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Staphylococcus aureus is a Gram-positive bacterium that causes varieties of serious infections, and thus remains a significant threat to public health. The Mu50 glycopeptide-resistant *S. aureus* strain has reduced susceptibility to vancomycin, a glycopeptide antibiotic that is heavily relied on for the treatment of infections caused by *S.aureus*. The two component system GraSR genes from Mu50 glycopeptide-resistant *S.aureus* have been identified as a mediator in a rapid signal transduction cascade that occurs when the bacteria cell wall is stressed by antibiotics. Mu50 *S. aureus*, GraS and GraR were successfully cloned, expressed and purified using Glutathione Sepharose 4B for GraS which is designed for a single step purification of GST fusion protein, while GraR was purified using Fast Protein Liquid Chromatography with an anion exchanger resin DEAE Sepharose. The proteins are now ready for subsequent kinetics studies to investigate their role in cell wall stress by antibiotics, and regulation in cell wall biosynthesis.

The Analysis of the Native Molecular Weight of the Citrate/Methylcitrate Synthase *mmgD* from strain 168" of *Bacillus subtilis*, a rod shape gram-positive bacterium, capable of forming endospore during inadequate supply of nutrients, and the mother cell metabolic gene (*mmg)* is one operon that is expressed during this

sporulation. The *mmgD* protein is one of the six open reading frames (ORFs) of the *mmg* operon and was shown previously to be a combined citrate/methylcitrate synthase. The goal of this work was to determine the native molecular weight of this protein. Gel filtration chromatography is one of the methods use in determining the native state of a protein by comparing to known standard proteins molecular weights. The previously cloned *mmgD* gene was expressed, purified and subjected to gel filtration chromatography to establish its apparent native molecular weight. The analysis showed that a major fraction of the protein existed as a monomer while a very small fraction eluted from this column showed an aggregation of proteins.

CLONING AND PURIFICATION OF THE TWO COMPONENT SYSTEM GraSR INVOLVED IN GLYCOPEPTIDE ANTIBIOTIC RESISTANCE IN *STAPHYLOCOCCUS AUREUS* (MU5O), AND CHARACTERIZATION OF THE CITRATE/METHYLCITRATE SYNTHASE FROM *BACILLUS SUBTILIS* STRAIN 168

By

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Approved by

_____
Committee Chair

DEDICATION

To God almighty who makes a way where there seems to be none, my loving family who are always there with their support and finally, to everyone that dreams and diligently works to achieve it.

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

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Date of Acceptance by Committee

_____

Date of Oral Examination

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CHAPTER I

INTRODUCTION

I.A. Background View of *Staphylococcus aureus* (Mu50 strain)

Staphylococcus aureus is a Gram-positive spherical bacterium that occurs in microscopic yellow clusters resembling grapes. It is a major human pathogen and a significant public health threat that causes a variety of serious and suppurative (pus forming) infections hence normally referred to as hospital acquired (nosocomial) infection of surgical wounds. This pathogen has been particularly efficient at developing strains that are highly resistant to antibiotics (Timothy R. & Robin A. Howe (2002).Strains of methicillin-resistant *Staphylococcus aureus* (MRSA) have been a problem in hospitals for many years and have recently begun spreading outside hospital settings and within the community. Recent studies have shown that *S. aureus* is capable of mounting a response to antibiotics that target cell wall peptidoglycan biosynthesis (Antoaneta B. & Golemi-Kotra (2008). Until recently, Vancomycin, which is a glycopeptide antibiotic that was introduced in the mid-1950s, was believed to have retained activity against all strains of *S.aureus*. Therefore the spread of MRSA led to a heavily reliance on vancomycin, hence has led to an increase in the number of intermediate resistance strains VISA (vancomycin intermediate *staphylococcus aureus).* The first clinical strain of *S. aureus* resistant to vancomycin (MU50) was isolated in 1997 from the pus of a Japanese male baby with a surgical wound infection that did

not respond to vancomycin (Kuroda M, et al (2001). Similar strains have been reported from many other countries around world, irrespective of the controversy surrounding the definition of this type of resistance as MIC (Minimal Inhibitory Concentration), which varies in different countries it is used and also in the methods to detect it. There are some phenotypic differences between vancomycin resistant strains from different geographical areas; a common characteristic of these strains appears to be the accelerated synthesis of a grossly fortified cell wall.

1.B. The Resistant Mechanism of VISA/hVISA (Mu50)

Evidence regarding the mechanism for vancomycin resistance derives from studies on clinical and laboratory isolated strains of *S. aureus* with various levels of resistance. These resistant strains share the characteristics of a thickened cell wall with reduced levels of peptidoglycan cross-linking, that leads to an increase in free D-ala-D-ala side chains to which vancomycin can bind (Timothy R. & Robin A. Howe (2002)). The thickened peptidoglycan prevents the passage of vancomycin hence clogging it within the matrix. This forms a meshwork which hinders other drug molecules from reaching the site of cell wall biosynthesis at the plasma membrane. Using differential hybridization, the two component system GraSR, which is constitutively activated in Mu50 (VISA) and Mu3 (hVISA) but strongly depressed in vancomycin-susceptible *S. aureus* (VSSA) (Hui-min Neoh, et al (2008), were identified as glycopeptide-resistant genes.

1.C. The Two Component Systems GraSR

Whole genome array studies of Mu50 (VISA) have been identified, among other overexpressed genes, the Two Components Regulatory System (TCRS) referred to as GraSR. These participate in a phosphotransfer-mediated signaling pathway that require histidine protein kinase (HK) that is conserved in GraS, and a regulatory protein GraR. These two genes associated with glycopeptide resistance are known to respond to cell wall stress (Longzhu et al, 2009) through regulation of cell wall biosynthesis. The protein GraS which senses the environmental stimuli such as antibiotic drugs like vancomycin, is capable of undergoing autophosphorylation at a conserved histidine residue, and through a phosphorelay cascade, transfers the phosphoryl group to a conserved aspartic residue of response protein GraR, thus making it active to alter specific target gene transcription. GraR functions as a transcription regulator and when phosphorylated at a conserved asparate residue, forms the biologically active GraR- dimer species.

These two-component systems have been implicated in vancomycin resistance in *Enterococcus faecium* (VanSR) and bacitracin in *Bacillus subtilis* (LiaSR). Interestingly, vancomycins are bacterial cell wall synthesis inhibitors (B. Antoaneta & Golemi-Kotra, 2007). The GraSR system plays a central role in maintaining the integrity of the cell wall peptidoglycan and coordinates the *S. aureus* response to cell wall damage, much like an antibiotic resistance mechanism.

1.D. The Research Objectives

The overall goal of this study is to understand how the GraSR system coordinates the response to stress to the cell wall and subsequently maintains the integrity of the cell wall peptidoglycan when damaged by antibiotics like vancomycin. This is significant because this regulatory pathway is similar to other antibiotic resistance mechanisms and understanding this mechanism would lead to better formulation of these antibiotics for effective results.

The objectives of this thesis work were as follows

1. To clone the GraS and GraR genes from Mu50 *S. aureus*.
2. To express and purify the GraS and GraR proteins.
3. To measure autophosphorylation kinetics of GraS by γ^{32}P-ATP.
4. To measure phosphotransformation kinetics between GraS→GraR.

CHAPTER II

MATERIAL AND EXPERIMENTAL METHODS

II.A. Gene Identification and Characterization

Identification of the GraSR genes was done using their respective reference gene numbers from previously described materials. Using reference gene SA0615 from *S.aureus* strain N315 (Hui-min Neoh et al, 2008), the nucleotide sequence from coding region 708912→ 709952, of strain Mu50 was identified as the potential GraS homolog. An NCBI BLAST query using this sequence returned GraS (Mu50) as a match with 100% sequence identity and similarity. The protein coded by this gene consists of 346 amino acids. The protein characteristics were analyzed on the Expasly tools website, using Scanprosite software, which showed a Histidine Kinase domain profile from 127-332aa, and the theoretical isoelectric point and molecular weight was 6.29 and 31727.22 Da respectively. Finally, the structure predicted that the protein possesses two transmembrane domains as follows;

Sequence Length: 346
Sequence 1-16 inside
Sequence 17-34 TM helix
Sequence35 – 43 outside
Sequence 44 - 63 TM helix
Sequence 64 – 346 inside

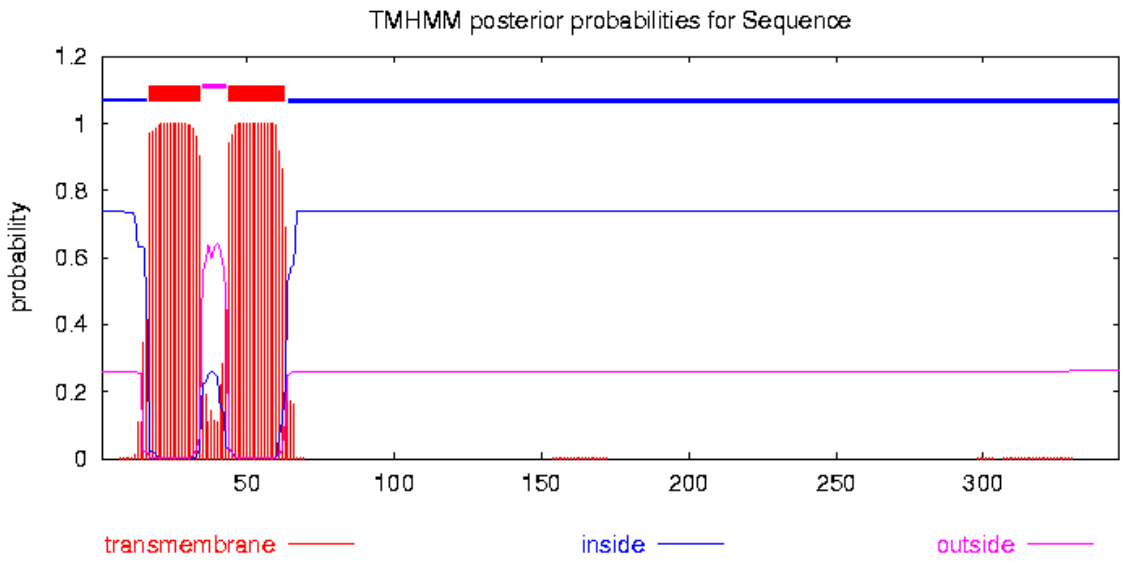

Figure 1: GraS sequence profile showing two transmembrane domains

The reference gene SAV0695 (Mu50 strain) with coding region at base pair numbers 732494- 733168 (Neoh et al, 2008) was identified as the GraR gene. The gene has 675 base pairs that is coding for about 225 amino acids. Investigation with Expasly tool software shows no transmembrane protein, no stop codons inside the sequence and the theoretical isoelectric point (pI) and molecular weight (MW) were computed to be 5.26 and 55207.25 Da, respectively.

II.B. Primer Design of GraS and GraR Genes

The GraSR primers were designed with Sigma genome/DNACalc software and ordered from Sigma-Aldrich. The restriction enzymes were obtained from New England Biolabs and their cognate nucleotide sequences were included in the calculation of the primers. The cystoplasmic nucleotide sequence (SA0615-GraS) coding from 77-346aa

was cloned with glutamate (N-end rule) as penultimate amino acid after methionine, below is the protein sequence.

