVDF	Polyvinylidene fluoride
rec	Recombinant
RIA	Radioimmunoassay
RLA	Receptor ligand assay
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC media	Super optimal broth media
Tet	Tetracycline
TFA	Trifluoroacetic acid

ABBREVIATIONS (cont.)

TGF β	Transforming growth factor β
TSH	Thyroid stimulating hormone
Tyr	Tyrosine
UV	Ultraviolet
Val	Valine
VEGF	Vascular endothelial growth factor
Vis	Visible
Wt	Wild-type
w/v	weight/volume
WBB	Western blot buffer

1. INTRODUCTION

In females, pituitary gonadotropins act on the ovary to drive follicular development to completion. In human, folliculogenesis begins during embryonic development in which the germ cells undergo mitosis to produce a finite number of oocytes (1). A single layer of squamous granulosa cells surrounds each oocyte to form a primordial follicle. These oocytes enter meiosis, but arrest in the diplotene stage of prophase I (2, 3). From birth to puberty, groups of primordial follicles simultaneously initiate growth every 2-4 weeks. The most mature of these reach preantral size but then undergo atresia (4), a process involving apoptosis beginning in granulosa cells and extending to the oocyte itself. Until puberty, none of the developing follicles reach the preovulatory follicle stage, as there is insufficient circulating FSH to support these follicles through the later stages of development (3). Follicular development from primordial to late secondary stages is known as gonadotropin-independent growth. Antrum formation, recruitment, selection of the dominant follicles and ovulation are gonadotropin-dependent steps that rely primarily on adequate FSH. As the FSH and LH pulsatile release pattern develops during puberty, growing follicles at the proper stage of development are rescued and continue to grow (5). Gonadotropin-dependent growth is characterized by proliferation of granulosa cells and increased oocyte size. FSH stimulates follicles to develop into tertiary follicles, a stage characterized by antrum formation. One or more follicles from the tertiary follicle pool are selected to become dominant follicles that will undergo ovulation. Granulosa cells in dominant follicles acquire LH receptors induced by FSH, which enable them to respond to the preovulatory rise in LH. The rest of the tertiary follicles will undergo atresia (6).

FSH and LH are released in a pulsatile fashion by the anterior pituitary gland under the episodic influence of gonadotropin-releasing hormone (GnRH)(7). Microarray studies of gene

expression in Sertoli cells showed that, compared with control, after 2 h FSH induced 224 genes; 4 h, 246 genes; 8 h, 271 genes; and 24 h, 98 genes. The number of genes repressed by FSH were as follows: 2 h, 40 genes; 4 h, 42 genes; 8 h, 66 genes, and 24 h, 41 genes (8). An *in vitro* study of FSH-regulated gene expression in pig granulosa cells showed that FSH up-regulated 25 genes (9). Among them were genes for catalytic activity (i.e. COX-2), genes for DNA binding (*HP1-BP74*), nucleic acid binding (*CCT3*), and lipid binding (*annexin V*), and metal ion binding (*matrin*), genes for signal transducer activity (*SCARB1*), genes for anti-oxidant activities (*GPX3*), and genes for structural molecules (type IV collagen). During the late luteal/early follicular phases, an increase in circulating FSH stimulates exponential growth of granulosa cells. FSH also stimulates granulosa cells to differentiate and produce steroid hormones, including de novo synthesis of progesterone and conversion of theca cell-derived androgens to estrogen via induction of the enzyme aromatase. Granulosa cells also produce other enzymes such as plasminogen activator (10, 11) and regulatory hormones, such as activin and the inhibins (12). Subsequently, a progressive decrease in FSH concentration in blood causes most of the growing follicles to undergo atresia except for those selected to become dominant follicles by LH. Ovulation is triggered by the LH surge, which increases gene expression that leads to granulosa and theca cell differentiation and follicular rupture (13).

FSH is a member of the glycoprotein hormone family, which also includes thyroid-stimulating hormone (TSH), LH, and chorionic gonadotropin (CG) (14). Of these members, FSH, LH and CG are gonadotropins, while TSH is a metabolic hormone (15). Glycoprotein hormones are heterodimers produced by the anterior pituitary gland or placenta. They are composed of an α and a β subunit of which the α subunit is common to all four hormones (16).

The β subunit is different for each glycoprotein hormone, and it determines the function of each hormone.

The glycoprotein hormone family belongs to an even larger protein family, the cystine-knot growth factor superfamily, which includes: platelet-derived growth factor (PDGF) (17), nerve growth factor (NGF) (18), and vascular endothelial growth factor (VEGF) families (19), as well as the transforming growth factor β (TGF β) superfamily (20, 21). In addition to the well-known members of the cystine knot superfamily, several other proteins such as mucins, von Willebrand factor (22), Norrie disease protein (23) and bone morphogenetic protein antagonists (gremlin, cerberus, differential screening-selected gene aberrant in neuroblastoma, and sclerostin) (24, 25) display the cystine knot motif in their structure.

The cystine-knot motif consists of three disulfide bonds. If the 6 Cys residues involved in these bonds are numbered 1-6, Cys residues 2-5 and 3-6 form the ring, while Cys residues 1-4 penetrate the ring, thereby creating the “knot”. The cystine knot defines the fold of the protein, which consists of two parallel loops (L1 and L3) above the ring and one loop (L2) below (26). Gonadotropins differ from other cystine-knot growth factors in that both subunits are glycosylated, possess a large L2 loop, and are synthesized as pre-hormones lacking the N-terminal pro domain, while most of the others are synthesized as pre-pro-hormones.

The common α -subunit has ten highly conserved cysteine residues that are all involved in disulfide bond formation (14). Two disulfide bonds, 28-82 and 32-84 form the cystine ring, while 10-60, ties the knot. The three resulting loops, α L1, α L2, and α L3, form the bulk of the α subunit structure; α L1 and α L3 are parallel hairpin loops on one side of the knot while the α L2 loop is on the opposite side of the knot. As part of the heterodimer, the α L2 loop possesses both beta sheet structure and the only alpha helical region found in either hCG or hFSH, but is

completely disordered in the isolated α subunit (27). The relatively large size of the α L2 and β L2 loops also distinguish the glycoprotein hormones from the rest of the Cys knot superfamily. The other two α subunit disulfide bonds bridge the N- and C- terminal ends of the subunit. Neither of these disulfide bonds is essential for dimer formation or for biological activity (28, 29).

While the folding pathways for hCG β , LH β , and the common α subunit have already been studied (30-35), disulfide bond formation in FSH β remains to be examined. However, since FSH β is similar to hCG β in its structure (36), it is reasonable to assume that FSH disulfide bond formation follows a pathway similar to that observed for hCG β , although perhaps speculative, because FSH β possesses fewer Pro residues than LH and CG β subunits. The FSH β subunit has 12 highly conserved Cys residues that are homologous to the 12 Cys residues in LH and CG β subunits. Based on hCG β folding, the first putative hFSH β folding intermediate may possess 2 disulfide bonds formed between Cys residues 28-82 and 32-51, which are homologous to disulfide bonds 35-89 and 37-58 present in the first folding intermediate of hCG β (30). These create a Cys knot disulfide bond cluster that will eventually rearrange to form the Cys knot ring in the mature subunit (the FSH β Cys numbering differs from that of the hCG β subunit due to the absence of 7 amino acid residues at the amino terminus of hFSH β). The second folding intermediate may possess 2 additional disulfide bonds formed between Cys residues 3-84 and 17-66. The former is assumed to complete the transient Cys knot disulfide cluster, while the latter connects loops β L1 and β L3. The third folding intermediate may possess a disulfide bond between Cys residues 87-94 that stabilizes the determinant loop, which is essential for heterodimer formation with the α subunit. Subunit association may occur immediately before, simultaneously with, or immediately after the 87-94 disulfide bond forms. The hypothesis that α

subunit acts as a chaperone for β subunit folding is partly based on the coincidence of subunit association with formation of this disulfide bond. Next, a disulfide bond may form between Cys residues 20-104, latching the seat-belt loop that probably stabilizes the heterodimer structure of FSH by embracing the L2 loop in the α subunit. The final step may be a disulfide rearrangement such that disulfide bonds Cys³-Cys⁸⁴ and Cys³²-Cys⁵¹ switch partners to make disulfide bonds Cys³²-Cys⁸⁴ and Cys³-Cys⁵¹. Once these disulfide bonds rearrange, cystine knot formation is complete. It is interesting to note that the Cys knot forms first in the rapidly folding α subunit, but last in the slow-folding β subunit (32). Slow folding on the part of hCG and LH β subunits may be a consequence of the high Pro content of these proteins. As rotation at these residues is restricted (37), their presence may impede folding. Because both FSH and TSH β subunits possess fewer Pro residues, their folding may be more rapid, more like that observed for the common α subunit. Indeed, both FSH and TSH β subunits are more sensitive to protease digestion when denatured (38, 39) than either CG or LH β subunits (unpublished data from our laboratory).

FSH is a glycoprotein hormone that classically has two N-linked oligosaccharides attached to both the α and β subunits (40). Glycosylation facilitates folding, extends the half-life of the glycoprotein in the serum, as well as activating the receptor (41-43). The α subunit glycosylation sites are conserved in all vertebrate species (44). The α subunit residues Asn⁵⁶ and Asn⁸², are glycosylated in all mammals except humans, in which the homologous residues are Asn⁵² and Asn⁷⁸ due to the absence of four N-terminal amino acid residues as compared with all other mammalian subunits (15). Asn^{52/56} oligosaccharide is important for both LH and FSH biological activity (45-47). The glycosylation site at Asn⁷⁸ functions in proper folding and α subunit stabilization (46). In this regard the glycan appears to substitute for the disulfide bond,

Cys¹⁷-Cys⁶⁶ found in the β subunits. The latter probably contributes to the more rigid structure of the hormone-specific subunit (48). The Asn⁷⁸ glycan appears to be the only one that may be directed toward the cell surface in the hormone-receptor complex (49, 50). Paradoxically, it appears to have no effect on FSH biological activity (47, 51).

Due to N-terminal heterogeneity involving residues 1 and 2, possibly representing alternative signal peptidase cleavage (52), hFSH β glycosylation sites are reported to be either at Asn⁵ and Asn²² or at Asn⁷ and Asn²⁴. FSH β oligosaccharides appear to determine the metabolic clearance rate for FSH. In a study of recombinant hFSH preparations in which both α and β subunit glycosylation sites were eliminated by mutagenesis, the clearance rates for α subunit mutants were not significantly altered (45). Single FSH β mutants were cleared 2-5 times faster than wild-type hFSH, while the double FSH β mutant was cleared 10 times faster than recombinant wild type protein (53). Conflicting results were reported for the effects of eliminating β subunit glycosylation on biological activity, with one report indicating no effect and the other showing a 215% increase in biological activity (47, 51). Studies involving isolated hFSH glycoforms revealed 10- to 25-fold increased receptor binding activity of di-glycosylated hFSH as compared with tetra-glycosylated hFSH (unpublished data from our laboratory).

A long held assumption is that FSH exists as a family of isoforms differing in their oligosaccharide structure (54, 55). Techniques used for separation of these isoforms include isoelectric focusing, chromatofocusing, and zone electrophoresis in agarose suspension (56-58), which separate FSH based on their charge differences, resulting in acidic-basic isoforms. Such studies have indicated that FSH isoform abundance differs in FSH present in serum, pituitary extracts and urine (58). Sialic acid analysis of hFSH isoforms, separated by isoelectric chromatofocusing followed by anion exchange chromatography, indicated that the pI differences

between isoforms were correlated with the sialic acid content (59). Mass spectrometry analysis of glycopeptides derived from hFSH isoforms showed significant overlap in the glycan populations derived from hFSH isoform preparations obtained by chromatofocusing (38). Therefore, the long-held assumption that FSH isoforms differ solely on the basis of glycosylation may be erroneous.

