Purification and Characterization of Putative Glutathionylspermidine synthetase, *YgiC* from *Escherichia coli*

by

Sirisha Gadapa

Submitted in partial fulfillment of the Requirements

For the Degree of

Master of Science

in the

Chemistry Program

Youngstown State University

May 2011

Purification and Characterization of Putative

Glutathionylspermidine synthetase, *YgiC* from *Escherichia coli*

Sirisha Gadapa

I hereby release this thesis to public. I understand that this thesis will be made available from the Ohio LINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature: _____

Sirisha Gadapa Date

Approvals:

_____

Dr. Nina V. Stourman, Thesis Advisor Date

_____

Dr. Michael A. Serra Date

Associate Professor, Committee Member

_____

Dr. Josef B. Simeonsson Date

Assistant Professor, Committee Member

_____

Dr. Peter J. Kasvinsky, Ph.D., Date

Dean of Graduate Studies and Research

ABSTRACT

Glutathione (γ-Glu-Cys-Gly) is a tripeptide-, and a primary thiol found in most organisms. It regulates intracellular thiol levels and maintains redox balance. In *Escherichia coli* glutathione reacts with polyamine spermidine (*N*-(3-amino) propyl-1, 4-diaminobutane) to form a conjugate glutathionylspermidine (G-Sp). This reaction is catalyzed by an ATP-dependent bifunctional enzyme glutathionylspermidine synthetase/amidase. Genes *ygiC and yjfC* in *E. coli* genome are associated with putative glutathionylspermidine synthetase activity. The purpose of our research was to characterize the glutathionylspermidine synthetase homologue, *YgiC* (45 kD). Overexpression and purification of *YgiC* protein were attempted in order to perform the activity studies. Several different growth conditions were utilized to achieve the expression of *YgiC* protein in BL21(DE3) *E. coli* cells. Chromatographic techniques namely ion exchange, hydrophobic interaction and gel filtration chromatography were employed for protein purification.

Acknowledgements

First, I would like to express my immense gratitude to my research advisor Dr. Nina Stourman for her constant support and encouragement throughout my research work and preparation of the thesis. I would like to thank my committee members Dr. Michael Serra and Dr. Josef Simeonsson for their valuable advices and encouragement.

I would like to thank my family, especially my mother and my father for their emotional support and encouragements during this period. I owe a lot to my brother and sisters for always calling to find out how life was treating me in a far away land. I would like to give special regards to my husband and all my friends who encouraged me in all aspects of life.

I truly believe that all these would have not come to pass without the financial support of the YSU School of Graduate Studies and the esteemed faculty members of YSU Chemistry Department.

Table of Contents

List of Figures

List of Abbreviations

ATP……..……..Adenosine triphosphate

AMPPNP..... Adenylyl imidodiphosphate

ADP……..……..Adenosine diphosphate

DEAE……..……..Diethylaminoethyl

dsDNA……...……..Double stranded DNA

DTT……..……. Dithiothreitol

DNA.....Deoxyribonucleic acid

EDTA……..Ethylenediaminetetraacetic acid

GPX.....Glutathione peroxidase

GST..... Glutathione transferase

GCL .....Glutamate-cysteine ligase

GSSG ..... Glutathione disulfide

GSH..... Glutathione

γ- GC..... γ-Glutamylcysteine

GSS..... GSH synthetase

HPLC..... High performance liquid chromatography

HEPES……..Hydroxyethyl piperazineethanesulfonic acid

IPTG……..…..Isopropyl-beta-D-thiogalactopyranoside

LB-media……Luria-Bertani media

OD ..... Optical density

$O_2^{\bullet-}$…….……Superoxide

pI.....Iso-electric point

ROS..... Reactive Oxygen Species

RNA..... Ribonucleic acid

rpm..... Revolution per minute

SOD.....Superoxide dismutase

SDS..... Sodium dodecyl sulfate

SDS-PAGE..... Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SOC.....Super optimal culture's media

TAE…….…...…….Tris-Acetate-EDTA

TEMED…….Tetramethylethylenediamine

TG-SDS……..…Tris-Glycine-Sodium dodecyl sulfate

TRIS……...……..Tris (hydroxymethyl) aminomethane

UV……..……...……Ultra violet

CHAPTER I: INTRODUCTION

Life of an aerobic organism depends on the oxygen molecule. Its presence in the environment helps the organism to generate energy in the form of ATP (adenosine triphosphate). Aerobic organisms acquire energy by reduction of an O_2 molecule to water. Free radicals of oxygen are harmful products of normal cellular metabolism affecting living organisms. Reactive oxygen species (ROS) are harmful free radicals that cause many oxidation-reduction (redox) reactions. These reactions damage the cellular metabolism of any organism. Reactive products like H_2O_2 (hydrogen peroxide), free radicals like superoxide ($O_2^{\bullet -}$) and the hydroxyl radical ($OH^{\bullet}$) are examples of stress causative species. In eukaryotes, damage induced by ROS occurs mainly in the presence of transition metals. Reduced iron or copper are capable of catalyzing homolytic cleavage of H_2O_2 in Fenton reaction (Reaction 1-1). This reaction leads to the formation of the reactive hydroxyl radical [1].

$$Fe\ (II) + H_2O_2 \rightarrow Fe\ (III) + OH^- + OH^{\bullet} \qquad \text{(Reaction 1-1)}$$

Reactive species play a positive role by participating in cellular signaling pathways and a negative role by damaging cellular macromolecules, causing changes in signal transduction and gene expression and affecting cell proliferation and differentiation [2]. Potential biological damage termed as oxidative stress, is considered as important harmful effect of ROS.

In living organisms oxidative stress occurs under conditions like overproduction of ROS, deficiency in cellular antioxidant systems, or sometimes both. Excessive ROS can cause damage to DNA, cellular lipids and proteins and also inhibit their biological functions. There are many effects of ROS impairing the functions of cellular components. Interaction of ROS with purine and pyrimidine bases and the deoxyribose backbone components of the DNA are the conditions stimulating mutagenesis and carcinogenesis [3]. Proteins with cysteine and methionine amino acid residues are susceptible to oxidation by the action of ROS. Oxidation of thiol groups in cysteine residues may cause the formation of intra- or inter-protein disulfides and result in dysfunction of structurally or functionally important sites of proteins [21].

Role of Antioxidants

Antioxidants maintain redox homeostasis and fight against oxidative damage. These substances neutralize free radicals by accepting or donating an electron [4]. Antioxidant systems perform their action both enzymatically and non-enzymatically. Cells have evolved a series of enzymatic antioxidant batteries acting under impaired conditions. Superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase act as direct enzymatic scavengers of ROS [3]. Low molecular weight α-tocopherol (vitamin E), ascorbate (vitamin C), glutathione (GSH), flavonoids, carotenoids and ubiquinol are non-enzymic in action [3].

Superoxide dismutases (SOD's) play an important role in removing $O_2^{\bullet-}$ by accelerating its conversion to H_2O_2. Conversion of H_2O_2 to water and oxygen is catalyzed by catalase [34]. Low doses of $O_2^{\bullet-}$ radical or H_2O_2 initiated resistance mechanism in bacteria helping to survive in the presence of higher doses [1]. Glutathione reductase, dehydroascorbate reductase and thioredoxin reductase are enzymes involved in the regeneration of reduced forms of molecular antioxidants and the maintenance of protein thiols [22]. All of these antioxidant systems act in parallel in preventing the generation of ROS, free radical chain termination, detoxication of radicals and repair of damaged elements.

Polyamines and glutathione are molecules found abundantly in bacteria in millimolar concentrations. Bacterial cells respond to stress conditions by utilizing these molecules for maintaining cell homeostasis and growth. Both polyamines and glutathione are involved in regulating many biochemical processes. Glutathione is involved in the regulation of the intracellular thiol redox balance and in the defense against oxidant or other chemically induced damage. Polyamines play an essential role in cell growth, cell division and cell differentiation [3, 29]. The availability of these molecules inside the cell depends on cell status which varies with many environmental conditions. Investigations of endogenous glutathione and the polyamine spermidine in *Escherichia coli* revealed that cells lacking either spermidine or glutathione are less sensitive than the wild type to certain antibiotics. This suggests that these endogenous molecules react to stress conditions [33].

Glutathione

Glutathione is a low molecular weight thiol found in both eukaryotes and prokaryotes. In prokaryotes, it is mainly found in gram-negative bacteria, *e.g. Escherichia coli*. Glutathione is usually absent in gram-positive bacteria with the exception of *Streptococcus* and *Enterococcus sp* [6, 12]. The thiol moiety of the molecule is important for its activity in protecting cellular macromolecules.

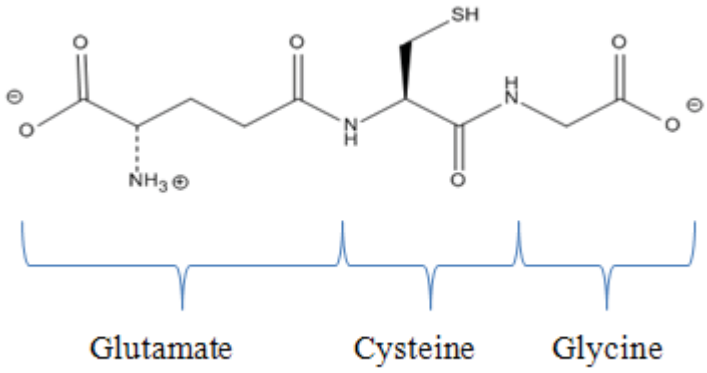


Figure 1-1. Structure of GSH

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine, molecular mass 307 daltons). At physiological pH, it carries two negatively charged carboxyl groups and a positively charged amino group. GSH contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. The presence of the γ-glutamyl bond protects the tripeptide from degradation by intracellular peptidases. The sulfhydryl group of cysteine serves as an electron donor allowing elimination of free radicals.

The GSH levels in a cell are determined by the concentration of total glutathione and the ratio between its oxidized and reduced forms [21]. Glutathione reductase maintains 98% of intracellular glutathione in its reduced form [7]. Glutathione reduces endogenously produced hydrogen peroxide using selenium-dependent GSH peroxidase [7]. Oxidation of two GSH molecules leads to a disulfide bond forming glutathione disulfide (GSSG). GSSG is an oxidized form of GSH. The ratio of GSH to GSSG normally exceeds 10**:**1. A concentration of 0.17-0.33% of GSSG in the total intracellular glutathione, a range of 300-600 reduced/oxidized (GSH/GSSG) ratios and not more than 1% of mixed disulfides were reported [21]. A high ratio of GSH/GSSG indicates a high level of reduced glutathione available for antioxidant activity.

