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ABSTRACT

GnRH is a neuropeptide known to regulate reproduction in vertebrates. The purpose of this study was to design and produce recombinant gonadotropin-releasing hormone associated peptide (rGnRH/GAP) as an alternative of the previous GnRHs and native extracted hormone from tissue, to induce final maturation in fish. Decapeptide as well as GAP area sequences were compared between GnRH1, GnRH2, and mGnRH from *Acipenser sp* and *Huso* huso, respectively. Considering the conserved amino acids and the replacement of un-stable amino acids with those that were more stable against proteolytic digestion as well as had a longer half-life, the sequence was designed. The sequences of decapeptide and GAP region were synthesized and then cloned on pET28a expression vector and transformed into expression host *Escherichia coli* BL21(DE3). The supernatant of cultured recombinant bacteria was used for purification using TALON Metal affinity resin. The purity of the GnRH/GAP was confirmed by single 8 kDa band on SDS-PAGE and western blot. Bioinformatics studies were performed for evaluation of homology between GnRH protein sequences and prediction of 3D protein structure using Swiss Model. The result showed that the structure prediction of the recombinant GnRH decapeptide was relatively similar to decapeptide of GnRH2 from Beluga (*Huso huso*). The GAP structure was similar to GAP1 of Nile tilapia (*Oreochromis niloticus*) and sturgeon and GnRH2 of Chinese sturgeon (*Acipenser sinensis*). The mass analysis showed that the sequence was exactly the same as designated sequence. Biology activity of rGnRH/GAP was tested in mature goldfish (*Carassius auratus*) and results showed that rGnRH/GAP had a positive effect in final maturation. Indeed 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) was increased 17 h and 24h after injection with rGnRH/GAP and spawning stemmed from that injection. These novel findings introduce the potential of utilizing rGnRH/GAP in aquaculture.

Introduction

Gonadotropin-releasing hormone (GnRH) belongs to conserved neurohormonal gene family that has 30 different isoforms, including 15 isoforms in vertebrata and 15 isoforms in non-vertebrates (1). GnRH is a decapeptide with 10 amino acids found in fish as well as other animals which are synthesized primarily on neurons in the brain (2). Two forms of GnRH (mammalian GnRH and chicken GnRH-II) are found in fish as same as other vertebrates, but there are nine forms of GnRH in fish that are distinct from other vertebrates. The release of GnRH from hypothalamus regulates reproductive and sexual functions in vertebrates (3, 4).

Primary structure of GnRH protein precursor is composed of a signal peptide in the N-terminal region (approximately 28 amino acids), a section of ten amino acids (decapeptide), which is an active region of the peptide and is almost completely conserved in all fishes. Thereafter, there are three amino acids; Glycine, Lysine and Arginine, which are conserved in all species of fish and where the protease digestion occurs in the sequence of this peptide. Finally, in C-terminal, there is also a GnRH-associated peptide (GAP) that has about 60 amino acids, which is less conserved in fish than other regions of the peptide, and has different sequences and sizes (5, 6). Studies have shown that GAP has a role in the stability of GnRH structure and unlinked to the biological activity of the peptide (7). Nikolic et al (8) reported that the inhibition of PRL basal secretion obtained by GAP was comparable to that reported for dopamine (9). It was shown that GAP was co-secreted with GnRH into the hypophyseal portal blood of ovariectomized sheep (10). In addition, some reports showed that GAP could stimulate gonadotropins release in rats *in vitro* and *in vivo* (8, 11-15). The previous studies indicated that GAP could have a physiological significance in the regulation of pituitary function in mammals. However, there are also reports where these GAP effects were not observed, as for instance in human pituitary prolactinoma *in*

vitro (16) or in sheep *in vivo* (17). To the best of our knowledge, a study by Planas et al. (18), on the effects of human GAP on PRL release in tilapia, has been the only attempt so far to investigate the possible biological function of GAP in non-mammalian vertebrates.

One of the major goals of aquaculture practices is to artificially stimulate spawning via the manipulation of the hormonal axis. Different endocrine approaches have been considered to induce final sexual maturation in breeding fish (19). Pituitary extract, human chorionic GTH, hCG and different analogs of GnRH are used to induce spawning in finfish reproduction (19, 20). There are significant advantages to using GnRH as compared with gonadotropin-based preparations such as fish pituitary and synthetic gonadotropins. Synthetic GnRH eliminated the risk of the transmission of infectious diseases and allowed the possibility of applying exact doses of GnRH. Another important factor is the high degree of interspecies similarity between GnRH peptides allowing to be used for more than one fish species (21). Due to the structure of GnRH peptide, this hormone is exposed to a variety of endopeptidases in the body that reduced its half-life (21).

This is a major challenge because GnRH peptide is digested by proteolytic enzymes shortly after the treatment (22, 23). The synthesized GnRHs are decapeptides that are designed to resist to enzymatic degradation in blood circulation. However, they had only a very short half-life period in fish (up to 23 minutes in gilthead seabream,*Sparus aurata L*) (21, 22, 24). Failure of single-stage injection protocols to stimulate final maturation in females is probably the result of short residence of GnRH in circulation. It is important to improve hormone formulations that have more resistance to enzymatic degradation with no requirement for further frequent use of hormone. The latter is a priority in development of spawning induction therapies.

There have been different strategies used for increasing the protein's half-life in blood in which one of them is changing the amino acid sequences (25-27). Naturally, GnRH has a half-life of 2 to 4 minutes, which is the result of breaking the bound between amino acids Trp^3-Ser^4 and Tyr^5-Gly (23, 28). Typically, substitution of Gly at position 6 with a D-amino acid (D-Ala and D-Arg) in GnRH or with His and Lys increases the metabolic stability (28). Studies have shown that GAP has a role in the stability of GnRH structure.

Recent advances in the construction of cDNA encoding target protein provided the potential for the production of recombinant peptides (29). The recombinant hormones of fish have been produced in various systems such as yeast Pichia. pastoris, Drosophila S2, silk worm Bomyx mori and bacterial expression system and their biological activity has been evaluated in vitro and in vivo (19,29, 30-31). The production of rGnRH /GAP with acceptable biological activity in fish species can introduce an effective hormone therapy in aquaculture and it is used as specific homologous for the treatment of reproductive disorders in new species. Considering the positive effects mentioned in relation to rGnRH/GAP as an induction of final maturation in breeding fish. In this study, the stable sequence of decapeptide GnRH was designed based on all native as well as synthetic and GAP sequences was added to decapaptide. After suitable expression of the peptide, purification was performed using affinity tag chromatography. Finally, biology activity of rGnRH/GAP was tested in its capacity of regulating the final maturation in goldfish.

