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Identification and characterization of a novel Old Yellow Enzyme from *Bacillus subtilis str.168*

Xiqian Sheng, Ming Yan*, Lin Xu, Miao Wei

College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing, 211816, People's Republic of China

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ABSTRACT

We identified and characterized YqiG, a novel Old Yellow Enzyme (OYE) from *Bacillus subtilis str.168*, as a member of the "thermophilic-like" subfamily. It is most related to XenA from *Pseudomonas putida*, with 37.9% identity, but it exhibits certain differences in sequence and enzyme properties. The YqiG can reduce various activated alkenes and exhibits high temperature (60 °C for 12 h) and pH stability (pH 4.0 and 9.0 for 12 h), which indicates that it has a great potential for biocatalysis. However, it shows low tolerance toward organic solvents. Also the YqiG shows high activities towards maleimide, dimethyl maleate and acyclic ketones (55.5 U/mg, 6.22 U/mg and 2.97 U/mg, respectively). Besides, The YqiG can catalyze citral to (S)-citronellal with a low rate but high ee% (>99%).

1. Introduction

Old Yellow Enzyme [EC 1.6.99.1] was the first enzyme shown to contain flavin as a prosthetic group and was originally isolated from brewer's bottom yeast by Warburg and Christian in 1933. In recent years, the OYE family has grown rapidly, and numerous OYE homologues with different characteristics have been identified [1]. Over the past 80 years of research, the OYEs have exhibited undeniably great potential for asymmetrically reducing a broad range of activated alkenes because they can generate up to two chiral centers when catalyzing C=C bond reductions [2,3]. The OYEs are widely distributed in nature [4], especially in plants [5], fungi and bacteria [6–9]. These enzymes feature a broad substrate spectrum, including α, β-unsaturated aldehydes, ketones, nitriles, imides, nitro aromatics and carboxylic acids [1,8,10]. The enoate reductase stereospecifically reduces the C=C bond through hydrogen atom anti-addition [2,11].

In 2010, Toogood et al. classified the OYEs into two categories: the "classical" family and the "thermophilic-like" family [1]. The "thermophilic-like" family often exhibited higher enzyme properties than the "classical" family [12], such as higher stability of pH and temperature. One difference between the two subfamilies is that the "classic" family usually forms monomers or dimers such as OYE1 from *Saccharomyces pastorianus* [13], 12-oxophytodienoate reductase from plants and MR from bacterica. but the "thermophilic-like" family often forms oligomers [1,14]. Until now, the vast majority of OYE enzymes discovered belong to the "classic" family [5]. Only a few OYE members are "thermophilic-like" enzymes, including YqjM (P54550) from *Bacillus subtilis* [15], XenA (AAF02538) from *Pseudomonas putida* [6], Chr-OYE3 (KJ019329) from *Chryseobacterium* sp. CA49 [16], TOYE (ZP_00777979) from *Thermoanaerobacter pseudethanolicus* E39 [17], GeoOYE (Q5KXG9) from *Geobacillus kaustophilus* [18] and CrS (YP_004203660) [19] from *Thermus scotoductus*. Based on the above sequences, we found a novel "thermophilic-like" OYE referred to as YqiG. Here, we identify and characterize YqiG from *Bacillus subtilis*; this is a new "thermophilic-like" OYE identified from *Bacillus subtilis* after YqjM (the first characterized "thermophilic-like" family OYE).

2. Materials and methods

2.1. Chemicals and solvents

The chemicals and solvents used as substrates are analytical grade and from Aladdin. All of the enzymes used for cloning (including DNA polymerase, T4 DNA ligase and restriction enzymes) were purchased from Takara Biotechnology. The pET-22b (+) was from our own lab.

* Corresponding author.
E-mail address: yanming@njtech.edu.cn (M. Yan).

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2.2. Cloning the yqiG gene from Bacillus subtilis subst. subtilis. str. 168

The *Bacillus subtilis* strain used as a PCR template was purchased from ATCC, and the yqiG DNA sequence was obtained from the UNIPROT database. The PCR experiments were performed using PrimerStar® DNA polymerase (forward primer, pET-22b (+): 5′-GGAATTCCATATGAATCCTAAGTATAAGCCA-3′; reverse primer: 5′-CATGCCATGGTTAATCTTTATAAGGCACCCA-3′). The reaction began with an initial denaturing step for 300 s at 95 °C followed by 30 cycles of 10 s at 98 °C, 15 s at 56 °C, and 90 s at 72 °C with a final extension step at 72 °C for 300 s. The yqiG gene was integrated into pET-22b (+) using the restriction sites NdeI and NcoI. The constructed vectors were transformed into *E. coli* DH5α. The plasmids were also transformed into *E. coli* BL21 (DE3) for expression after the vectors were sequenced

2.3. YqiG expression and purification

Five milliliters of Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) supplemented with 100 mgL^{-1} ampicillin was inoculated with a single YqiG colony for 12 h at 37 °C. The YqiG-containing cells were then subcultured using 100 ml LB medium supplemented with 100 mgL^{-1} ampicillin at 37 °C. Induction was initiated with the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD_{600} of 0.6–0.8, and the cells were incubated for another 12 h at 30 °C.

