*Sindh Univ. Res. Jour. (Sci. Ser.) Vol.44 (2) 171-176 (2012)*

**SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)**

# Molecular cloning, expression and purification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH-1) of *Neisseria meningitidis*

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Received 27th January 2012 and Revised 26nd March 2012)

**Abstract:** Glyceraldehyde 3-phosphate dehydrogenases (GAPDHs) are classical cytoplasmic glycolytic enzymes, which despite lacking predicted export signals, have also been shown to be expressed to the surface of a number of bacteria (some eukaryotic organisms), and shown to play additional roles in pathogesis of these organisms. *N. meningitidis* is an obligate human nasopharyngeal commensal which can cause life-threatening infections including septicaemia and meningitis. *N. meningitidis* genome possess two genes (GapA-1 and GapA-2) encoding GAPDH enzymes. This study was aimed to characterize GapA-1 of *Neisseria meningitidis.* Gene encoding GapA-1 was cloned, over-expressed, and purified to near homogeneity. In summary, GapA-1 was shown to be expressed and purified as a full-length protein

**Keywords:** *Neisseria meningitidis*, GAPDH, cloning, over-expression, purification

## 1. INTRODUCTION

Glycolytic enzymes, which along with other enzymes involved in central metabolism, are sometimes called 'housekeeping enzymes' are classical cytoplasmic proteins that are found in all cell types. These enzymes are known to be constitutively expressed and localised mainly to the cytoplasm of the cell. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a classical cytoplasmic glycolytic enzyme which catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate (Kim and Dang, 2005). A number of recent studies have demonstrated that in addition to its central role in metabolic function, GAPDH has also been found at unexpected locations in the cell performing non-glycoltic biological roles in pathogenesis of the organisms (Kim and Dang, 2005; Sirover, 1999). There is growing evidence that several so-called housekeeping enzymes including fructose bisphosphate aldolase (FBA) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) are often exported to the surface of bacterial pathogens. Whilst their intracellular functions are well known, it is unclear whether they perform any additional functions on the bacterial surface.

The expression of GAPDH on the surface of a pathogen was first reported in *S. pyogenes*, a Gram-positive pathogen, (Lottenberg *et al.*, 1992; Pancholi 2003). Later on, GAPDH was shown to be exported to the surface of other Gram-positive bacteria such as staphylococci (Modun and Williams, 1999; Modun *et al.*, 2000, *agalactiae*, Seifert *et al.*, 2003) and *Listeria monocytogenes* (Schaumburg *et al.*, 2004). Furthermore, surface exposed GAPDH of enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* has been demonstrated to bind to human plasminogen and fibrinogen, suggesting a possible role in the pathogenesis of these bacteria (Egea *et al.*, 2007). The surface-exposed GAPDH of *Mycoplasma genitalium* has also been shown to play important in adhesion to human mucin (Alvarez *et al.*, 2003), and GAPDH of *Lactobacillus plantarum* has been reported to be involved in adherence to gastric mucin and Caco-2 cells (Kinoshita *et al.*, 2008; Ramiah *et al.*, 2008).

Besides bacteria, GAPDH has been shown to be expressed on the cell surface of Fungi, such as, the surface exposed GAPDH of *Candida albicans* and is shown to be involved in mediating adhesion to fibronectin, laminin and plasminogen (Gil-Navarro *et al.*, 1997; Gozalbo *et al.*, 1998; Jonathan *et al.*, 2003). GAPDH has also been found

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on the surface of *Trichomonas vaginalis* (the single-celled protozoan) and is shown to bind extracellular matrix components, including fibronectin (Lama *et al.*, 2009). Therefore, GAPDH is a moonlighting protein expressed on the surface of several Gram-positive pathogens and fungi, which performs various roles unrelated to the glycolysis cycle, thus, may act as virulence factor (Egea *et al.*, 2007). The aim of this study was to clone, overexpress and purify the rGAPDH of *N. meningitidis* to facilitate identification of the additional roles in pathogenesis and subsequently in vaccine production against the meningococcal disease.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial strains, growth condition, and media

Bacterial strains and plasmids used in this study are listed in (Table 1). *E. coli* strains (TOP10F′ and BL21 (DE3) pLysS) were grown at 37°C in L B broth or on LB agar. Where appropriate, antibiotics were used at the following concentrations: ampicillin (100 µg $ml^{-1}$), kanamycin (30 µg $ml^{-1}$). Meningococcal strains (Table 1) were grown on Brain Heart Infusion (BHI) agar and/or Brain Heart Infusion Broth or Muller-Hinton Agar (MHA) or Muller-Hinton Broth (MHB) or Dulbecco's Modified Eagle Medium (DMEM) supplemented with Vitox (Oxoid) at the concentration suggested by the manufacturer (Oxoid) or 2% Fetal calf serum (Invitrogen) and incubated in an atmosphere of 5% $CO_2$ at 37 °C with or without shaking.

