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# Molecular cloning and characterization, and prokaryotic expression of the *GnRH1* gene obtained from Jinghai yellow chicken

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**ABSTRACT.** The gonadotropin-releasing hormone (GnRH) plays an important role in the control of reproductive functions. Recent studies have reported the occurrence of *GnRH* molecular variants in numerous species. In this study, the *GnRH1* gene from Jinghai yellow chicken was cloned by reverse transcriptase-polymerase chain reaction and transformed into BL21 (DE3) competent cells. The *GnRH1* gene and amino acid sequences were subjected to bioinformatic analyses. The *GnRH1* gene nucleotide sequence was discovered to be 352 bp long, containing a coding, promoter, and section of the 3'-regions. The *GnRH1* gene shared 93, 81, 54, 58, 61, 76, 76, 59, 76, and 66% sequence identity with *Meleagris gallopavo*, *Columba livia*, *Homo sapiens*, *Bos taurus*, swines, *Capra hircus*, *Ovis aries*, *Pantholops hodgsonii*, *Equus caballus*, and *Rattus norvegicus*, respectively. The *GnRH1* gene

 

showed conserved domains. The GnRH1 protein was a secreted protein comprising 92 amino acids, with a molecular weight of 10205.6 Da and a theoretical pI of 5.67. Most of the amino acid residues were observed to be hydrophilic, indicating water solubility. The predicted secondary structures of proteins included α-helices (h; 23.08%), β-extensions (e; 10.92%), and random coils (c; 66.0%). The successful construction of prokaryotic expression vector pET32a-*GnRH1* was confirmed by restriction and sequence analysis. SDS-PAGE analysis showed the successful expression of recombinant plasmid in *Escherichia coli* BL21 (molecular weight = 25-28 kDa). Larger quantities of protein were expressed in supernatant, indicating greater expression in soluble form. Western blot analysis confirmed the expression of the target protein.

**Key words:** Jinghai yellow chicken; Cloning; Bioinformatic analysis; Prokaryotic expression; *GnRH*

## INTRODUCTION

Gonadotropin-releasing hormone (*GnRH*), the central initiator of the reproductive hormonal cascade, was first isolated from the mammalian hypothalamus as a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$) (Schally et al., 1971; Matsuo et al., 1971; Baba et al., 1971). *GnRH* is processed from a precursor polypeptide in specialized neurons in the hypothalamus by enzymatic processing, and packaged in storage granules that are transported through axons to the external zone of the median eminence (Seeburg et al., 1987; Fink, 1988). The hormone is released in synchronized pulses from the nerve endings of approximately 1000 neurons to the hypophyseal portal system every 30-120 min, in order to stimulate the biosynthesis and secretion of luteinizing hormone (*LH*) and follicle-stimulating hormone (*FSH*) in pituitary gonadotrophs (Fink, 1988). *GnRH* is well documented to be the primary hypothalamic regulator of LH release in both spontaneous and induced ovulators (Bakker and Baum, 2000) in mammals. *GnRH* also plays a pivotal role in the control of avian reproduction. Sexually active birds have been reported to express more immunoreactive *GnRH* neurons and fibers than in sexually inactive or photo-refractory birds (Sharp et al., 1990; Hahn and Ball, 1995; Parry et al., 1997; Cho et al., 1998).

Earlier, *GnRH* was considered to be a unique molecular form; however, subsequent studies have reported the occurrence of *GnRH* molecular variants in numerous species. The *GnRH* family currently includes over 25 different isoforms: 14 and 11 in the representative vertebrate and invertebrate species, respectively. Most phylogenetic analyses support the existence of three paralogous groups of *GnRH* in gnathostomes (jawed vertebrates): *GnRH1*, *GnRH2*, and *GnRH3* (Tostivint, 2011). The *GnRH1* group contains the hypophysiotropic GnRH forms that are expressed by neurons located in the pre-optic area. *GnRH1* regulates gonadal maturation through the stimulation of pituitary gonadotropins. The *GnRH2* group, which is structurally highly conserved from fish to mammals, contains all *GnRH* forms expressed by the midbrain neurons. *GnRH2* is believed to modulate sexual and feeding behavior. Finally, the *GnRH3* group, which is currently known only in teleost fish, contains *GnRH* forms that are expressed by neurons located in the ventral forebrain. *GnRH3* has been demonstrated to exert neuromodulatory functions (Fernald and White, 1999; Okubo and Nagahama, 2008).

Previous studies have demonstrated the isolation of two *GnRH* molecular variants from chicken brains: *cGnRH1* (King and Millar, 1982; Miyamoto et al., 1983) and *cGnRH2* (Miyamoto et al., 1984). Of the two subtypes, *GnRH1* is directly involved in the control of reproduction in domestic chickens (Sharp et al., 1990). The *GnRH1* gene is located on chromosome 22 in chickens, and codes for the 92 amino acid GnRH1 protein, which is eventually processed to the *GnRH1* decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-$NH_2$).

So far, despite the extensive studies on the structure of the *GnRH1* gene in chicken, limited information is available on the molecular regulation of reproduction in chicken. As most studies relating to the *GnRH1* gene have been conducted on mammals and fish (and not chicken), and because of the important role of the *GnRH1* gene, it is essential to elucidate the role of the *GnRH1* gene in chicken. We have, therefore, studied the same in Jinghai yellow chicken. For this purpose, we have cloned the cDNA for the *GnRH* gene obtained from the Jinghai yellow chicken, because of the evolved number of variants. In addition, our studies on the *GnRH1* genes in the Jinghai yellow chicken would assist in the understanding of the regulatory mechanism of the *GnRH1* gene. In this study, the cDNA of the *GnRH1* gene from Jinghai yellow chicken was cloned by reverse transcriptase-polymerase chain reaction (RT-PCR), followed by bioinformatic analysis. The *GnRH1* gene was cloned to a pET32a vector for expression in BL21.

## MATERIAL AND METHODS

### Chicken strains

Jinghai yellow chicken is a small, high-quality broiler, which displays consistent body conformation, excellent performance, and stable genetic traits. The chickens were cultivated by traditional genetic breeding of the local yellow chicken from Nantong city, Jiangsu Province, China, after five generations. Both were used for cultivation. The details of breeding were shown in Figure 1 (Wang et al., 2013). The hypothalamus was collected from 20-week-old Jinghai yellow chicken and stored at -80°C.

