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Rational Design of Catechol-2, 3-dioxygenase for Improving the Enzyme Characteristics

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Abstract Catechol-2, 3-dioxygenase (C23O) from *Pseudomonas* sp. CGMCC2953 identified in our laboratory, which is one of the key enzymes responsible for phenanthrene biodegradation, was expected to get better characteristics tolerant to environment for its further application. With the aim of improving the enzyme properties by introducing intermolecular disulfide bonds, X-ray structure of a C23O from *Pseudomonas putida* MT-2, a highly conserved homologous with the C23O from *Pseudomonas* sp. CGMCC2953, was directly used to find the potential sites for forming disulfide bonds between two monomers of the target C23O. Two sites, Ala229 and His294, were identified and mutated to cysteine, respectively, by using site mutagenesis. The expected disulfide bond between these two CYS residues was confirmed with both molecular modeling and experimental results. The optimum temperature of the mutated enzyme was widened from 40 to 40~50 °C. The mutated C23O became more alkalescency stable compared with the wild-type enzyme, e.g., 75% of the maximal enzyme activity retained even under pH 9.5 while 50% residue for the wild-type one. Improvement of thermostability of the mutated C230 with the redesigned disulfide was also confirmed.

Keywords Catechol-2,3-oxygenase (C23O) · Computer simulation · Disulfide bond · Gene site mutagenesis · Stability

Introduction

Environment pollution in oilfield area is a serious problem. Of all the contamination in oilfield, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and hazardous pollutants generated from direct emission and incomplete combustion of petroleum. Soil bacteria are

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able to degrade a variety of aromatic compounds, which can be applied for degradation of chemical wastes in environment. A soil bacterium with phenanthrene-degrading ability, *Pseudomonas* sp. CGMCC2953, was isolated from petroleum-polluted soil sample [1].

The gene encoding catechol-2, 3-dioxygenase (C23O) has been cloned from chromosomal DNA of *Pseudomonas* sp. CGMCC2953 and expressed in *Escherichia coli*. The enzyme exhibited some better characteristics than its homologs reported previously [2–5]. This enzyme is a member of the superfamily of extradiol dioxygenases [6] and catalyzes the conversion of catechol to 2-hydroxymuconic semialdehyde by breaking the C–C bond at 2,3-(meta) position [7]. Tests for enzyme activities showed that the majority of the isolates growing with PAHs as sole source of carbon and energy had an active catechol 2, 3-dioxygenase [8]. Therefore, C23O is the key enzyme of the bacterial pathways for the degradation of phenanthrene [9]. The C23O is a tetramer that is composed of four identical subunits, each of which contains a ferrous iron atom [10]. It is an unstable enzyme as exposure to oxidizing agents such as oxygen or substrate analogs such as alkyl- or chlorocatechols [11, 12]. As C23O is the key enzyme during the biodegradation process, improvement of the enzyme characteristics would make this enzyme more applicable in biodegradation.

The disulfide bond was proved to have great effect on the construction of protein [13] with special advantages. First, formation or reduction of a disulfide bond is a localized, two-state, and structurally well-defined change. Second, disulfide species are stable, covalent intermediates, which may be isolated and characterized structurally. Third, the rates of disulfide-bond formation and reduction can be varied without significantly altering other interactions [14].

In this work, the protein three-dimensional structure of C23O from *Pseudomonas putida* MT-2, a highly conserved homolog of the target C23O from CGMCC2953, was used to guide the design of thermostability improvement of the protein. With introduction of a disulfide bond in the protein structure, the thermal and alkalescence stability of C23O enzyme was improved to some extent, indicating that the strategy is available.

Materials and Methods

Microorganism

The microorganism containing gene c23o, isolated from petroleum-polluted soil sample in our laboratory [1], was identified as *Pseudomonas* sp. and deposited in China General Microbiological Culture Collection Center (CGMCC) with number CGMCC2953.

