Research Article

Cloning and Expression of Iranian Turkmen-thoroughbred Horse Follicle Stimulating Hormone in *Pichia pastoris*

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Received: September 27, 2014; **Revised:** February 01, 2015; **Accepted:** April 17, 2015

Background: Follicle stimulating hormone (FSH) plays an essential role in reproductive physiology and follicular development.
Objective: A new variant of the equine *fsh* (*efsh*) gene was cloned, sequenced, and expressed in *Pichia pastoris* (*P. pastoris*) GS115 yeast expression system.
Materials and Methods: The full-length cDNAs of the *efshα* and *efshβ* chains were amplified by reverse transcription polymerase chain reaction (RT-PCR) using the total RNA isolated from an Iranian Turkmen-thoroughbred horse's anterior pituitary gland. The amplified *efsh* chains were cloned into the pPIC9 vector and transferred into *P. pastoris*. The secretion of recombined eFSH using *P. pastoris* expression system was confirmed by Western blotting and immunoprecipitation (IP) methods.
Results: The DNA sequence of the *efshβ* chain accession number JX861871, predicted two putative differential nucleotide arrays, both of which are located in the 3′UTR. Western blotting showed a molecular mass of 13 and 18 kDa for eFSHα and eFSHβ subunits, respectively. The expression of desired protein was confirmed by protein G immunoprecipitation kit.
Conclusions: eFSH successfully expressed in *P. pastoris*. These findings lay a foundation to improve ovulation and embryo recovery rates as well as the efficiency of total embryo-transfer process in mares.

Keywords: Follicle stimulating hormone; Horse; Molecular cloning; *P. pastoris*

1. Background

Superovulation in horses is much less successful than in other domestic species. In addition to superovulation rate, the number of embryos are typically negligible (1). Hence, there is a great demand to develop products that induce multiple ovulations in mares due to changes in the governance of the horse reproduction industry (2). Follicle stimulating hormone (FSH), a member of the glycoprotein hormone family, plays an essential role in this process (3). Generally, the anterior pituitary gland is the tissue that is responsible for releasing this hormone (4-8), and granulosa cells in the ovary are the target cells for the numerous actions of FSH (6, 8-11). Similar to other members of the glycoprotein hormone family, including luteinizing hormone (LH), chorionic gonadotropin hormone (CGH), and thyroid stimulating hormone (TSH), FSH consists of two heterodimer non-covalently linked subunits: a common alpha subunit and a hormone-specific beta subunit (6, 12). An increase in the exogenous FSH concentration in the circulatory system during follicular development may leads to increase in the number of ovulatory follicles, which results in an increase in the number of mature follicles, the ovulatory rate, and the rate of embryo transfer (3). Currently, this method is applicable for both human infertility and animal breeding programs(13). Some gonadotropin sources with FSH-like activity, such as pituitary extraction from domestic animals, pregnant mare serum gonadotropin (PMSG), and FSH extraction

from the urine of postmenopausal women, have been used for the same purpose. Despite the success of some of these treatments in many domestic species, these sources are less effective in mares (14-18). It has been reported that recombinant eFSH (reFSH) shows a much more specific effect in increasing the number of total preovulatory follicles than other therapeutic methods (3). Many of the recombinant proteins that have been successfully produced using the yeast expression system have been used in basic laboratory research and the therapeutic industry (19). The methylotrophic *P. pastoris* yeast is a suitable host for the production of many recombinant proteins because of its easy genetic manipulation, expression of intracellular or extracellular foreign protein (20), and capability to perform eukaryotic protein modifications, such as glycosylation, disulfide bond formation, and proteolytic processing (20-22).

2. Objectives

The purpose of the present research was to express the recombinant horse FSH using the *P. pastoris* expression system and to detect this protein in the secretion media. This study describes the first production of the horse FSH in the yeast expression system using the Iranian native mare *fsh* gene.