### Extraction of total RNA and RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer protocol, and treated with 2 µL DNase (10 U/µL) (Sangon Biotechnology Co., Ltd., Shanghai, China), in order to remove DNA contaminants. First-strand cDNAs were synthesized using 2 µg total RNA with a cDNA synthesis kit (TaKaRa, Otsu, Japan) as per the user manual. A pair of primers was designed based on the published cDNA nucleotide sequence of the *Gallus gallus GnRH1* gene. The primers (*GnRH1*-forward: 5'-CAGCGGGAAGAGTTGGAG-3'; *GnRH1*-reverse: 5'-CTGGGTTTGTTGATGGTGTT-3') were synthesized by Sangon Biotech (Sangon Biotechnology Co., Ltd.). The reaction mixture for PCR was composed of a total volume of 20 µL, containing 2 µL 10X PCR buffer, 0.8 µL dinucleotide triphosphate mix (2.5 mM), 2.2 µL $MgCl_2$, 0.5 µL Taq DNA polymerase (5 U/µL), 1 µL of each primer, 1 µL first-strand cDNA, and 11.5 µL deionized water (Sangon Biotechnology Co., Ltd.). The PCR cycle conditions were set as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, ending with an

extension at 72°C for 10 min. PCR products were separated by electrophoresis on 1% agarose gels and visualized by scanning with the Nanodrop 1000 system (Thermo Fisher Scientific, Waltham, MA, USA).

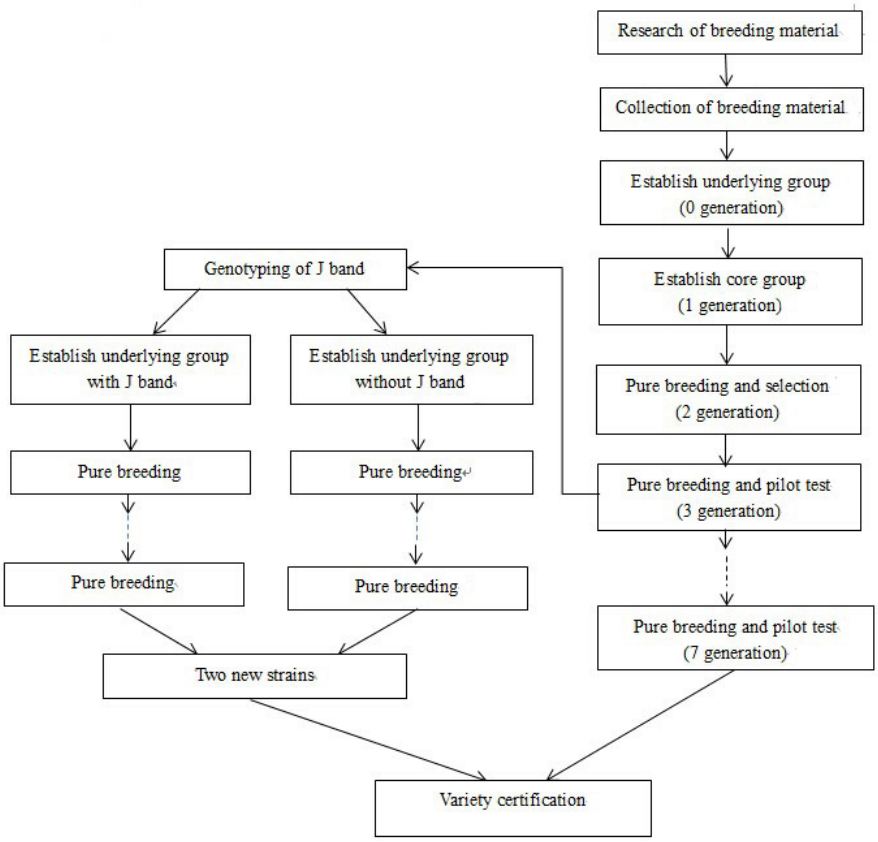


**Figure 1.** Selection and breeding process of Jinghai yellow chicken. Jinghai yellow chicken was cultivated using both traditional breeding method and molecular breeding method. The DNA fingerprinting J band was used in genotyping.

## Cloning of the *GnRH1* gene in Jinghai yellow chicken

RT-PCR products were purified using the SanPrep Column Gel DNA Fragment Recovery Kit (Sangon Biotechnology Co., Ltd.) and inserted into a pUCm-T vector (Sangon Biotechnology Co., Ltd.) according to the manufacturer protocol. The recombinant plasmid was transformed into competent *Escherichia coli DH5α* cells. White colonies were picked after 12-16 h, and the inserts analyzed by restriction analysis using *Eco*RI and *Hin*dIII. Five positive recombinant clones were sequenced by Sangon Biotechnology Co., Ltd.

## Sequence analysis

The open reading frame (ORF) of the Jinghai yellow chicken *GnRH1* gene was pre-

dicted using the DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA), leading to the deduction of the amino acid sequence. The resulting sequences were confirmed using the Basic Local Alignment Search Tool (BLASTx) on the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Four phylogenetic trees were constructed using the ClustalX and DNAMAN programs. The phosphorylation sites on the GnRH1 amino acid sequences were forecasted using the NetPhos2.0 tool (http://www.cbs.dtu.dk/services/NetPhos/). The hydrophilicity of the GnRH1 amino acid sequences from the Jinghai yellow chicken was analyzed by ProtScale (http://web.expasy.org/protscale/); SignalP4.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to analyze the potential signal peptide cleavage sites (Petersen et al., 2011); the simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/) was used to predict the conserved domains in amino acid sequences. The basic physicochemical properties of GnRH1 proteins were analyzed using the ProtParam tool (http://web.expasy.org/protparam/); TargetP (http://www.cbs.dtu.dk/services/TargetP/) and PSORT II Prediction (http://psort.hgc.jp/form.html) tools were used for the prediction of GnRH1 protein subcellular localization, while the Hopfield neural network (HNN) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) was used to predict the secondary structure of the GnRH1 protein (Combet et al., 2000).

## Construction of prokaryotic expression vector pET32a-*GnRH1*

Based on the sequencing results, the codons of the *GnRH1* gene were optimized and the signal peptides removed. Two restriction sites, *Kpn*I and *Xho*I, were added to both ends of the *GnRH1* gene, and a 222-bp gene sequence was obtained. The gene was synthesized by Sangon Biotechnology Co., Ltd. The synthesized gene and the pET32a vector were subjected to double digestion by *Kpn*I and *Xho*I restriction enzymes. The gene was ligated into the pET32a vector by T4-DNA ligase (Sangon Biotechnology Co., Ltd.), at 16°C for 16 h. The recombination vector was then transduced into *E. coli DH5α* competent cells treated with $CaCl_2$. Finally, the pET32a-*GnRH1* plasmid was transduced into BL21 (DE3) competent cells for expression. The positive plasmids were checked by restriction enzyme digestion. Positive plasmids were also sequenced by Sangon Biotech Co., Ltd. in order to confirm the presence of the insert in the recombinant.

## Induced expression of pET32a-GnRH1

The positive host strain (30 μL) was inoculated into 2 mL Luria-Bertani (LB) liquid medium containing ampicillin (100 mg/mL), and the culture was incubated at 37°C overnight. This seeding medium (1 mL) was transferred to 50 mL fresh LB liquid medium at a 1:50 ratio, and cultured at 37°C until an OD (600 nm) of 0.4-1.0 was obtained. In order to obtain the appropriate inducible concentration for expression, the bacterial culture was divided and induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sangon Biotechnology Co., Ltd.), at 15°, 25°, and 30°C for overnight cultures and at 37°C for a 5-h culture. The bacteria in each group were clarified by microcentrifugation at 13000 *g* for 30 s. These were then collected and lysed by Bacterial Lysis Buffer (Sangon Biotechnology Co., Ltd.), and both the supernatant and precipitant of the cell lysates were denatured by boiling for 5 min with 2X sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Sangon Biotechnol-

ogy Co., Ltd.). The samples were then loaded on 12% SDS-PAGE.

