Optimization of expression and purification steps of LasI synthase, a N-acetyl homoserine lactone (AHL) autoinducer enzyme for bacterial quorum sensing

Jesse Keeler, Remington Turner, Miranda Ghali, Dr. Rajesh Nagarajan
Research Experience for Undergraduates at Boise State University; Northwest Nazarene University

Abstract
Bacteria communicate, via quorum sensing, using small chemical molecules called autoinducers, to take a census count of neighboring bacteria in a colony. Quorum sensing helps bacteria to work as a unit to form biofilms, an extracellular matrix designed to protect them from antibiotic attack. Pseudomonas aeruginosa, an opportunistic pathogen implicated in cyclic fibrosis, meningitis and pneumonia, use 3-oxo-decanoyl homoserine lactone autoinducers (AHL) to facilitate intercellular communication and in the formation of biofilms. Before contact, antibiotics and the body’s immune system have little or no effect on bacteria covered by their biofilm, so cells continue to thrive while harming the host with virulence factors and toxins. LasI AHL synthase enzyme uses 3-oxo-decanoyl-ACP and 3-oxo-decanoyl-methionine (SAM) to synthesize 3-oxo-decanoyl homoserine lactone autoinducer. However, little is understood about mechanism of LasI enzyme and thus enzyme must be obtained in order to run enzymatic studies. The goal of this research is to optimize cell growth, storage conditions, protein expression and purification for LasI to enable future kinetic studies on this enzyme. The process of obtaining pure enzyme begins with the plasmid transformation of S. aureus. Then, the plasmid was induced in LB medium 25°C for 5 hours. Cells were then used to supernatant 75% size exclusion chromatography and the purity of LasI will be verified by SDS-PAGE gel electrophoresis. With better understanding of the function of this enzyme, an inhibitor for LasI can be designed and synthesized to limit the ability of these bacteria to form biofilm, which will increase the efficacy of antibiotic therapy in the future.

Introduction
The LasI vector used for expression was an S1A303 tope plasmid, which was transformed from P. aeruginosa bacteria into E. coli bacteria by Prof. Mark Church, University of Colorado, Denver. The plasmid also contained two other key elements that contribute to purity and manipulation of the genes. First, the plasmid contains genetic information to light the antibiotic kanamycin. With kanamycin resistance, lasI contain E. coli are able to grow in antibiotic LB broth solution when all other microorganisms die. Second, the plasmid is restricted by the lacZ2 restriction pathways. This allows the plasmid to only be transcribed if lactose is present in the cell. Since normal lacZ2/lacT regulation is controlled by a negative feedback loop, a substrate called IPTG is used to induce the pathway to continue to replicate. This forces the bacteria to make the LasI enzyme. The structure of LasI allows for two substrates to bind in a way that the substrates can bond together. A portion of lasI has a 3D structure like a tunnel, which creates space for the long carbon chain of 3-oxo-decanoyl-ACP (Figure 11). Other shorter chains are not able to fit into the tunnel for the conjugation of LasI. This allows for substrate specificity to longer chain ACPs, which may be used for more complex AHLs. The proposed reaction for AHL synthase is as shown in Figure 2. The first step is SAM and ACP to bind with lasI. Next an acylation reaction is performed between the two substrates, which releases the desired product, 3-oxo-C12-ACP (AHL). The quorum sensing pathway leads to the deadly formation of biofilms. The goal of this research is to find an inhibitor molecule that will prohibit the quorum sensing pathway, and shutdown biofilm formation.

Methods
Cell Preparation: isolated colonies of S1A303 on kanamycin LB agar plates were used to inoculate LB plus kanamycin, and IPTG was added to the plates. The cells were then harvested after 8 hours of expression.
Lysis and Purification: Cells were thawed on ice with 1 mL lysis buffer (20 mM Tris-HCl, 0.5 mM NaCl, pH 7.9, 10 mM imidazole per 250 mL of initial culture) and chilled to -20°C. The entire sample was then sonicated for 5 minutes and filtered through 0.2 μm filter. The supernatant was loaded onto the column and the flow-through was collected. 10 mL of wash buffer (200 mM Tris-HCl, 0.5 mM NaCl, pH 7.9, 40 mM imidazole) was added and one wash fraction was taken at 0.5 mL, fabric filter and stored on ice. Next, 5 mL of elution buffer (200 mM Tris-HCl, 0.5 mM NaCl, pH 7.9, 200 mM imidazole) was added and five 5 mL fractions were collected in Nalgene tubes and stored on ice until gel analysis. SDS-PAGE gels were performed before and after Superdex 27 size exclusion column.