### 2.2 DNA manipulation

Chromosomal DNA of *N. meningitidis* was extracted by using a DNeasy Blood & Tissue kit (Qiagen) using the protocol for bacterial cells recommended by the manufacturer. The plasmid purification was carried out from 5 ml overnight cultures using a QIAprep Spin Kit (Qiagen) according to the manufacturer's instructions. *Taq* polymerase, Restriction enzymes and T4 DNA ligase were purchased from Roche. All enzymatic reactions were carried out according to the manufacturer's instructions. The concentration of purified plasmid and genomic DNA was quantified using a NanoDrop (ND-1000) spectrophotometer (Agilent Technologies). PCR products/ DNA constructs were sequenced in both directions using specific primers at the School of Biomedical Sciences (University of Nottingham) on an ABI 377 automated DNA sequencer.

### 2.3 Polymerase chain reaction (PCR) and agarose gel analysis

All PCR reactions were performed in a 25 µl final volume using sterile 0.2 ml thin-walled PCR tubes and cycled using a C1000 model Thermal Cycler (BIO-RAD). An agarose gel (0.8-1%) was prepared according to the standard protocol. DNA ladders 2-Log (New England BioLabs) and 0.5 kb (Fermentas) were run alongside the samples to enable analysis of DNA fragment size in the samples. Gels were viewed using an Uvitec gel documentation system.

### 2.4 Construction of pSAT-5 expression vector

A 1,032-bp DNA fragment corresponding to the GapA-1 coding sequences was amplified by PCR using genomic DNA of *N. meningitidis* strain MC58 and the primer pair GapA1_E1 5'CGC**GGATCC**ATGGGCATCAAAGTCGCCATC-3' and GapA-1_E2 5'-CGC**GTCGAC** TTATTT GAGCGGGCGCACTTC-3'. The bold sequences identify the restricticton sites of *Bam*HI and *Sal*I. The DNA fragment was purified and ligated to the pCR-T7/NT-TOPO expression vector (Invitrogen) according to the manufacturer's instructions. Screening for positive clones/transformants was performed by colony PCR (Sheu *et al*., 2000) amplification of the desired DNA fragment and DNA sequencing. A plasmid construct containing correctly oriented GapA-1 was chosen and used to transform *E. coli* BL21 (DE3) pLysS according to the manufacturer's instructions. The pCR-T7/NT-TOPO - based constructs drive expression of the recombinant protein with N-terminal 6-histidine tag under the control of phage T7 promoter.

### 2.5 Purification of rGAPDH-1 under denaturing conditions

Protein purification was achieved by re-suspending the *E. coli* culture pellet obtained from 20 ml culture in 1 ml lysis buffer B (100 mM $NaH_2PO_4$, 10 mM Tris-Cl, and 8 M Urea, pH 8.0) and sonicated (20 s pulses for 5 min on ice) using a (SoniPrep sonicater MSE 150) to disrupt the cells. The lysate from disrupted cells was centrifuged at 8,000 × *g* for 30 min at 4°C. The supernatant was collected in a sterile Eppendorf tube for purification of the recombinant protein. Purification was carried out using a Ni-NTA spin kit (Qiagen). Firstly, Ni-NTA spin columns were equilibrated with 600 µl of buffer B, and centrifuged at 700 × *g* for 2 min to drain the buffer and then 600 µl of supernatant collected from the lysate of disrupted cells was loaded into pre-equilibrated columns and centrifuged at 700 × *g* for 2 min. Secondly, the spin columns were washed twice with approximately 600 µl of buffer C (100 mM $NaH_2PO_4$, 10 mM Tris-Cl, and 8 M Urea, pH 6.3). The third step was the elution of pure proteins bound

to spin column with buffer E (100 mM $NaH_2PO_4$, 10 mM Tris-Cl, and 8 M Urea, pH 4.5). The protein elutes were stored at -20°C.

