Original Article

Biochemistry

Buffalo Pituitary Gonadotropins: Characterization of Bacterially Expressed Recombinant Alpha and Hormone Specific Beta Subunits

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ABSTRACT [ENGLISH/ANGLAIS]

Buffalo pituitary gonadotropin alpha subunit was expressed in E.coli as a recombinant protein from its cDNA constructed from pituitary RNA. Similarly the beta subunits of buffalo pituitary follicle stimulating hormone (FSH), Luteinizing Hormone (LH) and Thyroid stimulating hormone (TSH) were cloned and expressed in E.coli. The bacterially expressed non-glycosylated recombinant proteins were purified on Ni-affinity columns and characterized to some extent for physico-chemical properties. All the proteins were expressed without their respective signal peptides.

Keywords: Buffalo gonadotropins, bacterial expression, alpha and beta cDNAs

RÉSUMÉ [FRANÇAIS/FRENCH]

Buffle hypophysaire gonadotrope sous-unité alpha a été exprimée dans E. coli comme protéine recombinante à partir de son ADNc construite à partir d'ARN hypophysaires. De même, les sous-unités bêta de l'hormone de l'hypophyse buffle folliculo-stimulante (FSH), l'hormone lutéinisante (LH) et l'hormone stimulant la thyroïde (TSH) a été cloné et exprimé dans E. coli. Les exprimées par la bactérie non glycosylées des protéines recombinantes ont été purifiées sur colonnes d'affinité Ni- et caractérisé dans une certaine mesure pour les propriétés physico-chimiques. Toutes les protéines ont été exprimées sans que leurs peptides signaux respectifs.

Mots-clés: Gonadotrophines Buffalo, l'expression bactérienne, alpha et bêta. ADNc

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INTRODUCTION

The pituitary FSH and LH are the main regulatory hormones for normal growth, sexual development and reproductive function. Together with TSH and placental CG they form the thyrotropin hormone family. All of them happen to be glycoproteins. These are composed of two dissimilar, non-covalently associated subunits, designated as α and β subunits [1-3] The alpha subunit is species specific where as the beta subunit is hormone specific. These subunits are glycosylated at specific residues, 2 N- glycosylation sites in the common alpha, FSH β and CG β but only one site in TSH β and LH β. Alone the subunits exhibit weak binding, if any, to the receptor. But they regain their full binding upon reconstitution. Also, it has been shown that carbohydrate moieties are not required for the binding with the receptor. However deglycosylated hormones show slightly higher binding to the receptor with a concomitant decrease, strangely, in the level of signal transduction [4]. A fraction of alpha subunit produced in the pituitary does not combine with any beta subunit and hence not used in the formation of hypophyseal glycoprotein hormone [5]. The free TSH beta subunits have been shown to be degraded before they are secreted [6]. However, this free subunit is secreted in a pulsed manner synchronously with LH into the peripheral blood stream. The level of the free subunits in the blood depends on a number of factors including age, sex and in females also on the stage of the reproductive cycle [7]. These subunits have been reported to be differently glycosylated than the native hormonal subunits [8]. The physiological role of the free subunits has received very little attention. It has been reported that free alpha subunit stimulates differentiation of lactotrophs in immature pituitaries [9]. It has also been reported to stimulate PRL secretion from placenta derived decidual cells. Level of free alpha subunit in plasma may be used as

a tumor marker as it has been shown to increase during some pathological conditions particularly pituitary adenomas [10].

During the purification of gonadotropins the free subunits have been observed to be present in the pituitaries. These freely occurring subunits give a band in SDS PAGE around 14-20 kDa [5]. Also these subunits were reportedly hypoglycosylated as compared to the native subunits [11]. However these subunits are less studied. Since the deglycosylation methods available are not 100% efficient, the alpha and beta subunits of the glycoprotein hormones were separately cloned in the bacterial system. These subunits were expressed as N-terminal histidine tagged proteins and purified by Ni-affinity chromatography. *E.coli* has earlier been used as a host to express glycoprotein hormones [12] although without their glycan portions. Further, in the present work, the free and native subunits (subunits from intact hormone) from the pituitaries were also purified and segregated, and studied for their in Cibacron Blue is a popular textile dye and has been used for purification of the glycoprotein hormones [13]. In the present study the interaction of the different subunits of the thyrotropin family of hormones and the intact hormone with Cibacron Blue has been studied.