3. Materials and Methods

The chemical and biological laboratory reagents and instruments that were used in this study include NucleoSpin RNA kit (Macherey Nagel, Düren, Germany); Revert AidTM H minus first-strand cDNA synthesis kit (Fermentas, Massachusetts, USA); pTZ57R/T vector (Fermentas, Massachusetts, USA); DNA sequencing analysis (MWG, Ebersberg, Germany); *Pichia* expression kit, (Invitrogen, Massachusetts, USA); *P. pastoris* cell culture media (Invitrogen, Massachusetts, USA); Gene-Pulser (Bio-Rad, callifornia, USA); sorbitol (Invitrogen, Massachusetts, USA); Amplicon Ultra 3000 MWCO (Millipore Bedford, Massachusetts, USA); Primary antibodies against the eFSHα and eFSHβ subunits (Santacruz, Texas, USA); horse radish peroxidase (HRP)-conjugated secondary antibodies against eFSHα and eFSHβ subunits (Sigma Aldrich, Missouri, USA); nitrocellulose membranes (Amersham, london, UK); enhanced chemiluminescence (ECL) reagent (Amersham ECL, London, UK); Aautoradiography GBX developer (Sigma Aldrich, Missouri, USA); Amersham Hyperfilm ECL instrument (Amersham, london, UK); and protein G immunoprecipitation kit (Sigma Aldrich, Missouri, USA).

3.1. Strains and Media

The *E. coli* DH5α strain and *P. pastoris* yeast in a *Pichia* expression kit were used as the competent cells. Luria Bertani (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, pH 7.0) was used as the bacterial growth culture at 37°C (25). The *P. pastoris* cell culture media was composed of yeast extract peptone dextrose (YEPD) medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, and 0.1% (w/v) ampicillin), minimal dextrose (MD) medium (1.34% (w/v) yeast nitrogen base with ammonium sulfate without amino acids (YNB), 4×10^{-5}% (w/v) biotin, 2% (w/v) dextrose, 0.05% (w/v) ampicillin, and 1.5% (w/v) agarose), buffered glycerol-complex (BMGY) medium (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, 1.34% (w/v) YNB, 1% (v/v) glycerol, 4×10^{-5}% (w/v) biotin, and 100 mM potassium phosphate buffer pH 6.0) and buffered methanol-complex (BMMY) medium, supplemented with 0.5% (v/v) methanol.

3.2. Cloning of the efshα and efshβ cDNAs into Expression Vector

The total mRNA from the Iranian Turkmen-thoroughbred mare's anterior pituitary gland was freshly extracted using a NucleoSpin RNA kit. The cDNA synthesis was then performed with 1 µg of total mRNA using a Revert Aid™ H minus first-strand cDNA synthesis kit and an oligo (dt) primer that, followed by RT-PCR.

The primers carrying the *Avr*II site (Table 1) were used to amplify the *efshα* and *efshβ* cDNA sequences by polymerase chain reaction (PCR).

The PCR mixture for each subunit consisted of a total volume of 25 µL that included 2 units of *Taq* DNA polymerase, 2 µL of RT-cDNA, 2.5 µL of 10× PCR buffer, 1 µL of 10 mM dNTP, 1.5 µL of 50 mM

Table 1. Oligonucleotide sequences that were designed as the forward and reverse primers for the *efshα* and *efshβ* genes. The *AvrII* restriction sites have been underlined

Primer	Sequence
Forward horse *fshα*	5'CCTAGGAGGAGAGCTATGGATT 3'
Reverse horse *fshα*	5' CCTAGGCACTTGGTGAAACC 3'
Forward horse *fshβ*	5'CCTAGGCCAGGATGAAGTC 3'
Reverse horse *fshβ*	5'CCTAGGGTACACACAGACATCT 3'

$MgCl_2$, and 0.5 µL of 10 pmoles of each forward and reverse primer. The PCR thermocycling steps for *efshα* and *efshβ* genes were as follow in Table 2.

The amplified PCR products were separately cloned through the T/A cloning method using the pTZ57R/T vector and frozen DH5α competent cells. The recombinant bacterial colonies were identified by colony PCR, plasmid DNA digestion using *Avr*II, and DNA sequencing analysis. Ultimately, the gel-purified products were subcloned by ligation into the *Avr*II site of the linearized pPIC9 plasmid and then transformed into the DH5α competent cells through the standard protocol (23).