## Purification of recombinant protein and Western blot

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed using a radioimmunoprecipitation assay lysis buffer (Applygen Technologies Inc., Beijing, China) for 15 min on ice. Cell lysates were then clarified by microcentrifugation at 12,000 *g* for 10 min at 4°C. Equal amounts of protein (40 μg) were quantified and separated on 12% SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Bedford, MA, USA) by electrophoresis. Membranes were then blocked with PBS containing 0.05% Tween 20 and supplemented with 5% non-fat dry milk, for 1 h at room temperature.

Since the recombinant protein carried an N-terminal His-tag, the GnRH1 protein could be purified using the HisTrap HP column charged with $Ni^{2+}$ ions (GE Healthcare, Little Chalfont, UK). Before elution of the target protein, the nonspecific proteins were washed from the column using the binding buffer (20 mM imidazole, 500 mM NaCl, and 20 mM $NaH_2PO_4$). The target proteins were eluted using the elution buffer (500 mM NaCl, 20 mM $NaH_2PO_4$, at a linear gradient of 50-300 mM imidazole). The purified proteins were analyzed using SDS-PAGE and Western blot (Jian et al., 2013).

# RESULTS

## Cloning of the *GnRH1* gene

Based on the published cDNA sequence of *G. gallus* obtained from GenBank, a pair of primers was designed to amplify the coding sequence of the *GnRH1* gene in Jinghai yellow chicken. The results of RT-PCR demonstrated the presence of specific bands in the 352-bp region (Figure 2), which is consistent with the expected results. Double-digestion analysis of recombinant plasmid by *Eco*RI and *Hin*dIII indicated the successful insertion of the *GnRH1* gene into the pUCm-T vector (Figure 3).

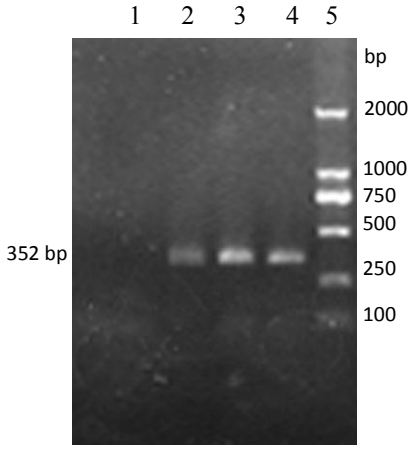


**Figure 2.** Gel electrophoresis results of *GnRH1* RT-PCR products. *Lane 1* = negative control; *lanes 2*, *3*, *4* = RT-PCR products of the *GnRH1* gene; *lane 5* = DL2000 DNA marker.

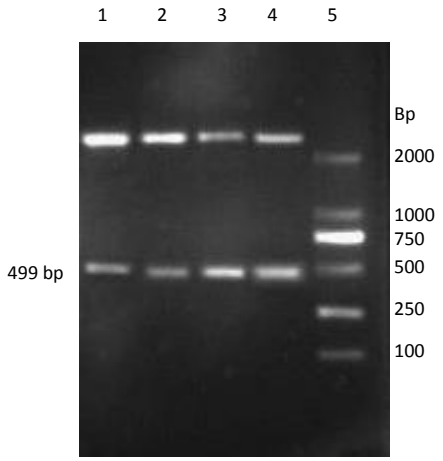


**Figure 3.** Restriction analysis of recombinant plasmids. *Lanes 1*, *2*, *3*, and *4* = double-digestion products of recombinant plasmids; *lane 5* = DL2000 DNA marker. As the restriction sites were used on the vector, the products of double digestion were longer than the RT-PCR products.

## Sequencing and sequence analysis of the *GnRH1* gene

The sequencing result showed the successful cloning of 352 bp of the *GnRH1* gene, which contained the coding (CDS) region, promoter region, and a section of the 3'-region (Figure 4). The amino acid sequence was also determined using the DNAMAN program. A 279-bp ORF coded for a protein 92 amino acids long. The results of the BLAST analysis showed that the *GnRH1* gene nucleotide sequence in Jinghai yellow chicken shared 93, 81, 54, 58, 61, 76, 76, 59, 76, and 66% identity with *Meleagris gallopavo*, *Columba livia*, *Homo sapiens*, *Bos taurus*, swines, *Capra hircus*, *Ovis aries*, *Pantholops hodgsonii*, *Equus caballus*, and *Rattus norvegicus*. Five phylogenetic trees were constructed using the ClustalX and DNAMAN programs (Figures 5, 6, 7, 8, and 9). The bootstrap value was set to 2000. The clustering results obtained from the NJ, draw, observed divergency, Kimura, and maximum likelihood methods were consistent. The genetic distance between Jinghai yellow chicken and *G. gallus* was observed to be the shortest, in contrast to the genetic distance between Jinghai yellow chicken and *Rana catesbeiana*. Jinghai yellow chicken initially clustered together with *G. gallus*, followed by *M. gallopavo*, *C. livia*, and others.

## Protein structure analysis of GnRH1

### *Analysis of physical and chemical properties*

Analysis by ProtParam showed that the GnRH1 protein was composed of 92 amino acids. The molecular weight and theoretical pI were determined to be 10205.6 Da and 5.67, respectively. The GnRH1 protein contained 20 types of amino acids; Glu accounted for the highest proportion of 13.0%, while Asp and Trp accounted for the lowest proportion at 1.1%. The instability index was computed to be 50.05, which classified the GnRH1 protein as being unstable. Furthermore, the aliphatic index was observed to be 84.89, grand average of hydropathicity was -0.403, and the estimated half-life was 30 h.

```
          10         20         30         40         50         60
1         CAGCGGGAAGAGTTGGAGCGATTCTGCAGCCTCTCTCAGCAAACAGAATGGAGAAGTCCA
                                                      M  E  K  S

          70         80         90         100        110        120
61        GGAAGATCTTGGTCGGTGTCCTCCTGTTCACCGCATCTGTGGCAATCTGCTTGGCTCAAC
          R  K  I  L  V  G  V  L  L  F  T  A  S  V  A  I  C  L  A  Q

          130        140        150        160        170        180
121       ACTGGTCTTATGGCCTGCAACCAGGAGGAAAAAGGAACGCTGAAAATCTAGTGGAATCAT
          H  W  S  Y  G  L  Q  P  G  G  K  R  N  A  E  N  L  V  E  S

          190        200        210        220        230        240
181       TTCAAGAGATCGCAAACGAAATGGAAAGTCTAGGAGAAGGGCAGAAGGCTGAATGCCCTG
          F  Q  E  I  A  N  E  M  E  S  L  G  E  G  Q  K  A  E  C  P

          250        260        270        280        290        300
241       GCTCTTACCAGCATCCCAGGCTGAGTGATCTGAAGGAAACCATGGCAAGCCTGATCGAAG
          G  S  Y  Q  H  P  R  L  S  D  L  K  E  T  M  A  S  L  I  E

          310        320        330        340        350
301       GAGAAGCCAGAAGAAAGGAGATTTAAATCCACAACACCATCAACAAACCCA
          G  E  A  R  R  K  E  I  *
```

**Figure 4.** Nucleic acid and amino acid sequences of GnRH1 obtained from Jinghai yellow chicken. 1-47 bp, the promoter region; 48-326, the coding region; 327-351, the 3-prime region.

