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# Cloning, expression and purification of alkylhydroperoxidase C, a crucial respiratory antioxidant in *Brucella abortus*

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During infection, *Brucellae* face extensive oxidative pressure inside host macrophages in the form of oxidative burst and reactive oxygen species (ROS). Alkylhydroperoxidase C (AhpC) is the primary scavenger of these ROS in *Brucella* and alkylhydroperoxidase D (AhpD) helps it to regain its reduced state after the catalytic reaction. In this study, we attempted to crystallize the AhpC protein from *B. abortus*. Extensive crystallization attempts were carried out but crystals could not be obtained. *B. abortus AhpC* (*BaAhpC*) gene was cloned and expressed in *Escherichia coli*, followed by protein purification using affinity and gel filtration chromatography. BaAhpC was found to be a dimer in solution. Protein sequence alignment analysis shows that BaAhpC share about 50% identity with *Mycobacterium tuberculosis* AhpC (MtbAhpC) protein and has 3 conserved cysteine residues which are directly involved in catalytic activity. The homology model of BaAhpC structure was prepared using MtbAhpC as a template which show it may exist in the form a dodecamer.

**Keywords:** Alkylhydroperoxidase C, *Brucella abortus*, *BaAhpC* gene cloning

## Introduction

Brucellosis is a highly contagious zoonosis which mainly affects cattle but can also infect humans through animal carcass, infected milk and exposure in slaughter house. It is also known as the undulant fever or malta fever. It is caused by a Gram negative bacterium of *Brucella* sp. *Brucellae* are non-capsulated, non-motile and facultative intracellular coccobacilli. Brucellosis occurrence in traditional livestock husbandry practice makes the knowledge about the disease considerably important in reducing the economic and public health impacts of the disease[1].

*Brucella* species is susceptible to reactive oxygen species (ROS) generated during the oxidative burst in host macrophages. Once *Brucella* enters the host macrophages, it triggers an oxidative burst. When macrophages are activated by tumour necrosis factor α (TNF-α) or interferon γ (IFN-γ), they diffuse molecular oxygen from cytosol to phagosome via NADPH oxidase (phagocyte oxidase complex). This complex releases the reactive oxygen species ($O^{2-}$) into the phagosomal lumen by transferring electron from NADPH to oxygen[2-3]. The ROS i.e. $O^{2-}$, can react with nitric oxide to form peroxynitrile ($ONOO^-$) or can accept protons to form hydrogen peroxide ($H_2O_2$)[4-8]. Hydrogen peroxide is the most favourable species that is formed by $O^{2-}$. Thus, reactive oxygen $O^{2-}$ is considered lethal due to its formation of downstream oxidizing agents. *Brucella* has another way of coming across ROS i.e. through aerobic metabolism. NADH dehydrogenase complex is the primary producer of $H_2O_2$ in *Brucella* during aerobic respiration[9-10]. There are five antioxidative enzymes produced by *Brucella* that include: catalase, alkylhydroperoxide reductase, bacterioferritin comigratory protein, thiol peroxidase and rubrerythrin.

Alkylhydroperoxide reductase C (AhpC) is a peroxiredoxin enzyme that catalyzes the reduction of hydrogen peroxide to hydrogen and water. They are not dependent on co-factors like $Fe^{2+}$ or $Mn^{2+}$, instead they have cysteine residues for reducing the peroxide molecules[11-12]. This gene is ubiquitously present in yeast, plant cells, mammalian cells, archae and bacteria[13-14]. Steele *et al*[15] showed that AhpC is the primary antioxidant used to detoxify $H_2O_2$ produced endogenously during respiratory metabolism by *B. abortus*[15]. There are two genes in *B. abortus* 2308 genome that encode for alkyl hydroxyperoxidase complex namely BAB1_0531 and BAB1_0532 (AhpCD). Iron responsive regulator protein (Irr) is highly upregulated in *Brucella* containing vacuole which is formed when *Brucella* invades macrophages

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wherein the expression of AhpCD in response to $H_2O_2$ is upregulated as well. Irr has been speculated to be the regulator responsible for enhancing the expression of AhpC in macrophages[16-17]. Another regulator, OxyR family is conserved among prokaryotes and plays an extensive role in regulation of genes pertaining to hydrogen peroxide levels. *oxyR* genes have been shown to possess overlapping promoters with other genes wherein all the genes shared are different. OxyR has a conserved function of protecting the cell from external lethal levels of hydrogen peroxide even though the genes regulated are different[18-20].