EIEE IKHKDLAETP FQRHTVDYLY RQISAHKEKV VEQQLQLNMHEQTITEFVHD KTPVTAMKLLIDQE
KNQERKQALLYEWSR INSMLDTQLY ITRLESQRKDMYFDYVSLKR MVIDEIQLTR HISQVKGIGF
DVDFKVDDYVYTDTKWCRMI IRQILSNALKYSENFNIEIG TELNDQHVSL YIKDYGRGIS KKDMPRIFER
FTSTANRNE TTSSGMGLYLVNSVKDQLGI HLQVTSTVGK GTTVRLIFPL QNEIVERMSE VTNLSF

Including the restriction enzymes nucleotides of Bam H1 for forward primer [5'*ggatccGAAATAGAAGAAATTAAACATAAAGAT*3'] and Xho1 for reverse primer [5'*ctcgag+TGT+TTAAAATGACAAATTTGT*3']. Three (ACA) nucleotides were added to the 3' end of the template nucleotide sequence after the stop TAA (5'TGACAAATT TGTCATTTTAA + ACA 3') and hence appeared as TGT in the complementary strand of the reverse primer. This was done to increase the melting point of the reverse primer to a close range with the forward primer melting point; this will prevent any denaturation that might occur due to huge temperature difference between the primers.

The nucleotide sequence of GraR (SAV0695), from region 732494- 733168 which codes for the protein sequence below, was cloned.

MQILLVEDDN TLFQELKKEL EQWDFNVAGI EDFGKVMDTF ESFNPEIVIL DVQLPKYDGF YWCRKMREVS
NVPILFLSSR DNPMDQVMSM ELGADDYMQK PFYTNVLIAK LQAIYRRVYE FTAEEKRTLT WQDAVVDLSK
DSIQKGDDTIFLSKTEMIIL EILITKKNQI VSRDTIITAL WDDEAFVSDN TLTVNVSRLR KKLSEISMDS
AIETKVGKGYMAHE

The primer was designed with the inclusion of the restriction enzymes nucleotides, NdeI for the direct primer [5'ACG*catatg*ATGCAAATACTACTAGAAGATGAC3'] and Hind III for the reverse primer [5'ACG*aagctt*TTATTCATGAGCCATATATCC3'].

II.C. Gene Amplification Reaction (PCR)

The polymerase chain reaction was conducted with the following mixture containing dNTP, 10x Taq buffer, genomic DNA, Taq polymerase, direct and reverse primers and DD H_2O. The reaction was programmed to run as follows:

Table 1: PCR stages, temperature and time.

Stages	temperature	time	
Separation	95^{o}C	5mins	
Melting	95^{o}C	30sec	
Annealing	55^{o}C – 61^{o}C	1min	35x
Synthesis	72^{o}C	1.5min	
Extension	72^{o}C	10mins	

II.D. Cloning, Overexpression and Purification

Following the successful amplifications of GraS and GraR, the amplified DNAs were respectively sub cloned first into pSTBlue-1 vector (Novogen) before cloning into their destination vectors. pSTBlue-1 vector is a multipurpose cloning vector made up of3851bp featuring a versatile multiple cloning region with blue/white screening, dual opposed T7/SP6 promoters and dual kanamycin/ampicillin resistance. This end conversion mix produces perfectly blunt ends. The Perfectly Blunt Ends Cloning Kit was used and the manufacturer's protocol followed accordingly. Two different vectors were used as the destination vector for GraS and GraR genes, GraS was cloned into pGEX 4T-1 (GE Healthcare), which is a 4.9 kb plasmid with a glutathione S transferase (GST) gene

fusion tag which aids in the purification and detection of proteins produced in *E.coil.* The GraR was cloned into pET26b (Novogen), a 6194bp expression vector with multiple cloning sites, that is useful for the production of recombinant protein in *E.coli* strain BL21(DE3) cells for biochemical studies. Following cloning and overexpression of the genes, all the different plasmids containing the insert were sequenced by SeqWright-DNA Technology Service.

II.D.1. Cloning, Overexpression and Purification of GraS

The amplified DNA from PCR was cut from the gel and purified using the QIA Quick Gel Extraction Kit protocol. The concentration was determined at 260 nm using the formula [50 µg/ml *absorbance @260 nm *dilution factor (100)], 2 µL of the DNA was cloned intopSTBlue-1, transformed into a single cell mixture and plated on 88 mm LB medium plate that has kanamycin as selecting marker, X gal and IPTG for blue and white screening of recombinant DNA and grown for 16 hrs @37^0C. Plasmid cultures from the white colonies in the master plate were grown for 16 hrs and the plasmid was purified and sequenced.

II.D.1a. Cloning GraS into pGEX 4T-1 Vector

On confirmation of the sequenced plasmid, the plasmids pGEX 4T-1 and pSTBLUE/GraS were digested with restriction enzymes Bam HI and XhoI. Since the two restriction enzymes react effectively in the same buffer (NEBuffer 3), the samples were digested by both enzymes simultaneously for 1 hr. The mixtures were prepared by mixing the plasmid (pGEX4T-1), Bam HI, XhoI, 10x BSA, NEBuffer 3 and ddH_2O.The same

mixture was also prepared for plasmid pSTBlue/GraS. The digestion was confirmed by gel electrophoresis, the result observed under UV light, and the gene of interest and the digested pGEX4T-1 plasmid were sliced from the gel. The two samples were purified with QIA Gel Extraction and Purification Kit, according to the manufacturer's protocol. The concentrations were also calculated by first determining the absorbance @260 nm, using the formula: 50 µL/ml *absorbance @ 260 * dilution factor (100 µL).

The GraS gene was ligated into the pGEX 4T-1 vector, a vector with a glutathione S transferase (GST) gene fusion tag which aids in the purification and detection of proteins produced in *E.coil*. The ligation mixture was prepared in the ratio of 1:5 insert and vector, respectively, and incubated at room temperature for 30mins. The ligation sample was prepared by mixing Insert (SA0615), vector (pGEX4T-1), T4 Ligase, 10x T4 Ligase buffer and DD H_2O. The ligation mixture was transformed into 100 µL of competent cells [BL21 (DE3)], and plated on 100 µg/ml ampicillin agar plate and incubated @ 37^0C for 16 hrs.

II.D.1b. Expression of pGEX4T-1/GraS

A liquid LB culture of 600 ml was grown to mid-log phase (OD_{600nm}= 0.6) and induced with IPTG at a final concentration of 1 mM and incubated at 37^oC with constant shaking at 150 rpm overnight.

II.D.1c. Purification of GraS-GST

The protein was subjected to a batch purification process and protocol for the Glutathione Sepharose 4B affinity (GE Healthcare) resin. For first time use, the resin was prepared using the following procedure:

The bottle was shaken to mix the resin with the storage buffer, one ml of the slurry was transferred to 2 mL on a rack and left to settle. The solution at the top was removed with a pipette. The resin was equilibrated with PBS buffer (@ pH7.4) after it was washed for 5 mins with PBS buffer five times. The GraS/pGEX 4T-1pellet (2 g) was suspended in 15 ml of PBS buffer atpH 7.4, sonicated to liberate the protein and then centrifuged at 25,000Xg (13 000 rpm) for 60 mins. The supernatant containing the protein was transferred to a 15ml tube and the washed and equilibrated resin was added. The tube was placed in a rotating mixer and incubated for two hours at room temperature. After two hours, the tube was placed in a rack to settle. The supernatant carrying unbound proteins was removed from the top of the resin. PBS buffer was added to the resin and mixed, allowed to stand for ten minutes and then the solution was removed from the top of the resin. This step was repeated seven times. The GST-GraS was eluted by adding 10 mM Reduced Glutathione (Sigma, cat# G4251-25G) and 50 mM Tris at pH 8.0. This was mixed and allowed to stand for ten minutes at 25^0C. The solution was removed from the top; this is fraction one. The procedure was repeated likewise for seven more times. Each fraction was analyzed by 12.5% SDS-PAGE, and the fractions that contained

the most pure protein were combined. The concentration of the protein was determined by the Bradford assay.

II.D.2. Cloning, Overexpression and Purification of GraR

The amplified gene from PCR was cut from a 1% agarose gel and purified with the QIA Quick PCR Purification Kit. The purified gene was cloned into pSTBlue-1, transformed into competent E.coil (NovaBlue single cell) and plated on Agar/ kanamycin (50 µg/ml) plate with 35 µl of 50 mg/ml Xgal and 200 µl of 10 mM IPTG for recombinant screening and left to grow for 16 hrs. The plasmid was isolated from white colonies, purified by growing in LB culture media, and sequenced.

II.D.2a. Cloning GraR into pET26b Vector

On confirmation of the pSTBlue /GraR cloning from sequencing, the pSTBlue/GraR plasmid and the destination vector (pET26b) was digested with the selected restriction enzymes NdeI and HindIII. The two reactions were set up for simultaneous digestion since the enzymes NdeI and HindIII are 100% effective in the same buffer (NEBuffer 2). The reaction sample was prepared as follows, 2 µL plasmid DNA, 1 µL NdeI, 1 µL HindIII, 3 µL NEBuffer 2 and 12 µL DD H_2O. The digestion mixture was left for 2 hrs and the result was investigated on 1% agarose gel electrophoresis. The GraR gene and the vector were cut from the gel, purified, and subsequently ligated. Based on the spectrophotometer calculation, the PCR product was found to be 25 ng/µL and the vector 32 ng/µL; 4 uL of the insert was used against 1.5 µl of the vector to make 1: 5 ligations. The ligation mixture was incubated for 30mins at room temperature, and

transformed into BL21 (DE3) competent cells, plated on kanamycin/ agar plate and incubated at 37^0C for 16 hrs. The cells that were observed afterwards were platted on a master plate.

II.D.2b. Expression of pET26b/GraR

A 600 ml expression culture of LB media was induced with 1 mM IPTG incubated at 22^oC with constant shaking at 150 rpm overnight. The culture was centrifuged after 16 hrs, and the pellet stored at -80^oC prior purification.

II.D.2c. Purification of pET26b /GraR

Fast protein liquid chromatography (FPLC) was used in the purification of GraR. The pellet was suspended in loading buffer (10 mM Tris/$MgCl_2$ at pH 7.0), sonicated at 30% power with 60% pulse in five cycle of one minute each. The resulting supernatant after centrifugation was loaded onto a DEAE Sepharose column (Tricorn 5/200 mm) that was equilibrated with the loading buffer. The flow rate was set at 2.5 ml/min, washing unbound fraction with 2 column volume buffer. The protein was eluted with 8 column volumes of elution buffer (500 mM Tris/5 mM $MgCl_2$ at pH 7.0) and fractions of the elution were collected. The fractions of the peaks that appeared in the chromatogram were investigated for the presence of GraR on SDS-PAGE, and the fractions that showed the presence of GraR on the gel were combined and desalted by passing the protein through a desalting column (Hiprep^{Tm} 26/10 Desalting). The desalted protein was loaded directly onto a Heparin sepharose column (Hiprep^{Tm} Heparin FF 16/10), an affinity binding and ion exchange ligand for a wide range of biomolecules, including DNA

binding proteins like GraR. The column was equilibrated with the loading buffer (10 mM Tris/5 mM$MgCl_2$ at pH 7.0) and the protein eluted with the elution buffer (500 mM Tris/ 5 mM $MgCl_2$ at pH 7.0). The fraction containing pure GraR were combined, concentrated and stored for further characterization of the protein.

CHAPTER III

RESULTS AND DISCUSSION

III.A. Amplification, Cloning and Overexpression of GraS

GraS (SA0615-Mu50) was successfully amplified via the polymerase chain reaction (PCR) (figure 2). The amplified gene lies between 0.5 - 1.0Kb, which is consistent with expected size of 816 kb.