FSH analysis by Western blot using monoclonal antibodies, such as hFSH β -specific RFSH20 or hFSH β peptide-specific P03, revealed two gel bands, one at 21 kDa and another at 24 kDa (52, 60). Mass spectrometry analysis of 21 kDa hFSH β revealed two peaks, one at m/z 12,566 and the other at m/z 12,361, which corresponded closely to the residue 1-111 and 3-111 isoform peptide formula weights, respectively (52). The 24 kDa hFSH β preparation provided a series of ions ranging from 13,450-13,900, consistent with the presence of carbohydrates. Amino acid sequence analysis of each band confirmed that the 21 kDa form had no carbohydrates attached, while the 24 kDa form had both carbohydrates attached. The mass spectrometry analysis of hFSH β revealed significantly lower masses than the relative molecular weights indicated by SDS-PAGE for both hFSH β variants.

Human FSH samples collected from the pituitaries of two sets of females, one group of three individuals, 21-24 years of age, and a second group of three individuals, aged 71-81 years, suggested that the di-glycosylated hFSH was more abundant than tetra-glycosylated hFSH in all the younger females of reproductive age while tetra-glycosylated hFSH was more abundant in the older, postmenopausal females (Fig. 1). Sample sizes were small because of the difficulty of obtaining pathologic samples from suitable individuals, nevertheless, the difference was highly statistically significant. Other studies have shown that FSH extracted from urine of postmenopausal women possesses more of the more acidic isoforms than are found in

reproductive aged women (60). Therefore, our working hypothesis is that less acidic forms may represent di-glycosylated hFSH or are enriched for di-glycosylated hFSH, while more acidic forms may represent tetra-glycosylated or tetra-glycosylated-enriched hFSH. Pituitary glycoform abundance was also observed to vary in a cycle stage-specific manner (61). Analysis of purified di- and tetra-glycosylated hFSH glycoform preparations indicated that di-glycosylated hFSH had at least 10-fold greater receptor binding affinity and 88-fold greater biological activity than tetra-glycosylated hFSH (unpublished data from our laboratory). Moreover, the receptor-binding activities of combinations of both glycoforms reflected the amount of di-glycosylated hFSH present, demonstrating the functional significance of glycoform ratios.

The overall hypothesis of our lab is that di-glycosylated and tetra-glycosylated FSH are the two functionally significant hFSH glycoforms in the pituitary. Di-glycosylated FSH has oligosaccharides attached only to the α -subunit while none are attached to the β -subunit. Tetra-glycosylated FSH has oligosaccharides attached to both α and β subunits. Because di-glycosylated hFSH is significantly more active than tetra-glycosylated hFSH, the loss of the more active form of the hormone may contribute to declining fertility following age 35 (62).

Human FSH has been primarily isolated from pooled pituitary tissue or pooled postmenopausal urine. The amount of di-glycosylated hFSH recovered from these sources is limited because it only represents 15-20% of the total hFSH and because it is difficult to separate from the more abundant tetra-glycosylated hFSH. Therefore, we wanted to develop a system in which we could synthesize sufficient di-glycosylated FSH for biological studies. Several laboratories have attempted to use eukaryotic systems to make recombinant di-glycosylated FSH by mutating the β subunit glycosylation sites, Asn⁷ and Asn²⁴, and co-transfecting the mutant gene with the α gene into Chinese hamster ovary or COS-7 cells. However, genetically mutating

the coding sequence for hFSH β can change the conformation of the protein (47, 63) and significantly reduces glycoprotein hormone yield (64). Therefore, we proposed to use an alternative approach and express wild-type recombinant FSH β in a prokaryotic expression system, combine it with an α subunit preparation, and isolate the resulting heterodimer to prepare semisynthetic, di-glycosylated hFSH.

Expressing recombinant, non-glycosylated FSH β using a bacterial system has several advantages. First, prokaryotes, such as *E. coli*, lack the ability to perform N-glycosylation. Therefore, the wild-type protein sequence can be maintained, thereby eliminating the problem of potential changes in protein conformation. Second, the expression system is low in cost and can be scaled up more rapidly than when using a eukaryotic cell line. A potential pitfall using bacteria is that unless the proteins are directed to the periplasmic space, the redox potential of the cytoplasmic compartment is not favorable for disulfide bond formation owing to their rapid reduction by the combined action of thioredoxins and glutaredoxins (65). To circumvent this problem, we use a mutated *E. coli* strain called the *Origami* strain. This strain has mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, which reportedly alter the cytoplasmic redox potential in favor of oxidation, and thereby enhance disulfide-bond formation in the cytoplasm of the bacterial host (66). Studies involving hCG β suggest that it may be possible to fold bacterially expressed β subunit (67). On the other hand, only negligible folding was detected when the readily folded α subunit was expressed in bacteria (68). Progress has been made since the earlier study involving recombinant α subunit. One critical improvement is the choice of the redox buffer, which is important for *in vitro* folding (69). Studies involving recombinant hCG β folding suggest a redox buffer consisting of cysteamine and cystamine is most effective in folding hCG β , while other redox buffers such as combinations

of oxidized and reduced glutathione seem to be much less effective. The addition of the enzyme, protein disulfide isomerase, also improves the folding rate and yield of native hCG β .

2. Purpose of Study:

The purpose of this study was to produce milligram quantities of non-glycosylated hFSH β , which could be combined with human pituitary gonadotropin α subunit preparations to prepare di-glycosylated hFSH for our studies. Studying recombinant di-glycosylated hFSH will allow us to address two questions relating to this novel glycoform. First, is the absence of β subunit carbohydrate alone responsible for the high biological activity of di-glycosylated hFSH or are there additional posttranslational modifications yet to be discovered? This leads into the second question, is the FSH β protein alone sufficient to account for the difference in relative molecular weight defined by SDS-PAGE and the actual mass determined by mass spectrometry? Mouse FSH β subunit migrates as 26 and 24 kDa bands, human FSH β migrates as 24 and 21 kDa bands, while horse FSH β migrates as 21 and 18 kDa bands, yet all three subunits are composed of 111 residues with predicted molecular weights of 12.4, 12.5 and 12.3 kDa, respectively. Comparison of the mobilities of these naturally occurring glycoproteins with recombinant hFSH β will indicate if the protein sequence alone determines the anomalous electrophoretic mobility following SDS-PAGE.

3. MATERIALS AND METHODS

3.1 Construction of Expression Vectors

The plasmid, pKR8, containing the cDNA sequence for hFSH β was provided by Dr. James A. Dias (Wadsworth Center, Albany, NY) (70). We designed three pairs of forward and reverse primers that allowed us to amplify FSH β for three different expression constructs: 1) the hFSH β mature sequence along with its signal peptide and a His-tag at each end to facilitate

isolation (pre-hFSH β +his), 2) the mature hFSH β sequence and a single N-terminal His-tag (hFSH β +his), and 3) mature hFSH β lacking any modification except f-Met and a glycine amino acid at the amino terminus replacing wt residues, Asn¹-Ser² (hFSH β). The forward primers were: 5'-GAATTCgatgaagacactccagttttc-3', 5'- GCTAGCaatagctgtgagctgaccaaca-3', and 5'-CCATGGaatagctgtgagctgaccaaca-3'; and the reverse primers were 5'-GTCGACTtctttcatttcaccaaagga-3', 5'- AAGCTTtattctttcatttcaccaaaggagc-3', and 5'-AAGCTTtattctttcatttcaccaaaggagc-3', respectively. The upper case letters in the primers highlight restriction enzyme sites used to facilitate cloning. PCR was performed for 30 cycles using a denaturation temperature of 95°C, annealing temperature of 40°C, and an elongation temperature of 72°C. The annealing temperature was low to minimize nonspecific amplification. The hFSH β cDNAs were first ligated into pGEM-T Easy vector (Promega, Madison, WI). The ligation reactions were incubated at 4°C overnight. The ligation products were transformed into *E. coli* XL1-Blue. Selection of recombinant clones was based on blue and white selection. The transformation procedure was performed as follows: competent *E. coli* XL1-blue cells were incubated with ligation product for 30 min on ice. The bacteria were subjected to heat shock for 50 sec at 42°C immediately followed by 2 min incubation on ice. Finally, 900 μ L of SOC media (rescue media) were added and the bacteria incubated at 37°C for 30 min to 1 hr. A 200 μ L sample of transformed bacteria was spread onto an LB ampicillin plate that was pre-soaked with 100 μ L of 100 IPTG (100 mM) and 40 μ L of XGAL (25mg/ml). The LB plate was incubated at 37°C overnight. Several white colonies observed on the plate the next morning were isolated and grown in LB broth supplemented with ampicillin overnight at 37°C on a shaker set at 250 rpm. Plasmids were isolated to test for the presence of hFSH β gene insert in the pGEM-T Easy vector. Plasmid isolation was performed using a TENS plasmid mini prep protocol as follows: 1.5 mL

overnight culture was transferred to a microcentrifuge tube. The cells were pelleted by centrifugation at 13,000 rpm for 1 min. Most of the supernatant was removed from the microfuge tubes leaving only about 50-100 μL of liquid in each tube. The cell pellet was resuspended followed by the addition of 300 μL TENS buffer (10 mM Tris pH 8.0, 1 mM EDTA, 0.1 N NaOH, and 0.5% SDS). The cells were lysed by gently inverting the tubes several times. Next, 150 μL of 3 M NaOAc, pH 5.2, was added and immediately mixed by inverting the tube. The microcentrifuge tubes were centrifuged for 3 min at 13,000 rpm. The supernatant (~600 μL) was carefully transferred to a clean tube and 900 μL 95% ethanol were added. The tubes were centrifuged for 3-5 min at 13,000 rpm to precipitate DNA and the supernatant discarded. The DNA pellet was washed 2x with 70% ethanol, then air-dried, and resuspended in 50 μL 10 mM Tris-HCl, pH 8.0, in the presence of 20 $\mu\text{g/ml}$ RNase. DNA digestion was performed with restriction enzymes to confirm that at least one of the selected colonies contained the hFSH β gene. Digestions were performed with *Bgl*III at 37°C for 1 h. The *Bgl*III restriction enzyme site was generated by a silent mutation in the hFSH β coding sequence (71). The digestion products were electrophoresed on a 0.8% agarose gel using 0.5X TBE buffer. Agarose electrophoresis was carried out at 100 V for 1 hr and DNA stained with 0.5 mg/mL ethidium bromide.

Once we confirmed that a colony contained the pGEM-T Easy-hFSH β construct, the plasmid was then sent to the DNA sequencing facility at the University of California, Berkeley, to confirm that no mutation in the sequence had occurred during amplification before proceeding to the next step, which was cloning hFSH β into the pET-33b(+) expression vector. Once the absence of mutations in the gene sequence was confirmed, pre-hFSH+his, hFSH β +his, and hFSH β , were released from pGEM-T Easy vector by EcoRI + Sall, NheI + HindIII, and NcoI +

HindIII digestions, respectively. The same restriction enzymes were used to digest pET-33(b+) under the same conditions to make complementary ends in the plasmid. To isolate hFSH β and pET33-b(+) fragments, the digested products were loaded onto a 0.8% agarose gel and electrophorized at 100 V for 50 min in 0.5x TBE buffer. hFSH β and pET33-b(+) fragments were cut out from the gel and recovered by QiaQuick Gel Extraction kit (Qiagen) following the manufacturer's protocol. pET33-b(+) and hFSH β fragments were ligated using T₄ DNA ligase (New England Biolabs) at 4°C overnight. The ligation products were used to transform *E. coli* XL1-blue strain, and transformants were selected using LB Kan⁺Tet⁺ plate. Finally, the insertion of hFSH β fragment into the expression vector was confirmed by restriction enzyme digestion with *Bgl*III. The constructed expression vectors were then purified from the XL1-blue cultures, and used to transform *Origami* expression strain.

