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Substitution of His260 residue alters the thermostability of *Pseudoalteromonas carrageenovora* arylsulfatase

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Abstract

This study aimed to improve the thermostability of arylsulfatase from *Pseudoalteromonas carrageenovora*. A library of *P. carrageenovora* arylsulfatase mutants was constructed by introducing random mutagenesis using error-prone PCR. After screening, two mutants of H260L and D84A/H260L showed enhanced thermal stability than the wild-type predecessor (WT). Site-directed mutagenesis demonstrated that only amino acid residue at Position 260 plays an important role in the thermostability of *P. carrageenovora* arylsulfatase. Thermal inactivation analysis showed that the half-life ($t_{1/2}$) values at 55°C for H260L, H260I, H260Q, H260F and H260R were 40.6, 48.4, 30.9, 29.1 and 34.5 min, respectively, while that of WT was 9.1 min. Structure modeling demonstrated that the additional hydrogen bonds and/or optimization of surface charge-charge interactions could be responsible for the increased thermostability imparted by H260L, H260I, H260Q, H260F and H260R.

Key words: arylsulfatase, *Pseudoalteromonas carrageenovora*, directed evolution, error-prone PCR, thermostability

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1 Introduction

Arylsulfatases catalyze the hydrolysis of arylsulfate ester bonds to release free sulfonates, which are widespread in nature and usually found in microorganisms, plant seeds, and most animal or human tissues (Stressler et al., 2016b). A number of arylsulfatases have been characterized from microorganisms, such as *Flammeovirga pacifica* (Gao et al., 2015), *Klebsiella aerogenes* (Okamura et al., 2008), *Klebsiella pneumoniae* (Miech et al., 1998), *Kluyveromyces lactis* (Stressler et al., 2016a), *Marinomonas* sp. (Wang et al., 2015), *Pseudoalteromonas carrageenovora* (Kim et al., 2005), *Pseudomonas aeruginosa* (Marino et al., 2013), *Salmonella typhimurium* (Henderson and Milazzo, 1979), *Serratia marcescens* (Murooka et al., 1980), *Sphingomonas* sp. (Kim et al., 2004), and *Thermotoga maritima* (Lee et al., 2013). Arylsulfatases have potential applications in many fields, such as cancer detection (Niu et al., 2012), increasing the inorganic sulfate of soil (Blum et al., 2013), improving the quality of agar (Wang et al., 2015), doping analysis and food processing (Stressler et al., 2016b).

Agar, the main cell wall structure polysaccharide of red algae, is widely used in biotechnology, food and pharmaceutical industries. It is composed of agarose and agaropectin (Duckworth and Yaphe, 1971). The presence of sulfate groups in agar leads to a low gel strength due to the avoidance of a cross-linked structure during gelation (Arnott et al., 1974). Two possibilities to obtain high-quality agar with increased gel strength are either separation of agarose from agaropectin (Duckworth and Yaphe, 1971; Izumi, 1970) or removal of sulfate groups from agaropectin (Guiseley, 1970). The former method resulted in poor extraction yields and high commercial costs (Cregut and Rondags, 2013). The latter is a traditional method by using alkaline treatment usually accompanied with some drawbacks, such as the reduction of agar yields, having difficulty to control the agar quality, and the environmental pollution. Hence, new methods are urgently needed in producing high-quality agar. Some arylsulfatases have been reported to have good potentials in agar desulfation and quality improvement (Kim et al., 2004; Lee et al., 2013; Lim et al., 2004; Wang et al., 2015). The enzymatic method has many advantages, such as high specificity, mild reaction conditions, and low environmental pollution (Cregut and Rondags, 2013). Thus, the efficient and specific enzymatic desulfation of agaropectin is a promising alternative to the current techniques used in agar industry.

The recombinant arylsulfatase from *P. carrageenovora* has

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activity towards *p*-nitrophenyl sulfate (*p*NPS) and activity in desulfation of agar (Kim et al., 2005; Lim et al., 2004). The thermostability of this enzyme is not reported (Kim et al., 2005). Previously, the arylsulfatase gene (984 bp) from *P. carrageenovora* DSM No. 6820 was cloned in our laboratory, and we found that its sequence was exactly the same as the arylsulfatase gene *astA* from *P. carrageenovora* (Barbeyron et al., 1995; Lim et al., 2004). We further expressed this gene by using pET-28a(+) vector in *E. coli*, and found that the recombinant enzyme was not stable at and above 45°C. In order to use *P. carrageenovora* arylsulfatase as a biocatalyst in agar desulfation, it is desirable to improve its thermostability.