Materials & methods

Bioinformatics studies

The gene sequences and amino acid sequences related to different fish pre-pro GnRH was acquired from the National Center for Biotechnology Information, (http://www.ncbi.nlm.nih.gov/nucleotide/), Uniprot (https://www.uniprot.org/) and Expasy

(https://web.expasy.org/docs/swiss-prot) databases. Sequences of the decapeptide region as well as the GAP area that contributed to the sustainability of the pro-protein were compared among GnRH1 Amur sturgeon (*Acipenser schernckii*) (GenBank: AOW41585.1), GnRH1 Chinese sturgeon (*Acipenser sinensis*) (GenBank: AGK30598.1), GnRH2 Chinese sturgeon (GenBank: AGK30597.1), mGnRH Beluga (*Huso huso*) (GenBank: ABR18540.2), GnRH2 Beluga (GnBank: EF534706.2). Considering the preserved amino acids in each of the species cited and the best displacement in amino acids in sequence that play a role increasing the half-life and the stability of the peptide against proteolytic digestion, the decapeptide sequence was selected. The relative half-life in the biological environment and the peptide stability were checked at ProtParam (https://web.expasy.org/protparam/) database.

The selected decapeptide (GnRH peptide) was translated to gene sequence using translate tools (www.expasy.org/tools/translate). The GAP region was added due to increase in sustainability of the pro- protein, as well as for ease of decapeptide purification due to the small size of the decapeptide consequently, was selected based on GnRH2 sequence of beluga.

Three-Dimensional (3D) structure of the protein was constructed by comparative modeling according to Swiss Model (http://www.swissmodel.expasy.org/spdbv/) corresponding to its amino acid sequence. Among all current theoretical approaches, comparative modeling is the only method that can reliably generate a 3D model of a protein from its amino acid sequence.

Gene synthesis and cloning

In order to facilitate cloning and increase the stability of the translated sequences, the start and stop codons as well as His-tag were added on the gene sequence and it was synthesized by Shainegene Company (Shanghai, China). The synthesized gene was cloned in an expression

vector (pET28a^{+}). The designed protein contained 77 amino acids, so the expected molecular weight was around 8 kDa.

Transformation of recombinant vector

E. coli BL21 (DE3) became competent as a host for cloning and expression based on the protocol of Sambrook and Russell (2001). Cell suspension was dispensed into 500 µl aliquots stored at -80 $^{\circ}$C. For transformation, 10 ng of recombinant vector (pET28a^{+}/GnRH) was transferred into one tube of competent cells and tapping gently. It was incubated on ice for 30 min and heated at 42°C for 30 seconds then quickly placed on ice. One ml of pre warmed LB medium was added to the vial, and it was placed in a shaking incubator and shaked at 37°C for 1 h at 225 rpm. One hundred µl of grown recombinant bacteria were poured on a LB plate containing kanamycin antibiotic (50 µg/mL) and incubated at 37°C overnight. After growth of bacterial colonies on the selective plate, a single colony was taken and transformation was confirmed using colony PCR with specific primer (table 1). The PCR were performed on a PTC-200 thermal cycler (BioRad, USA) by denaturation at 94°C for 10 min, followed with 30 cycle of amplification at 94°C for 1 min, 57°C for 1 min and 72°C for 90 s and an additional elongation at 72°C for 10 min after the last cycle. The PCR products were checked by electrophoresis on 1.5 % agarose gel containing ethidium bromide

Gene expression and protein extraction

A single recombinant colony was cultured in 100 mL LB media containing kanamycin (50 µg/mL) at 37°C overnight. Then 5 mL of starter culture were transferred into fresh LB media (2 L) containing kanamycin (50 µg/mL) and grown up to a cell density of OD_{600} = 0.5–0.6. Cell density was determined by measuring the OD of culture at 600 nm with a spectrophotometer. The culture was induced by adding IPTG to a final concentration of 1 mM and allowing further growth at 20°C overnight. The induced cells were centrifuged at 5000 g for 10 min. The cell

pellet was suspended in lysis buffer (100 mM Tris, pH 8.0, 100 mM Nacl, 10 mM Imidazole, 0.5 % (v/v) Triton x-100,) and 1X EDTA-Free Protease Inhibitor Cocktail. The cell suspension was subjected to sonication for disruption (3 times for 30 s with 50% pulses), then it was centrifuged at 10000 g for 25 min in 4°C, after which the supernatant was taken for protein production analysis using SDS-PAGE. The supernatants of induced and un-induced cells were taken on 15% SDS-PAGE. Proteins band were developed by Coomassie brilliant blue staining.

Purification of recombinant GnRH/GAP

The supernatant was purified using Batch/Gravity-Flow column purification with TALON metal affinity resin. The solubilized protein was loaded on Co^{2+}- CMA, TALON resin (Clontech). 3 mL of resin suspension was transferred to a sterile column and washed with DD water then equilibrated with 20 bed volume of equilibration buffer containing; 50 mM Tris, 300 mM NaCl, 10 mM Imidazole and 0.05% Triton x-100 at pH 8.0. The solubilized sample (pH adjusted to 8) was loaded on to the column and shacked at 4°C for 1 h. After that the column was washed with 10 bed volumes with washing buffer that was the same as equilibration buffer. Finally, the His-tagged protein was eluted with 5 bed volumes elution buffer (50 mM Tris, 150 mM Imidazole and 1 % (v/v) Triton x-100, pH 7.5) at a flow rate of 1 mL/min. The elute samples collected in 500 µl fractions. The majority of the His-tagged protein was recovered in the first one bed volume. The fractions were analyzed using 15% SDS-PAGE. The fractions containing rGnRH were dialyzed in sodium chloride serum (0.09%, pH 7.0) with slow stirrer at 4°C for 18 h. The dialyzed sample was lyophilized and stored at -20°C for further use.

Western blotting

Purified protein from SDS-PAGE was transferred to nitrocellulose membrane using transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% SDS, 20% methanol) by Bio-Rad Mini

Protean Tetra Cell System. The membrane was incubated in the blocking buffer of 3% BSA/phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, pH7.3) for 2 h. The membrane was then incubated in a monoclonal anti-6xHis tag antibody (at 1:3000 dilution in PBST/BSA (PBS containing 1% Tween 20 and 2% BSA) by gentle shaking at 4 °C overnight. The membrane was washed with PBS/T three times and then incubated with HRP-conjugated goat anti mouse IgG (Sigma Aldrich, USA) as secondary antibody diluted 1:5000 in PBST/BSA with gentle shaking for 1 hour for 1 h at 37 ◦C. The membrane was washed three times with PBS/T and detection was carried out using HRP staining solution. Chromogenic reaction was stopped by rinsing the membrane twice with distilled water.

Determination of protein concentration

Protein concentration was determined by Bradford's method (1976) with bovine serum albumin as a standard (0, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0 and 60.0 µg/mL). The lyophilized peptide was dissolved in saline (0.09%, pH 7) and assayed with Bradford buffer and the absorbance of samples measured at 595 nm with spectrophotometer.

Mass spectroscopy analysis

The purified peptide was studied using MS/MS (MALDI-TOF/TOF) mass spectrometer to reveal amino acid sequence. Sample preparation for Mass spectroscopy was performed by Jean-Baptiste Vincourt in a scientific platform in Nancy, France. 500 ng of the lyophilized protein were suspended in SDS-loading buffer, and it was run on SDS-PAGE. Proteins band were developed by Coomassie brilliant blue staining. The corresponding band was excised and processed as follows for protein content identification:

Cysteines were reduced in 100 mM DTT, and they were prepared in 100 mM ammonium bicarbonate (BA), then they were alkylated in iodoacetamide, 100 mM in BA, each for 45 min.