Biosynthesis of Glutathione

Two successive enzymatic reactions are required to synthesize glutathione. Coupling of glutamate and cysteine catalyzed by glutamate-cysteine ligase (GCL, EC 6.3.2.2; formerly γ-glutamylcysteine synthase) results in the formation of γ-glutamylcysteine (γ-GC). The second reaction couples γ-GC with glycine and is catalyzed by GSH synthetase (GSS, EC 6.3.2.3). Both enzymes consume one molecule of ATP per catalytic cycle [7]. Synthesis of GSH is limited by bioavailability of L-cysteine and is initiated by the addition of cysteine or its precursors into the medium [13].

Oxidation of Glutathione

Interactions of GSH with free radicals generates the thiyl radical (GS•). This radical reacts with another GS• radical to form glutathione disulfide (GSSG) undergoing a single electron redox reaction [21]. Another redox reaction involves the two-electron oxidation of GSH with hydroperoxides involving glutathione peroxidase enzyme. This reaction leads to the formation of glutathione disulfide (GSSG) or a mixed disulfide [7, 21]. Glutathione also participates in thiol–disulfide exchange. This reaction plays a key role in the formation of protein disulfides (GSSR) and may be an important element in the regulation of biological processes [14, 21].

Functions of Glutathione

The sulfhydryl group of cysteine in glutathione serves as electron donor providing reducing properties. GSH functions as a cellular redox buffer and has been suggested to be involved in cell proliferation [7]. Because of its abundance, GSH protects cell from toxicity arising during the exposure to excessive amounts of endogenous and exogenous electrophiles [7]. In the presence of enzymes from glutathione transferase (GST) family, glutathione undergoes conjugation reactions with electrophilic endogenous compounds and foreign chemicals allowing their safe elimination from the cell [8]. Glutathione is involved in drug detoxification and amino acid transport in the γ-glutamyl cycle [15]. It is also involved in selenium metabolism [18].

Extracellular glutathione protects cell against toxic substances. In gram-negative bacteria, it plays a role in the regulation and transport of intracellular K^+ (potassium ions) [30]. Accumulation of intracellular potassium ions is important in maintaining the turgor pressure of the cell and intracellular pH [30]. GSH is involved in the mobilization of metal ligands, acts as a cofactor in redox reactions involving metals, and is involved in the transport of metals across cell membranes and acts as a source of cysteine for metal binding [7].

Spermidine (Sp)

Polyamines are polycationic compounds found in all bacterial and animal cells [9]. Polyamines act as growth factor for many microorganisms, stabilize membrane structures of bacteria, as well as the structure of ribosomes, some viruses and DNA of many organisms [16]. Spermidine is derived from amino acids arginine and methionine. The specific role of polyamines is not clear, but their intracellular concentrations are tightly regulated by several feedback, recycling, and export/import mechanisms [17]. Gram-negative bacteria like *E. coli* possess high levels of polyamines [23]. Spermidine synthase is an enzyme required to catalyse the reaction between putrescine and decarboxylated adenosylmethionine which results in spermidine and thiomethyladenosine as products. According to Fukuchi, accumulation of spermidine in the stationary phase resulted in a loss of cell viability [16]. Cell wall incorporation, biosynthesis of siderophores, acid resistance and protection from free radical are processes involving polyamines [23].

Glutathionylspermidine (GSp conjugate)

Glutathionylspermidine (GSp conjugate) is a common metabolite found in both *Trypanosomas* protozoal parasite and *Escherichia coli* bacteria. Glutathione and spermidine are involved in the formation of the GSp conjugate. Glutathionylspermidine synthetase/amidase (GSp synthetase) is the enzyme that catalyzes the reaction using ATP as energy source. In *Escherichia coli*, bifunctional glutathionylspermidine synthetase/amidase catalyzes both the ATP-dependent formation of an amide bond between $N^1$of spermidine and the glycine carboxylate of glutathione and the opposing hydrolysis of this amide bond [17]. The role of the GSp conjugate in protecting DNA against radical or oxidant-induced damage is still unclear [11].

N^1-monoglutathionylspermidine was initially detected in *Escherichia coli* under stationary or anaerobic growth conditions [7]. In pathogenic protozoa of genera *Trypanosoma* and *Leishmania,* N^1, N^8-bis (glutathionyl) spermidine conjugate, termed as trypanothione, was identified [10]. Trypanothione is absent in *E. coli* as the enzyme catalyzing its synthesis is not found [7, 11]. In trypanosomatids, trypanothione synthase catalyzes the addition of the second GSH molecule to the free primary amine of glutathionylspermidine to form trypanothione. Trypanothione maintains redox balance in protozoal parasites because they lack catalase and the GSH peroxidase and GSH reductase enzyme and depend on trypanothione. Therefore, trypanothione metabolism has been considered as possible drug target for new antiparasitic drugs [10, 11].

Glutathionylspermidine synthetase/amidase (GSP synthetase)

Glutathionylspermidine synthetase/amidase bifunctional enzyme was identified four decades ago [19, 25]. GSP synthetase couples ATP hydrolysis with the formation of an amide bond between $N^1$of spermidine and glycine carboxylate of GSH. Glutathionylspermidine synthetase/amidase is a homodimer of 70 kD monomers, with each monomer containing 619 amino acids [31]. Each monomer possess two separate domains with different catalytic activities. A 50 kD carboxy terminal domain (C-terminal) constituting 431 amino acids possesses the synthetase activity, producing glutathionylspermidine conjugate. A 25 kD amino terminal (N-terminal) domain (1 - 225 amino acids) catalyzes the hydrolysis of glutathionylspermidine to glutathione and spermidine [25, 28]. A linker region composed of residues 196 - 205 connects two domains [24]. The amino acid residues Cys59 and His131 are catalytically active and highly conserved in GSP synthetase enzymes throughout organisms [24].

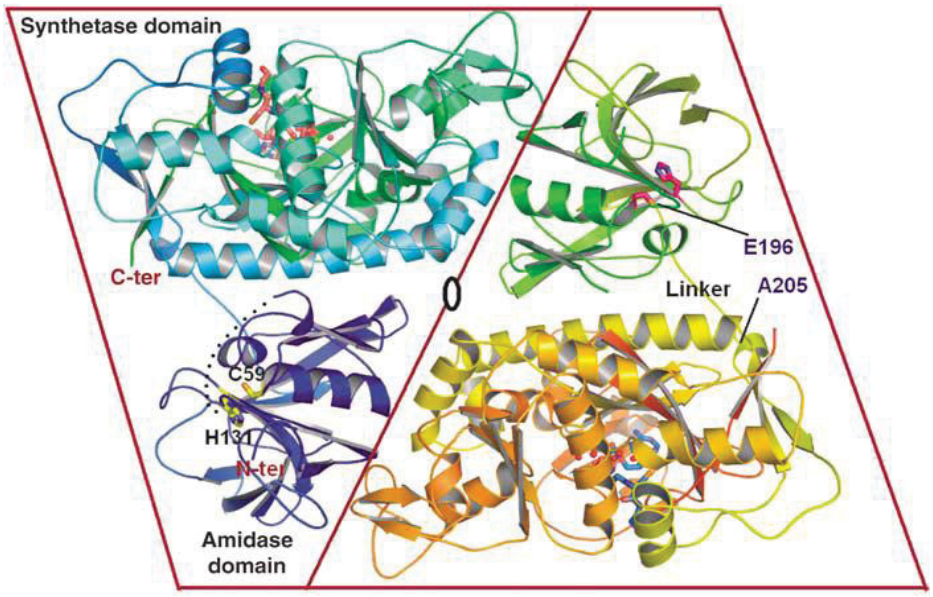
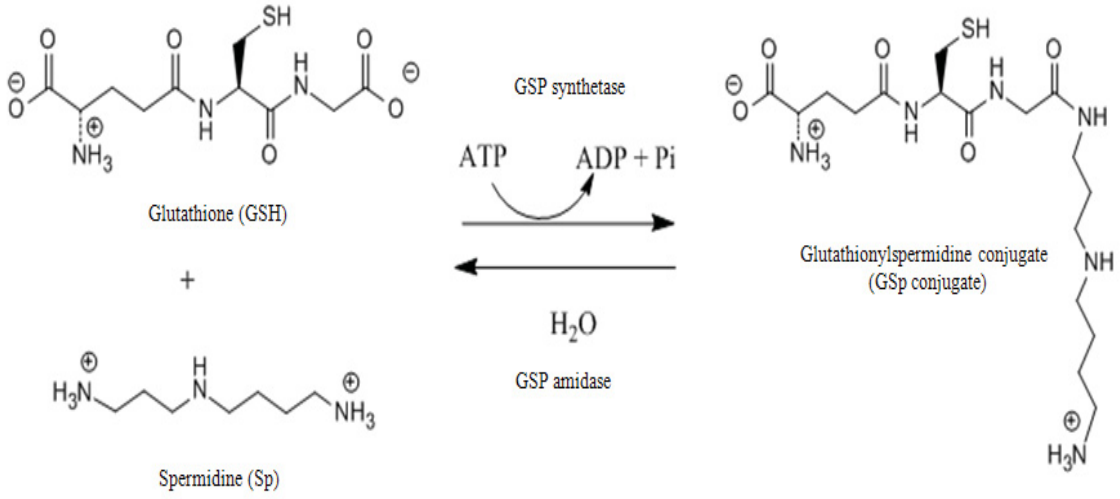


Figure 1-2. Overall structure of glutathionylspermidine synthetase/amidase enzyme. The regions of enzyme show synthetase and amidase domains, linkers and catalytic residues Cys59 and His131 (From reference 24).

Crystal structures of *Escherichia coli* glutathionylspermidine synthetase interacting with substrate analogs like AMPPNP, GSH/ADP, inhibitor and ADP complexes have been reported in recent studies [24]. These structures are helpful in predicting a mechanism and sites of action on GSH and spermidine. The C-terminal synthetase domain of the GSP enzyme belongs to the ATP-grasp super family and comprises a hydrophobic pocket that contains the ATP-binding site [25].

