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Purification and characterization of arsenite oxidase from *Arthrobacter* sp.

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Abstract The chemolithoautotroph, *Arthrobacter* sp.15b oxidizes arsenite to arsenate using a membrane bound arsenite oxidase. The enzyme arsenite oxidase is purified to its homogeneity and identified using MALDI-TOF MS analysis. Upon further characterization, it was observed that the enzyme is a heterodimer showing native molecular mass as ~100 kDa and appeared as two subunits of ~85 kDa LSU and 14 kDa SSU on SDS–PAGE. The V_{max} and K_m values of the enzyme was found to be 2.45 μM (AsIII)/min/mg) and 26 μM, respectively. The purified enzyme could withstand wide range of pH and temperature changes. The enzyme, however, gets deactivated in the presence of 1 mM of DEPC suggesting the involvement of histidine at the binding site of the enzyme. The peptide analysis of large sub unit of the enzyme showed close match with the arsenite oxidases of *Burkholderia* sp. YI019A and arsenite oxidase, Mo-pterin containing subunit of *Alcaligenes faecalis*. The small subunit, however, differed from other arsenite oxidases and matched only with 2Fe–2S binding protein of *Anaplasma phagocytophilum*. This indicates that Rieske subunits containing the iron–sulfur clusters present in the large as well as small subunits of the enzyme are integral part of the protein.

Keywords Arsenite oxidase · *Arthrobacter* sp.15b · MALDI-TOF MS

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Introduction

Arsenic cycling in soil, sediment and natural water systems is driven by several possible microbial transformations, including both reduction and oxidation (Oremland and Stolz 2003; Mukhopadhyay et al. 2002; Silver and Le Phung 2005). The biological oxidation of arsenite using bacteria is of particular interest for decontamination of arsenic-contaminated waste or ground water. Microbial oxidation of arsenite is a critical link in the global As cycle by converting more toxic arsenite into less mobile and less toxic arsenate species (Ehrlich 2001). Phylogenetically diverse arsenite-oxidising bacteria have been isolated from various aquatic and soil environments. It has been shown to be induced in the presence of arsenite (Ilyaletdinov and Abdrashitova 1981; Oremland et al. 2002; Phillips and Talylor 1976; Salmassi et al. 2002; Weeger et al. 1999; Macur et al. 2004; Rhine et al. 2006). Although numerous studies have been reported on arsenite oxidation, the gene encoding arsenite oxidase has shown great degree of divergence

(Inskeep et al. 2007). All known aerobic arsenite oxidases so far reported are heterodimeric in nature having molybdopterin and Rieske-like subunits with 3Fe–4S at the center in large sub unit and 2Fe–2S in the small sub unit (Rhine et al. 2007; Santini et al. 2004). This enzyme is actively involved in electron transport where Mo serves to cycle the electrons via Mo^{IV} and Mo^{VI} valence states (Ellis et al. 2001). Although the function of the molybdopterin ligand has not yet been conclusively established, interaction of this ligand with coordinated metal is sensitive to the oxidation state, indicating that the molybdopterin may be directly involved in the enzymatic mechanism (Kisker et al. 1997).

Recently, arsenite oxidases purified from a chemolithoautotrophic arsenite oxidizer NT-26, a member of α-Proteobacteria, and *Hydrogenophaga* sp. str. NT-14 has been studied in detail where the enzyme is located in the periplasmic space (Santini et al. 2000; Vander et al. 2004). Among the heterotrophic arsenite oxidizers, *Alcaligenes faecalis* and ULPAs1, members of *β*-Proteobacteria, the enzyme anchored to the periplasmic face of the inner membrane (Anderson et al. 1992; Muller et al. 2003). Homology between the sequences at the amino acid level varied from 72% to less than 52% among the purified enzymes derived from these three organisms. Owing to low homology in the sequence and differences in the localization of the enzyme within the cell, enzyme arsenite oxidase may have evolved separately in the two types of metabolically different organisms—autotrophs and heterotrophs.

We have isolated and characterized a chemoautotrophic bacterium from a sewage treatment plant site, capable of growing in arsenite containing medium supplemented with HCO_3^-. The organism is designated as *Arthrobacter* sp.15b, a member of Firmicutes. (16S rDNA accession no. AM 491801). The report here describes the purification scheme and further characterization of the enzyme arsenite oxidase from the above strain.

Materials and methods

Cell free extract preparation

Bacterial isolate was cultured in 1 l of MM-1 containing $(NH_4)_2SO_4$ 1.0 g; KH_2PO_4 0.5 g; KCl 0.05 g; $Ca(NO_3)_2$ 0.1 g; $NaHCO_3$ 0.5 g and $NaAsO_2$ 1.0 g, Yeast extract 0.04%. All the chemicals were procured from Sigma Chemicals until and unless mentioned otherwise.

Trace element stock solution: HCl (25%)—6.5 ml (Merck), $FeCl_2{\cdot}4H_2O$—1.5 gm (Merck), H_3BO_3—60 mg, $MnCl_2{\cdot}4H_2O$—100 mg, $CoCl_2{\cdot}6H_2O$—120 mg, $ZnCl_2$—70 mg, $NiCl_2{\cdot}6H_2O$—25 mg, $CuCl_2{\cdot}2H_2O$—15 mg, $Na_2MoO_4{\cdot}2H_2O$ (Merck)—25 mg.