After 2 washing, cycles in BA and BA/acetonitrile, 1:1, 15 min each, gel bands were dried in a speed-vac for 1 hour, and they were digested with 100 ng trypsin in 20 ul BA overnight. Peptides were extracted twice consecutively in 25 ul acetonitrile, 80%, TFA 1% for 10 min under sonication in a water bath sonicator. Extracted peptides were pooled, dried in a speed-vac and resuspended in 10 ul 2% acetonitrile, 0.1% TFA.

HPLC was performed using Ultimate 3000 equipment (Thermo). Peptides (6.4 ul) were injected in the micro liter pickup mode onto a desalting column and loaded onto a 15 cm Acclaim pep map RSLC C18 column (Thermo) and eluted over a 30 min run by a 2-45% acetonitrile linear gradient and 170 fractions were collected onto a 1536 TF anchorchip MALDI plate via a Proteineer FcII fractionator (Bruker) and mixed with α-Cyano-4-hydroxycinnamic acid (HCCA) directly upon deposition. Sample acquisition in TOF and TOF/TOF modes were performed automatically using WARP-LC as a pilot software on an Auto flex speed MALDI mass spectrometer (both from Bruker). Peptide assignments, protein identification and scoring were managed on a Proteins cape server allowing a 50 ppm tolerance for mass measurements through interrogation of the whole swiss prot database of a local Mascot server. In addition, as the protein of interest was recombinant, its theoretical artificial sequence was used to assign non-naturally occurring peptides.

In vivo biological activity

Mature female goldfish (*Carassius auratus)* were obtained from Fish Hatchery Center (Sari, Mazandaran, Iran) and transferred to wet lab located at the Faculty of animal science, Sari, Iran. The fish were acclimated to the experimental conditions into two 250 L tanks and fed with a commercial carp diet for two weeks. After 2 weeks of acclimation and gradual temperature increase, selected fish were randomly divided into two experimental groups (n = 6). Photoperiod for this indoor experiment was set at 12hL:12hD cycle and temperature was 25.2 ± 0.3 $^{\circ}$C. To

supply needed water, water was used after 24 h aeration. Air stone aeration was connected to a central air pump (Iran Pash, Urmia, Iran) in each aquarium.

The first treatment consisted in the injection of the recombinant GnRH (20 µg/kg body weight) dissolved in 0.9% NaCl and the second group of fish (control) received 0.9% NaCl at 1 ml/ kg of body weight. Blood samples were collected from 3 fish in each group before injection (0), 17 h after injection (ovulation time) and 24 h after injection for measurement of 17α, 20β-dihydroxy-4-pregnen-3-one (DHP). Spawning success was determined by the number of fish that spawned after the injection.

Enzyme-linked immune sorbent assay for 17α, 20β-dihydroxy-4-pregnen-3-one

DHP was determined by the enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Cat. No: CK-E91529, Eastbiopharm, USA). The assay range is from 1 ng/mL to 400 ng/mL and the kit sensitivity was 0.52 ng/mL.

Statistical analysis

Statistical analyses were performed using the STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Data are presented as a mean ± standard deviation of the mean (SD). Normality and homogeneity of variances were tested by the Kolmogorov–Smirnov and Bartlett methods, in order to comply with the prerequisites of analysis of variance (ANOVA). Within-group differences of DHP levels were analyzed by repeated-measures ANOVA, and between-group differences were analyzed using T- test at each sampling time.

Results

Bioinformatics study

GnRH/GAP protein sequences selected from GnRH1 Amur sturgeon, GnRH1 Chinese sturgeon, GnRH2 Chinese sturgeon, GnRH2 Beluga and mGnRH Beluga and they were aligned with

multiple sequence alignment by Clustal Omega (www.EBI.ac.uk/service/tools). The Clustal Omega result showed that pre-pro-GnRH contained a signal peptide, the conserved decapeptide, the cleavage site and the GnRH associated peptide (Fig. 1). Three regions of GnRH including decapeptide, proteolytic processing site and GnRH-associated peptide (GAP) at C-terminal (60 amino acids) were selected according to the preserve amino acids, and resistance to proteolytic digestion and higher half- life and peptide stability and considered for its gene synthesis (Fig. 2). The comparison of the signal peptide and GAP sequences of GnRH showed a very low conservation between different species of fish. The alignment of Beluga GnRH2 and GnRH2 of Chinese sturgeon was done. Resulting the GAP area selected from Beluga GnRH2 sequence with accession number in GeneBank, EF534706.2 that had up 99% sequence identity with GnRH2 of Chinese sturgeon (Fig. 3).

According to sequence analysis of designed peptide in Expasy/protparam, the estimated half-life was more than 10 h in *E.coli*, in vivo and up to 1 h in mammalian reticulocytes, in vitro. The instability index (II) was computed to be 10.92 that classified the peptide as stable. The instability index is a measure of proteins, used to determine whether it will be stable in a test tube. If the index is less than 40, then it is probably stable in the test tube. If it is greater than it is probably not stable.

Prediction of the three- Dimensional protein structure

Predicted tertiary protein structure of the rGnRH/GAP was obtained using spdbv in Swiss Model. 3D structure predicted in accordance with the decapeptide sequence designed and selected GAP sequence. Our result showed that the 3D structure prediction of rGnRH decapeptide was somewhat similar to GnRH2 from Beluga built with Swiss Model. The GnRH-associated peptide (GAP) structure was relatively similar to GAP1 of Nile tilapia and sturgeon as

well as GnRH2 of Chinese sturgeon. They had a Helix-Loop-Helix structure (HLH) but the recombinant hormone had a large helix and a small helix with a short loop between them (Fig. 4).

GnRH/GAP gene sequence and synthesis

The peptide sequence of GnRH/GAP designed was translated to nucleic acids using translate tools in Expasy and after synthesis and checking up for correct gene sequence, it was then cloned at pET28a. After gene synthesis, the primary sequence was confirmed s (ShineGene, China). Regarding GnRH sequences of various species of aquatic organism cited in databases and the ability to produce cDNA encoding of this hormone, it was possible to produce recombinant GnRH/GAP (rGnRH/GAP).

Transformation of recombinant vector and expression of the peptide

The Recombinant vector pET28a^{+} which contains the gene of GnRH/GAP was transformed into *E. coli* BL21 (DE3). After the growth of the *E. coli* cells containing the recombinant vector a single colony took, transformation was confirmed using colony PCR and a fragment of 185 bp detected at the expected size (Fig. 5). The recombinant bacterial cultured and induced by IPTG then proteins in cells extract from both un-induced and induced cultures checked by SDS–PAGE. The molecular weight of the expressed peptide was estimated at expected size of 8 kDa and it was detected in the induced culture (Fig. 6).

Purification of recombinant GnRH/GAP

The peptide was soluble in cytoplasm thus it was in cell lysate supernatant. The supernatant was purified using affinity chromatography. After purification the first fractions of elution loaded on a 15% SDS-PAGE and the purified recombinant peptide bands developed by Coomassie brilliant

299 blue staining and a band of about 8 kDa (expected size) was detected according to protein
300 molecular weight marker (Fig. 7).