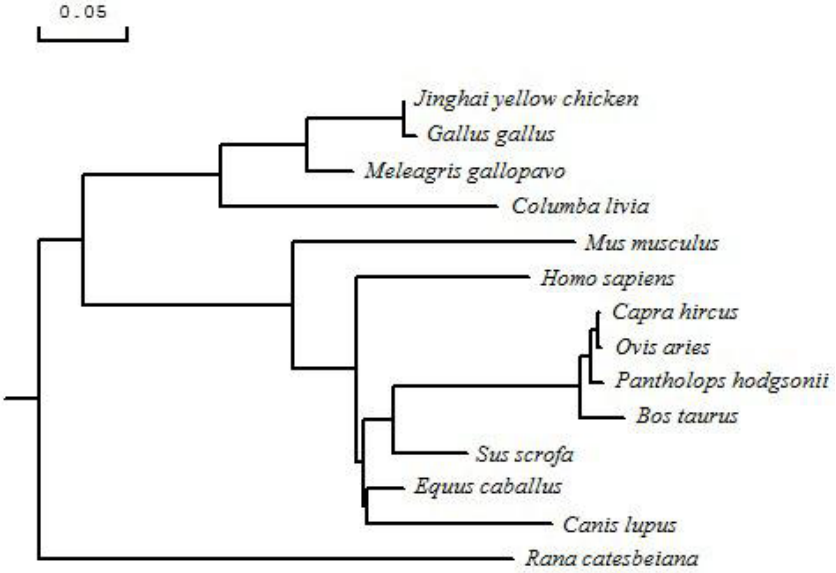


**Figure 5.** Phylogenetic tree constructed by draw method using the ClustalX program. Jinghai yellow chicken initially clustered together with *Gallus gallus*, followed by *Meleagris gallopavo*, *Columba livia*, and others.

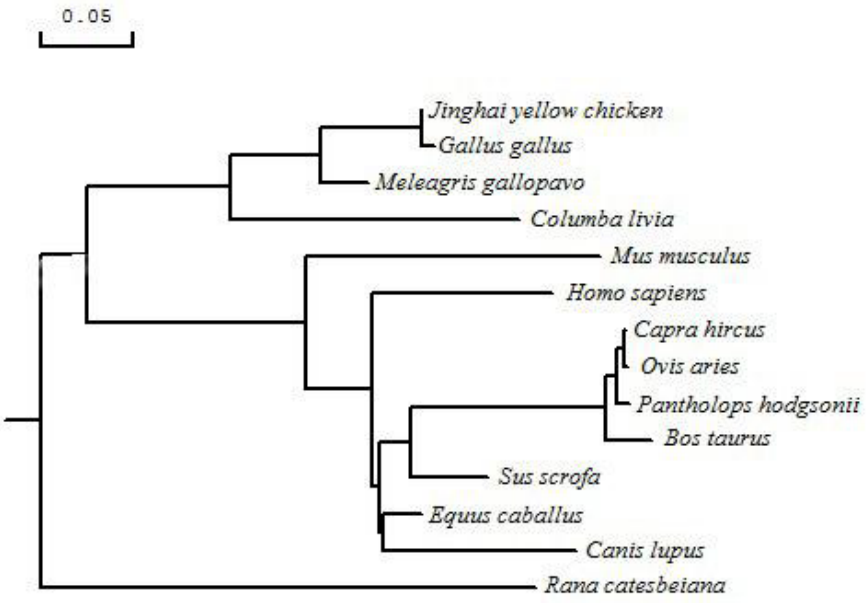


**Figure 6.** Phylogenetic tree constructed by NJ method using the ClustalX program. Jinghai yellow chicken initially clustered together with *Gallus gallus*, followed by *Meleagris gallopavo*, *Columba livia*, and others.

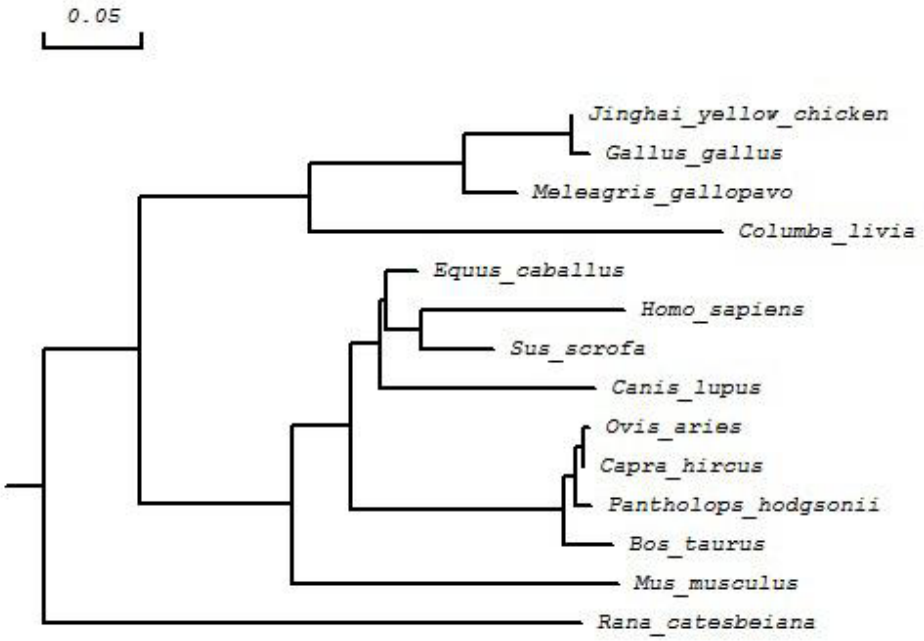


**Figure 7.** Phylogenetic tree constructed by the observed divergency method using the DNAMAN program. Jinghai yellow chicken initially clustered together with *Gallus gallus*, followed by *Meleagris gallopavo*, *Columba livia*, and others.

