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Characterization of a Recombinant Thermostable Arylsulfatase from Deep-Sea Bacterium *Flammeovirga pacifica*

Chao Gao[1†], Min Jin[2†], Zhiwei Yi[2], and Runying Zeng[2,3,4*]

[1]*XingHuaLing District Food and Drug Administration, Taiyuan 030000, P.R. China*
[2]*State Key Laboratory Breeding Base of Marine Genetic Resource; Third Institute of Oceanography, SOA, Xiamen 361005, P.R. China*
[3]*Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Xiamen 361005, P.R. China*
[4]*South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, Guangzhou 510000, P.R. China*

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*Corresponding author
Phone: +86-592-2195323;
Fax: +86-592-2195323;
E-mail: zeng@tio.org.cn

†These authors contributed equally to this work.

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A novel sulfatase gene, *ary423* (1,536 bp ORF), encoding a protein of 511 amino acids with a calculated molecular mass of 56 kDa, was identified from *Flammeovirga pacifica*, which was isolated from deep-sea sediments of west Pacific Ocean. Amino acid sequence analysis revealed that Ary423 possessed a conserved C-X-A-X-R motif, which was recognized as the sulfatase signature. Phylogenetic analysis suggested that Ary423 belonged to arylsulfatases. After heterologous expression in *Escherichia coli* cells, the recombinant Ary423 was purified with a Ni^{+} affinity column, and was shown to be highly active at a broad range of temperatures from 30° to 70°C, with maximum activity at 40°C. Furthermore, recombinant Ary423 retained more than 70% and 40% of its maximum activity after 12 h of incubation at 50°C and 60°C, respectively, exhibiting good thermostability at high temperatures. The optimal pH for Ary423 was determined to be 8.0 and the activity of Ary423 could be slightly enhanced by Mg^{2+}. The recombinant enzyme could hydrolyze sulfate ester bonds in *p*-nitrophenyl sulfate (NPS) and *Asparagus* crude polysaccharides with a specific activity of 64.8 U/mg and 25.4 U/mg, respectively. These favorable properties could make Ary423 attractive for application in the desulfating process of agar production.

Keywords: Arylsulfatase, deep sea, *Flammeovirga pacifica*, thermostability, characterization

Introduction

Sulfatase (E.C. 3.1.6.X) is a highly conserved family of enzymes found from prokaryotes to eukaryotes. It can cleave the sulfate ester bond to liberate inorganic sulfate and the corresponding alcohol [28]. Sulfatases act on a broad diversity of substrates ranging from complex glycoplipids and glycosaminoglycans to amino acids and sulfated hydroxyl steroids [32], which leads to their classification to at least three mechanistically distinct groups, named as arylsulfatases, dioxygenase, and alkylsulfatases [26, 33].

Arylsulfatases (E.C. 3.1.6.1) represent a class of hydrolytic enzymes involved in the hydrolysis of arylsulfate esters to aryl compounds and inorganic sulfate. Arylsulfatases are distributed in a wide range of organisms from bacteria to mammals and their primary and tertiary structures are highly conserved although they originate in different species [3, 13]. Notably, arylsulfatases undergo a unique post-translational modification that converts cysteine or serine to formylglycine in their active site [33]. These unique modifications are mediated by sulfatase-maturating enzymes through recognition of the conserved motifs (C/S)-X-P-X-R [8, 30] or (C/S)-X-A-X-R [2], which are regarded as the "sulfatase signature" and are described as being essential for the production of formylglycine and proper conformation of the arylsufatase active site.

To date, arylsulfatases from eukaryotes are the best studied [33]. In mammals, arylsulfatases are involved in various metabolic processes and are implicated in several diseases [1, 21, 27]. In contrast to the roles of arylsulfatases in mammals, the roles of bacterial arylsulfatases primarily

lie in the assimilation and dissimilation of sulfate [6], or response to sulfate starvation [5]. Furthermore, arylsulfatases are also widely applied in the desulfatation of agar in industry, since the existence of sulfate in agar causes weakened gel strength by interfering with the formation of cross-linked structure between the molecules [19].