Cloning and Nucleotide Sequencing of the Gene c23o Encoding C23O

The gene c23o encoding C23O was amplified by PCR (forward primer: 5'-GGAAAGCTT CAATGAAAAAAGGCGTA-3' with a *Hin*dIII site; reverse primer: 5'-GTCTCGAG TTAGGTCAGAACGGTCAT-3' with a *Xho*I site) with a program: initial denaturation at 94 °C for 4 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; and additional extension at 72 °C for 10 min after the cycles. The DNA fragment amplified was digested by *Hin*dIII and *Xho*I and ligated with pET-28a plasmid digested by the same enzymes. The recombinant plasmid pET-c23o, confirmed by restriction enzyme analysis and DNA sequencing, was transformed into *E. coli* BL21 (DE3) for expression.

Identifying Potential Sites of Introducing Disulfide Bond of C23O by Modip

The crystal structure of C23O from *P. putida* MT-2 (Protein Databank ID: 1MPY) [15] was directly used as the basis for identifying the potential sites of introducing disulfide bond of C23O from CGMCC2953 in view of the high homology (95.5%) between the two genes (Fig. 1). Modip (http://caps.ncbs.res.in/dsdbase//modip.html), a web-based disulfide bond prediction server, was used to predict the potential disulfide bond forming sites between monomers. According to its principle, the dihedral angle and atom distance between two cysteines were defined in Fig. 2. With Modip program, a specific residue was virtually mutated first. The above-described geometry characteristics of the structure were checked then. Finally, the credibility of the identified disulfide bond site were evaluated and ranked by means of method reported by Sowdhamini [16]. The grade of forming disulfide bond was listed in Table 1. After choosing the mutation position, the corresponding amino acids were virtually mutated to cysteine to check the formation of disulfide bonds.

Gene Site Mutagenesis of C23O

Four primers were designed (nucleotide in square is mutation position):

Forward primer:

22AF : GCTTCGCGCC[TGC]GACCTGATCTCCATGAC

28F : AC<u>AAGCTT</u>CAATGAAAAAAGGCGTAAT

Reverse primer:

22AR : GTCATGGGAGATCAGGTC[GCA]GGCGCGAAGC

22R : TG<u>CTCGAG</u>TTAGGTCAGAACGGTCATGAAACGTTCGTTGAGAATGCGGTC[GCA]GTAA

First, two fragments of c23o were amplified by PCR with 28F plus 22AR and 22AF plus 22R separately. Secondly, two amplified DNA fragments in the first step were amplified together by PCR with 28F plus 22R to produce the whole mutated gene c23om.

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          *:********:*****::::.** ** :******:**** ****************************************
target    MKKGVMRPGHIQLRVLNLESALAHYRDLLGLIEVDRDDFGRVYLKAWTEVDKFSLVLREADEPGMDFMGFKVVDEDALRQ    80
1MPY_A    MNKGVMRPGHVQLRVLDMSKALEHYVELLGLIEMDRDDQGRVYLKAWTEVDKFSLVLREADEPGMDFMGFKVVDEDALRQ    80

          ***** ******************************************:*:**************************
target    LERDLTAYGCAVEQLPAGELNSCGRRVRFQAPSGHHFELYADKEYTGKWGVNEVNPEAWPRDLKGMAAVRFDHALMYGDE   160
1MPY_A    LERDLMAYGCAVEQLPAGELNSCGRRVRFQAPSGHHFELYADKEYTGKWGLNDVNPEAWPRDLKGMAAVRFDHALMYGDE   160

          ************************************************************:*******************
target    LPATYDLFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFHLDTWEDLLRAADLISMTDTSID   240
1MPY_A    LPATYDLFTKVLGFYLAEQVLDENGTRVAQFLSLSTKAHDVAFIHHPEKGRLHHVSFHLETWEDLLRAADLISMTDTSID   240

          ****************************************************************
target    IGPTRHGLTHGKTIYFFDPSGNRNEVFCGGDYNYPDHKPVTWTTDQLGKAIFYHDRILNERFMTVLT   307
1MPY_A    IGPTRHGLTHGKTIYFFDPSGNRNEVFCGGDYNYPDHKPVTWTTDQLGKAIFYHDRILNERFMTVLT   307
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Fig. 1 Sequence alignment between 1MPY_A (A chain of the structure) and the target (C23O from *Pseudomonas* sp. CGMCC2953)