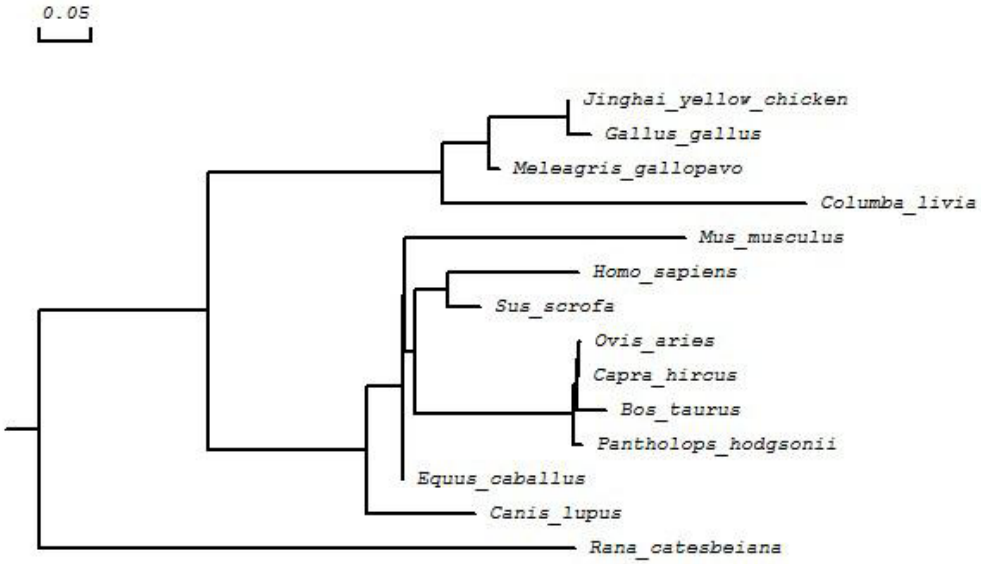


**Figure 8.** Phylogenetic tree constructed by Kimura method using the DNAMAN program. Jinghai yellow chicken initially clustered together with *Gallus gallus*, followed by *Meleagris gallopavo*, *Columba livia*, and others.

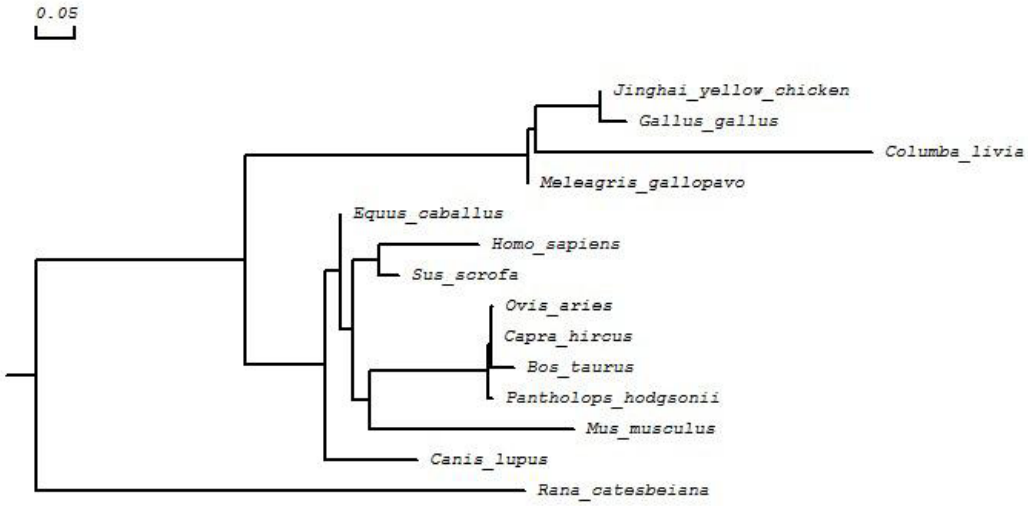


**Figure 9.** Phylogenetic tree constructed by maximum likelihood method using the DNAMAN program. Jinghai yellow chicken initially clustered together with *Gallus gallus*, followed by *Columba livia*, *Meleagris gallopavo*, and others.

## *Subcellular localization analysis*

Analysis by TargetP program showed that the GnRH1 protein was synthesized by ribosomes on the endoplasmic reticulum, and oriented to the target sites by signal peptide after synthesis. The GnRH protein sequence analysis using the PSORT II prediction program

indicated that the GnRH1 protein belonged to the secreted protein family, with the biological role being activated after being secreted to the outside of the cell (Table 1).

**Table 1.** Subcellular localization analysis of the GnRH1 protein.

| Program | Subcellular localization | Proportion (%) |
|---|---|---|
| TargetP | mTP | 3.5 |
| | SP | 92.7 |
| | Others | 5.2 |
| PSORT II | Outside | 82.0 |
| | Endoplasmic reticulum (membrane) | 10.0 |
| | Endoplasmic reticulum (lumen) | 10.0 |
| | Lysosome (lumen) | 10.0 |

## *Signal peptide splice site prediction of the GnRH1 protein*

The signal peptide splice site of the GnRH1 protein was predicted using SignalP-4.1. A cleavage site was observed between 23- and 24-amino acid regions, and the amino acids from 1 to 23 were observed to be the signal peptide (Figure 10).

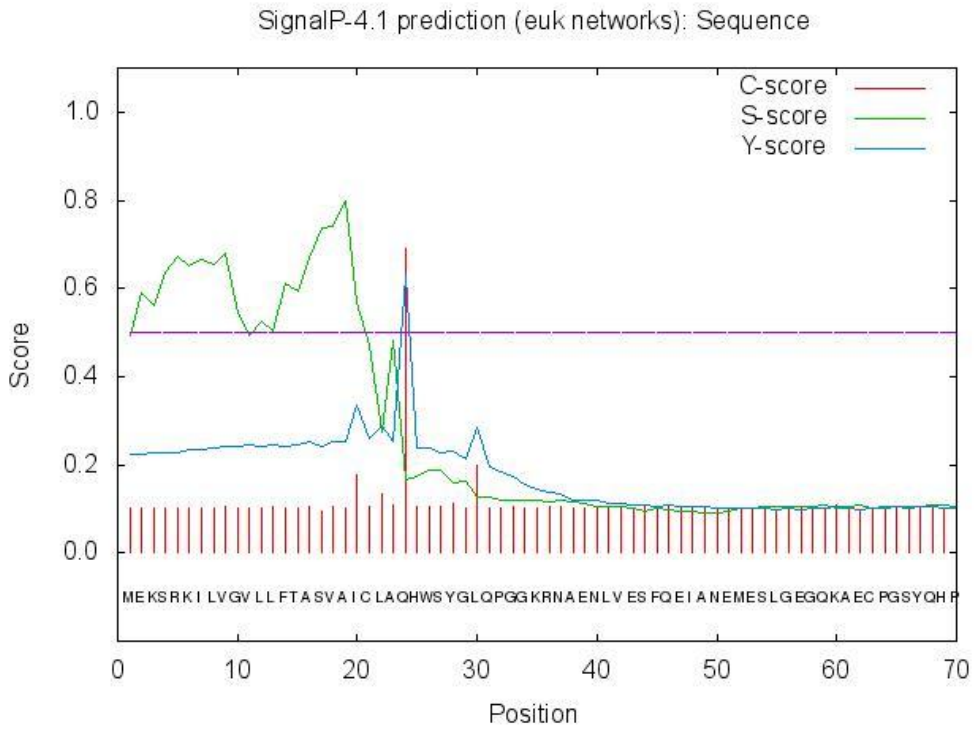


**Figure 10.** Signal peptide splice site prediction for the GnRH1 protein. A peak was detected between 23 and 24 amino acid, which maybe be a cleavage site.