So far, considerable advances have been made in revealing the role of arylsulfatases in the marine environments. In algae, arylsulfatases have been reported to facilitate the dissimilation and assimilation of sulfate in the cell [7]. In several marine animals that feed on algae, arylsulfatases were secreted as digestive enzymes to improve the digestion and absorption of marine polysaccharides such as carrageenan, fucoidan, and porphyran [14]. There have also been several studies reporting sulfatases isolated from marine bacteria [18, 19, 23, 26]. However, to our best knowledge, no arylsulfatases from deep-sea organisms have been extensively characterized, particularly the arylsulfatases from deep-sea bacteria. In our previous study, a novel algal polysaccharides degradation bacterium, *Flammeovirga pacifica*, was isolated from deep-sea sediments of west Pacific Ocean [35]. Although the bacteria of the genus *Flammeovirga* have been proved to be able to digest complex polysaccharides, little is known about the enzymatic ability of *Flammeovirga* sulfatase towards polysaccharides, including agar. In this context, characterization of *Flammeovirga* sulfatase may be helpful to reveal the mechanism underlying the unique polysaccharides digestion ability of *Flammeovirga*.

In our study, a novel arylsulfatase gene (*ary423*) was identified from *Flammeovirga pacifica*. After its overexpression in *E. coli*, the enzymatic properties of the purified recombinant Ary423 protein were characterized for desulfatation of *p*-nitrophenyl sulfate (NPS) and crude polysaccharides from *Asparagus*.

Materials and Methods

Overexpression and Purification of Recombinant Ary423

The isolation and identification of *F. pacifica* from the deep-sea sediments of west Pacific Ocean have been described in our previous report [35]. Notably, *F. pacifica* has been proved to produce a wide range of extracellular enzymes that are implicated in agar hydrolysis [4]. In a search throughout the scanned genome of *F. pacifica* [4], a putative sulfatase gene, designated as *ary423*, was identified. The *ary423* gene was amplified from the genomic DNA of *F. pacifica* with a forward primer (5′-GTACTCGAGCGG ATGAAAAGCTCTCT-3′) and a reverse primer (5′-ACGGGA TCCCGTTAATTCTGTGGTT-3′), which contained the recognition sequences for *Xho*I and *Bam*HI (underlined), respectively. The amplicon was digested with *Xho*I and *Bam*HI, and then inserted into vector pET-His (Biovector, China), which was linearized with the same restriction enzymes. For the overexpression and purification of 6×histine-tagged Ary423 fusion protein (11 amino acids were added to Ary423 protein), vector pET-His-*ary423* as well as the empty pET-His vector were transferred into *E. coli* BL21(DE3) cells, separately. The recombinant *E. coli*-pET-His-*ary423* and *E. coli*-pET-His cells were grown at 37°C in LB medium containing 100 μg/ml ampicillin with shaking, until the OD_{600} of the cultures reached 0.5–0.6. Then the protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at the final concentration of 1 mM for an additional 12 h at 16°C. The bacteria were harvested and sonicated for 20 min at a pulse frequency of 3 s/3 s. After centrifugation for 15 min at 20,000 ×*g*, the supernatant was collected and the recombinant proteins were purified with a Ni-NTA affinity column according to recommendations of the manufacturer (Qiagen, Germany). The obtained recombinant Ary423 protein was resolved by glycine-SDS-PAGE and stained with Coomassie brilliant blue for visualization.

Enzyme Activity Assay

Generally, the activity of Ary423 was assayed by measuring the amount of p-nitrophenol released from NPS. Briefly, 1 ml of diluted enzyme was incubated with 250 μl of NPS solution (25 mM, pH 8.0) for 30 min. The enzymatic reaction was stopped by addition of 1 ml of NaOH solution (0.5 M), and the amount of released *p*-nitrophenol was quantified spectrophotometrically at the wavelength of 410 nm. One unit of enzymes was defined as the amount of enzyme that produced 1 μmol *p*-nitrophenol per minute under standard experimental conditions.

The enzymatic activity of Ary423 towards *Asparagus* crude polysaccharide was determined using the $BaCl_2$-Gelatin method described by Dodgson [10]. Diluted enzyme (2 ml) was incubated with crude polysaccharide dissolved in 2 ml of Tris-HCl buffer (pH 8.0) at 40°C for 1 h. Then supernatant of the reaction solution (400 μl) was mixed with 3.6 ml of 3%TCA (dissolved in 1 M HCl) and 1 ml of 0.5% $BaCl_2$-Gelatin (Sangon Biotech, China). After incubating the mixture solution at room temperature for 15 min, the amount of sulfate produced was determined by measuring the absorbance at 360 nm.