One ml of trace element solution was added to 1 l medium and final pH was maintained to 7.8. The culture was incubated for 16 h at 28°C and harvested at 4,500 g for 5 m at 4°C in late log phase when absorbance of the growth medium at 600 nm reached to 0.35. Resulting pellet was washed three times with washing buffer containing 20 mM Tris–Cl, 0.1 mM PMSF and 0.6 mM EDTA, 0.9% NaCl pH 8.4 and centrifuged each time with the same speed. Finally pellet was suspended in to 50 ml of suspension buffer containing 20 mM Tris.Cl, 0.6 mM PMSF and 0.6 mM EDTA at pH 8.4 with 2 mg ml^{-1} lysozyme and incubated for 2 h at room temperature with occasional stirring. $MgSO_4$ and Mg $(CH_3COO)_2$ were added to give final concentration of 20 and 100 mM, respectively. DNase 100 µg and RNase 500 µg were added and incubated the extract at RT for another 30 min. Cell suspension thereafter was sonicated five times for 1.30 m burst with 10 min cool down interval. Broken cell suspension was heated at 60°C for 1 min in water bath and cooled immediately on ice to 4°C. Cell debris was removed by centrifugation of the lysate at 9,000*g* for 15 m. Clear supernatant was collected in fresh tube and pH was adjusted to 8.4 with 2 M NaOH.

Sphaeroplast preparation

In order to locate the presence of arsenite oxidase in the cells of the isolate, sphaeroplast was prepared as described by Anderson et al. 1992 with little modification. *Arthrobacter* sp.15b cells from a full grown culture were suspended in 20 mM Tris–HCl, 0.1 mM PMSF, 10 mM EDTA pH 8.4 containing 20% Sucrose. In order to isolate the sphaeroplasts, the outer membrane was lysed by treatment with 0.5 mg ml^{-1} (final conc.) lysozyme for 40 m at 25°C.

Purification of arsenite oxidase

The purification protocol was based on the method developed by Anderson et al. 1992 with modifications e.g., higher amount of lysozyme (2 mg ml^{-1}) was added, since bacteria were gram positive. The isolate was cultured in 6 l of MM1 at 28°C for 16 h and was harvested at 4,500 g for 5 min at 4°C in late log phase when absorbance of the growth medium at 600 nm reached 0.35. Resulting pellet was washed three times with washing buffer containing 20 mM Tris.Cl, 0.1 mM PMSF and 0.6 mM EDTA, 0.9% NaCl pH 8.4 and centrifuged each time. Finally the pellet was suspended in 50 ml of suspension buffer containing 50 mM Tris.Cl, 0.6 mM PMSF and 0.6 mM EDTA pH 8.4 with 2 mg ml^{-1} lysozyme and incubated for 1 h at room temperature with occasional stirring. $MgSO_4$ and Mg $(CH_3COO)_2$ were added to a final conc. 20 and 100 mM, respectively. DNase 50 µg and RNase 100 µg were added and incubated at RT for another 30 min. The Cell suspension was then sonicated five times, for 1 min each, with an interval of 1 min for cooling. Broken cell suspension was heated to 60°C for 1 m in water bath and cooled immediately on ice to 4°C. Cell debris was removed by centrifugation at 9,000*g* for 30 m. Total protein (2,500 mg) was precipitated with ammonium sulfate (20–85% saturation). Protein was dissolved into suspension buffer and subjected to dialysis for overnight at 4°C. Protein pH was maintained to 8.4 prior to loading onto the Ion-exchange column-High trap QFF (Amersham) which was pre-equilibrated with Tris.Cl at pH 8.4. Protein was eluted with linear gradient of 0–100% of 1 M NaCl. Active fractions were pooled and concentrated using centrifugal device Amicon 10 kDa. One ml of concentrated protein (20 mg) was further loaded onto the gel filtration column-Sephacryl S200 16/60 column equilibrated with buffer containing 50 mM Tris.Cl pH 7.0, 300 mM NaCl and 0.01% TritonX. Active fractions were pooled and further concentrated. 1.5 mg of protein was then loaded onto the High trap Phenyl FF affinity column pre equilibrated with 50 mM sodium phosphate buffer pH 7.0 containing 1 M ammonium sulfate. Protein was eluted with the same buffer against linear but decreasing gradient of 100–0% of ammonium sulfate. Active fractions were concentrated and SDS–PAGE was performed according to the procedure of Laemmli (1970). The protein content was determined by the Bradford method (Bradford 1976), where Bovine serum albumin was used as the standard. All column purification steps were carried out at 4°C using FPLC AKTA system (Amersham).

Enzyme assay for arsenite oxidation

The assay is based on reduction of an artificial electron acceptor DCIP (Anderson et al. 1992) in MES (morpholino ethelenediol sulfonic acid) buffer at pH 6.0. Kinetic value of the crude fraction as well as the column purified fraction was determined by the addition of the enzyme into 1 ml of assay buffer containing 60 µM DCIP (2,6-dichlorophenol indophenol), 200 µM of $NaAsO_2$ and 50 mM MES at pH 6.0. Change in absorbance/min was monitored at 600 nm for a period of 5 min. Protein boiled for 10 m (heat denatured) served as a control for this experiment.