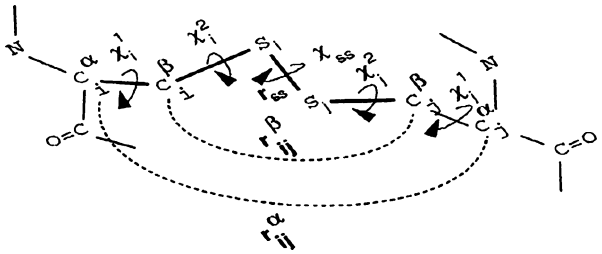

Fig. 2 Definition of the dihedral angle and atom distance r_{ij}^{α}, r_{ij}^{β}, and r_{ss} indicate the C alpha–C alpha distance, C beta–C beta distance, and S–S distance. x_{ss}, x_i^1, x_i^2, x_j^1, and x_i^2 indicate various dihedral angles in disulfide bond

Expression and Purification of the Mutated C23O in *E. coli*

The preculture of *E. coli* BL21 (DE3) containing the pET-c23om was prepared by inoculating a single colony into 5 mL LB medium supplemented with kanamycin (100 μg mL^{-1}) and incubated overnight at 37 °C with orbital shaking (200 rpm). Two milliliters of the overnight culture was transferred to 200 mL LB medium supplemented with kanamycin and further incubated at 37 °C. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mmol L^{-1} when the OD_{600} of the culture reached about 0.5. The incubation was carried out at 37 °C for another 3 h before the cells were harvested. The cell pellets were washed in a half volume of phosphate-buffered saline (PBS; 150 mmol L^{-1} NaCl, 3 mmol L^{-1} KCl, 10 mmol L^{-1} Na_2HPO, and 2 mmol L^{-1} KH_2PO, pH 7.4) and disintegrated by sonication in the PBS. The resultant supernatant was loaded onto a Ni-NTA-agarose column preequilibrated with PBS. The column was washed with the PBS containing 20 mmol L^{-1} imidazole to remove unbound proteins and then eluted with a "stepwise" gradient of imidazole (60 mmol L^{-1}, 100 mmol L^{-1}, 300 mmol L^{-1}, and 500 mmol L^{-1}) in PBS. Eluate was collected and analyzed by 12% SDS-PAGE. After dialysis against buffer A (20 mmol L^{-1} Tris–HCl, 150 mmol L^{-1} NaCl, pH 7.5), the purified enzyme was stored at −20 °C.

Table 1 Explanation of the feasibility grade.

Stereochemical parameter	Grade				
	A	B	C		D*
$r_{ij}^{\alpha} \leq 7$ Å and $r_{ij}^{\beta} \leq 4{,}7$ Å	√	√	√		√
1.6 Å $\leq \lvert r_{ss} \rvert \leq 2.4$ Å	√	√	×	√	×
$60° \leq \lvert x_{ss} \rvert \leq 120°$	√	√	√	×	×
$30° \leq \lvert x_i^1 \rvert \leq 90°$ or $150° \leq \lvert x_i^2 \rvert \leq 180°$	√	×	×		×
$30° \leq \lvert x_j^1 \rvert \leq 90°$ or $150° \leq \lvert x_j^2 \rvert \leq 180°$	√	×	×		×

The credibility of predicted disulfide bond decreases in an order of A, B, C, and D. D* denotes that the predicted result is incredible

**D* Sulfur could not be fixed*

Identification of Disulfide Bond

The solution containing the target protein was put onto the TLC plate first. Solution A (0.05 mol L^{-1} $Na_2[Fe(CN)_5NO]\cdot 2H_2O$, 0.1 mol L^{-1} HCl, 23.5 mol L^{-1} CH_3OH) was sprayed then. Solution B (0.4 mol L^{-1} NaCN 23.5 mol L^{-1} CH_3OH) was sprayed on the plate last. Sample with disulfide bond would display red with yellow background [17].