The structure of *B. abortus* AhpC (BaAhpC) is still unknown. Since it is the major anti-oxidant enzyme present in the *Brucella*, it could be exploited as a potential target for inhibitor study. Detailed three dimensional structure of this protein and its active site will provide a better picture for further studies. Therefore, we attempted to crystallize the *Brucella* AhpC protein. This study illustrates the cloning of *BaAhpC* gene into an expression vector and transformation of cloned vector in the *E. coli* expression system. Optimization of over-expression of recombinant BaAhpC protein in *E. coli* was done. The recombinant AhpC protein was purified after induction from *E. coli* BL21 cells, *via* Ni-NTA affinity chromatography and analysed for polymeric states by gel permeation chromatography. Though extensive crystallization trials were performed; crystals of recombinant BaAhpC protein were not obtained. Alternatively, we did the homology modelling of BaAhpC and analysed its active site.

## Materials and Methods

### Cloning of *Brucella abortus AhpC* Gene

The gene sequence of the *B. abortus AhpC* was identified and retrieved from National Center for Biotechnology Information (NCBI) database and Accesion no is CP000888.1. Forward primer with sequence 5'CTAGCTAGCATGCTCGGCATCGGC GACAAG3' and reverse primer 5'CCGCTCGAGGG CTGCCTTGAGCGTCTCACCAC3' containing the restriction sites of *Nhe*I and *Xho*I respectively, were obtained from Sigma (Sigma, USA). PCR was carried out using genomic DNA of *B. abortus* S19 as template. Gene specific primers were added to the mixture and extension was carried out at 72°C with Phusion DNA polymerase for 30 cycles with varying concentration of $MgCl_2$ (0.1 mM to 1 mM). The amplified PCR product was visualized on 0.8% agarose gel. Thereafter, *BaAhpC* gene PCR product and pET21c vector (Novagen) were subjected to double digestion by *Nhe*I and *Xho*I restriction enzymes at 37°C for 3 hrs. The digested vector was run on 1% agarose gel and the required fragments were eluted using gel extraction kit while digested PCR product was purified using PCR purification kit. The digested pET21c vector and *BaAhpC* gene product were taken in molar ratio 1:4 and ligated with the help of T4 DNA ligase in a reaction mix containing ligation buffer in final concentration of 1X. The ligation was carried out at 16°C for 16 hrs.

### Transformation and Clone Confirmation

The ligated product was mixed with 10 µL thawed DH5α cells (Novagen) and incubated on ice for 5 min. This mixture was then subjected to heat shock at 42°C for 90 s. Then, 1 mL of Luria Bertani (LB) media was added to the tube containing the mixture and kept for 1 hr at 37°C in the incubator shaker. The mixture was then spread evenly into an ampicillin (100 µg/mL) supplemented LB agar plate and kept overnight at 37°C. Positive colonies were initially identified with colony PCR using previously established method. Single colonies from overnight grown plates were picked using a sterile pipette tip and were first streaked onto ampicillin containing LB agar plate (master plate). The same tip was then dipped into 25 µL chilled master mix prepared for PCR. The PCR was carried out for 30 cycles using this mixture keeping the parameters similar as mentioned above. The PCR product was analysed on 1% agarose gel. Positive colonies as identified by colony PCR were inoculated in 10 mL of LB broth supplemented with 100 µg/mL ampicillin and allowed to grow on 37°C for overnight. The overnight grown culture was then used for plasmid extraction using the alkaline lysis method. The purified plasmid was then subjected to restriction digestion with *Nhe*I and *Xho*I enzymes similar to the method discussed previously. The digested plasmid was visualized on 0.8% agarose gel for confirmation of clone.

### Expression and Purification of the Protein

The general physiochemical parameters of the BaAhpC protein were computed using ProtParam tool of Swiss Institute of Bioinformatics (SIB) ExPASy bioformatics resources portal[21]. For, expression of the *AhpC* gene, the recombinant pET21c-BaAhpC plasmid was transformed into the *E. coli* BL21 (DE3) cells (Novagen). The transformed cells were plated on

ampicillin containing LB agar plates and incubated overnight at 37°C. For primary culture, a single colony of pET21c-BaAhpC plasmid containing BL21 cells was inoculated in a test tube containing 10 mL LB media and ampicillin. The culture was allowed to grow overnight at 37°C with continuous shaking. Later, 4 conical flasks containing 50 mL LB broth each were inoculated with 0.5 mL overnight grown culture and kept on incubator shaker at 37°C for 3 hrs. After the culture reached the $OD_{600}$ of ~0.5, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added in 3 conical flasks in varying final concentrations of 0.5 mM, 1.0 mM and 2.0 mM while one was kept uninduced. The culture was again allowed to grow at same temperature for 4 hrs. After that, 0.5 mL of culture was aliquoted from each flask into 2 mL microcentrifuge tube and 1% sodium dodecyl sulphate (SDS) was added directly into the tube. The culture was allowed to lyse and the lysate was then loaded onto the SDS-polyacrylamide gel electrophoresis (PAGE) to check for optimum concentration of IPTG required for induction.

