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RESEARCH

Expression of Two Old Yellow Enzyme Homologues from *Gluconobacter oxydans* and Identification of their Citral Hydrogenation Abilities

Bo Yin · Xuepeng Yang · Guodong Wei · Yushu Ma · Dongzhi Wei

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Abstract Two genes that encode proteins which share 30–35% sequence identity with yeast OYE (Old Yellow Enzyme, an NAD(P)H FMN-oxidoreductase), the well-studied archetype of the OYE protein family, have been identified in *Gluconobacter oxydans* M5. The two genes are localized in the chromosome and plasmid, respectively. Comparison of the deduced amino acid sequences of the enzymes with database entries revealed 75.1% similarity and 64.9% identity to that of the *Pseudomonas syringae pv. glycinea* NAD(P)H-dependent 2-cyclohexen-1-one reductase. The two proteins were expressed as His-tag fusion proteins in *Escherichia coli* and purified. The ability of the purified proteins to hydrogenate citral was identified. The results showed that the α,β-double bond of citral *cis*-isomer 'neral' could be stereoselectively reduced to produce citronellal by the purified OYE homologues.

Keywords *Gluconobacter oxydans* · Old yellow enzyme · Citral · NADH FMN-oxidoreductase · Citronellal

Introduction

Old yellow enzyme (OYE, an NAD(P)H oxidoreductase), whose name came from its colour (due to bound FMN), was the first recognized flavoprotein purified from brewers' bottom yeast by Warburg and Christian in 1932 [1]. In 1991, the first OYE gene, OYE1 gene, was cloned from the *Saccharomyces carlsbergenesis* (brewers' bottom yeast) by Saito [2]. Subsequently, the new OYE genes were cloned from *S. cerevisiae*, which were designated as OYE2 and OYE3 gene, respectively [3, 4]. Recently, the genes of OYE homologues in other species such as plants [5] and bacteria [6] have been cloned and expressed. More recently, the new members of the family have been discovered through genome sequencing projects. The rapidly growing family provides available resource for researching its physiological function and exploring biocatalytic process.

As the rate of oxidation of OYE oxidase by molecular oxygen was low, it was not considered as a physiological reaction [3]. However, several isolated or expressed old yellow enzymes and their homologues have been reported to reduce the carbon double bond of α, β-unsaturated carbonyls recently [3, 6, 7]. Although the substrates are not natural for the enzymes, the enzymatic asymmetric alkene reduction by them, which can be employed in chiral building block production, might be a useful biocatalytic process. For example, the stereoselective enone could be reduced by *S. carlsbergensis* old yellow enzyme [8], and a double chiral compound, (4R,6R)-4-hydroxy-2,2,6-trimethylcyclohexan-one, could be produced via a two-step enzymatic asymmetric reduction, where the old yellow enzyme of *S. cerevisiae*, a homologue of the *S. carlsbergensis* old yellow enzyme, was employed [9].

Gluconobacter oxydans is well known for its oxidative fermentation [10]. The organism possesses a large set of intracellular soluble $NAD(P)^+$-dependent oxidoreductases and the enzymes of the oxidative pentose-phosphate system and the Entner–Doudoroff pathways [11]. It is noteworthy that these enzymes produce NAD(P)H in the

B. Yin · G. Wei · Y. Ma · D. Wei (✉)
State key Lab of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, China
e-mail: dzhiwei@ecust.edu.cn

X. Yang
College of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou 450002, China

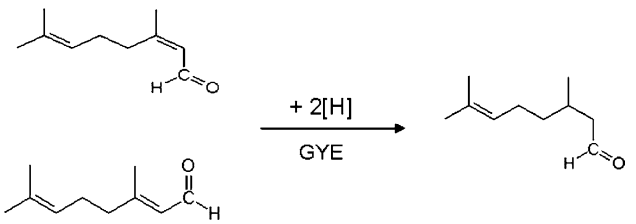

Fig. 1 Biotransformation of citral (geranial and neral) into citronellal

course of their reactions. It is still a matter of debate, how this cofactor is reoxidized in this strain. In the present work, two OYE homologues (NAD(P)H oxidase), which were designated as GYE1 and GYE2, were identified in the organism by sequence analysis. The corresponding genes, *gye1* and *gye2*, were successfully cloned and overexpressed as His-tag fusion proteins in *Escherichia coli* and purified. The ability of the purified GYEs to hydrogenate citral was identified. The results showed that the α, β-double bond of citral *cis*-isomer 'neral' could be stereoselectively reduced to produce citronellal by the purified OYE homologues, GYEs (Fig. 1).

Materials and Methods

Citral (a mixture of the *trans*-isomer geranial and the *cis*-isomer neral), citronellal, NADH and NADPH were purchased from Sigma-Aldrich.

Enzymes

The restriction enzymes, *Nde*I, *EcoR*I, *Nhe*I and *Hind*III, T4 DNA ligase, and LA Taq DNA polymerase were purchased from Takara Biotech Co. Ltd. (Dalian, China).

Bacterial Strains and Plasmids

Gluconobacter oxydans M5 was used for the preparation of genomic DNA. *Escherichia coli* DH5α (*rec*A$^-$ *end*A$^-$) (Stratagene) was used as the host for gene manipulation. BL21 (DE3) (Novagen) was used as the host for overexpression of the 6 × His tagged GYE proteins. The pGEM-T vector was purchased from Promega. The pET-28a was from Invitrogen. Luria–Bertani (LB) medium and Y-S medium (8% D-sorbitol, 2.4% yeast extract and other factors, pH 6.0) were prepared as described [12].

