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An Arsenate Reductase Homologue Possessing Phosphatase Activity from Sweet Potato (*Ipomoea batatas* [L.] Lam): Kinetic Studies and Characterization

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ABSTRACT: A cDNA encoding a putative arsenate reductase homologue (IbArsR) was cloned from sweet potato (Ib). The deduced protein showed a high level of sequence homology (16−66%) with ArsRs from other organisms. A 3-D homology structure was created based on AtArsR (PDB code 1T3K) from *Arabidopsis thaliana*. The putative active site of protein tyrosine phosphatase ($HC(X)_5R$) is conserved in all reported ArsRs. IbArsR was overexpressed and purified. The monomeric nature of the enzyme was confirmed by 15% SDS-PAGE and molecular mass determination of the native enzyme via ESI Q-TOF. The IbArsR lacks arsenate reductase activity but possesses phosphatase activity. The Michaelis constant (K_M) value for *p*-nitrophenyl phosphate (pNPP) was 11.11 mM. The phosphatase activity was inhibited by 0.5 mM sodium arsenate [As(V)]. The protein's half-life of deactivation at 25 °C was 6.1 min, and its inactivation rate constant K_d was 1.1×10^{-1} min^{-1}. The enzyme was active in a broad pH range from 4.0 to 11.0 with optimum activity at pH 10.0. Phosphatase would remove phosphate group from nucleic acid or dephosphorylation of other enzymes as regulation signaling.

KEYWORDS: sweet potato (*Ipomoea batatas* [L.] Lam), three-dimensional homology structure (3-D homology structure), expression, arsenate reductase, arsenate [As(V)], phosphatase, *p*-nitrophenyl phosphate (pNPP)

INTRODUCTION

Sweet potato grows easily and is widely distributed in Taiwan. It is considered as one of the cash crops sustaining the agricultural economy. This local crop is highly regarded in Taiwan. Several components from sweet potato have been shown to exhibit antioxidative effects.[1−4] Although sweet potato shows physiological activities with potential medical applications,[5] several scientific studies have been reported. This encourages us to search active enzymes or components from sweet potato for use.

Arsenic is considered as a global contaminant. Arsenic exposure is known to be associated with increased risk of a variety of diseases, including cancer and diabetes.[6,7] Phytoremediation is considered to be one of the most cost-effective treatments, although it is currently under development. In order to study whether sweet potato can be used as a good candidate for phytoremediation, it is necessary to investigate the metabolism of arsenic in sweet potato. Arsenic in the environment exists in two inorganic oxidation states, arsenate [As(V)] and arsenite [As(III)]. As(V) and As(III) enter plant cells via phosphate transporters and aquaglyceroporins, respectively.[8] Once taken up, As(V) is reduced to As(III), catalyzed largely by arsenate reductases (ArsRs), members of the superfamily of protein tyrosine phosphatase (PTPase).[9,10] As(III) can then be complexed with glutathione (GSH) or phytochelatins (PCs). Thus, arsenate reductase was our first choice of enzyme to study. An ArsR homologue cDNA from sweet potato was cloned base on the sequence homology of ArsR from other sources.

Here, we report cloning of a putative IbArsR cDNA from sweet potato. The coding region of the cDNA was introduced into an *E. coli* expression system. The target protein was overexpressed and purified and its properties investigated. We expected to see the participation of the enzyme in the reduction of arsenate [As(V)] via recycling of the GSH (reduced glutathione) pool. However, this enzyme lacks arsenate reductase activity but showed phosphatase activity as demonstrated by its ability to convert pNPP to *p*-nitrophenol (pNP).

MATERIALS AND METHODS

Sweet Potato (*Ipomoea batatas* [L.] Lam). Sweet potato was grown in a field located in the northern part of Taiwan.