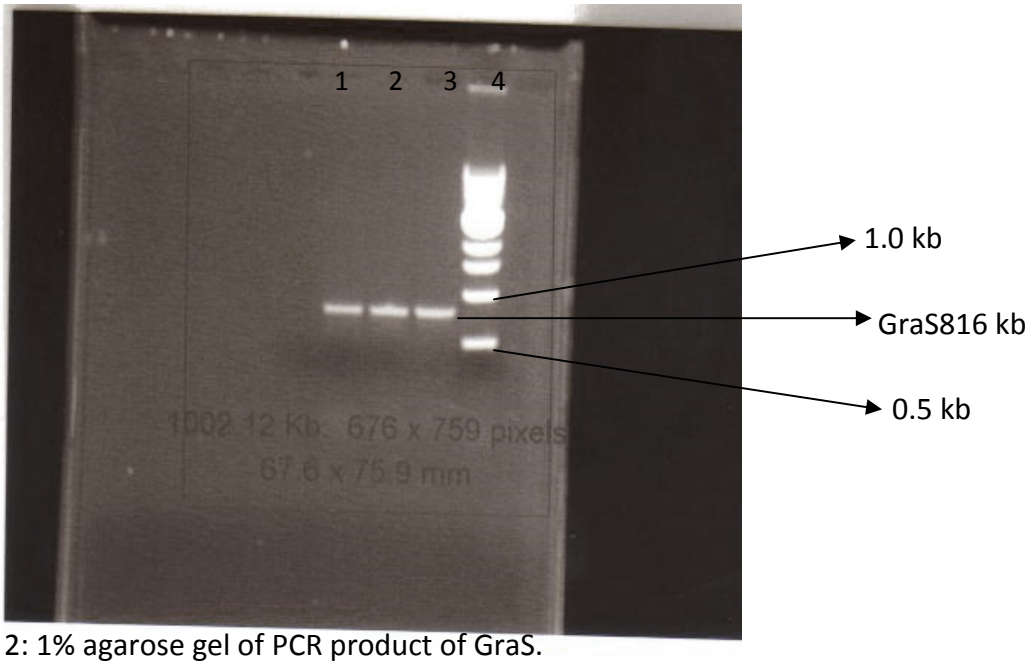

Figure 2: 1% agarose gel of PCR product of GraS.

From left to right: Lane 1-3: amplified GraS and lane 4: 1kb DNA ladder.

The amplified GraS was cut from the gel, and purified using QIA Quick Gel Extraction Kit. The purified gene was ligated by T4 DNA Ligase into pSTBlue-1, a perfectly blunt end vector, transformed into single cell mixture and plated on agar/kanamycin plate with the addition of Xgal and IPTG for recombinant screening. The plate was incubated @37^{o}C for 16 hrs.

GraS/ pSTBlue sequence result.

```
seq             NNAAAANGNNNGNCANTGNATNNNNNNGCCATTTNGNNNNCNCTNTAGAATNCAGCGGCN 60
Sa0615          ------------------------------------------------------------


seq             GCGAGNTCGGGCCCCCACACGTGTGGTCTAGAGCTAGCCTAGGCTCGAGAAGNNNTNGAC 120
Sa0615          ------------------------------------------------------------


seq             GANTTCAGATGGNTNCGAAATAGAAGAAATTAAACATAAAGATTTAGCGGAAACGCCATT 180
Sa0615          ----------------GAAATAGAAGAAATTAAACATAAAGATTTAGCGGAAACGCCATT 44
                                ******************************************


seq             TCAACNTCATACAGTTGATTATTTATNTCGTCAAATCTCAGCGCACAAAGAAAAGGTTGT 240
Sa0615          TCAACGTCATACAGTTGATTATTTATATCGTCAAATCTCAGCGCACAAAGAAAAGGTTGT 104
                ***** ******************** ********************************


seq             TGAGCAACAGTTACAATTGAACATGCATGAACAAACCATTACAGAATTTGTGCACGACAT 300
Sa0615          TGAGCAACAGTTACAATTGAACATGCATGAACAAACCATTACAGAATTTGTGCACGACAT 164
                ************************************************************


seq             AAAAACACCTGTGACAGCCATGAAATTATTAATTGATCAAGAAAAAAATCAAGAAAGAAA 360
Sa0615          AAAAACACCTGTGACAGCCATGAAATTATTAATTGATCAAGAAAAAAATCAAGAAAGAAA 224
                ************************************************************


seq             ACAGGCATTACTATATGAATGGTCTCGTATAAACTCGATGCTGGATACACAGCTGTATAT 420
Sa0615          ACAGGCATTACTATATGAATGGTCTCGTATAAACTCGATGCTGGATACACAGCTGTATAT 284
                ************************************************************


seq             TACTAGATTAGAATCTCAACGCAAAGATATGTATTTTGATTACGTGTCACTTAAACGCAT 480
Sa0615          TACTAGATTAGAATCTCAACGCAAAGATATGTATTTTGATTACGTGTCACTTAAACGCAT 344
                ************************************************************


seq             GGTCATTGATGAAATACAATTAACAAGACATATTAGTCAGGTTAAAGGTATTGGTTTTGA 540
Sa0615          GGTCATTGATGAAATACAATTAACAAGACATATTAGTCAGGTTAAAGGTATTGGTTTTGA 404
                ************************************************************


seq             TGTTGACTTTAAAGTGGATGATTATGTTTATACAGATACAAAATGGTGTCGTATGATTAT 600
Sa0615          TGTTGACTTTAAAGTGGATGATTATGTTTATACAGATACAAAATGGTGTCGTATGATTAT 464
                ************************************************************


seq             TAGACAGATTTTGTCAAACGCATTGAAATATAGTGAGAATTTTAATATTGAAATTGGGAC 660
Sa0615          TAGACAGATTTTGTCAAACGCATTGAAATATAGTGAGAATTTTAATATTGAAATTGGGAC 524

seq             AGAATTAAATGATCAACATGTTTCGTTATATATTAAAGACTATGGCAGAGGTATTAGTAA 720
Sa0615          AGAATTAAATGATCAACATGTTTCGTTATATATTAAAGACTATGGCAGAGGTATTAGTAA 584
                ************************************************************
```

```
seq             AAAAGATATGCCGCGAATATTTGAACGAGGATTTACGTCAACGGCTAACAGAAATGAAAC 780
Sa0615          AAAAGATATGCCGCGAATATTTGAACGAGGATTTACGTCAACGGCTAACAGAAATGAAAC 644
                ************************************************************

seq             GACGTCTTCAGGTATGGGTCTATATTTAGTAAATAGTGTAAAGGATCAATTAGGTATTCA 840
Sa0615          GACGTCTTCAGGTATGGGTCTATATTTAGTAAATAGTGTAAAGGATCAATTAGGTATTCA 704
                ************************************************************

seq             CCTGCAAGTCACGTCGACTGTTGGTAAGGGGACAACTGTCAGATTGATTTTCCCATTACA 900
Sa0615          CCTGCAAGTCACGTCGACTGTTGGTAAGGGGACAACTGTCAGATTGATTTTCCCATTACA 764
                ************************************************************

seq             AAATGAAATTGTTGAACGCATGTCGGAAGTGACAAATTTGTCATTTTAAACACTCGAGAT 960
Sa0615          AAATGAAATTGTTGAACGCATGTCGGAAGTGACAAATTTGTCATTTTAAACA-------- 816
                ************************************************

seq             CACAAATTCTGGATCCGATACGTAACGCNTNNNCAGCNNNNNNNNNNN 1008
Sa0615          -------------------------------------------------
```

Seq represent the cloned gene while Sa0615 is the original sequence template.

The restriction enzymes BamHI and XhoI were used to digest the pSTBlue-1/GraS plasmid on 100% sequenced result confirmation. Figure 3 below depict restriction digestions result showing the inserted gene, cut and uncut plasmids.

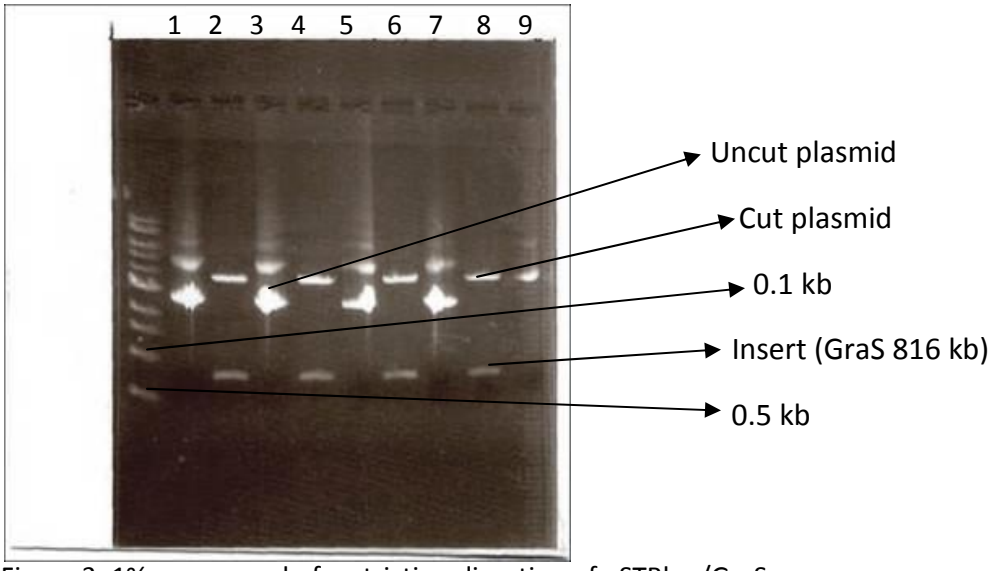

Figure 3: 1% agarose gel of restriction digestion of pSTBlue/GraS.
From Left to right, Lane1: 1Kb DNA ladder, Lane 2, 4, 6, and 8: Uncut plasmids and Lane 3, 5, 7, and 9: cut plasmids.

The GraS genes were cut from the gel and purified using the QIA Quick Extraction Kit and protocol according to the manufacturer used, and the concentration was measured on a spectrophotometer. The purified gene was ligated into the destination vector pGEX4T-1 that was digested with the same enzymes BamHI and XhoI. The ligation products were transformed into BL21 (DE3) and plated on agar/ampicillin plate, then incubated at 37^{o}C for sixteen hours.