### *Transmembrane helix structure prediction and conservative domain analysis of GnRH1*

Transmembrane structure was predicted using the TMHMM Server v.2.0. The amino acids from 7 to 29 were discovered to be possible transmembrane helices (Figure 11), whereas prediction by TMpred indicated that the amino acids from 7 to 26 were possible transmembrane helices (Figure 12). The Smart software speculation showed that no conservative domains were found in the GnRH1 protein, and that amino acids from 7 to 29 were possible transmembrane helices. Therefore, we hypothesize that amino acids from 7 to 26 were possible transmembrane helices of the GnRH1 protein.

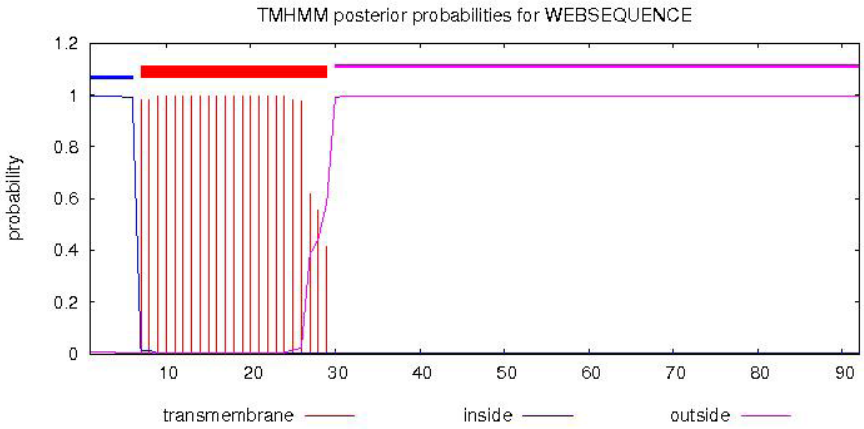


**Figure 11.** Transmembrane helix structure prediction by TMHMM. Horizontal ordinate was the amino acid position and vertical coordinate was the probability of transmembrane helices. Red was the transmembrane region.

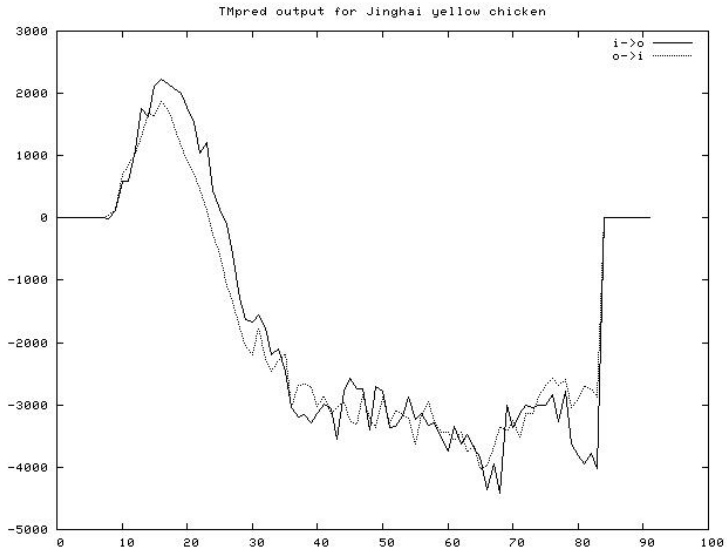


**Figure 12.** Transmembrane helix structure prediction by TMpred. Horizontal ordinate was the amino acid position and vertical coordinate was the score of transmembrane helices.

## *Analysis for potential phosphorylation sites in GnRH1*

The presence of phosphorylation sites was determined by NetPhos 2.0. We discovered that the amino acid sites 44-Ser, 54-Ser, 73-Ser, and 81-Ser could be the potential protein kinase phosphorylation sites of the GnRH1 protein from Jinghai yellow chicken (Figure 13).

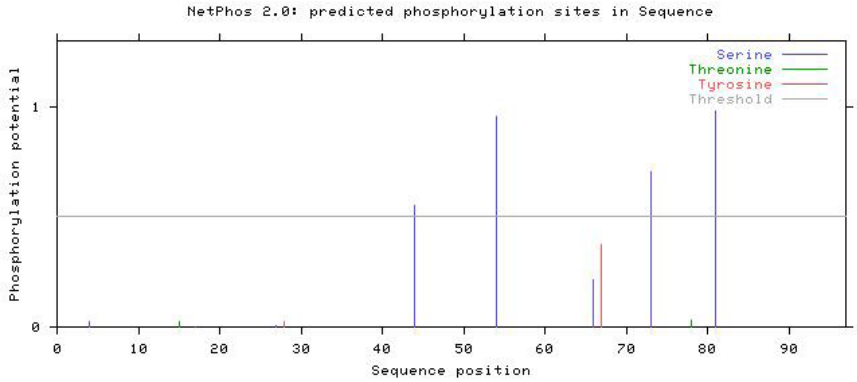


**Figure 13.** Analysis for potential phosphorylation sites in the GnRH1 protein.

## *Hydrophobicity and hydrophilicity analysis of GnRH1*

ProtScale from the expert protein analysis system (ExPASy) domain was used to analyze the hydrophobicity of the GnRH1 protein (Figure 14). Most of the amino acid residues were discovered to be hydrophilic; therefore, the GnRH1 protein might be a water-soluble protein.

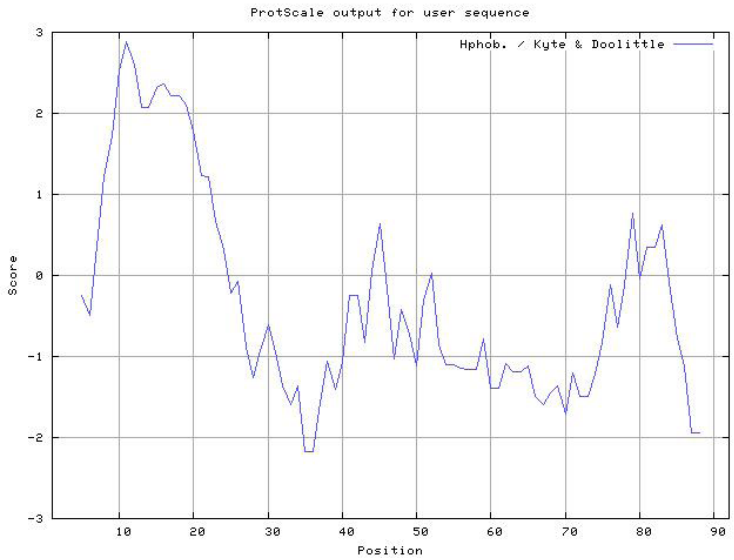


**Figure 14.** Hydrophobicity and hydrophilicity analysis of GnRH1. Horizontal ordinate was the amino acid position and vertical coordinate was the score of hydrophobicity and hydrophilicity. Score less than 0 indicated the hydrophilic region.