Total RNA Preparation and cDNA Synthesis. Tuberous root of sweet potato (wet weight 30 g) was frozen in liquid nitrogen and ground to powder in a ceramic mortar. PolyA mRNA (20 μg) was prepared using Straight A's mRNA Isolation System (Novagen). Three micrograms of mRNA was used in 5′-RACE-Ready cDNA and 3′-RACE-Ready cDNA synthesis using Clontech's SMART RACE cDNA Amplification Kits.

Isolation of ArsR cDNA. Using the 3′-RACE-Ready cDNA of sweet potato as a template and UPM primer (universal primer A mix, purchased from BD biosciences) and a degenerate primer (5′-GCC

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ATC RTY GAY GTC AGG-3′), a 0.55 kb fragment was amplified by PCR. The degenerate primer was designed based on the conserved sequences of ArsR from AtArsR (*Arabidopsis thaliana*, Nucleotide accession no. NM_120425, Protein accession no. NP_568119), OsArsR (*Oryza sativa*, Nucleotide accession no. AY860059, Protein accession no. AAX54896), PvArsR (*Pteris vittata*, Nucleotide accession no. DQ310370, Protein accession no. ABC26900), HlArsR (*Holcus lanatus*, Nucleotide accession no. AY704470, Protein accession no. AAU11500). The 0.55 kb fragment was subcloned and sequenced. On the basis of this DNA sequence, a reverse primer (5′-CCA GCC ATT ATA ACC ACG-3′) near the 5′ end of the 0.55 kb fragment was synthesized. The reverse primer allowed sequence extension to the 5′ end of the 0.55 kb fragment. Using the 5′-RACE-Ready cDNA of sweet potato as a template, a 0.45 kb fragment was amplified by PCR. This DNA fragment was subcloned and sequenced. Sequence analysis revealed that the combined sequence of the 0.45 and 0.55 kb covered an open reading frame of IbArsR cDNA (699 bp, EMBL accession no. GQ465431). The identity of IbArsR cDNA clone was assigned by comparing the inferred amino acid sequence in various databanks using the basic local alignment search tool (BLAST).

Computational Analysis. The BLAST program was used to search homologous protein sequences in the nonredundant database at the National Center for Biotechnology Information, National Institutes of Health (http://www.ncbi.nlm.nih.gov/). Multiple alignments were constructed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Structural modeling was carried out by using the SWISS-MODEL program[11] (http://swissmodel.expasy.org/SWISS-MODEL.html) to create a 3-D homology model based on the known X-ray structure of AtArsR (PDB code 1T3K) [12] from *A. thaliana*. The modeling data was then superimposed with the ArsR-like ScCdc25 (PDB code 3FS5)[13] from *S. cerevisiae* by the SPDBV_4 program.

Construction of the Coding IbArsR cDNA in an Expression Vector. The coding region of the IbArsR cDNA was amplified using gene-specific flanking primers. The 5′ upstream primer contains *Eco*RI recognition site (5′-GAATTCG ATG ACT CGG AGC ACC ACC-3′), and the 3′ downstream primer contains His6-tag and *Xho*I recognition site (5′-CTCGAG GCT TTG ATT TGA TTG TTG- 3′). Using 0.2 μg of IbArsR cDNA as a template, and 10 pmol of each 5′ upstream and 3′ downstream primers, a 0.42 kb fragment was amplified by PCR. The fragment was ligated into pCR4 and transformed into *E. coli* ECOS 101. The recombinant plasmid was isolated and digested with *Xho*I and *Eco*RI. The digestion products were separated on a 1.5% agarose gel. The 0.42 kb DNA insert was gel purified and subcloned into *Eco*RI and *Xho*I sites of pET-20b(+) expression vector (Novagen) and introduced into *E. coli*. The recombinant protein was overexpressed in *E. coli* Rosetta (DE3) pLysS and its function identified by activity assay as described below.

