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Research Paper

Cloning and Expression of Gonadotropin-releasing Hormone to Develop an Immunological-based Sterilization Vaccine

Mozhan Javidi[1], Alireza Gholami[2*], Parvaneh Maghami[1]

1. Department of Biology, Faculty of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran.
2. R&D Department, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran.

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ABSTRACT

Background: Gonadotropin hormone-releasing hormone (GnRH) is a peptide involved in mammals' fertility and may also serve as a candidate target for cancer immunotherapy. Immunonsterilization is known as a proper alternative to surgical castration and has been advocated by European countries in recent years. Immunization with GnRH can effectively inhibit the secretion of gonadotropins and cause infertility in both genders of mammals. In this study, a recombinant trimer of GnRH is designed and expressed in a prokaryotic system.

Materials and Methods: A construct containing GnRH trimer was designed and prepared using gene synthesis. A cloning site was embedded and connected to the GnRH using a linker to further clone any protein of interest. The construct was subcloned into a pET-32a+ plasmid vector. The recombinant vector was transferred to BL21, an *Escherichia coli* strain, and the expression was induced using isopropyl β- d-1-thiogalactopyranoside (IPTG). The cell lysate was prepared using lysis buffer and nickel affinity chromatography purification. The GnRH expression was evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis after protein purification.

Results: The cloning was verified using a polymerase chain reaction (PCR) followed by sequencing the recombinant vector. The result of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis verified the recombinant protein's expression and the purification process's function.

Conclusion: The GnRH was properly cloned and expressed in BL21. The results also verified the reliability of the purification process. The construct design allows the researchers to express another peptide sequence attached to the GnRH using the embedded linker to improve the stability and antigenicity. A stable recombinant GnRH would be applied in immunocastration and cancer immunotherapies.

*** Corresponding Author:**
Alireza Gholami
Address: *R&D Department, Research and Production Complex, Pasteur Institute of Iran, Tehran, Iran.*
Phone: +98 (912) 3486080
E-mail: *algo2212@gmail.com*

Introduction

Controlling the population of stray dogs has always been one of the burdens of public health. These dogs are the main reservoir of various diseases, such as rabies in other animals as well as humans [1]. The increase in the population of dogs is considered a serious threat to the preservation of biodiversity [2]. The surgical methods used to sterilize dogs present problems, such as high costs and logistical difficulties in trapping the dogs [3]. Non-surgical castration options include utilizing the clamps, rings, and banders as bloodless castration, besides immunological castration methods [4]. Gonadotropin-releasing hormone (GnRH) is a decapeptide secreted from the hypothalamus that induces the secretion of gonadotropins. Immunization against GnRH can effectively induce infertility in male and female mammals [5]. In recent years, GnRH and its receptor have also been studied as a candidate target for hormone-dependent cancer immunotherapy [6]; it has been known as a regulator of cell proliferation. The GnRH receptor activates a phosphotyrosine phosphatase and reduces cancer cell proliferation. It has been well-studied in breast, ovarian, endometrial, and glioblastoma cancer cells [7-9]. Due to the ovarian suppressive function of GnRH its analogs have also been proposed as prophylaxis to the toxic effects of radiotherapy and chemotherapy on ovarian cells [10].

Since the GnRH decapeptide is relatively unstable, it has been proposed to be used along with various carriers, to induce proper immunity [11]. The current immunonsterilization vaccines need to be applied in two doses which brings some limitations [12]. Accordingly, more research should be carried out to develop a more potent vaccine. On the other hand, a stable recombinant GnRH could be further evaluated as a candidate for cancer immunotherapy drugs [13]. This study examines the cloning and expression of a fragment containing a triple GnRH sequence along with a linker and some specific sites of restriction enzymes for further cloning of a carrier protein to improve the GnRH stability.