In-gel assay

Cell free extract or purified protein (20 µg) is loaded directly on a native PAGE after mixing with loading dye and run was carried out at 4°C in dark cold room. Gel was equilibrated in 50 mM MES pH 6.0 for 15 min in shaking condition followed by staining in 100 mM of DCIP in 50 mM MES pH 6.0 for 1 h at 4°C. Gel was briefly rinsed with 50 mM MES pH 6.0 and flooded with 200 µM of sodium arsenite for 3–5 min until achromatic or clearing zone developed in the gel.

Effect of metal ions and chemical agents on enzyme activity

Effect of different metal ions as well as chemical agents on the activity of purified fraction was tested with metal salts like $CaCl_2$, $FeSO_4$, $MgCl_2$, NaCl, $CoCl_2$, $ZnCl_2$, Na_2Mo_4, EDTA, Iodoacetic acid, *Para* chloromercuribenzoic acid (*p*CMB) and DEPC. Stock solution (100 mM) of these salts were prepared. Change in absorbance was recorded prior to adding salts and after adding the salt stock solution (10 µl) in 990 µl reaction buffer (final conc. 1 mM) containing DCIP 60 µM, 200 µM of $NaAsO_2$, 50 mM MES pH 6.0 along with 10 µg of arsenite oxidase. Change in

absorbance was calculated which was translated as the percent change in activity.

Effect of pH on purified arsenite oxidase activity

Purified arsenite oxidase from *Arthrobacter* sp.15b was added in assay buffer. Concentration of DCIP and $NaAsO_2$ remained same i.e., 60 and 200 μM, respectively. Buffering condition for kinetic study in reaction buffer was achieved using different buffer. Citrate (50 mM) was used to maintain pH 3 and 4, similarly MES (50 mM) for pH 5 and 6, Tris.Cl (50 mM) for pH 7 and 8, finally Borate (50 mM) for pH 9. Relative activity of arsenite oxidase at different pH was calculated.

Effect of heat and temperature on purified arsenite oxidase activity

The effect of heat on the purified fraction was evaluated by incubating the enzyme at different temperature (5–50°C with 5°C increments) for 1 h followed by the addition of reaction mixture containing DCIP 60 μM, 200 μM of $NaAsO_2$ and 50 mM MES at pH 6.0. Similarly to, monitor the effect of temperature on purified enzyme, reaction buffer was incubated in water bath for 5 m at different temperatures ranging from 5 to 50°C followed by the addition of enzyme. Change in absorbance was recorded immediately after mixing the enzyme in buffer. Absorbance was monitored by spectrophotometer (Varian, Cary 300 Bio). Maximum change in absorbance was considered as 100% activity. Thus relative activity of arsenite oxidase at different temperatures and heat were calculated.

MALDI TOF MS

The CBB stained protein bands of interest were sliced from SDS–PAGE gel and washed in 100 μl destaining solution containing 1:1 100 mM NH_4HCO_3 and 100% ACN (Acetonitrile) till the bands appear colorless. Finally the gels were dehydrated in 100% ACN. Reduction of proteins were carried out in100 μl of 10 mM DTT in 50 mM NH_4HCO_3 for 45 min at 56°C. Alkylations of proteins were done in 100 μl of 55 mM Iodoacetamide (IAA) prepared in 50 mM NH_4HCO_3. Digestion was carried out with 10 μl of trypsin (10 ng) and incubated at 37°C for 12–16 h. Ten micro liter of 0.1% TFA (Triflouroacetic acid) and 10 μl of 100% ACN were added to the samples followed by sonication for 15 min at RT. Matrix was prepared by dissolving alpha cyano-hydroxy cinnamic acid (Bruker daltonics, Breman, Germany) in 1:2 ACN and 0.1%TFA to make a 1 mg ml^{-1} solution. It was sonicated for 15 min and solution was centrifuged at 9,000*g* for 5 m. Peptide mixtures (2 μl) were mixed with equal amount of matrix and 0.5 μl of this was spotted on MALDI ground steel target plate (Bruker daltonics, Germany). Mass spectra were obtained on a Bruker autoflexII MALDI-TOF mass spectrometer equipped with a pulsed N_2 laser (337 nm). Around 500 laser shots were collected from one spot from five different positions with 100 shots per position. Peptides mass fingerprinting (PMF) spectra were searched online against NCBI and MSDB using the mascot search engine (Matrix sciences, London) with 100 ppm mass tolerance and 0–2 missed cleavage.

Results

Localization of arsenite oxidase

The presence of arsenite oxidase was tested in different fractions during the sphaeroplast preparation (Table 1). Approximately 10% of total activity was found in released content from the periplasmic space. Thus the protein seems to be located in the membrane fraction showing 90% of the activity.