Expression and Purification of the Recombinant IbArsR. The transformed *E. coli* Rosetta (DE3) pLysS containing the IbArsR was grown at 32 °C in 100 mL of Luria−Bertani containing 55 μg/mL ampicillin and 12.5 μg/mL chloramphenicol until A_{600} reached 0.6. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. The culture was incubated at 130 rpm for an additional 24 h at 15 °C. The cells were harvested and soluble proteins extracted in 20 mM Tris-HCl (pH 7.0)/5 mM dithiothreitol with glass beads as described before.[14] The recombinant IbArsR was purified by Ni-NTA affinity chromatography (elution buffer 20 mM Tris-HCl/5 mM dithiothreitol (pH 7.0) containing 60−100 mM imidazole) as per the manufacture's instruction (Qiagen). The purified protein was checked by 15% SDS-PAGE. Protein bands on the gel were detected by staining with Coomassie Brilliant Blue R-250. Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

Molecular Mass Analysis via Electrospray Ionization Quadrupole-Time-of-Flight (ESI Q-TOF). The protein sample (125 μg/250 μL) in 0.5 mM Tris-HCl (pH 7.0)/0.1 mM dithiothreitol containing 0.05% glycerol was shipped to Yao-Hong Biotechnology Co. (Taiwan) for molecular mass determination using an ESI Q-TOF mass spectrometer (Micromass, Manchester, England).

Arsenate Reductase Activity Assay. Arsenate reductase was assayed by measuring NADPH oxidation according to Shi et al.[15] with some modification. The reaction mixture contained 5 mM MOPS/MES buffer (pH 7.0), 3 μg of IbGR (*I. batatas* glutathione reductase,[16]), 0.6 μg of IbGrx (*I. batatas* dithiol glutaredoxin, homemade), 1 mM GSH, and 10 μg of IbArsR. The reaction was started by addition of 0.2 mM NADPH/50 mM sodium arsenate. The reaction was followed by a decrease in A_{340} due to oxidation of NADPH and reduction of As(V). This assay method is generally used to determine the arsenate reductase activity.

Phosphatase Activity Assay, Kinetic and Inhibition Studies. The phosphatase activity of the recombinant IbArsR was tested at 25 °C by monitoring the amount of *p*-nitrophenol (pNP) produced at A_{405} as *p*-nitrophenyl phosphate (pNPP), a phosphotyrosine analog, became dephosphorylated.[17] The reaction mixture contained 20 mM Tris-HCl (pH 7.9) and 10 μg IbArsR in a total volume of 100 μL. The reaction was started by addition of 20 mM pNPP. The reaction progress was followed by the increase in A_{405} due to the dephosphorylation of pNPP for a period of 3 min. The kinetic properties of the IbArsR (10 μg) were determined in a total volume of 100 μL assay mixture containing 20 mM Tris-HCl (pH 7.9) and various concentrations of pNPP (2.85 to 40 mM) with the fixed amount of either 0.5 or 1.5 mM sodium arsenate as an inhibitor. The absorbance at 405 nm was recorded between 30 s and 3 min. The absorption coefficient of pNP at 405 nm is ε =18 000 M^{-1} cm^{-1}. K_M, V_{max}, and k_{cat} were calculated from Lineweaver−Burk plots. Inhibition kinetics of phosphatase activity by sodium arsenate was performed using various concentrations of pNPP (2.85−40 mM) with two fixed amount (0.5 or 1.5 mM) of sodium arsenate as inhibitor. The residue activity was monitored by the increase in A_{405}.

Enzyme Characterization. The phosphatase activity of recombinant IbArsR was tested under various conditions. Aliquots of IbArsR were subjected to various treatments as described below and then assayed for activity.

(1) Effect of heat on enzyme activity. Enzyme samples were preheated at 25 °C for 2, 4, 8, or 16 min, respectively.
(2) Effect of pH on enzyme activity. Enzyme sample was adjusted to the desired pH by adding a one-half volume of buffer with different pHs: 0.2 M citrate buffer (pH 2.5 or 4.0), 0.2 M potassium phosphate buffer (pH 6.0, 7.0, or 8.0), or 0.2 M CAPS buffer (pH 10.0 or 11.0). Each sample was incubated at 4 °C for 30 min.