### *Secondary structure prediction of GnRH1*

The secondary structure prediction of GnRH1 was performed by HNN (Figure 15). In the figure, the upper line denoted amino acid sequence, while the lower line denoted the secondary structure, where *h* represents α-helix, *e* represents β-extended, and *c* represents random coils. The prediction software indicated that α-helix (h) accounted for 23.08%, β-extended (e) accounted for 10.92%, and random coils (c) accounted for 66.0% of the total secondary structures of the target protein.

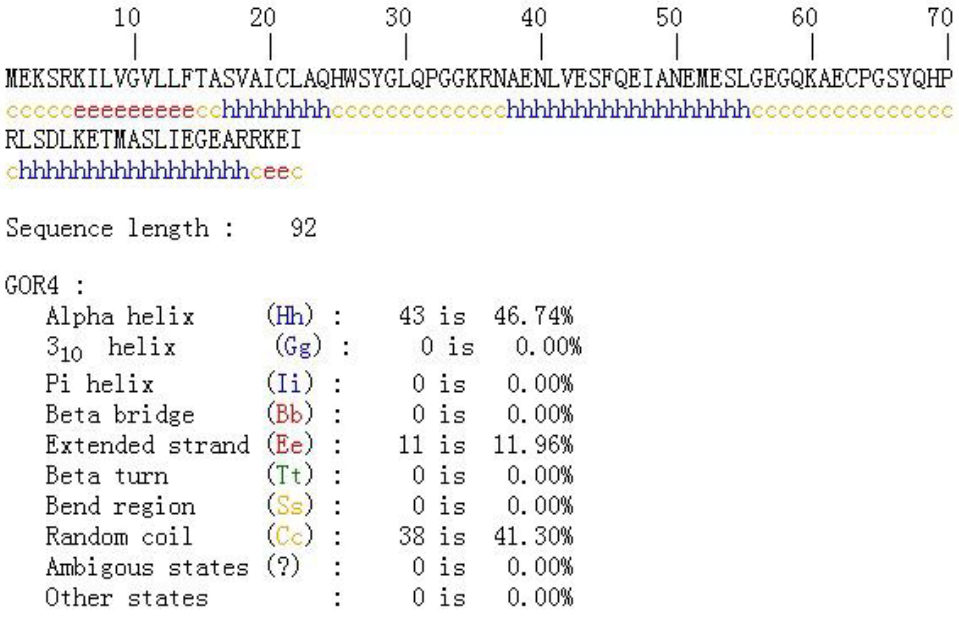


**Figure 15.** Prediction of secondary structure of the GnRH1 protein by HNN.

## Construction of the recombinant plasmid and its expression

The recombinant plasmid was identified by restriction endonucleases *Kpn* and *Xho*I. We observed a specific band of 222 bp, which indicated the successful construction of the prokaryotic expression vector, pET32a-*GnRH1* (Figure 16). The expression of positive bacteria containing the pET32a-*GnRH1* recombinant plasmid was induced using IPTG. The inducing conditions were listed in Table 2. The supernatant and precipitant were detected by SDS-PAGE (Figure 17). SDS-PAGE analysis demonstrated the detection of specific protein bands between 25 and 30 kDa, with the best inducing condition determined to be 37°C for 5 h. Protein expression was detected in both the supernatant and precipitates. In all five treatment strategies, a larger amount of protein was detected in the supernatant, compared to the precipitates, which indicated that the protein was chiefly expressed in soluble form. The protein bands detected by SDS-PAGE were transferred to a PVDF membrane in order to conduct to a Western blot analysis. Specific bands were detected at 30 kDa, both in the supernatant and precipitates, and the quantity of protein expression in the supernatant was observed to be higher than that in

precipitates (Figure 18). The GnRH1 protein was discovered to be expressed successfully in BL21 cells. The protein was expressed in soluble form with a higher protein activity.

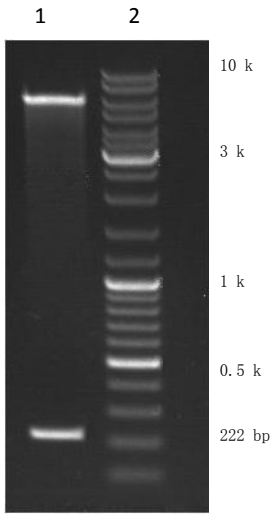


**Figure 16.** Electrophoresis results of restriction-digested recombinant plasmid pET32a-*GnRH1*. *Lane 1* = restriction-digested products of recombinant plasmid pET32a-*GnRH1*; *lane 2* = DNA ladder mix marker.

**Table 2.** Conditions inducing the expression of pET32a-*GnRH*1-containing BL21 cells.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Supernatant | No IPTG | 15°C, overnight with IPTG | 25°C, overnight with IPTG | 30°C, overnight with IPTG | 37°C, 5 h with IPTG |
| Precipitant | No IPTG | 15°C, overnight with IPTG | 25°C, overnight with IPTG | 30°C, overnight with IPTG | 37°C, 5 h with IPTG |

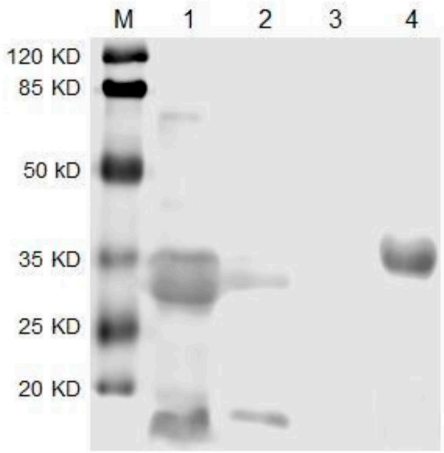


**Figure 17.** Results of Western blot analysis of recombinant proteins. *Lane M* = protein marker; *lane 1* = supernatant; *lane 2* = precipitate; *lane 3* = negative control; *lane 4* = positive control.

