Enzyme and Microbial Technology 119 (2018) 10–16

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Improved thermostability and catalytic efficiency of overexpressed catalase from *B. pumilus* ML 413 (KatX2) by introducing disulfide bond C286-C289

Mugisha Samson[a], Taowei Yang[a,b,*], Mukama Omar[a], Meijuan Xu[a], Xian Zhang[a], Uliho Alphonse[a], Zhiming Rao[a,b,*]

[a] The Key Laboratory of Industrial Biotechnology, School of Biotechnology, Jiangnan University, Wuxi, 214122, PR China
[b] Jiangnan University (Rugao) Food Biotechnology Research Institute, Rugao, Jiangsu Province, PR China

ARTICLE INFO

Keywords:
Catalase
Site-directed mutagenesis
Disulfide bond
Thermo-stability
Catalytic efficiency

ABSTRACT

Catalase catalyzes the decomposition of hydrogen peroxide to water and oxygen. The main role of this enzyme is to prevent cell damage caused by reactive oxygen species (ROS). However, endogenous catalase is sensitive to high temperature and possesses limited activity. To satisfy requirements for this critical bottleneck, in this work, we improved the thermo-stability of a heme-catalase (KatX2) from a high oxidative stress resistance *Bacillus pumilus* ML413 through the construction of a disulfide bond between S286C and D289C. After the site-directed mutagenesis targeting the disulfide bond between S286C and D289C into the wild-type catalase, a potential improvement of thermo-stability half-life at 60 °C was increased by 48 min compared to the wild-type half-life. Unexpectedly, a catalytic efficiency of KatX2 S286C/D289C mutant was increased by 40% when compared to the wild-type KatX2. More importantly, this unprecedented highly stable KatX2 recombinant mutant S286C/D289C exhibits higher catalytic efficiency and thermo-stability with no change on the catalase secondary structure. Thus, this rational design based KatX2 could be adopted as a potential biocatalyst in industry.

1. Introduction

Catalase enzyme (hydrogen-peroxide: hydrogen peroxide oxidoreductase, EC1.11.1.6) is found in various aerobic organisms namely animals, plants, bacteria, archaea, and fungi. It mediates the breakdown of hydrogen peroxide (H_2O_2)-produced from reactive oxygen species (ROS) like oxygen ions and hydroxyl radicals- to water and oxygen. ROS are produced in the normal cell process of electron transport chain in mitochondria. However, excess ROS and free radicals can cause cell oxidative damage and/or death [1,2]. During this redox imbalance, catalase plays a pivotal role in cell detoxification by catalyzing hydrogen peroxide decomposition [3]. Catalase is also widely used in food products processing, textile and clinical assays [4,5] owing to its higher turnover number to convert thousands of hydrogen peroxide molecules to water and hydrogen per second, making it useful industrially [5].

Catalases are categorized into three main groups based on their functional features: heme-containing monofunctional, bifunctional catalase-peroxidases, and Mn-containing catalases [6,7]. Among them, monofunctional catalases have received the most interest due to their commercial applications and thus an ever-increasing in its identification, production and purification from various organisms [8–11]. To promote its usage, more research has been done on targeting identification, homologous and heterologous overexpression as well as the exploration of new natural catalase genes [12,13]. Overexpression of catalases has shown a great specific activity and low turnover rate based on the source of the gene [14]. However, few reports were developed for new engineered catalases [15]. Currently, protein engineering strategies have been used to enhance the performance of target proteins. Site-directed mutagenesis assisted by structure modeling and analysis methods have been applied in studies over the past few years for rational engineering [16–18]. Furthermore, various enzymes were engineered and optimized using site-directed mutagenesis to achieve catalytic and thermos-stability improvement [19,20]. Even though, powerful approaches in protein engineering have been employed, no further attention has been paid to catalase engineering [5].