Directed evolution is mainly to simulate natural mutation and selective evolution under the Darwinian principle in the laboratory (Boersma et al., 2007). The main advantage of this method is that it does not require knowledge of the structure and function of the enzyme, and it is easy to improve the function and properties of the enzyme (Kumar and Singh, 2013). Among the directed evolution methods, error-prone PCR based mutation is a widely used method due to its simplicity and versatility (Ben Mabrouk et al., 2013; Lin et al., 2016). In this study, we used error-prone PCR based directed evolution to improve the thermostability of *P. carrageenovora* arylsulfatase, and then site-directed mutagenesis was carried out for illustration of the relationship between enzyme's microstructure and property.

2 Materials and methods

2.1 *Strain and nucleotide sequence*

Pseudoalteromonas carrageenovora DSM No. 6820 was purchased from Leibniz-Institut DSMZ. The nucleotide sequence of the arylsulfatase gene from *P. carrageenovora* is available in the GenBank database with accession number of KJ509595.

2.2 *Construction of E. coli containing the wide-type P. carrageenovora arylsulfatase gene*

Domain search was performed using SMART (Letunic et al., 2015), and *P. carrageenovora* arylsulfatase was found to contain a signal peptide with 18 amino acids length. We cloned the 930 bp of arylsulfatase gene except the signal peptide gene part and aimed to increase the solubility of the recombinant arylsulfatase. The gene was amplified from the bacterial genomic DNA by PCR with pfu DNA polymerase (TaKaRa) and a primer pair of the forward ars-F (5′-CGCGGATCCTTTACGTTTAACGGCAGC-3′, where the underline indicates the *Bam*HI site) and the reverse ars-R (5′-CCCAAGCTTGCGTTTTAGTTCGTAAC-3′, the underline indicates the *Hin*dIII site). After digestion with *Bam*HI and *Hin*dIII, the amplicon was inserted into the pET-28a(+) vector (Novagen) and the resulting construct was named pET-28a-ars. Once the sequence of the gene was confirmed by sequencing, the recombinant plasmid was introduced into *E. coli* BL21 (DE3) for gene expression.

2.3 *Construction of random mutagenesis library*

Random mutagenesis was performed by the error-prone PCR method using the primer pair (ars-F and ars-R) described as above and the plasmid pET-28a-ars as the template. PCR was conducted in 50 μL volume containing 5 μL 10× buffer (100 mmol/L Tris-HCl, pH 8.3, 500 mmol/L KCl), 0.2 μmol/L primers, 0.5 mmol/L dTTP, 0.5 mmol/L dGTP, 0.1 mmol/L dATP, 0.1 mmol/L dCTP, 1 U r*Taq* DNA polymerase (TaKaRa), 7 mmol/L $MgCl_2$, and 2 ng template DNA. The thermal cycling parameters were: 95°C for 5 min; 94°C for 45 s, 50°C for 45 s, 72°C for 1 min (35 cycles); and 72°C for 10 min. After digestion with *Bam* HI and *Hin* dIII, the PCR products were inserted into the pET-28a(+) vector to generate a recombinant plasmid library. After that, the plasmids were transformed into freshly prepared *E. coli* BL21 (DE3) competent cells to generate the mutant library.

2.4 *Screening the library for mutant with improved thermostability*

A two-step screening protocol was used for the selection of arylsulfatase mutant with improved thermostability. First, the transformants in the library were replicated on Luria-Bertani (LB) agar plates containing 50 μg/mL kanamycin, and incubated at 37°C for 16 h. Then, these transformants were transferred individually using toothticks to two sets of LB agar plates and incubated at 37°C for 10 h. One set of plates for screening were supplemented with 50 μg/mL kanamycin and 0.05 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG), and another set of plates for storage were supplemented with 50 μg/mL kanamycin. After the screening plates were treated at 50°C for 90 min, 0.4% (w/v) agar containing 5 mmol/L *p*NPS was covered on the plates. The transformants showing arylsulfatase activity (resulting in yellow colonies) were selected.

In the second-round screening, the above selected individual variant was selected from the above storage agar plates and grown in 5 mL LB medium with 50 μg/mL kanamycin at 37°C. After the OD_{600} reached 0.6, enzyme expression was induced by adding 0.05 mmol/L IPTG. After incubation at 16°C for 16 h, the cells in 5 mL culture were harvested by centrifugation (6 000× *g*, 5 min) at 4°C and washed with 50 mmol/L Tris-HCl (pH 7.5). The cell pellet was resuspended in 1 mL 50 mmol/L Tris-HCl (pH 7.5), and then the cells were lysed by intermittent sonication on ice. After centrifugation at 10 000× *g* for 20 min at 4°C, the supernatant was collected and treated at 55°C for 30 min. The residual activity of arylsulfatase in the supernatant was determined by the method reported by Kim et al. (2004). The mutant in which the residual activity was at least 10% higher than that of the wild-type enzyme was preliminarily considered to have improved thermostability. The enzyme gene sequence of the mutant was sequenced by Invitrogen Trading (Shanghai) Company.