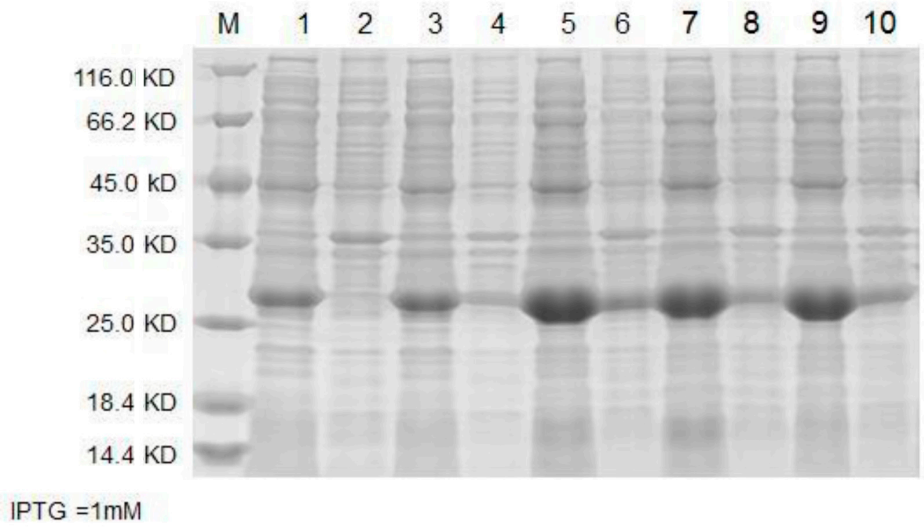


**Figure 18.** SDS-PAGE detection of GnRH1 protein expression. *Lane M* = markers; *lane 1* = control, no IPTG (supernatant); *lane 2* = control, no IPTG (precipitant); *lane 3* = 15°C, overnight (supernatant); *lane 4* = 15°C, overnight (precipitant); *lane 5* = 25°C, overnight (supernatant); *lane 6* = 25°C, overnight (precipitant); *lane 7* = 30°C, overnight (supernatant); *lane 8* = 30°C, overnight (precipitant); *lane 9* = 37°C, 5 h (supernatant); *lane 10* = 37°C, 5 h (precipitant).

## DISCUSSION

In this study, the cDNA of the *GnRH1* gene obtained from the Jinghai yellow chicken was cloned by RT-PCR, with subsequent sequence analysis. The recombinant vector pET32a-*GnRH1* was constructed and transformed into *E. coli* BL21 (DE3) host cells for expression. We concluded from the results that *GnRH1* could be produced in a prokaryotic expression system.

GnRH, which is produced in the hypothalamus, is a neuropeptide consisting of 10 amino acids, and is a primary factor in the regulation of gonadotropin hormone synthesis and secretion (Kumakura et al., 2004). FSH and LH are glycoprotein hormones synthesized, following stimulation by GnRH. These hormones are known for the regulation of gametogony and gonadal steroid synthesis in Sertoli (FSH) and Leydig (LH) cells, respectively (Schulz et al., 2001; Li et al., 2005). The chicken *GnRH1* gene was first characterized in 1982 by King et al. The *GnRH1* gene contains four exons and three introns. The second, third, and a portion of the fourth exon encode for the pre-prohormone, which includes a signal peptide (21-23 amino acids), the GnRH (10 amino acids), a cleavage site (Gly-Lys-Arg), and the GnRH-associated peptide (GAP, 40-60 amino acids) (King and Millar, 1982, 1997). The sequence of the second exon is the most conserved, whereas the other exons show high variability. As a consequence, the signal peptides and the GnRHs are well conserved. However, the GAPs show less homology among different species.

In Jinghai yellow chicken, the cDNA of the *GnRH1* gene was cloned and analyzed by bioinformatic analysis. The cloned *GnRH1* gene of Jinghai yellow chicken was discovered to be 352 bp long, containing a CDS region, promoter region, and a section of the 3'-region. The ORF included 279 bp nucleotides coding for a protein of 92 amino acids, which agreed

with the results of previous studies. BLAST results showed that the *GnRH1* gene nucleotide sequence in Jinghai yellow chicken shared 93, 81, 54, 58, 61, 76, 76, 59, 76, and 66% identity with *M. gallopavo*, *C. livia*, *H. sapiens*, *B. taurus*, swines, *C. hircus*, *O. aries*, *P. hodgsonii*, *E. caballus*, and *R. norvegicus*. The *GnRH1* gene was found to be conserved. Five phylogenetic trees indicated that the genetic distance between Jinghai yellow chicken and *G. gallus* was the shortest, while the genetic distance between Jinghai yellow chicken and *R. catesbeiana* was the longest. The species were clustered into three categories: aves, mammalia, and amphibian; this showed that the phylogenetic trees were reliable.

Further analysis of the GnRH1 of Jinghai yellow chicken included the analysis of the amino acid sequence using bioinformatic programs. Analysis using ProtParam showed that the GnRH1 protein included 92 amino acids. The molecular weight and theoretical pI were discovered to be 10205.6 Da and 5.67, respectively. The GnRH1 protein sequence displayed 20 types of amino acids, among which Glu accounted for the highest proportion at 13.0%, while Asp and Trp accounted for the lowest at 1.1%. The instability index was computed to be 50.05, which classified the GnRH1 protein as being unstable. Furthermore, the aliphatic index was 84.89, the grand average of hydropathicity was -0.403, and the estimated half-life was 30 h. The signal peptide splice site of the GnRH1 protein was predicted by SignalP-4.1. We discovered a cleavage site between the 23rd and 24th amino acid residues, and the amino acids from 1 to 23 were thus classified as the signal peptide. This result agreed with those of studies conducted by King and Millar (1982, 1997).

Analysis using the TargetP program showed that the GnRH1 protein was synthesized by ribosomes on the endoplasmic reticulum, and oriented to the target sites by the signal peptide after synthesis. Analysis of the protein by PSORT II prediction program indicated that the GnRH1 protein belonged to the secreted protein family, playing a biological role after being secreted to the outside of the cell. Transmembrane structures were predicted by TMHMM Server v.2.0. Using this program, the amino acids from 7 to 29 were discovered to be possible transmembrane helices. Prediction by TMpred also indicated the same. The Smart software speculation showed the absence of conserved domains in the GnRH1 protein, and also that amino acids from 7 to 29 were possible transmembrane helices. Therefore, we speculate that amino acids from 7 to 26 were possible transmembrane helices of GnRH1 protein.

Phosphorylation sites were analyzed by NetPhos 2.0. The 44 Ser, 54 Ser, 73 Ser, and 81 Ser amino acid sites in the GnRH protein of Jinghai yellow chicken were discovered to be potential protein kinase phosphorylation sites. ProtScale was used to analyze the hydrophobicity of the GnRH1 protein. Most of the amino acid residues were discovered to be hydrophilic; therefore, we theorized that the GnRH1 protein might be a water-soluble protein. Secondary structure prediction of GnRH1 was conducted by HNN. The prediction indicated the presence of an α-helix (h; 23.08%), β-extended sheet (e; 10.92%), and random coils (c; 66%).

In order to learn more about the GnRH1 protein, further research on prokaryotic expression was carried out, based on the results of the bioinformatic analyses. The recombination vector was constructed and transduced into *E. coli DH5α* competent cells. The expression products were identified by SDS-PAGE and Western blot. SDS-PAGE analysis detected specific protein bands between 25 and 30 kDa, and the best inducing condition was discovered to be 37°C for 5 h. Proteins were expressed either in supernatant or in the precipitates. A greater quantity of protein was detected in the supernatant in all five treatments, indicating that the proteins were expressed in soluble form. Western blot analysis detected the specific bands at approximately 30 kDa, both in the supernatant and precipitation; the quantity of proteins

expressed was higher in supernatant than that in precipitates. The GnRH1 proteins were therefore successfully expressed in BL21 cells. The expression showed higher protein activity in soluble form.

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