EXPRESSION AND STRUCTURAL INVESTIGATION OF ACID TOLERANT ARSENITE OXIDASE WITH BIOSENSOR POTENTIAL

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ABSTRACT

Groundwater contamination by the two dominant toxic arsenic species, arsenite (As(III)) and arsenate (As(V)) has caused a global epidemic of arsenic poisoning effecting over 100 million people. Currently there is no commercially available As(III) biosensor to specifically detect this most toxic inorganic compound. Arsenite oxidase (Aio) catalyzes the oxidation of As(III) to As(V) by two electron transfer. As a redox enzyme, Aio is potentially suitable for construction of enzyme based electrochemical biosensor to detect As(III) specifically. A moderate acidophilic arsenite oxidizer, *Thiomonas delicata* DSM 16361 was used as source of Aio in this study. To obtain Aio sufficient for biosensor construction, it was first necessary to optimize the expression and purification of recombinant Aio in *E. coli* strain. Full length *aioBA* gene was isolated and deposited to GenBank under accession number KX792110. Expression of the recombinant Aio was successfully performed in ZYM-5052 autoinduction medium at 20 °C for 48 hours in *E. coli* strain C43(DE3). The Aio was purified to homogeneity with purity $> 90\%$ and characterized. The purified Aio was found to be heterodimeric with subunits of 91 and 21 kDa (17 kDa without signal peptide) in size, respectively. Specific activity of purified Aio was 4 U/mg, with substrate K_m of 14 μM. The temperature-activity profile of purified Aio was found optimum at 55 °C and Aio retained nearly 45% of its initial activity after pre-incubation at 60 °C for 1 hour. The enzyme was stable in acidic pH ranging from pH 2.5 to 6. Activity of Aio was retained in the presence of 10 mM metal ions (K^+, Li^+, Co^{2+}, Ca^{2+}, Ni^{2+}, Mn^{2+}, and Zn^{2+}) and anions (NO^{2-}, SO_4^{2-}, and Cl^-). In order to elucidate the acidophilic adaptation of Aio, homology model of Aio was constructed using X-ray crystallized structure of Aio from *Alcaligenes faecalis* (PDB ID: 1G8K) owing to the high sequence identity of 64%. The homology model was compared to the two structures from alkaliphilic sources, *A. faecalis* and *Rhizobium* sp. NT-26. Notable difference between the structures and model was observed on the surface of the enzyme as well as the existence of a unique loop region in *T. delicata* Aio. Several structural features that might be implicated in the acidophilic adaptation of *T. delicata* Aio were: (a) a higher proline content in Aio, (b) positively charged surface protein, (c) a decreased number of salt bridges and hydrogen bonds, and (d) introduction of polar and charged residues distal to catalytic site. When Aio was incorporated in biosensor construction, the DCPIP/Aio electrochemical assay was capable of detecting 10 to 500 ppb As(III) by using carbon screen printed electrode. This revealed the potential of Aio as a biosensing material for determining safe level of As(III) in water systems. This is the first characterization study of acid tolerant Aio from *T. delicata* DSM 16361 with biosensor potential.