3.3. Recombinant P. pastoris Transformants

As shown in Figure 1, the recombinant pPIC9-eFSHα and pPIC9-eFSHβ vectors were linearized with *Sal*I and *Sac*I, respectively (*Pichia* expression kit). These recombinant vectors were co-transformed (5 µg) into the host *P. pastoris* GS115 His^+ Mut^+ cells (containing *aox1* gene) by co-electroporation in a Gene-Pulser with a voltage of 1900 V, capacitance of 25 µF, and resistance of 400 ohms (24).

The yeast transformants were resuspended in 1 M sorbitol, and the cell mixture was then spread and cultured on selective MD medium plates (histidine-restricted media) at 29.5ºC for four days. The single colonies for His^+ transformants were inoculated into the YEPD liquid medium and incubated for 48 h at 29.5ºC with 290-rpm agitation. The presence of both *efshα* and *efshβ* genes in *P. pastoris* genome was confirmed by PCR using forward and reverse *aox1* primers. The pPIC9 vector without any insert and the recombinant pPIC9-eFSHα and pPIC9-eFSHβ vectors were used as control groups (*Pichia* expression kit).

3.4. Secretion of the Recombinant eFSH

The positive single-recombinant colonies carrying both recombinant pPIC9-eFSH*α* and pPIC9-eFSHβ vectors were selected and cultured in BMGY medium (25 mL) on a rotary incubator for 18 h at 29.5°C with 290-rpm agitation (25) to reach an OD_{600} value of approximately 3.0 (24). The yeast cells were harvested by centrifugation at 3000 ×g for 5 min and subsequently resuspended and cultivated in BMMY medium (100 mL). The incubation was continued at 29.5ºC for five days. Methanol was added every 24 h to a final concentration of 0.5% to maintain the induction phase. The samples from five consecutive days (days 0 to 5) were collected at 0, 6, 24, 30, 48, 54, 72, 78, 96, 102, and 120 h after induction. The supernatant of the suspension was separated by centrifugation at 13000 ×g for 3 min and stored at -70ºC for use in the biochemical confirmatory tests. The negative controls, which

Table 2. Thermocycling conditions for *efshα* and *efshβ* genes

Stage	Temperature (°C)		Time	The number of cycle	
	efshα	*efshβ*		*efshα*	*efshβ*
Initial DNA denaturation	95	95	5 min	1	1
DNA denaturation	94	94	30 sec	30	35
Annealing	56	51	30 sec		
Extension	72	72	45 sec		
Final extension	72	72	5 min	1	1

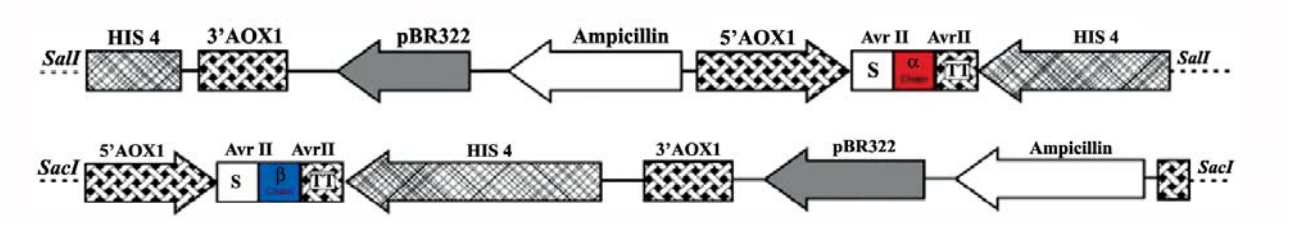

Figure 1. Schematic of linearized the recombinant pPIC9-eFSHα and pPIC9-eFSHβ vectors at the *SalI* and *SacI* restriction sites, respectively. The integration site of each subunit into the pPIC9 plasmid was generated with *AvrII*. TT: transcription termination sequences

included GS115 (His$^+$ Mut^s) cells expressing extracellular albumin, GS115 (His$^+$ Mut$^+$) cells expressing intracellular β-galactosidase, and GS115 (His$^+$ Mut$^+$) cells transformed with non-recombinant pPIC9, were cultured and collected in the same manner (*Pichia* expression kit).