Enzymatic Assays and Kinetics

The enzyme activity was assayed in buffer A. To determine the influence of temperature on enzyme activity, 5.4×10^{-3} mg purified protein was preincubated separately at 30, 40, 50, 60, and 70 °C for 10 min in buffer A, and then the reaction was performed at the corresponding temperatures by adding catechol to a final concentration of 50 mmol L^{-1}. The activity was determined by GC analysis as described as following. Sample was quantified in a gas chromatograph (GC model 6890 N, Agilent, Palo Alto, CA, USA) equipped with a DB5-5% phenyl methyl siloxane capillary column (30 m×0.25 mm×0.25 μm). The injector and transfer line temperature were set at 290 °C, and the temperature program was as follows: 100 °C for 2 min and then increased to 280 °C with a rate of 20 °C min^{-1}. Aliquot (1 μL) was injected at a split of 1:13 (*v*/*v*). Five different buffers (with pH 5.5, 6.5, 7.5, 8.5, and 9.5, respectively) were also used to test the effect of pH on the enzyme activity at 30 °C. One unit of specific activity was defined as the amount of enzyme that converted 1 μmol substrate per minute per milligram protein.

Results and Discussion

Potential Disulfide Bond Forming Sites Identified by Modip and the Following Virtual Mutations

Modip identified only one possible pair of potential disulfide bond forming site between monomers (i.e., Ala229 and His 294). In Fig. 3, it displayed the three-dimensional structure of virtually mutated enzyme (A229C and H294C). By measuring the geometry characteristics of the mutant enzyme (Table 2), we found that only r_{ij}^{α}, r_{ij}^{β}, and r_{ss} met the standards required by Sowdhamini [16]. So, the feasibility level of this potential pair of residues was grade C, suggesting that existence of the newly formed disulfide bond between the two identified residues was credible.

Virtually mutating both residues (i.e., Ala229 and His 294) to cysteine, however, could produce a structure in which a new intermolecular disulfide bond was created (Fig. 3). This guaranteed the credibility of the identified potential disulfide-forming sites.

Expression of Mutated C23O in *E. coli* BL21

As marked in the DNA sequence above, the 229th and 294th amino acid were mutated to CYS via PCR. The site mutagenesis was then confirmed by DNA sequencing. Recombinant plasmid pET-c23o and/or pET-c23om harboring the target genes was constructed and transformed into *E. coli* BL21 (DE3) for expression (Fig. 4).

As shown in Fig. 2, the recombinant mutated C23O was expressed in *E. coli* BL21 (DE3) with 24.6% of the total proteins after inducement for 3 h at 37 °C. The purity of the target protein in supernatant after sonication could reach to 53.5%. The over-expressed