ABSTRAK

Pencemaran air bawah tanah oleh dua spesies arsenik yang dominan, arsenit (As(III)) dan arsenat (As(V)) telah mengakibatkan wabak keracunan arsenik global melibatkan lebih 100 juta orang. Pada masa kini tiada biosensor komersial yang dapat mengesan sebatian arsenik bukan organik yang paling tosik secara khusus. Arsenit oksidase (Aio) adalah pemangkin untuk pengoksidaan dari As(III) ke As(V) dengan dua pemindahan elektron. Sebagai enzim redoks, Aio berpotensi untuk digunakan dalam pembinaan enzim biosensor elektrokimia untuk mengesan As(III) secara khusus. *Thiomonas delicata* DSM 16361 yang merupakan mikrob berciri asid serdehana digunakan sebagai sumber Aio dalam kajian ini. Untuk mendapatkan Aio yang cukup untuk pembinaan biosensor, ekspresi dan penulenan Aio rekombinan optimum amat diperlukan. Jujukan penuh gen *aioBA* dipencilkan dan didepositkan ke GenBank bawah nombor pencapaian KX792110. Ekspresi optimum untuk Aio rekombinan dilaksanakan dalam keadaan aerobik di dalam autoinduksi media ZYM-5052 pada suhu 20 °C selama 48 jam dengan *E. coli* C43(DE3). Aio ditulenkan ke homogenan dengan 90% ketulenan dan dicirikan. Aio tulen adalah heterodimerik dan mengandungi subunit dengan berat molekul 91 dan 21 kDa (17 kDa tanpa isyarat peptida) masing-masing. Aktiviti spesifik Aio tulen adalah 4 U/mg, dengan K_m substrat 14 μM. Profil suhu-aktiviti Aio tulen adalah optimum pada suhu 55 °C dan Aio mengekalkan 45% aktiviti asalnya setelah dieramkan pada suhu 60 °C selama sejam. Enzim ini stabil dalam pH berasid dari pH 2.5 ke 6. Aktiviti Aio dapat dikekalkan dalam 10 mM ion logam (K^+, Li^+, Co^{2+}, Ca^{2+}, Ni^{2+}, Mn^{2+}, dan Zn^{2+}) dan juga anion (NO^{2-}, SO_4^{2-}, dan Cl^-). Untuk memahami adaptasi keasidan, model homologi Aio telah dibina dengan menggunakan struktur kristal X-ray Aio daripada *Alcaligenes faecalis* disebabkan oleh identiti jujukan yang tinggi, 64%. Homologi model tersebut dibandingkan dengan dua struktur dari sumber alkalifilik, *A. faecalis* dan *Rhizobium* sp. NT-26. Perbezaan yang ketara antara templat dengan model diperhatikan pada permukaan enzim dan kewujudan gelung yang unik dalam Aio *T. delicata*. Ciri-ciri struktur tersendiri yang mungkin dikaitkan dengan adaptasi keasidan Aio *T. delicata* dikenalpastikan seperti berikut: (a) pertambahan bilangan proline dalam Aio, (b) permukaan protein bercaj positif, (c) pengurangan bilangan jambatan garam dan ikatan hidrogen, dan (d) penambahan bilangan asid amino berkutub dan bercaj berjauhan dengan tapak pemangkin. Assay elektrokimia DCPIP/Aio menunjukkan keputusan yang baik dalam pengesanan As(III), dan dapat mengesan daripada 10 ke 500 ppb As(III) dengan menggunakan elektrod cetakan skrin karbon. Ini mendedahkan potensi Aio untuk dijadikan sebagai pengesan biologi untuk menentukan kepekatan As(III) dalam sistem air. Ini adalah kajian pencirian pertama Aio yang toleran asid daripada *T. delicata* DSM 16361 dengan potensi biosensor.

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LIST OF ABBREVIATIONS

Amp	-	ampicillin
As(III)	-	arsenite
As(V)	-	arsenate
bp	-	base pair
BLAST	-	Basic Local Alignment Search Tool
BSA	-	bovine serum albumin
C-terminus	-	carboxyl terminus
CV	-	column volume
DCPIP	-	2,6-dichlorophenolindophenol
DNA	-	deoxyribonucleic acid
DOPE	-	discrete optimized protein energy
E. coli	-	*Escherichia coli*
EDTA	-	ethylene diamine teraacetate
His	-	histidine
IMAC	-	immobilized metal ions affinity chromatography
IPTG	-	isopropyl-β-D-thiogalactopyranoside
kDa	-	kilo-Dalton
LB	-	Luria-Bertani media/broth
mAU	-	mili absorbance unit
MGD	-	molybdopterin guanidine dinucleotide
MES	-	2-(N-morpholino)ethanesulfonic acid
min	-	minute(s)
Mo	-	molybdenum
MWCO	-	molecular weight cut off
N-terminus	-	amino terminus
Ni-NTA	-	nickel-nitrilotriacetic acid
nm	-	nanometer

OD	-	optical density
PAGE	-	polyacrylamide gel electrophoresis
PCR	-	polymerase chain reaction
PDB	-	Protein Data Bank
PES	-	polyethersulfone
PISA	-	Protein Interfaces, Surfaces and Assemblies
RMSD	-	root mean square deviation
rpm	-	revolutions per minute
SDS	-	sodium dodecyl sulfate
sp.	-	species
T.	-	*Thiomonas*
T4 PNK	-	T4 polynucleotide kinase
TAE buffer	-	tris-acetate-EDTA buffer
Tris-HCl	-	tris-(hydroxymethyl)-aminomethane hydrochloride
UV	-	ultra violet