3.5. Western Analysis

The secretion media from the recombinant *P. pastoris* GS115 strain (His$^+$ Mut$^+$) and the control groups were concentrated five-fold using an Amplicon Ultra 3000 MWCO at 13000 ×g. The concentrated samples were electrophoresed in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) apparatuses through the Laemmli method and blotted 16 h overnight onto nitrocellulose membranes. The membranes were incubated in the blocking solution (1 M TBS-Tween-20 plus 3% non-fat milk) for 2 h with shaking at 22ºC. The primary antibodies against the eFSHα (Sc-292422) and eFSHβ (Sc-18222) subunits were diluted at ratios of 1:750 and 1:800, respectively. Each membrane was incubated with a specific primary antibody for 2 h at 22ºC. The membranes were then washed three times with 1 M TBS-Tween-20 solution for 15 min. The corresponding horse radish peroxidase (HRP)-conjugated secondary antibodies against eFSHα (A5420) and eFSHβ (A0545) subunits were diluted at ratios of 1:125000 and 1:500000, respectively. Each membrane was incubated with the corresponding specific diluted secondary antibody for 1.5 h at 22ºC. The membranes were washed five times for 15 min and treated with an enhanced chemiluminescence (ECL) reagent to reveal the immunoreactive bands through an equilibration technique using. The signals were detected on an Amersham Hyperfilm ECL instrument.

3.6. Immunoprecipitation

A protein G immunoprecipitation kit was used to detect the expressed recombinant eFSH in the *P. pastoris* BMMY medium. This enrichment was accomplished by binding the recombinant eFSH with a primary specific β-subunit antibody followed by the precipitation of the immune complexes with protein G. The concentrated sample obtained 54 h after induction was chosen to confirm the previous experiments. First, 600 µL of the sample was mixed with 0.5 to 1 µg of the primary antibody, and the mixture was shaken for 14 h at 4ºC. After the sample was transferred to a filter column, 30 µL of G-protein was added to the column, and the mixture was shaken for 14 h at 4ºC. Subsequently, the obtained purified recombinant eFSHβ sample and the control sample were analyzed using the Western blotting.

4. Results

4.1. Nucleotide Sequence Analysis of efsh Subunits

After the amplification of the *efshα* and *efshβ* genes from cDNA (Figure 2), the sequence analysis of these amplified gene fragments showed that the *efshα* gene had the expected size of 387 base pairs, which confirmed the information included in the GenBank. However, the *efshβ* gene was a fragment with 465 bp. Our sequence analysis was revealed that *eFSHβ* reported here was a bit different (accession no. JX861871**)** to what was in GenBank at its 3′-UTR (Figure 3) with 3 more nucleotides within the 413-to-418 (GCC → GCTGCC) and having a nucleotide substitution at 443 base pair region (TAT instead of TAC).

4.2. Genomic Integration of the pPIC9-eFSHα and pPIC9-e FSHβ Vectors

The recombinant pPIC9-eFSHα and pPIC9-eFSHβ vectors were co-inserted randomly into the genome of *P. pastoris* cells (*Pichia* expression kit). The agarose gel electrophoresis showed that which of the *P. pastoris* GS115 (His$^+$ Mut$^+$) transformants containing both recombinant pPIC9-eFSHα and pPIC9-eFSHβ vectors (Figure 4). Based on the standard protocol (*Pichia* expression kit), the expected size of the *P. pastoris* genomic *aox1* gene and the integrated *efshα* and *efshβ* genes were approximately 2.2 kb, 891 bp and 969 bp respectively. These colonies were then selected and cultured in specific media for expression of the recombinant protein.

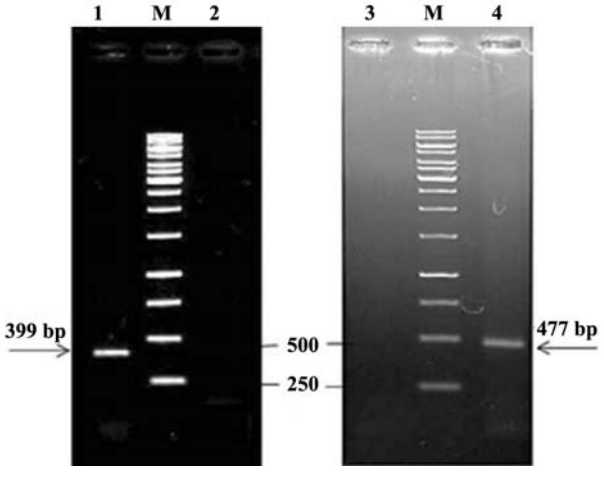

Figure 2. PCR amplification of the nucleotide sequences that encode the *efshα* (left side) and *efshβ* (right side) genes. Lanes 1 and 4 represent the amplified cDNAs for the *efshα* and *efshβ* genes respectively, and lanes 2 and 3 are the negative PCR controls