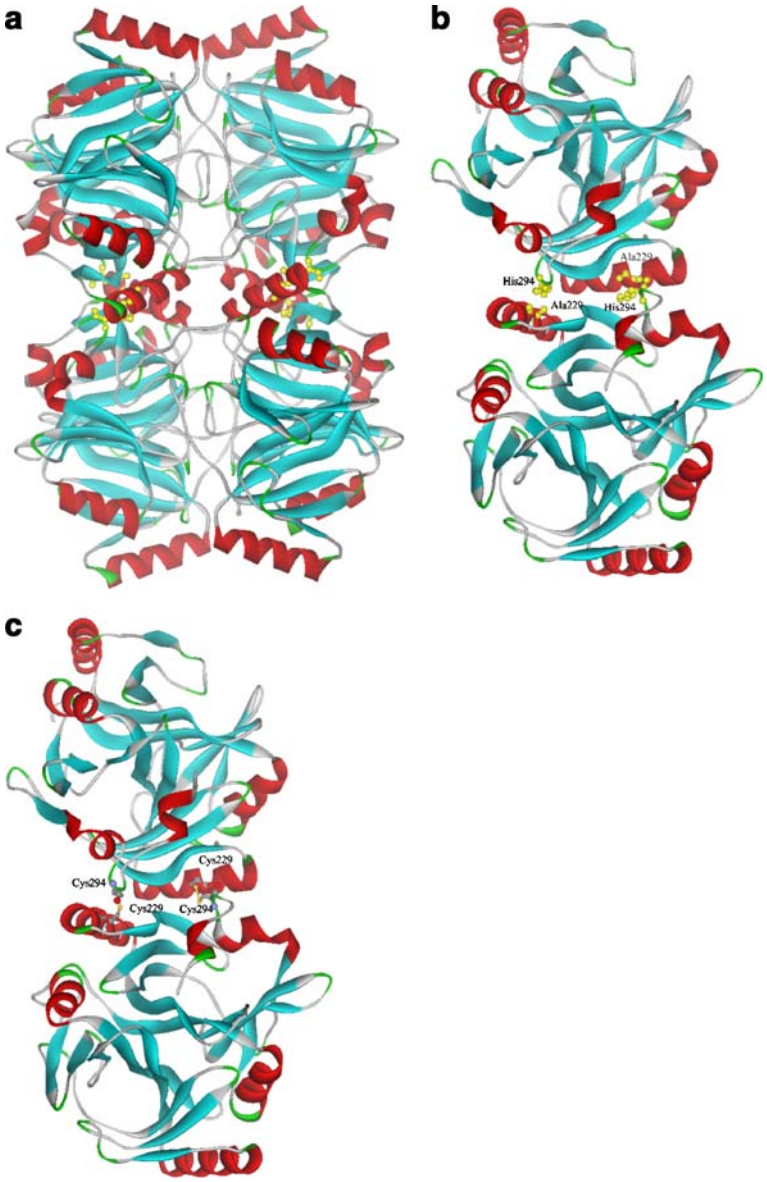

Fig. 3 Three-dimensional structure of C23O from *P. putida* MT-2 (1MPY) and its virtual mutation structure. **a** Three-dimensional structure of C23O from *P. putida* MT-2 (1MPY). The structure is composed of four identical subunits. Subunit in the *right bottom* is named A. The one in the *upper right* is named B. The one in the *left bottom* is named C. The one in the *upper left* is named D. The potential disulfide bond forming sites were displayed and colored *yellow*. **b** Side view of the A and B subunits of 1MPY. The potential disulfide bond forming sites were displayed, labeled, and colored *yellow*. **c** Side view of the virtual mutant of A and B subunits of 1MPY. New disulfide bond were formed between the identified sites by Modip

Table 2 Data of two mutated CYS of C23O.

r_{ij}^{α}	6.017 Å
r_{ij}^{β}	4.429 Å
r_{ss}	2.290 Å
x_{ss}	177.12°
x_i^1	57.39°
x_i^2	−111.74°
x_j^1	175.69°
x_j^2	−124.56°

protein could be purified by using Ni-NTA-agarose column chromatogram with a "stepwise" gradient elution of imidazole solution, which leads to a 96.7% purity of the target protein. In addition, there is no obvious difference between expression level of the wild type (data not shown) and the mutated C23O.

Kinetic Parameters

Initial velocity of the enzymatic reaction was investigated at pH 7.5 and 30 °C. The K_m values of the wild type and mutated C23O were obtained as 10 and 40 μM, respectively, and the k_{cat} values were estimated as 52 and 192 s^{-1}, respectively. Consequently, the k_{cat}/K_m 4.8 of the mutant was slightly lower than k_{cat}/K_m 5.2 of the wild type, indicating that the mutation has little effect on catalytic efficiency.

Identification of Disulfide Bond

According to the classic color reaction about sulfur compounds, three possible reaction results were shown in Table 3.

Both the wild-type and mutated C23O were tested meanwhile. Sample with the mutated C23O turned red with a background of yellow. The others, otherwise, turned yellow only.

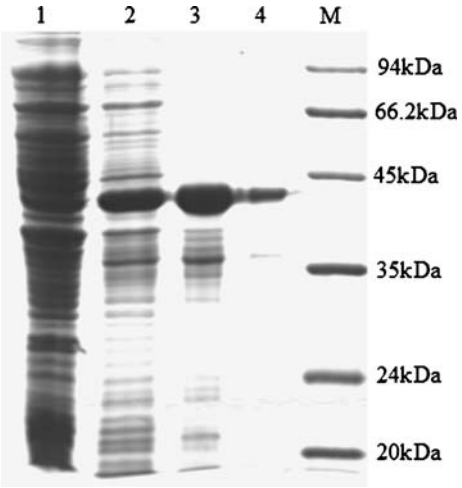

Fig. 4 SDS-PAGE analysis of the over-expressed mutated C23O *Lane 1* Lysate before inducement. *Lane 2* Lysate after inducement. *Lane 3* Supernatant after sonication. *Lane 4* Purified the mutated C23O. *Lane M* Markers (94, 66, 45, 35, and 24 kDa)

Table 3 Results of the color reaction with compounds containing different chemical group.