Expression
LB broth from Fisher scientific was used for broth and agar plates. 100 μL of kanamycin stock solution (10 mg/mL) per 20 mL broth was used for 1L culture. The E. coli cells were given to use from Herbert Schweizer from Colorado State, Fort Collins. Fisher scientific instruments were used for sterilization (incubation loop, Bunsen burner and isopropanol alcohol squeeze bottle). The mini centrifugation instruments are from Eppendorf, while the large centrifuge is a Servall Evolution (model: SLA-3000). Bottles and flasks are from Nalgene.

Buffers for Lysis and Purification
The Tris-HCl and NaCl were from Fisher, but the imidazole was from Sigma-Aldrich. A stock buffer (A) was made to 200 mM Tris-HCl, 0.5 mM NaCl, pH 7.9. Lysis buffer used buffer A and 5 mM imidazole, wash buffer was made with buffer A and 40 mM imidazole, elution buffer was made with buffer A and 200 mM imidolate.

Lysis
A freshly made lysisoyte stock from Sigma was made to 40 mg/mL and 160 μL. Then PMSF from Sigma was made to 33 mg/mL isopropanol alcohol and 40 μL was used. An equilibrated 1 mL Ni-NTA resin (Qiagen) was packed into a 5 mL Qiagen gravity column. Falcon flasks and Eppendorf tubes were used to catch the fractions. In a 4°C cold box with ring stand and three finger clamps.

Analysis of Purity
15% SDS-PAGE gels were made to the recipe in Chem-Raf book using BIO-RAD chemical solutions. The electrophoresis equipment was a BIO-RAD mini-protein tetra system.

Results
Figure 1: SDS-PAGE profile of all the 15 bands in culture. Lane 1 is 100bp Bio-Rad ladder; lane 2 is crude lysate; lane 3 has crude lysate. Lane 4 has crude lysate and 5% SDS-PAGE. Lane 6 has crude lysate and 10% SDS-PAGE. Lane 7 has crude lysate and 15% SDS-PAGE. Lane 8 has crude lysate and 20% SDS-PAGE. Lane 9 has crude lysate and 30% SDS-PAGE. Lane 10 has crude lysate and 40% SDS-PAGE.

Figure 2: Comparison of the different bands with their molecular weight. A lane 1 is 100bp Bio-Rad ladder; lane 2 is crude lysate; lane 3 has crude lysate. Lane 4 has crude lysate and 5% SDS-PAGE. Lane 5 has crude lysate and 10% SDS-PAGE. Lane 6 has crude lysate and 15% SDS-PAGE. Lane 7 has crude lysate and 20% SDS-PAGE. Lane 8 has crude lysate and 30% SDS-PAGE. Lane 9 has crude lysate and 40% SDS-PAGE. Lane 10 has crude lysate and 50% SDS-PAGE.

Figure 3: SDS-PAGE gel of Ali culture. Lane 1 has bioreactor grown crude lysate; Lane 2 has crude lysate; Lane 3 has crude lysate. Lane 4 has crude lysate and 5% SDS-PAGE. Lane 5 has crude lysate and 10% SDS-PAGE. Lane 6 has crude lysate and 15% SDS-PAGE. Lane 7 has crude lysate and 20% SDS-PAGE. Lane 8 has crude lysate and 30% SDS-PAGE. Lane 9 has crude lysate and 40% SDS-PAGE. Lane 10 has crude lysate and 50% SDS-PAGE.

Discussion
Figure 5 shows optimized purification and expression time because the strong visible band occurred at 24 hr. Figure 6 shows that 6 hours of expression and 250ml, pellet is not as good as 8 hr. In figure 5, a longer expression time (8hr) makes for more protein in the elutions. Figures 5 and 6 use Qiagen Ni-NTA resin (1ml), whereas Figure 8 uses thermo Ni-NTA spin column (20mL). Figure 8 contain large amounts of impurities because Ni-NTA spin column, but these have visible banding above 24L. However, Qiagen gels show much more purity by having less visible bands above 24L. Figure 7 shows the elutions used in Figure 5 can be further buffer washed, and an additional SDS-PAGE gel was successful because the gel picture shows less higher weight molecular bands, which suggests even greater purity.

Conclusion
Purification and expression steps of LasI AHL synthase were optimized and found to be around 8 hours. Qiagen gravity column (1mL) was shown to be better at removing higher molecular weight contaminants, than the Thermo spin column (100mL). Using a 15% acrylamide solution is better than 12% for SDS-PAGE gel analysis because lasI has a relatively low molecular weight.

Future Work
The next steps to be taken in this research would be to concentrate the proteins samples after the size exclusion with amicon concentrator. Then the size of cultures should be increased, so that protein samples can be stored in various conditions and analyzed for optimal storage conditions. Also, larger batches of expressed culture must be made in order to have enough las on hard to perform assays to better understand the kinetics of LasI.

References

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