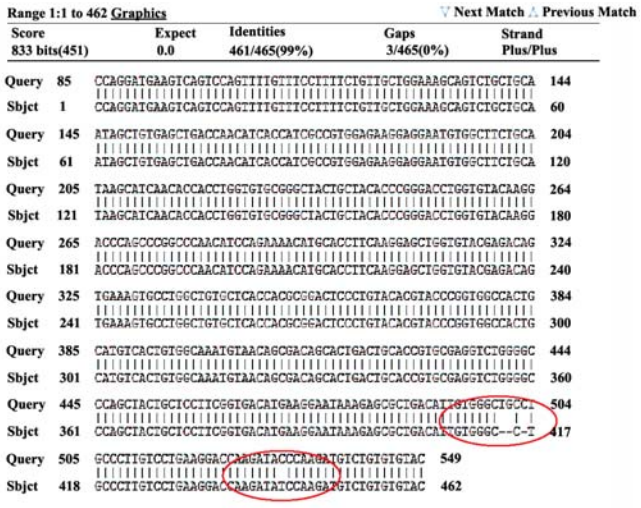

Figure 3. Nucleotide sequence alignment of the Iranian Turkmen-thoroughbred *efshβ* gene. The nucleotide variations are shown with red circular marks: GCC → GCTGCC in the region between base pairs 413 and 418 and TAC → TAT in the region between base pairs 442 and 444

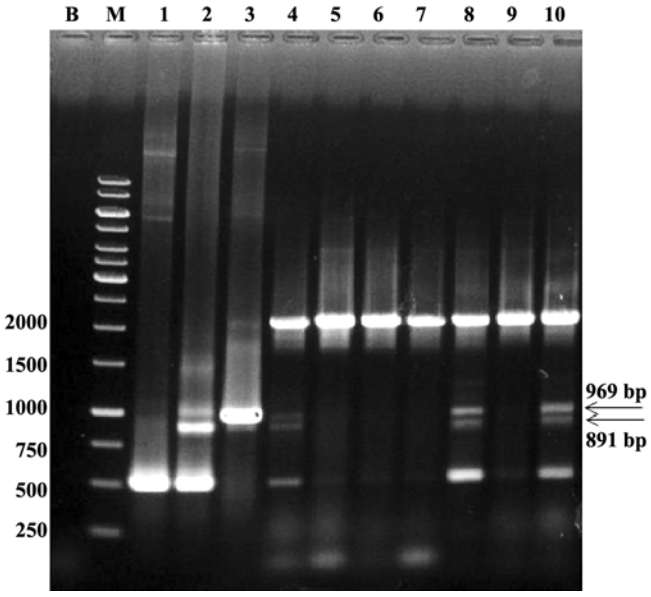

Figure 4. PCR analysis of the transformed *P. pastoris* genome using the *aox1* primers (5′GACTGGTTCCAATTGACAAGC 3′ and 5′ GCAAATGGCATTCTGACATCC 3′). Lane 1 shows the pPIC9 vector carrying the *aox1* gene without any insert (506 base pairs for *aox1* gene), lane 2 shows the pPIC9 vector carrying *efshα* gene (506 base pairs for the *aox1* gene + 387 base pairs for *efshα* gene), and lane 3 shows the pPIC9 vector carrying the *efshβ* gene (506 base pairs for the *aox1* gene + 465 base pairs for *efshβ* gene). The analysis of the *P. pastoris* recombinants in lanes 4 to 10 revealed that single *P. pastoris* colonies in lanes 4, 8, and 10 contain the recombinant vectors for both *efshα* and *efshβ* subunits, because two separated bands were detected in the sites that were equal to those found in control lanes 2 and 3. The observing of a band in the 2.2 kb in lanes 4-10 revealed the wild-type of *aox1* gene that existed naturally in the genome of *P. pastoris* GS115 (His^+ Mut^+)

