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Enhancing the thermostability of fumarase C from *Corynebacterium glutamicum* via molecular modification

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ABSTRACT

Fumarases have been successfully applied in industry for the production of L-malate. However, the industrialization of fumarases is limited by their low thermostability. In this study, the thermostability of fumarase C from *Corynebacterium glutamicum* was enhanced through directed evolution, simulated mutagenesis, site-directed mutagenesis and saturated mutagenesis. Mutant 2G (A411V) was initially constructed through directed evolution. Its half-life at 50 °C ($t_{1/2,\ 50°C}$) increased from 1 min to 2.2 min, and the T_{50}^{15} (temperature at which the activity of enzyme decreased by 50% in 15 min) increased from 44.8 °C to 47.2 °C. Besides, several different mutants were obtained by site-directed mutation. Among them, mutant 3G (A227V) showed significant improvement in thermostability with a 3.3-fold improvement of $t_{1/2,\ 50°C}$ and a 3.6 °C increase in T_{50}^{15} compared to the wild-type enzyme. Then, 2/3G (A227V, A411V) was obtained by combining the mutant 2G with the mutant 3G, for which the $t_{1/2,\ 50°C}$ and T_{50}^{15} increased to more than 768 min and 52.4 °C, respectively. Finally, site-saturated mutagenesis was employed on amino acid residues 175-Glu, 228-Gly, 297-Gly, 320-Lys and 464-Glu to maximize the thermostability of mutant 2/3G. The most thermostable mutant 175G with amino acid substitutions (A227V, A411V, E175K) was isolated. Its $t_{1/2,50°C}$ increased to more than 2700 min while that of wild-type enzyme was only 1 min and T_{50}^{15} was 9.8 °C higher than the wild-type enzyme. The thermostable mutated enzymes generated without affecting the activity in this study would be an attractive candidate for industrial applications.

1. Introduction

Fumarase or fumarate hydratase (EC 4.2.1.2) are found in prokaryotes and eukaryotes [1,2] which catalyzes the reversible hydration of fumarate to L-malate in the tricarboxylic acid (TCA) cycle [3]. According to the relevant subunit arrangement, thermal stability and metal requirements, fumarases are classified into two distinct classes. Class I fumarases including fumarase A and fumarase B, are oxygen-sensitive, thermolabile dimeric proteins of 120 kDa containing a 4Fe-4S cluster. Class II fumarases (fumarase C) are oxygen-insensitive, thermostable and iron independent homotetramers of 200 kDa [4].

Industrially, fumarases with high activities from mesophilic microorganisms, such as *Lactobacillus brevis* [5], *Brevibacterium flavum* [6] and *Corynebacterium glutamicum* [7] are the preferred biocatalysts for the production of L-malate using fumarate as a substrate. More economical methods using the immobilised microbial cell [8] or enzymes alone [9] are applied in continuous reaction. Yamamoto has achieved continuous industrial production of L-malate through the immobilization of *Brevibacterium flavum* [10]. Giorno has studied an enzyme membrane reactor with immobilised fumarase for production of L-malate [9].

The reaction temperature for the production of L-malate is usually set between 40 and 60 °C in order to minimize the generation of the unwanted by-product succinic acid [11]. However, at these temperatures, those fumarases from mesophilic microorganisms will quickly deactivate [7]. Some fumarases from thermophilic bacteria have good thermal stability and their optimum reaction temperature can be as high as 85 °C. However, When the reaction temperature of the enzymes from these thermophiles is reduced to 40–60 °C, the enzyme activity is only 10%–20% of the highest enzyme activity [12]. Moreover, it will consume enormous energy if maintain a reaction temperature of 85 °C when the production scale is large. Therefore, the thermostable fumarase between 40 and 60 °C is currently required for the production of L-malate.