After the optimization of IPTG concentration and preliminary checking of over expression of BaAhpC protein, large scale culture was grown by inoculating 1% of primary culture in 1000 mL LB broth with 100 μg/mL ampicillin as selectable marker. Media was grown at 37°C for 4 hrs till the $OD_{600}$ reached ~0.5. IPTG was added to a final working concentration of 0.5 mM in the culture and was further allowed to grow at 37°C for 4 hrs.

**Protein Extraction and Purification**

The induced culture was transferred to 500 mL GSA tubes and centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the cell pellet was suspended in lysis buffer containing 50 mM Tris HCl pH 8.0, EDTA 0.5 mM, β-mercaptoethanol 5 mM and 0.1% triton X-100. The re-suspended cells were subjected to 3 cycles of freeze and thaw in liquid nitrogen and water bath at 37°C, respectively. After freeze-thaw, sonication was done to completely lyse the cells using a sonicator equipped with a microtip for 3.0 min. The cell lysate obtained after sonication was centrifuged at 12,000 rpm for 10 min at 4°C. Pellet was discarded and supernatant was carefully collected for protein purification. Supernatant was passed through the nickel-nitrilotriacetic acid (Ni-NTA) sepharose beads (GE healthcare) packed in glass column (Sigma) pre-equilibrated with binding buffer (50 mM Tris HCl pH 8.0, 0.5 mM EDTA and 5 mM β-mercaptoethanol). The column was then washed twice with wash buffers 1 and 2 containing 5 mM and 25 mM imdiazole, respectively. Finally, the protein fractions were eluted using elution buffer (50 mM tris HCl pH 8.0, 0.5 mM EDTA and 5 mM β-mercaptoethanol) containing 250 mM imidazole. The eluted fractions obtained were then checked on 12% SDS-PAGE. The protein fractions that contained sufficiently pure proteins were concentrated in Amicon centricons (Millipore) with exclusion limit of 10 kDa and finally subjected to gel filtration chromatography.

**Gel Filtration Chromatography**

Gel filtration chromatography was performed on a HiLoad Superdex 75G 16/60 column (GE Healthcare) fixed on Akta purifier at a flow rate of 0.5 mL $min^{-1}$. Before the sample run the column was calibrated using standard globular protein markers blue dextran (2000 kDa), bovine serum albumin (BSA) (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (23.2 kDa) and RNaseE (13.7 kDa). The column was then pre-equilibrated with the buffer containing 50 mM Tris HCl pH 8.0, 1 mM EDTA, 10 mM β-mercaptoethanol and 5% glycerol following which the concentrated BaAhpC protein was injected into the column. The peak fractions were collected manually in 1 mL aliquots. The elution peak volume obtained for BaAhpC protein was plotted on the standard curve and the corresponding molecular weight was calculated. The purity of the purified protein was assessed on SDS-PAGE. The collected aliquots were then concentrated using Amicon centricon tubes of 10 kDa cutoff and the protein was stored for further crystallization trials.

**Crystallization Trials**

Purified and concentrated BaAhpC protein was subjected to extensive crystallization trials. Crystallization was setup using the Mosquito™ automated robot (TTP Labtech) in 96 well plates using the index screen, crystal screens (Hampton Research, USA) and morpheus screens (Molecular Dimensions, UK) mixing the 1.0 μL of protein with 1.0 μL buffer solution. The plates were stored at both 4°C and 16°C incubator and monitored at regular intervals.

**Modelling and Structural Analysis**

The BaAhpC protein sequence (Accession no ACD73998.1) was subjected to homology modelling in Swiss model[22] using the crystal structure of *M. tuberculosis* AhpC protein (PDB id 2BMX)[23]. The

modelled structure was verified using the Procheck software[24]. The structures were superposed over one another using PyMol software (Schrödinger, LLC) and the active site residues were also traced.