RESULTS

Cloning, Sequence Alignment, and Structure Modeling of a cDNA Encoding IbArsR. A putative IbArsR cDNA clone was identified based on the consensus pattern and sequence homology to the published ArsRs. The IbArsR cDNA (699 bp, EMBL accession no. GQ465431) contains an open reading frame which encodes a protein of 139 amino acid residues with a predicted molecular mass of 15.5 kDa. Figure 1A shows the amino acid sequence alignment of the putative sweet potato IbArsR with ArsRs from several sources. The IbArsR shared 66%, 57%, 54%, 38%, and 16% sequence identity with ArsR from AtArsR (*A. thaliana*, Nucleotide accession no. NM_120425, PDB code 1T3K), HlArsR (*H. lanatus*, Nucleotide accession no. AY704470, Protein accession no. AAU11500), OsArsR

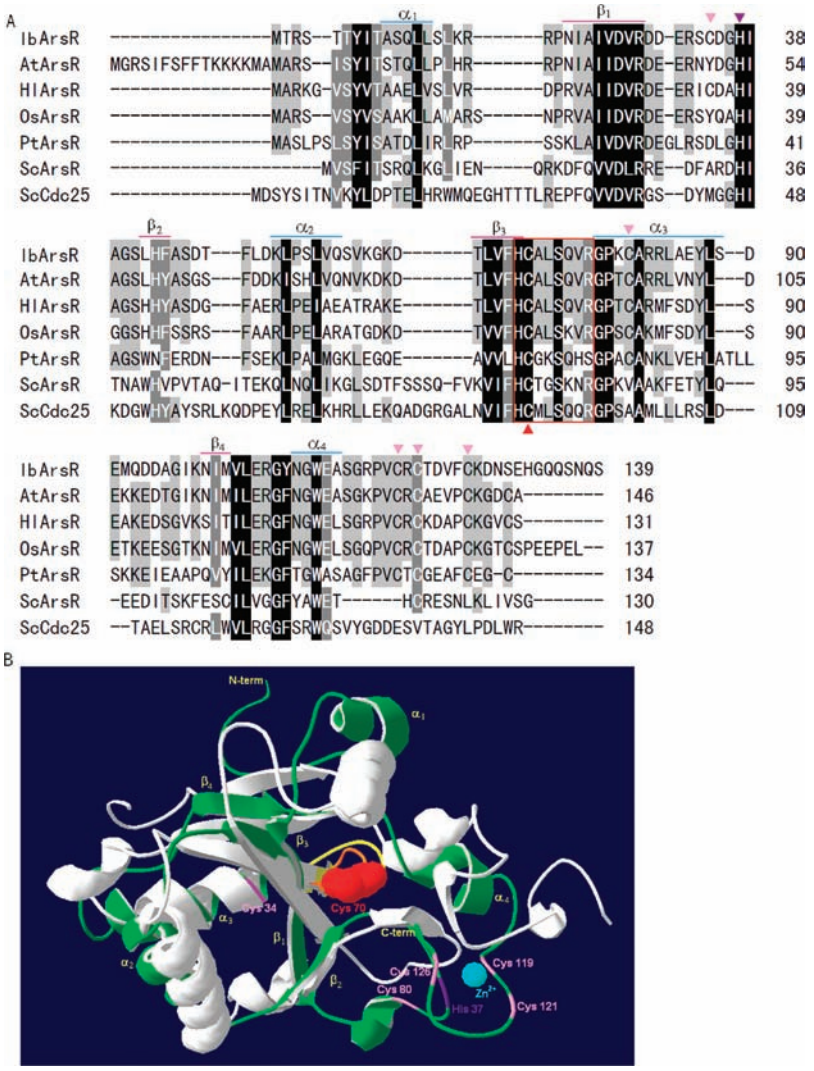

Figure 1. Optimal alignment of the amino acid sequences of IbArsR from various sources and homology structure prediction. (A) Alignment of amino acid sequence of IbArsR (present study), AtArsR (*A. thaliana*), HlArsR (*H. lanatus*), OsArsR (*Oryza sativ*), PvArsR (*P. vittata*), ScArsR (*S. cerevisiae*), and ScCdc25 (ArsR-like) (*S. cerevisiae*). Conservative replacements are shaded gray. The protein secondary structure was predicted by the SWISS-MODEL program and represented as α helices and *β* strands. $HC(X)_5R$ domain (red box) denotes the amino acids catalytic site. (B) Three-dimensional homology model of IbArsR. The model was created by SWISS-MODEL based on the known X-ray structure of AtArsR. They were superimposed with the SPDBV_4 program. Superimposition of IbArsR (green) and ArsR-like ScCdc25 (white) is shown using protein solid ribbon. Upper red ball (Cys^{70}) represents the active site. Pink triangles denote Cys residues that might involve intramolecule disulfide bond formation.

(*O. sativa*, Nucleotide accession no. AY860059, Protein accession no. AAX54896), PvArsR (*P. vittata*, Nucleotide accession no. DQ310370, Protein accession no. ABC26900), ScArsR (*S. cerevisiae*, Protein accession no. Q06597), ArsR-like ScCdc25 (*S. cerevisiae*, Nucleotide accession no. NM_001181332, PDB code 3FS5). A highly conserved motif $HC(X)_5R$ was observed in the alignment. This is a