**2.6 Protein purification by gel electro-elution**

The partially purified samples of rGAPDH-1 were resolved in a single lane on 10% SDS-PAGE. The gel was stained briefly with Simply Blue SafeStain (Invitrogen). The band corresponding to the recombinant protein of interest was identified according to its size estimated by comparing with the molecular weight markers. The protein band with an apparent molecular mass of *ca.* 37-kDa demonstrating rGAPDH-1 was excised out from gel and further cut into small slices. For electro-elution, the gel slices were transferred to a D-tube (D-Tube Dialyzer midi, MWCO 6-8 kDa, Novagen) containing filter sterile 1×SDS-running buffer and electro-elution was performed at constant voltage of 110 volts for 2 h in a agarose gel casting tank filled with 1 × SDS running buffer. The eluted protein was aspirated from the D-Tubes and dialysed in clean D-tubes against PBS for 48 h at 4°C and then concentrated using centrifugal filter device (Microcon, Amicon Bioseparation, 5,000Da MWCO).

**2.7 Raising a rabbit polyclonal antiserum against purified rGAPDH**

The work was done according to the Guidance on the Operation of the Animals (Scientific Procedures) act of 1986 Rabbit polyclonal antibodies against the denatured purified recombinant GAPDH-1 protein were raised in New Zealand white female rabbits. Before injecting purified proteins, blood sample was taken to collect pre-immune serum. Typically, rabbits were immunized three times at 2-week intervals with 30 µg of protein emulsified in Freud's Complete (first immunization) or incomplete adjuvant. After two doses, the rabbits were test bled, boosted once more, and sacrificed 10 days later.

**2.8 SDS-PAGE and Western blot analysis**

The recombinant proteins were electrophretically separated by 10% SDS-PAGE using Mini- Protean III equipment (BIO-RAD) following the method of Laemmli (Laemmli, 1970). Proteins separated by SDS-PAGE were visualized by staining with SimplyBlue SafeStain (Invitrogen) or PageBlue$^{TM}$ Protein Stain Solution (Fermentas) according to the manufacturer's instructions. Gels were scanned using a GS-800 calibrated densitometer (BIO-RAD). For western blot analysis, the protein mixtures separated by 10% SDS-PAGE were transferred onto a nitrocellulose membrane (Amersham Biosciences) in semi-dry blotting buffer (5.82 g Tris base, 2.93 g Glycine, 3.75ml 10% SDS, 200 ml methanol) using a Trans-Blot SD semidry transfer cell (BIO-RAD) at a constant currant of 14 mA for approximately 30 min. The membranes were incubated in 1% Bovine serum albumin (BSA) (Sigma) for 30 min. The membranes were then probed with mouse anti-pentahistidine antibody (Qiagen) or rabbit primary antibody diluted 1:10,000 and 1:1000, respectively, in blocking buffer (5% [wt/vol] non fat dry milk, 0.1% [vol/vol ] Tween 20 in 1 × phosphate-buffered saline PBS) and incubated overnight with shaking at 4°C. The following day, the membrane was washed three times 15 min each with PBS containing 0.05% Tween-20 (PBS-Tween), and incubated into secondary antibody (anti-mouse IgG conjugated to alkaline-phosphtase or anti-rabbit conjugated to alkaline phosphtase) (Sigma) at the concentration of 1:30,000 blocking buffer for 2 h at room temperature. After 2 h the membrane was washed thrice with PBS-Tween for 15 min each time. The membrane was then developed using the BCIP (5-bromo-4-chloro-3-indolylphosphate) – Nitro Blue Tetrazolium liquid substrate (PerkinElmer$^{TM}$). The membrane was finally washed with $dH_2O$ and a digital image of the membrane was taken using a GS-800 calibrated densitometer (BIO-RAD).

**Table 1. Bacterial strains and plasmids used in this study**

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| ***E. coli* strains** | | |
| JM109 | *end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 ($r_K^-r_K^-$) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F′ *tra*D36 *pro*AB *laq*I$^q$ZΔM15] | Promega |
| TOP10F′ | F′*lac*Iq*Tn*10(TetR) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G | Invitrogen |
| BL21(DE3)pLysS | F- *ompT hsd*SB (rB-mB-) *gal dcm* (DE3) pLysS (CamR) | Invitrogen |
| **Plasmids** | | |
| pCR-T7/NT-TOPO | Expression vector encoding resistance to ampicillin | Invitrogen |
| pSAT-5 | MC58 GapA1 gene cloned in pCR-T7/NT-TOPO | This study |

## 3. RESULTS AND DISCUSSION

### 3.1 Cloning of GapA-1 of *N. meningitidis* strain MC58.

The GapA-1 gene consisting of 1,032 bp, (which encodes a protein of a 344 amino acids) was amplified by PCR (Fig 1), purified and then ligated with the pCR-T7/NT-TOPO expression vector followed by transformation of *E. coli* TOP10F′ cells for plasmid propagation and purification. The screening for the successful clones was attempted by colony PCR and restriction digestion. A number of clones were subjected to restriction digestion analysis. The restriction sites *BamH*I and *Sal*I (present in GapA1_E1 and GapA1_E2 primers respectively) were used to confirm the ligation of the desired insert. In order to to ascertain the orientation of GapA-1, the plasmid DNA was sequenced. The plasmid containing GapA-1 in correct orientation was designated as pSAT-5 and used for expression of rGAPDH-1 protein.