Characterization of Recombinant Ary423

The optimum temperature for maximum Ary423 activity was determined by conducting the enzyme activity assay at various temperatures (30°C–80°C) in Tris-HCl buffer (50 mM, pH 8.0). The thermostability of recombinant Ary423 was evaluated by measuring the residual enzyme activity after incubating the enzyme in Tris-HCl buffer (50 mM, pH 8.0) at different temperatures in the absence of substrate for various time durations (1, 2, 4, 8, and 12 h).

The optimum pH was determined by incubating Ary423 in the following buffers at 40°C: 50 mM Na_2HPO_4/citric acid solution (pH 4.0–7.0), 50 mM Tris-HCl buffer (pH 7.0–9.0), or 50 mM Gly/

NaOH buffer (pH 9.0–11.0) [25, 34].

To assess the effects of various additives, including metal ions, chelators, and reducing reagents, on Ary423 activity, various ions and reagents of different final concentrations (10 and 100 mM) were added to the reaction mixture, and the residual activity of Ary423 was assayed under the standard conditions as described above. The following agents were used for this purpose: metal ions (Ca^{2+}, Mn^{2+}, Cu^{2+}, K^{+}, Na^{+}, Fe^{3+}, Mg^{2+}, and Zn^{2+}), reducing reagents (urea, DTT and β-mercaptoethanol (β-ME)), and chelators (EDTA).

Sequence Analysis

The similarities of DNA and protein sequences were analyzed with BLASTN and BLASTP programs, respectively (http://www.ncbi.nlm.nih.gov/BLAST). The comparison of sequence homology was carried out using the ClustalW program ver. 2.1. The MEGA program ver. 5.1 (DNAstar, USA) with neighbor-joining method was used to generate the phylogenetic tree based on sulfatase amino acid sequences.

Accession Numbers

The DNA and amino acid sequences of Ary423 are available in the GenBank database under the accession number KP642149. The strain *F. pacifica* providing the target gene has been deposited in BCCM/LMG (LMG26175), DSMZ (DSM 24597), and China Marine Culture Collection Center (MCCC, Accession No. 1A06425).

Results

Sequence Analysis of Ary423

According to gene prediction, a 1,536 bp ORF (GenBank Accession No. KP642149, designated as *ary423*), encoding a potential sulfatase of 512 amino acids with a calculated molecular mass of 56 kDa, was identified in the genome of *F. pacifica*, which was isolated from deep-sea sediments of west Pacific Ocean. Although the DNA sequence of *ary423* had no significant similarity with existing DNA sequences in the NCBI database (less than 50% identity), the encoded Ary423 protein was highly homologous to known sulfatases. The amino acid sequence of Ary423 shared 80%, 79%, 78%, and 75% identities with those of *Echinicola pacifica*