## Results

### Protein Sequence Alignment of *Brucella* AhpC

AhpC is a conserved alkylhydroperoxide reductase that is encoded by an open reading frame (ORF) with 555 bp in *B. abortus* S-19 genome. Since *Brucella* encounters oxidative environment in macrophages, AhpC is one of the important proteins for its survival inside macrophages. BaAhpC is a 183 amino acid (aa) long protein with molecular weight of 20.4 kDa enzyme (Table 1). The amino acid sequence alignment with other known structures of AhpC e.g. *E. coli*, *M. tuberculosis*, *S. typhimurium* (Fig. 1a) has revealed that the *E. coli* and *S. typhimurium* have conventional 2 active site cysteine residues. However, *Brucella* AhpC has 3 conserved cysteine residues, C57, C170 and C172 similar to the *M. tuberculosis* AhpC in which all three cysteine residues C61, C174 and C176 are directly involved in the catalytic activity[25]. We also studied the amino acid sequence alignment of AhpD of *B. abortus* S19 with AhpD of *M. tuberculosis* H37Rv. Similar to AhpC, AhpD also shared more than 50% identity with its counterpart in *M. tuberculosis,* which indicate similarity in structure and function of both the proteins (Fig. 1b). No result was obtained in basic local alignment search tool (BLAST) search of *Brucella* genome with *E. coli AhpF* nucleotide sequence as query indicating absence of *AhpF* gene in *Brucella*. *M. tuberculosis* also lacks any homologous

Table 1 — *Brucella abortus* AhpC protein parameters as calculated by ProtParam.

| | |
|---|---|
| Number of amino acids | 183 |
| Molecular weight (Da) | 20398.97 |
| Theoretical pI | 5.00 |
| Ext. coefficient | 22585 |


Fig. 1 — Multiple sequence alignment of BaAhpC and AhpD. a) BaAhpC protein sequence is aligned with AhpC sequences from *E. coli*, *S. typhimurium* and *M. tuberculosis* with Clustal Omega. The conserved cysteine residues are highlighted in red. b) BaAhpD protein sequence is aligned with AhpD protein sequence from *M. tuberculosis* using Clustal Omega showing a high similarity to the latter.

*AhpF* gene and instead both these organisms have *AhpD* gene which helps in reducing the AhpC after the reaction to make it ready for another catalytic cycle. The total length of recombinant BaAhpC protein along with 6X-His tag was ~21 kDa.

**Cloning of *BaAhpC* Gene**

*BaAhpC* gene was amplified by PCR using genomic DNA as template with varying $MgCl_2$ concentrations. Analysis of PCR product on agarose gel revealed that 0.3 mM $MgCl_2$ concentration was optimum for the amplification of *AhpC* gene which was then used in subsequent PCR reactions (Fig. 2a). The double digested PCR product and pET21c vector were ligated and transformed into *E. coli* DH5α cells. Positive colonies were confirmed by colony PCR (Fig. 2b). Plasmid pET21-AhpC was isolated from these colonies and the clones were further confirmed by double digestion with *Nhe*I and *Xho*I enzymes which generated a smaller fragment of about 552 bp corresponding to the length of *BaAhpC* gene (Fig. 2c). The clones were also verified by DNA sequencing using T7 promoter specific primers and no undesired mutations were seen.

**Optimization of BaAhpC Over Expression**

To check the over expression of BaAhpC protein and optimum IPTG concentration for induction small amount of culture was induced with different IPTG concentrations and induced culture time was set at 4 hrs. The induced culture lysate were subsequently analyzed on SDS-PAGE. SDS-PAGE analysis of induced and un-induced cells revealed a distinct thick band indicating the high expression of ~21 kDa protein in all induced samples irrespective of IPTG concentration (Fig. 3a). The expression of BaAhpC was observed in soluble fraction of the induced cell lysate. For subsequent large scale cultures, 0.5 mM concentration of IPTG was used for induction.

**Recombinant protein purification**

Recombinant BaAhpC-6X-His tagged protein was purified from cell lysate of induced *E. coli* BL21 (DE3) cells. Protein purification was carried out using Ni-NTA affinity chromatography exploiting the affinity of 6X-His tag for $Ni^{2+}$ ions. The protein was eluted at a concentration of 250 mM imidazole in elution buffer. The eluted fractions from Ni-NTA column were analysed on SDS-PAGE and the 20.5 kDa BaAhpC protein was seen in all the eluted fractions along with some impurities (Fig. 3b). The BaAhpC protein was purified at a concentration of 12 mg/mL from 1 litre of induced culture. The concentration was determined by examining the OD at 260 nm.