Bacillus pumilus exhibits high oxidative stress resistance compared to other microbial organisms with various antioxidant enzymes [21,22]. Similarly, in this study, *Bacillus pumilus* ML413 was used as a potential source of catalase gene. However, *Bacillus subtilis 168* is widely used for ectopically express catalase due its potential to yield and secrete enormous quantity of proteins. And being generally recognized as safe (GRAS), it is also known to be a major cell factory for secreted target

* Corresponding author at: The Key Laboratory of Industrial Biotechnology, School of Biotechnology, Jiangnan University, Wuxi, 214122, PR China.
E-mail addresses: yangtw@jiangnan.edu.cn (T. Yang), raozhiming@jiangnan.edu.cn (Z. Rao).

https://doi.org/10.1016/j.enzmictec.2018.08.002
Received 4 February 2018; Received in revised form 25 June 2018; Accepted 8 August 2018
Available online 09 August 2018
0141-0229/

proteins, thus serving as an industrial competent strain [23,24].

Based on that, in this study, *Bacillus subtilis 168* was used for catalase expression as well explore its novel features when genetically engineered to circumvent its high temperature sensitivity as previously reported [25,26]. Therefore, a set of site-directed mutagenesis and disulfide bond inclusion has been performed on the endogenous protein aiming to enhance *Bacillus pumilus* ML413 heme-catalase (KatX2) properties. By fine-optimizing the catalase gene, a unique thermostability of 268 min' half-life at 60 °C and a 40% catalytic efficiency were successfully achieved. This high stability of catalase allows for the real-time decomposition of hydrogen peroxide, providing a promising use in healthcare and industrial processes.

2. Materials and methods

2.1. Bacterial strains, plasmids and materials

Bacillus pumilus ML413 isolated from Chinese soil provided the gene. *E. coli* JM109 was used for plasmid construction while *Bacillus substilis* 168 was used as a host strain. The plasmids pMD18-T and pMA5 were used as cloning and expression vectors, respectively (TaKaRa Co., Dalian, China). This company also provided PrimeSTAR® enzyme, ExTaq DNA polymerase, T4 DNA ligase, and restriction enzymes (*BamH*I and *Mlu*I). The DNA extraction, mini plasmid isolation and mini rapid purification kits were purchased from (Sangon Biotech Co., Ltd., Shanghai, China). HisTrap™HP column for protein purification was from (GE Healthcare, Inc., Little Chalfont, U.K). Other high analytical grade chemicals were obtained from commercial sources.

2.2. Construction of recombinant strains and site-directed mutagenesis

Bacillus pumilus ML 413 (KT963080) genomic DNA was used as template for catalase gene amplification using primers F and R (Table1). Cloning and expression of catalase were performed as reported in our previous work [14]. The obtained recombinant plasmid was used as a template for site-directed mutagenesis, whereby an overlap-extension PCR method [27] was carried out for catalase site-directed and selected mutations with specific designed primers (Table1). Mutated plasmids were then transformed into *E. coli* JM109 for selection of positive mutants.

Prior for disulfide bond introduction within the catalase wild-type, Design 2, a web-based software, was used for disulfide bonding prediction. To verify the created disulfide bond in the mutated protein, samples were subjected to SDS-PAGE in the presence or absence of 1,4-dithiothreitol (DTT), a reducing agent, with a working concentration of 100 mM, as previously described [28]. The mutated recombinant plasmids were ligated into pMA5 plasmid similarly digested with *BamH*I and *Mlu*I. All positive transformants were confirmed by sequencing (Sangon Biotech Co., Ltd., Shanghai, China). The selected catalase mutants harboring disulfide bond were then transformed into *Bacillus subtilis* 168. The wild type *Bacillus subtilis* 168/pMA5-KatX2 recombinant was used as control.