3.2 Expression of recombinant hFSH β

3.2.1 Small Scale Expression of rec hFSH β

A colony of *Origami* cells containing either pET-33(b+)-hFSH β , pET-33(b+)-hFSH β +his, or pET-33(b+)-pre-hFSH β +his was grown in 3 mL Kan⁺Tet⁺ LB broth overnight at 37°C on a shaker at 250 rpm. The overnight culture was added to 50 mL Kan⁺Tet⁺ LB broth at a ratio of 1:50. The culture continued to grow at 37°C on the shaker until the OD₆₀₀ reached 0.8-1.0. The culture was then induced with 1 mM IPTG for 3 hrs at 30°C with constant shaking. Once induction was completed, a 1.5 mL sample was collected and centrifuged at 13,000 RPM for 1 min. The expression of recombinant protein was detected by Western blot analysis of total cell lysate.

3.2.2 Western Blot analysis

Western blots were performed using an anti-peptide monoclonal antibody PO3, which was provided by Dr. Jean-Michel Bidart (Institute Gustav-Roussy, Paris, France). The antibody was raised against a synthetic peptide representing residues 28-50 of the mature sequence (50). The peptide sequence was: CAGYCYTRDLVYKDPARPKIQKT.

Western blotting was performed using Millipore Corp (Bedford, MA) Immobilon-P polyvinylidene difluoride (PVDF) membranes. Proteins were transferred to the PVDF membranes using a Mini-PROTEAN II apparatus for 2 h at 100 V at 4°C. The transfer buffer was composed of 25 mM Tris-HCl, pH 7.4, 190 mM glycine, and 20% methanol. After transfer was completed, the PVDF membrane was washed 3 times with water for 5 min each, then air-dried overnight. The dry PVDF membrane was incubated for 1 h with FSH monoclonal antibody PO3 (diluted 1:1000) in 5% (w/v) nonfat dry milk suspended in Western blotting buffer (WBB). WBB was composed of 150 mM sodium chloride, 1 mM EDTA, 50 mM Tris-HCl, and 0.05% Tween 20. The membrane was then washed 3 times for 5 min each with WBB. The washed membrane was incubated for 30 min with 1:1000 anti-mouse HRP-linked secondary antibody in WBB containing 5% nonfat dry milk. After incubation, the membrane was washed once with WBB and twice with Milli-Q water. FSH β bands were detected by treating the membrane with ECL Plus chemiluminescence reagent (GE Healthcare, Piscataway, NJ) for 5 min. Immunoreactive bands were detected with a Versa Doc 4000 (BioRad Hercules, CA).

3.2.3 Large Scale Expression of recombinant hFSH β

Although we prepared three expression vector constructs, only the pET-33b(+)*hFSH β* construct that encoded the mature *hFSH β* sequence was used for large scale expression experiments. The conditions for large scale induction were similar to small scale analysis in which an overnight culture was transferred to 200 mL Kan⁺Tet⁺ LB broth at a ratio of 1:50 and

incubated for approximately 3.5 hr at 37°C until the OD₆₀₀ reached 0.8-1. The culture was then induced with 1 mM IPTG for 3 hours at 30°C. Following induction, the culture was transferred to a 250 mL flat-bottomed centrifuge bottle and centrifuged at 8,000 RPM for 10 min to pellet the cells. The cell pellet was resuspended in 10 mL PBS, pH 7.4, and centrifuged for 10 min at 3,200 rpm. The supernatant was decanted and the cell pellet resuspended in 10 mL BugBuster reagent (Novagen, San Diego, CA) and incubated for 20 min at room temperature with slow shaking. The lysed cells were centrifuged at 17,000 x g for 10 min at 4°C. The supernatant contained soluble recombinant hFSHβ. The inclusion bodies in the pellet were then subjected to further purification (67). First, the pellet was homogenized twice in 14 mL of 50 mM ammonium acetate, pH 5.0, containing 1 mM EDTA, and 1% Triton-X 100 in a Wheaton homogenizer and recovered by centrifugation at 17,000 x g for 10 min at 4°C. Second, the pellet was homogenized once in 14 mL 16 mM Tris-HCl, pH 8.0, containing 4 mM EDTA and 2% deoxycholate. The suspended pellet was placed on a shaker for 15 min at room temperature and then centrifuged at 17,000 rpm at 22°C for 10 min. Finally, the pellet was washed with 14 mL 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA and centrifuged at 33,000 rpm at 4°C for 10 min. The resulting pellet was stored at -80°C.

3.3 Purification of rec hFSHβ

3.3.1 Purification of insoluble rec hFSHβ fraction

The hFSHβ in the inclusion body fraction was subjected to reverse-phase HPLC. The inclusion body pellet was solubilized in urea under reducing conditions. Accordingly, the pellet was homogenized in 1.5 mL, argon-sparged, 8 M urea in 50 mM Tris-HCl (pH 8.7). Freshly prepared 50 mM dithiothreitol (DTT) was added to the solution. The reaction tube was blanketed with argon and incubated for 1 hr at 37°C. The solution was then centrifuged at 17,000 x g for 10

min at 22°C. The supernatant was applied directly to a 4.6 mm X 30 cm Vydac (Hesperia, CA) C4, 300 Å pore, reverse-phase HPLC column that was equilibrated with 10% Solvent A (water containing 0.1% TFA) and 90% Solvent B (80% acetonitrile/water containing 0.1% TFA). The flow rate was 0.5 ml/min and UV absorbance was monitored at 210 nm. The HPLC system was a Waters (Milford, MA) model 625 quaternary gradient HPLC pump and Waters model 486 UV/Vis programmable absorbance monitor, which were controlled by the Waters Empower control/data acquisition software.

3.3.2 Purification of soluble rec hFSH β fraction

Purification of soluble recombinant hFSH β was accomplished using Affi-Gel Blue affinity chromatography (BioRad, Hercules, CA). Two ml of Affi-Gel Blue bead suspension were added to a 20 mL disposable column. The column was first equilibrated with washing buffer (0.1 M NaPO₄, 0.15 M NaCl, pH 8.6). The column was connected to a peristaltic pump, which removed buffer from the bottom of the column and directed it to the flow cell of a GE Healthcare (Piscataway, NJ) Uvicord S detector. The soluble recombinant hFSH β recovered in BugBuster® Protein Extraction Reagent was applied to the column and washed with starting buffer to remove solubilization buffer components and unbound protein. The bound fraction was eluted with elution buffer composed of 0.1 M NaPO₄, 0.4 M KCl, pH 8.5, collected in an Amicon (Millipore, Billerica, MA) Ultra-15 (10,000 MW cutoff) and concentrated to a final volume of 100-200 μ l by centrifugation for 20 min at 3500 rpm in the HL-6000 rotor of a Sorvall (Thermo Fisher Scientific, Waltham, MA) RC-3B centrifuge at 4°C. The concentrated bound fraction was then applied to a 10 x 300 mm Superdex 75 (GE Healthcare, Piscataway, NJ) high performance gel filtration column. The chromatogram was developed with 0.2 M ammonium bicarbonate containing 20% acetonitrile at a flow rate of 0.4 ml per min for 75 minutes. Fractions

were collected by hand based on the absorbance at 210 nm and protein recovered by evaporation in a ThermoSavant (Waltham, MA) SpeedVac.

3.4 Recombinant hFSH β folding and assembly with N⁵²dg-hCG α

Reagents for folding and assembly of rec hFSH β were prepared in argon-saturated Milli-Q water. Recombinant hFSH β was diluted to 1.28 μ M (0.02 mg/mL) in 50 mM Tris-Cl, pH 8.7, 1 mM EDTA, containing 0-2 M urea. Folding was initiated by the addition of 6.4 mM cysteamine and 3.6 mM cystamine that had been prepared as 10-fold concentrated stocks in 10 mM HCl immediately before use. Reactions were incubated for 4 h at room temperature and folding was quenched by the addition of 100 mM iodoacetamide in 20 mM sodium phosphate buffer, pH 8.5. Folded, recombinant hFSH β was separated from unfolded recombinant hFSH β by gel filtration chromatography using a Superdex 75 column as described above.

Conditions for associating recombinant hFSH β with hCG α were similar to folding conditions. Recombinant hFSH β was diluted to 1.28 μ M (0.02 mg/mL) in 50 mM Tris-HCl, pH 8.7, containing 1 mM EDTA. N⁵²dg-hCG α was diluted to 3.84 μ M (0.06 mg/mL), a 3-fold greater concentration than that of recombinant hFSH β . Accordingly, N⁵²dg-hCG α was added to the reaction tube before adding recombinant hFSH β (69). Cysteamine (6.4 mM) and cystamine (3.6 mM) were used to initiate the assembly reaction. These were prepared as 10-fold concentrated stocks in 10 mM HCl immediately before use. When incubation was finished, the tubes were pooled and concentrated in an Amicon Ultra-4 to reduce the volume to less than 200 μ L. All of the concentrated subunit association mixture was subjected to Superdex 75 gel filtration chromatography as described above.

3.5 FSH Dimer-Specific Radioimmunoassay

Fractions recovered from Superdex-75 gel filtration chromatography were analyzed by radioimmunoassay to determine the extent of assembly of recombinant hFSH β with hCG α . To generate a standard hormone curve, a 10 μ g sample of tetra-glycosylated hFSH was dissolved in RIA buffer (0.05 M Phosphate buffer, pH 7.5, 7.7 mM sodium azide and 0.5% (w/v) bovine serum albumin) and serially diluted to the following concentrations with RIA buffer: 1000, 464, 215, 100, 46.4, 21.5, 10, 4.64 and 2.15 ng in 0.1 mL. The assay was carried out in duplicate 12 X 75 mm polypropylene tubes. In each tube, add 0.1 mL containing either tetra-glycosylated hFSH or recombinant hFSH β /hCG α hybrid, 0.1 mL containing 30,000 cpm 125 I-tetraglycosylated hFSH, and 0.1 mL FSH heterodimer-specific monoclonal antibody FSH-10 (diluted 1:1000) (72). The reaction tubes were mixed by vortexing and incubated at 4°C overnight. The following day, 0.4 mL decomplexed fetal calf serum (diluted 1:3 in RIA buffer) were added to each tube followed by 1 mL 20% polyethylene glycol 8000 suspended in RIA buffer. Each assay tube was mixed by vortexing for 30 sec, and then centrifuged for 50 min at 3,500 rpm at 4°C. Radioactivity remaining in each tube was measured using a Packard Instruments (Meriden, CT) Cobra II gamma counter. Data were corrected for non-specific binding, which was determined by adding excess cold FSH. The specific binding activity was defined as the ratio of total binding minus the non-specific binding to the total counts added to the assay. Competitive dose-response curves were plotted using KaleidaGraph (Synergy Software, Reading, PA)

3.6 FSH Radioligand Receptor Assay

Radioligand receptor assay was performed using rat testicular tissue and 125 I-hFSH tracer. Tetra-glycosylated hFSH was used as the reference preparation. A 10 μ g sample of tetra-glycosylated hFSH was dissolved in 1 mL of RIA buffer (0.1 M Tris-HCl, pH 7.4, 0.02% sodium azide, and 0.1% BSA), then diluted to the following series of concentrations in RIA

buffer: 1000, 464, 215, 100, 46.4, 21.5, 10, 4.64 and 2.15, 1.0, 0.465, 0.215, and 0.1 ng in 0.1 mL. Recombinant hFSH β /N⁵²dg-hCG α was dissolved in RLA buffer and serially diluted, 1:10, 1:100, and 1:1000. The tracer, ¹²⁵I-tetra-glycosylated hFSH, was prepared according to the procedure of Hunter and Greenwood (73) and diluted to a final concentration of 2.5 ng/mL. Rat testes were decapsulated, weighed, and homogenized in glass homogenizer with 3 volumes of RLA buffer using 20 strokes of the tight pestle. The homogenate was then filtered through eight layers of cheesecloth and diluted to a final concentration of 125 mg/mL. In each 12 X 75 mm polypropylene assay tube, 100 μ L RLA buffer, 100 μ L unlabeled hormone, 100 μ L ¹²⁵I-tetraglycosylated hFSH, and 200 μ L rat testes homogenate were added sequentially. Tubes were mixed by vortexing and incubated at 37°C for 2 hours with gentle shaking in a water bath. Receptor-bound tracer was separated from unbound tracer by centrifugation at 3,500 RPM in a Sorvall RC-3 centrifuge for 20 minutes at 4°C. The supernatant was aspirated and the tubes were counted in the Cobra II gamma counter. Data were corrected for non-specific binding, which was determined by adding 2000-fold excess cold FSH. The specific binding activity was defined as the percent of total binding minus the non-specific binding to the total counts that were added to the reaction. Competitive dose-response curves were compiled using KaleidaGraph (Synergy Software, Reading, PA).