Materials and Methods

The nucleotide sequence of GnRH was extracted from The National Center for Biotechnology Information (Homo sapiens, Accession Number: NG_016457.1), and codon optimized for BL21, an *Escherichia coli* strain suggested for recombinant protein expression. pET-32a+ was chosen to express the recombinant protein attached to a HisTag for further purification. NcoI (ER0571, Thermo, USA) and BamHI (ER0051, Thermo, USA) restriction enzymes were chosen for cloning the construct in pET-32a+. Two unique restriction sites, including NheI (ER0971, Thermo, USA), and SnabI (ER0401, Thermo, USA) were embedded in the fragment for a further cloning step to improve the stability of the recombinant trimer of GnRH. The final sequence was reconsidered for expected frameshifts after cloning into the pET-32a+ + expression vector.

The synthesized fragment was received in pUC57 and was transferred to Top 10 *E. coli*. The Favorgen (FAPDE 001, YTA, Iran) kit was used for plasmid extraction according to the kit's protocol. The designed fragment, including GnRH, was primarily amplified using newly designed primers (F: GTGGAATTGTGAGCGGATAAC, R: GCTGCAAGGCGATTAAGTTG) to reach a high concentration of the fragment (481 base pair [bp]) for cloning. The polymerase chain reaction (PCR) amplification was carried out using Ampliqon mastermix (A140301, Ampliqon, Denmark). The reaction temperature cycles were as follows: Denature for 30 s at 94 ˚C, anneal primers for 30 s at 64 ˚C, and extension of amplification for 30 s at 64 ˚C. Both the PCR product and pET-32a+ were digested using NcoI, and BamH1 enzymes. Briefly, one unit of the restriction enzymes was used per µg of the PCR product and the pET vector, and the reaction was incubated at 37 ˚C for 12 h. To avoid self ligation the digested pET vector was separated by gel electrophoresis and extracted from the gel. The ligation process was carried out using T4 DNA ligase (EL0011, Thermo, USA). The ligation process was carried out using 1 unit of the enzyme at room temperature for 1 h. The sequence of recombinant pET-32a+ (rpET) was verified by the Sanger sequencing method (Pishgam, Iran) using S-tag and T7 terminator primers. The rpET was transferred to BL21 using cold calcium chloride heat shock transformation and the protein expression was induced by isopropyl β- d-1-thiogalactopyranoside (IPTG). The BL21(DE3) strain is widely used for recombinant protein production because of its engineered capacity to produce T7 polymerase and its deficiency in some proteases The induction was carried out at OD600=0.6 with two concentrations of IPTG (0.1, and 0.5 mM IPTG).

The culture was harvested at 2 h and 4 h after induction to compare them. The harvest was lysed and centrifuged at 18000 relative centrifugal force for 10 min. The supernatant was used to be assessed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method using ExcelBand protein marker covering a wide range of 5-245 kDa (PM2700, SMOBIO, Taiwan).

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Figure 1. The designed fragment to express GnRH in the prokaryotic system using pET-32a+

Notes: Three repetitions of the GnRH decapeptide sequence were attached to a rigid linker sequence (EAAAK) to be used for further cloning of any gene of interest.

The purification was carried out using a Ni-NTA column (R90101, Thermo, USA). A total of 1 mL of Ni-NTA resin was packed in a 5 mL empty poly prep gravity flow columns (7311550, Bio-Rad, USA) and equilibrated with an equilibration buffer containing 50 mM sodium phosphate buffer, pH 8.0, 500 mM sodium chloride, 0.5% triton X-100, 10% glycerol and 10 mM imidazole. The clarified bacterial lysate was passed through the column and the flow through was collected in a separate tube. The resin was washed with 3 column volumes of the equilibration buffer. The column was then eluted with an elution buffer similar to the equilibration buffer containing 250 mM imidazole.

Results

The gene synthesis was carried out by GeneScript company and the designed fragment was received in the pUC57 (rpUC57) vector (Figure 1). The plasmid was transferred to TOP10 *E. coli* for the next steps of cloning. The designed fragment was 131 bp in length.