Numerous molecular modifications have emerged in recent years to improve the adaptation of existed biocatalysts to reaction condition [13]. In this work, the specific enzyme activitivies of five recombinant fumarase C from *Corynebacterium glutamicum*, *Escherichia coli* BL21 (DE3) [14], *Streptomyces lividans* TK54 [11], *Streptomyces thermovulgaris*

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[15] and *Thermus thermophilus* [12] were determined at 40 °C. The recombinant fumarase C from *C. glutamicum* (cgFumC),[1] which has the highest specific enzyme activity, was selected and the thermostability was improved with different molecular modifications in this study. Firstly, a high-throughput screening method was successfully established after adjustment and optimization of the detection methods of L-malate reported in literatures. Based on the established high-throughput screening methods, directed evolution was chosen. Fumarase C is an ancient and conserved protein, the researchers evidenced a high degree of structural conservation among the fumarases proteins from different species [16]. Multiple sequence alignments and simulated mutation in modeled structure were then used to predict potential pots for site-directed mutagenesis. Saturation mutagenesis has become known as an essential tool for improving the thermal stability of enzymes [17]. Furthermore, alanine scanning was used to predict sites that may be crucial for the thermostability of cgFumC, then each of the sites was randomized by saturation mutagenesis to form different libraries of mutants.

2. Materials and methods

2.1. Bacterial strain, plasmid and reagents

C. glutamicum 13032, *E. coli* DH5α, *E. coli* BL21 (DE3) and the plasmid pET-28a (+) were cultured in our laboratory. The genome was extracted from *C. glutamicum* 13032 and *E. coli* BL21 (DE3) using AXY™ Bacterial Genomic DNA Miniprep kit according to the manufacturer's instructions. *E. coli* DH5α was used as the amplification host for pET-28a (+). *E. coli* BL21 (DE3) was used as the host for the expression of *fumC* gene from *C. glutamicum* (*cgFumC*),[2] *E. coli* BL21 (DE3) (*ecFumC*),[3] *S. lividans* TK54 (*slFumC*),[4] *S. thermovulgari* (*stFumC*)[5] *and T. thermophilus* (*ttFumC*).[6] All other reagents were of biochemical grade or the highest purity.

2.2. Gene cloning and construction of recombinant bacteria

Primers used to amplify *fumC* genes from *C. glutamicum* 13032 and *E. coli* BL21 (DE3) were designed based on the 5′ and 3′ sequences of the annotated *fumC* gene in their genome. The forward primers were 5′-GGTCGCGGATCCGAGCAGGAAT-3′ and 5′-TCGCGGATCCATGAATA CAGTACGCAG-3′, respectively. The reversed primers were 5′-TGCAA GCTTTTAGAACTTGTTCTCGCGCT-3′ and 5′-TGCAAGCTTTTAA

CGCCCGGCTTTCATAC-3′, respectively. *BamH* I and *Hind* III restriction sites (underlined) were introduced into the primers. The amplified fragments were double digested with *BamH* I and *Hind* III, ligated with T4 DNA ligase to the vector pET-28a (+) to obtain the recombinant plasmid pET28a (+)-*cgFumC* and pET28a (+)-*ecFumC*. In addition, the recombinant plasmid pET28a (+)-*slFumC,* pET28a (+)-*stFumC* and pET28a (+)-*ttFumC* were constructed by Shanghai Generay Biotech Co., Ltd. Then, the recombinant plasmid were transformed into the *E. coli* BL21 (DE3), generating recombinant *E. coli* BL21 (DE3)-pET28a (+)-*cgFumC*, *E. coli* BL21 (DE3)-pET28a (+)-*ecFumC*, *E. coli* BL21 (DE3)-pET28a (+)-*slFumC, E. coli* BL21 (DE3)-pET28a (+)-*stFumC* and *E. coli* BL21 (DE3)-pET28a (+)-*ttFumC*.

2.3. Expression and purification of enzyme

The recombinant bacteria were cultured for 8–10 h, then inoculated in 50 mL fresh Luria-Bertani (LB) medium containing 50 mg/L kanamycin and cultured at 37 °C until the OD_{600} reaches 0.5–0.6. Protein expression was induced by Isopropyl-beta-D-Thiogalacto-pyranoside (IPTG) at a final concentration of 0.5 mM. The cells were transferred to a shaking incubator of 30 °C, 200 rpm for 10–12 h before harvested by centrifugation and disrupted by ultrasonic wave in potassium phosphate buffer (pH 7.3). The soluble protein fractions were purified by Ni affinity column. The column was washed by wash buffer (50 mM potassium phosphate buffer, pH 7.3, 0.5 M NaCl, 60 mM imidazole) and then eluted by elution buffer (50 mM potassium phosphate buffer, pH 7.3, 0.5 M NaCl, 500 mM imidazole). The protein eluted was immediately dialyzed and analyzed by SDS-PAGE.