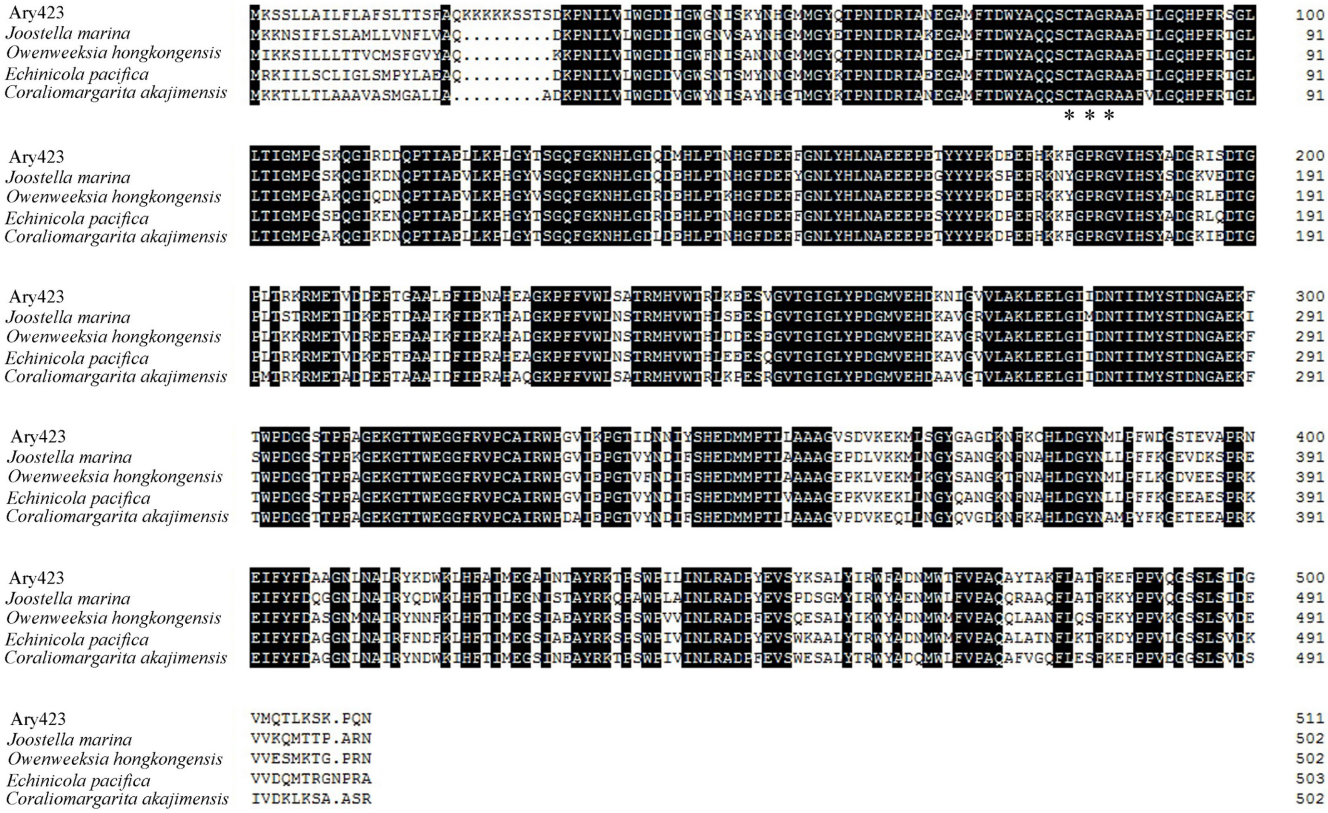

Fig. 1. Alignment of amino acids of Ary423 and arylsulfatases of other bacteria.

Multiple alignments of amino acid sequences of sulfatase derived from different bacteria are shown. The sulfatases aligned are from *F. pacifica* (GenBank Accession No. KP642149), *Joostella marina* (gi|495888632), *Owenweeksia hongkongensis* (gi|503966432), *Echinicola pacifica* (gi|648544026), and *Coraliomargarita akajimensis* (gi|502808952). The identical residues through all aligned sequences are shaded black. The conserved C-X-A-X-R motif is indicated with asterisks (X, denotes positions without a clear residue consensus).

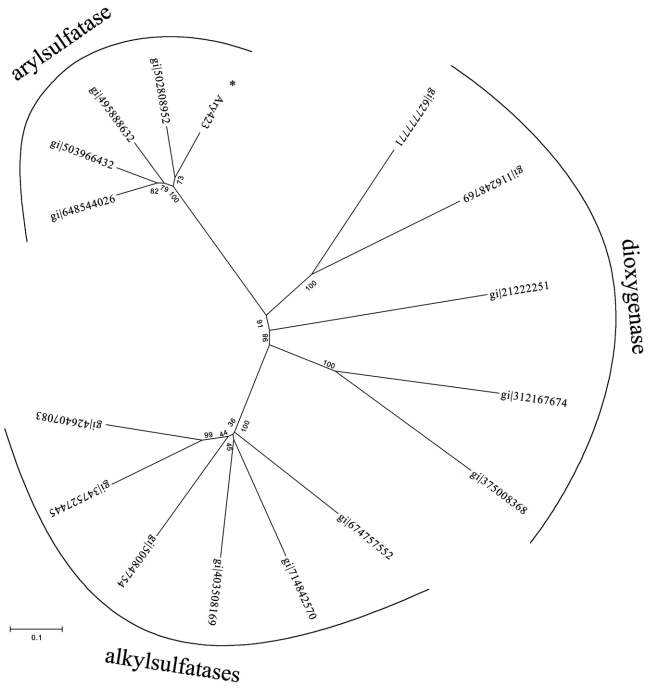

Fig. 2. Phylogenetic tree of sulfatases based on amino acid sequences.