Cells harvested by centrifugation were suspended in 100 mM potassium phosphate (pH 7.0) and then broken with high pressure homogenizer (APV, Holland). Cell debris was removed by centrifugation at 12,000 rpm for 30 min at 4 °C.And then the supernatant was purified on ÄKTAprime plus (USA) with HiTrap DEAE FF column. The column was equilibrated with 20 mM Tris-HCl buffer (pH7.0) containing 1M NaCl.

The purified YqiG was stored at 4 °C.

2.4. Protein analysis

Protein concentration was determined with the BCA Protein Assay Kit (CWBIO, China). The molecular mass and the purity of the purified protein were estimated by SDS-PAGE. Free flavin was also determined by the Spectra Max M2. Free flavin would be released after incubating in 100 °C boiling water bath for 10 min. Molecular mass of native YqiG was determined using an ÄKTA purifier system with a Superdex 200 pg 16/600 column. The flow rate for protein elution is 0.8 ml/min. Thyroglobulin (670 kDa), globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), VB12 (1.35 kDa) were used as molecular mass standards.

2.5. Enzyme assay

The dimethyl maleate (DMM) was used as substrate for the determination of the enzymatic properties, and all measurements were used with purified YqiG on a Multimode Plate Reader (Spectra Max M2, Molecular Devices Corporation). And these experiments were implemented at least in triplicate.

The enzyme activity was detected by monitoring NAD(P)H oxidation at 340 nm. These reactions were performed in 200 μl system containing 10 mM substrate, 1 mM NADPH, 5 μl YqiG and phosphate buffer (100 mM, pH 7.0) at 30 °C. To avoid a false positive NADPH consumption, we added two controls: the same reaction mixture without substrates and the same reaction mixture without YqiG. One unit of enzyme activity was defined as 1 μmol of NADPH oxidized per minute.

To determine the influence of pH, we used sodium acetate (pH 4–6), potassium phosphate (pH 6–8), Tris-HCl (pH 8–9) and sodium carbonate (pH 10) to determine the pH optima and the pH stability. And a temperature range of 20–60 °C was used for the determination of the optimum temperature. The effect of organic solvents was studied using ethanol, butyl acetate, isopropanol, n-hexanol, n-amyl alcohol, n-butanol, ethyl acetate, methanol, dimethyl sulfoxide (DMSO), iso-pentol alcohol and n-hexane. The kinetic parameters were determined using the Michaelis-Menten equation, and 1 mM NADPH remained a constant with the substrate added at between 0.25–2 mM. We used maleimide as substrate when determined the kinetic parameters of NADPH and NADH.

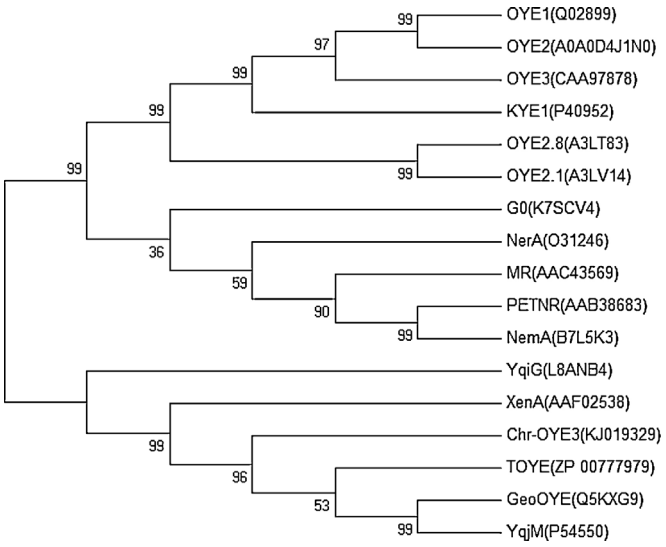

Fig. 1. Phylogenetic relationships between YqiG homologies and other OYEs with known functions. Amino acid sequences were used to generate the distance tree using ClustalX (Version 1.83). A distance neighbor-joining tree was then created using MEGA (Version 4.0). The OYE names are indicated to the right of the phylogenetic tree with NCBI accession numbers in parentheses.

2.6. Analytical procedures

The product and enantiomeric excess (e.e%) were analyzed using the Aglient 7820 gas chromatograph (GC) equipped with a flame ionization detector (FID) and the FS-column HYDRODEX β-TBDAC (25 m,0.25 mm).

3. Results

3.1. Sequence and phylogenetic analyses

The YqiG amino acid sequence was identified in the National Center for Biotechnology information (NCBI) databank using the basic local alignment search tool (BLAST). A phylogenetic analysis was performed for the YqiG with sixteen known OYE enzymes (Fig. 1). The results show that the YqiG did not cluster with other OYE enzymes but shared 37.9% identity with XenA from *Pseudomonas putida*. A phylogenetic tree shows that the YqiG belongs to the “thermophilic-like” subclass.

A sequence analysis was performed for YqiG with twelve known OYE enzymes (Fig. 2). The YqiG consists of 373 amino acids with the predicted molecular weight 40 kDa. The sequence results showed certain shared invariant residues only within this subfamily, such as M37, Y40 and L98 []. But the YqiG still showed different positions with the “thermophilic-like” subclass, such as C38, R353 and R391. The data indicate that YqiG may be more similar to the “thermophilic-like” subclass than the “classic” class.