Reaction catalyzed by Glutathionylspermidine synthetase/amidase



Reaction 1-2. Reaction between glutathione and spermidine catalyzed by the bifunctional enzyme glutathionylspermidine synthetase. (From reference 25)

In the conjugation reaction of GSH with spermidine the C-terminus of GSH is initially phosphorylated by the γ-phosphate of ATP to form an acylphosphate. This acylphosphate is subjected to nucleophilic attack by the $N^1$of spermidine [26, 27]. The resulting tetrahedral adduct breaks down to form an amide bond and further disrupts bond of the phosphate leading to the formation of GSp. Inorganic phosphate and ADP are other products released after catalysis [24]. Amidase activity initially identified in *E. coli* was also speculated in trypanosomal species [5]. In *E. coli* GSP enzyme synthetase and amidase activities were separately targeted by potent slow-binding inhibitors that induce time dependent inhibition [25]. Reaction with aldehyde substrate analogs -

resulting in glutathionyl acyl-enzyme intermediate formation and the loss of amidase activity. Selective inhibition of GSP amidase activity was reported in *E. coli* bacteria [28]. Inhibition of amidase activity resulted from oxidation of thiol of Cys59 (at N-terminus) to sulfenic acid increased the concentration of intracellular GSp conjugate [28].

Gene information - ygiC

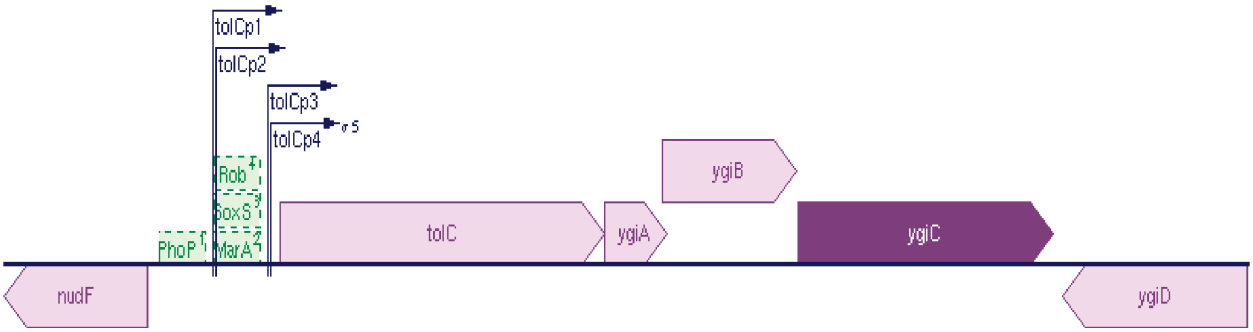


Figure 1-3. Gene local context of *ygiC* gene b3038. (From reference 32)

YgiC (*ygiC* gene product) is a conserved protein with unknown function. *E. coli GSP* shows significant similarity (25 -28% identity) in its C-terminal domain with *YgiC* [31]. In *E. coli* genome, chromosomal *tolC* locus contains *tolC*, *ygiB* and *ygiC* genes expressed in a single operon [20]. The functional link between *YgiB* and *YgiC* proteins and TolC is unknown [20].

STATEMENT OF THE RESEARCH PROBLEM

Characterization of putative glutathionylspermidine synthetase *YgiC* in *E. coli* is the major research goal. *YgiC* and *YjfC* share significant homology with the C-terminal domain of *E. coli GSP* that is associated with glutathionylspermidine synthetase activity [5]. *YgiC* is a hypothetical protein of 45 kD, consisting of 386 amino acids. High conservation of catalytically important residues in *YgiC* suggested that this protein would show activity similar to *GSP* synthetase. The research project focuses on the overexpression and purification of *YgiC* protein to near homogeneity for use in characterization studies.

CHAPTER II: MATERIALS AND METHODS

Materials:

E. coli BL21(DE3) cells from Stratagene, LB media, ampicillin, streptomycin sulfate, HEPES free acid, sodium phosphate (dibasic) heptahydrate (Na_2HPO_4. $7H_2O$), potassium phosphate (monobasic) (KH_2PO_4), ammonium chloride (NH_4Cl), magnesium sulfate ($MgSO_4$), molecular weight protein marker, calcium chloride ($CaCl_2$), isopropyl-thio-2-D-galactopyranoside (IPTG), TRIS-HCl, TRIS, EDTA, dithiotheritol (DTT), Acryl/Bis 29:1 40%, TEMED, glycerol 99%, TG-SDS 10X liquid concentrate, sodium dodecyl sulfate (SDS), EZ-Vision loading dye, coomassie brilliant blue G-250 were purchased from Amresco. D-Glucose anhydrous was from Mallinckrodt, sodium chloride (NaCl) was from VWR chemicals, Toyopearl Butyl-650 was from Tosch Biosciences, DNA Plasmid purification kit was from Qiagen, *Hind* III restriction enzyme was from Promega. Sephadex - 200 was from Sigma.

Expression of *YgiC* protein from *Escherichia coli*

Transformation of E. coli BL21(DE3) strain with pET20b-ygiC plasmid

Preparation of BL21(DE3) competent cells

E. coli BL21(DE3) cells were initially streaked onto an LB/agar plate and grown overnight at 37°C. A single colonie was used for inoculation into 10 mL of LB media. The culture was grown overnight with shaking in an incubator at 37°C. 0.5 mL of overnight culture was diluted using 50 mL of LB media. The cell culture was grown to an optical density $O.D_{600}$ of 0.5. Cells were kept on ice for 20 min and then centrifuged at 4000 × *g* for 20 minutes at 4°C. The pellet was resuspended in 25 mL of an ice cold solution of 0.1 M $MgCl_2$. Cells were kept on ice for 10 min and were centrifuged at 4000 × *g* for 5 minutes at 4°C. The pellet was resuspended in 5 mL of an ice cold solution of 0.1 M $CaCl_2$ and was kept on ice for 20 min. The cells were centrifuged at 4000 × *g* for 5 minutes at 4°C and were resuspended in 3 mL of 85 mM $CaCl_2$ / 15% glycerol solution. 150 µL aliquots of cells were distributed into 1.5 mL microcentrifuge tubes and stored at -80°C.

Transformation of competent cells

A sterile culture tube was chilled on ice, 50 µL of BL21(DE3) competent cells thawed on ice and 1 µL of pET20-*ygiC* were added to the bottom of the tube. The transformation reaction was incubated on ice for 20 min. The cells were then heat shocked at 42°C for 45 sec. The tube was placed on ice for a minute and 950 µL of SOC media was added at room temperature. The tube was incubated with shaking for 45 min at 37°C. 100 µL of culture was plated on to LB/agar plate with 100 µg/mL of ampicillin and incubated overnight at 37°C.

Preparation of the stock of BL21 (DE3)/ pET20-ygiC cells

Eight colonies of successful transformants were selected. Each transformant was inoculated into 3 mL of LB media containing 100 μg/mL of ampicillin and grown overnight at 37°C with shaking at 100 rpm. 50 μL from each overnight culture was diluted with 5 mL of LB with 100 μg/mL of ampicillin. Diluted cultures were grown to an optical density $O.D_{600}$ of 0.6-0.8, the exponential phase of cell growth. To analyze the protein expression, 1 mL from each culture was collected into 1.5 mL microcentrifuge tubes, collected samples were harvested by centrifuging at 14,000 rpm for 1 minute at 4°C. Cell pellets were resuspended in 50 μL of 2X SDS loading dye. Cultures continued to grow for period of 6 hours until stationary phase and another 1 mL of sample was collected. All samples were analyzed using SDS-PAGE. Cultures resulting in better expression of protein of approximately 45 kD size in SDS-PAGE analysis were aliquoted in 1:1 proportion with 75% glycerol into 1.5 mL microcentrifuge tubes and stored at -80°C.

Plasmid DNA isolation and digestion

BL21(DE3)/pET20-*ygiC* cells were grown in 10 mL of LB media containing 100 μg/mL of ampicillin overnight at 37°C with shaking at 100 rpm. Plasmid purification was performed using the QIAprep^{R} Spin Miniprep Kit and followed the procedure outlined by the manufacturer. Using 990 μL of water and 10 μL of DNA sample absorbance at 260 nm, 280 nm and 400 nm was measured. The concentration of DNA was calculated using the equation -

C (ng/ μL) = (A_{260} – A_{400}) X 50 (extinction coefficient of dsDNA) X 100 (dilution factor), where C is the concentration of the sample and A_{260} and A_{400} are absorbances at 260 nm and 400 nm respectively.

The digestion reaction was performed in a 1.5 mL microcentrifuge tube containing 16 μL of the purified DNA sample, 2 μL of Promega Buffer E, 1 μL of *Hind* III and 1 μL of *BamH* I restriction enzymes. A control tube contained 16 μL of pET-20b plasmid and the same volumes of buffer and restriction enzymes. The reaction mixtures were incubated at 37°C for 3 hrs and were analysed using 1% agarose gel. 20 μL of each digestion reaction were mixed with 4 μL of EZ Vision dye and were loaded on to 1% agarose gel containing TAE buffer. The gel was run at 100 volts for approximately 55 min and the bands were visualized under UV light.

A. Protein Expression

Protein expression was performed using different media to determine the one which gave the best expression of *YgiC* protein. All media contained 100 μg/mL ampicillin. We attempted growing BL21(DE3)/pET20-*ygiC* cells in LB media, minimal media MM9 containing 0.02M glucose and MM9 media containing 0.02M glucose, 5% of casamino acids. Cultures were grown under two different temperatures 25°C and 37°C.

3 mL of LB media was inoculated with BL21(DE3)/pET20-*ygiC* cells. The culture was grown overnight with shaking at 100 rpm at 37°C. 50 μL of overnight cultures were diluted using 5 mL of LB media, 5 mL of MM9 media containing 0.02 M glucose or 5 mL of MM9 media containing 0.02M glucose and 5% of casamino acids. Culture tubes were shaken at 100 rpm at 25°C. Another 50 μL of overnight cultures were diluted using 5 mL of LB media, 5 mL of MM9 media containing 0.02 M glucose or 5 mL of MM9 media containing 0.02 M glucose and 5% of casamino acids and shaken at 100 rpm at 37°C. When cells reached an $O.D_{600}$ of 0.6-0.8, 1 mL from each culture was collected. Protein expression was induced by addition of 0.3 mM of IPTG and the culture continued to grow for 6 - 8 hours until the cells reached stationary phase. Another 1 mL of each culture was collected at this time. Protein expression in all samples was analyzed by SDS-PAGE as described earlier. Based on the results of protein expression in cells grown under different conditions of media were chosen for expression of *YgiC* in larger quantities.

Expression of YgiC protein in LB media

An initial culture of 100 mL of LB media was inoculated with BL21(DE3)/pET20-*ygiC* cells. The culture was grown overnight with shaking at 100 rpm at 37°C. 60 mL of overnight culture was diluted with 6 liters of LB media. Diluted cultures were grown with shaking at 100 rpm at 37°C to an exponential phase of cell growth ($O.D_{600}$ of 0.6-0.8). 1 mL of culture was collected for analysis. The 6 L culture was allowed to grow for additional 6 hours until stationary phase and another 1 mL sample was collected for analysis. Cultures were centrifuged at 10,000 × *g* for 10 minutes at 4°C, and the cell pellets were stored in the freezer at -20°C.