Due to the small size of the synthesized fragment, it could barely be extracted from agarose gel. Thus, the GnRH coding fragment was amplified using designed primers from the rpUC57 plasmid. The resulting fragment was 481 bp in length. The PCR product was analyzed by 1.5% agarose gel (Figure 2).

The PCR product was digested and the resulting 131 bp fragment was extracted from agarose gel using Expin™ Combo GP kit (Cat. No. 112-150 / 112-102, GeneAll, Korea). The cloning was verified by bacteria colony PCR using S-tag and T7-terminator primers which are universal primers used for sequencing the PET vector. In case of a successful cloning, a 321 bp band can be expected and the original pET vector would result in a 190 bp band. The results from sequencing using the abovementioned primers also verified the cloning and the intactness of the GnRH coding sequence (Figure 3).

The SDS-PAGE analysis showed no difference between the two employed concentrations of IPTG; however, the expression of an expected 29 kDa band was noticeably higher in the harvests collected at 4 h after induction compared to those at 2 h. The BL21 lysate was used as the negative control. A higher concentration

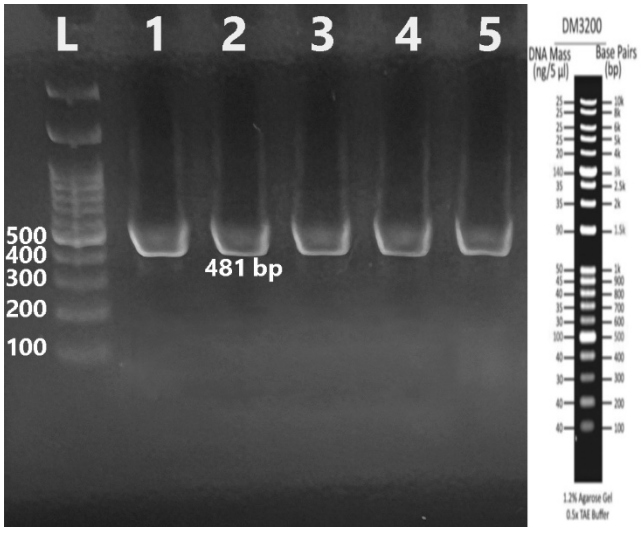

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Figure 2. The synthesized fragment amplified from the delivered construct

Notes: A 481 base pair band is the PCR amplified band of interest. Lanes 1 to 5 show PCR fragments amplified from the rpUC57 using the designed primers and L shows a ladder 10 kb.

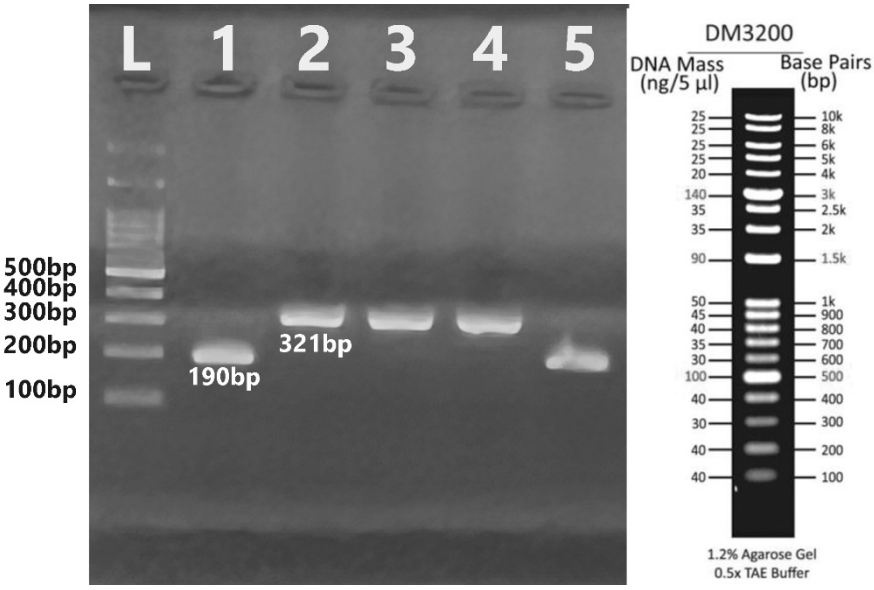

Figure 3. The verification of cloning using PCR conducted on recombinant plasmids

