Production of a Fusion Protein Containing GnRH as a Contraceptive Antigen

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1. Abstract:

Our laboratories have been developing and testing recombinant micro-organisms that can be administered as reformulated USDA approved vaccines to feral cats as part of a baiting program. Published research has demonstrated that when gonadotrophin releasing hormone (GnRH) is appropriately formulated and injected into male or female cats, infertility ensues. As a first step in producing a recombinant vaccine, a plasmid was created by cloning the following genes to encode a fusion protein consisting of a histidine-tag(6xHIS)::flagellin (FliC)::enhanced green fluorescent protein (EGFP)::GnRH. The gene encoding the fusion protein (FEG) was placed under the control of an inducible promoter (T7 RNA polymerase) and the plasmid transformed into the gut bacterium *Escherichia coli.* Following induction of FEG in *E. coli*, a cell lysate was prepared and the fusion protein purified to approximately 90% purity by nickel affinity chromatography. The purified fusion protein was shown to be specifically reactive with antibodies that recognized either the His-tag, FliC, EGF, or GnRH. There are several positive attributes of the fusion protein: 1) the HIS-tag allows for one step purification; 2) the GFP portion causes the culture as well as the affinity column to glow green and validates expression and binding respectively; 3) the FliC portion has been shown to enhance immunogenicity at least 1000 fold; and 4) the estimated cost (materials and labor) of the bacterial produced FEG is about 10-fold cheaper than a commercially synthesized version. The recombinant FEG is being tested as an injectable contraceptive vaccine in mice (VA Tech) and cats (Auburn University). If infertility is induced, the gene encoding the recombinant FEG will be moved into feline herpes virus for expression studies and further anti-fertility testing in cats.

2. Introduction

Immunocontraception is a concept that has received a great deal of attention in the last decade as a less invasive substitute for surgical sterilization. Gonadotropin-releasing hormone (GnRH) is a neuropeptide systhesized in the hypothalamus with tropic effects on the anterior pituitary. It is a key molecule in the maturation of gametes and in the maintenance of gonads [1]. GnRH is a decapeptide which, when secreted into the cardiovascular system, begins a cascade of hormonal effects to ultimately control the maturation of sperm and egg cells [2]. As its name implies, GnRH is responsible for the release of gonadotropins—lutenizing hormone (LH) and follicle-stimulating hormone (FSH)—from cells in the anterior pituitary called gonadotrophs. These gonadotropins give rise to cortical sex hormones (testosterone, estrogen, progesterone) in the gonads which in turn are responsible for sexual maturity. In a GnRH immunocontraception study on male cats, it was found that in addition to reduced ejaculate size and sperm count, reduced levels of GnRH also lead to atrophy of the testes [3].

To trigger the desired immune response against GnRH, we have developed a recombinant plasmid encoding a fusion protein containing FliC, EGFP and a 14 repeat GnRH sequence. FliC, the *Salmonella* flagellin protein, stimulates the immune system by binding to Toll-like Receptor-5 (TLR-5) to act as an adjuvant. As a result, antibodies are efficiently produced against the neighboring GnRH peptide sequence. Since no differentiation between the body's natural GnRH and the introduced GnRH of the FEG construct can be detected, natural GnRH is targeted by a specific antibody and ultimately destroyed; since LH and FSH become deficient, then the gonadal tissues fails to develop and infertility ensues.

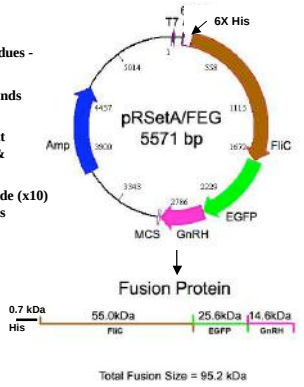
Purification of the fusion protein was accomplished via His-tag nickel column chromatography. Our cloning vector, pRSetA, includes a T7 promoter, β-lactamase (ampicillin resistance), and a 6x-Histidine tag [4].