301 ***Western blot analysis of recombinant GnRH/GAP***

302 In Blotting analysis with anti-His-Tag antibodies, a band of 8 kDa developed in the membrane.
303 The six Histidine tag was designed at carboxyl terminal of recombinant peptide for affinity
304 purification and the His-Tag sequence was not isolated from recombinant peptide (Fig. 8).

305 ***Protein concentration***

306 The concentration of recombinant GnRH/GAP peptide determined after dialysis in sodium
307 chloride serum and then lyophilized. The peptide powder dissolved in the same buffer and the
308 concentration of the recombinant peptide was 3.5 mg/mL.

309 ***Mass spectrometry analysis***

310 The results of the Mass spectroscopy on this peptide showed that the sequence of the
311 recombinant peptide was exactly consistent with the designed sequence (Fig. 9, 10).

312 ***In vivo biological activity***

313 The results showed that no significant difference of DHP level was observed between treatments
314 before injection ($P>0.05$). A significant difference of DHP level between GnRH/GAP injected
315 fish and control fish was observed 17 h and 24 h after the injection ($P<0.01$). DHP levels
316 increased 17 h and subsequently decline at 24 h after injection in the fish group that received 20
317 µg/kg body weight of recombinant GnRH/GAP but no change was observed in DHP level 17 h
318 after injection in the control fish (Fig. 11). All females injected with recombinant GnRH/GAP
319 spawned, while none of the control fish spawned.

320

321 **Discussion**

GnRH is a decapeptide produced in the hypothalamus with a regulatory function in the reproductive system (32-34). Different analogs of GnRH with different half-life exist (22). These analogs are decapeptides and some amino acids are substituted at the native GnRH to increase half-life. Due to the structure of GnRH peptide, this hormone is exposed to a variety of endopeptidases in the body that reduced its half-life (21). Synthetic GnRH has only a half-life of 23 minutes in *vivo* because of degradation by enzymatic degradation (22, 35). In this study, the design of bioactive sites was based on interspecies conserved amino acids at position 2, 3, 4, 9 and 10 that remained unchanged. In contrast, the amino acids at positions 1, 5, 7 and 8 were varying among different species of fish (36, 37). There were His or Tyr at position 5, Trp or Leu at position 7 and Arg or Tyr at position 8 in examined species. Thus, in order to increase the stability and half-life of the peptide His, Leu and Try were chosen at positions 5, 7 and 8, respectively. There was Glutamine at position 1 in most fishes but there was Glutamic acid instead of Glutamine in some fish species such as Russian sturgeon (*Acipenser gueldenstaedti*), (GenBank: AAB34379.1) and in other vertebrates and synthetic GnRH. Glu was selected rather than Gln because of differences in the stability index in favor of Glu. Amino acid at position 6 (Gly) was high conserved in different fish species but can be a target site for proteolytic digestion and thus potentially reduces the half- life so, we used serine instead of Gly (28, 38). Typically, substitution of Gly at position 6 with a D-amino acid in GnRH or with His and Lys increases its metabolic stability (28). In our design, serine increased more the stability than His and Lys. Substitution of Gly at position 6 with a D-amino acid (D-Ser) in LHRH increases its metabolic stability and the binding affinity to LHRH receptor (28). Amino acids of Pro^{9} and Gly^{10} are conserved in all types of GnRH sequences. Since the presence of Gly at the end of C-terminal (position 10) even after proteolytic digestion of the precursor hormone was important

for the biological function of the hormone so we did not make replace this the amino acid. Two of the most used analogues in the market are mGnRHa (pGlu-His-Trp-Ser-Tyr-DAla-Leu-Arg-Pro-Net) and sGnRH (pGlu-His-Trp-Ser-Tyr-DArg-Trp-Leu-Pro-Net). These analogues used D-Ala and D-Arg at position 6 (instead of Gly) to increase the stability and ethyl amide at position 10 instead of glycine to increase the binding of the receptor to the ligand (28, 39). Despite the changes in the amino acid sequence in these analogues, the half- life of them was not so high (22). After the decapeptide the proteolytic site is highly conserved and consist of three amino acids (Gly, Lys and Arg) where the protease digestion occurs in the sequence of this peptide and separates it from GAP area (36). The functional part of GnRH protein is decapeptide (bioactive site) that is processed from the precursor by removal of the signal peptide and cleavage at the dibasic amine acid to separate GAP region (5). The signal peptide and GAP sequences showed a very low conservation between species (36). It is presumed that GAP domain involved in detecting the receptors for the precursor protein. Although still controversial, the function of the GAP is believed to be restricted to providing the correct secondary structure for precise processing of the GnRH precursor (5). Several methods of peptide modification have been discovered to prolong its half- life and do not require repeated injection of the hormone. These techniques include chemical and genetic methods that increase the half- life of the polymers, such as polyethylene glycol (PEG) and add lipid to the peptide which reduces the rate of renal excretion (28). It is also possible to change the amino acids in amino and carboxyl ends after chemical synthesis of peptide to increase binding to the cell surface receptors. In genetic engineering, methods with displacement of amino acids that are important in the stability of the structure can increase peptide stability against protease digestion (40). The tertiary structure of GnRH in protein data bank (PDB) only for Gonadotropin-releasing hormone agonist (GnRH1,

LHR.H) from *homosapiens* was recorded (Legrand, p. 2015, http://www.rcsb.org/structure/4D5M). Since the recombinant GnRH sequence with beluga GnRH2 that had up 99% sequence identity, the predicted tertiary structure of rGnRH was compared with the predicted 3-D structure of the peptide of this fish. There was Helix-Loop-Helix structure (HLH) in GAP of rGnRH/GAP, Nile tilapia and sturgeon as well as GnRH2 of Chinese sturgeon. There was a large helix and a small helix with a short loop between them in the recombinant hormone. In sturgeon there were two identical lengths of alpha helices with long loop between them but, in Nile Tilapia and Chinese sturgeon, there were one long and one short alpha helix with a long loop between them (9). Also, the HLH structure was predicted for GAP1 sequences of the vertebrate species analyzed. Some variations were observed in the length of the alpha helices, as well as in the length of the loop (9). Sirkin et al (9) predicted 3D structure of GAP2, a Helix-loop-Helix structure, with long alpha helices, observed in some species like shark and other teleost. However, some other GAP2 structure showed one or very short or no alpha helix such as in a sauropsid (alligator), in a chondrostean (sturgeon) and in a teleost (arowana). Comparison of the GAP sequences between GnRH2, mGnRH, GnRH1 and GnRH2 from fish showed the low conservation suggesting that GAP may participate in the folding and processing of GnRH prohormone and the regulation of gonadotropin secretion as suggested by) Bond (41) and Seeburg (42)..