2.5 *Gene expression and protein purification*

E. coli BL21 (DE3) harbouring the recombinant plasmid was cultured in 200 mL of LB medium containing 50 μg/mL kanamycin at 37°C until the OD_{600} reached 0.8, then IPTG was added to a final concentration of 0.05 mmol/L. After incubation at 16°C for 16 h, the induced cells were harvested by centrifugation at 6 000× *g* for 5 min at 4°C. Purification of the His-tagged protein was conducted using Ni sepharose 6 Fast Flow (GE Healthcare Life Sciences) affinity chromatography under the native conditions according to the method described previously (Zhu et al., 2015). The eluate was dialyzed twice against 50 mmol/L Tris-HCl buffer (pH 7.5). The protein concentration was determined by Bradford method (Bradford, 1976) with bovine serum albumin as the standard. The homogeneity of the purified enzyme and its molecular mass were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained by Coomassie Brilliant Blue.

2.6 *Arylsulfatase activity assay*

Arylsulfatase activity was assayed according to the method described by Kim et al. (2004) with slight modifications. Purified enzymes were used unless otherwise mentioned. In brief, the reaction mixture contained 20 μL of enzyme solution (0.4 μg) and 80 μL of substrate solution which was 50 mmol/L Tris-HCl buffer

(at enzyme's optimal pH value) containing 20 mmol/L *p*NPS. After incubation at 55°C for 10 min, the reaction was terminated by adding 25 μL of 5 mol/L NaOH. The amount of *p*-nitrophenyl released was determined by measuring the absorbance at 410 nm with a spectrophotometer (Cary 50, USA). One unit of arylsulfatase activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenyl per minute under the assay conditions.

2.7 *Influence of temperature on the activity and stability of the enzyme*

The optimal temperature for arylsulfatase was determined by measuring the enzyme activity at different temperatures ranging from 30 to 80°C with *p*NPS as the substrate. To investigate the thermal stability, the enzyme (0.4 μg) was incubated at 45, 50, 55 and 60°C over the period of 10–60 min, respectively. After the heat treatment, the samples were cooled on ice immediately. The residual activity was measured at 55°C by the standard method described above. The activity of the enzyme without heat treatment was taken as 100%.

2.8 *Effect of pH on enzyme activity*

To determine the optimal pH of arylsulfatase, enzyme activity at different pH values was determined at 55°C with *p*NPS as the substrate. The buffer systems were 50 mmol/L of citrate phosphate buffer (pH 5.0–7.0), Tris–HCl buffer (pH 7.0–9.0), and glycine–NaOH buffer (pH 9.0–11.0).

2.9 *Determination of K_m and V_{max} values*

Enzyme activity was measured with arylsulfatase (0.4 μg) and *p*NPS at different concentrations (0.1, 0.5, 1, 1.5, 2, 2.5 and 3 mmol/L) under optimal conditions for 10 min, respectively. The kinetic parameters of K_m and V_{max} were calculated by linear regression analysis of Lineweaver-Burk double-reciprocal plot.

2.10 *Sequence and structure analysis of the mutant derivatives*

The gene and protein sequence were analyzed by using DNAMAN 5.1 software (Lynnon BioSoft, Canada). The three-dimensional structural model of arylsulfatase was generated by the Modeller 9.16 software (Webb et al., 2007) using two protein crystal structures (PDB accession codes 2cbn and 4gcw) as the templates. The model quality was analyzed using the PROCHECK program (Laskowski et al., 1993). The PyMol Molecular Graphics System (DeLano Scientific LLC, San Carlos, CA, USA) was used for the visualization and analysis of the structure.

2.11 *Site-directed mutagenesis*

Site mutagenesis was conducted using the Site Directed Mutagenesis Kit (TaKaRa). The recombinant plasmid, pET-28a-ars, was used as the template. Once the sequence of the mutant enzyme gene was confirmed by sequencing, the recombinant plasmid with the mutant gene was transformed into *E. coli* BL21 (DE3).

2.12 *Statistical analysis*

All the experiments were conducted in replicates of three and the results are presented as mean±standard deviation (SD).

3 Results

3.1 *Construction and screening of random mutant library of P. carrageenovora arylsulfatase*

The randomly mutated arylsulfatase genes were generated by error-prone PCR. A mutant library with about 2×10^5 *E. coil* clones was constructed by inserting the mutant genes into pET-28a(+) vector. In the first-round library screening, about 1 000 clones were observed to display yellow color on the screening plates, which were subjected to the second-round screening for further identification. After two-step screening, two clones named 4-138 and 4-153 were observed to display higher arylsulfatase thermostability than the wide-type clone. Compared with parent arylsulfatase gene, one base and two bases were found different for 4-138 and 4-153 mutant enzyme genes, respectively. The mutant arylsulfatase 4-138 had a single amino acid substitution of H260L, and 4-153 had two alterations including D84A and H260L. The two mutant enzymes had the same alteration at Position 260. Mutant 4-138, mutant 4-153 and wild-type arylsulfatases were named H260L, D84A/H260L and WT, respectively.