GraS/pGEX4T sequence result below;

```
Query  43   GAAATAGAAGAAATTAAACATAAAGATTTAGCGGANNNNNNNNNNNNAACGTCATACAGTT  102
            ||||||||||||||||||||||||||||||||||           ||||||||||||||||
Sbjct  1    GAAATAGAAGAAATTAAACATAAAGATTTAGCGGAAACGCCATTTCAACGTCATACAGTT  60

Query  103  GATTATTTATATCGTCAAATCTCAGCGCACAAAGAAAAGGTTGTTGAGCAACAGTTACAA  162
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GATTATTTATATCGTCAAATCTCAGCGCACAAAGAAAAGGTTGTTGAGCAACAGTTACAA  120

Query  163  TTGAACATGCATGAACAAACCATTACAGAATTTGTGCACGACATAAAAACACCTGTGACA  222
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTGAACATGCATGAACAAACCATTACAGAATTTGTGCACGACATAAAAACACCTGTGACA  180

Query  223  GCCATGAAATTATTAATTGATCAAGAAAAAAATCAAGAAAGAAAACAGGCATTACTATAT  282
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCCATGAAATTATTAATTGATCAAGAAAAAAATCAAGAAAGAAAACAGGCATTACTATAT  240

Query  283  GAATGGTCTCGTATAAACTCGATGCTGGATACACAGCTGTATATTACTAGATTAGAATCT  342
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GAATGGTCTCGTATAAACTCGATGCTGGATACACAGCTGTATATTACTAGATTAGAATCT  300

Query  343  CAACGCAAAGATATGTATTTTGATTACGTGTCACTTAAACGCATGGTCATTGATGAAATA  402
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CAACGCAAAGATATGTATTTTGATTACGTGTCACTTAAACGCATGGTCATTGATGAAATA  360

Query  403  CAATTAACAAGACATATTAGTCAGGTTAAAGGTATTGGTTTTGATGTTGACTTTAAAGTG  462
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CAATTAACAAGACATATTAGTCAGGTTAAAGGTATTGGTTTTGATGTTGACTTTAAAGTG  420

Query  463  GATGATTATGTTTATACAGATACAAAATGGTGTCGTATGATTATTAGACAGATTTTGTCA  522
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GATGATTATGTTTATACAGATACAAAATGGTGTCGTATGATTATTAGACAGATTTTGTCA  480

Query  523  AACGCATTGAAATATAGTGAGAATTTTAATATTGAAATTGGGACAGAATTAAATGATCAA  582
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  AACGCATTGAAATATAGTGAGAATTTTAATATTGAAATTGGGACAGAATTAAATGATCAA  540

Query  583  CATGTTTCGTTATATATTAAAGACTATGGCAGAGGTATTAGTAAAAAAGATATGCCGCGA  642
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  CATGTTTCGTTATATATTAAAGACTATGGCAGAGGTATTAGTAAAAAAGATATGCCGCGA  600
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Query  643  ATATTTGAACGAGGATTTACGTCAACGGCTAACAGAAATGAAACGACGTCTTCAGGTATG  702
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  ATATTTGAACGAGGATTTACGTCAACGGCTAACAGAAATGAAACGACGTCTTCAGGTATG  660

Query  703  GGTCTATATTTAGTAAATAGTGTAAAGGATCAATTAGGTATTCACCTGCAAGTCACGTCG  762
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  GGTCTATATTTAGTAAATAGTGTAAAGGATCAATTAGGTATTCACCTGCAAGTCACGTCG  720

Query  763  ACTGTTGGTAAGGGGACAACTGTCAGATTGATTTTCCCATTACAAAATGAAATTGTTGAA  822
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  ACTGTTGGTAAGGGGACAACTGTCAGATTGATTTTCCCATTACAAAATGAAATTGTTGAA  780

Query  823  CGCATGTCGGAAGTGACAAATTTGTCATTTTAA  855
            ||||||||||||||||||||||||||||||||
Sbjct  781  CGCATGTCGGAAGTGACAAATTTGTCATTTTAA  813
```

Query represents the cloned gene while Sbjct represent the original sequence template.

A seed culture was made with the harvested plasmid and after 16 hrs was used to inoculate an expression culture of 600 ml that was induced at mid log phase (O.D= 0.6) with 600 µL of 1 M IPTG. The pellet of cells (2 g) containing the GST-GraS were subjected to a batch purification process which involves Glutathione Sepharose 4B that is designed for a single step purification of glutathione S- transferase (GST) fusion proteins produces using the pGEX series of expression vectors.

Below is the result of the purification showing pure GST-GraS, protein of about 57 kDa lying between 50– 60 kDa.

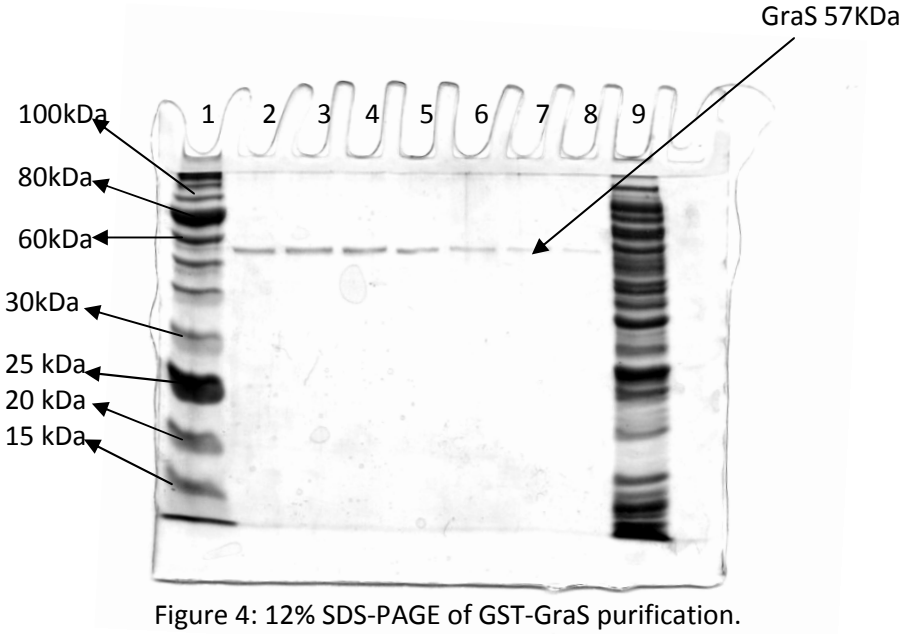

Figure 4: 12% SDS-PAGE of GST-GraS purification.
From left to right: Lane1 DNA ladder, Lane2-8: GST-GraS and Lane 9: crude extract.

III.B. Amplification, Cloning and Overexpression of GraR

GraR (SAVO695) was successfully amplified via the polymerase chain reaction (PCR). Fig 5 depicts the result of GraR amplification gene which lies between 0.5-1.0 kb and the result is consistent with the expected size of GraR 675 Kb.

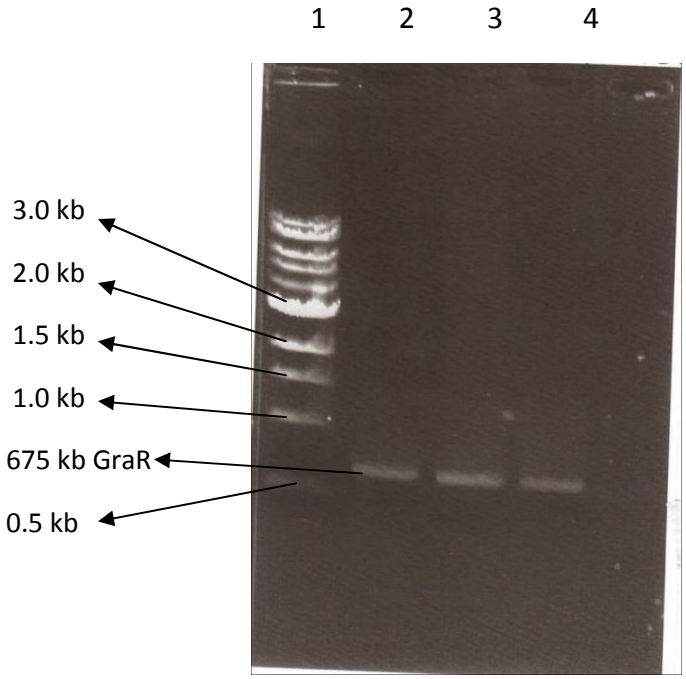

Figure 5: 1% agarose gel of PCR product of GraR.
From left to right: Lane1: 1kb DNA ladder, Lane 2-4: amplified GraR

The amplified GraR was cut from the gel, purified using the QIA quick gel extraction kit. The purified gene was ligated by T4 DNA Ligase into pSTBlue-1, transformed into NovaBlue single cell mixture and plated on agar/kanamycin plate with the addition of Xgal and IPTG for recombinant screening. The plate was incubated @37^{o}C for 16 hrs and the white colony picked afterward and used in making a seed culture. The plasmid was sequenced for confirmation and below is the result.

pSTBlue/GraR Sequence result

```
Query  336   ATGCAAATACTACTAGTAGAAGATGACAATACTTTGTTTCAAGAATTGAAAAAAGAATTA  395
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     ATGCAAATACTACTAGTAGAAGATGACAATACTTTGTTTCAAGAATTGAAAAAAGAATTA  60

Query  396   GAACAATGGGATTTTAATGTTGCTGGTATTGAAGATTTCGGCAAAGTAATGGATACATTT  455
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    GAACAATGGGATTTTAATGTTGCTGGTATTGAAGATTTCGGCAAAGTAATGGATACATTT  120

Query  456   GAAAGTTTTAATCCTGAAATTGTTATATTGGATGTTCAATTACCTAAATATGATGGGTTT  515
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GAAAGTTTTAATCCTGAAATTGTTATATTGGATGTTCAATTACCTAAATATGATGGGTTT  180

Query  516   TATTGGTGCAGAAAAATGAGAGAAGTTTCCAACGTACCAATATTATTTTTATCATCTCGT  575
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   TATTGGTGCAGAAAAATGAGAGAAGTTTCCAACGTACCAATATTATTTTTATCATCTCGT  240

Query  576   GATAATCCAATGGATCAAGTGATGAGTATGGAACTTGGCGCAGATGATTATATGCAAAAA  635
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   GATAATCCAATGGATCAAGTGATGAGTATGGAACTTGGCGCAGATGATTATATGCAAAAA  300

Query  636   CCTTTCTATACCAATGTATTAATTGCTAAATTACAAGCGATTTATCGTCGTGTCTATGAG  695
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   CCTTTCTATACCAATGTATTAATTGCTAAATTACAAGCGATTTATCGTCGTGTCTATGAG  360

Query  696   TTTACAGCTGAAGAAAAACGTACATTGACTTGGCAAGATGCTGTCGTTGATCTATCAAAA  755
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   TTTACAGCTGAAGAAAAACGTACATTGACTTGGCAAGATGCTGTCGTTGATCTATCAAAA  420

Query  756   GATAGTATACAAAAAGGTGACGATACGATTTTTCTATCCAAAACAGAAATGATTATATTA  815
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   GATAGTATACAAAAAGGTGACGATACGATTTTTCTATCCAAAACAGAAATGATTATATTA  480

Query  816   GAAATTCTTATTACCAAAAAAAATCAAATCGTTTCGAGAGATACAATTATCACTGCATTA  875
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   GAAATTCTTATTACCAAAAAAAATCAAATCGTTTCGAGAGATACAATTATCACTGCATTA  540

Query  876   TGGGATGATGAAGCATTTGTTAGTGATAATACGTTAACAGTAAATGTGAGTCGTTTACGA  935
              |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  541   TGGGATGATGAAGCATTTGTTAGTGATAATACGTTAACAGTAAATGTGAATCGTTTACGA  600

Query  936   AAAAAATTATCTGAAATTAGTATGGATAGTGCAATCGAAACAAAAGTAGGAAAAGGATAT  995
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   AAAAAATTATCTGAAATTAGTATGGATAGTGCAATCGAAACAAAAGTAGGAAAAGGATAT  660

Query  996   ATGGCTCATGAATAA  1010
              |||||||||||||||
Sbjct  661   ATGGCTCATGAATAA  675
```

Query represents the cloned gene while Sbjct represent the original sequence template.

The restriction enzymes NdeI and HindIII were used to digest the pSTBlue-1/GraR plasmid after the sequence was confirmed. Figure 6 below shows the restriction digestion result, the first two bands in lane 2 and 3 are the insert (GraR) while the last bands of the same lanes are the cut plasmids.