Expression of YgiC protein in minimal media MM9 containing 0.02 M glucose and 5% casamino acids

The initial culture was prepared by the same procedure as described in "Expression of *YgiC* protein in LB media." 60 mL of overnight culture was diluted with 6 liters sterile minimal media MM9 containing 0.02 M glucose, 5% of casamino acids. Diluted cultures were grown to $O.D_{600}$ of 0.6 - 0.8 (an exponential phase of cell growth). 1 mL of culture was collected for analysis. The 6 L culture was allowed to grow for additional 6 - 8 hours until stationary phase and another 1 mL sample was collected for analysis. Cultures were then centrifuged at 10,000 × *g* for 10 minutes at 4°C, and the cell pellet was stored at -20°C.

Expression of YgiC protein in minimal media (MM9) media containing 0.02 M lactose

The initial culture was prepared as described in "Expression of *YgiC* protein in LB media." 1 mL of overnight was diluted using 100 mL sterile minimal media MM9 containing 0.02 M lactose and 5% casamino acids. Diluted culture were grown to an O.D $_{600}$ of 0.6 - 0.8 with shaking at 100 rpm at 37°C. 1 mL of sample was collected after reaching an O.D $_{600}$ of 0.6 - 0.8. The 100 mL culture was allowed to grow overnight with shaking at 100 rpm at 37°C and another 1 mL sample was collected for SDS-PAGE analysis.

Protein purification

Protein purification was performed using different chromatographic techniques to determine the procedure which would give the best purification of *YgiC* protein. We attempted protein purification using three chromatographic techniques: ion exchange chromatography, hydrophobic interaction chromatography and gel filtration chromatography. All steps of the purification were performed at 4°C unless otherwise specified. All buffers contained (in addition to the buffering component) 1 mM dithiothreitol, and the pH of each was adjusted at room temperature.

Initial purification by streptomycin sulfate precipitation

Cell pellets previously stored at -20°C were resuspended in 50 mM of HEPES buffer, pH 7.5, 1 mM EDTA, and 1 mM DTT to a final volume of 100 mL. The cells were lysed on ice using a sonicator probe for 30 sec with 2 min stirring intervals. Sonication was performed for 6 -7 cycles. The lysate after sonication was centrifuged at

10,000 × *g* for 20 min and the supernatant was brought to 1% (w/v) of streptomycin by dropwise addition of solution of streptomycin sulfate in 50 mM of HEPES with stirring. The solution was centrifuged at 10,000 × *g* for 20 min and the supernatant was stored at 4°C. To analyze initial purification, 20 µL of supernatant and pellet after each step were collected, resuspended in 20 µL of 2X SDS loading dye and analyzed using SDS-PAGE.

Based on the results from SDS-PAGE analysis, purification of *YgiC* protein was continued. After the initial purification, we attempted protein purification using chromatographic techniques in two ways i.e. by loading protein on to an ion exchange column followed by a hydrophobic interaction column or an ion exchange column followed by gel filtration purification. The purification procedures and conditions for both are described below.

Purification using chromatographic techniques

All steps of the purification process were performed at 4°C. All buffers contained (in addition to the buffering component) 1 mM dithiothreitol, except for the buffer used for the hydrophobic column, and the pH of each was adjusted at room temperature. The supernatant obtained after streptomycin treatment was dialyzed against 0.05 M sodium phosphate buffer, pH 7.5 overnight at 4°C and was loaded on the ion exchange column as described further in ”Ion exchange chromatography”.

Ion exchange chromatography

A column (9.0 cm x 3.5 cm) was filled with DEAE cellulose matrix. The column was equilibrated with 500 mL of 0.05 M sodium phosphate buffer, pH 7.5. The supernatant obtained after streptomycin precipitation was dialyzed against 2 L of 0.05 M sodium phosphate buffer, pH 7.5 overnight at 4°C. Dialyzed protein sample was briefly centrifuged, loaded on the column and a flow through was collected. 500 mL of 0.05M sodium phosphate buffer, pH 7.5 was run through the column as a wash solution. The protein was eluted with a 500 mL gradient of 0 - 400 mM NaCl in 0.05 M sodium phosphate buffer, pH 7.5 and 5 mL fractions were collected. Absorbances of the fractions were measured at 280 nm. Fractions showing high absorbance and a band of 45 kD on SDS-PAGE were pooled together and dialysed against 0.05 M TRIS buffer, pH 7.5 overnight at 4°C. All protein solutions were stored at 4°C.

Hydrophobic interaction chromatography

A column (7.5 cm x 2.0 cm) was packed with Toyopearl-Butyl-650 matrix. Approximately 30 mL of dialyzed protein after ion exchange column was concentrated to volume of 1-2 mL using an Amicon concentrator with filter size of 10 kD. Ammonium sulfate was added to the concentrated protein to a final concentration of 1.7 M. The column was equilibrated with 150 mL of 0.05 M TRIS buffer, pH 7.5 containing 1.7 M ammonium sulfate. Concentrated protein with 1.7 M ammonium sulfate was loaded on the column and flow through was collected. 150 mL of 0.05 M TRIS buffer, pH 7.5 containing 1.7 M ammonium sulfate was run through the column

as wash solution. The protein was eluted with a 30 mL gradient of 1.7 - 0 mM ammonium sulfate in 0.05 M TRIS buffer, pH 7.5 and 2 mL fractions were collected. Absorbance of the fractions was measured at 280 nm. Fractions showing high absorbance and a band of 45 kD on SDS-PAGE were pooled together and dialysed against 0.05 M sodium phosphate buffer, pH 7.5 overnight at 4°C.

Gel filtration chromatography

A column (62 cm x 2.0 cm) was filled with Sephadex-200 matrix. Protein fractions with a high absorbance at 280 nm and a band of 45 kD on SDS-PAGE after the ion exchange column were pooled and dialyzed in 0.05 M sodium phosphate buffer, pH 7.5 overnight at 4°C. Dialyzed protein sample was concentrated to 1 mL using an Amicon concentrator with cut-off filter of 10 kD. The column was equilibrated with 200 mL of 0.05 M sodium phosphate buffer, pH 7.5 and was loaded with 1 mL of concentrated protein. Protein elution was performed using 200 mL of 0.05 M sodium phosphate buffer, pH 7.5 containing 0.15 M NaCl. 1 mL of fractions were collected and the absorbance of each fraction at 280 nm was measured. Fractions showing high absorbance and a band of 45 kD on SDS-PAGE were pooled, dialysed against 0.05 M sodium phosphate buffer, pH 7.5 and stored at 4°C.

Analysis by SDS-PAGE

Samples collected throughout all purification steps were analysed by SDS-PAGE. 12% polyacrylamide gels were used for analysis. 20 μL of each sample was mixed with 20 μL of 2X SDS-PAGE loading buffer. The mixture was heated for 10 minutes at 80°C. The gels were loaded with 15-20 μL of sample. The gel was run at 200 Volts for approximately 55 minutes approximately. All gels were stained in Coomassie brilliant blue R-250 for 30 min and destained.

CHAPTER III: RESULTS

Cell transformation

The pET20b-*ygiC* plasmid was introduced into BL21(DE3) *E. coli* cells using the heat shock method. Cells transformed with pET20b-*ygiC* plasmid were grown on LB-agar plates containing ampicillin (LB-agar/ampicillin plate) under incubation at 37°C. As shown in Figure 3-1, BL21(DE3) cells transformed with pET20b-*ygiC* plasmid grew on LB-agar/ampicillin plate.

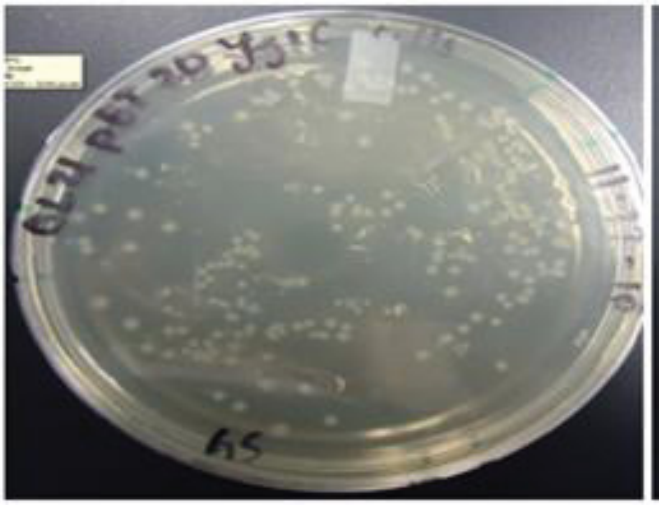


Figure 3-1. Transformed cells grown on LB-agar/ampicillin plate

Analysis of the digested plasmid using agarose gel electrophoresis

Transformed cells were subjected to plasmid DNA isolation and purified plasmid was digested using restriction enzymes *Hind III* and *BamH I*. Analysis by 1% agarose gel electrophoresis resulted in two bands about 4 kbp and 1 kbp for the digested pET20b-*ygiC* plasmid that corresponds to the size of pET20b vector and *ygiC* gene (Figure not shown).

Expression of YgiC protein in E. coli

E. coli has been used extensively as the host for protein expression due to its rapid growth rate, capacity for continuous fermentation and relatively low cost. Expression of *YgiC* protein was performed in the BL21 (DE3) strain of *E. coli* cells.

Effect of the various growth conditions on protein expression

BL21(DE3)/pET20*ygiC* cells were grown in LB media, minimal media MM9 containing 0.02M glucose and MM9 containing 0.02M glucose, 5% of casamino acids to determine the optimal conditions for expression of *YgiC* protein. Protein expression was investigated by SDS-PAGE analysis.