MATERIALS AND METHODS

Tissues and Biologicals

The buffalo pituitaries were procured from a local abattoir. TRI-reagent was purchased from Sigma Co., St. Louis, Missouri, USA. Oligo-dT primers were purchased from B'lore Genei, India. The cloning vector pGEMT-Easy was purchased from Promega Life Sciences and the bacterial expression vectors pET 28a, pET 15b were purchased from Novagen. The *E.coli* strain DH5a and BL21(DE3)pLysS were purchased from Gibco BRL and Novagen respectively. All restriction enzyme and other enzymes were purchased from New England Biolabs (NEB), Beverly, MA, USA or MBI Fermentas. All the other reagents were obtained either from Sigma Chemical Company, USA or purchased locally.

Total RNA Purification from Buffalo Pituitary Glands

All the glassware and plasticware were treated with 0.1% DEPC (DiEthyl PyroCarbonate) (Sigma) for 8-12 h, autoclaved and dried. Fresh buffalo pituitaries were removed from the buffalo within 30 min of slaughter. The pituitaries were cut into pieces of about 100 mg and rinsed twice in RNase free DEPC treated water and quickly frozen in liquid nitrogen. The frozen tissue was thawed in TRI REAGENT™ (Sigma T9024, 1 mL/100 mg tissue) and homogenized in a porcelain mortar and pestle on ice. The rest of the procedure was according to standard protocol [14] The protocol was adopted from the manufacturer (Sigma Chemicals). RNA samples were quantitated by nanodrop (Absorbance at 260 and 280 nm) spectrophotometer and the ratio was recorded. Purity of the samples was checked by agarose-gel electrophoresis. Samples showing A_{260}/A_{280} in a range of 1.8-2.0 were used for first strand cDNA synthesis.The integrity of RNA samples was analyzed by horizontal agarose gel electrophoresis [14].

First Strand cDNA Synthesis

The first strand cDNA was synthesized using 2 ug of total RNA, 500 ng of oligo(dT)18 and DEPC treated water were added to make a final volume of 15 uL in a PCR tube and incubated at 55°C for 5 min. The tube was quickly chilled on ice. To this tube, a mix of 2.5 uL 10X RT buffer, 1.25 uL of dNTP (10 mM) mix, 0.5 uL of MMLV reverse transcriptase (200U/uL) and 5.25 uL DEPC water were added to make a 25 uL reaction mixture. The mixture was incubated at 37°C, for 1 h and reaction was terminated at 85°C for 5 min and stored at -20°C till further use.

Polymerase Chain Reaction (PCR)

The first strand cDNA was used to amplify specific genes by polymerase chain reaction (PCR). PCR were carried out in 20 uL reaction containing 2 uL Taq polymerase buffer (10X), 0.2 mM dNTP mix (10 mM), 25 pmoles each of forward and reverse primer (25 pmoles/uL), 0.2 uL Taq polymerase (5 U/uL, NEB) and deionized water. PCR was performed by denaturing at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min. 2 uL of each PCR was electrophoresed on 1.5% agarose in 1X TAE at 75-80V. Images were captured using quantity one in trans-UV light. The amplification of the required gene product by PCR was analyzed by horizontal agarose gel electrophoresis.

Ligation

A cohesive-end ligation of the amplified PCR product and vector was done. The ligation mix was incubated at 55°C for 5 min and then immediately chilled on ice to avoid any secondary structure hindrance for ligation. To this 1

of T4-DNA ligase was added and the reaction was left undisturbed for 16 h at 22°C. Competent bacterial Cell Preparation and Transformation were conducted as per standard protocols.[14] The method of Blue-White screening of the transformants is based on the selection of clones by a-complementation of lac Z gene [14]. Plasmids were isolated using modified alkaline-lysis method [14].Plasmids when needed in large amounts were isolated using gentle Lysis method with some modifications.

Subcloning of the different Subunits in the pET Vectors

pET 28a(+) vector was purified at large scale by midiprep plasmid isolation and desalted using Sephadex G-50 resin. This vector was digested with selected pair of the restriction enzymes and the vector after digestion was resolved in 1% LMP agarose, excised and purified.

The linearized vector and specific subunit insert were ligated at a concentration of 100 femtomoles each using T4 DNA ligase at 16°C for 16 hours. *E. coli* DH5a competent cells were transformed by ligation mix. Colonies were inoculated in 5 mL LB media in the presence of Kanamycin (100 pg/ mL) and grown for 12-14 h. Plasmids were isolated as mentioned earlier. The clones were screened by restriction enzyme digestion and also by PCR amplification.