**Gel Permeation Chromatography of Purified Recombinant *Brucella* AhpC**

The concentrated Ni-NTA fraction containing BaAhpC protein was subjected to the gel filtration chromatography on pre-equilibrated HiLoad Sepharose 75 column. The protein was eluted in two distinct peaks. First larger peak was obtained at 55 mL and second peak was obtained at 74 mL (Fig. 4a). On comparison to standard curve (Fig. 4b),

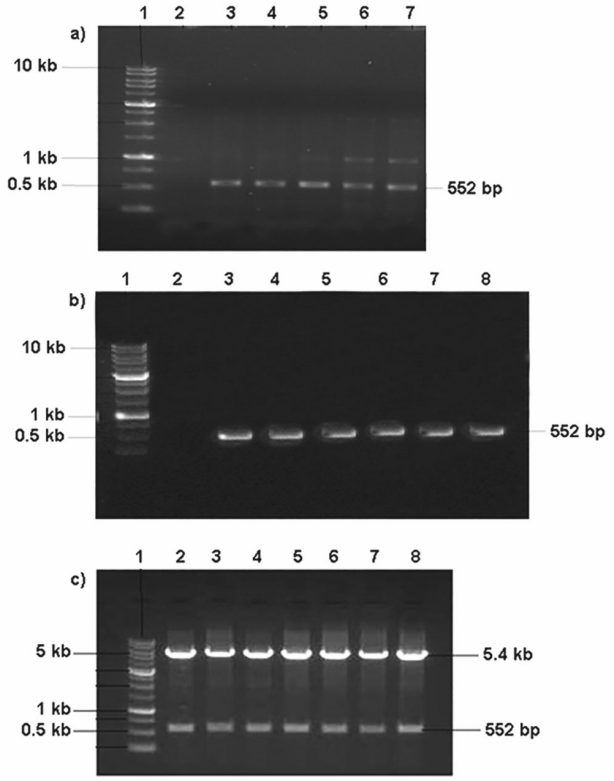


Fig. 2 — Cloning of *BaAhpC* gene. a) PCR amplification of *BaAhpC* gene with varying $MgCl_2$ concentrations. Lane 1 show 1 Kb DNA ladder, Lane 2 is negative control, lane 3-7 show amplification of 552 bp *BaAhpC* gene in increasing $MgCl_2$ concentrations viz 0.1 mM, 0.2 mM, 0.3 mM, 0.5 mM and 1.0 mM. b) Colony PCR of pET21-*BaAhpC* transformed colonies. Lane 1 show the 1 Kb DNA ladder, Lane 2 is negative control, Lane 3-8 show PCR amplification of *BaAhpC* gene from positive colonies. c) Double digestion of PET21-*BaAhpC* purified plasmid from positive colonies with *Nhe*I and *Xho*I restriction enzymes. Lane 1 show 1 Kb DNA Ladder, lane 2-8 shows double digested vector and an insert of 552 bp corresponding to *BaAhpC* gene.