In this study, *E. coli* was used for expression system to produce the recombinant protein. Several cell types both prokaryotic (bacteria) and eukaryotic (animal, fungi, etc.) are used to produce recombinant proteins. Bacteria are a good host for production of recombinant peptides and are used for proteins that do not have post-translation changes such as GnRH. They also have multiple benefits, including easy cultivation, low cost and high production potential, thus

allowing production from laboratory to industrial scale (43). *E. coli* as a factory was used for production of recombinant GnRH in some studies as well as the current study (44, 45). Generally, proteins with molecular weight below 60 kDa are successfully expressed in soluble forms of *E. coli* (46). Weber et al. (47) isolated three forms of GnRH from native tissue of pituitary gland of tilapia . Extraction from the pituitary gland is accompanied by some problems including the transmission of the disease from the source to the hormone receiver, the process of extraction is time-consuming and the amount of production is low, requiring more fish to euthanize (19). Therefore, the technology of recombinant production or peptide synthesis is more beneficial than extraction of pituitary tissue. In previous study reported the chemical synthetic of GnRH (GnRHa) designed to withstand enzymatic degradation in the blood stream had a higher resistance than normal GnRH against enzymatic degradation and had a half-life of 23 minutes (21). Further studies to estimate the half-life of this recombinant GnRH in the plasma of sturgeon and the kinetics of GnRH enzymatic degradation is under way.

In this study, the expressed peptide was soluble in cytoplasm and there was not any detection in insoluble precipitation (data not shown). Purification process was performed in native condition and there was no need of refolding process of the purified peptide. Xu et al. (45) reported that chimeric peptide (recombinant GnRH) was expressed in an inclusion body and the target peptide purified in denaturing condition (45). Two fusion proteins, GnRH-P53 and GnRHIII-p53 were expressed intracellularly and dissolved in 8M urea and the purification process was performed in denaturing condition with Ni-NAT affinity chromatography (48).

The results of the in *vivo* study showed that DHP level increased 17 h and 24 h after injection by recombinant GnRH/GAP but no change was observed in DHP level in the control fish. All females injected with recombinant GnRH/GAP spawned, while none of the control fish spawned.

The ovulation-induction effect of GnRH was observed in common carp (*Cyprinus carpio*) (49). The ability of mGnRHa to induce ovulation goldfish is similar to several species (50). The potential of DHP to induce final maturation was demonstrated in several fish species (51-54). In this study, recombinant GnRH/GAP stimulated a surge of blood DHP occurring 17 h after injection subsequently decline at 24 h. Podhorec (50) reported that GnRH treatments stimulated a surge of DHP detected at 12 h post-injection in tench (*Tinca tinca*). Pinillos et al (55) reported a peak of DHP occurring 6 h later with the subsequent decline seen at 24 h in tench. The effectiveness of GnRH/GAP injection to promote DHP elevation and spawning is the demonstration of the biological activity of the recombinant peptide.

Conclusion

The recombinant GnRH/GAP was successfully produced in *E.coli* expression system in soluble form and then purified and a significant concentration of the peptide obtained. The specificity was checked at both the DNA and protein levels using PCR, SDS-PAGE and western blot respectively. Mass analysis confirmed that the sequence of the recombinant peptide was exactly the same as the designed sequence. Due to the negative effects of multiple hormone injections in fish, it is now necessary to develop a hormonal therapy that has a higher half-life and that does not require multiple injections to induce sexual maturation in fish. All females goldfish injected with recombinant GnRH/GAP spawned which is promising to use this recombinant peptide to induce spawning in other teleost fish species of interest for aquaculture.

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Mass spectrometry was performed at the proteomics core facility of UMS 2008, UL-CNRS-INSERM, IBSLor, http://umsibslor.univ-lorraine.fr"

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Table 1

Primer pairs used in the amplification of rGnRH.

Gene		Primer Sequence (5' - 3')	Size (bp)
GnRH	Forward	CCATGGGAACATTGGAGCCATAGC	186
	Revers	AAGCTTCTTCTTCCTCTGCATCTG	

.

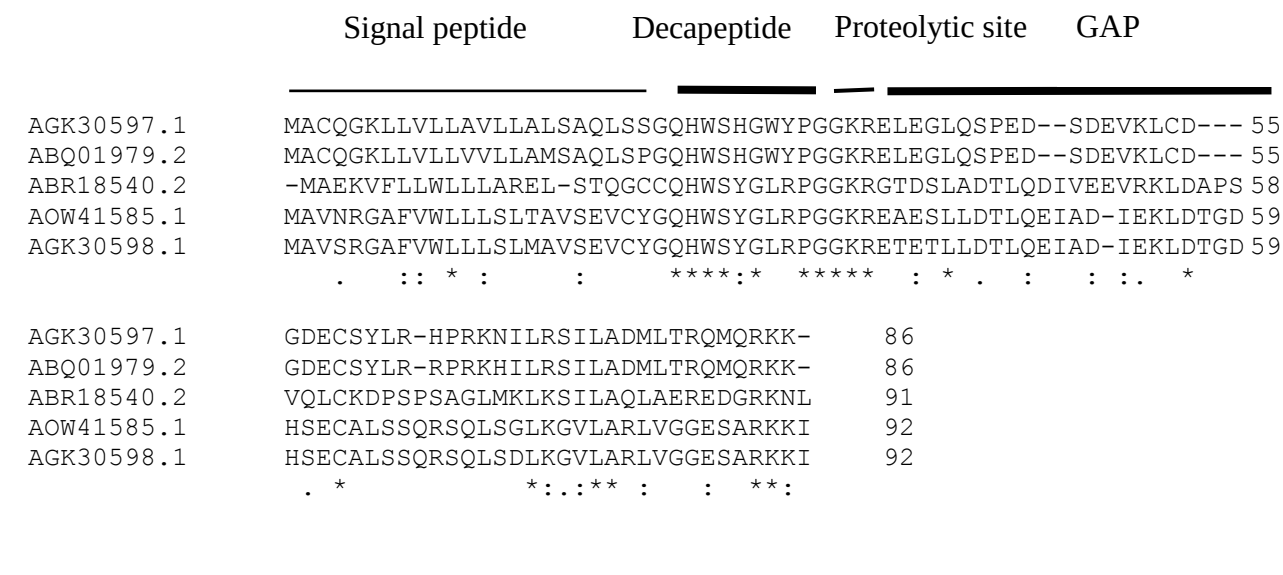


Fig. 1. Multiple alignment of GnRHs from fish. Signal peptide, amino acids 1-24; Decapeptide, amino acids 25-34; Proteolytic site, amino acids 35-37; GnRH-associated peptide, amino acids 38-93.

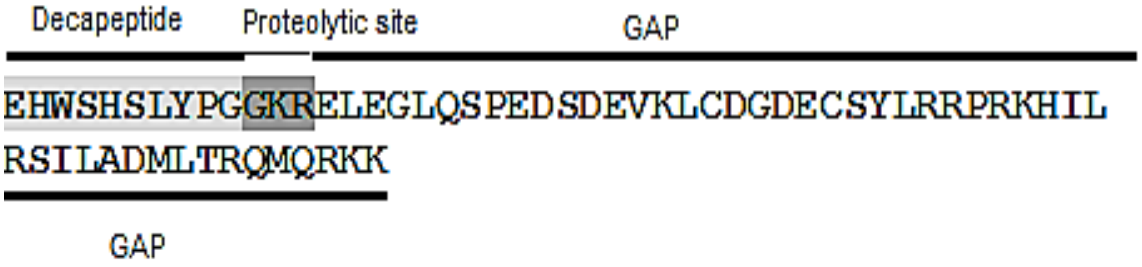


Fig 2. The amino acid sequences of GnRH designed. Decapeptide, the grey color (10 aa), proteolytic site, the black color (3 aa); GAP,no highlight (49 aa).