3.7 Protein sequence determination

Samples of rec hFSH β were sent to Dr. Elliott Bedows, University of Nebraska Medical Center, Omaha, NE, who arranged for analysis by automated Edman degradation in the core facility at that institution.

3.8 Digestion of Di- and Tetra- glycosylated hFSH with λ Protein Phosphatase

Samples consisting of 50 ng/ μ L di- and tetra-glycosylated hFSH were incubated with λ protein phosphatase (New England BioLabs, Ipswich, MA) at 30°C for 2 hrs. The reaction was inactivated with 50 mM EDTA and incubated at 65°C for 1 hr. The samples were dried in a SpeedVac and analyzed by Western blotting.

4. RESULT

4.1 Construction of Expression Vectors

The hFSH β gene was amplified from the plasmid, pKR8 (70). Primers were designed such that they amplified hFSH β coding region to construct pre-hFSH β +his, hFSH β +his, or hFSH β only.

A putative mature recombinant hFSH β band was observed between the 10 and 15 kDa MW markers in the Coomassie-stained SDS gel after 1 and 3 hr induction (Fig 3A). Western blot analysis of recombinant hFSH β from the 3 hr induced total cell lysate, as well as the soluble and insoluble fractions derived from a 3-hr, 45-ml culture revealed a 12.6 kDa band. In the soluble fraction, faint 24 and 60 kDa bands were detected. As aggregated immunoactivity was observed at the tops of the lanes for the large-scale expression samples, we assumed the faint bands also represented aggregated protein. Sample loads for Fig. 3 had been adjusted to provide the same amounts of hFSH β immunoactivity. Total cell lysate and the insoluble fraction samples were diluted 1:100, while the soluble fraction sample was only diluted 1:5. Thus, the majority of recombinant hFSH β expressed by bacteria was recovered from the insoluble fraction. Comparing the recombinant hFSH β preparations with pituitary hFSH β clearly indicated the size differences between these two preparations. Two pituitary hFSH β bands migrated at the expected relative molecular weights of 21 and 24 kDa while recombinant hFSH β migrated as a single 12.6 kDa band.

4.2.1 Purification of insoluble rec hFSH β fraction

We first attempted to purify hFSH β from solubilized inclusion bodies by reverse-phase HPLC (Fig. 6A). We used a C4 reverse-phase column to purify recombinant hFSH β , under the assumption that at pH 3, intermolecular disulfide bond formation would be impeded, as was reported during folding studies of conotoxins (74), a family of cystine knot peptides with a different topology from the Cys knot growth factor superfamily (75). The chromatogram was divided into 15 fractions, and samples subjected to analysis by SDS-PAGE and Western blotting. Despite the low pH of the mobile phase, solubilized hFSH β reduced Cys residues apparently formed intermolecular disulfide bonds leading to the many different peaks observed in the chromatogram. This was confirmed by Western blot results showing FSH β immunoreactivity associated with molecular weight bands at the top, bottom and in between.

Following reduction and alkylation, reverse-phase HPLC of insoluble fraction proteins produced a chromatogram dominated by one major peak and several minor ones (Fig. 6B). Six column fractions were collected and analyzed by SDS-PAGE and Western blotting. SDS-PAGE indicated that all six fractions contained a 13 kDa band that corresponded to the fastest migrating component in the insoluble fraction sample (Fig. 6B, top inset). Western blotting with hFSH β -specific monoclonal antibody (PO3) confirmed that the major component in each fraction was hFSH β (Fig. 6B, bottom inset). Aggregated hFSH β was detected at the top of the Western blot and a 21 kDa band was noted in fraction B (Fig. 6B, lower inset, lane 3) and 24 kDa was observed in fractions D and E (Fig. 6B, lower inset, lanes 5 & 6).

Because hFSH β appeared to be the only protein present in the insoluble fraction, we attempted to fold it into its native structure and associate the folded protein with hCG α . Freshly expressed rec hFSH β was separated into soluble and insoluble fractions, and the insoluble

fraction pellet was washed as described in Materials and Methods. The washed pellet was solubilized under reducing conditions. Folding was carried out in a cysteamine/cystamine redox buffer in the presence of urea at room temperature for 3 hours. Assembly of the putative folded hFSH β with N⁵²dg-hCG α was carried out in the same cysteamine/cystamine redox buffer lacking urea. The combined subunits were incubated for 3 hours at room temperature. Once incubation was completed, the reaction mixtures containing each subunit alone or combined subunits were subjected to Superdex 75 gel filtration chromatography. Fig. 7 shows three chromatograms aligned with one another. The only peaks associated with the reduced and alkylated recombinant hFSH β preparation, were eluted around 47 minutes, where salts emerge. No peak emerged at or around 31.6 min, where 21 kDa hFSH β is known to elute. N⁵²dg-hCG α was eluted at 31 minutes, and this peak was preceded by a small peak with a 29-minute retention time. Products of the association of recombinant hFSH β with N⁵²dg-hCG α consisted largely of free α subunit emerging in a peak at 31.6 min. A peak at 27 min, which represented either a potential hFSH dimer fraction or else the 29-min component associated with N⁵²dg-hCG α .

4.2.2 Purification of soluble rec hFSH β fraction

Recombinant hFSH β was purified from the soluble fraction by Affi-Gel Blue affinity chromatography. We assumed that since the cytoplasm of the *Origami* strain has a higher oxidizing potential than wild-type *E. coli*, recombinant hFSH β in the soluble fraction was at least partially folded. Because the first 4 disulfide bonds likely to form in FSH β stabilize the Cys knot configuration, the parallel loops β L1 and β L3 were likely to be organized, thereby enabling dye intercalation. Accordingly, we used Affi-Gel Blue chromatography to purify hFSH β from the soluble fraction. Fig. 4 shows a typical chromatogram. Most of the soluble protein failed to bind the resin as indicated in Fig 5A lane 2 and Fig 5B lane 1. Bound proteins eluted with elution

buffer were readily detected. After concentration by ultrafiltration, aliquots of recombinant hFSH β were fractionated by Superdex 75 gel filtration chromatography (Fig. 5). The first aliquot produced four column fractions, which were dried in a SpeedVac prior to SDS-PAGE analysis (Fig. 5A). Fractions I-V were recovered from the column, but only fractions II-V were characterized. The Commassie-stained SDS-PAGE gel indicated that fraction V possessed a single major band, but its apparent molecular weight was less than expected (Fig. 5A, inset). Fractions II, III, and IV had much less protein than fraction V, and no ~12 kDa rec hFSH β band was detectable in any of these fractions. Subsequent aliquots produced chromatograms like that shown in Fig. 5B. The largest fraction now emerged at 19 min (Fraction I). As we did not analyze this fraction, we do not know if the same protein found in fraction V in panel A was now present in this fraction. The fraction V peak was not as large as its counterpart in panel A. Samples of the second set of column fractions were subjected to Western blot analysis (Fig. 5B, inset). The blot showed that hFSH β immunoreactivity was detected in column fractions II, III, and IV as indicated by an immunoreactive bands with relative molecular weights of 12.5, 30, and 60 kDa. Fraction V on SDS-PAGE showed a major band at ~ M_r 10 kDa, but in a Western blot only a faint 21 kDa band was observed. The flow through fraction possessed a major 21 kDa band as well as the 30 kDa band observed in fractions II-IV.

4.4 FSH Radioligand Receptor Assay

Protein recovered from the Superdex 75 column 27 min peak (Fig. 7) was used to examine the receptor-binding activity of rec hFSH. Due to limited availability of recombinant FSH, a complete dose response curve could not be generated. Recombinant hFSH showed progressive displacement of tracer from FSH receptors with increasing concentration, however, the difference in slope between the inhibition curves suggested chemical non-equivalence.

4.5 FSH Dimer-Specific Radioimmunoassay

To confirm the receptor-binding activity of rec hFSH, pituitary tetra-glycosylated hFSH and recombinant, di-glycosylated hFSH were compared in a radioimmunoassay. The monoclonal antibody employed in the assay (FSH-10) was reportedly specific for the heterodimeric form of FSH (76). The slope of dose-dependent curve of rec hFSH was very shallow as compared with that for pituitary tetra-glycosylated hFSH (Fig 9A). This indicates that the two molecules were only partially related immunologically. Another RIA was carried out to determine if the immunological activity in the putative recombinant hFSH preparation was due to the N⁵²dg-hCG α (Fig 9B). The slope of the N⁵²dg-hCG α preparation dose-response curve was shallow, like that observed for the putative recombinant hFSH preparation.

4.6 Protein sequence determination

Since recombinant hFSH β failed to associate with the α subunit, we speculated that a protease cleaved the recombinant hFSH β while in the bacterial cytoplasm. This would explain the failure of conditions reported to facilitate hCG β folding to support hFSH β folding, as protein fragments seldom fold properly. When we compared rec hFSH β with a sample of degraded urinary hFSH in a Western blot, the immunoreactive bands for both subunits displayed identical electrophoretic mobilities. Moreover, in the recombinant hFSH β lane, there was some immunological activity migrating near the position of intact pituitary hFSH β . The P03 antibody was raised against a peptide corresponding to FSH β residues 28-50. A protease-sensitive site exists in FSH β near residues Tyr³⁸-Lys³⁹ (77), making it possible that we were detecting either the N- or C-terminal fragment. Automated Edman degradation of hFSH β derived from the inclusion body fraction yielded a very low level sequence consistent with the recombinant

hFSH β amino terminus. It was believed that the low yield resulted from recombinant hFSH β retaining most of the N-terminal formyl-Met, which does not react with the Edman reagent. When we tried to confirm the fragment nature of recombinant hFSH β using mass spectrometry, neither the hFSH β preparation nor tryptic peptides derived from it were detectable.

4.7 λ Protein Phosphatase Digestion of Di- and Tetra- glycosylated hFSH

Because we initially assumed the PO3 epitope was in the C-terminal half of the subunit, the N-terminal sequence suggested another post-translational modification was responsible for the retarded migration of non-glycosylated pituitary hFSH β . As phosphorylation is a common modification that can retard electrophoretic mobility, we examined the sequence for potential phosphorylation sites. An EMBL database search for phosphorylation sites on FSH β revealed three potential PKC phosphorylation sites and two CK2 phosphorylation sites. We treated samples of di- and tetra-glycosylated hFSH with λ protein phosphatase. However, this treatment did not affect the migration rate of either hFSH β subunit variant (Fig. 11). Thus, phosphorylation does not appear to be responsible for the different migration pattern between pituitary hFSH β and rec hFSH β . The most likely explanation remained proteolysis.

5. DISCUSSION

The goal of this project was to express milligram quantities of non-glycosylated hFSH β that could be combined with pituitary human α subunit preparations to prepare large amounts of semi-synthetic di-glycosylated hFSH. This highly active glycoform is extremely interesting because it is more abundant than tetra-glycosylated hFSH in pituitaries of women in their twenties, much lower in abundance in pituitaries from postmenopausal women, and intermediate and highly variable in abundance in perimenopausal women (61). The high receptor-binding

activity of di-glycosylated hFSH as compared with tetra-glycosylated hFSH has been attributed to the absence of β subunit N-glycans in the former. However, the anomalous electrophoretic mobility of non-glycosylated hFSH β that yields a relative molecular weight of 21,000 for a protein with a formula weight of 12,500, suggests other post-translational modifications are possible. Any such modifications must be low in mass, as monoisotopic masses predicted for the full-length and truncated non-glycosylated hFSH β isoforms were only 81.4 and 77.0 mass units, respectively, less than the m/z values determined by MALDI-TOF-MS (52). Moreover, the small differences between theoretical and actual masses were within experimental error for the instrument employed in the earlier study.