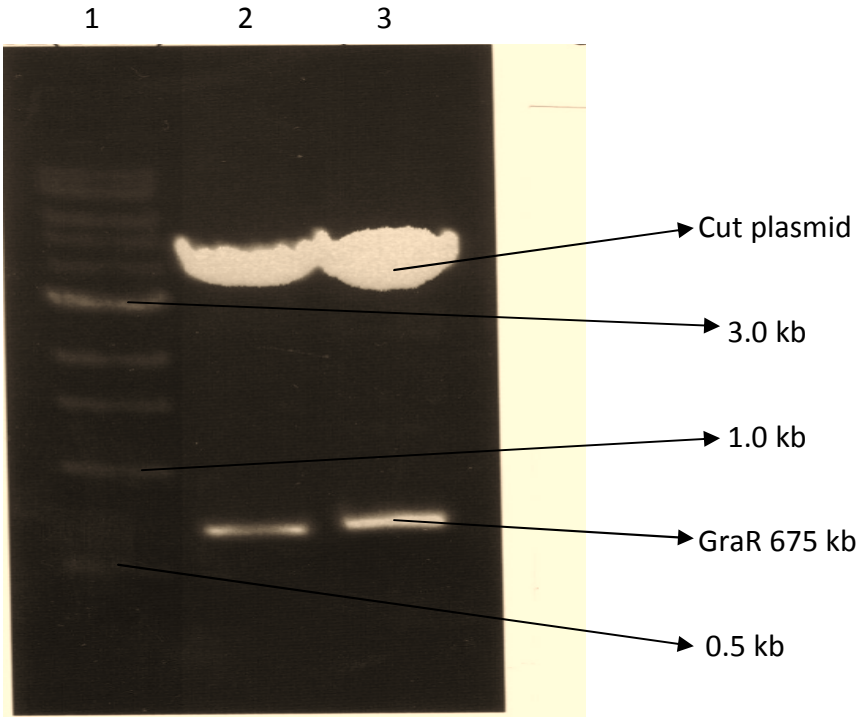

Figure 6: 1% agarose gel of pSTBlue/GraR restriction digestion.
From Left to Right, Lane1: 1Kb DNA ladder, Lane2 and 3 cut plasmids with insert (GraR).

The GraR genes were cut from the gel and purified using the QIA Quick Extraction Kit and the concentration was measured on the spectrophotometer at 260nm.The purified gene was ligated into the destination vector pET26b that has been

digested with the same enzymes NdeI and HindIII. The ligation products were transformed into BL21 (DE3) and plated on agar/kanamycin plate and incubated at 37^{o}C for 16 hrs.

GraR/pET26b sequence result

```
Query  60   TTATTCATGAGCCATATATCCTTTTCCTACTTTTGTTTCGATTGCACTATCCATACTAAT  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  TTATTCATGAGCCATATATCCTTTTCCTACTTTTGTTTCGATTGCACTATCCATACTAAT  616

Query  120  TTCAGATAATTTTTTTCGTAAACGACTCACATTTACTGTTAACGTATTATCACTAACAAA  179
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  615  TTCAGATAATTTTTTTCGTAAACGATTCACATTTACTGTTAACGTATTATCACTAACAAA  556

Query  180  TGCTTCATCATCCCATAATGCAGTGATAATTGTATCTCTCGAAACGATTTGATTTTTTTT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555  TGCTTCATCATCCCATAATGCAGTGATAATTGTATCTCTCGAAACGATTTGATTTTTTTT  496

Query  240  GGTAATAAGAATTTCTAATATAATCATTTCTGTTTTGGATAGAAAAATCGTATCGTCACC  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  GGTAATAAGAATTTCTAATATAATCATTTCTGTTTTGGATAGAAAAATCGTATCGTCACC  436

Query  300  TTTTTGTATACTATCTTTTGATAGATCAACGACAGCATCTTGCCAAGTCAATGTACGTTT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  TTTTTGTATACTATCTTTTGATAGATCAACGACAGCATCTTGCCAAGTCAATGTACGTTT  376

Query  360  TTCTTCAGCTGTAAACTCATAGACACGACGATAAATCGCTTGTAATTTAGCAATTAATAC  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  TTCTTCAGCTGTAAACTCATAGACACGACGATAAATCGCTTGTAATTTAGCAATTAATAC  316

Query  420  ATTGGTATAGAAAGGTTTTTGCATATAATCATCTGCGCCAAGTTCCATACTCATCACTTG  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  ATTGGTATAGAAAGGTTTTTGCATATAATCATCTGCGCCAAGTTCCATACTCATCACTTG  256

Query  480  ATCCATTGGATTATCACGAGATGATAAAAATAATATTGGTACGTTGGAAACTTCTCTCAT  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  ATCCATTGGATTATCACGAGATGATAAAAATAATATTGGTACGTTGGAAACTTCTCTCAT  196

Query  540  TTTTCTGCACCAATAAAACCCATCATATTTAGGTAATTGAACATCCAATATAACAATTTC  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  195  TTTTCTGCACCAATAAAACCCATCATATTTAGGTAATTGAACATCCAATATAACAATTTC  136

Query  600  AGGATTAAAACTTTCAAATGTATCCATTACTTTGCCGAAATCTTCAATACCAGCAACATT  659
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  135  AGGATTAAAACTTTCAAATGTATCCATTACTTTGCCGAAATCTTCAATACCAGCAACATT  76

Query  660  AAAATCCCATTGTTCTAATTCTTTTTTCAATTCTTGAAACAAAGTATTGTCATCTTCTAC  719
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75   AAAATCCCATTGTTCTAATTCTTTTTTCAATTCTTGAAACAAAGTATTGTCATCTTCTAC  16

Query  720  TAGTAGTATTTGCAT  734
            |||||||||||||||
Sbjct  15   TAGTAGTATTTGCAT
```

A seed culture of LB media was made and after 16hrs was used to inoculate an expression culture of 600 ml that was induced at mid log phase ($O.D_{600}$ nm= 0.6) by 1 mM IPTG. The purification of GraR was done with fast protein liquid chromatography (FPLC); using 180 ml column. The supernatant from lysis of the expression pellet containing the crude protein was applied to a 180 ml DEAE sepharose column that was equilibrated with loading buffer (10 mM Tris/5 mM $MgCl_2$ at pH 7.0). The un-bound protein fractions were collected as the flow rate ran at 2.5 ml/min. The protein was eluted with 8 column volumes of elution buffer (500 mM Tris/5 mM $MgCl_2$ at pH 7.0) while collecting 5 ml fractions.

Figure 7 shows the chromatogram of the DEAE sepharose purification.

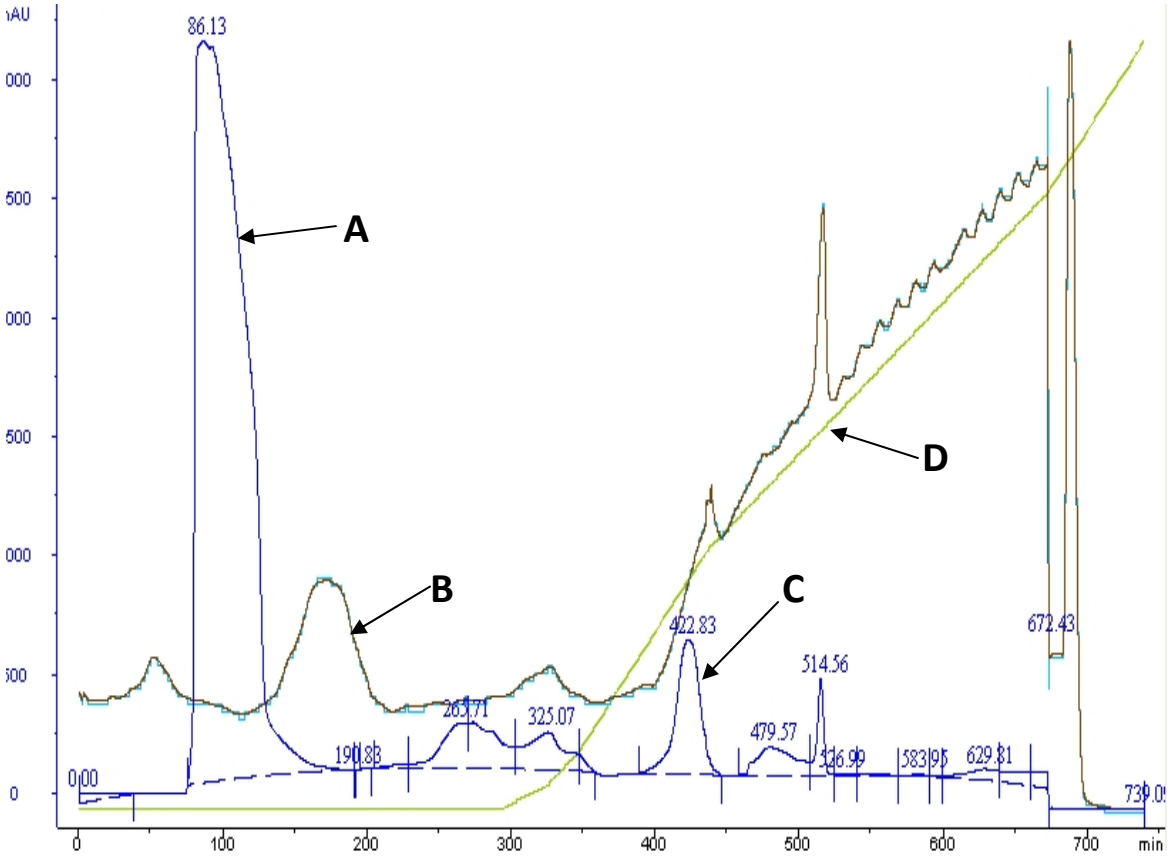

Figure 7: DEAE chromatogram of GraR purification.

A: UV peak

B: Conductance

C: Peak containing GraR fractions

D: 100% linear gradient of buffer B

The fractions under the UV peaks in the DEAE chromatogram were collected and analyzed on 12% SDS-PAGE for the presence of GraR.

The SDS-PAGE result of the DEAE fractions is show below.

Wells no	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Fractions no	152	154	159	162	165	168	Crude extract	Ladder DNA	76	79	84	87	114	Ladder DNA

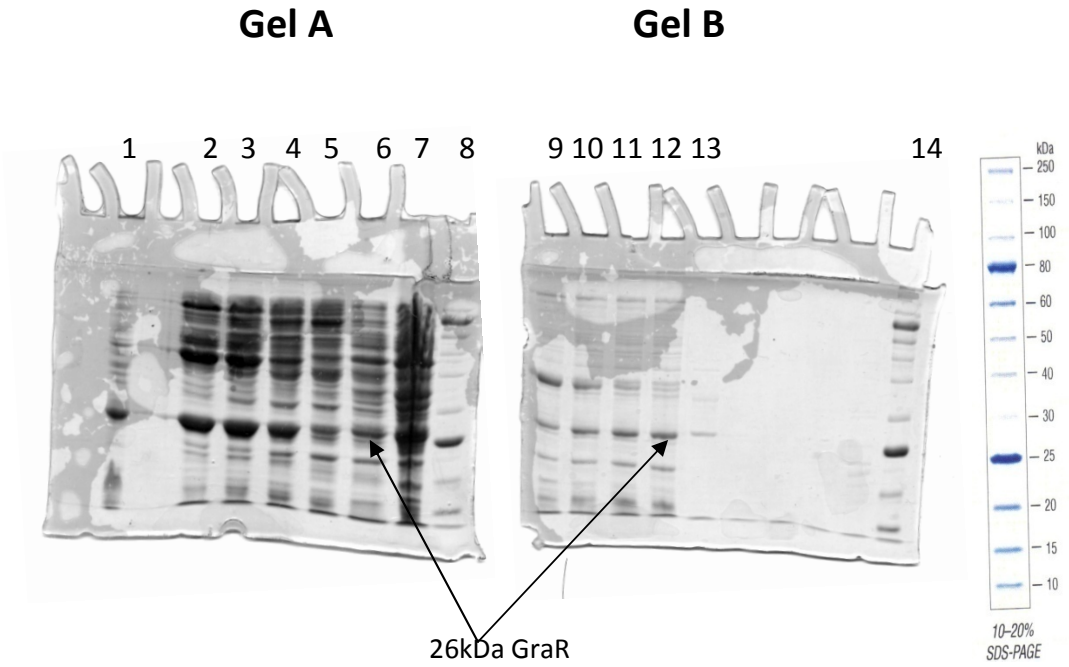

Figure 8: 12% SDS-PAGE gel of fractions from DEAE sepharose chromatography of GraR. GraR is a protein of 26 KDa and the rest of the bands are considered impurities,

The fractions showing the presence of GraR (fractions 79– 87 & fraction 113–116) were desalted by passing through a desalting column, concentrated and passed through heparin column afterwards to further purify GraR, since GraR is a DNA binding protein.