3. Objectives of the study were to :

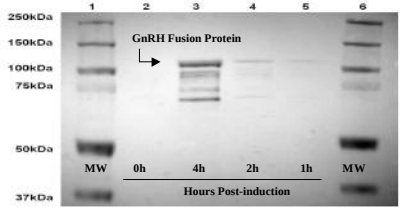
1. Construct a fusion gene encoding histidine-tag(6xHIS):: flagellin(FliC):: enhanced green fluorescent protein (EGFP)::GnRH
2. Clone the fusion gene into a plasmid vector
3. Express the fusion protein in *E. coli*
4. Purify the fusion protein by affinity chromatography

4. Features of fusion gene & corresponding fusion protein

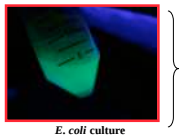
- **His-tag = 6 his residues - binds nickel**
- **FliC = flagellin - binds TLR 5, adjuvant**
- **EGFP = fluorescent protein - adjuvant & marker**
- **GnRH = decapeptide (x10) - induces specific Abs**



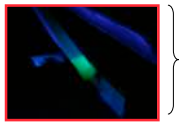
5. Immunoblot of Induced GnRH Fusion Protein in *E. coli*



6. Rationale for Affinity Column Purification:

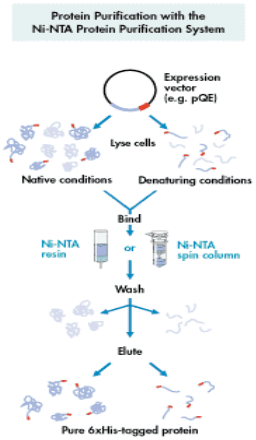


E. coli culture

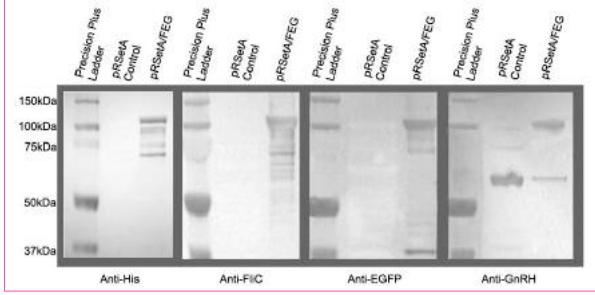


Extract on Affinity Column

When the cellular extract is passed through the Ni* affinity column, only the histidine tagged proteins have an affinity to nickel and bind to the column. After washing the column to ensure that only his-tag proteins remain in the column, elution is performed by washing with a lower pH buffer.



7. Immunoreactivity of Affinity Purified GnRH Fusion Protein: 50 mg/ 50 ml culture



8. Estimated Cost of Recombinant versus Synthesized GnRH Fusion Protein

Source	50 mg	Min. Dose*	Cost/Animal
Synthesized	$30,575.	50 ug	$ 30.58
Recombinant	$ 3,637.	50 ug	$ 3.64

* Based on research by J. Levy *et al*., (1)

9. Conclusions: A GnRH fusion protein was:

1. **Produced from a fusion gene encoding 4 distinct protein motifs in *Escherichia coli*.**
2. **Immunoreactive with antibodies specific not only for GnRH but also Flic, EGFP and the His-tag; the later motifs facilitate purification and also act as adjuvants.**
3. **Calculated to be approximately 10X fold less costly to produce as a recombinant protein as opposed to being commercially synthesized.**

10. Ongoing Research:

- **The GnRH fusion protein is being tested in mice (Va Tech) and cats (Auburn University) in the form of an injectable for its ability to stimulate GnRH antibodies and induce infertility.**
- **The synthetic gene encoding the GnRH fusion protein is being engineered into a feline herpes virus USDA approved vaccine strain. This will allow the testing of the FHV as a means to contracept feral cats.**

11. References

1. Martini, Frederic H., Fundamentals of Anatomy & Physiology, 6th ed, San Francisco: Benjamin Hall, 2004.
2. http://www.fertility.com/international/treatment/Female_Treatments/Second Step/Gonadotropin_releasing_Hormone.jsp
3. Levy *et al.*, Theriogenology 62: 1116-1130, 2004
4. Invitrogen. pRSet A, B and C Users Manual version E. Carlsbad, Ca.

12. Acknowledgements

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