Our primary goal was to express sufficient quantities of di-glycosylated hFSH for structure-function studies. The results of our studies allowed us to address two questions. First, was the absence of β subunit carbohydrate responsible for the high biological activity of diglycosylated hFSH? Second, was the FSH β protein alone sufficient to account for the difference in relative molecular weight defined by SDS-PAGE and the actual mass determined by mass spectrometry?

Three expression vectors containing various hFSH β constructs were prepared in the course of these studies. Initially, we designed primers that allowed us to clone pre-hFSH β cDNA into the expression vector to generate His-tags at the N- terminal and C-terminal ends of the recombinant protein, which also included the signal peptide. The His-tags were added to provide an affinity tag for rapid enrichment of both the folded and unfolded forms of the protein enabling isolation of either form. Expression of this cDNA construct yielded a 20.8 kDa band following Western blot analysis. However, the protein did not appear to bind to a nickel affinity column for reasons we do not understand. If the His-tag had been located at the mature N-terminus, then one

could rationalize that access to the His-tag might be blocked by the immediately adjacent hFSH β , however, the signal peptide provided a linker that should have made the His-tag readily accessible. At the same time, an alternative affinity purification procedure was provided by Dr. Bedows. The topology-directed ligand affinity chromatography method had the advantage of selecting folded β subunit. Accordingly, we eliminated the His-tag and expressed the pre-hFSH β alone. After the initial small-scale expression experiment, we did not perform any further experiments with the pre-hFSH β construct because Dr. Bedows later advised us, based on ongoing work in his laboratory, that the signal peptide appeared to interfere with protein folding *in vitro* by directing unfolded preprotein to the inclusion body. Although the signal peptide is important in eukaryotes for directing secreted preprotein precursors to the endoplasmic reticulum for proper folding (78), glycosylation, and association with the α subunit; in prokaryotes, different signal peptides direct proteins to the periplasmic space, which performs some of the analogous functions (signal peptide cleavage, folding, and disulfide bond formation, but not N-glycosylation) for bacterial proteins (79). Therefore, we designed another set of primers to amplify the mature hFSH β cDNA lacking the signal peptide and proceeded with large-scale expression studies using vectors expressing this form of the subunit.

Previous studies indicated that CHO cell expression of β mutants lacking both N-linked glycans was inefficient, apparently reflecting inability to secrete into the media (80). The carbohydrates residues on α subunit dictate the secretion of the hormone (53, 80). Biological activities of rec hFSH N-glycosylation deletion mutants yielded conflicting results in two reports (47, 53). Both studies substituted the codon for asparagine (Asn) with the codon for glutamine (Gln) and found that they were equivalent *in vitro* bioactivity of rechFSH mutants expressing α 78/ β rechFSH, α /7 rechFSH, or α /24 rechFSH. Mutant cell lines expressing α 52/ β rec hFSH

showed loss in bioactivity that was 50% or more comparing to the wild-type. However, in the study by Bishop *et al*, the removal of both carbohydrate residues from the β subunit resulted in significantly increased bioactivity (2.16 vs. 1); while Flack *et. al.*, reported that these mutants have lower bioactivity than wild-type (0.71 ± 0.22 vs 1). Neither report quantified the yield of rec hFSH made by CHO cells (47, 51).

Despite our effort to minimize the expression of rec hFSH β in the inclusion body by using a genetically engineered *E. coli* strain, the majority of expressed protein was recovered from inclusion bodies. This result was not surprising based on results reported for the folding pathway of hCG β (30, 31, 69) In order for hCG β to form biologically active conformation, the disulfide bonds between cysteines 23 and 72 and between 9 and 90 must form first. The formation of these two disulfide bonds is the rate-limiting event in the folding pathway of hCG β *in vitro*. If hFSH β folding is similar to that of hCG β , then the disulfide bonds between cysteines 3 and 84 and between 87 and 94 must form first. It may be the oxidative environment in *E. coli* cytoplasm was not sufficient to support the disulfide bond formation.

Association of recombinant, non-glycosylated hFSH β with α subunit *in vitro* has not been studied, but recombinant, non-glycosylated hCG β was readily associated with α subunit (81). Using non-glycosylated hCG β expressed in CHO cells as a model, we attempted to replicate the same effect on hFSH β folding by performing the reaction in the presence of α subunit *in vitro*. In this case, we used a partially deglycosylated hCG α (dgAsn⁵²hCG α) preparation in folding and assembly reactions since the absence of carbohydrate at this site facilitates heterodimer formation (46, 82-84).

Several redox conditions that facilitate disulfide bond formation have been studied, such as, low concentrations of reducing agents or mixtures of oxidized and reduced glutathione or

cysteamine and cystamine. Addition of protein disulfide isomerase (PDI) to these buffers is known to assist in disulfide formation (85-87). Since there are six intramolecular disulfide bonds in hFSH β , exogenous addition of thiols is required to promote correct disulfide bond formation of recombinant proteins from *E. coli* by thiol-disulphide interchange. Cystamine has also been proposed as the oxidizing equivalent of the endoplasmic reticulum (ER), since the mixed function oxidase that catalyzes the oxidation of cysteamine to cystamine is located on the ER near the sites of protein synthesis. Furthermore, cystamine is much more effective than oxidized glutathione in forming a mixed disulfide with the free thiol of bovine serum albumin (88), suggesting that cystamine would be a more effective reagent in the mechanism of disulfide bond formation via thiol-disulfide exchange. Glutathione, on the other hand, is the major redox buffer in eukaryotic cells (89), but genetic evidence in yeast has demonstrated that glutathione is dispensable for disulfide formation and instead functions as a net reductant in the ER (90). Cystamine/cysteamine mixtures have been found to be most effective in facilitating *in vitro* folding of hCG β when 3.63 mM cystamine was combined with 6.37 mM cysteamine (69). We employed this redox system in our folding studies involving rec hFSH β as well as for association of rec hFSH β with N⁵²dg-hCG α . Our results suggested that at best, a limited amount of FSH activity emerged from these experiments as detected by RIA, using monoclonal antibody FSH10, which recognized the FSH heterodimer (91).

The limited FSH activity appeared to be associated with the hCG α derivative preparation as indicated by the slopes of the inhibition curves for these preparations in FSH receptor-binding and radioimmunoassays. The ¹²⁵I-hFSH tracer was displaced from FSH receptors only at the highest concentrations of recombinant hFSH employed, nevertheless, the inhibition curve slope was significantly different from that for pituitary hFSH, indicating chemical nonequivalence.

FSH dimer-specific RIA also indicated some FSH immunoactivity present in these experiments. Our lab reported that α subunit from eLH has receptor-binding activities (>1%) using rat testicular homogenate with [¹²⁵I]eLH as radioligand (92), while 3% and 12% of FSH receptor-binding activities have been reported for eLH α in a calf testicular homogenate with [¹²⁵I]oFSH as radioligand (93) or a porcine granulosa cell system using [¹²⁵I]hFSH as radioligand (94), respectively. The biological activities of these α subunit preparations were the result of contamination with LH. However, LH derived from serum expressed dual LH and FSH activities (95, 96). As the result, our hCG α preparation may have some FSH contamination which contribute to the biological activity as indicated slope inhibition curve.

The lack of FSH activity attributable to a semisynthetic hFSH may also suggested that the 12 kDa band represented a fragment, rather than the complete β subunit. Proteolysis of hFSH β was encountered during isolation of hFSH from crude pituitary extracts (Singh and Bousfield, unpublished data from our laboratory) and recently resurfaced during characterization of urinary hFSH (61). Moreover, proteases might co-aggregate in the inclusion bodies and cause proteolytic degradation during solubilization (97). When samples of nicked urinary hFSH and recombinant hFSH β were compared on the same Western blot, the mobilities of both immunoreactive bands were identical. Automated Edman degradation of two samples of recombinant hFSH β indicated the amino terminal sequence rather than the anticipated C-terminal sequence. This unexpected outcome triggered a re-evaluation of the potential epitope for monoclonal antibody P03. This anti-peptide antibody was raised against a peptide corresponding to hFSH β residues 28-50. A β L2 loop nick reported at Val³⁸-Tyr³⁹ for pituitary hFSH β (50) would occur in the middle of peptide sequence, -C²⁸AGYCYTRDLV³⁸-Y³⁹KDPARPKIQKT⁵⁰- used to generate the P03

antibody. Since the Edman results were consistent with the 12 kDa band including the N-terminal sequence, the P03 epitope must reside within residues 28-38.

Because we had assumed that the PO3 epitope was within residues 39-50, we had considered the alternative hypothesis that the 12 kDa recombinant hFSH β band was the full-size β subunit. Consideration of alternative modifications that could alter electrophoretic mobility led to an evaluation of the hFSH β sequence for potential phosphorylation sites. Using PROSITE search in EMBL database, hFSH β sequence revealed three potential PKC phosphorylation sites, two CK2 phosphorylation sites, one myristyl, and two glycohemone beta chain along with two N-glycosylation sites (Fig 12). However, phosphatase digestion of tetra-glycosylated hFSH and di-glycosylated hFSH resulted in no difference in migration rate between the phosphatase treated and untreated samples (Fig. 11).

Expression of recombinant hFSH β in bacterial cells yielded largely hFSH β fragments. These were difficult to detect because the immunoreactive fragment migrated with an apparent MW of 12 kDa. Other investigators in our laboratory have observed a 10 kDa band associated protease nicking of pituitary hFSH β using a different antibody that recognizes an epitope near or including residues 67-72. The Laemmli buffer system is inappropriate for analyzing peptides, as the putative 38 residue hFSH β N-terminal peptide migrates with an apparent molecular weight of 12 kDa, while the 73-residue C-terminal peptide produced a 10 kDa band. While mass spectrometry should have been able to detect both fragments, neither the recombinant hFSH β nor tryptic peptides derived from it were detectable under conditions that readily detected a similar sized protein.

Based on our results, we were unable to answer our two questions, whether the absence of β subunit carbohydrate responsible for the high biological activity of diglycosylated hFSH,

and whether the FSH β protein alone sufficient to account for the difference in relative molecular weight defined by SDS-PAGE and the actual mass determined by mass spectrometry. Although the data from folding and association experiments indicated that rec hFSH appeared to have some biological activities, the yield of the protein was not sufficient to allow us to carry out further experiments that would allow us to answer the above questions. In our study, we encountered the difficulties of proteolysis and folding that limited our yield of rec hFSH. For further studies, we are considering to express mutant hFSH β along with α subunit in eukaryotic cells in which the Asn codons are mutated by substituting with other amino acids. Although the yield is limited, but it will provide us with enough quantities of nonglycosylated hFSH β to addresses two questions above.