Purification and characterization of arsenite oxidase

The crude extract was subjected to precipitation with (20–85%) $(NH_4)_2SO_4$ and dialyzed with 12 kDa molecular weight cut off membrane. This step

Table 1 Localisation of Arsenite oxidase

Treatment (Lysozyme-EDTA)	Sp. activity
Supernatant	0.01 μM As(III)/mg protein/min
Pellet	0.1 μM As(III)/mg protein/min

Table 2 Purification steps of arsenite oxidase from *Arthrobacter* sp.15b

Purification of arsenite oxidase	Total protein (mg)	Total activity μMAs(III)/min	Sp. activity μMAs(III)/min/mg	Purification fold
Cell free extract	2,500	25	0.01	1
20–85% (NH)2So4	1,750	20	0.011	1.1
Anion-exchange QFF	20	5	0.25	25
Sephacryl S200	1.5	4	2.66	26
Phenyl FF	0.125	2	16	160

yielded 1.4 fold purification with specific activity 0.114 μM As(III)/min/mg of preotein. The protein was then subjected to anion exchange chromatography and showed a specific activity of 0.25 μM As(III)/min/mg with 2.5 fold purification. Active fractions from ion-exchange column were loaded on Gel filtration Sephacryl S200, 16/60 column. Specific activity of protein at this stage was 2.66 μM As(III)/min/mg with 26 fold purification. Eluted active protein fractions were pulled, concentrated and loaded on Phenyl FF hydrophobic column for further purification. Specific activity was measured as 16 μM As(III)/min/mg with 160 fold over all purification (Table 2). Purity of the active fraction was checked on SDS–PAGE. The protein was found to be a heterodimer with large subunit 85 kDa and small subunit 14 kDa (Fig. 1a). The native form of heterodimeric enzyme exhibited mol.wt ~100 kDa when it was resolved on PAGE. The native protein was also subjected to in-gel assay using DCIP reagent and showed a zone of clearing near 100 kDa thus confirming the presence and the size of the native enzyme (Fig. 1b).

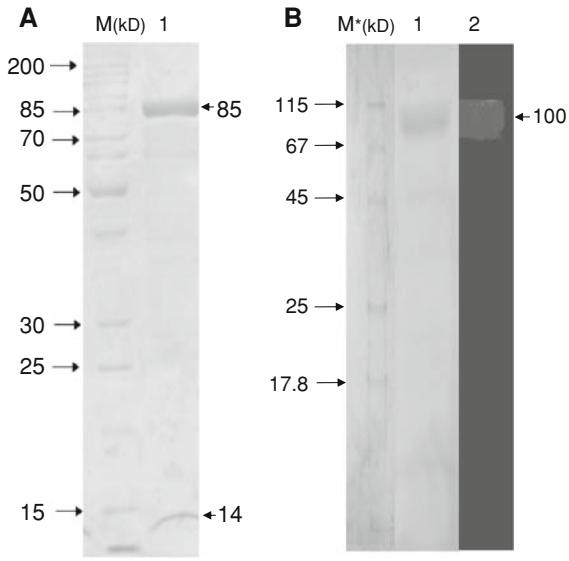

Fig. 1 **a** SDS–PAGE of purified arsenite oxidase from *Arthrobacter* sp.15b. *Lane* M = protein ladder (MBI fermentas). *Lane* 1 purified Arsenite oxidase. **b** Native PAGE and Zymogram of purified protein of isolate 15b. *Lane* M is protein marker of different mol wt. *Lane* 1 and 2 is loaded with column purified protein. *Lane* M and 1 is stained with CBB R250, *Lane* 2 is stained with DCIP. *Lane* M* = Mol.mass standards, Nitrate reductase inactivator (115 kDa), Albumin bovine(67 kDa), Albumin egg(45 kDa), ChymotripsinogenA (25 kDa), Myoglobin equin (17.8 kDa)

Effect of metal ions and chemical agents

The effect of potential inhibitor/activator metal ion, on purified arsenite oxidase activity is shown (Table 3). The enzyme activity was not inhibited by treatment with 1 mM of Na^{+}, Ca^{2+}, Fe^{2+}, Mo^{6+} and EDTA. Co^{2+} and Zn^{2+}, however, inhibited the arsenite oxidase activity and reduced the activity by 98.5 and 98%, respectively. Sulfhydryl (-SH) group quencher like Iodoacetic acid and *para*-chloromercuribenzioc acid reduced the activity of arsenite

Table 3 Effect of metal ions on purified arsenite oxidase

Metal ions and chemicals (1 mM)	Percentage activity retained
Ca^{2+}	92
Mg^{2+}	100
Fe^{2+}	90
Na^{+}	100
Co^{2+}	1.5
Zn^{2+}	1.8
EDTA	100
Mo^{6+}	100
Iodoacetic acid	85
*p*CMB	90
DEPC	5