GAP

```
ABQ01979.2      MACQGKLLVLLVVLLAMSAQLSPGQHWSHGWYPGGKRELEGLQSPEDSDEVKLCDGDECS 60
AGK30597.1      MACQGKLLVLLAVLLALSAQLSSGQHWSHGWYPGGKRELEGLQSPEDSDEVKLCDGDECS 60
                ***********.****:***** ************************************

ABQ01979.2      YLRRPRKHILRSILADMLTRQMQRKK    86
AGK30597.1      YLRHPRKNILRSILADMLTRQMQRKK    86
                ***:***:******************
```

GAP

Fig. 3. Pairwise alignment of two GnRH protein sequence from *Huso huso* (ABQ01979) and *Acipenser Sinensis* (AGK30597). The grey color shows the GAP area.

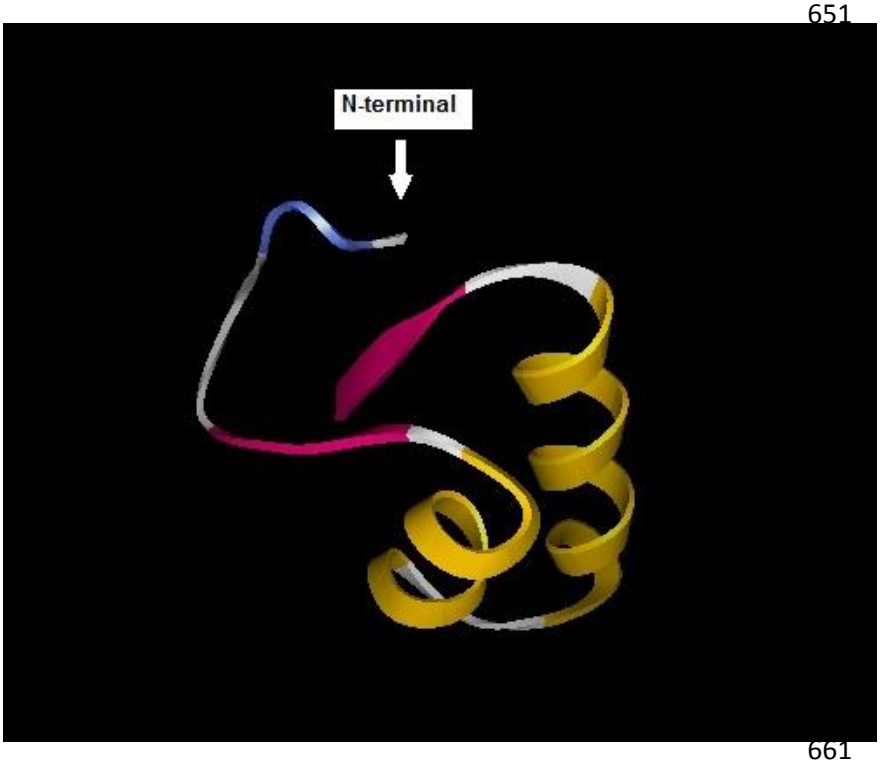


Fig 4. 3D-structure model constructed according to Swiss-Pdb-Viewer in Swiss-Model. The N-terminal shows decapeptide. In yellow appears alpha helices and the white loops and in pink β-turns.

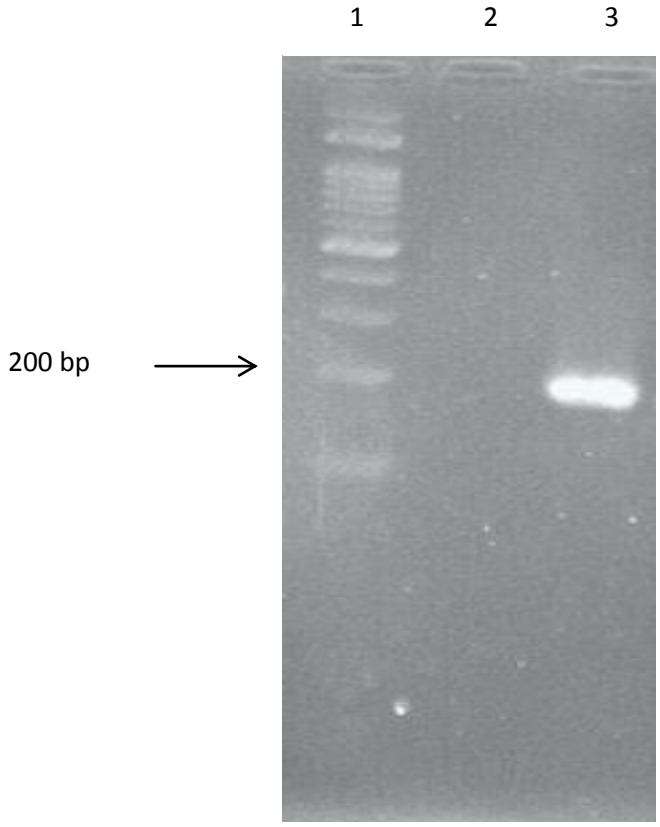


Fig. 5. Agarose gel electrophoresis of PCR colony. Line 1, DNA molecular weight marker (CinnaGen, SL7041, PR911653); line 2, negative control; line 3, the band of 186 bp of GnRH/GAP gene.