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APPENDICES

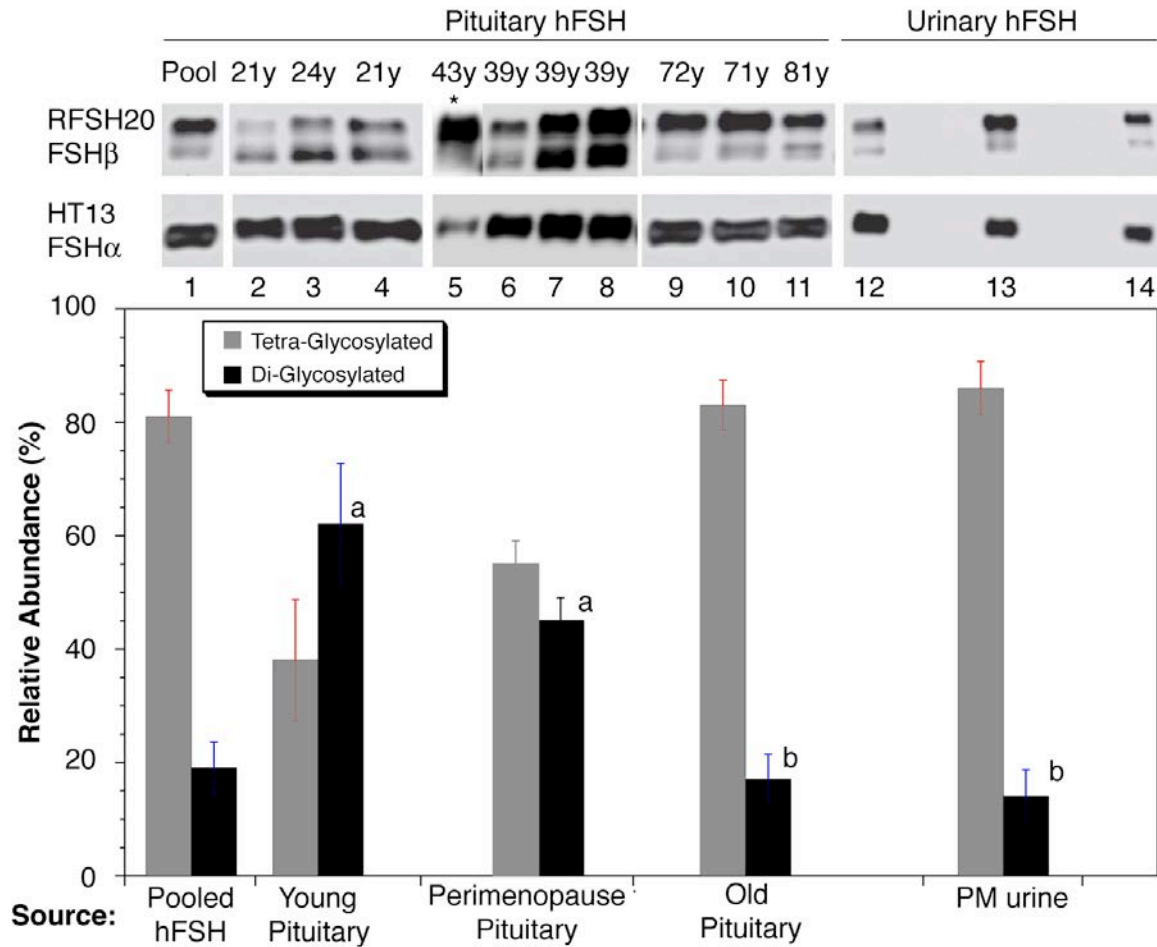


Figure 1. FSH glycoform abundance in individual pituitary glands and pooled postmenopausal urinary preparations. Top panel shows Western blot analysis of FSH β subunits derived from hFSH isolated from individual pituitaries from women of known age (lanes 2-11) and three urinary gonadotropin preparations (12-14). A hFSH β -specific monoclonal antibody RFSH20 was used to detect all but the sample in lane 5, for which an anti-hFSH β peptide monoclonal antibody P03 was employed. The slower band represents the glycosylated 24 kDa band. The faster migrating band represents the non-glycosylated 21 kDa band. The middle panel shows the α subunit blots for the same preparations that were probed with an α subunit-specific monoclonal antibody HT13. The bottom panel shows the average relative abundance for both glycoforms in each group of three hFSH

preparations. Pooled hFSH is a purified hFSH preparation (AFP4161B) available from the National Hormone and Pituitary Program. FSH sources: Young pituitary, hFSH from 21-24 year old women in lanes 2-4, as indicated; Perimenopausal pituitary, hFSH from 39 year old women in lanes 6-8 (sample in lane 5 analyzed with anti-peptide antibody P03 not included in average); Old pituitary, hFSH from 71-81 year old women; PM urine, three lots of urinary gonadotropin preparation Pergonal.

A.
Pre-hFSH β +his

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1   atg ggc agc agc cat cat cat cat cat cac agc agc ggc ctg gtg
    M  G  S  S  H  H  H  H  H  H  S  S  G  L  V
46  ccg cgc ggc agc cat atg gct agc atg act ggt gga cag caa atg
    P  R  G  S  H  M  A  S  M  T  G  G  Q  Q  M
91  ggt cgg gat ccg aat tcg ATG AAG ACA CTC CAG TTT TTC TTC CTT
    G  R  D  P  N  S  M  K  T  L  Q  F  F  F  L
136 TTC TGT TGC TGG AAA GCA ATC TGC TGC AAT AGC TGT GAG CTG ACC
    F  C  C  W  K  A  I  C  C  N  S  C  E  L  T
181 AAC ATC ACC ATT GCA ATA GAG AAA GAA GAA TGT CGT TTC TGC ATA
    N  I  T  I  A  I  E  K  E  E  C  R  F  C  I
226 AGC ATC AAC ACC ACT TGG TGT GCT GGC TAC TGC TAC ACC AGA GAT
    S  I  N  T  T  W  C  A  G  Y  C  Y  T  R  D
271 CTG GTG TAT AAG GAC CCA GCC AGG CCC AAA ATC CAG AAA ACA TGT
    L  V  Y  K  D  P  A  R  P  K  I  Q  K  T  C
316 ACC TTC AAG GAA CTG GTA TAT GAA ACA GTG AGA GTG CCC GGC TGT
    T  F  K  E  L  V  Y  E  T  V  R  V  P  G  C
361 GCT CAC CAT GCA GAT TCC TTG TAT ACA TAC CCA GTG GCC ACC CAG
    A  H  H  A  D  S  L  Y  T  Y  P  V  A  T  Q
406 TGT CAC TGT GGC AAG TGT GAC AGC GAC AGC ACT GAT TGT ACT GTG
    C  H  C  G  K  C  D  S  D  S  T  D  C  T  V
451 CGA GGC CTG GGG CCC AGC TAC TGC TCC TTT GGT GAA ATG AAA GAA
    R  G  L  G  P  S  Y  C  S  F  G  E  M  K  E
496 gtc gac acg ctt gcg gcc gca ctc gag cac cac cac cac cac cac
    V  D  T  L  A  A  A  L  E  H  H  H  H  H  H
541 tga
    *

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hFSH β +his

```

1   atg ggc agc agc cat cat cat cat cat cac agc agc ggc ctg gtg
    M  G  S  S  H  H  H  H  H  H  S  S  G  L  V
46  ccg cgc ggc agc cat atg gct agc AAT TCG TGT GAG CTG ACC AAC
    P  R  G  S  H  M  A  S  N  S  C  E  L  T  N
91  ATC ACC ATT GCA ATA GAG AAA GAA GAA TGT CGT TTC TGC ATA AGC
    I  T  I  A  I  E  K  E  E  C  R  F  C  I  S
136 ATC AAC ACC ACT TGG TGT GCT GGC TAC TGC TAC ACC AGA GAT CTG
    I  N  T  T  W  C  A  G  Y  C  Y  T  R  D  L
181 GTG TAT AAG GAC CCA GCC AGG CCC AAA ATC CAG AAA ACA TGT ACC
    V  Y  K  D  P  A  R  P  K  I  Q  K  T  C  T
226 TTC AAG GAA CTG GTA TAT GAA ACA GTG AGA GTG CCC GGC TGT GCT
    F  K  E  L  V  Y  E  T  V  R  V  P  G  C  A
271 CAC CAT GCA GAT TCC TTG TAT ACA TAC CCA GTG GCC ACC CAG TGT
    H  H  A  D  S  L  Y  T  Y  P  V  A  T  Q  C
316 CAC TGT GGC AAG TGT GAC AGC GAC AGC ACT GAT TGT ACT GTG CGA
    H  C  G  K  C  D  S  D  S  T  D  C  T  V  R
361 GGC CTG GGG CCC AGC TAC TGC TCC TTT GGT GAA ATG AAA GAA TAA
    G  L  G  P  S  Y  C  S  F  G  E  M  K  E  *

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hFSH β

```

1   atg ggc AAT TCG TGT GAG CTG ACC AAC ATC ACC ATT GCA ATA GAG
    M  G  N  S  C  E  L  T  N  I  T  I  A  I  E
46  AAA GAA GAA TGT CGT TTC TGC ATA AGC ATC AAC ACC ACT TGG TGT
    K  E  E  C  R  F  C  I  S  I  N  T  T  W  C

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```

91   GCT GGC TAC TGC TAC ACC AGA GAT CTG GTG TAT AAG GAC CCA GCC
    A  G  Y  C  Y  T  R  D  L  V  Y  K  D  P  A
136  AGG CCC AAA ATC CAG AAA ACA TGT ACC TTC AAG GAA CTG GTA TAT
    R  P  K  I  Q  K  T  C  T  F  K  E  L  V  Y
181  GAA ACA GTG AGA GTG CCC GGC TGT GCT CAC CAT GCA GAT TCC TTG
    E  T  V  R  V  P  G  C  A  H  H  A  D  S  L
226  TAT ACA TAC CCA GTG GCC ACC CAG TGT CAC TGT GGC AAG TGT GAC
    Y  T  Y  P  V  A  T  Q  C  H  C  G  K  C  D
271  AGC GAC AGC ACT GAT TGT ACT GTG CGA GGC CTG GGG CCC AGC TAC
    S  D  S  T  D  C  T  V  R  G  L  G  P  S  Y
316  TGC TCC TTT GGT GAA ATG AAA GAA TAA
    C  S  F  G  E  M  K  E  *

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B

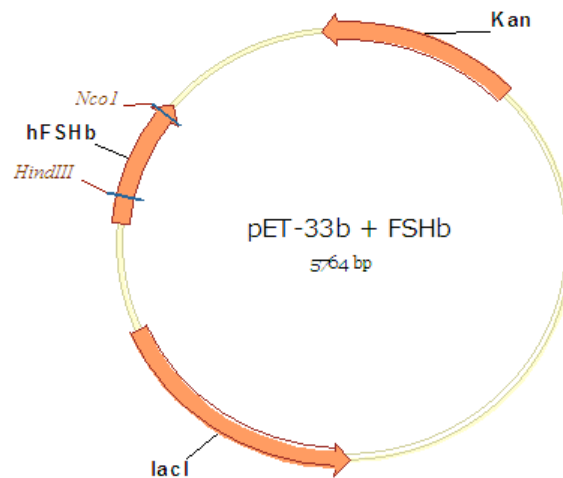


Figure 2. (A) Sequences of three hFSH β constructs. Codons in capital letters are the coding sequence of hFSH β confirmed by DNA sequencing. The sequences were sequenced to confirm that they had no mutations before they were subcloned into the expression vector pET-33b(+); codons in lower case letters represented the DNA sequences from pET-33b(+) vector that are expected to express along with hFSH β . Pre-hFSH β +his has 6xHis at both the N- and C- termini of the sequence. The hFSH β construct had a modified f-Met and a Gly amino acid residue appended to the N-terminus. (B) Schematic representation showing insertion of hFSH β sequence

into expression vector pET-33b(+). The diagram represents mature hFSH β based on sequencing results from pGEM-Teasy vector before it was subcloned into pET-33(b+) vector.

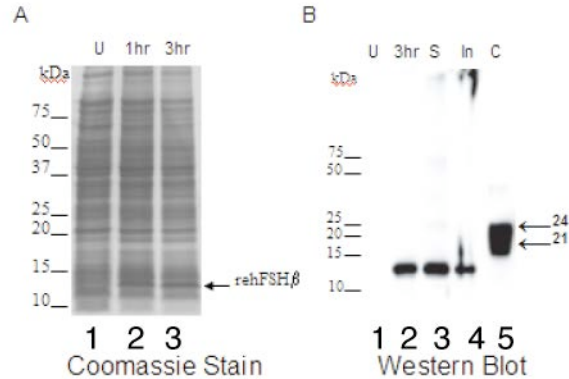


Figure 3. Induction of rec hFSH β expression in *E. coli*. (A) SDS-PAGE of expressed protein. Samples were collected from 50 mL of bacterial cell cultures induced with 1 mM IPTG for 3 hours. The soluble fraction was diluted 1:5, while uninduced total cell extracts, inclusion bodies and induced total cell extracts were diluted 1:100. Lane 1, .02% total uninduced cell extract; lane 2, .001% cell extract after 1 h induction; lane 3, .001% cell extract after 3 h induction. The arrow indicates the expected size of rec hFSH β based on its mass. (B) Western blot of rec hFSH β . The primary antibody was anti-hFSH β peptide monoclonal antibody PO3. Lane 1, 0.01% cell extract from uninduced cells; lane 2, 0.01% cell extract after 3 h induction; lane 3, .2% soluble fraction after 3 h induction; lane 4, 0.01% insoluble fraction after 3 h induction; lane 5, 1 μ g pituitary hFSH and the arrow indicates the 21 and 24 $\times 10^{-3}$ relative molecular weight bands that correspond to di- and tetra-glycosylated hFSH, respectively.