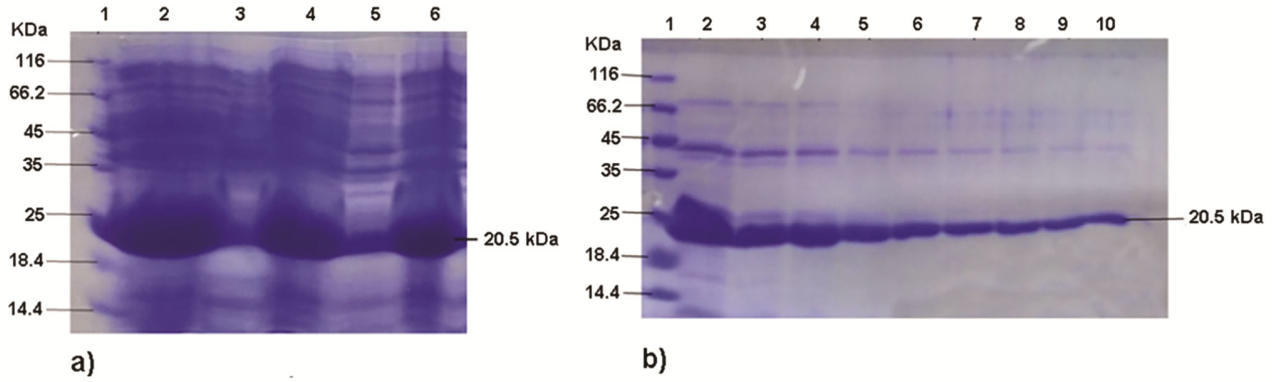


Fig. 3 — Over-expression and purification of BaAhpC protein. a) 12% SDS-PAGE showing the over-expression of BaAhpC protein in different IPTG concentrations. Lane 1 show the protein marker. Lane 2 show the over-expression at 0.5 mM IPTG, Lane 3 is un-induced, Lane 4 shows over-expression at 1.0 mM IPTG, Lane 5 is Un-induced, Lane 6 shows over-expression at 2.0 mM IPTG concentrations. b) 12% SDS-PAGE showing the Ni-NTA purification of BaAhpC protein. Lane 1 shows the protein marker and Lane 2-10 shows the Ni-NTA purified fractions.

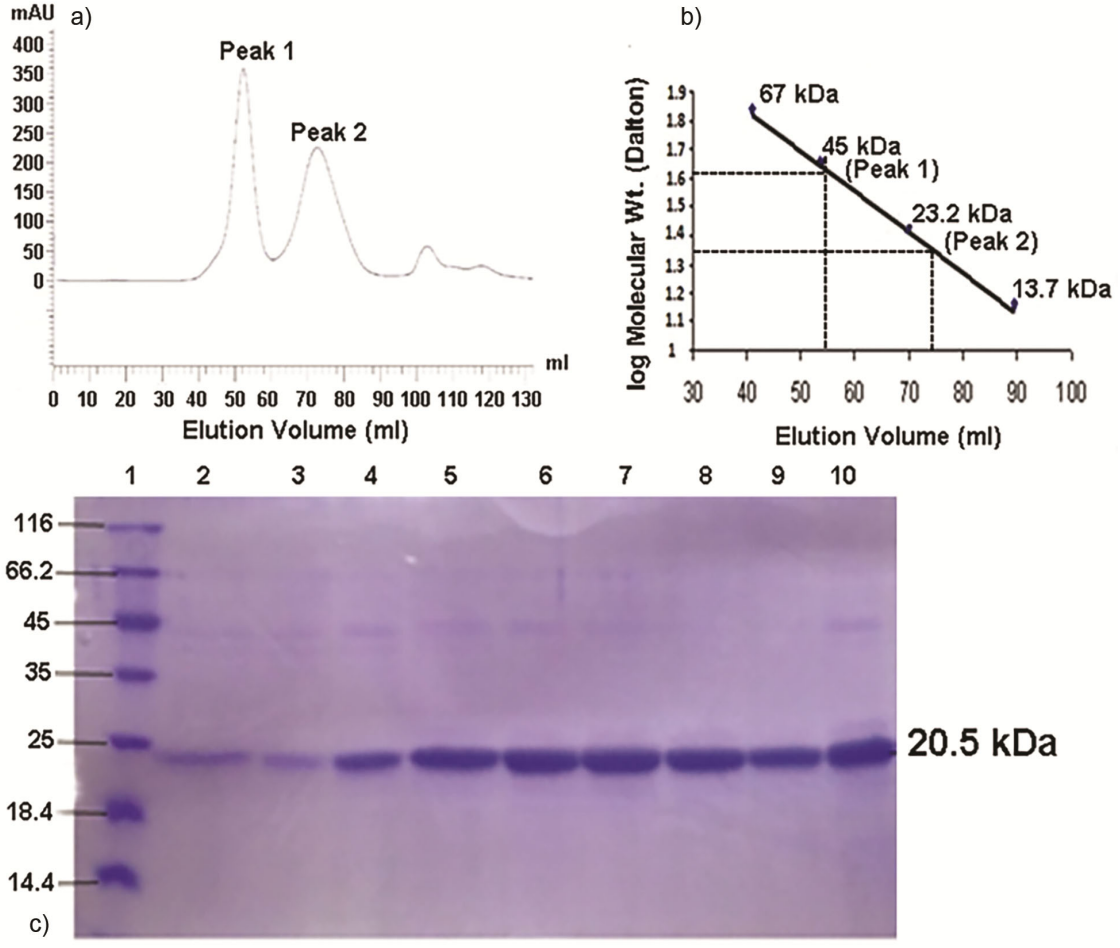


Fig. 4 — Gel Filtration profile of BaAhpC. a) Gel elution profile of BaAhpC on HiLoad Superdex 75G 16/60 column (GE Healthcare) shows two distinct peaks at 55 mL and 74 mL. b) Standard graph for HiLoad Superdex 75G 16/60 column showing the peak at 74 mL corresponding to ~20.5 kDa monomeric BaAphC protein while the peak at 55 mL corresponding to ~41 kDa dimeric BaAhpC protein. c) 12% SDS-PAGE of the elution fractions from the both the peaks show purified BaAhpC protein.