Amino acid sequences of sulfatases belonging to different types were retrieved from the NCBI database. The tree was constructed with MEGA 5.1 software by the neighbor-joining method. Numbers showing bootstrap values of 1,000 trials are presented. The scale bar indicates 10 nucleotide substitutions per 100 nucleotides. The position of Ary423 is indicated with an asterisk. The analyzed sulfatases were from *Coraliomargarita akajimensis* (gi|502808952), *Echinicola pacifica* (gi|648544026), *Owenweeksia hongkongensis* (gi|503966432), *Joostella marina* (gi|495888632), *Rubrobacter radiotolerans* (gi|627777771), *Burkholderia rhizoxinica* HKI 454 (gi|312167674), *Streptomyces coelicolor* A3 (gi|21222251), *Rhizobium leguminosarum* bv. *viciae* 3841 (gi|116248769), *Geobacillus thermoleovorans* CCB_US3_UF5 (gi|375008368), *Ralstonia pickettii* (gi|674757552), *Burkholderia* sp. MSHR4009 (gi|714842570), *Sphingobium* sp. SYK-6 (gi|347527445), *Nocardiopsis alba* ATCC BAA-2165 (gi|403508169), *Acinetobacter* sp. ADP1 (gi|50084754), and *Pseudomonas* sp. UW4 (gi|426407083).

(gi|648544026), *Coraliomargarita akajimensis* (gi|502808952), *Joostella marina* (gi|495888632), and *Owenweeksia hongkongensis* (gi|503966432), respectively (Fig1). As shown in Fig. 1, Ary423 possesses the conserved C-X-A-X-R motif (Cys82, Ala84, Arg86), which is regarded as the "sulfatase signature" and proposed to be responsible for the activation and proper conformation of the active site of sulfatases [2, 9].

Based on their enzyme activities and substrate specificities, sulfatases can be divided into at least three mechanistically distinct groups [26]. To determine the subgroup of Ary423, amino acid sequences of representative sulfatases belonging

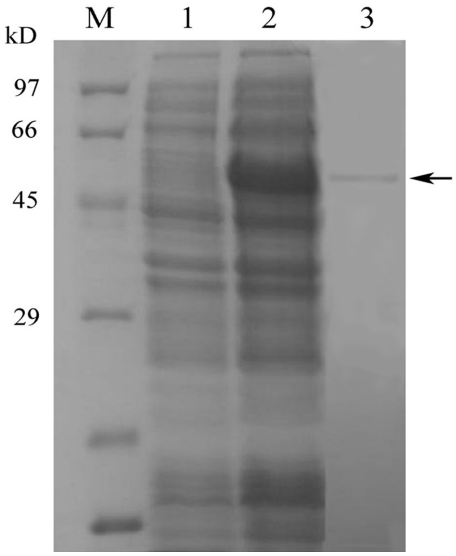

Fig. 3. Overexpression and purification of recombinant Ary423.

SDS-PAGE of overexpressed and purified recombinant Ary423 is shown. Lane M, protein marker; Lane 1, *E. coli*-pET-His-*ary423* cell extract, non-induced; Lane 2, *E. coli*-pET-His-*ary423* cell extract, induced. Lane 3, purified recombinant Ary423. The arrow indicates the protein band corresponding to recombinant Ary423.

to different groups were retrieved from the NCBI database and a phylogenetic tree was further constructed to compare the sequence homology. As shown in Fig. 2, Ary423 clustered with representative arylsulfatases in the phylogenetic tree, suggesting that Ary23 is one of the arylsulfatases.