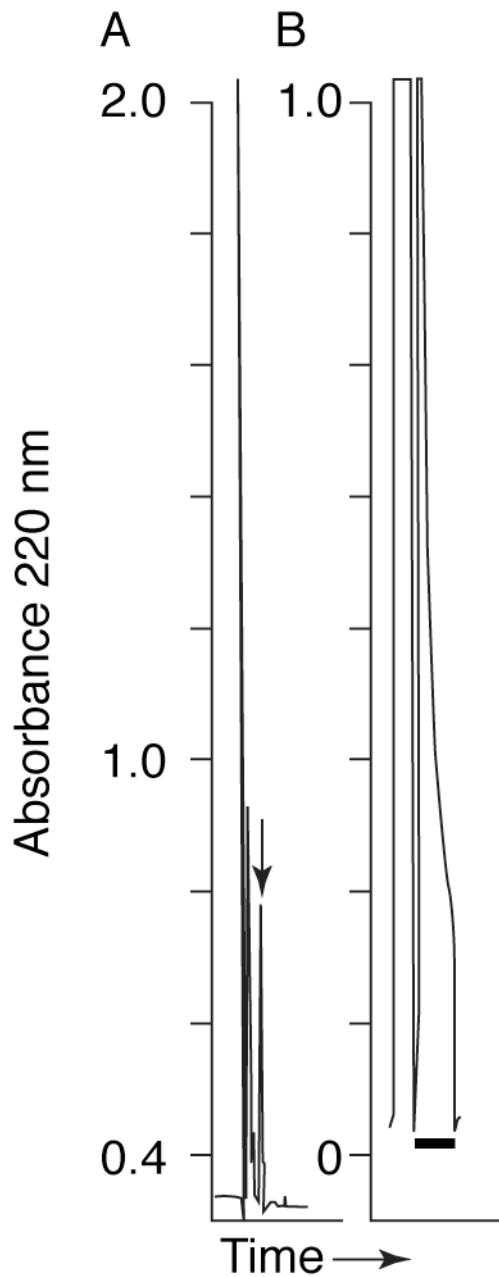
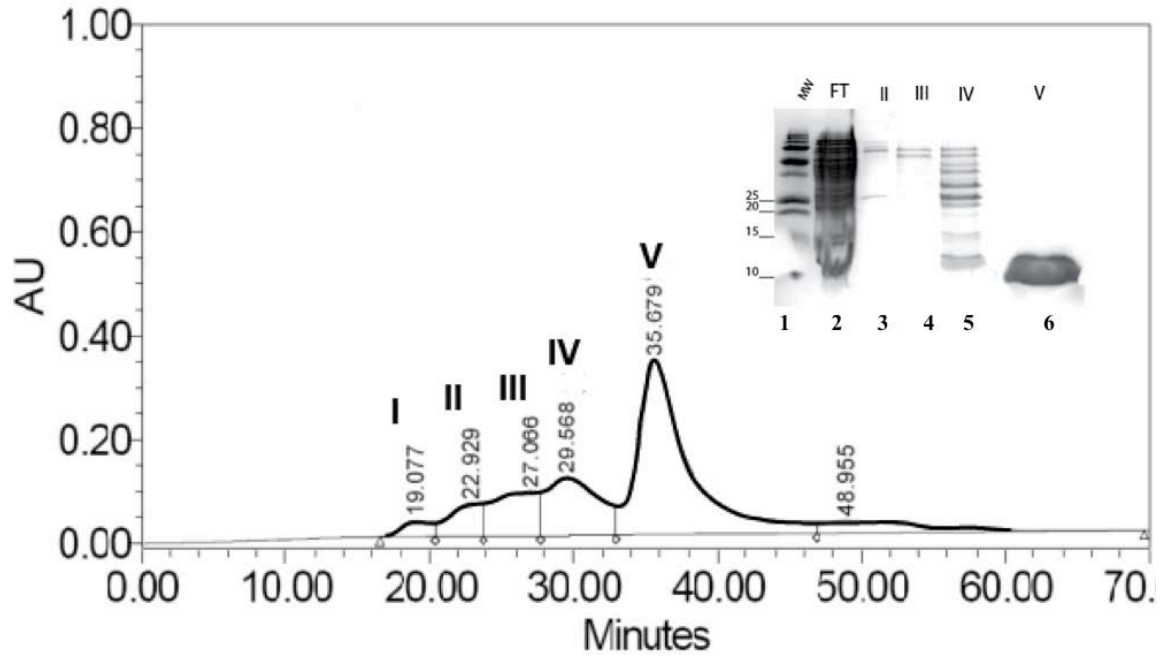


Figure 4. Affi-Gel Blue chromatography of soluble *E. coli* expressed hFSH β . The soluble fraction was applied to a 1-ml Affi-Gel Blue column equilibrated with 0.1 M NaPO₄, 0.15 M NaCl, pH 8.6, buffer at a flow rate of 1 ml/min. After washing with the same buffer, bound protein was eluted with 0.1 M NaPO₄, 0.4 M KCl, pH 8.5. Absorbance was recorded using a dual-pen chart recorder. For clarity, each plot was placed in a separate panel. A. Full-scale 2 absorbance unit output of the detector at 206 nm. The arrow indicates the affinity bound fraction. B. Recorder sensitivity set to 1 absorbance unit full scale. The bar indicates the portion of the chromatogram pooled to obtain recombinant hFSH β .

A.



B.

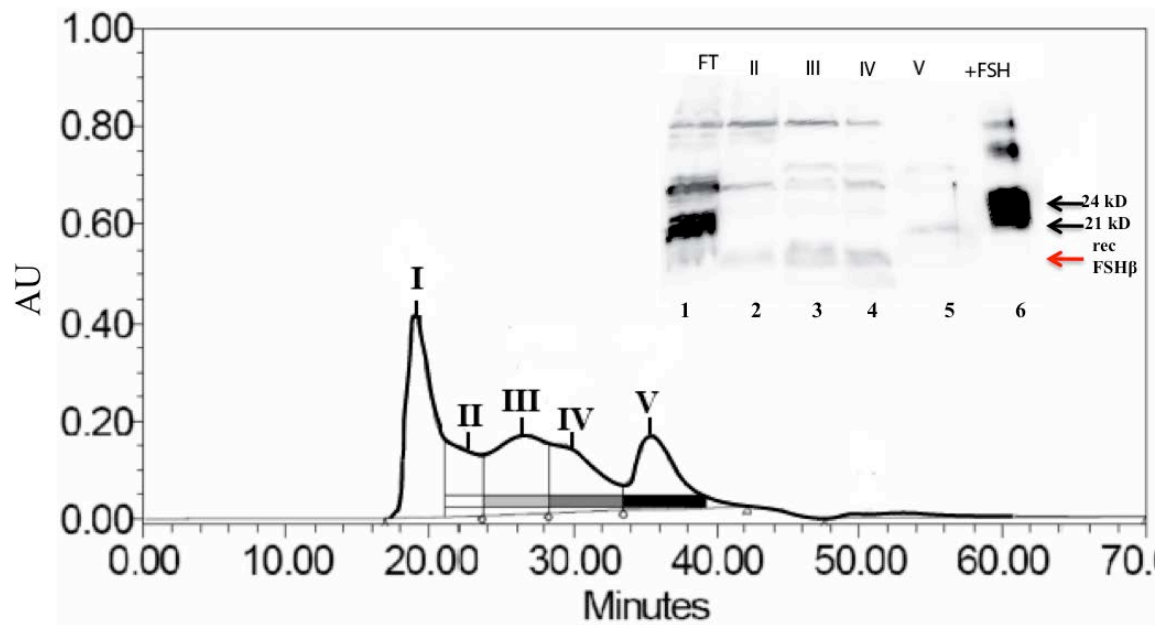


Figure 5. Superdex 75 gel filtration of soluble rec hFSH β derived from the Affi-Gel Blue chromatogram shown in Fig. 4. The column was equilibrated with 0.2 M ammonium bicarbonate containing 20% acetonitrile at a flow rate of 0.4 ml/min, at 25°C. Bound soluble

fraction from Affi-Gel Blue chromatography was concentrated in an Amicon Ultra-15 and 200 μ l aliquots applied to a 10 x 300 mm Superdex 75 gel filtration column. Panel A shows results of the first injection. Fractions I-V were collected, but only fractions II-V were concentrated in SpeedVac and analyzed on a 15% Laemmli SDS-PAGE gel. Lane 1, MW standard, lane 2, 20 μ l of flow through sample from Affi-Gel Blue purification (Fig 4), lanes 3-6 correspond to fractions II-V, respectively. Panel B shows the second 200 μ l injection of soluble fraction. Fractions I-IV were collected similar as above, but fractions II-IV were analyzed by Western blotting instead SDS-PAGE. Lane 1, 1% of flow through sample from Affi-Gel Blue purification (Fig 4); lanes 2-5 correspond to fractions II-V in the chromatogram; lane 6, 300 ng pituitary hFSH. Black arrows highlight the 21 and 24 kDa bands, as indicated. Red arrow shows the expected size for rec hFSH β based on its formula weight. The primary antibody was anti-hFSH β peptide monoclonal antibody, P03.

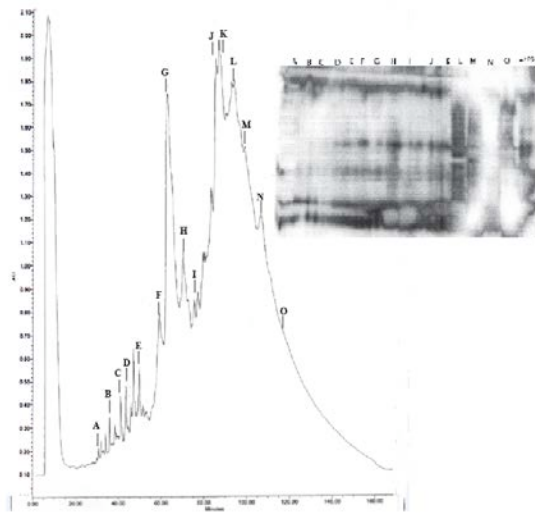
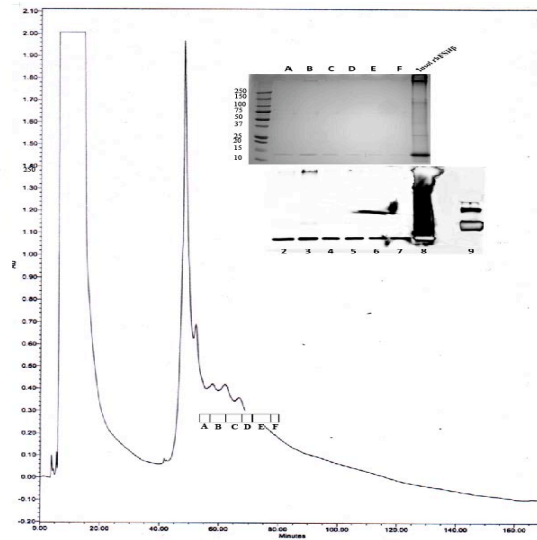
A**B**

Figure 6. Reverse-phase HPLC of inclusion body proteins. A. Reduced, solubilized inclusion bodies derived from 100 ml induced culture were loaded directly to a C4 reverse phase column. The HPLC was equilibrated with 10% water/acetonitrile containing 0.1% TFA at 0.5 ml/min at 25°C. Following a 5 minute wash, an 8-80% acetonitrile gradient was developed over 150 min. The peaks labeled A-O were selected for Western blot using anti-hFSH β peptide antibody, P03. Inset: Western blot of the selected fractions, as indicated. B. Solubilized inclusion bodies treated with 20mM iodoacetamide prior to reverse-phase HPLC under identical conditions. The open bars indicate portions of the chromatogram pooled to obtain fractions A-F. Inset: Upper panel; Coomassie blue-stained SDS gel. Lane 1; MW marker; lanes 2–7 correspond to 5% of fractions A–F, respectively; lane 8 was insoluble rec hFSH β without iodoacetamide treatment. Lower panel; Western blot under the same conditions as in panel A. Lanes 2-7 correspond to fractions A-F, respectively; Major band associated with rec hFSH β migrated at ~12.5 kDa, and appeared in all fractions A through F; lane 3 which corresponds to fraction B, had a slight faint at molecular weight of 21 kDa, the same apparent molecular weight associated with pituitary hFSH β preparations; lane 8, insoluble fraction rec hFSH β ; lane 9, 300 ng of pituitary hFSH.