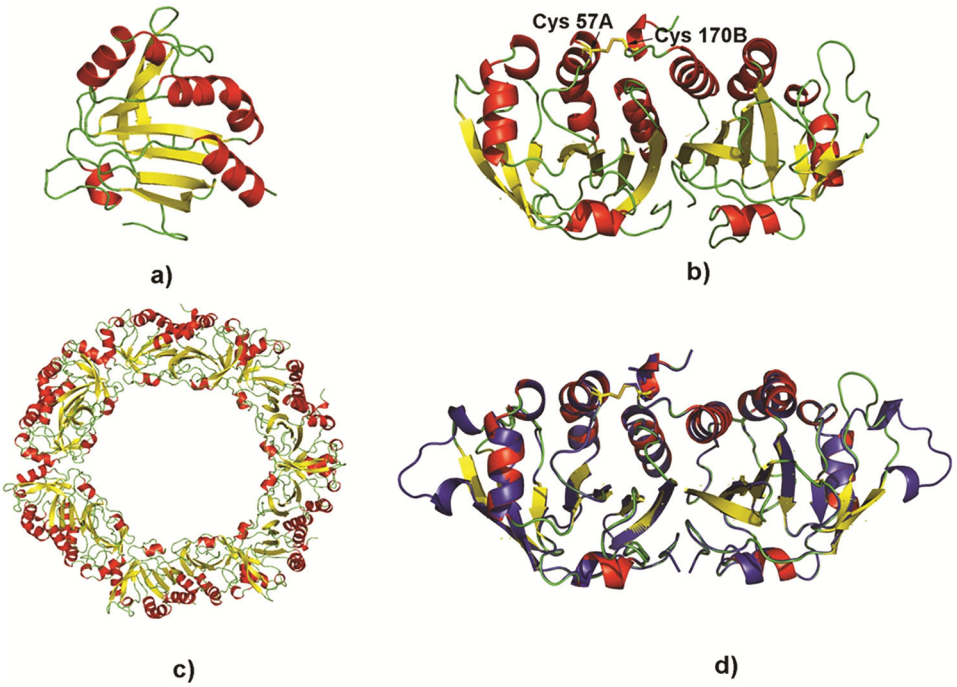


Fig. 5 — Homology modelling of BaAhpC using MtbAhpC crystal structure (PDB id 2BMX) as template, a) Monomer of BaAhpC showing the core of 7 β sheets (yellow) flanked by 5 α helices (red). b) Dimer of BaAhpC showing the disulfide bridge between Cys57 of subunit A and Cys170 of B subunit. c) Hexameric arrangement of BaAhpC. d) Structural alignment of BaAhpC (yellow and red) and MtbAhpC (blue) dimers.

it was observed that first larger peak corresponds to dimeric molecular weight (~ 41 kDa) of BaAhpC and second smaller peak corresponds to monomeric BaAhpC protein. BaAhpC is also physiologically known to form dimers. The SDS-PAGE of the eluted fractions from both peaks show almost pure BaAhpC protein (Fig. 4c). The protein was finally concentrated to about 6 mg/mL and stored for future studies.

#### Crystallization Trials

Purified BaAhpC protein at a concentration of 6 mg/mL was subjected to extensive crystallization trials using pre-formulated screens as well as manual conditions, but no crystals were observed. Majority of the drops precipitated. Drops were also kept with lower concentration of 4 mg/mL but no visible improvement was recorded. Further crystallization trials are planned.

#### Homology model of BaAhpC

Homology model of BaAhpC made using the MtbAhpC crystal structure (PDB id 2BMX) consists of 183 amino acids in one monomer. Ramachandran plot analysis of the model shows all residues in allowed regions. The monomer of BaAhpC shows highly similar structural features as that of MtbAhpC (Fig. 5a). Three cysteine residues Cys 57, 170 and 172 are conserved and are at same position as that of MtbAhpC Cys 61, 174 and 176 out of which two cysteine residues (Cys57A and Cys170B) make a disulfide bond (Fig. 5b). The BaAhpC model is shown in an unusual hexameric arrangement similar to that of MtbAhpC (Fig. 5c). Both BaAhpC and MtbAhpC dimers show the rmsd of only about 0.113 A for 294 amino acids (Fig. 5d). The super positioning of both the structures reveal that the active site cysteine residues are conserved in BaAhpC and probably follow similar pattern of enzyme action as that of MtbAhpC.