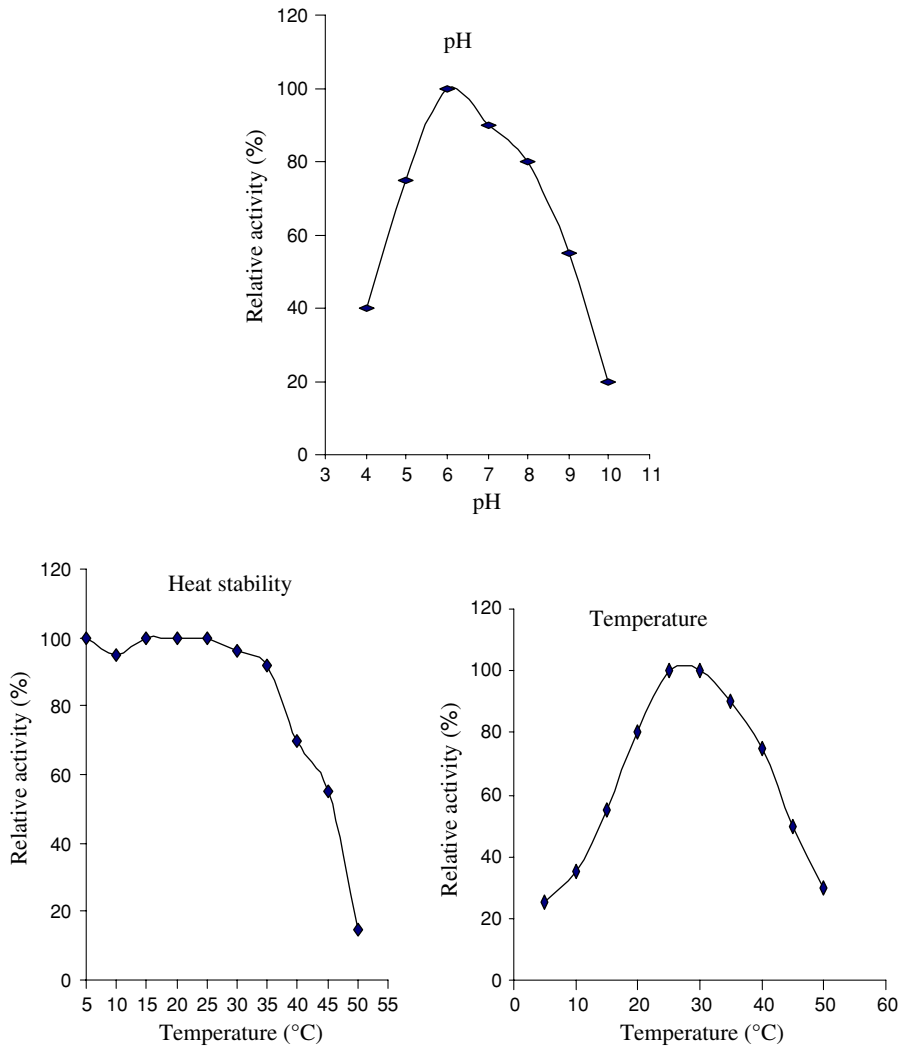

Fig. 2 Effect of pH, Heat stability and temperature on purified arsenite oxidase

oxidase by 15 and 10% only. The enzyme when treated with 1 mM DEPC (diethylpyrocarbonate), retained only 5% of activity.

Effect of pH

The effect of pH on purified arsenite oxidase was carried out in enzyme assay buffer (DCIP 60 μM, 200 μM of $NaAsO_2$ and 50 mM MES pH 6.0; Fig. 2a). Enzyme exhibited maximum activity at pH 6. Purified enzyme remains active at neutral (pH) acidic (pH 5), and alkaline (pH 8) region by retaining 85, 78 and 82%, respectively. Significant loss of activity could be noticed at pH 4 and pH 10. Since enzyme activity at pH 6 remained maximum, this pH was considered optimal for enzyme activity assay of arsenite oxidase.

Heat stability

The enzyme remained active between 5 and 35°C and retained activity to 100–92%. However, free enzyme retained 70% of activity during incubation for 1 h at 40°C. Only 55% of activity was observed while enzyme was incubated at 45°C and only 15% when it was incubated at 50°C for 1 h (Fig. 2b).

Temperature effect

Effect of temperature on enzyme activity i.e., reduction of DCIP by sod.arsenite in MES buffer at pH6 has been determined. Optimum activity of arsenite oxidase of *Arthrobacter* 15b was found at 25°C. Gradual loss in activity was observed in the assay buffer at below 25°C

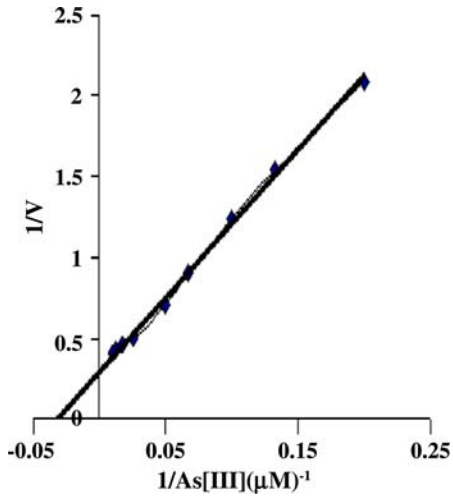

Fig. 3 Lineweaver-Burk plot of purified arsenite oxidase activity of *Arthrobacter* sp.15b

and above 35°C (Fig. 2). Our results show optimum temperature for enzyme activity at 25°C.

Determination of K_m, V_{max}

The kinetic parameters for the purified enzyme were determined by double reciprocal (Lineweaver-Burk) plot with sodium arsenite as a substrate (Fig. 3). K_m value of arsenite oxidase was found to be 26 μM and V_{max} was 2.45 μMAs(III)/min/mg of protein.