Expression of His-tagged Subunits in pET 28a(+) Vector

E. coli BL21 (DE3) pLysS strain was initially selected for the expression of buffalo thyrotropin family hormone subunit-pET28a (+) construct as per standard protocols [14].

Purification of the Recombinant His-tagged Subunits

Induced bacterial cells were harvested by centrifugation at 5,000g for 2 min at room temperature. 10 mL of lysis buffer (20 mM Phosphate buffer (pH 7.8), 500 mM $NaCl_2$ and 1 mM PMSF) was added. Cells were suspended by vigorous vortexing. Lysozyme was added to a final concentration of 1 mg/mL, mixed by gentle vortexing and kept on ice for 1 h with intermittent shaking. 1% Triton X-100 was added and the mixture was further incubated on ice for 30 min. It was then centrifuged for 20 min at 14,000g at 4°C. Supernatant was collected (in case of the soluble protein) and loaded on Ni- NTA matrix pre-equilibrated with loading buffer (Buffer A; 20 mM Phosphate buffer (pH 7.8), 500 mM NaCl). Matrix was washed with 10 column volumes of loading buffer. Elution was carried out sequentially with 20 mM Phosphate buffer (pH 6.0), 500 mM NaCl, 50 mM imidazole (Buffer B) and 20 mM Phosphate buffer (pH 6.0), 500 mM NaCl, 250 mM imidazole (Buffer C). All the fractions were analyzed by resolving equal volumes of each on 13% SDS-PAGE. Also the absorbance at 280 nm was measured for all the fractions followed by measuring the immunoreactivity. Protein was estimated according to protocol given by Bradford [15]. Proteins were essentially resolved by high resolution SDS-Polyacrylamide slab gel electrophoresis system [16].

Cibacron Blue Sepharose Chromatography

A 3 mL column of Cibacron Blue 3GA agarose gel was prepared, equilibrated with 810 bed volumes of 0.05 M phosphate buffer, pH 7.0. The column was maintained at a constant flow rate of 20-25 mL/h. The sample dissolved in the equilibration buffer was loaded onto the gel. After the sample entered the gel the flow was stopped. Thirty min later the gel was washed with the same buffer at a constant flow rate to wash all the unbound proteins. Subsequently the bound proteins with ionic interactions were eluted with 0.05 M PB, pH 7.0 containing 1 M NaCl. Finally the column was washed with 80% Ethylene Glycol, to elute out the hydrophobically bound protein to the column. Fractions (2 mL) of unbound and bound protein were collected and their absorbance was measured at 280 nm.

In a separate experiment to elute bound proteins, 0.3 M a-methyl-D- mannoside in 0.05 M PB, pH 7.0 was initially used. Following this, two other buffers were used, the order of elution being 0.05 M PB, pH 7.0 containing 1 M NaCl followed by 80% ethylene glycol in 0.05 M PB, pH 7.0.

ELISA

Immunoreactivity of all protein fractions from Cibacron Blue column chromatography was measured in triplicates using direct binding ELISA. Highly purified homologous buLH was used to obtain standard immunoreactivity curve. Primary antibody developed against bLH a and P subunits was used at the dilution of 1:15,000 (standardisation data not shown). Goat anti-rabbit IgG-HRP conjugate was diluted to 1:1,500. A solution of ortho phenylene diamine (1 mg/mL) and 0.06% H_2O_2 in 0.05 M citrate buffer, pH 5.5 was used as substrate. Plates were read at 490 nm in an ELISA reader (ECIL, India).

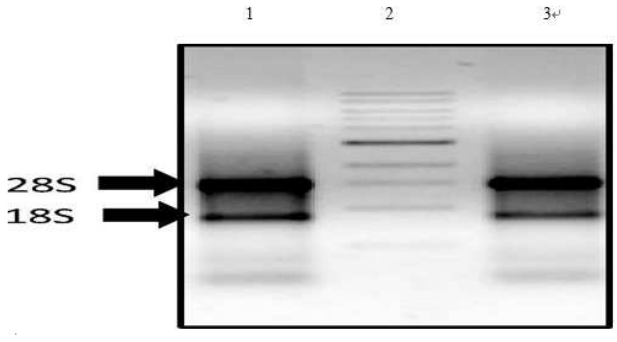
Percentage immunoreactivity was calculated using the following formula:

$$\text{Percentage immunoreactivity (\%)} = \frac{\text{Pooled A490 of a fraction}}{\text{Additive A490 of all the bound and eluted fractions}} \times 100$$