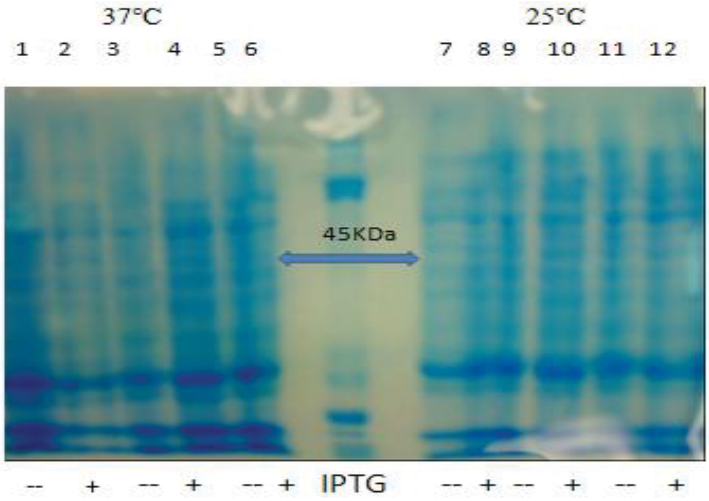


Figure 3-2. Effect of the temperature on the expression of *YgiC* protein. Lane 1, 2 and 7, 8: cells grown in LB media at 37°Cand 25°C without (-) and with (+) IPTG, Lane 3, 4 and 9, 10; cells grown in MM9 media with 0.02M glucose at 37°C and 25°C without (-) and with (+) IPTG; Lane 5, 6 and 11, 12: cells grown in MM9 media with 0.02 M glucose and 5% casamino acids at 37°C and 25°C without (-) and with (+) IPTG. Protein marker is in the middle lane.

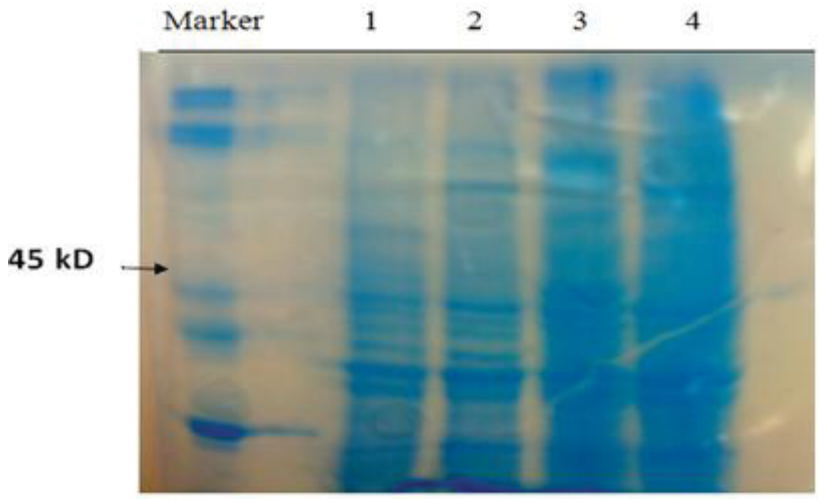


Figure 3-3. Expression of *YgiC* protein in LB and MM9 media. Lane 1: cells grown in LB media at 37°C, Lane 2: cells grown in MM9 media with 5% casamino acids at 37°C, Lane 3: cells grown in LB media at 25°C, Lane 4: cells grown in MM9 media at 25°C.

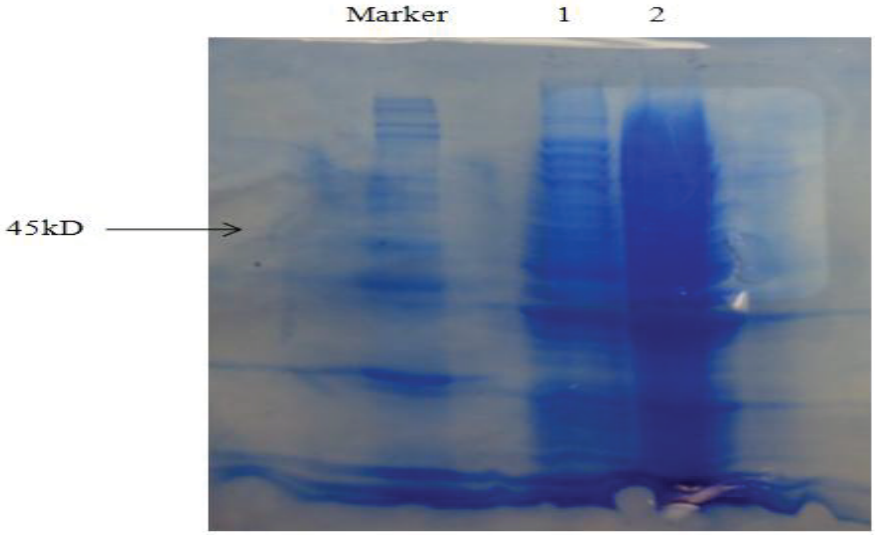


Figure 3-4. Expression of *YgiC* protein in MM9 media with 0.02 M lactose. Lane 1: cells grown to exponential phase (O.D $_{600}$ ~0.6- 0.8), Lane 2: overnight culture.

Protein expression was observed in three different media (as described in effect of temperature in Material and Methods chapter, Chapter II) at two temperatures: 37°C and 25°C. As shown by SDS-PAGE analysis (Figure 3-2), protein expression was better at 37°C than at 25°C. Also cell growth at 25°C was slow when compared to 37°C. As shown by SDS-PAGE (Figure 3-3), minimal media without casamino acids resulted in poor expression with prolonged cell growth. Figure 3-4 shows that MM9 media containing 0.02 M lactose did not improve the expression.

Overexpression of YgiC protein

Based on the analysis of protein expression, MM9 media containing 0.02 M glucose, 5% casamino acids and LB media were selected for protein expression and 37°C was the temperature chosen for the culture growth. Below are the results of protein expression in LB media (Figure 3-5) and MM9 media containing 0.02 M glucose, 5% casamino acids (Figure 3-6) after induction by 0.3 mM IPTG when cells were grown in a six liter culture.

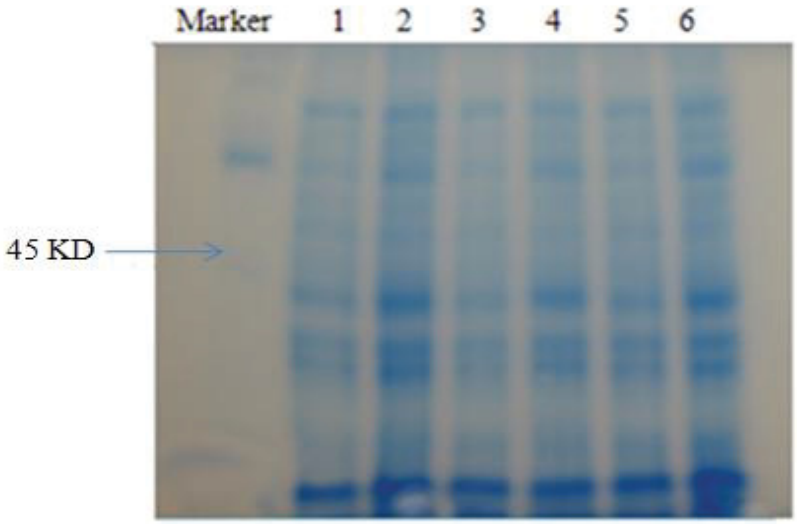


Figure 3-5. IPTG induced expression of *YgiC* protein in LB media. Lane 1, 3 and 5: before IPTG addition and Lane 2, 4 and 6: after IPTG addition.

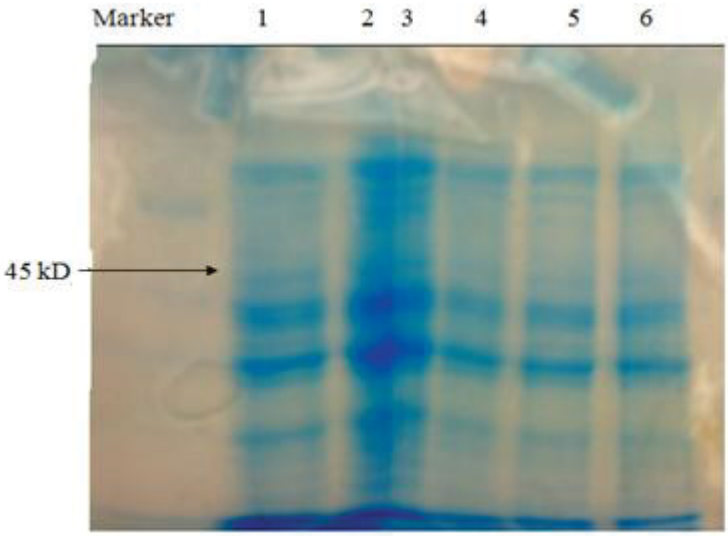


Figure 3-6: IPTG induced expression of *YgiC* protein in MM9 media containing 0.02 M glucose and 5% casamino acids. Lane 1, 3 and 5: before IPTG addition and Lane 2, 4 and 6: after IPTG addition.

Through SDS-PAGE analysis, large scale expression of *YgiC* protein revealed a band at 45 kD. As shown in Figure 3-5 and 3-6, LB media and MM9 containing 0.02 M and 5% casamino acids gave sufficient expression of *YgiC* protein to proceed to further purification.

Protein purification

Cell cultures from large scale expression were centrifuged and the cell pellets were used for further purification of *YgiC*.

Initial purification of YgiC protein by addition of streptomycin sulfate

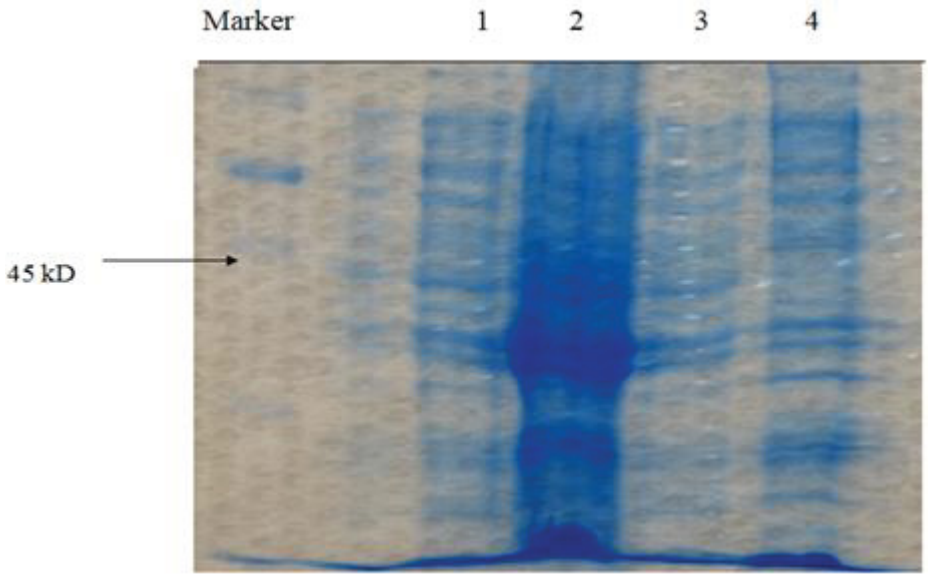


Figure 3-7. Initial purification of *YgiC* protein from cells grown in LB media. Lane 1: supernatant after sonication, Lane 2: pellet after sonication, Lane 3: supernatant after streptomycin addition and Lane 4: pellet after streptomycin addition.