3.2 *Key amino acid residue for arylsulfatase thermostability*

Mutagenesis was used to investigate the effect of amino acid alteration on enzyme's thermostability. Residue of Asp84 was substituted by Ala with the primer pair of D84A-F and D84A-R shown in Table 1. The wild-type and the mutant arylsulfatases were expressed and purified from *E. coli* as His-tagged fusion proteins. Protein bands of WT and mutant enzymes (D84A, H260L, D84A/H260L) were clearly detected on 12% SDS-PAGE (Fig. 1). After incubation at different temperatures, H260L and D84A/H260L could retain higher residual activity than WT, while D84A maintained lower residual activity than WT (Fig. 2). These results indicated that amino acid residue at Position 260 plays an important role in the thermostability of *P. carrageenovora* arylsulfatase.

In order to further confirm the influence of His260 site for thermostability, saturation mutagenesis of this site was carried out by site-directed mutagenesis. Eighteen pairs of primers were designed and used to generate the different amino acid alterations at Position 260 (Table 1). After incubation at 55°C for 30 min, mutants of H260L, H260F, H260Q, H260I and H260R re-

Table 1. The primers for mutant arylsulfatase genes

Primer	Sequence (5′ to 3′)
D84A-F	CGCAGTCTTTTTAAGTCACTTA
D84A-R	ACATTTTTTATGTCTAACCCAACTT
H260A-F	TAAACATCTTTCGAAGCACCAC
H260R-F	TAAACATCTTTCGAAGCACCAC
H260D-F	TAAACATCTTTCGAAGCACCAC
H260C-F	TAAACATCTTTCGAAGCACCAC
H260Q-F	TAAACATCTTTCGAAGCACCAC
H260E-F	TAAACATCTTTCGAAGCACCAC
H260I-F	TAAACATCTTTCGAAGCACCAC
H260G-F	TAAACATCTTTCGAAGCACCAC
H260N-F	TAAACATCTTTCGAAGCACCAC
H260K-F	TAAACATCTTTCGAAGCACCAC
H260M-F	TAAACATCTTTCGAAGCACCAC
H260F-F	TAAACATCTTTCGAAGCACCAC
H260P-F	TAAACATCTTTCGAAGCACCAC
H260S-F	TAAACATCTTTCGAAGCACCAC
H260T-F	TAAACATCTTTCGAAGCACCAC
H260W-F	TAAACATCTTTCGAAGCACCAC
H260Y-F	TAAACATCTTTCGAAGCACCAC
H260V-F	TAAACATCTTTCGAAGCACCAC
H260-R	ATGCCAATAAACTTACCCTTAGGCA

Note: The nucleosides underlined represent the coding bases of the amino acid alterations.

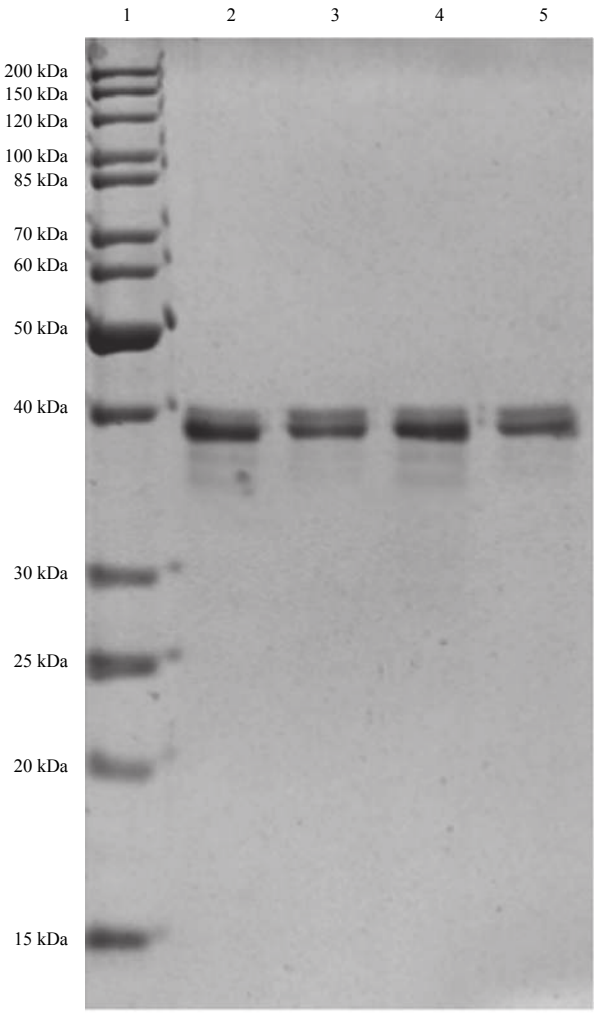

Fig. 1. Purified fusion proteins of arylsulfatase. Molecular weight markers (Lane 1), D84A (Lane 2), H260L (Lane 3), D84A/H260L (Lane 4) and WT (Lane 5).