## Discussion

*Brucella* virulence is largely dependent on its replication and survival in host macrophages where it resides in *Brucella* containing vacuole (BCV) which resembles an endoplasmic reticulum structure[26]. Reactive oxygen species (ROS) like superoxide $O^{2-}$ and hydrogen peroxide $H_2O_2$ have been suggested to play an important role in anti-*Brucella* activities of both non-cytokine and TNF-α/IFN-γ activated host macrophages[27]. As *Brucella* is an aerobic bacterium, it is dependent on respiratory metabolism for its energy production, which in turn contributes to the

production of endogenous ROS[28]. AhpC has shown to play a role in virulence of several important pathogens e.g. *Helicobacter pylori*, *M. bovis*, and *Staphylococcus aureus*[29-30]. AhpCD is the complex containing the alkylhydroxyperoxide reductase enzyme AhpC and AhpD where AhpD is a peroxiredoxin reductase that employs reducing equivalents generated by cellular metabolism to revive the activity of AhpC[31].

Peroxidases that play an important role in defending the pathogenic cells against oxidative and nitrite stress are categorized as peroxiredoxins (Prx). Peroxiredoxins are divided into two categories based on number of conserved cysteine residues involved in enzyme activity: 1 Cys Prx and 2 Cys Prx[32-33]. In case of 1 Cys Prx, the conserved cysteine residue is present in N-terminal of the enzyme which forms the cysteinyl-sulphenic acid during the catalytic reaction[34-35]. However, in case of 2 Cys Prx, another cysteine is present in the C-terminal region which forms the inter subunit disulphide bond with the N-terminal cysteine during the catalytic reaction[36]. A flavin dependent enzyme is involved in reducing both the cysteine sulphenic acid in 1 Cys-Prx and the inter subunit disulphide bond in 2 Cys-Prx, which helps in reviving the Prx for the next catalytic reaction[11-12].

The structure of AhpC of *M. tuberculosis* H37Rv has been elucidated by Guimaraes *et al* (2005) wherein Cys176 has been mutated to a serine and the protein is trapped in an intermediate state. They have reported that without mutation, i.e. wild type AhpC protein did not form stable crystals and its crystal structure could not be elucidated. Through the crystal structure of AhpC of *M. tuberculosis*, they have shown that AhpC exists as a hexamer of dimers, wherein the structure of functional dimer is similar to that of 2-Cys Prx. The disulphide bond is formed between the Cys 61 and Cys174 of different subunits. Similar to the above mentioned case of wild type MtbAhpC crystallization in our attempts to crystallize the recombinant wild type *Brucella* AhpC, majority of the drops precipitated and yield no results. It is possible that the BaAhpC is behaving similarly to the wild type MtbAhpC and may require site specific modification of one of the cysteine. We are further trying to optimize the conditions to grow diffraction quality crystals. Thus, we report here the expression and purification of *Brucella* AhpC along with homology modelling and alignment studies, which help in determining the next step in designing the strategy for elucidating the crystal structure of BaAhpC and which in turn, will help in designing the inhibitors for the same. Chauhan & Mande (2002) have reported that all the three cysteines are directly involved in the catalytic activity of AhpC in *M. tuberculosis*. Similarly, *Brucella* AhpC possesses three cysteines in its sequence which might be involved in the catalytic activity of AhpC. Another striking similarity in *Brucella* and *Mycobacterium* where AhpC is concerned is that *Brucella* also lacks any sequence homologous to AhpF family which act as a reductase of AhpC in *S. typhimurium*. Instead, *Brucella* has AhpD protein which is under the same promoter as AhpC protein. It has been speculated that AhpD protein plays a role in reducing the AhpC protein in *Brucella* as well (Steel *et al*, 2010). Both AhpC and AhpD proteins of *B. abortus* have more than 50% amino acid sequence identity with that of the *M. tuberculosis*. The gel permeation chromatography of purified recombinant BaAhpC revealed that it typically forms dimers which form via an inter subunit disulphide linkage between a C-terminal cysteine of one subunit and an N-terminal cysteine of another subunit. The homology model of BaAhpC with *M. tuberculosis* AhpC as template showed that it has 49% identity and about 61% similarity with the MtbAhpC structure. In our gel permeation chromatography result, we have concluded that recombinant BaAhpC was purified as a dimer which harbours the inter subunit disulphide bond, although it may exist as a dodecamer in its active state as shown in the homology model of BaAhpC using MtbAhpC as the template.

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