LIST OF SYMBOLS

Å	-	Amstrong(= 10^{-10} nm)
α	-	alpha
β	-	beta
γ	-	gamma
°C	-	degree Celsius
C_α	-	carbon alpha
σ	-	sigma
μg	-	microgram
mg	-	milligram
L	-	litre
μM	-	micromolar
mM	-	millimolar
x g	-	relative centrifugal force
ppb	-	part per billion
ppm	-	part per million
v/v	-	volume per volume
w/v	-	weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Arsenic is classified as one of the notorious carcinogen by the United States Environmental Protection Agency (EPA). Arising from both natural and anthropogenic causes, arsenic is widely spread in the environment. Changes in the oxidation state of arsenic have implications for its mobility and toxicity to living cells. The toxicity of arsenic follows the order: Inorganic As(III) species $>$ Organic As(III) species $>$ Inorganic As(V) species $>$ Organic As(V) species $>$ Elemental Arsenic (Akter and Ali, 2011). The two arsenic species which generally exposed to human and microorganisms are arsenite As(III) and arsenate As(V), with As(III) is considered 100 times more toxic (Rosen, 2002).

As(III) and As(V) species can interchange oxidation state depending on redox potential, pH and biological processes from the microbial activities. Various groups of As(III) oxidizing bacteria have been isolated from diverse arsenic contaminated environments, such as cattle-dipping fluids, hot springs, mine tailing and drainage water. The As(III)-oxidizing bacteria isolated to date can be divided into two groups: (i) chemolithoautotrophs (utilize As(III) as the electron donor) or (ii) heterotrophs (growth in the presence of organic matter).

Arsenite oxidase (Aio) catalyzes the oxidation of As(III) to As(V) by a two-electron transfer. Belonging to a member of the dimethylsulfoxide (DMSO) reductase family of molybdenum-containing enzymes, Aio consists of two heterodimeric subunits. The large subunit (AioA) shares similarities to the α subunit of the formate dehydrogenase and assimilatory nitrate reductase. The small subunit (AioB) on the other hand belongs to the structural class of the Rieske subunit of the cytochrome bc1 complex. Both the subunits have homologues in enzyme families differing

significantly from each other with respect to both redox cofactors and function. Hence, Lebrun *et al.* (2003) suggests Aio to be the ancient bioenergetic enzyme before the divergent of Bacteria and Archaea. As an ancient enzyme which utilizes As(III), a highly toxic metalloid as substrate, characterization of Aio is of special interest for bioremediation and biosensor construction. To date, two Aio have been extensively characterized which were originated from a chemolithoautotrophic *Alphaproteobacteria Rhizobium* sp. NT-26 (Santini and vanden Hoven, 2004) and heterotrophic *Betaproteobacteria Alcaligenes faecalis* (Anderson *et al.*, 1992). Both bacteria grow optimally at slightly alkaline pH 8, which can be categorized as alkaline-tolerant bacteria.

In mining environments, particularly acid mine drainage (AMD), inorganic As(III) is widespread as a result of the bioleaching of arsenic-bearing minerals. Under low pH condition (pH $<$2), As(III) oxidizers from these extreme environment are commonly isolated with acid-tolerant properties with an optimum growth pH near neutrality with the exception of *Thiomonas* strains (Hallberg and Johnson, 2003; Battaglia-Brunet *et al.*, 2006; Katayama *et al.*, 2006; Duquesne *et al.*, 2008; Bryan *et al.*, 2009; Arsène-Ploetze *et al.*, 2010), that grow optimally around pH 5. Microbial metabolism in AMD causes natural attenuation process to occur whereby *Thiomonas* strains are suggested to have acted as the As(III) oxidizer (Casiot *et al.*, 2003; Duquesne *et al.*, 2003; Morin *et al.*, 2003; Bruneel *et al.*, 2006; Battaglia-Brunet *et al.*, 2011; Bertin *et al.*, 2011). Oxidation of As(III) plays a pivotal role in the natural bioremediation since it could contribute to an improved immobilization of arsenic in helping to mitigate arsenic contamination. *Thiomonas* strains are therefore essential for maintaining the arsenic biogeochemical cycle in AMD. Freel *et al.* (2015) showed that several phylogenetic groups of *Thiomonas* strains populated along Regious creek AMD for more than a decade even though in low abundance with differences in the As(III) oxidation capabilities. Accumulation of evolutionary changes in the harsh environment of AMD may promote unique adaptive phenotypes especially in the *Thiomonas* Aio.