tained the residual activity of 62%, 54%, 53%, 64% and 59%, respectively (Fig. 3). Meanwhile, WT only maintained 21% of residual activity (Fig. 3). These results revealed that these five mutants of H260L, H260F, H260Q, H260I and H260R exhibited higher thermostability than wild-type arylsulfatase. The thermostability of the mutants except H260L at different tempertures was also investigated. H260F, H260Q, H260I and H260R could maintain higher residual activity than WT after incubation at 50, 55 and 60°C (Fig. 4). The half-life ($t_{1/2}$) values at 55°C for H260L, H260I, H260Q, H260F and H260R were 40.6, 48.4, 30.9, 29.1 and 34.5 min, respectively, whereas that of WT was 9.1 min (Table 2).

3.3 *Other enzyme properties of arylsulfatase*

Comparisons of other enzyme properties between wild-type and mutant arylsulfatases were shown in Table 2. The optimal temperatures of all mutants were the same as that of WT. H260L, H260I, H260Q and H260F had the maximum activity at pH 8.0, while H260R and WT had optimal pH of 7.5. The specific activities of mutants and WT had no much difference. The K_m of H260L, H260Q and H260R was similar to that of WT indicating similar substrate binding ability. The K_m of H260I and H260F was lower and higher than that of WT, respectively. For these five mutants, the changes of V_{max} were similar to that of K_m.

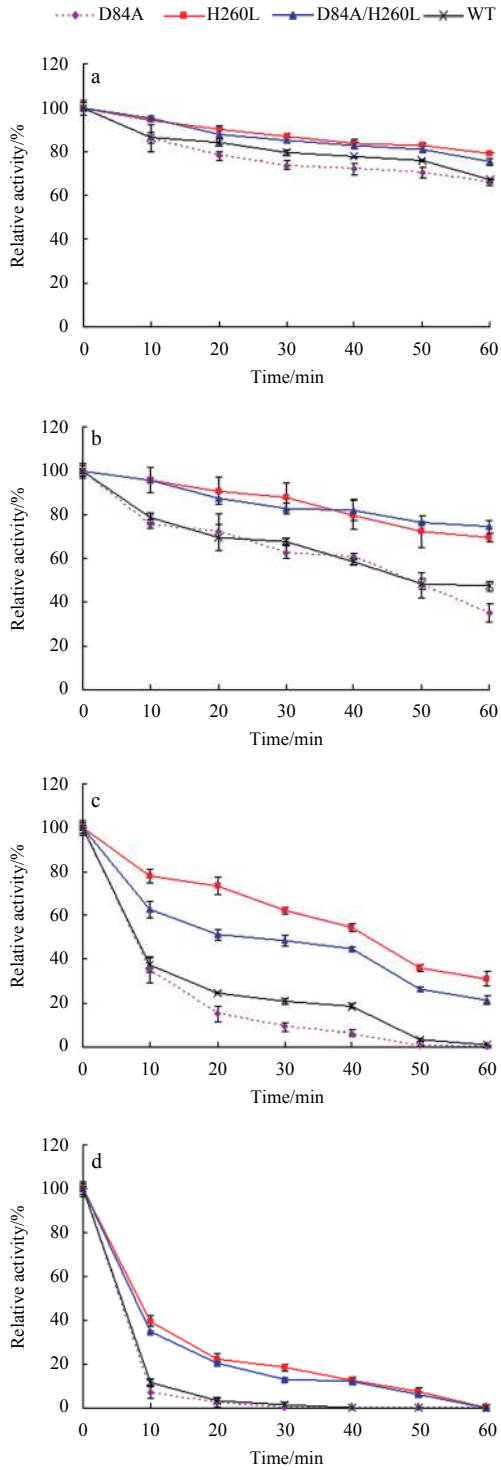

Fig. 2. Thermal stability of D84A, H260L, D84A/H260L and WT at 45°C (a), 50°C (b), 55°C (c) and 60°C (d). Activity of the enzyme before incubation was defined as 100%. Data are presented as mean±SD from three independent experiments.

