Cloning, Expression and Purification of His-tagged Firefly Luciferase

Sameer Jafari, Holly Blumenberg, Lachezara Rangelova, Valery Bognar, and Suma Somasekharan
School of Theoretical and Applied Science, Ramapo College of New Jersey, Mahwah, NJ, 07430

Introduction
Firefly luciferase is the enzyme responsible for the phenomenon of bioluminescence in insects such as firefly (Photinus pyralis), glowworm beetles and click beetles. Luciferase catalyzes the oxidation of luciferin resulting in the emission of yellow light 550nm to 620nm. Firefly luciferase is a 62kDa protein whose structure reveals a protein with two domains N and C. The active site lies in the C terminal domain. The large N terminal domain is made up of a β barrel and an αβββα structure. The C terminal has a fold called the αββββ structure found in many hydrolases. The enzyme belongs to a superfamily of adenylyl-forming enzymes which depend on ATP in the adenylation reaction. The goal of the project is to generate a recombinant DNA construct of a His-tagged luciferase gene in a pET expression vector, overexpress luciferase in E.coli and purify the protein using Metal-Chelate Affinity purification.

Experimental Design
In order to facilitate proper insertion of the luciferase coding sequence the following primer pair was designed for PCR:
Forward primer with a BamHI restriction site (green):
GAGGATCATGAGGACGCAAAAAAC
Reverse primer with a SacI restriction site (green):
GTGAGCTTCAGAATTTGGACGCTCCG

The pET30a vector, a strong expression vector, consists of a T7 promoter, N and C-terminal Histidine (His) tags, transcription initiation site, ribosome binding site for translation and are IPTG (isopropyl β-D-thiogalactopyranoside) inducible. The lac I repressor expressed by the pET vector will bind the lac operator and block gene expression. IPTG can block the repressor and enable expression of the genes under the control of the T7 promoter. IPTG allows for proper expression of His-tagged proteins which can be purified with affinity chromatography. The luciferase gene was sourced from pGEM-Muc (Promega).

Experimental Methods
Polymerase chain reaction (PCR) was utilized to amplify the luciferase gene found in pGEM-luc using Forward and Reverse primers to introduce restriction sites for BamHI and SacI on either sides of the coding sequence of luciferase. The luciferase PCR fragment and the pET30a vector were digested using BamHI and SacI. The digested luciferase fragment and linearized pET30a were gel purified and subsequently ligated using T4 DNA ligase. Bacterial transformation (heat shock at 42°C for 1 min) was done with control and ligated recombinant plasmids using DH5α cells plated on LB agar media with kanamycin antibiotic. After overnight incubation plasmid purification (Geneset Miniprep kit) was performed to purify recombinant plasmid DNA. A diagnostic digest was performed to identify the recombinant pET30a carrying luciferase (pET30a-luc). pET30a-luc plasmids were then transferred into BL21 DE3 E. coli cells, which are ideal for overexpression of the protein. After overnight incubation of the BL21 DE3 cells, colonies were picked and grown overnight in 4mL liquid culture. They were then transferred to 250mL LB agar media with kanamycin and grown until an optical density (OD) of 0.6 was reached. Expression of luciferase was done through fast induction by incubating cells with IPTG (final concentration 1mM) for 3 hours. After fast induction, the liquid culture was centrifuged for 5 min at 7000rpm in 4°C and the pellet bacterial cells were stored at -20°C.

Im mobilized Metal Affinity Chromatography

Immobile Metal Affinity Chromatography (IMAC) is a commonly used versatile protein purification technique that can yield a very high purity protein. The technique is commonly used to purify recombinant proteins carrying a polyhistidine tag. IMAC uses columns filled with matrices which have a transition metal such as Ni²⁺ or Co²⁺ immobilized on the matrix. The electron donor groups in the side chain of Histidine form coordination bonds with the transition metal. This therefore binds the recombinant protein on the column and all other proteins are washed out by a wash buffer. Eventually the protein can be eluted from the columns by using increasing concentrations of Imidazole in the elution buffer. Imidazole has the same structure as the side chain of Histidine and hence competes with the Histidine for the Ni²⁺ ions.

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References: 1) Crystal structure of firefly luciferase throws light on a superfamily of adenylyl-forming enzymes, https://doi.org/10.1036/S0096-2126(06)00033-0