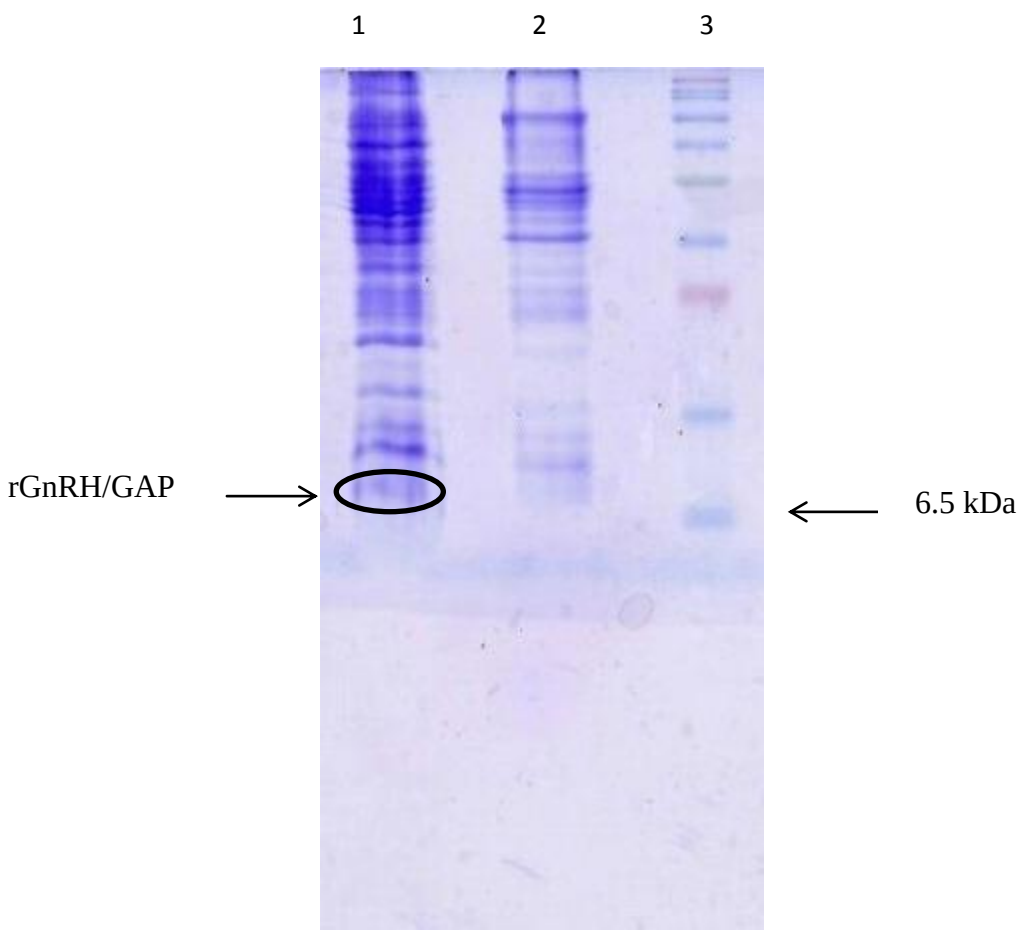


Fig. 6. SDS-PAGE gel electrophoresis of protein extraction of the recombinant bacteria cells. Line 1, induced bacterial cells; line 2, un-induced bacterial cells; line 3, Protein molecular weight marker (GeneDirex, PM001-0500). The arrow inside the picture represents 8 kDa of the expressed recombinant peptide.

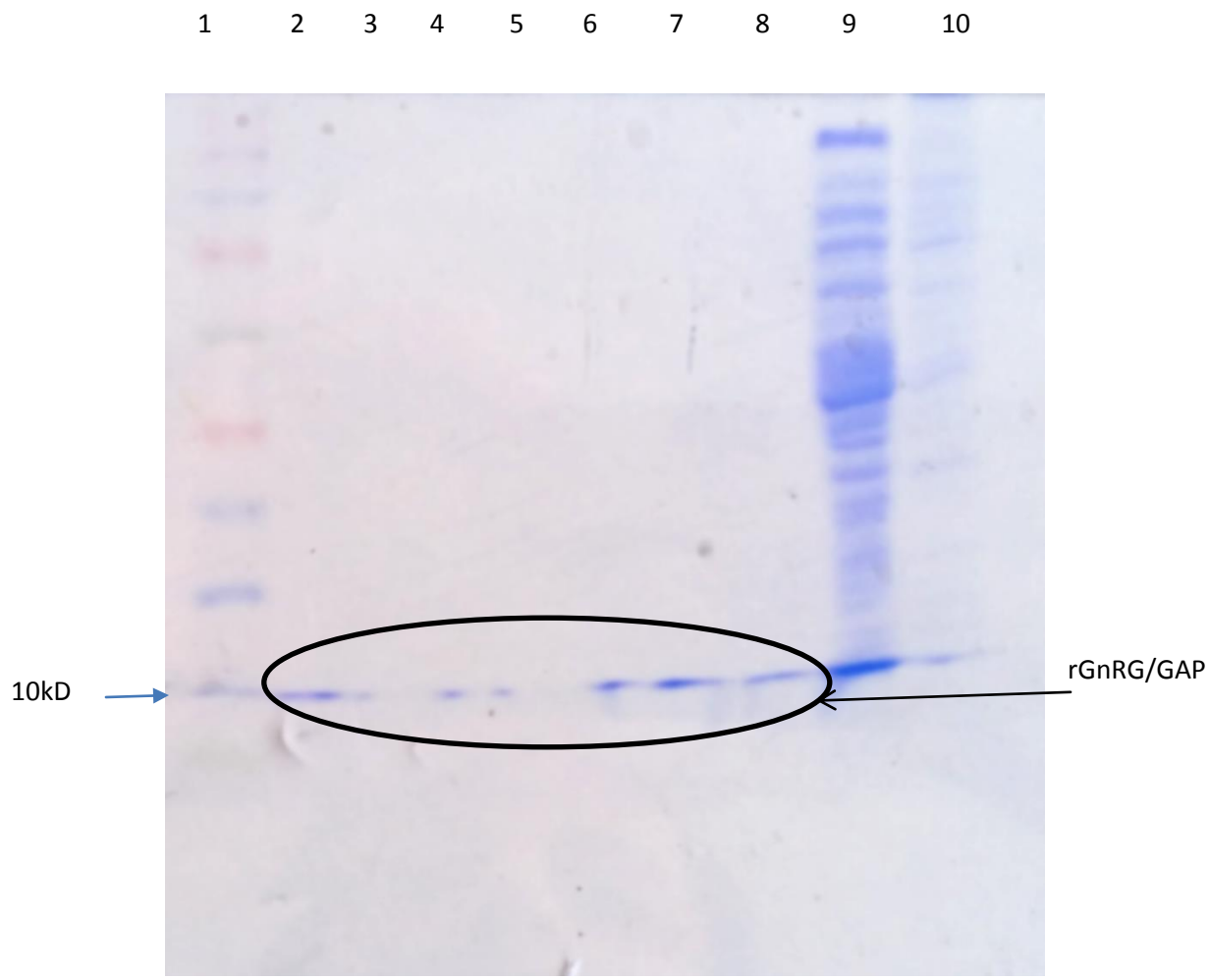


Fig. 7. SDS-PAGE gel electrophoresis of the recombinant peptide. Line1, Protein molecular weight marker (Bio-Rad, Model: 1610374); line 2 to 8, the bond of 8 kDa of purified recombinant peptide eluted fron affinity chromotography; line 9, induced recombinant bacterial cells lysate; line 10, un-induced recombinant bacterial cell lysate.