Heterologous Expression and Purification of Recombinant Ary423 Protein

The *ary423* gene was cloned into the pET-His expression vector and was heterologously overexpressed in *E. coli* BL21 (DE3) cells as an N-terminally His-tagged recombinant protein. In comparison with non-induced cells, the induced *E. coli*-pET-His-*ary423* cultures revealed the presence of a new protein with an approximate molecular mass of 58 kDa, which corresponded to the recombinant Ary423 protein (Fig. 3, lane 2), suggesting the successful overexpression of recombinant Ary423 protein. After purified with a Ni^{+} affinity column, the recombinant Ary423 was observed as a single band on the SDS-PAGE gel (Fig. 3, lane 3). Purified Ary423 was shown to be enzymatically active against NPS and crude polysaccharides with a specific activity of 64.8 U/mg and 25.4 U/mg, respectively, indicating its possible application in the desulfatation of crude polysaccharides.

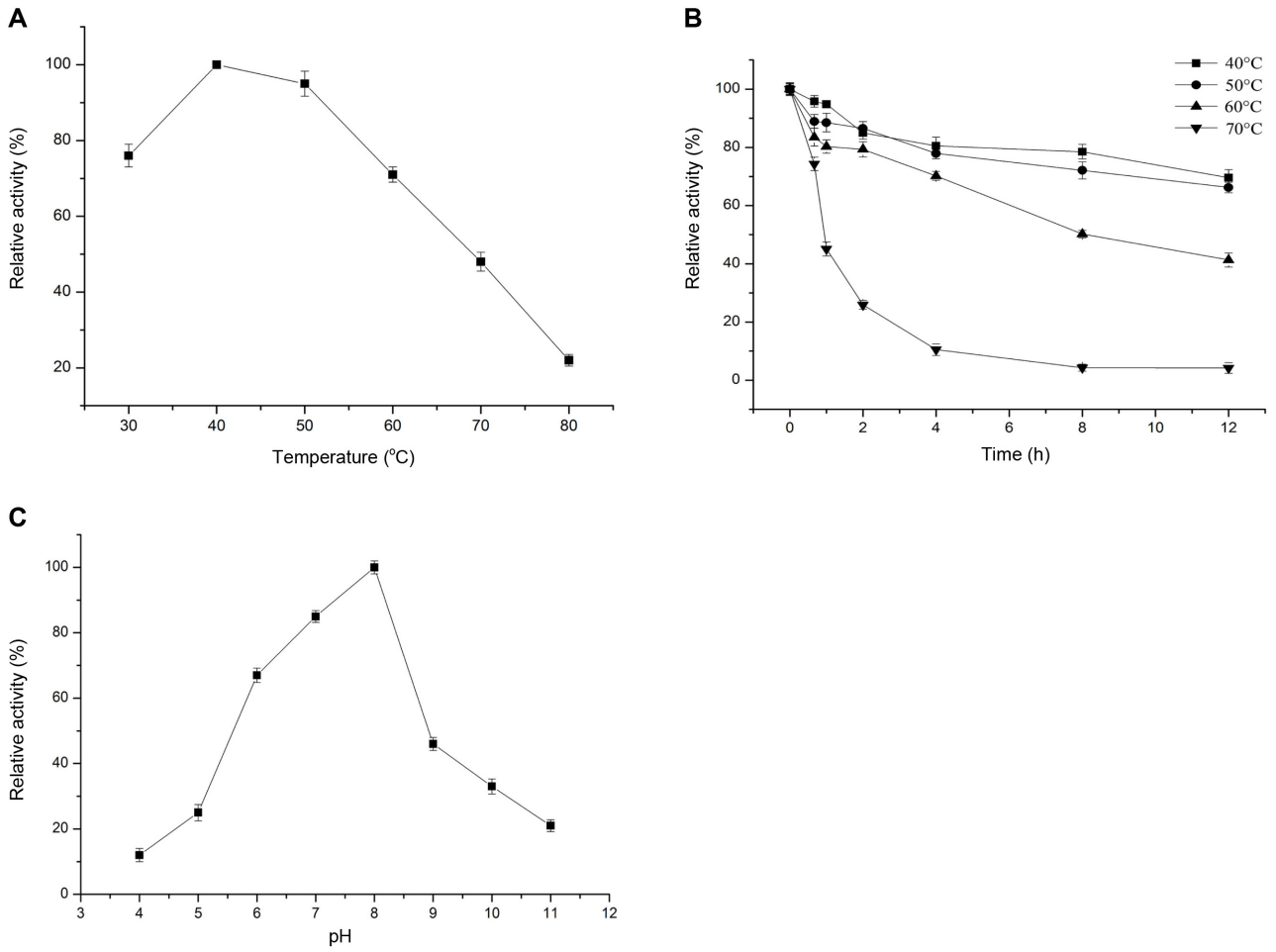

Fig. 4. Temperature and pH effects on the activity and stability of recombinant Ary423.
(**A**) Temperature effects on the activity of Ary423. The activity of Ary423 was measured at various temperatures ranging from 30°C to 80°C under standard assay conditions. Values are shown as percentages of the maximum activity of Ary423 (taken as 100%) and are expressed as the mean of triplicate independent experiments with standard deviation. (**B**) Temperature effects on the stability of Ary423. The residual activity of Ary423 was determined after incubating Ary423 in the absence of substrate at 40°C, 50°C, 60°C, or 70°C for different time durations. (**C**) pH effects on the activity of Ary423. The optimum pH was determined by incubating Ary423 in the following buffers at 40°C: pH 4.0 to 7.0, Na_2HPO_4/citric acid solution; pH 7.0 to 9.0, Tris-HCl buffer; pH 9.0 to 11, Gly/NaOH buffer.

Characterization of the Recombinant Ary423