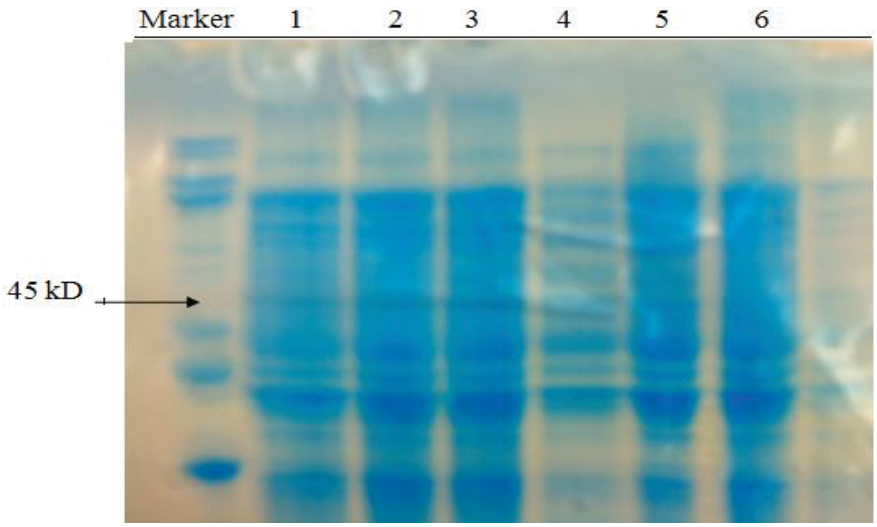


Figure 3-8. Initial purification of *YgiC* protein from cells grown in MM9 media. Lane 1: pellet after sonication, Lane 2, 3: supernatant after sonication, Lane 4: pellet after streptomycin addition, Lane 5: supernatant after streptomycin addition, Lane 6: dialyzed supernatant after streptomycin addition.

As shown by SDS-PAGE analysis (Figure 3-7 and 3-8), after sonication significant amount of *YgiC* protein was found in the supernatant solution. Figure 3-7 and 3-8 show an intense band at 45 kD in the supernatant after streptomycin addition and a light band at 45 kD found in the pellet.

Purification of YgiC protein using chromatographic techniques

Supernatant after streptomycin precipitation was further purified by chromatographic techniques. Protein samples were dialysed against buffer before each step of purification.

Ion exchange chromatography

Ion exchange chromatography separates proteins based on their charge and was used for further purification of *YgiC*. The absorbance at 280 nm of fractions eluted off DEAE column was measured to determine the fractions containing high protein concentration. Figure 3-9 shows the change of absorbance at 280 nm of collected fractions with a gradual increase in the concentration of NaCl from 0 to 400 mM. Fractions with high absorbance at 280 nm were analysed by SDS-PAGE.

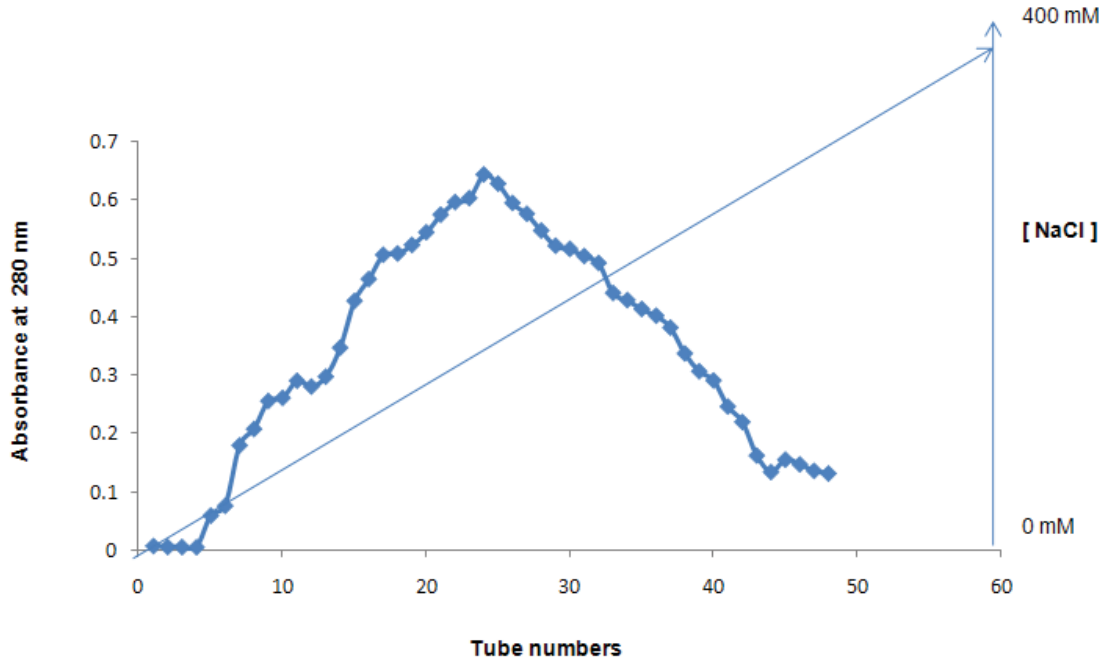


Figure 3-9. Graph of absorbance versus the tube number for fractions after DEAE-cellulose purification.

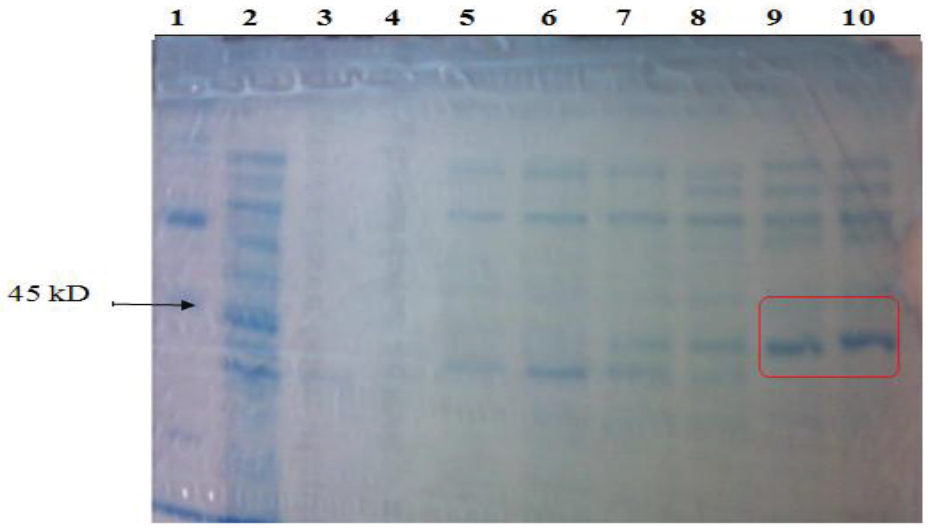


Figure 3-10. Purification of *YgiC* protein from cells grown in LB media by DEAE-cellulose. Lane 1: marker, Lane 2: protein supernatant loaded on DEAE column (protein load), Lane 3: flow through, Lane 4: wash and Lane 5-10: fractions collected off DEAE column. Fractions in lanes 9 and 10 show an intense band at 45 kD.

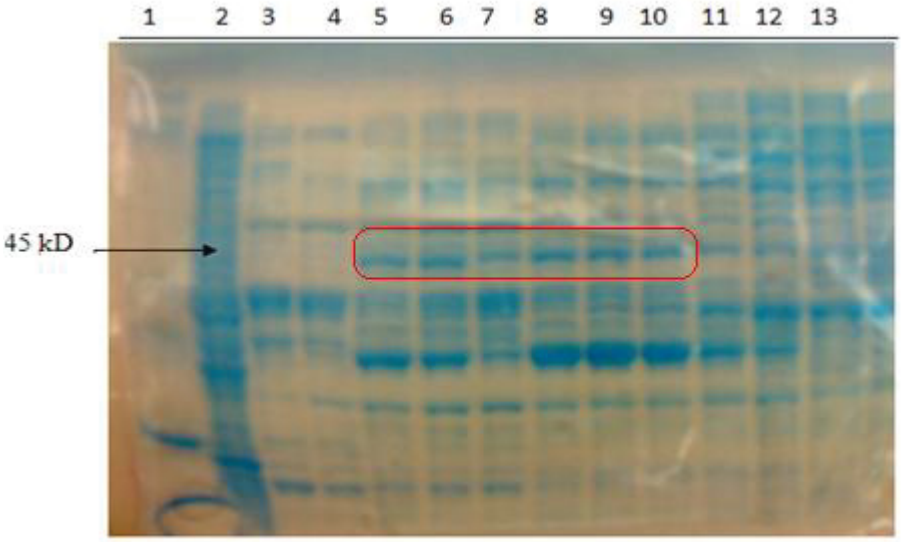


Figure 3-11: Purification of *YgiC* protein from cells grown in MM9 media by DEAE-cellulose. Lane 1: marker, Lane 2: protein load and Lanes 3-13 fractions collected off DEAE column. Fractions in lanes 5-10 show intense band at 45 kD.

As shown by SDS-PAGE analysis, several fractions after ion exchange contained a band at 45 kD. Figure 3-10 shows two fractions 9 and 10 contain intense band at 45 kD. Fractions in lanes 5-10 from Figure 3-11 show intense bands at 45 kD.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography utilizes hydrophobicity to separate proteins. High ionic strength buffers increase the hydrophobic interaction between protein molecules and the matrix. The fractions collected off DEAE-cellulose that contained 45 kD protein were further purified using a Toyopearl Butyl-650 column. Figure 3-12 shows the change of absorbance at 280 nm of collected fractions with the decrease in ammonium sulfate concentration from 1.7 mM to 0 mM.