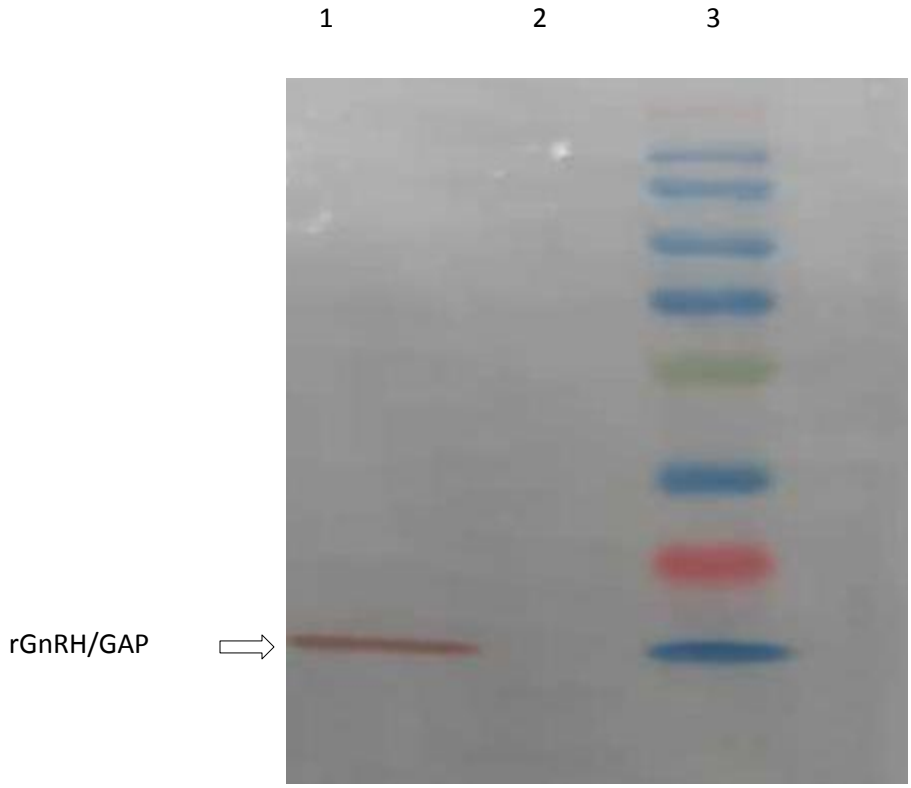


Fig 8. Western blot analysis of rGnRH/GAP after purification**.** Line1, The band of 8 kDa of rGnRH/GAP; line2, negative control; Line3, protein molecular weight marker (GeneDirex, PM001-0500)

MS/MS Peptide Matches

m/z meas.	z	Rt [min]	Score	P	Sequence	Modification	Range
1454.6863	1	26.80	60.5085	0	EHWSHSLYPGGK.R		1-13
1496.704	1	28.00	30.1565	0	EHWSHSLYPGGK.R	Acetyl: 1	1-13
1610.8025	1	26.20	59.3213	1	EHWSHSLYPGGKR.E		1-14
1652.8146	1	27.25	34.0074	1	EHWSHSLYPGGKR.E	Acetyl: 1	1-14
1674.7773	1	26.65	45.1752	0	R.ELEGLQSPEDSDEVK.L		15-29
3043.3531	1	30.25	59.6737	1	R.ELEGLQSPEDSDEVKLCDGDECSYLR.R	Carbamidomethyl: 17, 22	15-40
1387.577	1	26.50	18.1585	0	K.LCDGDECSYLR.R	Carbamidomethyl: 2, 7	30-40
1019.5616	1	28.60	22.9119	0	R.SILADMLTR.Q		49-57
1035.5691	1	28.60	23.3763	0	R.SILADMLTR.Q	Oxidation: 6	49-57
1594.8156	1	23.65	2.01982	1	R.SILADMLTRQMQR.K	Oxidation: 6, 11	49-61
1537.802	1	23.80	63.5183	1	K.KLAAALEHHHHHH.-		64-76
1579.7577	1	27.40	14.1284	1	K.KLAAALEHHHHHH.-	Acetyl: 1	64-76
1409.7122	1	22.90	56.7461	0	K.LAAALEHHHHHH.-		65-76

Fig. 9. MALDI- MS/MS data.

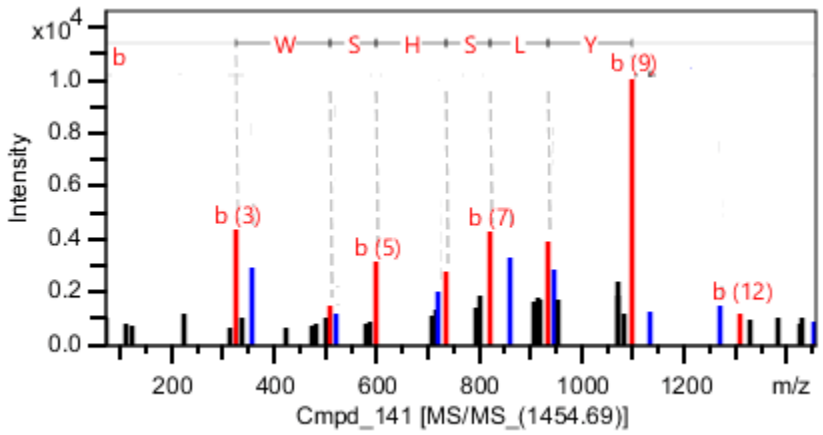
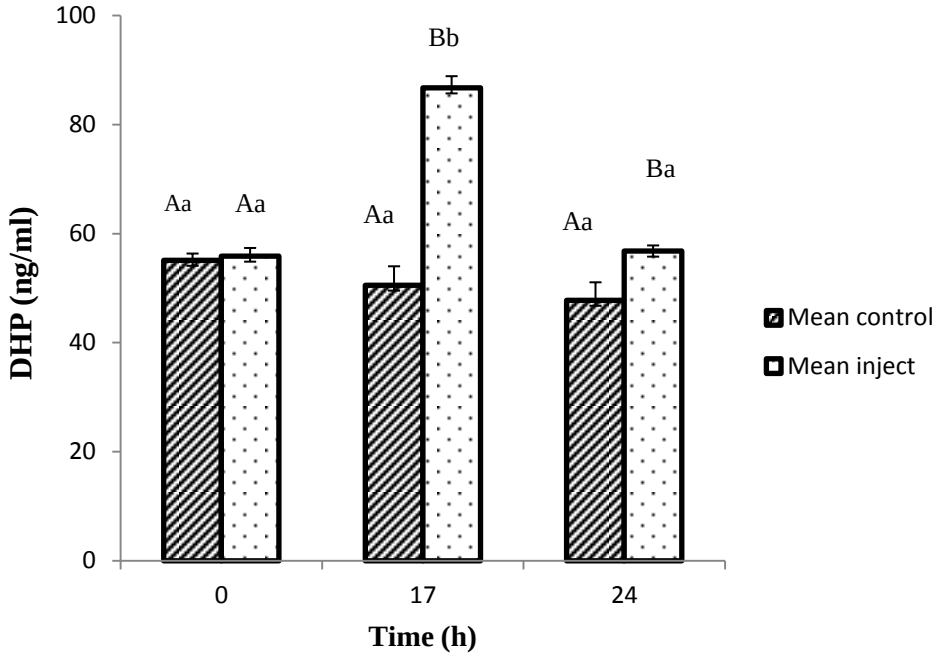


Fig 10. Spectra of recombinant GnRH decapeptide sequence (six sequences and the other spectral data was not shown).



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739

740 Fig. 11. Effects of recombinant GnRH/GAP, and 0.9% NaCl on 17α, 20β-dihydroxy-4-pregnen-
741 3-one (DHP) release in goldfish. Data are expressed as the mean ± SD. Uppercase letters indicate
742 significant differences among treatments at the same sampling time. Lowercase letters indicate
743 significant differences among sampling times within a group ($P < 0.05$).

744