consensus-active site motif conserved in members of PTPases superfamily,[9,18] where H at position 69 is a conserved histidine residue, C at position 70 is the catalytic cysteine residue, the five X residues at position 71−75 form a loop, and R at position 76 is a conserved arginine. A structural model of IbArsR was created based on the known structure of ArsR from *A. thaliana* (PDB code 1T3K) via the SWISS-MODEL program and superimposed with ArsR-like ScCdc25 (PDB code 3FS5) via the SPDBV_4 program to obtain a better structure. Superimposition of IbArsR (green) and ArsR-like ScCdc25 (white) was shown using a protein solid ribbon. The upper red ball represents the putative active site (Figure 1B).

Expression and Purification of the Sweet Potato IbArsR. The cDNA was introduced into the *E. coli* Rosetta (DE3) pLysS expression system as described in the Materials and Methods. The recombinant IbArsR protein was overexpressed and the total cellular proteins analyzed by 15% SDS-PAGE (Figure 2). The IbArsR fusion protein was purified by Ni-NTA affinity chromatography. The progress of purification was monitored by SDS-PAGE as shown in Figure 2. The results showed that the recombinant IbArsR protein was purified to near homogeneity in a single step. A double band with an apparent molecular mass of about 18.1 kDa was observed in eluted fractions (Figure 2, lanes 4−9). It is believed that Cys^{34}, Cys^{70}, Cys^{80}, Cys^{119}, Cys^{121}, and Cys^{126} are far apart enough to form intramolecular disulfide bonds (Figure 1B). Therefore, it is possible that the lower band represents a more compact recombinant IbArsR with intramolecular disulfide bonds

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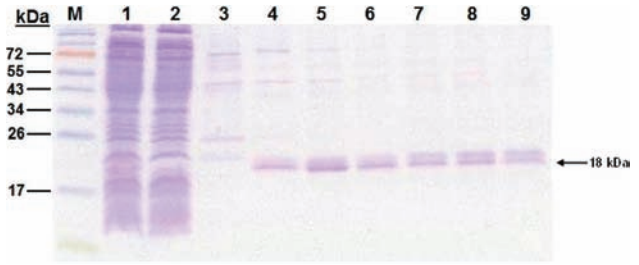

Figure 2. Coomassie Brilliant Blue R-250-stained SDS-polyacrylamide gel showing the purification of IbArsR. Fifteen microliters of each fraction was loaded per lane. Lane 1, crude extract from *E. coli* Rosetta (DE3) pLysS expressing IbArsR; lane 2, flow-through proteins from the Ni-NTA column; lane 3, wash; lanes 4−9, bound IbArsR eluted from Ni-NTA column by 60−100 mM imidazole. Molecular masses (in 18 kDa) of standards are shown on the left.