2.3. Culture media, conditions and catalase purification

The basal experimental media was composed of growth medium. For cloning, Luria Bertani (LB) made of yeast extract 5 g/L, NaCl 10 g/L, tryptone 10 g/L at 7.0 pH containing 100 g/mL of ampicillin was used for wild type and recombinant strains overnight cultivation in 10 mL of LB at 37 °C,180 rpm. Subsequently, the recombinants *Bacillus subtilis* 168 strains were transferred into 50 mL of the fermentation medium (glycerine 47 g/L, NH_4Cl 1.5 g/L, corn steep liquor 15 g/L, K_2HPO_4 2.7 g/L, KH_2PO_4 2.1 g/L, $MgSO_4.7H_20$ 1.9 g/L, NaCl 5 g/L, yeast extract 35 g/L, $FeSO_4.7H_2O$ 0.0025 g/L, 100 g/mL of kanamycin, pH 7.0) and cultured at 37 °C for 54 h. The cultured cells at the exponential phase (OD_{600}, 280 rpm) were harvested and centrifuged (10,000 × g for 20 min at 4 °C), and then the supernatant was filtered using a 0.22 mm syringe filter for further analysis. Protein purification was performed as previously described in our study [14]. Finally, the presence of disulfide bond in S286C/D289C mutant was analyzed by 100 mM 1,4-dithiothreitol (DTT), a redox agent, treatment as previously described [28].

2.4. Enzyme activity assay

A total volume of 3 mL composed of a standard reaction mixture of 50 mmol/L phosphate buffer (KH_2PO_4-K_2HPO_4/pH 7.0), 10 mmol/L H_2O_2 and 100 µL of the enzyme solution was used to monitor catalase enzyme activity. The decomposition of hydrogen peroxide to water and oxygen was measured using UV–vis spectrophotometer (UV-2450) at 240 nm for every 60 s at 37 °C. The ε value of H_2O_2 at 240 nm (ε240, 43.6 $mm^{-1}\,cm^{-1}$) was used to calculate the enzyme activity [29]. One unit of catalase was defined as the enzyme needed to decompose 1 µmol of hydrogen peroxide to water and oxygen per minute at 37 °C.

2.5. Catalase enzyme characterization

2.5.1. Kinetic parameters of catalase (K_m, V_{max} and K_{cat})

High concentration of H_2O_2 (> 100 mM) inhibits catalase enzyme activity. Thus, the effect of catalase activity was assessed within concentration range of 10 to 90 mM of substrate (hydrogen peroxide) in 50 mM PBS buffer (pH 7.0) at 37 °C. Michaelis constant (K_m) and maximal velocity (V_{max}) were then evaluated by linear regression from double reciprocal plots based on Linewaever and Burk theory [30]. The turnover number value (K_{cat}) was also determined.

2.5.2. pH, temperature and stability conditions

The optimal pH reaction for all mutants and wild-type recombinants was determined from buffers of various pHs at 37 °C. The 50 mM reaction buffers of sodium citrate pH 4–6, PBS pH 6–8, Tris − HCl pH 8–10 and glycine-NaOH pH: 10–13 were used. The pH stability was assessed by incubating the enzyme at 4 °C for 24 h. These several buffers and the remaining activity were determined using the enzyme assay delineated earlier. For the optimum temperature, the obtained optimum pH buffer was used in the reaction mixture within a temperature range from 10 °C to 60 °C. The thermostability was determined by incubating prepared

Table 1
Nucleotide sequences of primers used for site-directed mutagenesis in this study.

Primers	Sequence 5'-3'
Wild-type F	GTGT**GGATCC**ATGACAAATTCAAATCATAAAAATTTGACAACG
Wild-type R	GTGT**ACGCGT**TTAGTGGTGGTGGTGGTGTTTCATGCTTCCTTGAAG ATATGAGCG
S286C F	CCGACAAAGACGTGG**TGT**GAGGAAGATTATCCG
S286C R	CGGATAATCTTCCTC**ACA**CCACGTCTTTGTCGG
D289CF	ACGTGGAGCGAGGAA**TGT**TATCCGCTGCAAAAA
D289CR	TTTTTGCAGCGGATA**ACA**TTCCTCGCTCCACGT

Restriction sites are shown in bold and the underlined-bolded are nucleotide sequences corresponding to the mutant sites.