3.4 *Homology modeling*

The three-dimensional structural model of arylsulfatase was modeled by the Modeller 9.16 software. The model quality analysis showed that 98.7% residues were in the core and allowed regions according to the Ramachandran plot (not shown). The mutation points, D84A and H260L, were located in the inner and

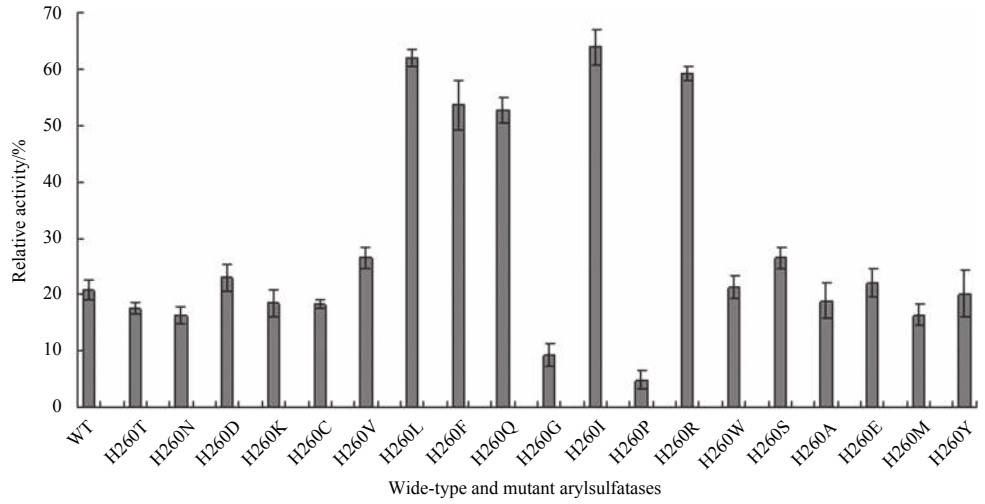

Fig. 3. Effect of amino acid residue at Position 260 on the thermal stability of arylsulfatase. After the enzyme was incubated at 55°C for 30 min, residual activity was measured to evaluate the enzyme's thermostability. Activity of the enzyme before incubation was defined as 100%. Data are presented as mean±SD from three independent experiments.

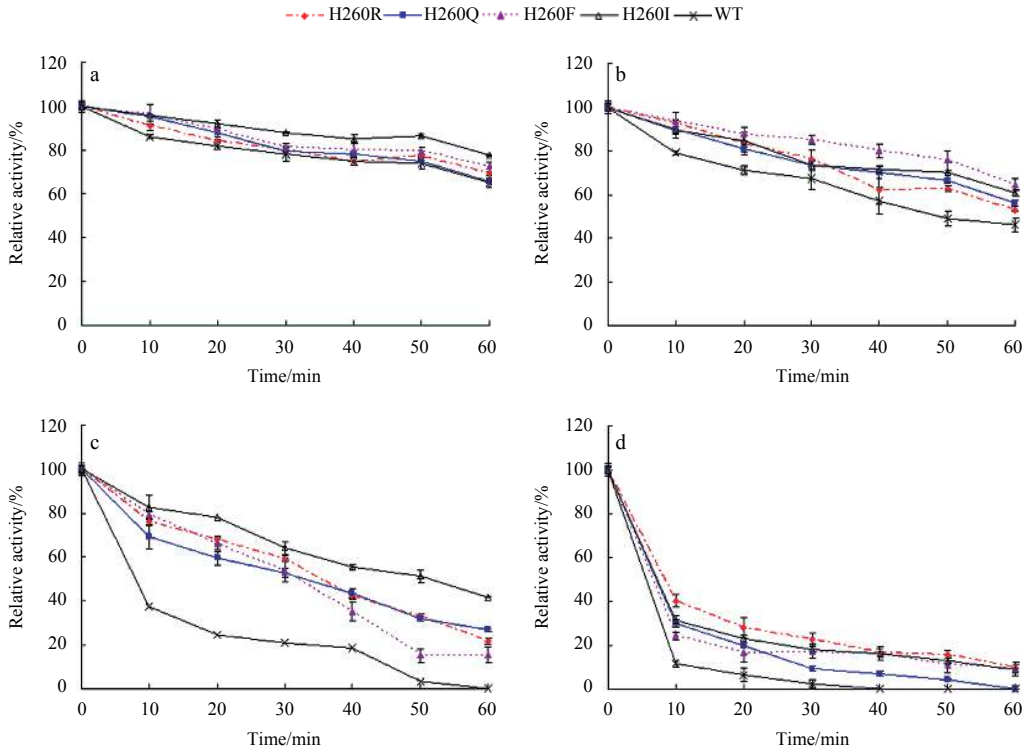

Fig. 4. Thermal stability of H260R, H260Q, H260F, H260I and WT at 45°C (a), 50°C (b), 55°C (c) and 60°C (d). Activity of the enzyme before incubation was defined as 100%. Data are presented as mean±SD from three independent experiments.

surface of the protein, respectively (Fig. 5).