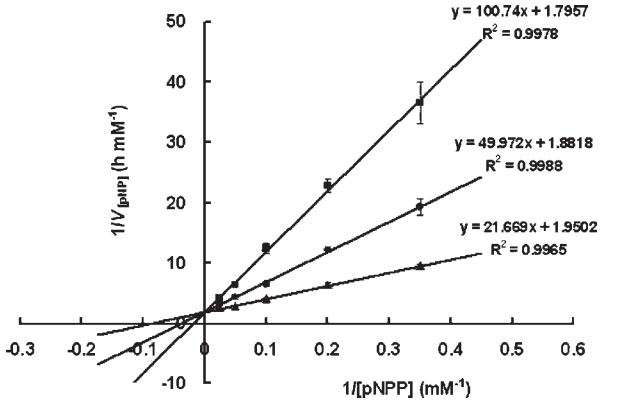

Figure 3. Double-reciprocal plot showing the effect of competitive inhibition on IbArsR activity. The initial rate of the enzymatic reaction was measured at pNPP concentration varying from 2.85 to 40 mM in the absence of inhibitor or two inhibition concentrations (0.5 or 1.5 mM sodium arsenate). K_M, V_{max}, and k_{cat} were calculated from Lineweaver−Burk plots.

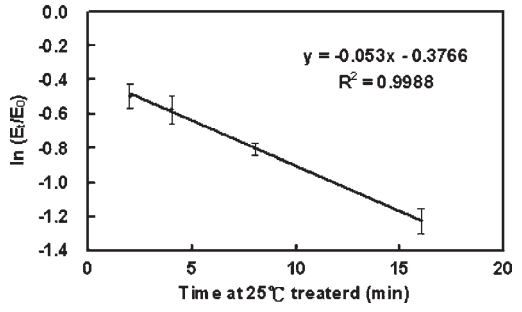

Figure 4. Effect of temperature on the purified IbArsR. The enzyme sample was at 25 °C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8, or 16 min and then assayed for phosphatase activity (10 μg of protein/reaction). Plot of inactivation kinetics. E_0 and E_t are original activity and residual activity at 25 °C for different time intervals. Data are means of three experiments.

formed between pairs of these Cys residues. The mass of native recombinant IbArsR was 18.1 kDa as analyzed using ESI Q-TOF. The result demonstrated that the IbArsR protein is monomeric under the conditions of 0.5 mM Tris-HCl (pH 7.0)/0.1 mM

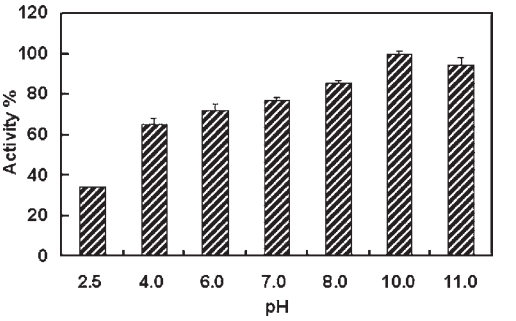

Figure 5. Effect of pH on the purified IbArsR. The enzyme samples were incubated in buffers with different pH values at 4 °C for 30 min and then assayed for phosphatase activity (10 μg of protein/reaction). Data are means ± SD of three similar experiments. A significant increase activity in the level of pH with increasing pH from 4.0 to 10.0 was observed ($p < 0.01$).

dithiothreitol/0.05% glycerol. The yield of the purified IbArsR was 1.1 mg per 100 mL of culture.

Kinetic and Inhibition Studies of the Purified IbArsR. As shown in Figure 3, the Lineweaver−Burk plot of the velocity ($1/V$) against 1/pNPP in the absence of inhibitor gave K_M = 11.11 mM, V_{max} = 147 nmol min^{-1}mg^{-1}, and k_{cat} = 0.03 s^{-1}. The plot of the velocity ($1/V$) against 1/pNPP for two inhibitor concentrations gave K_i = 0.39 and 0.41 mM, respectively.