2.4. Protein quantitation and enzymatic assays

The protein concentration was measured using Pierce BCA Protein Assay Kit (Shanghai, Thermo Fisher Scientific Co., Ltd.). Fumarase activity was determined spectrophotometrically by monitoring the fumarate disappearance at 250 nm as described [18] with a few modifications. The standard mixture contained 0.1 M potassium phosphate buffer (pH 7.3), 5 mM fumarate and fumarase with appropriate concentration in the total volume of 1 mL. The reaction was initiated by adding fumarase. One unit of enzymatic activity is defined as the amount of enzyme that converts 1 μmol fumarate per minute.

2.5. High throughput screening

Malate was heated in the sulfuric acid with orcinol, dehydrated and removed a carboxyl group to form 7-hydroxyl-5-methyl coumarin. The 7-hydroxyl-5-methyl coumarin has a strong fluorescence at the excitation wavelength of 369 nm and the absorption wavelength of 445 nm, the concentration of fluorescent substance is proportional to the fluorescence intensity in a certain reaction condition and concentration range. Huang Zhixian and Dai Renze used fluorescence to detect the content of malate in wine at 25 mL system [19].

A high-throughput screening method was established on the basis of the detection methods and systems mentioned in the literature for proper optimization, so that it could be operated on a 96-well-plate. And the thermostability of wild-type cgFumC and mutants were determined by measuring the catalytic activity of whole cell after heat-treatment. Ten wild-type recombinant bacteria and fifty mutants were piked to the shallow 96-well-plate containing 500 μL LB broths with 50 μg/mL kanamycin in each well (mother plate). Mother plates were placed in a shaking incubator of 37 °C, 200 rpm for 8 h to provide seed liquid. Then, 50 μL of seed liquid was drawn from each well of the mother plate to the corresponding deep well 96-well-plate containing 600 μL LB broths with 50 μg/mL kanamycin in each well (daughter plate). After incubation at 37 °C, 200 rpm for 2.5 h, IPTG was added to each well at a final concentration of 0.5 mM. The daughter plates were transferred to a shaking incubator of 30 °C, 200 rpm for 12 h. Centrifuged at room temperature, then discarded the supernatant and resuspend the cells with 100 μL of potassium phosphate buffer (pH 7.3). The resuspended cells were heat treated in a 55 °C or other suitable temperature water bath for 45 min and then rapidly cooled and added 600 μL disodium fumarate at a concentration of 1 mg/mL. After reaction at 40 °C and 200 rpm for 15 min, 2 μL supernatant was added to polytetrafluoroethylene shallow 96-well-plate containing 40 μL sulfuric acid with 5 mg/mL orcinol in each well (reaction plate). The reaction plates were placed in steam for 80 min, then rapidly cooled by ice bath and added 458 μL, 80% sulfuric acid to each well. Finally, the content of L-malate in each well was determined by measuring the fluorescence intensity of the mixed solution at the excitation wavelength of 369 nm and the absorption wavelength of 445 nm using a full-wavelength multi-functional microplate reader (Shanghai, Thermo Fisher Scientific Co., Ltd.). The mutants with higher residual activity than wild-type cgFumC were isolated and assayed.

[1] Abbreviation: cgFumC, recombinant fumarase C from *C. glutamicum*.
[2] Abbreviation: *cgFumC, fumC* gene from *C. glutamicum*.
[3] Abbreviation: *ecFumC, fumC* gene from *E. coli* BL21 (DE3).
[4] Abbreviation: *slFumC, fumC* gene from *S. lividans* TK54.
[5] Abbreviation: *stFumC, fumC* gene from *S. thermovulgari*.
[6] Abbreviation: *ttFumC, fumC* gene from *T. thermophilus*.