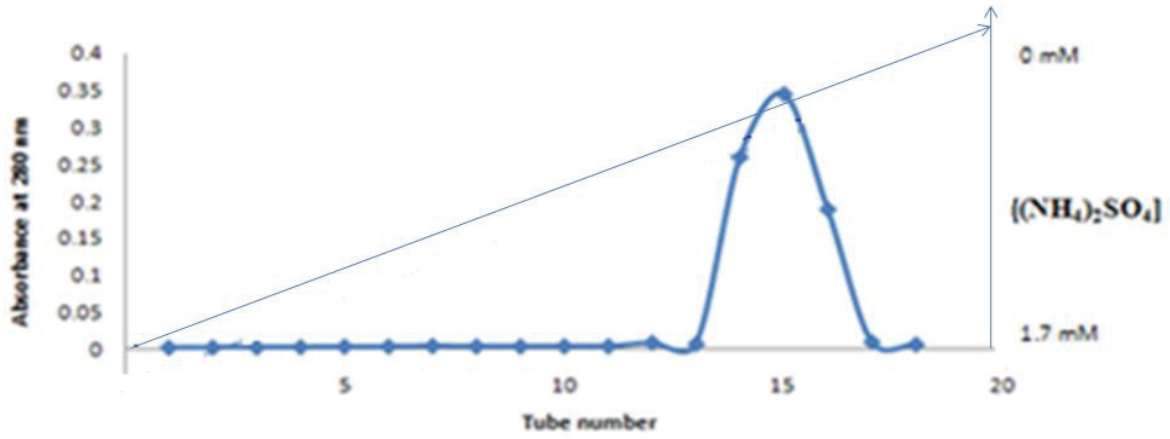


Figure 3-12. Graph of absorbance versus the tube number for fractions after Toyopearl Butyl-650 purification.

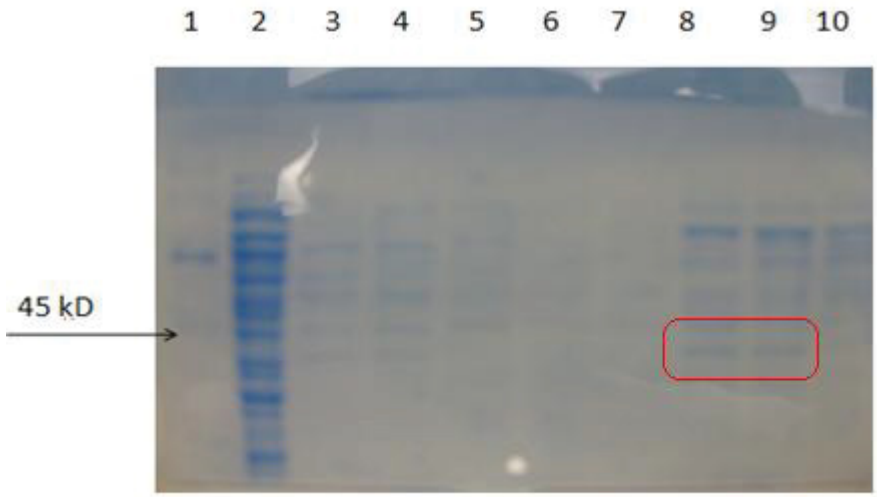


Figure 3-13. Purification of *YgiC* protein from cells grown in LB media by Toyopearl Butyl-650. Lane 1: marker, Lane 2: protein loaded on Toyopearl Butyl-650 column and fractions in lanes 8 and 9 show band at 45 kD.

Figure 3-14. Purification of *YgiC* protein from cells grown in MM9 by Toyopearl Butyl-650. Lane M: marker, Lane 1: protein load and Lane 2-10 fractions collected off Toyopearl Butyl-650. Fractions in lanes 9 and 10 show band at 45 kD.

SDS-PAGE analysis indicated that some samples after hydrophobic interaction column contain a band at 45 kD. Two fractions from lanes 8 and 9 on Figure 3-13 show faint bands at 45 kD from cells grown in LB media. Fractions from lanes 9 and 10 (Figure 3-14) show a faint band at 45 kD from cells grown in MM9 media.

Gel filtration chromatography

Gel filtration chromatography is a technique which separates molecules based on their size. The fractions after ion exchange column that contained a 45 kD protein were further purified using a Sephadex - 200 column followed by SDS-PAGE analysis. Figure 3-15 shows high absorbance at 280 nm for fractions under peak 3 and 4. SDS-PAGE analysis of fractions under these peaks showed a band at 45 kD (Figure 3-17).

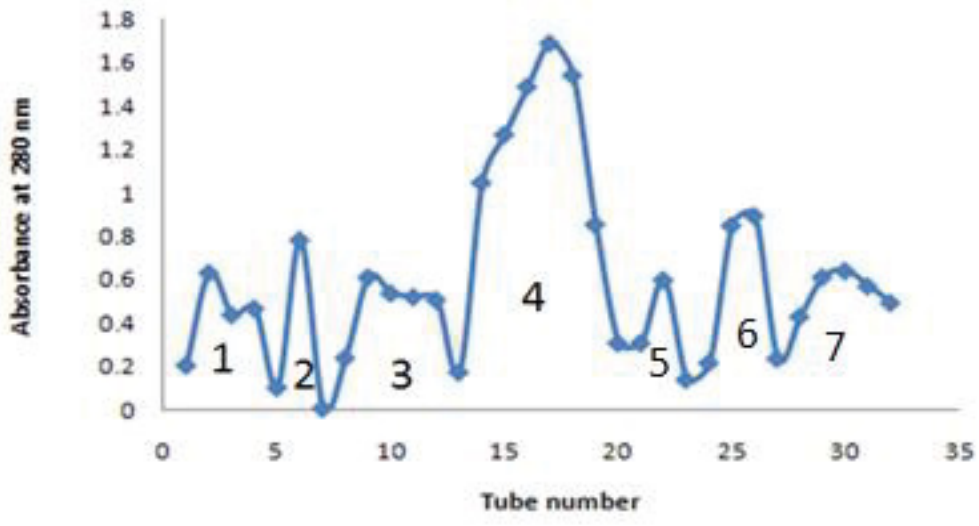


Figure 3-15. Graph of absorbance at 280 nm versus the tube number for fractions collected from Sephadex - 200.

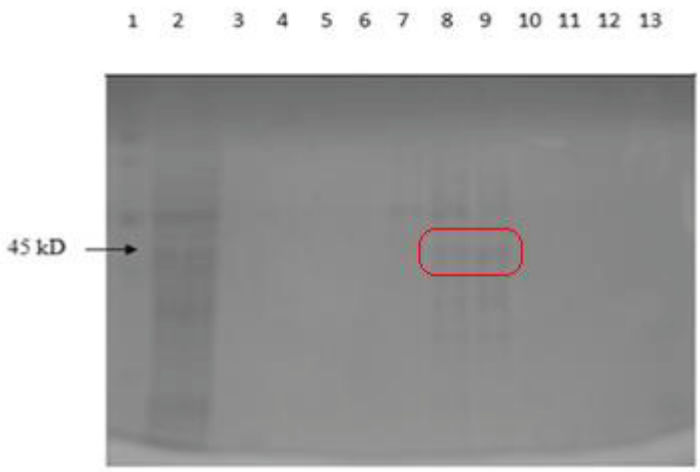


Figure 3-16. Purification of *YgiC* protein from cells grown in LB media using Sephadex – 200 column. Lane 1: marker, Lane 2: protein load and fractions in lanes 3-13 were eluted from the Sephadex-200. Fraction in lanes 8 and 9 show a band at 45 kD.

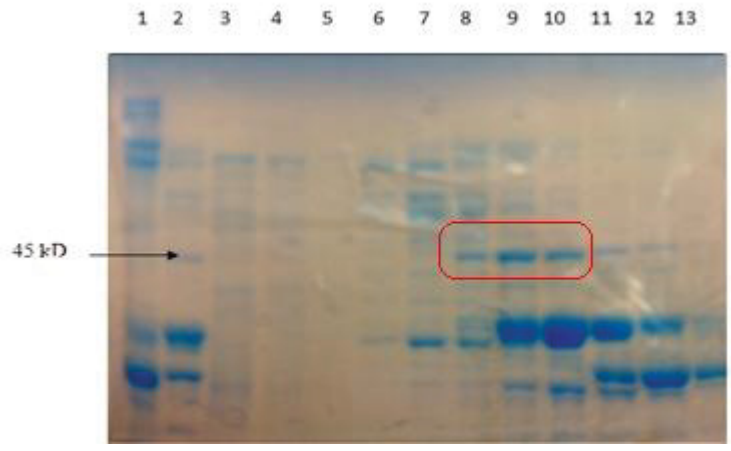


Figure 3-17. Purification of *YgiC* protein from cells grown in MM9 media by Sephadex - 200 column. Lane 1: marker, Lane 2: protein load and fractions in lanes 3-13 were eluted from Sephadex - 200. Fractions in lanes 8, 9 and 10 show an intense band at 45 kD.

Analysis of samples after gel filtration showed two fractions 8 and 9 (Figure 3-16) with faint bands at 45 kD from cells grown in LB media. Fractions in lanes 8, 9 and 10 from (Figure 3-17) show an intense band at 45 kD proteins from cells grown in MM9 media.

CHAPTER IV: DISCUSSION

GSP is associated with glutathionylspermidine synthetase activity. The C-terminal domain of *GSP* protein shows 25-28% homology with two *E. coli* proteins, *YgiC* and *YjfC.* Therefore, *YgiC* and *YjfC* proteins are expected to have glutathionylspermidine synthetase activity. In this research study, overexpression and purification of *YgiC* protein were attempted in order to perform activity studies on the protein. *Escherichia coli* is one of the most successful vehicles for over-expression of both prokaryotic and eukaryotic proteins [37]. *E. coli* strain BL21 (DE3) has been shown to be an effective expression host. T7 RNA polymerase is produced from λ-lysogen DE3 in the host bacterium. It is particularly useful to express proteins since it enables expression of T7 RNA polymerase by control of the IPTG-inducible *lac* UV5 promoter [37]. This strain is used in conjunction with pET vectors. The pET expression system is most commonly used in production of recombinant proteins. The pET20b plasmid containing *ygiC* gene was a gift from Dr. Armstrong (Vanderbilt University, Nashville, TN). Transformation of the plasmid pET20b-*ygiC* into *E. coli* BL21(DE3) strain was performed using the heat shock method. Analysis of expression of *YgiC* protein in BL21(DE3)/pET20b-*ygiC* cells grown under different conditions indicates that cells grown at 37°C in LB media and MM9 media containing 0.02 M glucose with 5% casamino acids provided the best *YgiC* expression. IPTG, a non fermentable analog of lactose was used to induce protein expression. IPTG inactivates the *lac* repressor and stimulates the T7 RNA polymerase that usually leads to the overexpression of the genes carried by pET20 plasmid. However, addition of IPTG did not show any effect on the expression of *YgiC* protein.