Figure 9 below shows the chromatogram of the heparin purification.

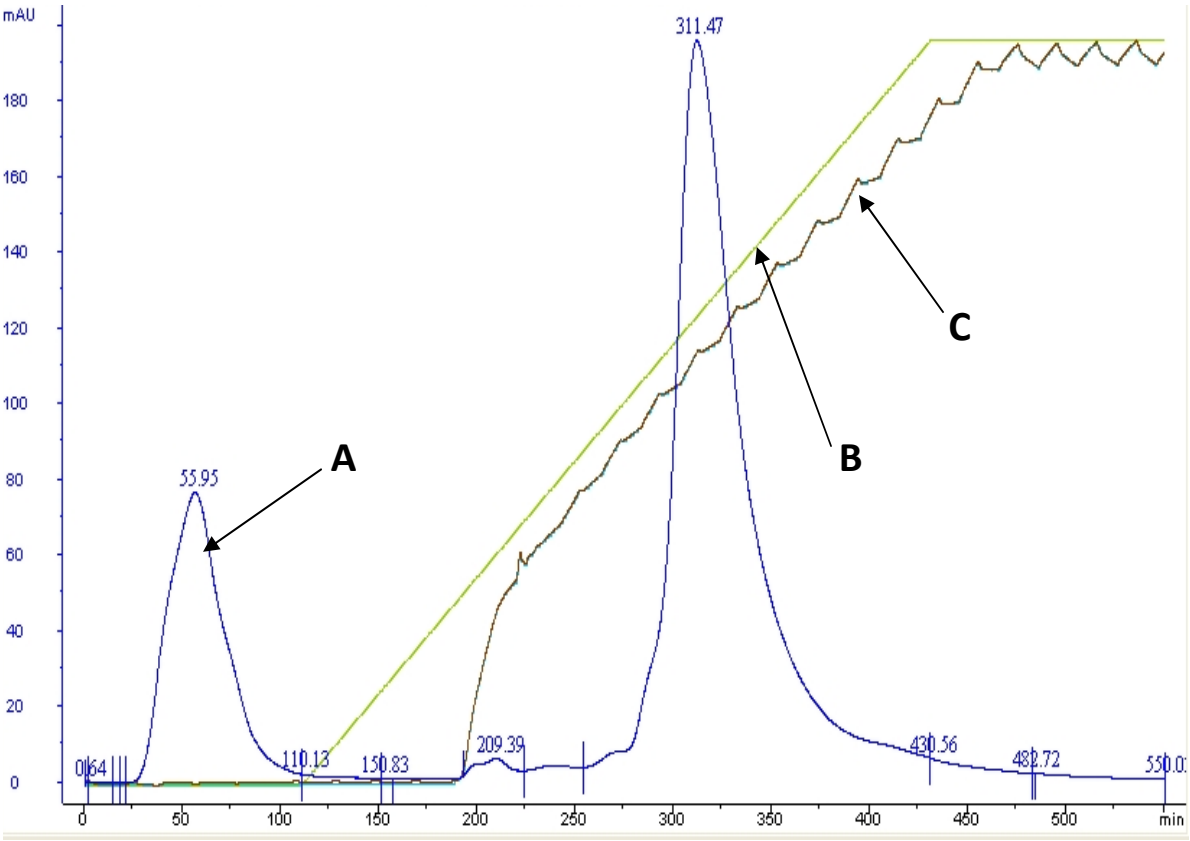

Figure 9: Heparin affinity chromatogram of GraR

A: UV peak

B: 100% linear gradient of buffer B

C: Conductance

The fractions showing peaks in the chromatogram was collected and concentrated down to 2 ml using a 10,000 Dalton ultrafiltration tube, the filtrate discarded while the proteins on top of the filter was investigated for the presence of pure GraR on 12% SDS-PAGE gel.

The picture below shows the result of the investigation.

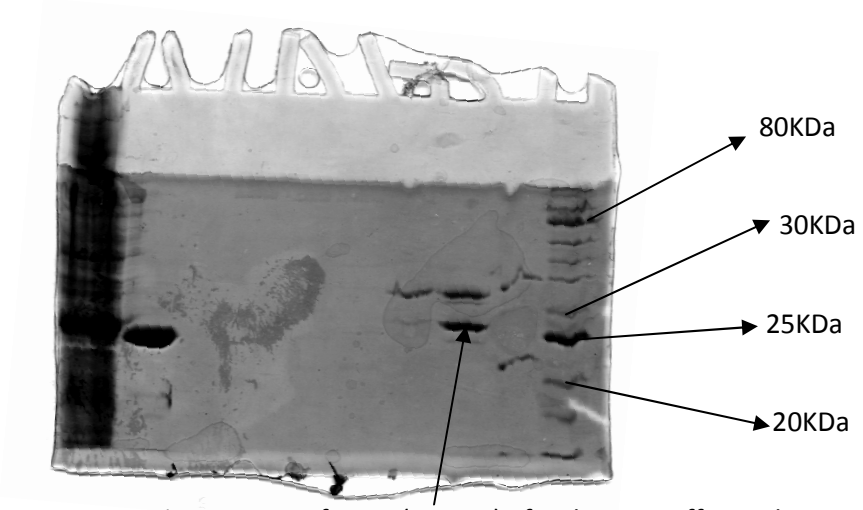

Figure 10: 12% SDS-PAGE of GraR (27 KDa) after heparin affinity chromatography

III.C. Trypsin Digestion

The bands in the SDS-PAGE gel of GraR and GraS that are consistent with their expected sizes were sliced from the gel and subjected to trypsin digestion. Two identifications were made which are consistent with response regulator in [*Staphylococcus aureus* subsp. aureus Mu50].Tables 2 and 3 below are the results of the trypsin digestions and mass spectral analyses for GraR and GraS respectively. In the tables are peptide sequences of the digestions with their nominal masses. The peptide sequences were respectively identified in the protein sequence of GraR and GraS and the matched peptides are in bold color.

Table 2: Peptide Sequence of GraR Trypsin Digestion

Start-End	Mr (calc)	Sequence by MS/MS
19 - 35	1995.9214	ELEQWDFNVAGIEDFGK
68-80	1459.8035	EVSNVPILFLSSR
111-116	762.4388	LQAIYR
118-127	1270.6193	VYEFTAEEKR
174	2794.2927	DTIITALWDDEAFVSDNTLTVNVSR
69-81	1459.8035	EVSNVPIIFLSSR

1 MQILLVEDDN TLFQELKK**EL EQWDFNVAGI EDFGK**VMDTF ESFNPEIVIL
51 DVQLPKYDGF YWCRKMR**EVS NVPILFLSSR** DNPMDQVMSM ELGADDYMQK
101 PFYTNVLIAK **LQAIYR**R**VYE FTAEEKR**TLT WQDAVVDLSK DSIQKGDDTI
151 FLSKTEMIIL EILITKKNQI VSR**DTIITAL WDDEAFVSDN TLTVNVSR**LR
201 KKLSEISMDS AIETKVGKGY MAHE
GraR protein sequence with matching peptides.

Table 3: Peptide Sequence of GraS Trypsin Digestion

Start- End	Mr (calc)	Sequence by MS/MS
85-93	1075.5298	DLAETPFQR
94-101	1065.4927	HTVDYLYR
151-160	1292.6827	KQALLYEWSR
152-160	1164.5927	QALLYEWSR
161-173	1566.8076	INSMLDTQLYITR
326-337	1469.8242	LIFPLQNEIVER

1 MNNLKWVAYF LKSRMNWIFW ILFLNLLMLG ISLIDYDFPI DSLFYIVSLN
51 LSLTMIFLIL TYFKEVKLYK HFDKDKEIEE IKHK**DLAETP FQRHTVDYLY**
101 **R**QISAHKEKV VEQQLQLNMH EQTITEFVHD IKTPVTAMKL LIDQEKNQER
151KQALLYEWSR INSMLDTQLY ITRLESQRKD MYFDYVSLKR MVIDEIQLTR
201 HISQVKGIGF DVDFKVDDYV YTDTKWCRMI IRQILSNALK YSENFNIEIG
251 TELNDQHVSL YIKDYGRGIS KKDMPRIFER GFTSTANRNE TTSSGMGLYL
301 VNSVKDQLGI HLQVTSTVGK GTTVR**LIFPL QNEIVER**MSE VTNLSF
GraSprotein sequence with matching peptides in bold red color.

The matching peptide sequence from the data of the trypsin digestion confirms the purification of GraS and GraR.

III.D. Phosphorylation Kinetics

With the successful purification of pure samples of GraS and GraR, the proteins are to be subjected to phosphosrylation kinetics which will establish the autophosphorylation capability of GraS and its phosphotransfer assay to GraR. The autophosphorylation activity of GST-GraS in the presence of with γ^{32}P-ATP (3000 Ci/mmol) is expected to be a time dependent experiment indicating a progressive reaction as a graph of band intensities against time. This will also show that GraS is capable of transducing a stress signal before *S. aureus* starts to duplicate (duplication time of *S. aureus* is 30 mins) (Antoaneta Belcheva & Dasantila Golemi-Kotra (2008). Subsequent incubation is expected to result in a rapid transfer of the phosphate from GST-GraS-P to GraR. It is expected that 70% of the phosphoryl group will be transferred in 30 seconds. This will be analyzed by 12% SDS-PAGE electrophoresis and autoradiography.

III.E. Conclusion and Future Experiments

GraS and GraR were successfully cloned and purified as was established by the sequencing results and the Trypsin digestion with MS. Bioinformatics suggested that the GraS protein has an N- terminal transmembrane domain and the C- terminal domain is the kinase for GraR. GraR is an expected two domain response regulatory protein that is

also made up of N-terminal regulatory domain and a C- terminal DNA binding domain referred to as the effector domain. The kinetics of the GraS autophosphorylation and its subsequence phosphotransfer (which is expected to happen within 30sec) is expected to indicate that *S. aureus* (Mu50) is capable of a rapid response to antibiotics that damage the cell wall of the bacteria. The signaling transduction process is mediated through these phosphorylation events. The phosphorylation kinetics of GST-GraS in the presence of ATP is to suggest that the autophosphorylation is time dependent and a progressive reaction. These queries are expected to establish that the GraSR two component systems is the main pathway through which rapid signal transduction is conducted as a response to bacterial cell wall damage and identification of these two proteins will lead to further experiments, designed to study their roles in cell wall damage repair.