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Notes: In the case of insertion of the fragment a 321 base pair band was expected (lanes 2, 3, and 4), and a 190 bp band resulted from the original cloning vector (lanes 1, and 5). Meanwhile, L shows a ladder of 10 kb.

of protein bands was observed at the expected area (29 kDa) in recombinant samples (Figure 4).

The purification was properly carried out using the Ni-NTA column. The results of SDS-PAGE verified the 29 kDa band of interest in recombinant BL21 culture while the negative controls (BL21 bacteria lysed and purified with Ni-NTA clolumn) showed no similar band (Figure 5).

Discussion

The increase in stray animal populations has become a significant global burden that has many negative effects on society, the environment, and public health [1]. The most common standard methods to control the population of these animals include surgical sterilization and non-surgical contraceptive methods [14]. New non-surgical methods include anti-fertility immunogenic agents and hormone-based contraception [15]. One of

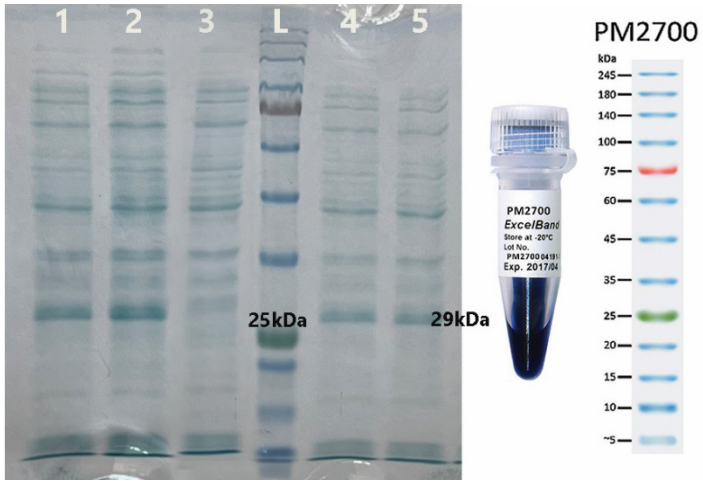

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Figure 4. The results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis verified the GnRH expression

Notes: Lanes 1 and 2 show the harvest at 4 h after the induction with 0.1 and 0.5 mM IPTG, respectively. Lane 3 shows negative control (BL21). Meanwhile, lanes 4 and 5 show the harvest at 2 h after the induction with 0.1 and 0.5 mM IPTG, respectively. L shows the protein ladder (PM2700, 5-245 kDa).

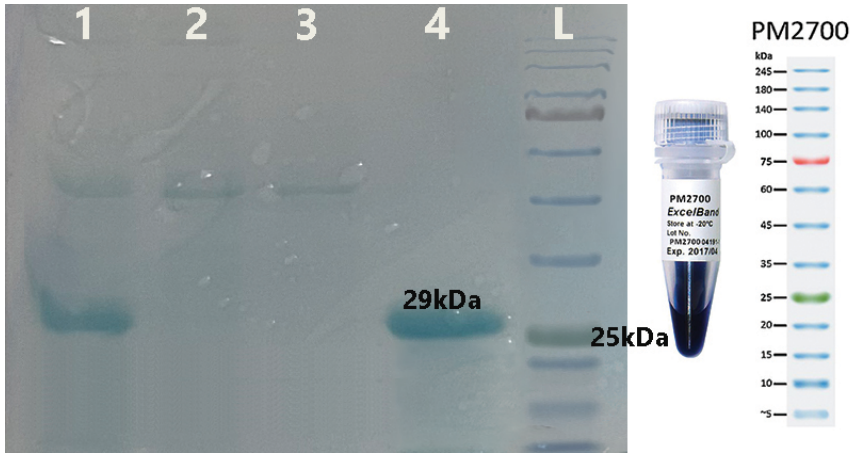

Figure 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis after purification