Protein mass identification by MALDI-TOF analysis

The protein band resolved in SDS–PAGE was subjected to MALDI-TOF MS analysis. The large subunit of molecular weight ~ 85 kDa generated

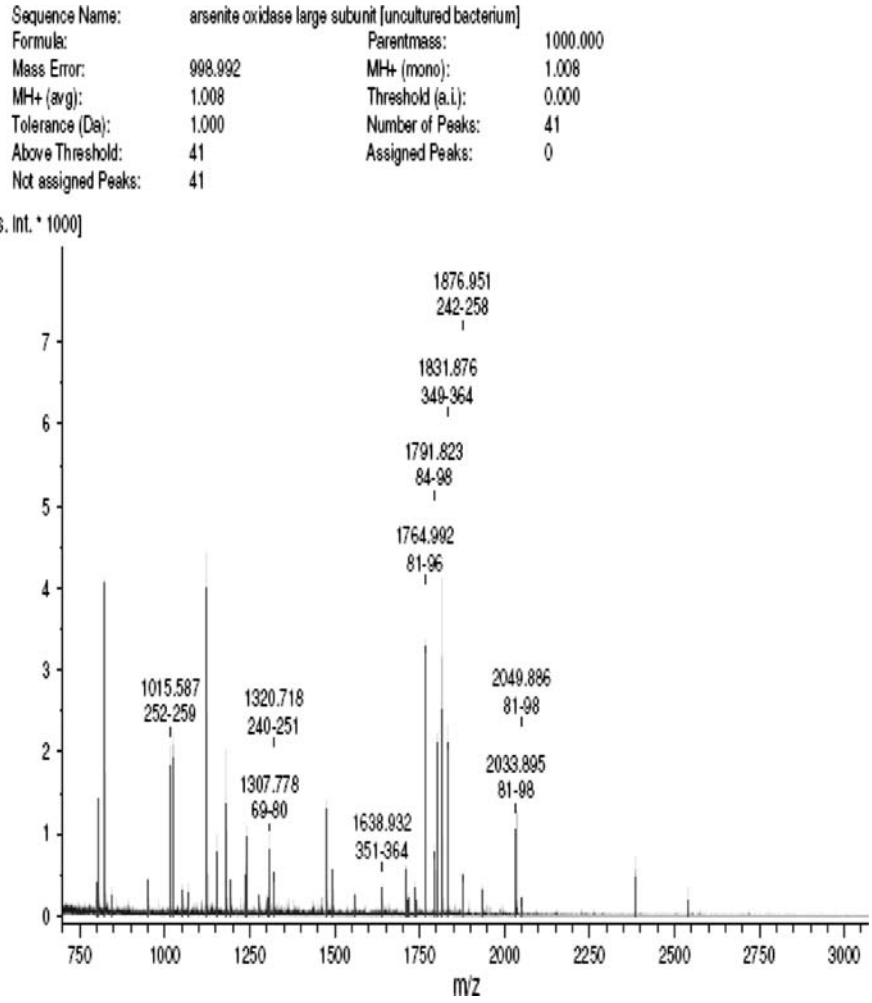

Fig. 4 MALDI-TOF analysis of large subunit of arsenite oxidase (~ 85 kDa) protein band of SDS–PAGE