4.3. Western Analysis

The presence of reFSH in each collected medium sample was confirmed by Western analysis. The Western blotting with specific polyclonal antibodies for each subunit revealed two bands of approximately 13 and 23 kDa, for eFSHα subunit, and two bands of approximately 18 and 29 kDa, corresponding to the eFSHβ subunit (Figure 5). The bands began appearing 24 h after methanol induction, and strong bands were observed 54 h after methanol induction.

4.4. Immunoprecipitation

The IP analysis with a rabbit polyclonal antibody against the FSHβ subunit (a common antibody that is used for both human and equine species) showed a similar pattern between the bands that appeared in the concentrated sample obtained 54 h after induction and the pure human FSH sample (positive control). As shown in Figure 6, two similar bands were found in

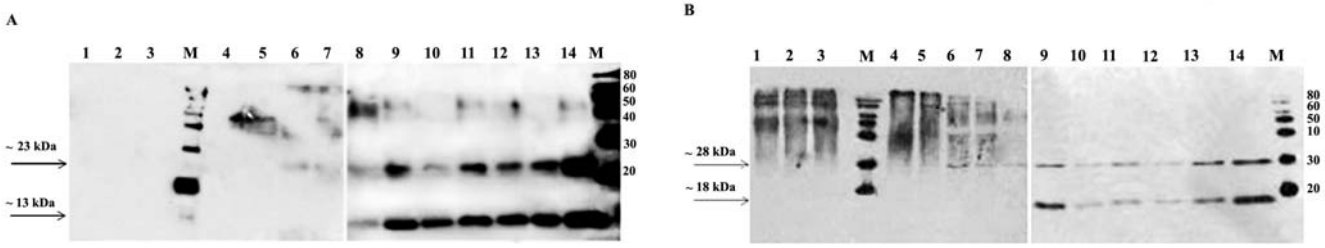

Figure 5. A: Characterization of the *P. pastoris*-expressed recombinant eFSHα and B: eFSHβ subunits by Western blotting analysis. The culture supernatants of the methanol-induced *P. pastoris* transformants were used for protein detection using a 15% SDS-PAGE gel and antibodies raised against the eFSHα and eFSHβ subunits. Lanes 1 to 3 are the control samples that contain *P. pastoris* GS115 cells secreting recombinant albumin protein, *P. pastoris* GS115 cells expressing intracellular recombinant β-galactosidase, and *P. pastoris* GS115 cells containing the expression vector without the *efsh* subunits, respectively. Lanes 4 to 14 are samples collected at different times after methanol induction (0, 6, 24, 30, 48, 54, 72, 78, 96, 102, and 120 h, respectively). The protein bands detected using specific antibodies for each subunit are marked in A and B

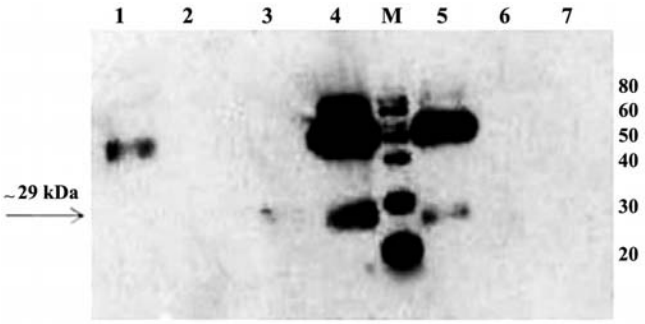

Figure 6. Western blotting analysis of the IP samples using an antibody against the eFSHβ subunit. The sample was obtained from *P. pastoris* secretion medium at 54 h after methanol induction. Lanes 1, 4 and 5 are the IP samples from the *P. pastoris* culture medium secreting the recombinant albumin protein (negative control), *P. pastoris* culture medium secreting the recombinant eFSH protein, and the recombinant human FSH (positive control), respectively. Lanes 2 and 7 are the samples obtained after the addition of a specific antibody to the filter columns containing recombinant eFSH and the recombinant human FSH sample (positive control), respectively. Lanes 3 and 6 are the samples that were collected after washing buffer solution was passed through the sample columns for the recombinant eFSH and the recombinant human FSH, respectively

each lane at the same position: the first band is approximately 29 kDa, and the second band is 50 kDa. We predicted that the smaller band corresponds to the FSHβ subunit as it could not be detected in the negative control, and the larger band can be corresponded either to the G-protein or our product.