Characterization of the Purified IbArsR. Thermal effect of the recombinant IbArsR was examined as described in the Materials and Methods. The enzyme appeared to be heat labile. The enzyme's inactivation kinetics at 25 °C fitted the first-order inactivation rate equation $\ln(E_t/E_0) = -K_d t$, where E_0 and E_t represented the original activity and the residual activity after treating for time t, respectively. The inactivation rate constant (K_d) calculated for the enzyme at 25 °C was 1.1 × 10^{-1} min^{-1}, and the half-life of inactivation was 6.1 min (Figure 4). The recombinant IbArsR has an optimal pH of 10.0 (Figure 5).

DISCUSSION

This study reported the first cloning and expression of an important enzyme, recombinant ArsR, from sweet potato. The recombinant IbArsR has been successfully expressed in *E. coli*. Rosetta (DE3) pLysS. On the basis of the sequence homology, the enzyme belongs to arsenate reductase, a member of the PTPase superfamily. However, the enzyme lacks arsenate reductase activity under our assay conditions but possesses PTPase activity. Our initial aim was to clone an ArsR cDNA that can express its arsenate reductase activity for investigating the metabolism of arsenic in sweet potato and possibly be applied to phytoremediation. Although the IbArsR lacks arsenate reductase activity under the current assay condition, it shows the PTPase activity, dephosphorylation. We will continue searching arsenate reductase either by cloning new ArsR cDNA or by the site-direct mutagenesis technique to alter this ArsR cDNA. We scanned the IbArsR sequence against Swiss-Prot Prosite to locate potential post-translational modification sites. Although several potential phosphorylation sites (Ser^{15}, Ser^{33}, Ser^{59}, Ser^{73}, Ser^{89}, Ser^{130}, Thr^{5}, and Tyr^{7}) were found, further studies are required to determine if these post-translational modifications affect the structure and function of recombinant IbArsR.

We compared the kinetic data of the recombinant IbArsR with that of other available sources. As shown in Table 1, the IbArsR

Table 1. Kinetic Characterization of IbArsR and Other Published ArsRs[a]

protein	substrate	K_M (mM)	V_{max} (nmol $min^{-1}mg^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
IbArsR	pNPP	11.1	147	0.03	2.7
OsACR2.1	pNPP	15.4	180	0.50	32.4
OsACR2.2	pNPP	15.0	58	0.16	10.4
LinACR2	pNPP	36.0	230	0.06	1.7
ScAcr2i)	pNPP	l.8	1	3×10^{-4}	0.2

[a] The kinetic parameter was determined as described in the Materials and Methods. The K_M value for pNPP was determined at 2.85−40 mM pNPP. Data represent the mean (±SE) of three separate experiments. Values are from this work [IbArsR (*I. batatas* ArsR)] or from the literature OsACR2.1 and OsACR2.2 [*O. sativa* ArsR[20,21]], LmACR2 [*L. major* ArsR[18]], and ScAcr2p [*S. cerevisiae* ArsR[19]].

has the second lowest K_M value for pNPP (11.11 mM). Therefore, the IbArsR can use a lower concentration of pNPP to dephosphate efficiently.

The PTPase activity of the recombinant IbArsR was competitively inhibited by sodium arsenate. It has been reported that LmArsR and ScArsR were inhibited by sodium arsenate.[17,19] The enzyme is active at 25 °C with a half-life of 6.1 min at that temperature. It was evident that the recombinant IbArsR was active in a pH range from 8 to 11 with optimal pH at 10.0

In conclusion, we cloned an IbArsR cDNA and successfully expressed the enzyme in *E. coli* Rosetta (DE3) pLysS. The recombinant enzyme showed phosphatase activity and labile under at 25 °C.

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Author Contributions

[#]C.-Y.L. and J.-K.H. contributed equally to first author.

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