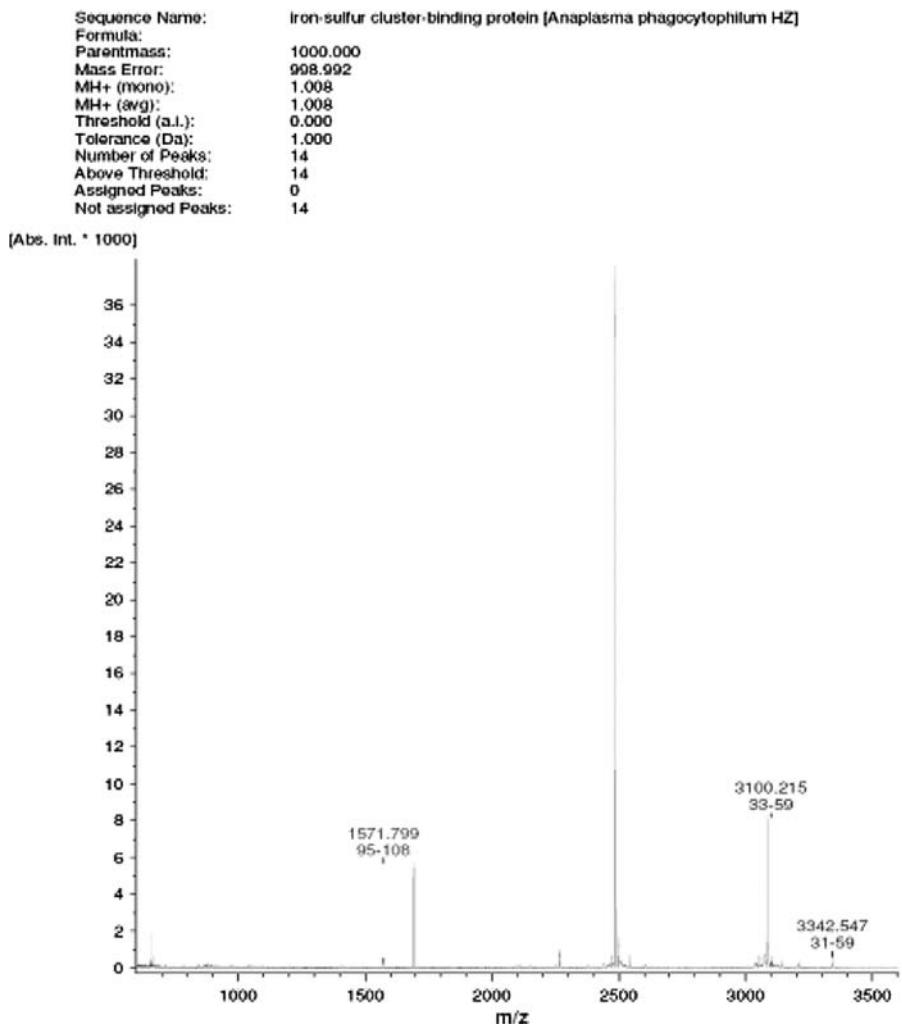

Fig. 5 MALDI-TOF analysis of small subunit of arsenite oxidase (~ 14 kDa) matching with 2Fe–2S cluster-binding protein

peptide fragments which matched with the large subunit (uncultured bacterium) of arsenite oxidase in the database (Fig. 4). Similarly, ~ 14 kDa protein band generated peptides which showed similarity with 2Fe–2S cluster binding protein of *Anaplasma phagocytophylum* HZ (Fig. 5).

Discussion

Arsenite oxidase purified from the isolate *Arthrobacter* 15b. was a membrane bound protein as observed in UPLAs1 and *A. faecalis* but differed with NT-14 and NT-26 where the enzyme was located in the periplasm (Table 4). As suggested by Lebrum et al. (2003), Arsenite oxidase is probably transported over the cytoplasmic membrane via the tat system and remains membrane attached by an N-terminal transmembrane helix of the Rieske subunit. Kinetics parameter like K_m and V_{max} of purified arsenite oxidase of *Arthrobacter* 15b was 26 µm, 2.45 µm As(III)/mg/min while for NT-14 it was 35, 6.1; for NT-26 it was 61, 2.4; and for *A. faecalis* 8, 2.8 respectively. V_{max} value showed close resemblance with the values of NT 26 and *A. faecalis*. We also observed higher substrate specificity for the enzyme of *Arthrobacter* compared to NT-14 and NT-26. The purified enzyme in non-denaturing condition showed molecular wt of 100 kDa (Fig. 1b).

Effects of metal ions on the stability of the purified enzyme revealed that Co^{6+} and Zn^{2+} were more toxic as evident for any oxidoreductase enzymes where as sulfhydral (-SH) group of cysteine suppressor like Iodoacetic acid and *p*CMB did not affect the activity

Table 4 Comparision of arsenite oxidases from *Arthrobacter* sp.15b, *Hydrogenophaga* NT-14, NT-26, and *Alcaligenes faecalis* NCIB 8687

	NT-14[a]	NT-26[b]	*A. faecalis*[c]	UPLAs1[d]	*Arthrobacter* sp.15b
Location	Periplasm	Periplasm	Membrane	Membrane	Membrane
Native mol.wt (kDa)	309	219	100	NR	100
Sub unit size	α 86	α 98	α 85	NR	α 85
	β 16	β 14	β 15		β 14
K_m (μm)	35	61	8	NR	26
V_{max} (μm (AsIII)/min/mg)	6.1	2.4	2.88	NR	2.45

NR Not reported

[a] Hoven et al. (2004); [b] Santini et al. (2004); [c] McNellis and Anderson Gretchen (1998); [d] Muller et al. (2003)

of arsenite oxidase suggesting that arsenite can not bind to cysteine residues in arsenite oxidase. However, enzyme activity was considerably suppressed on addition of DEPC that binds to histidine residue and the protein retained only 5% of its activity. This indicates that the histidine residue which serves as a binding site for the enzyme gets modified due to the presence of DEPC. This was in consensus with the findings of McNellis and Anderson (1998). Heat stability and pH data shows that arsenite oxidase is a versatile enzyme for bioremediation purposes as this can be used at moderate temperature up to 42°C and at wide range of pH from 5 to 8.0.

BLAST P analysis of a peptide sequence (PKASGQMPRTMHAYEK) identified by MALDI-TOF and Mascot search engine, showed homology

Fig. 6 BLASTP results of peptide sequence generated from LSU of *Arthrobacter* sp.15b showing match with Arthrobacter 15b arsenite oxidase nucleotide sequence deposited in the database (accession no. AM 492534)