The effect of temperature on the activity of recombinant Ary423 was investigated at various temperatures ranging from 30°C to 80°C (Fig. 4A). The maximum activity of Ary423 was observed at 40°C. Recombinant Ary423 was active over a wide range of temperatures from 30°C to 80°C, and the enzyme retained approximately 70% and 50% of its maximum activity at 60°C and 70°C, respectively, indicating that the enzyme was able to adapt to high temperature environments (Fig. 4A). Additionally, Ary423 retained more than 70% and 40% of its maximum activity after 12 h of incubation at 50°C and 60°C, respectively, exhibiting good thermostability at high temperatures (Fig. 4B). The pH profiles showed that Ary423 was active over a broad range of tested pH values from 4.0 to 11.0, with maximum activity observed at pH 8.0 (Fig. 4C).

The effects of various additives, including metal ions and other chemical reagents, on the activity of Ary423 are summarized in Table 1. Among the metal ions tested, Mg^{2+} could slightly enhance the activity of Ary423, and non-significant activated or inhibitory effect on Ary423 activity was observed by several metal ions (Na^+, K^+, Ca^{2+}). In contrast, the activity of Ary423 was strongly inhibited in the presence of several metal ions (Fe^{3+}, Zn^{2+}, Mn^{2+}, Cu^{2+})

Table 1. Effects of metal ions and chemical reagents on Ary423 activity.

Agent	Conc. (mM)	Relative activity (%)	Agent	Conc. (mM)	Relative activity (%)
Na^{+}	100	99.7	Cu^{2+}	10	66.8
K^{+}	100	99.6	Mn^{2+}	10	68.7
Mg^{2+}	100	100.5	Urea	10	66.1
Ca^{2+}	10	99.1	DTT	10	55.3
Zn^{2+}	10	78.1	β-Me	10	42.5
Fe^{3+}	100	65.6	EDTA	10	32.5

and chemical reagents (urea, DTT, β-Me, EDTA).

Discussion

So far, several sulfatase activities have been reported from various organisms in the marine environment, including algae [7], mollusks [14], and bacteria [18, 19]. However, little is known about the sulfatases isolated from deep-sea environments. Evidence has accumulated that extreme environments, particularly deep-sea environments, may breed novel microbes and enzymes with distinct activities and potential industrial value [15, 24]. Here, a thermostable arylsulfatase was isolated from a deep-sea bacterium, *Flammeovirga pacific* [35], and its enzymatic activities toward NPS and *Asparagus* crude polysaccharides were characterized. Therefore, our study represents what is believed to be the first report that characterizes an arylsulfatase derived from deep-sea environments. It provides us with a preliminary work to uncover the role of sulfatase in deep-sea environments.