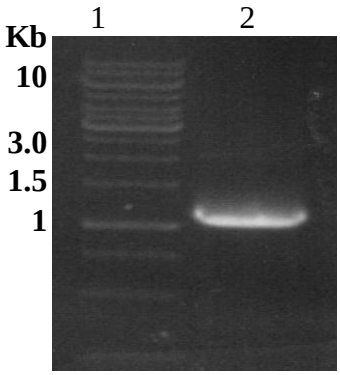



**Fig. 1. Agarose gel analysis demonstrating the PCR amplification of *gapA-1*. Lane 1, DNA markers, lane 2, PCR product of *gapA-1*.**

### 3.2 Expression and purification of rGAPDH

In order to express N-terminally 6 × His-tagged recombinant proteins, overnight culture of *E. coli* BL21 (DE3) harboring pSAT-5 were grown to mid log phase followed by induction with 1mM IPTG and further grown for 3 h. Samples were taken from induced and un-induced cultures at an hourly interval to monitor the optimal expression. Pre-induced and post-induced samples (cell **lysates**) were then analysed by SDS-polyacrylamide gel including negative control sample (*E. coli* BL21 (DE3) pLysS containing pCRT7/NT-TOPO vector without insert, which was treated under same expression conditions). A protein band of apparent molecular weight of *ca.* 37-kDa demonstrating rGAPDH-1 was observed in induced samples on SDS- PAGE **(Fig 2A)**. The resolved protein samples were subsequently transferred onto nitrocellulose membrane followed by probing with anti-penta antibodies. A strong immune-reactive band of predicted molecular mass of ca. 37-kDa corresponding to rGAPDH was detected by western blot analysis **(Fig 2B).** and also a weak band of the same size was present in un-induced samples suggesting the leaky expression as this protein band was absent in the negative control

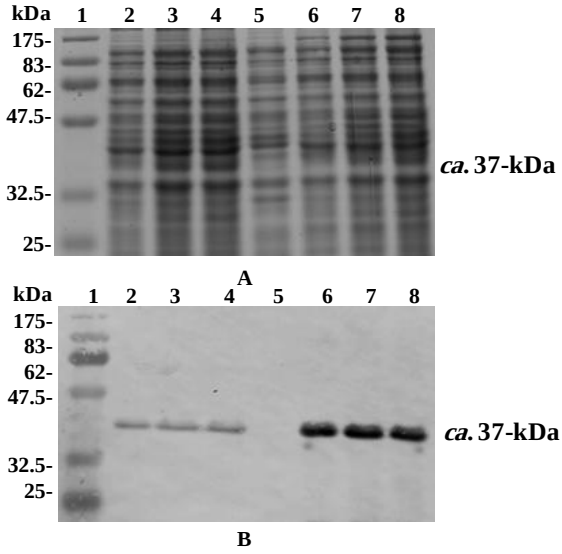



**Fig. 2. (A) SDS-PAGE (10%) analysis showing the expression of GAPDH-1 in induced and un-induced samples, Lane 1, protein markers, lane 2-4, un-induced 0-3 h sample, respectively, lane 5, control (*E. coli* harboring empty vector), and lane 6-8, induced 0-3 h sample, respectively. (B) Immunoblot analysis confirming expression of GAPDH-1in induced and un-induced samples, Lane 1, protein markers, lane 2- 4, un-induced whole cell proteins, lane 6, control (*E. coli* containing empty vector), lanes 6-8 whole cell proteins from induced samples.**

### 3.3 Determining the solubility of rGAPDH

In order to purify the recombinant protein under native condition, the protein should be in the soluble form. The solubility of the expressed rGAPDH was, therefore, determined by re-suspending the cell pellet in PBS. Following 30 min incubation at room temperature, cells were disrupted by sonication and centrifuged to separate soluble and insoluble fractions. Both fractions were collected separately and analysed by SDS-PAGE followed by immunoblot analysis using anti-penta histidine antibodies together with a positive control protein (Fig. 3). A strong/thick protein band corresponding to rGAPDH was detected in insoluble (pellet) fraction whereas a weak band was present in

soluble (supernatant) fraction of expressed protein suggesting that the rGAPDH protein was expressed largely as insoluble protein.