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Notes: Bands 1 and 4 are the recombinant bacteria that expressed the 29kDa band. Bands 2 and 3 are the negative controls. L shows protein ladder (PM2700, 5-245 kDa).

the most promising research areas for stray animal population control is the development of recombinant contraceptive vaccines [16]. The regulation of reproductive functions is centrally controlled by the hypothalamic decapeptide gonadotropin-releasing hormone. This peptide binds to specific GnRH receptors on pituitary gonadotrophs [17]. The activation of specific GnRH receptors leads to the secretion of luteinizing hormone and follicle-stimulating hormone, followed by the regulation of hormone-dependent structural mechanisms in the reproductive cycle [18]. The current commercial immunocontraceptive vaccines are effective with a longer duration of contraceptive effect after two doses of injection. GnRH is also a suitable candidate for developing immunotherapy drugs against cancer. Cell proliferation is inhibited by GnRH and its receptor [8, 19]. Access to a stable GnRH would be highly valuable for immunocastration and immunotherapy.

Due to the weak immunogenicity of GnRH decapeptide and its unstable structure, there was a need to design a recombinant structure for vaccines against GnRH. In a study by Jinshu et al., a GnRH-based recombinant vaccine was designed containing 3 repeats of GnRH decapeptide coding sequences along with the measles virus epitope. The anti-fertility effects of this vaccine were reported in male and female rats without using any adjuvants [20, 21]. They examined the optimal conditions for producing their recombinant GnRH vaccine on an industrial scale. The initial investigation focused on the expression of the GnRH construct with IPTG and lactose induction [22]. A study by Doroteu et al. on the comparison of the effect of injecting one and two doses of a commercialized anti-GnRH vaccine to Nelore bulls indicated the need to inject at least two doses of the vaccine to create a stable effect [12]. The higher efficiency of two-dose vaccination was also observed in white-tailed deer in another study [15]. While the potential fertility control capacity of GnRH decapeptide has been known for more than four decades, the successful development of a commercial product for animals has been limited by the lack of development of a suitable delivery system to induce adequate immunogenicity by requiring only a single dose injection.

In the current study, to develop a suitable sterilization vaccine, a sequence of three GnRH decapeptides was successfully expressed in a prokaryotic system and purified using a Ni-NTA column. Additionally, two additional distinct restriction sites were included with the EAAAK linker downstream of GnRH sequences. This cloning site could be further utilized to insert the desired carrier to enhance the stability of the recombinant GnRH. The expression level of the recombinant plasmid was improved by adjusting the concentration of IPTG and harvesting time. The need for a larger carrier to improve GnRH stability makes it a suitable candidate for expression in prokaryotic systems. The use of a non-pathogenic prokaryotic host, such as BL21, in addition to its harmlessness, has advantages such as easy cultivation steps, low cost, and high-scale production potential, which can be an advantage in industrial production of GnRH-based recombinant vaccines in the future.

Conclusion

In this study, it was possible to successfully clone and express the designed GnRH construct in the prokaryotic system. A stable GnRH would be an important step toward the development of immunocontraceptive vaccines

for fertility control or immunotherapy drugs for cancers. The designed construct used in this study can add a carrier attached to GnRH using the embedded linker. In future studies many carriers would be applied to improve the GnRH stability, simultaneously induce immunity against another antigen such as rabies glycoprotein, or trigger stronger immunity against cancer cells.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors' contributions

Conceptualization, investigation and formal analysis: Mozhan Javidi; Validation: Mozhan Javidi and Alireza Gholami; Resources: Parvaneh Maghami and Alireza Gholami; Project administration: Alireza Gholami; Supervision: Parvaneh Maghami; Methodology and writing the original draft: All authors; Review and editing: Parvaneh Maghami.

Conflicts of interest

The authors declared no conflict of interest.

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