5. Discussion

The eFSH protein has been used to improve ovulation and embryo recovery rates, which can most likely increase the efficiency of the total embryo-transfer process (26). Jennings *et al*. (2009) reported that the stimulation of follicles with reFSH may be more potent than the stimulation with purified eFSH, which would lead to an increase in the follicular growth rate in mares (3). Thus, it is obvious that the production of reFSH has a practical application in the equine reproduction industry. Therefore, the current study provides the first report of the utilization of the yeast *P. pastoris* expression system for the secretion of a new variant of the Iranian Turkmen-thoroughbred horse *fsh* gene. Saneyoshi *et al*. (2001) reported a 462 bp sequence for *efshβ* cDNA (27). The amino acid sequence that was deduced from their study is similar to the data that were reported by Fujiki *et al*. (1978) (3). However, our findings showed a 465 bp sequence for the *efshβ* with some significant variations in 3′-UTR. The heterologous expression of proteins with disulfide bonds often fails in bacteria (28, 29). As a eukaryotic expression system, *P. pastoris* was chosen because of its cost-effectiveness, potency for appropriate post translational modifications, secretion of foreign proteins into the culture medium (25), and a respiratory rather than a fermentative mode of growth that lowers the production of toxic waste materials in a high-cell-density environment (30). In fact, the expression of recombinant mammalian GTHs, such as porcine FSH (7), ovine FSH (3), and human chorionic gonadotropin (hCG) (31), in *P. pastoris* has shown equal biological activity compared with the native GTHs. Thus, *P. pastoris* was used for the expression of reFSH. We co-integrated the *efshα* and *efshβ* genes into the *P. pastoris* genome by separated signal peptide sequences. This secretion pattern is consistent with the native form, in which the two subunits are co-expressed using two independent expression constructs. Yu *et al*. (2010) reported that the abovementioned form of protein secretion is much more efficient than the use of a common signal peptide leader (25). The integration of a foreign gene into the *P. pastoris* genome is a preferential method (3) that causes more stable transformants (32) and thus leads to a high expression level of the heterologous gene (3). The recombinant gene constructs were co-electroporated into the *P. pastoris* genome based on different sites to increase the possibility of the co-insertion of both gene constructs into the genome. The analysis of the Western blotting results revealed that each subunit of the reFSH molecule was successfully synthesized and secreted into the *P. pastoris* culture medium; however, the results showed two specific bands for each subunit. Based on reports by Kasuto and Levavi-Sivan (2005), it is likely that the larger protein bands (23 and 29 kDa bands in the Figure 5) are highly glycosylated forms of the subunits or multimers (33). The existence of some bands greater than 50 kDa that immunoreacted with the eFSHα and even the eFSHβ antisera can be considered nonspecific bands that originated from the yeast (34). Although successful expression of recombinant FSH was reported in CHO by Fachal *et al*. (2010), the purification of this intracellular protein is often tedious and time consuming. In the current study, production of the recombinant eFSH was conducted in *P. pastoris* using extracellular secretion system. Application of this system resulted in secretion of the protein into the media which led to simple afterward purification. Post-translational modification is a concern in production of recombinant proteins in *P. pastoris*. However, this concern could be solved through application of

Glyco-Switc strain with the ability of post translational modifications same as mammalian cells (35-37). Meanwhile, further studies are required to compare the functionality of the *P. pastoris*-derived eFSH in mares or other species.

Acknowledgments

This study was substantially funded by the Royan Institute, Tehran, Iran. We express our appreciation to Gorgan University of Agricultural Sciences and Natural Resources and to the Royan proteomics lab for facilitating the research phases. In addition, the authors thank Zahra Mansouri and Najmeh Sadat Masoudi for their kind assistance.

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