CHAPTER IV

INTRODUCTION OF THE *mmgD* PROTEIN

The mother cell metabolic gene operon (*mmg*) is expressed during sporulation of *Bacillus subtilis*, a rod shape Gram-positive bacterium (J.J. Reddick& J. Williams, 2008). It provides a mechanism for regulating the metabolic state of the mother cell during development of the endospore. *mmgD* is one of the six open reading frames (ORFs) of *mmg*; its gene product has been shown to function as a citrate synthase (E.Bryan, et al, 1996) and also as a 2-methylcitrate synthase (Acharya, MS thesis. 2009). For better understanding of the *mmgD* protein, the detailed knowledge of the physical properties like the native molecular weight should be obtained. Gel filtration chromatography, also called size exclusion chromatography, is one of the common and widely used methods of determining apparent native protein molecular weight. The technique is based on the movement of proteins through a porous matrix to distinguished proteins of different sizes. The column of the gel filtration is made up of a solid stationary phase and a liquid mobile phase. The liquid mobile phase (buffer or solvent) which also contains the dissolved protein is allowed to flow through the solid phase (gel matrix) hence interacting with it, and based on their sizes, are separated. A mixture of protein flows through the column; the large proteins are excluded from the

pore and thus passed rapidly through the column between the beads with the mobile phase, and this volume

is known as the void volume (V_o). While the smaller proteins and small protein complexes can enter the pores of the matrix, their movement is retarded and requires more time to elute and the volume that is eluted is called the elution volume (V_e).

Our goal was to measure the native molecular weight of *mmgD* to ascertain whether it exists as a monomer, dimer, trimer or tetramer etc.

IV.A. Experimental Methods

IV.A.1. Expression & Purification of *mmgD* Protein

A liter culture of BL21(DE3) expressing *mmgD* from pET28a was expressed with induction by IPTG, from a glycerol stock in -80^{o}C and the pellet re-suspended in 20ml 1x binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl at pH 7.0, and 10% glycerol) and the cells sonicated and centrifuged afterwards to separate the debris. The supernatant was syringe filtered into a Ni-NTA column at room temperature. The unbound proteins were washed with wash buffer (60 mM imidazole, 500 Mm NaCl, 20 mM Tris-HCl at pH 7.0, and 10% glycerol). While the bound proteins were eluted with elution buffer (200 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl at 7.9, 10% glycerol). The elution was collected in 1ml fractions and the fractions checked for *mmgD* presence on a 12% SDS page gel. The elution fractions that showed the presence of *mmgD* were

dialyzed (7000 MWCO) overnight in 4 liters buffer containing 25 mM Tris-HCl, 10% glycerol at pH 7.5.

IV.A.2. Calibrating the Filtration Column

The gel filtration column used was a pre-packed column Superdex 200 10/300 GL (GE Healthcare) that was washed with 2 column volumes of degassed water and equilibrated with buffer (50 mM phosphate in 0.15 M of NaCl @ pH 7.2). Mixtures of purified proteins of varying known molecular masses were applied to the column. Below is the mixture of proteins that was used with their native molecular weight (in Da): Ribonuclease (13,700), carbonic anhydrase (29,000), ovalbumin (44,000), conalbumin (75,000), aldolase (158,000), ferritin (440,000), thyroglobulin (669,000). Blue dextran 2000 was used in determining the void volume.

Table 4: Protein standards, the concentrations and the quantities used.

Protein Molecules	conc. used mg/ml	M_r (Da)	Amt mixed in (200.68 µl
conalbumin (50mg)	3	75,000	34.8
Aldolase (50mg)	4	158,000	46.4
Ovalbumin (50mg)	4	44,000	46.4
Ferritin (15mg)	0.3	440,000	3.48
Thyroglobulin (50mg)	5	669,000	58
Ribonuclease A (50mg)	1	13,700	11.6
Blue Dextran 2000	1	N/A	120

IV.A.3. Preparation of Calibration Curve

To obtain the calibration curve, the partition coefficient K_{av} was calculated using the equation below:

$$K_{av} = \frac{V_e - V_o}{V_c - V_o}$$

Where $V_o$is the void volume, V_e is the elution volume and V_c is the geometric column volume. A plot of K_{av} versus log of molecular weight will give the calibration curve. The calibration curve can then be use in determining the native molecular weight of the sample protein after the K_{av} of the sample protein was calculated.

IV.B. Result

IV.B.1. Overexpression of *mmgD* Protein in BL21 (DE3)

An expression culture was made from the already cloned pET-28a/*mmgD*/BL21 (DE3) protein in glycerol stock in -80^{o}C, induced with IPTG and purified by Ni-NTA affinity chromatography afterwards. The result of the purification was analyzed on 12% SDS-PAGE electrophoresis. Figure 11 shows the result of the purification.

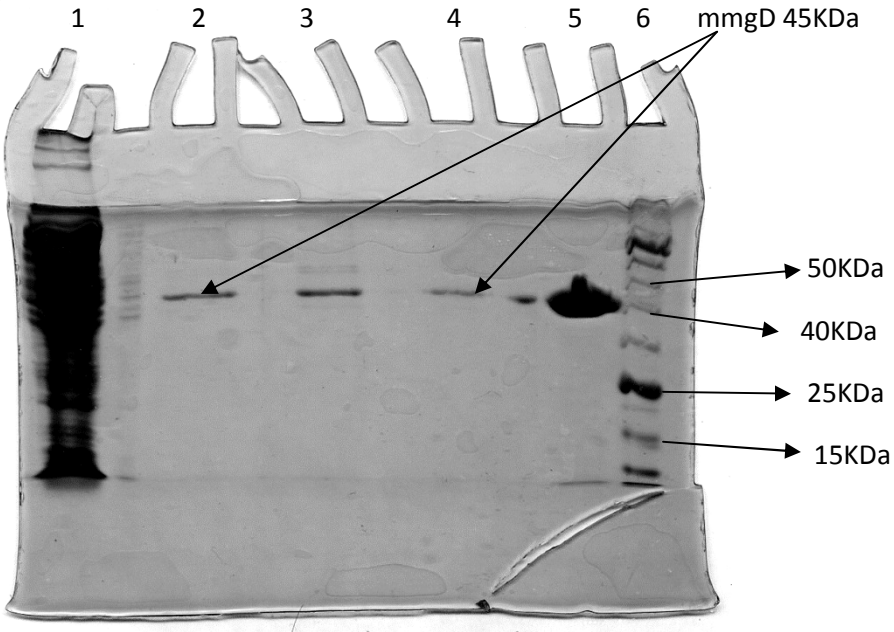

Figure 11: 12% SDS-PAGE Gel of *mmgD* Purification.
From Lane 1: Crude extract, Lane2 to 5: *mmgD* protein and Lane 6: protein marker.

IV.B.2.The Gel Filtration Result

The retention time of the protein standards were determined from their respective peaks in the chromatogram. The chromatogram of Blue dextran used in determining the void volume is depicted in the figure below.

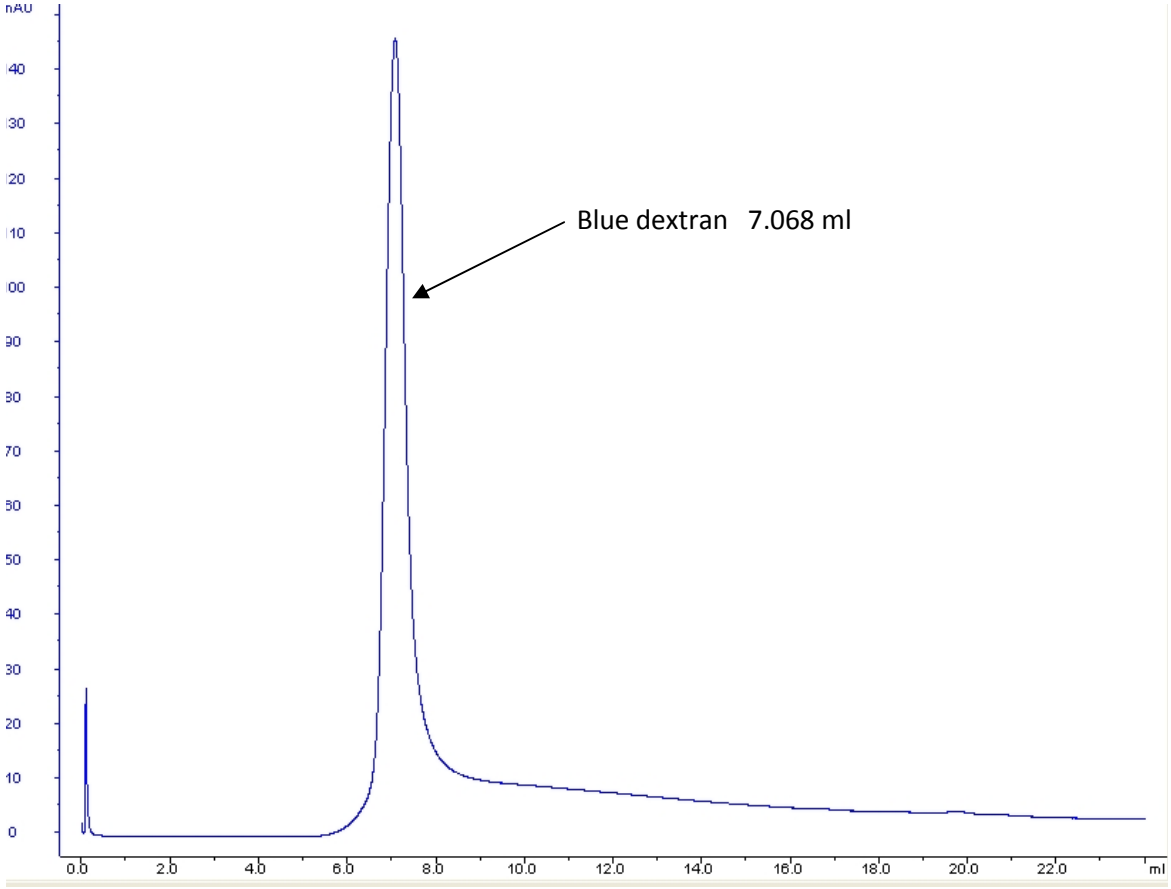

Figure 12: Blue dextran chromatogram with retention time of 7.068 ml

The standard proteins were mixed together and applied to the pre-packed size exclusion column that was equilibrated with buffer 50 mM phosphate in 0.15 M of NaCl at pH 7.2 and the flow rate adjusted to 0.5 ml/min. The diagram below shows the chromatogram of the gel filtration of the standard protein with the retention time.