RESULTS AND DISCUSSION

Pituitary glands obtained within half an hour of slaughter were immediately subjected to RNA isolation following the classical protocol [14] using Guanidine IsoThioCyanate (GITC) and also the Tri-reagent Sigma. The average yield was distinctly different by the two methods and so was the quality. The yield using GITC was in range of 0.8-1.3 pg/mg of the pituitary whereas by Tri-reagent it was 3.2-3.4 pg/mg. The quality of the RNA as assessed by the A_{260} : A_{280} ratio (data not shown) and the agarose gel electrophoresis was good. Two clear bands representing the 28S and 18S RNA in a ratio of about 2.5:1 could be seen (Fig 1). A total of about 10 pg of first strand cDNA was synthesized from a total of 2 pg RNA. Further the different pair of primers (Fig 2)used gave a gene specific amplification. The PCR product when analyzed on a 1.5% agarose gel yielded different amplicons sizes as follows Gonadotropin α 363bp (data not shown), FSH β 390bp, TSH β 417bp (data not shown) and LH β 426bp (Figure 3).

Figure 1: This figure shows a 1.5% agarose gel of total buffalo pituitary RNA preparation, run in TBE buffer. The two bands in lane 1 and 3 corresponds to 28S and 18S RNA.. Lane 2 has molecular weight markers of double stranded DNA of 1Kb.



These different PCR products were cloned in pGEMT Easy vector. The DH 5a competent cells were efficiently transformed and about 85% of white colonies by a complementation of the lac Z gene were obtained. The A^00 values for overnight culture of the bacteria could reach as high as 3 to 3.5. The plasmid concentration obtained in the miniprep was around 1pg/pL. The isolated plasmids were also checked for their quantitative and qualitative analysis by running an aliquot on a 1% agarose gel (data not shown). The isolated plasmids were subjected to a PCR using gene specific primers and also to the digestion by restriction enzyme. The PCR and restriction enzyme digestion both gave encouraging results as the band of PCR amplicons was similar to the band excised by restriction enzyme from the plasmid (data not shown). Further the plasmids were sequenced commercially using the universal T7 promoter and SP6 promoter primer. The sequences were also submitted to the NCBI database GenBank accession numbers **EU340285, FJ357420, FJ357421** (not shown). The sequenced plasmids were aligned to the other available sequences and there are very few differences in the coding sequences.

Figure 2: This figure shows list of primers used

Primers

Gonadotropin α subunit Forward

5' CC GAATTC ATG GAT TAC TAC AGA AAA 3' Reverse

5' CC GAATTC TTA GGA TTT GTG ATA ATA 3'

FSH β subunit

Forward

5' GGATCC CATATG AAG TCC GTC CAGTTC 3' Reverse

5' GAATTC TTCTCTGCTTTCACTGA 3'

TSH β subunit

Forward

5' GAATTC CAT ATG ACTGCTACCTTCCTGAT 3' Reverse

5' GAATTC TTAGATAGAAAATCCCACCAT 3'

LH β subunit

Forward

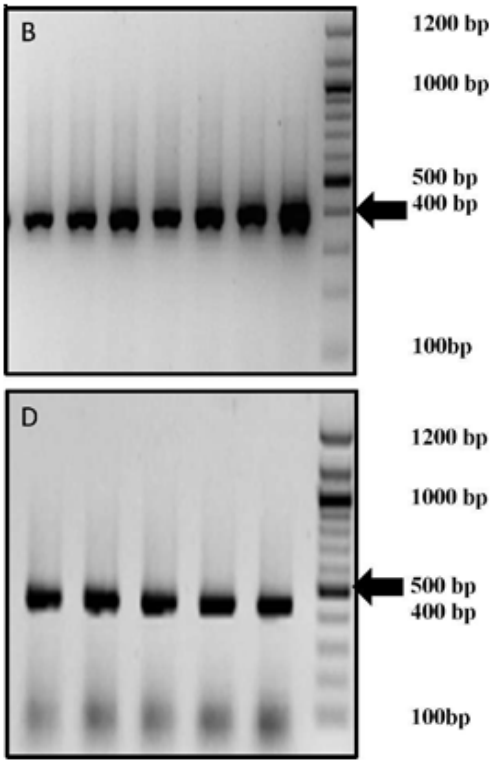
5' GAATTC CATATGGAGATGTTCCAGGGA 3' Reverse