Chemical group	Result
–SH	Turned red
Arginine	Turned from orange to blue
–S–S–	Turned red with a background of yellow

Because there is no CYS–CYS in the wild-type C23O, formation of the designed disulfide bond was confirmed on the basis of the classic color reaction [17].

Effect of Temperature on Enzyme Activity

As shown in Fig. 5, both the mutated and the wild-type C23O have almost the same highest specific activity at around 40 °C. Activity of the wild-type C23O decreased rapidly with temperature increasing from 40 °C. The mutated C23O, however, could keep its higher activity compared with the wild-type one. For instance, the residue enzyme activities of the mutated and the wild-type C23O were 90% and 45% of the highest activity, respectively, at 50 °C. It is obvious that formation of the disulfide in C23O via directed mutation widened the adaptable temperature range effectively.

Effect of pH on Enzyme Activity

As shown in Fig. 6, the optimal pH of the mutated C23O shifted slightly to alkaline direction compared with the wild-type C23O. Furthermore, the mutated enzyme became more stable in the higher pH environment, e.g., the mutated and the wild-type enzymes remained 75% and 50% of the optimum activity, respectively, at pH 9.5. It is suggested that the disulfide leads to an improvement of the target enzyme characteristic resistant against alkaline environment.

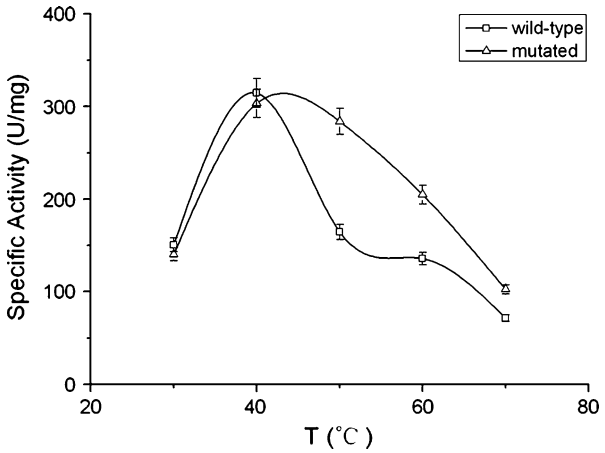

Fig. 5 Effect of temperature on the specific activity of the mutated and wild-type C23O

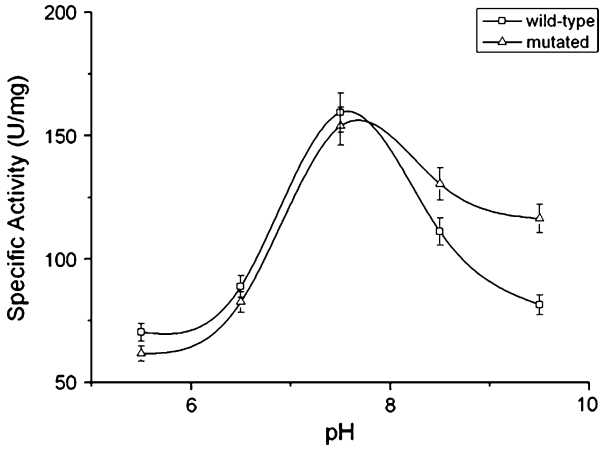

Fig. 6 Effect of pH on the specific activity of the mutated and wild-type C23O

Thermostability of the Mutated C23O

Compared with the wild-type C23O, replicates were made in different temperatures (30 and 50 °C) and continued for 1,200 min (Figs. 7 and 8). The wild-type C23O activity reduced to 38% of the maximum, while mutated C23O activity reduced to 45%, respectively, under 30 °C at the end of 1,200 min. When the reaction took place under 50 °C and continued for 80 min, the wild-type C23O activity reduced to 44% of the maximum while the mutated C23O activity to 52%. In addition, the experiments were also repeated under the other temperature, such as 40 °C, and similar trends were observed (data were not shown verbosely). Although the differences between the two types of C23O were not so huge, it is not neglectable that improvement of thermostability of the mutated C23O with the redesigned disulfide had been confirmed.