In order to overcome this problem we attempted to grow BL21(DE3)/pET20b-*ygiC* cells at 37°C in MM9 media containing 0.02 M lactose with 5% casamino acids. Expression of *YgiC* protein using 0.02 M lactose in media unfortunately, showed no difference compare to MM9 media containing 0.02 M glucose.

For *YgiC* purification cell cultures were grown in LB media and MM9 media containing 0.02 M glucose and 5% casamino acids. Cell lysate treated with streptomycin sulfate showed that most protein remains in the solution. We attempted treatment of the supernatant solution with ammonium sulfate initially at 40% saturation and then at 70% saturation. However, these attempts resulted in the loss of protein due to irreversible precipitation and were omitted in further purification experiments.

Chromatographic purification of *YgiC* protein was performed by the combination of two chromatographic columns anion-exchange followed by either gel filtration or hydrophobic column. In order to bind an anion-exchange column, the surface charge of the protein should be negative. Therefore, the pH of the buffer should be above the pI of the target protein. The pH of the buffers used with the DEAE column was above 4.6 (pI of *YgiC*).

Protein binding to hydrophobic interaction column adsorbents was promoted by moderately high concentrations of ammonium sulfate. Elution was achieved by a linear decrease of the concentration of salt in the elution buffer. However, hydrophobic interaction chromatography resulted in a significant loss of protein during the purification process.

Gel filtration chromatography using Sephadex - 200 column separated proteins based on their molecular sizes. This method resulted in partial purification of *YgiC* protein. The combination of purification by ion exchange column followed by gel filtration chromatography resulted in the best purification of *YgiC* which showed an intense band at 45 kD on SDS-PAGE gel.

Unfortunately, neither of chromatographic attempts purified *YgiC* protein to near homogeneity. Additional efforts for improvement of protein expression are currently under way. Fresh transformation of pET20b-*ygiC* plasmid into BL21(DE3) cells and application of buffers with different pH will be carried out to produce better results. Large amounts of purified protein would facilitate characterization studies of *YgiC*. Continuous spectrophotometric assay and high performance liquid chromatography (HPLC) techniques might be future experiments for enzymatic activity studies.

CONCLUSIONS

The purpose of our research was to characterize the glutathionylspermidine synthetase homologue, *YgiC* (45 kD). In this study *YgiC* protein was expressed in *E. coli* strain BL21(DE3). The analysis of protein expression indicates that most protein was produced by the cells grown at 37°C in LB and MM9 media containing glucose and casamino acids. Purification of *YgiC* protein using combination of ion-exchange and gel filtration chromatography resulted in partial purification of *YgiC* protein. However, neither combination of the chromatographic techniques purified protein to near homogeneity. Therefore, it would be prudent to find alternative approach to enhance the expression and purification of *YgiC* for characterization studies.

REFERENCES

1. E. C. Ziegelhoffer and T. J. Donohue; Bacterial responses to photo oxidative stress, *Nat Rev Microbiol.,* 7, 856 - 863, **2009**.
2. J. M. Dubbs and S. Mongkolsuk; Peroxiredoxins in bacterial antioxidant defense, peroxiredoxin systems, *Subcell Biochem.*, 44, 143 - 193, **2007.**
3. V. I. Lushchak: Oxidative stress and mechanisms of protection against it in bacteria; *Biochemistry,* 66, 476 - 489. **2001**.
4. H. Sies: Oxidative Stress; Oxidants and antioxidants*, Exp Physiol.,* 82, 291 -295, **1997.**
5. J. M. Bollinger, D. S. Kwon, G.W. Huisman, R. Kolter, C. T. Walsh; Glutathionylspermidine metabolism in *Escherichia coli*, *J. Biol Chem.,* 270*,* 14031 - 14041, **1995**.
6. R. C. Fahey, W. C. Brown, W. B. Adams, and M. B. Worsham; Occurrence of glutathione in bacteria*, J. Bacteriol.*, 133, 1126 - 1129, **1978.**
7. W. Wang and N. Ballatoria; Endogenous glutathione conjugates occurrence and biological functions, *J. Pharmacol Exp*, 50, 335 - 356, **1998**.
8. N. Allocati, L. Federici, M. Masulli and C.D. Ilio; Glutathione transferases in bacteria, *FEBS J.*, 1742 - 4658, **2008.**
9. C. W. Tabor and H. Tabor; Polyamines in microorganisms, *Microbiol Rev*, 49, 81 - 99, **1985.**
10. H. Shim and A. H. Fairlamb; Levels of polyamines, glutathione and glutathione-spermidine conjugates during growth of the insect trypanosomatid *Crithidia. fasciculate; J. Gen Microbiol.*, 134, 807 - 817, **1988.**

11. K. Smith, A. Borges, T. M. R. Ariyanayagam and A. H. Fairlamb; Glutathionylspermidine metabolism in *Escherichia coli*, *Biochem J.,* 312, 465 -469, **1995**.

12. G. L. Newton, K. Arnold, M. S. Price, C. Sherrill, S. B. Delcardayre, Y. Aharonowitz, G. Cohen, J. Davies, R. C. Fahey and C. Davis; Distribution of thiols in microorganisms, mycothiol is a major thiol in most actinomycetes, *J Gen Microbiol.,* 178, 1990 - 1995, **1996**.

13. P. C. Loewen; Levels of glutathione in *Escherichia coli, Biochem J*, 57, 107 -111, **1979**.

14. N. S. Kosower and E. M. Kosower; The glutathione status of cells, *Internat. Review of Cytology*, 54, 109 - 160, **1978**.

15. A. Meister; Selective modification of glutathione metabolism, *Science,* 220, 472 - 477, **1983**.

16. J. Fukuchi, K. Kashiwagi, M. Yamagishi, A. Ishihama, and K. Igarashi; Decrease in cell viability due to the accumulation of spermidine in spermidine acetyltransferase-deficient mutant of *Escherichia coli, J. Biol Chem.*, **270,** 18831 - 18835, **1995.**

17. C. H. Lin, D. S. Kwon, J. M. Bollinger and C. T. Walsh; Evidence for a glutathionyl-enzyme intermediate in the amidase activity of the bifunctional glutathionylspermidine synthetase/amidase from *Escherichia coli, Biochemistry,* 36*,* 14930 - 14938, **1997.**

18. J. Kessi and K.W. Hanselmann; Similarities between the abiotic reduction of selenite with glutathione and the dissimilatory reaction mediated by *Rhodospirillum rubrum* and *Escherichia coli*, *J. Biol Chem.*, 279, 50662 - 50669, **2004**.

19. H. Tabor and C.W. Tabor; Isolation, characterization and turnover of glutathionylspermidine from *Escherichia coli, J. Biol Chem.*, 250, 2648 - 2654, **1975**.

20. G. Dhamdhere and H. I. Zgurskaya; Metabolic shutdown in *Escherichia coli* cells lacking the outer membrane channel TolC, *Molecular Microbiology,* 1365 - 2958, **2010.**

21. G. V. Smirnova and O.N. Oktyabrsky; Glutathione in bacteria, *Biochemistry,* 70**,** 1199 - 1211, **2005**.

22. H. Sies; Strategies of antioxidant defense, *Eur J. Biochem,* 215*,* 213 -219, **1993.**

23. B. W. Wortham, C. H. Patel, M. A. W. Olivera; Polyamines in bacteria, pleiotropic effects yet specific mechanisms, *Adv Exp Med Biol.,* 603, 106 - 115, **2007**.

24. C. H. Pai, B. Y. Chiang, T. P. Ko, C. C. Chou, C. M. Chong, F.J. Yen, S. Chen, J.K Coward , A. H. J. Wang and C. H. Lin; Dual binding sites for translocation catalysis by *Escherichia coli*, glutathionylspermidinesynthetase, *EMBO J.*, 25**,** 5970 - 5982, **2006.**

25. C. H. Lin, S. Chen, D. S. Kwon, J. K. Coward and C.T. Walsh; Aldehyde and phosphinate analogs of glutathione and glutathionylspermidine, potent, selective binding inhibitors of *Escherichia coli*, bifunctional glutathionylspermidine synthetase/amidase, *Chem Biol.*, 4**,** 859 - 866, **1997.**

26. S. Chen and J. K Coward; Investigations on new strategies for the facile synthesis of polyfunctionalized phosphinates, phosphinopeptide analogues of glutathionylspermidine, *J. Org Chem.,* 63, 502 - 509, **1998.**

27. S. Chen, C. H. Lin, C. T. Walsh and J. K. Coward; Novel inhibitors of trypanothione biosynthesis, *Bioorg Med Chem Lett.,* 7, 505 - 510, **1997.**

28. B. Chiang, T. Chen, C. Pai, C. Chou, H. Chen, T. Ko, C. W. Hsu, W. Wu, W. Wang and C.H. Lin; Protein S-thiolation by glutathionylspermidine (gsp) the role of *Escherichia coli* gsp synthetase/amidase in redox regulation, *J. Biol Chem,* 285, 25345 - 25353, **2010.**

29. C. J. Bacchi and N. Yarlett; Polyamine metabolism, *Biochemistry and Molecular Biology of Parasites*, 119 – 131, **1995.**

30. M. Lluis, V. Karthik and G. George; Many faces of glutathione in bacteria; *Antioxid Redox Signal.,* 8, 753 - 762, **2006.**

31. D. S. Kwon, C. H. Lin, S. Chen, J. K. Coward, C. T Walsh and J. M Bollinger; Dissection of glutathionylspermidine synthetase/amidase from *Escherichia coli* into autonomously folding and functional synthetase and amidase domains, *J. Biol Chem.*, 272, 2429 - 2436, **1997.**

32. *Escherichia coli* K-12 substr MG1655, http://biocyc.org/ECOLI/NEWIMAGE?type=GENE&object=EG11165

33. G. Dhamdhere, G. Krishnamoorthy and H. I. Zgurskaya; Interplay between drug efflux and antioxidants in *Escherichia coli* resistance to antibiotics, *Antimicrob Agents Chemother.,* 54, 5366 - 5368, **2010.**

34. S. B. Farr, D. Richard and T. Danieli: Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase, *PNAS*, 83, 8268 - 8272, **1986.**

35. F. W. Studier and B. A. Moffat: Use of bacteriophage T7 RNA Polymerase to direct selective high-level expression of cloned genes, *J. Mol. Biol.,* 189,113-130, **1986.**

36. G Storz, L. A. Tartaglia, S. B. Farr and B. N. Ames: Bacterial defences against oxidative stress, *Trends Genet.,* 6, 363 – 368, **1990.**

37. B. Miroux and J. E. Walker: Over-production of proteins in *Escherichia coli*, mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels, *J. Mol Biol*., 260, 289 – 298, **1996.**