4 Discussion

Arylsulfatase is an ideal candidate used to remove sulfate groups in agar with a high recovery rate. Since the desulfation process of agar takes place at temperature of about 45°C with hydrodynamically complex substrates (Stressler et al., 2016b), good thermostability of arylsulfatase will facilitate the industrial application of the enzyme in the liquid form of agar. Directed evolution is a powerful approach to improve the enzymatic properties such as activity, thermostability, and substrate specificity (Kumar and Singh, 2013). In this study, error-prone PCR was employed in directed evolution of the thermostability of *P. carrageenovora* arylsulfatase. After the positive mutants of D84A/H260L and H260L were obtained, mutagenesis was applied to find out that only H260L was positive mutation for the arylsulfatase's thermostability.

In this study, the optimal reaction temperature of saturation mutants (including H260L, H260I, H260Q, H260F and H260R) and wide-type arylsulfatases was 55°C, which is higher than that

Table 2. Comparisons of some enzyme properties between wild-type and mutant arylsulfatases

Enzyme	Relative activity[1)]/%	K_m/ mmol·L^{-1}	V_{max}/μmol· mg^{-1}·min^{-1}	$t_{1/2}$ at 55 °C/min	T_{opt}/ °C	pH_{opt}
WT	100.0	0.71	12.76	9.1	55	7.5
H260L	99.8	0.66	11.47	40.6	55	8.0
H260I	92.2	0.38	10.79	48.4	55	8.0
H260Q	101.0	0.77	13.31	30.9	55	8.0
H260F	105.3	1.02	13.76	29.1	55	8.0
H260R	99.6	0.66	12.39	34.5	55	7.5

Notes: 1) Enzyme activity was determined by using the standard assay described in Section 2. The enzyme activity of WT was defined as 100%. T_{opt} stands for optimal temperature, and pH_{opt} optimal pH.

of the recombinant arylsulfatase from *Pseudoalteromonas carrageenovora* (45°C) characterized by Kim et al. (2005). These mutants not only had better thermal stability, but also maintained the enzyme activity well. The values of K_m, V_{max} and specific activity had no much difference between mutants (H260L, H260Q and H260R) and WT. The results indicated that these mutations affected the thermostability, and did not have much effect on the catalytic activity of the enzyme.

In wide-type arylsulfatase, two hydrogen bonds could be formed between His260 and its surrounding amino acid residues

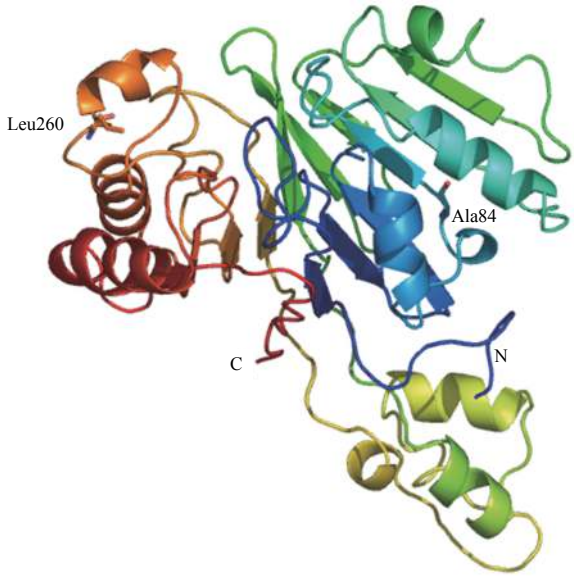

Fig. 5. Three-dimensional model of mutant arylsulfatase D84A/H260L. The substitution sites are represented in sticks. The letters N and C represent the respective termini of the mutant enzyme.