5' GAATTC TTAGAGGAAGAGGATGTCT 3'

The sequenced inserts (Figure 4) were subcloned in the expression vector pET 28a. The plasmids were again rechecked and were found to be positive both by PCR and restriction enzyme analysis (data not shown).The *E.coli* expressed different hormone subunits were checked by

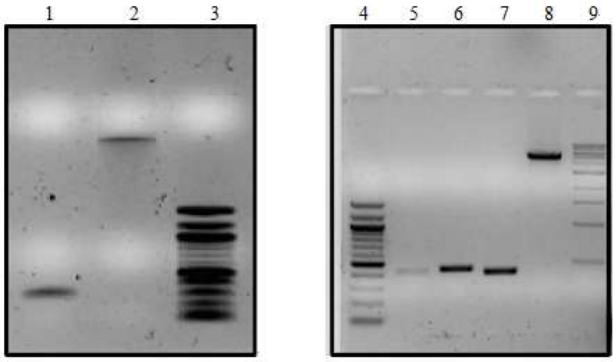
SDS-PAGE and western blot analysis (Figure 5). The alpha subunit could be seen a 14 kDa protein and was expressed in relatively good amounts. Similarly the LH β subunit was expressed as a 45 kDa protein and FSH β subunit was expressed as 20 kDa protein but the amounts were too low. The FSH β could be detected by a monoclonal His-tagged antibody only. However, the TSH β subunit could not be expressed in the bacterial system.

Figure 3: This figure shows PCR amplified products of different genes from buffalo pituitary first strand cDNA of FSH beta 390 bp (B) and LH beta 426 bp (D). A 100 bp DNA Marker is loaded along with to ascertain the exact size



The bacterial lysate was loaded on the Ni-NTA affinity matrix and the bound protein was differentially eluted. The A280 has been recorded and plotted as a function of fraction number The fractions showing peak absorbance were also analyzed by SDS-PAGE. The peak fractions were pooled and the total protein was estimated. The protein content for the LH β subunit was 1.5-2 mg/100 mL where as for the α subunit it was 2-2.3 mg/ 100 mL of the induced culture. The FSH β subunit expression was too low as the protein yield was even below 0.3 mg/100 mL of the bacterial culture. Further the elution from Ni-NTA column was standardized and it was found out that it is the introduction of the imidazole which necessarily elutes the bound protein from the column rather than the pH change. The peak fractions from the Ni-NTA chromatography were also checked on SDS-PAGE to see the purification profile. The 250 mM eluted protein was pooled and desalted on a G-50 column and again was shown to be a single band protein by SDS-PAGE (data not shown).The buffalo pituitary alpha subunit was expressed as an inclusion body protein. The inclusion bodies obtained after the lysis of the bacteria were further washed by centrifugation with the plain PB a number of times to remove the other contaminating protein. 6 M urea was used for solubilising the inclusion bodies followed by the purification of the protein over the Ni-NTA column.

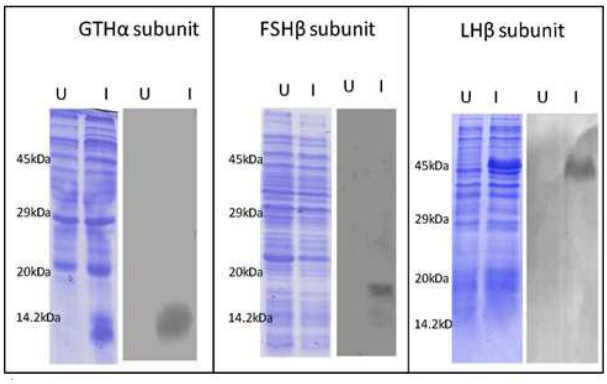
Figure 4: This figure shows the different inserts were purified from agarose gel after restriction enzyme digestion and were cloned in the pET 28a expression vector. Lane 1 has buGn α, Lane 2 has pET 28a plasmid, Lanes 3 and 4 represent 100bp Marker, Lane 5 is FSH β insert, Lane 6 is LH β, Lane 7 is TSH β, Lane 8 is linearized vector and Lane 9 is the 1 Kb marker.