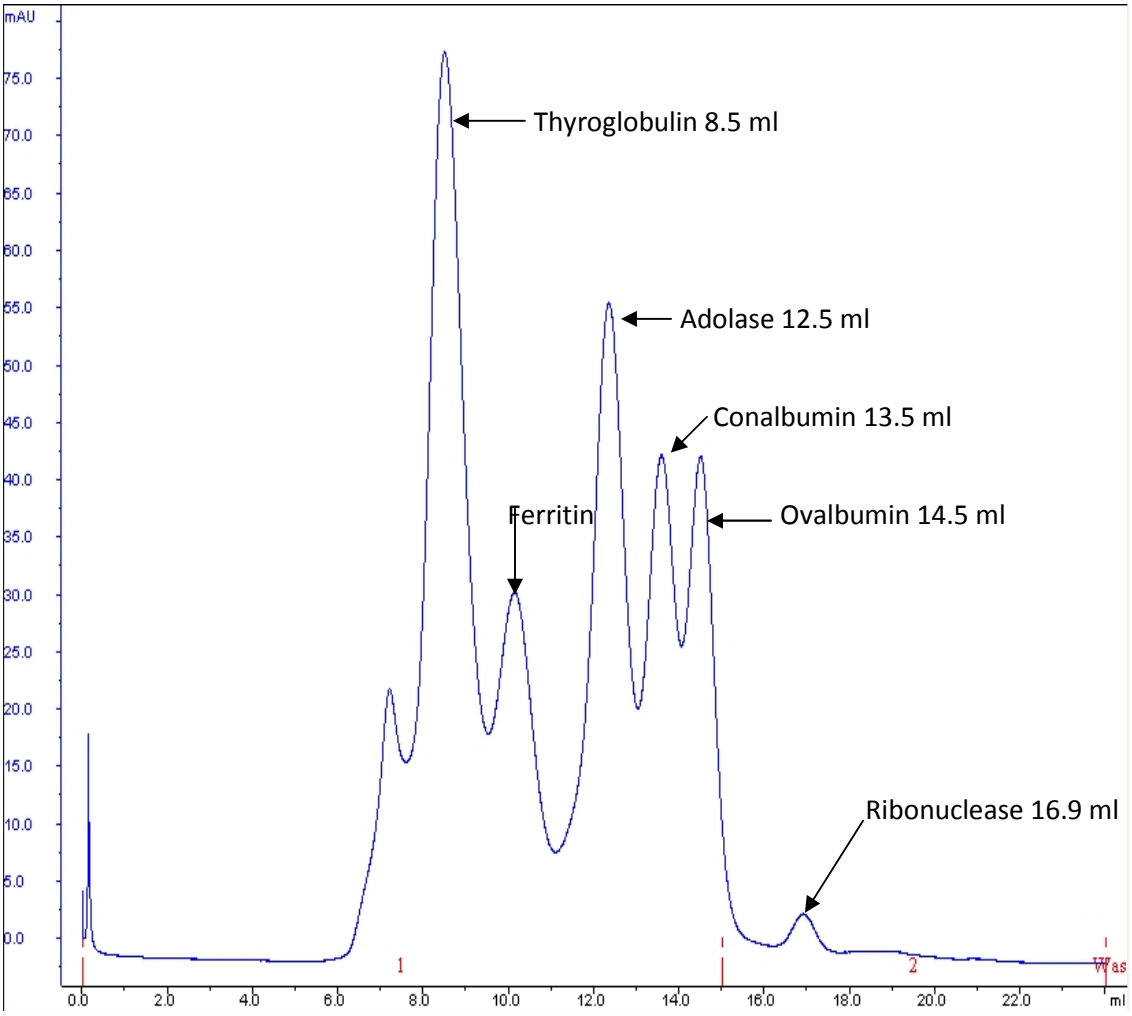

Figure 13: Chromatogram of the mixed protein standards with their retention time.

The purified *mmgD* protein was applied to the column and the chromatogram is shown in figure 14.

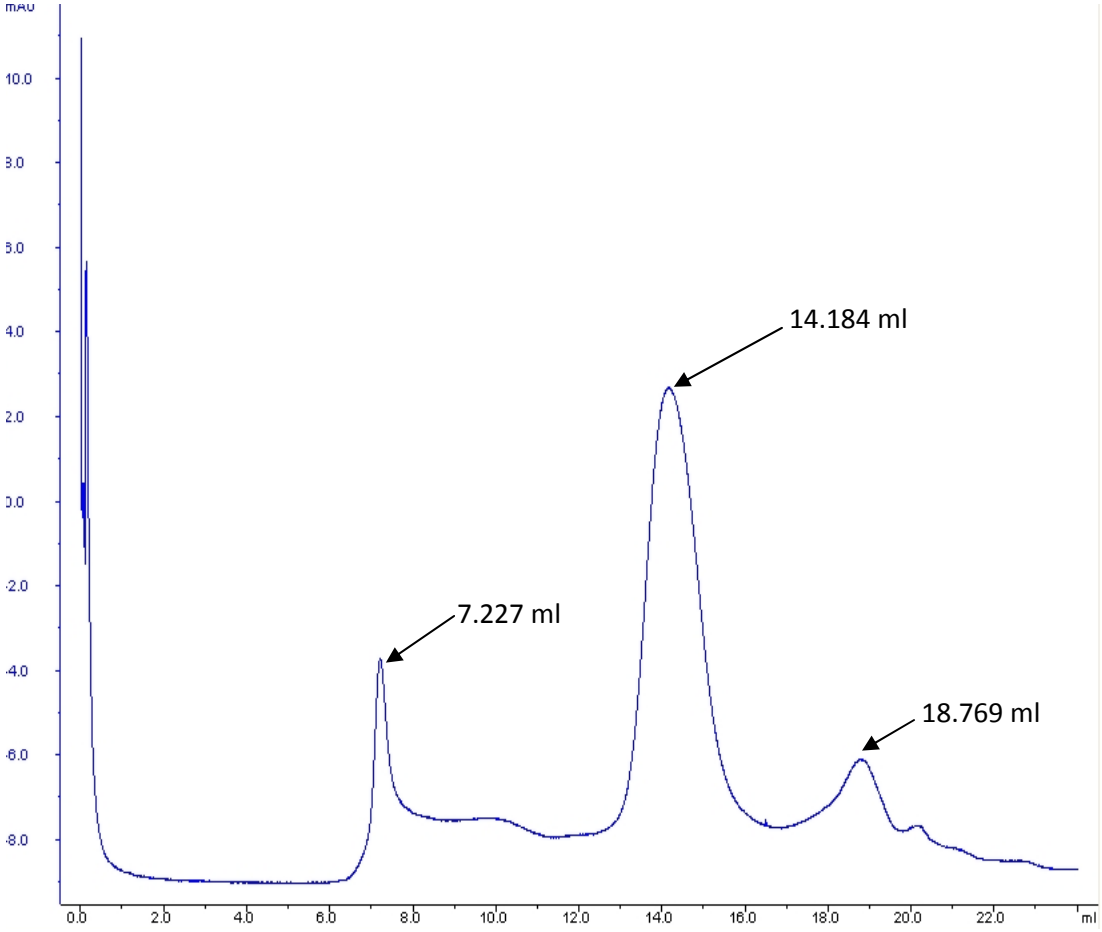

Figure 14: Chromatogram showing the retention time of *mmgD* protein

The retention time of the standard proteins were used in determining the partition coefficient K_{av} as shown in table 5.

Table 5: List of protein molecules showing their partition coefficient and Log M_r.

Protein Molecules	Elution Vol (V_o) ml	K_{av}	M_r (Da)	Log M_r
conalbumin (50mg)	13.543	0.38	75,000	4.88
Aldolase (50mg)	12.343	0.31	158,000	5.20
Ovalbumin (50mg)	14.500	0.44	44,000	4.64
Ferritin (15mg)	10.239	0.19	440,000	5.64
Thyroglobulin (50mg)	8.478	0.08	669,000	5.82
Ribonuclease A (50mg)	16.889	0.58	13,700	4.14
Blue Dextran 2000	7.068	N/A	N/A	N/A
mmgD	7.227	0.0094	1513561	6.18
mmgD	14.184	0.42	51,472	4.71
mmgD	18.789	0.69	5,495	3.74

The *mmgD* native molecular weight was calculated using a calibration curve of the graph of K_{av} against Log M_r for the standard proteins as shown in figure 15. Applied the partition coefficient K_{av} (y axis) *mmgD* proteins to the expression Y = -0.2791x + 1.735 and solved for X = Y- 1.735/0.2791, this resulted in apparent molecular weight of 51 Da

for the highest peak in the chromatogram which when compare with the theoretical molecular weight of 45 kDa falls within monomer range.

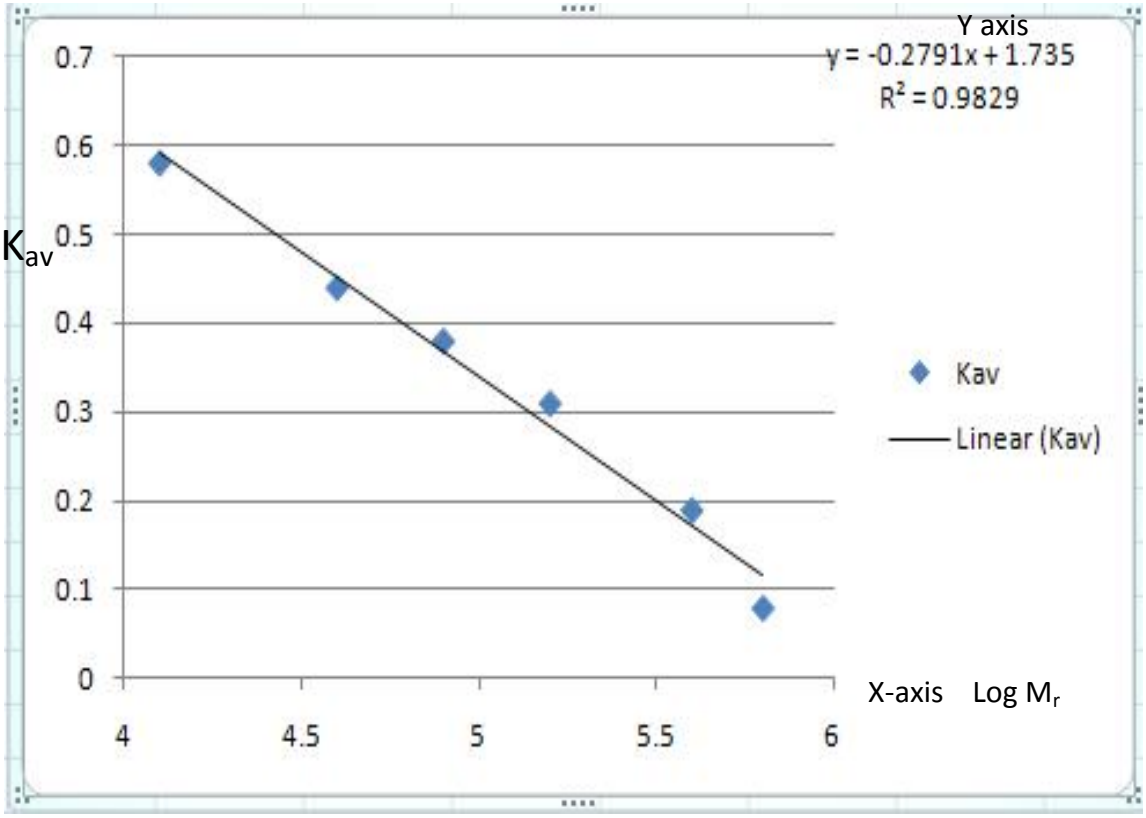

Figure 15: Calibration curve of the standard proteins.

IV.C. Conclusion

Obtaining a detailed knowledge of physical properties of a protein such as its native state, molecular weight etc is very essential to a biochemist as its helps in the full understanding of the functions of the protein in the cells. A protein can exist in its native state as a monomer, dimer, trimer, or higher aggregation of proteins. An analytical tool like gel filtration chromatography also known as size exclusion chromatography, is a widely used method in determining the native molecular weight of a protein by

comparing the elution profile of protein of interest with the elution patterns of a standard protein with known molecular weight.

mmgD was successfully grown and expressed from already cloned gene stored in -80^{o}C (cloned by Acharya, Rejwi (2009), purified and with the successful gel filtration which gave an apparent molecular weight of 51,472Da which is roughly consistent with *mmgD* (theoretical molecular weight of 45 kDa) as a monomer in its native state. *mmgD* also formed apparent aggregate as the first elution peak gave unreasonable high M_r (molecular weight) figure of (1513561 Da).

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