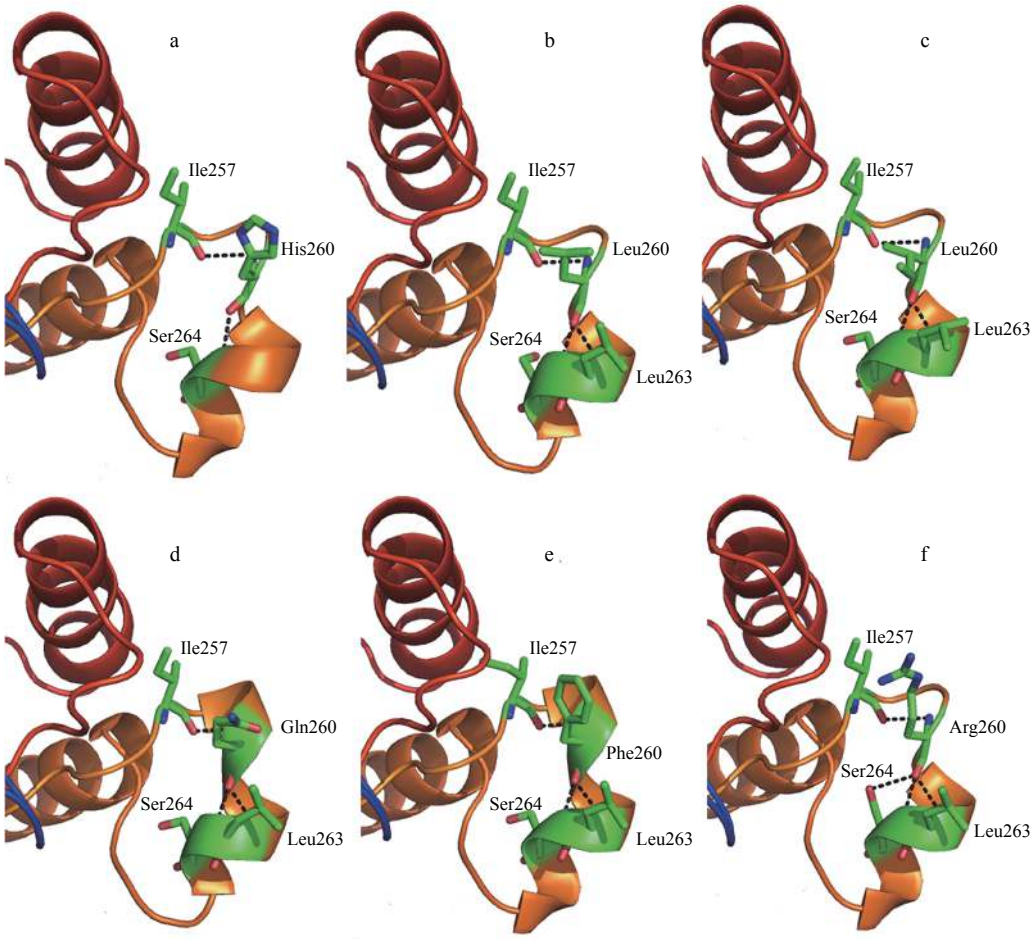

Fig. 6. The hydrogen bonds between amino acid residue 260 and its surrounding residues for wild-type (a), H260L (b), H260I (c), H260Q (d), H260F (e) and H260R (f).

(Ile257 and Ser264) (Fig. 6a). However, the number of hydrogen bond increased to three or four when His260 mutated to be Leu, Ile, Gln, Phe and Arg amino acid residues, respectively (Figs 6b–f). Because hydrogen bonding is considered to influence protein thermostability (Vieira and Degreve, 2009; Vogt et al., 1997), the additional hydrogen bonds in mutants of H260L, H260I, H260Q, H260F and H260R could account for their enhanced themostability. This is very consistent with previous studies which have proved that increasing the number of hydrogen bond can improve the thermostability of protein (Akbulut et al., 2013; Guo et al., 2015; Mohammadi et al., 2016; Zhou et al., 2015).

A rational-design approach has been proposed to optimize the surface charge-charge interactions to improve the thermal stability of protein (Schweiker and Makhatadze, 2009; Schweiker et al., 2007), which has been applied to enhance the thermostability of some enzymes such as lipase (Zhang et al., 2014) and endopolygalacturonase (Tu et al., 2015). As shown in Fig. 7, there were six surrounding amino acid residues with positive charge at His260 in wild-type arylsulfatase. Electrostatic repulsion existed due to the same charged amino acids. For mutants H260L, H260I, H260Q and H260F, His260 was mutated to be another uncharged amino acid. This might lead to optimization of surface charge-charge interactions and the mutant enzymes will become more favorable to increase their thermostability.

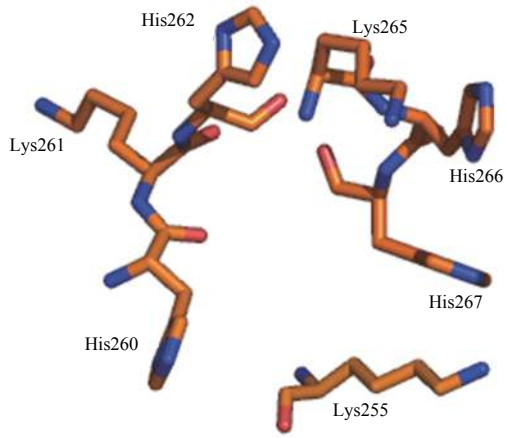

Fig. 7. The amino acid residues with positive charge surrounding Position 260 of wild-type arylsulfatase.

According to the present findings, it is reasonable to hypothesize that hydrogen bonding and optimization of surface charge-charge interactions resulted in improved thermostability of *P. carrageenovora* arylsulfatase. In addition, the single substitutions of H260L, H260I, H260Q, H260F and H260R were firstly reported responsible for thermostability improvement of *P. carrageenovora* arylsulfatase. This study may help for guiding molecular engineering of other arylsulfatases for improvement of the thermal tolerance.

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