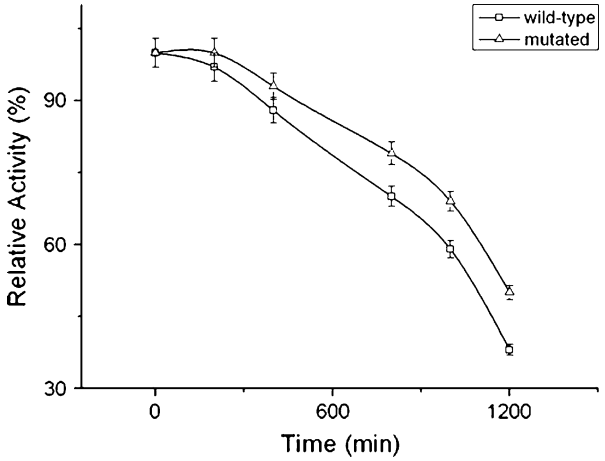

Fig. 7 The thermostability of mutated and wild-type C23O at 30 °C

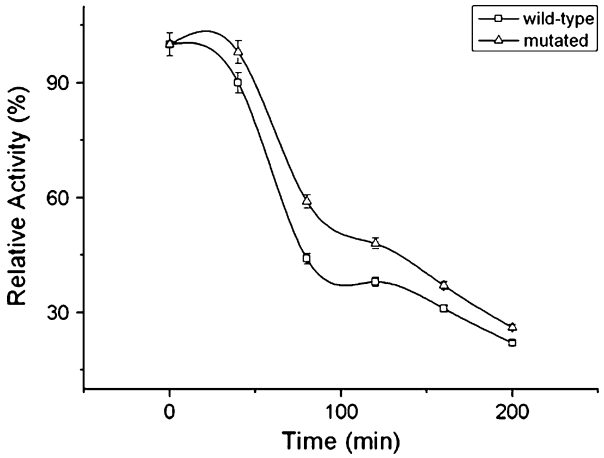

Fig. 8 The thermostability of mutated and wild-type C23O at 50 °C

Conclusion

In this work, a key enzyme (C23O) of the degrading pathway in the assimilation of phenanthrene was cloned from *Pseudomonas* sp. CGMCC2953. This enzyme shows some characteristics better than other reported C23O [1]. However, it could maintain its highest specific activity only under the temperature of 40 °C (Fig. 5). In order to improve its degrading characteristic on the basis of the original, X-ray structure of a C23O from *P. putida* MT-2, with 95.5% homology with the C23O from *Pseudomonas* sp. CGMCC2953, was directly used to find the potential sites for forming disulfide bonds between two monomers of the target C23O [18, 19]. As this enzyme could only exhibit degrading activity when keeping the structure of tetramer, it is very important to stabilize its structure for its degrading characteristic [20]. It has been shown for many naturally occurring proteins that disulfide bonds can enhance protein stability considerably [13]. With the simulation and calculation about two closest amino acids in two monomers, the couple of 229th and 294th amino acid which has the highest feasibility grade in this tetramer was mutated to CYS. It was confirmed that the expected disulfide bonds in protein were formed and the reconstruction was proved successfully.

It was concluded that the mutated C23O maintained higher activity than the wild-type one with increasing temperature. When the temperature was increased to 70 °C, a remarkable enzymatic activity of the mutated C23O could still be detected. There was no report on C23O that kept its catalytic activity at such a high temperature before. It is clear that the reconstruction played a positive role in improving thermal adaptability. The reason could be explained as that the disulfide bond formed between two monomers effectively reduced depolymerization of the tetramer under high temperature. Also, the result in the assay of thermostability (Figs. 7 and 8) proved that the reconstruction had a positive effect on improving thermostability.

On the other hand, the negative effect of pH on the mutated C23O (Fig. 6) was also reduced in alkaline condition. This could be derived from the two close CYSs that were more easily oxidized to form disulfide bond. As a result, more C23O tetramers could maintain the original conformation with the help of disulfide bond in alkaline condition than in acidic condition.

Further studies are needed to clarify the method used in this study being suitable in improving characteristics of other enzymes, especially with polymer structures. It may be a convenient way to make enzymatic reaction take place in a wider range of temperature and pH as expected.

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