The sequence analysis showed that the DNA sequence of *ary423* had no significant similarity with existing DNA sequences in the NCBI database (less than 50% identity), whereas the encoded Ary423 protein was highly homologous to known sulfatases, implying the distinct codon bias of *F. pacifica*, which may be related with the unique feature of deep-sea environments. The amino acid sequence analysis indicated that Ary423 bears a (C/S)-X-(A/P)-X-R motif, which is known to be conserved across all known members of the sulfatase family and is essential for sulfatase activities [2, 8, 30]. In this light, Ary423 can be identified as a typical member of the sulfatase superfamily. Our phylogenetic analysis results allowed the further classification of Ary423 as an arylsulfatase, which is consistent with the substrate specificity of Ary423 towards NPS and *Asparagus* crude polysaccharides.

As a mesophilic enzyme isolated from marine bacteria, Ary423 exhibited good thermostability. Our results showed that the enzyme was highly active at a broad range of temperatures from 30°C to 70°C, with a maximum activity at 40°C. Most strikingly, Ary423 retained more than 70% and 40% of its maximum activity after 12 h of incubation at 50°C and 60°C, respectively, which implied an overwhelming superiority over other known mesophilic arylsulfatases [16, 18, 19, 23]. In order to find clues for the thermostablity of Ary423, we attempted to compare the amino acid sequences between thermophiles and *F. pacifica* arylsulfatase. However, bacterial arylsulfatases, especially thermostable arylsulfatases derived from bacteria, are rare. To our best knowledge, only one thermostable bacterial arylsulfatase (arylsulfatase from *Thermotoga maritima,* NCBI Accession No. NP_229503) has been reported to date [22]. Sequence analysis showed that Ary423 shared only 10.08% amino acid identity with the arylsulfatase from *Thermotoga maritima*, which greatly hindered our search for clues of the arylsulfatase thermostability based on their protein primary sequence. Furthermore, we tried to seek clues of the thermostability from the 3D structure of Ary423 based on homology modeling using the Swiss-model program. Unfortunately, the most homologous template for Ary423 is the crystal structure of human placental arylsulfatase (PDB No. 1p49), which shared very low homology with Ary423 and thus led to a low-quality model of the Ary423 3D structure that was not fit for further analysis. In this context, further work such as crystal structural determination is merited to be performed in order to reveal the mechanisms underlying the arylsulfatase thermostability. According to pH optimum, arysulfatases derived from bacteria can be divided into two classes, with one class showing maximum activity at pH values of 6.5–7.1 and the other class with a higher optimal pH of 8.3–9.0 [17]. The purified recombinant Ary423 is active over a wide range of tested pH from 4.0 to 11.0 and displayed its maximum activity at pH 8.0, which thus falls into the second class of arylsulfatases.

According to the mechanism of sulfate ester cleavage proposed by Schmidt *et al*. [31], the hydrate form of the arylsulfatase active site FGly attacks the sulfate ester,

leading to break of the S-O bond and the formation of a covalent enzyme-sulfate intermediate. The strong inhibitory effect of EDTA on the Ary423 enzyme activity observed in this study is indicative that divalent cations (*i.e.*, Mg^{2+}) may participate in the catalytic process. This hypothesis was further supported by the observation that Mg^{2+} could slightly enhance the activity of Ary423 towards NPS. Mg^{2+} presumably stabilizes negative charges, which is developed in the sulfate during the nucleophilic attack by arylsufatase with the hydroxyl group of FGly.

In industry, arylsulfatase derived from bacteria can be applied to desulfate the sulfated polysaccharides, such as agar and agarose [19, 23]. It has been reported that sulfate content is an important quality criterion for agar and agarose [20], since the incorporation of a sulfate group in agar or agarose can result in weakness of the gel strength by blocking double helices formation between the molecules [11]. Although reduction of the sulfate content in agarose has been successfully achieved using the methods of fractionation and selective adsorption [12, 29], both of the methods result in a low yield of agarose. However, enzymatic hydrolysis of sulfate by arylsulfatase can simplify the agarose production process and increase the agarose recovery dramatically, making arylsulfatase a good candidate for agarose desulfatation in industry. Therefore, our study presents a novel arylsulfatase with the promising advantage of thermostablilty and wide range of operating temperatures, which may be attractive for application in agarose production under elevated temperature conditions.

Acknowledgments

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