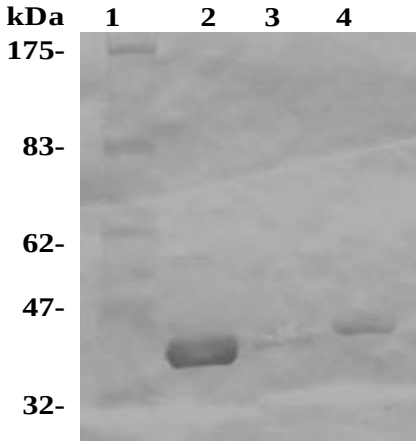



**Fig. 3. Immunoblot analysis showing solubility of the rGAPDH. Protein were resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. Lane 1, protein markers; lane 2, pellet (insoluble fraction; inclusion bodies); lane 3, supernatant fraction lane 4, rFBA protein as a positive control.**

### 3.4 Purification of rGAPDH-1 by D-tube Dialyzer

Following purification of the rGAPDH-1 by using metal affinity chromatography, the purified rGAPDH-1 was resolved on SDS-PAGE to confirm the purity and expected size based on predicted molecular weight. A *ca*.37-kDa band corresponding to rGAPDH was observed together with several additional protein bands, which were assumed to be non-specific co-purified contaminating proteins. Therefore, in order to obtain a purified rGAPDH-1 for raising polyclonal antiserum, the partially purified elutes containing rGAPDH-1were again resolved on SDS-PAGE and the protein band corresponding to GAPDH-1 was excised from the SDS-polyacrylamide gel and the gel pieces were used for purification by electro-eluation using D-tube dialyzers. The purified protein was analysed by SDS-PAGE and western blot analysis. One gel was stained with SimplyBlue SafeStain and subsequently destained with $dH_2O$ whilst another gel containing the same profile of proteins was used to transfer the resolved protein onto the nitrocellulose membrane for probing with either anti-penta histidine and/or Rα-GAPDH-1 antibodies. The SDS-PAGE demonstrated a single band with an apparent molecular weight of 37-kDa corresponding to rGAPDH-1. The western blot analysis demonstrated that Rα-GAPDH-1 (Fig 4A) anti-penta histidine (Fig. 4B) antibodies recognised the purified protein as rGAPDH.

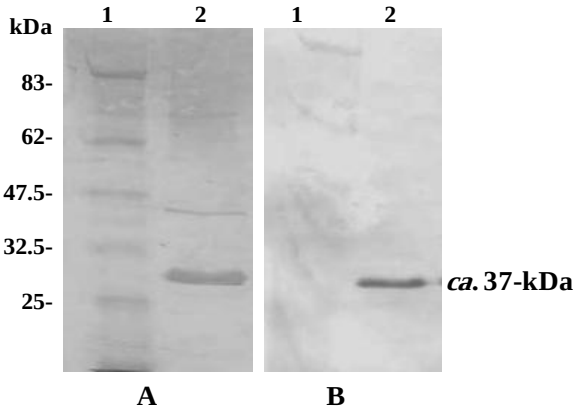



**Fig. 4. A) Western blot analysis demonstrating purity of rGAPDH Lane 1, Pre-stained protein markers, lane 2, purified rGAPDH probed with Rα-GAPDH-1 B) Lane 1, pre-stained proteinmarkers, lane 2, rGAPDH-1 probed with anti- pentahistidine antibodies.**

## 4. CONCLUSION

The present study describes the cloning of the GapA-1 gene of *N. meningitidis* strain MC58 and the expression and purification of rGAPDH protein for determining its additional roles in pathogenesis of meningococcal disease. The choice of gene for investigation in this study was based on the premise that glycolytic enzymes are often reported to be exported to the surface of a variety of bacterial and fungal pathogens and shown to have potential roles in adhesion to various host molecules. It was hypothesized that similar to glycolytic enzymes from other pathogens, meningococcal GAPDH may be a multi-functional protein, which performs additional roles unconnected to glycolysis in *N. meningitidis*.

In summary, the present study demonstrates the cloning of the GAPDH-1 enzyme and subsequent expression and purification of recombinant protein under denaturing conditions. Polyclonal antiserum also raised in rabbit against denatured rGAPDH-1 which was shown to react against the cognate purified proteins in immunoblot experiments.

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