Sequences producing significant alignments:		(Bits)	Value
gb|ABY19362.1|	arsenite oxidase large subunit [uncultured bac...	56.6	1e-07
gb|ABY19357.1|	arsenite oxidase large subunit [uncultured bac...	56.6	1e-07
gb|ABY19323.1|	arsenite oxidase large subunit [Burkholderia s...	49.8	1e-05
emb|CAM34515.1|	arsenite oxidase large subunit [Arthrobacter ...	49.8	1e-05
gb|AAQ19838.1|	arsenite oxidase Mo-pterin subunit [Alcaligene...	49.8	1e-05
pdb|1G8K|A	Chain A, Crystal Structure Analysis Of Arsenite Ox...	49.8	1e-05
sp|Q7SIF4|AOXB_ALCFA	Arsenite oxidase large subunit (AOI) >pd...	49.8	1e-05
gb|ABY19330.1|	arsenite oxidase large subunit [Pseudomonas sp...	49.4	1e-05
gb|ABY19328.1|	arsenite oxidase large subunit [Pseudomonas sp...	49.4	1e-05
ref|YP_001098817.1|	arsenite oxidase large subunit (AOI) [Her...	49.4	1e-05
gb|AAN05581.1|	arsenite-oxidase large subunit [Cenibacterium ...	49.4	1e-05
gb|ABY19350.1|	arsenite oxidase large subunit [uncultured bac...	48.6	3e-05
ref|YP_001585661.1|	arsenite oxidase, large subunit [Burkhold...	47.3	6e-05
ref|YP_001585636.1|	arsenite oxidase, large subunit [Burkhold...	47.3	6e-05
gb|ABY19355.1|	arsenite oxidase large subunit [uncultured bac...	44.3	5e-04
gb|ABY19329.1|	arsenite oxidase large subunit [Ralstonia sp. 22]	44.3	5e-04
gb|ABY19322.1|	arsenite oxidase large subunit [Alcaligenes sp...	44.3	5e-04
gb|ABY19321.1|	arsenite oxidase large subunit [Alcaligenes sp...	44.3	5e-04
gb|ABP63660.1|	arsenite oxidase Mo-pterin [Achromobacter sp. ...	44.3	5e-04
gb|ABY19319.1|	arsenite oxidase large subunit [Variovorax sp....	43.9	7e-04

Fig. 7 BLASTP search result of peptide sequence of SSU of arsenite oxidase identified by MALDI-TOF, matching with 2Fe–2S cluster-binding protein of Anaplasma

Sequences producing significant alignments:		(Bits)	Value
ref|YP_505261.1|	iron-sulfur cluster-binding protein [Anaplas...	93.1	1e-18
ref|YP_153890.1|	ferredoxin [2Fe-2S] adrenodoxin-like precurs...	76.1	1e-13
ref|YP_001937260.1|	putative ferredoxin, 2Fe-2S (andrenodoxin...	71.9	2e-12
ref|YP_001248702.1|	ferredoxin-like iron-sulfur cluster-bindi...	71.9	2e-12
emb|CAM76372.1|	Ferredoxin [Magnetospirillum gryphiswaldense ...	71.0	4e-12
ref|YP_427113.1|	ferredoxin [Rhodospirillum rubrum ATCC 11170...	70.2	8e-12
ref|YP_422386.1|	ferredoxin [Magnetospirillum magneticum AMB-...	70.2	8e-12
ref|ZP_00053067.1|	COG0633: Ferredoxin [Magnetospirillum magn...	70.2	8e-12
ref|YP_001234887.1|	ferredoxin [Acidiphilium cryptum JF-5] >g...	69.4	1e-11
ref|YP_506187.1|	iron-sulfur cluster binding protein [Neorick...	66.8	8e-11
ref|YP_745763.1|	ferredoxin, 2Fe-2s [Granulibacter bethesdens...	66.4	1e-10
ref|YP_191778.1|	ferredoxin, 2Fe-2S [Gluconobacter oxydans 62...	66.4	1e-10
ref|YP_303046.1|	ferredoxin [Ehrlichia canis str. Jake] >gb|A...	66.0	1e-10
ref|YP_180285.1|	ferredoxin, 2Fe-2S [Ehrlichia ruminantium st...	66.0	1e-10
ref|ZP_00545218.1|	Ferredoxin [Ehrlichia chaffeensis str. Sap...	66.0	1e-10
ref|ZP_01038925.1|	Ferredoxin, 2Fe-2S [Erythrobacter sp. NAP1...	63.0	1e-09
ref|ZP_02983457.1|	ferredoxin [Gluconacetobacter diazotrophic...	61.3	4e-09

with arsenite oxidase large subunit *Burkholderia* sp. YI019A and arsenite oxidase, Mo-pterin subunit *A. faecalis* (Fig. 6). However, BlastP results of peptide sequence of SSU (IDLEGACEGSMACSTCHLIVAPEWYSK) matched with 2Fe–2S cluster binding protein of *Anaplasma phagocytophilum* HZ (Fig. 7). Peptide mass of SSU did not show homology with other small subunit arsenite oxidase, possibly due to divergence of sequences at the amino acid level. This data suggests the presence of 3Fe–4S cluster in LSU and 2Fe–2S cluster in SSU of arsenite oxidase derived from *Arthrobacte*r sp.15b.

In conclusion our results suggest that arsenite oxidase isolated and characterized from a chemoautotrophic bacterium *Arthrobacter* sp.15b is of ~ 100 kDa in size and possess two sub units—LSU (85 kDa) and SSU (14 kDa). The enzyme shows stability with pH and temperature fluctuations. This enzyme is mostly localized in the membrane and perhaps involved in metabolism and not in detoxification process as has been proposed earlier for heterotrophs. Efforts are on to study the biotechnological application of this enzyme using immobilization techniques.

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