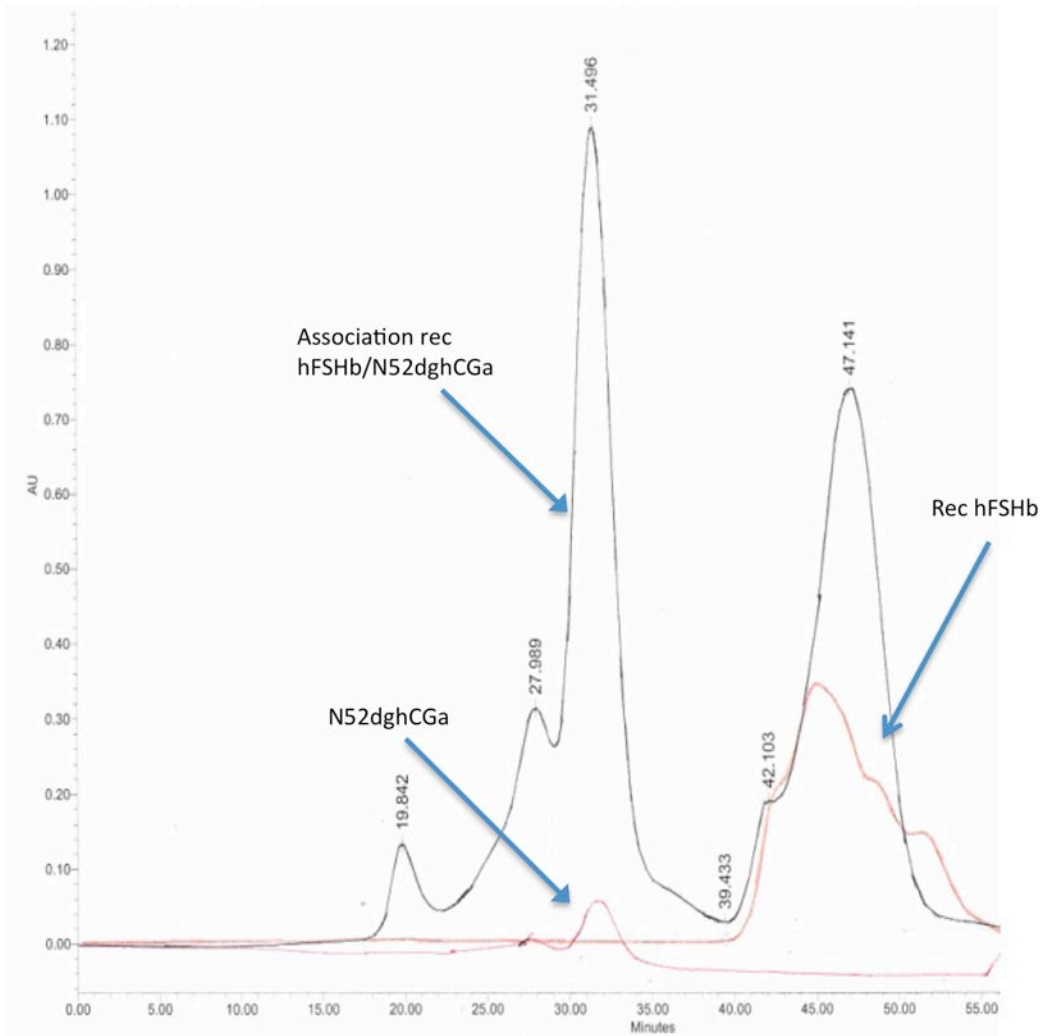


Figure 7. Superimposed Superdex 75 chromatograms following FSH subunit folding/association reactions. Samples of rec hFSH β , N⁵²dghCG α , and rec hFSH β /N⁵²dghCG α , as indicated by the arrows, were fractionated by gel filtration under the same conditions as in Fig. 6. The chromatograms were overlaid to facilitate comparison.

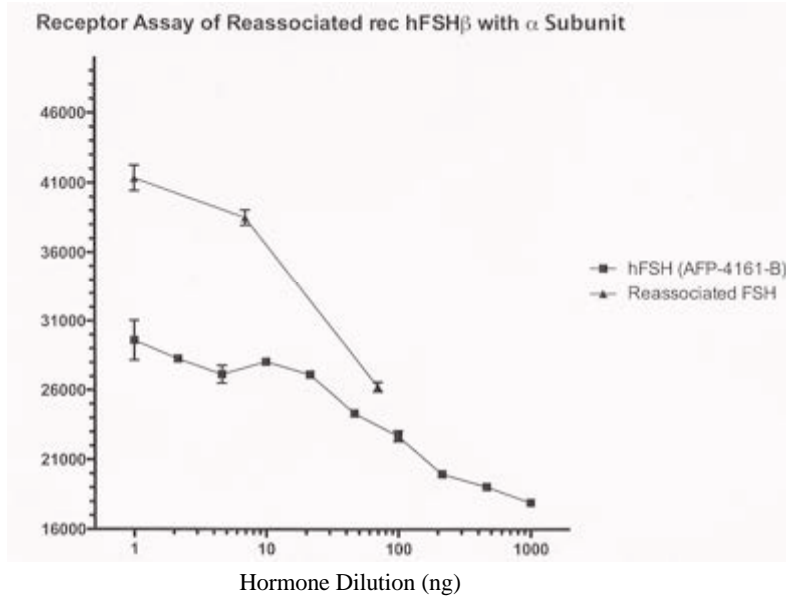


Figure 8. FSH radioligand receptor assay of rec hFSH β /hCG α . The assay was performed by using [125 I]eFSH tracer and rat testis homogenate receptor preparation. (■) Purified hFSH preparation AFP4161B. (▲) rec hFSH β /hCG α preparation from serial dilutions: 1:10, 1:100, and 1:1000.

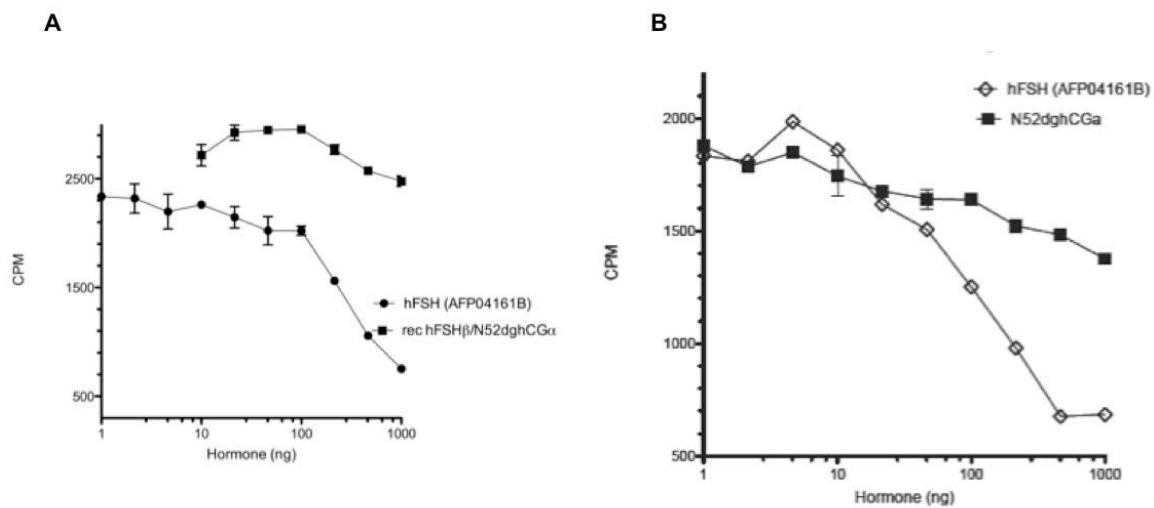


Figure 9. FSH radioimmunoassay of rec hFSH β /hCG α . FSH immunoactivity was detected by a heterodimer-specific monoclonal antibody, FSH-10. **(A)** The [125 I]hFSH tracer was displaced by increasing amounts of hFSH or rechFSH β /hCG α . Serially diluted concentrations of hFSH (AFP04161B) were prepared as described in materials and methods. **(B)** The [125 I]hFSH tracer was displaced by increasing amounts of hFSH or N 52 dg-hCG α .

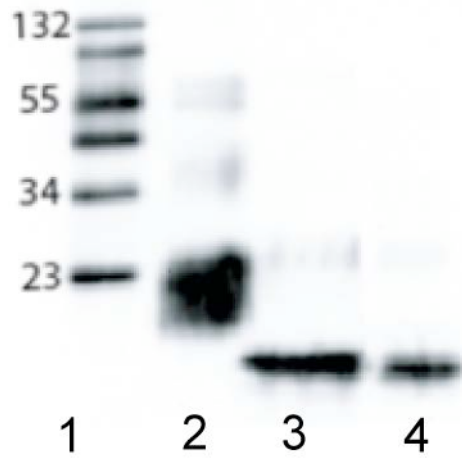


Figure 10. Western blot comparing rec hFSH β with degraded urinary hFSH sample. FSH β immunoactivity was detected with hFSH monoclonal antibody PO3. Lane 1, MW markers; lane 2, 500 ng hFSH; lane 3, degraded urinary hFSH sample; lane 4, rec hFSH β .



Figure 11. Protein phosphatase (PPase) treated hFSH. Western blot analysis of the samples probed with FSH β -specific polyclonal antibody, W556, to determine whether post translational phosphorylation affected the migration rate of FSH β . Lane 1, BioRad prestained MW markers (not visible by chemiluminescence); lane 2, mature, recombinant hFSH β ; lane 3, rec hFSH β +sp; lane 4, rec hFSH β +sp+his; lane 5, 200 ng tetraglycosylated hFSH; lane 6, 200 ng tetraglycosylated hFSH treated with PPase; lane 7, 200 ng diglycosylated hFSH; lane 8, 200 ng diglycosylated hFSH treated with PPase.

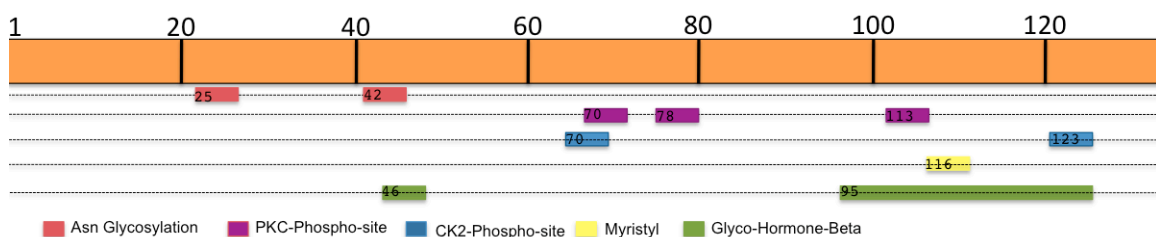


Figure 12. PROSITE search result in EMBL database for hFSH β peptide sequence. The search result revealed two N-glycosylation sites (orange), three protein kinase C (PKC) phosphorylation

sites (purple), Casein kinase 2 (CK2) phosphorylation sites (blue), one myristyl site (yellow), and two glyco-hormone beta chain motif (green).