Thiomonas delicata DSM 16361, isolated from Cheni gold mine, shows high As(III) oxidizing capability of 4 mg As(III) l^{-1} hr^{-1} which grows optimally at pH 4 to 7 as a moderate acidophile (Battaglia-Brunet *et al.*, 2006). Most interestingly, purification and detailed characterization of Aio from facultative chemolithoautrophic As(III) oxidizing *Thiomonas* strains have yet to be described. The characterization of the Aio is of great importance as we could further explore the enzymatic features that contribute to the irreplaceable role of *Thiomonas* strains as an As(III) oxidizer in AMD.

This could facilitate the engineering of enzymes and aid in construction of robust biosensor for monitoring arsenic in acid mining effluent. Therefore, in this study, by performing heterologous expression and purification of the Aio from *T. delicata* DSM 16361 strain deposited in German Collection of Microorganisms and Cell Cultures (DSMZ), we characterized the Aio in terms of its biochemical properties. Homology modeling of the enzyme was constructed to support the experimental findings. In order to explore the potential of Aio as biosensing material for As(III) detection, Aio was applied to the electrochemical device in this study. Since cyclic voltammetry is the most commonly used and versatile electroanalytical technique for the study of redox enzyme, thus it was employed to characterize the mediated Aio for biosensing ability. To the best of our knowledge, this is the first characterization study of Aio from a moderate acidophilic chemolithoautotrophic betaproteobacterium.

1.2 Problem statement

To date, there is no commercially available biosensor to detect As(III) which is the most toxic inorganic arsenic. Only one study of enzyme based As(III) biosensor has been reported using *Rhizobium* sp. NT-26 Aio (Male *et al.*, 2007). Currently, most of the arsenic test strips detect total arsenic content are expensive, low specificity and reproducibility. In arsenic remediation process, first stage pre-oxidation of As(III) is preferential to transform the arsenic species to the less soluble form As(V) for precipitation. Therefore, assessment of efficient conversion of As(III) is crucial. Moreover, As(III) is the prevalent species in anoxic drinking groundwater, hence specific detection of this bioavailable arsenic species is critical.

Additionally, molybdenum containing enzymes represent an essential group of enzyme in biology, Aio is one of it. Due to the difficulty to purify and express this exotic enzyme, to date only Aio from *Alcaligenes faecalis* and *Rhizobium* sp. NT-26 have been purified and characterized in details (Anderson *et al.*, 1992; Ellis *et al.*, 2001; Santini *et al.*, 2004). Biochemical characterization has been performed to Aio from *Arthrobacter* sp. 15b. However, no detailed studies has been presented for biochemical characterization of purified Aio from *T. delicata*, which is a dominant As(III) oxidizing genus found at AMD (Freel *et al.*, 2015). The absence of the three-dimensional structure for *T. delicata* Aio in PDB prompted us to construct its homology model for the acidophilic adaptation. By using *in silico* approach, a more comprehensive study about the structural characteristic of Aio can be obtained to further improve our knowledge concerning the adaptation of Aio from an acidophilic bacteria.

1.3 Objective of study

This study was carried out to investigate the characterization of Aio from *Thiomonas delicata* DSM 16361. The specific objectives were as following:

(i) to clone and express of arsenite oxidase gene *aioBA* in *E. coli*

(ii) to purify and characterize recombinant Aio

(iii) to model the recombinant Aio structure and elucidate its acidophilic adaptation through *in silico* analysis

(iv) to apply Aio for the construction of As(III) biosensor

1.4 Scope of study

In this study, *T. delicata* strain was purchased from DSMZ under DSM number 16361. The *aioBA* gene was amplified from genomic DNA of *T. delicata* by PCR to obtain full length gene sequence. After verification of the sequence, the *aioBA* was cloned to pET21a expression vector and transformed into *E. coli* cells. Placement of polyhistidine tag to *aioB* was then performed for the aid of affinity purification system. Optimization of expression was performed. After that, purification of recombinant Aio was carried out, which was affinity and gel filtration chromatography. Purity of recombinant Aio was verified using SDS-PAGE. Characterization of Aio was performed in terms of biochemical studies and *in silico* studies. Homology modeling of Aio was constructed to allow comparison with other crystallized Aio structure. Finally, application of Aio to biosensor construct was attempted to reveal the potential of Aio as a biosensing element.

1.5 Significance of study

In the present study, *aioBA* from *T. delicata* DSM 16361 was successfully amplified and cloned to *E. coli*. Expression of functionally active recombinant Aio allowed two-step purification of Aio. Biochemical properties of recombinant Aio were elucidated, which filled the research gap about the potential inhibitors of Aio. New insights of *T. delicata* Aio adaptation to low pH were revealed from the structural information of Aio through homology model. Comparison of *T. delicata* Aio

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