The recombinant subunits were loaded individually on Cibacron Blue column and eluted in tandem with elution buffer (0.05 M Phosphate buffer pH 7.0, 1 M NaCl and 80% Ethylene Glycol). All the eluted fractions were analyzed for their respective immuno reactivity. Based on the nature of the eluant, it can be inferred that the unbound fraction is an extremely hydrophilic and nonionic fraction, while the fraction eluted using 1 M NaCl is an ionic fraction. Finally the fraction eluted using ethylene glycol is an extremely hydrophobic fraction. The native LH *a* subunit appeared in all three fractions i.e. 48.5% as unbound, 39.1% as ionic and 12.35% as

hydrophobic fraction. In the case of freely occurring a subunit, the unbound fraction represented only 31% while there was an increase in the hydrophobic fraction from 12.35% to 29.7%, the same was observed for the recombinant alpha as there was higher percentage 30% of the hydrophobic fraction (Figure 6). Hence the native α subunits are predominantly hydrophilic molecules while the freely occurring and recombinant a subunit exhibits a little more hydrophobicity. In the case of β subunit, the native β subunit had 13.49% unbound, 37.5% ionic and 49% hydrophobic fractions. In the freely occurring β subunits also, there was an increase in the hydrophobic molecules as the percentage hydrophobic fraction of the β subunit increased from 49% to 59% (Figure 6) thereby further asserting the above mentioned hypothesis.

Figure 5: This figure shows the SDS-PAGE and western blot of the different subunits expressed in the bacterial system. The three subunits were expressed namely alpha, FSH beta and LH beta . Molecular weight markers are indicated on the left for the reference. U is uninduced and I is induced protein profile and western blot results.

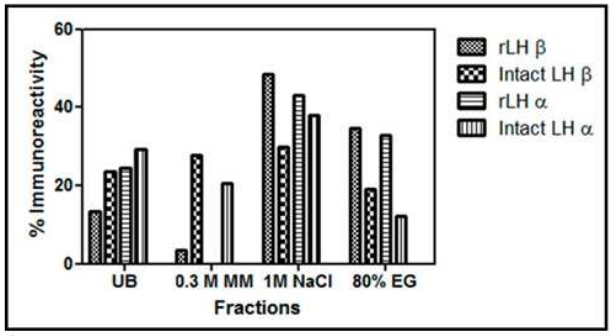


In the present study we report that cDNAs corresponding to the buffalo pituitary glycoprotein hormonal subunits have been cloned and expressed, and sequence was submitted to GenBank. The cDNAs and the expressed protein for different subunits of bubaline glycoprotein hormones into E.coli had the expected composition of the nucleotides and amino acids. These also had predictable physico-chemical properties same as that of the native pituitary derived subunit except increased hydrophobicity.

The different beta subunits also have a varied similarity at the level of cDNA sequences and the deduced amino acid sequences. At the cDNA level the general similarity is about 99% to 96% for the various P subunits and at the protein level the similarities range from 97-94%. That the glycoprotein hormonal subunits have well conserved sequences across the species is well accepted [1]. The beta subunits of the glycoprotein hormones, however, have been reported to be expressed at very lower level in *E.coli* because of a self attenuating region in the 5' sequence of the mRNA. The expression of the N-terminal deletion constructs of the bFSH β have been shown to increase in this case [17]..This may be a probable explanation of the lower expression of the FSH β subunit and non-expression of the TSH β subunit. Further there are very few attempts to express the TSH P subunit in the prokaryotic expression system.

The increase in the 'hydrophobic fraction' (i.e. eluted with 80% ethylene glycol) in the freely occurring subunit as observed in the present study enables us to attribute this to decreased glycosylation. Cibacron blue obviously could distinguish these two grossly different forms.

Figure 6: This figure shows Percentage Immunoreactivity profiles of pure intact LH and recombinant LH subunits loaded on Cibacron Blue 3GA agarose. Unbound protein was washed off using 0.05 M phosphate buffer pH 7.0 (UB), bound proteins were eluted with 0.3 M a-methyl-D- mannoside (0.3 M MM), 1 M sodium chloride (NaCl) and 80% ethylene glycol (EG). All the obtained fractions were assessed with anti- *a* and anti-P bLH antisera. The percentage immunoreactivity of fractions was calculated as given under methods section.



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CONFLICT OF INTEREST

Nil