Database Searches and Sequence Alignments

Database searches were performed with the BLAST server from the NCBI (National Center for Biotechnology Information) using the blastp and tblastn options. All non-redundant GenBank CDS (coding sequence) translations and the RefSeq Protein, PDB, SwissProt, PIR (Protein Information Resource) and PRF (Protein Research Foundation) databases were employed. Amino acid sequences were aligned with the VectorNTI program (InforMax,Vector NTI 8.0).

Cloning of gye1 and gye2 Genes by PCR

Oligonucleotide primer pools were designed based on the complete genome sequence of *G. oxydans* 621H (Genbank accession number CP000009). The sequences of GYE1 primers were 5'-CGA*CATATG*CCAACCCTGTTCGATC-3' (sense strand) and 5'-TTA*GAATTC*TCAGTTGGGGCC GGAGGTG-3' (antisense strand). The sequences of GYE2 primers were 5'-TA*GCTAGC*ATGACCAGC CTGTTTG AG-3' (sense strand) and 5'-GAC*AAGCTT*TCAGCGAG CAAGCGG ATAATC-3' (antisense strand). Restriction sites were denoted in italics The genomic- and plasmid-DNA isolated from *G. oxydans* M5 were used as template, respectively. Amplification of *gye*1 and *gye*2 gene sequence were performed by PCR as follows: initial denaturing at 95°C for 5 min, followed by 30 cycles of 45 s at 95°C, 45 s at 55°C, and 80 s at 72°C. The PCR-amplified products were cloned into pGEM-T vector, respectively.

Construction of Expression Vector and Production of the GYE Proteins

The expression vector was constructed using standard recombinant techniques. The recombinant cloning plasmids, pGEM-*gye*1 and pGEM-*gye*2, were transformed into *E. coli* DH5α and amplified. The recombinant plasmids obtained were digested (*Nde*I and *EcoR*I for *gye*1, and *Nhe*I and *Hind*III for *gye*2), and the fragment of about 1,100 bp was recovered using a Gel Extraction Kit (Qiagen). The fragments were ligated with pET-28a, digested with the same enzymes, separately. The resultant expression plasmids were designated as pET28a-*gye1* and pET28a-*gye2*, respectively.

The sequences of pET28a-*gye*1 and pET28a-*gye*2 were confirmed by direct sequencing of the plasmid DNA (Invitrogen, Shanghai, China). Each of the two expression plasmids was transformed into *E. coli* BL21 (DE3) by electroporation with the Gene pulser XCell (Bio-Rad). The target recombinant strains, designated as BL21/ pET28a-*gye*1 and BL21/ pET28a-*gye*2, respectively, were screened by Kan and confirmed by PCR. Optimal expression was reached at 30°C. Induction was performed with 0.5 mM IPTG (isopropyl beta-D-thiogalactoside) at an A_{600} of 0.6.

The cells were then incubated for another 15 h. After centrifugation at 6,000 rpm for 15 min at 4 °C, the bacterial pellet was washed with 50 mM Tris-HCl, pH 7.0, and resuspended in the same buffer.

Purification of the Recombinant His-tagged GYE Proteins

The cells were lysed by sonication and the supernatant was collected by centrifugation at 10,000 rpm for 30 min at 4°C. The target protein was purified using affinity chromatography with a HisTrap^{TM} FF crude column (Phamacia, USA) with Ni-NTA agarose. The column was preequilibarated with equilibrium buffer (50 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, pH 7.0). The sample was loaded with a flow rate of 1.0 ml/min. After washing with three bed volumes of equilibrium buffer, the bound target protein was eluted with elution buffer (50 mM Tris-HCl, 0.5 M NaCl, 200 mM imidazole, pH 7.0). The target protein fractions were pooled and desalted through a HiTrap^{TM} desalting column (5 ml) with storage buffer (20 mM potassium-phosphate buffer, pH 7.2, 250 mM NaCl, 0.01% Triton x-100, and 50% glycerol (v/v)). The protein fractions were collected and stored at −20°C. Protein purity was determined by SDS-PAGE [13]. Proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250. The elution volume of the different soluble GYE proteins was identified by a Superdex 200 column.

Biocatalysis Assay

The biocatalysis assay was carried out in a two-phase reaction with 0.9 ml aqueous phase and 0.1 ml organic solvent. The aqueous phase contained 50 mM sodium phosphate, pH 7.0 and a defined mass of purified enzyme with 0.1 M NADH or NADPH. The organic solvent contained acetonitrile with 0.4 M citral. The reaction was started by adding 0.1 ml organic solvent in the 0.9 ml aqueous phase. The mixture was transferred in 4 ml screw capped glass vials and agitated on an orbital shaker at 150 rpm and 30°C for 3 h. After 3 h, the total sample was extracted with 0.4 ml freshly prepared solution of 0.15% (v/v) 1-octanol (internal GC standard) in chloroform. After centrifugation, 0.4 ml of the lower organic phase (acetonitrile/chloroform mixture) was removed for GC analysis.

Analytical Methods

Biotransformation reactions were monitored by GC/MS (6890N/5975MSD) using DB-WAX column (0.25 mm × 30 m; Agilent Technologies, USA) with nitrogen as the carrier gas (1 ml/min) and a flame ionization detector (280°C) with hydrogen (40 ml/min) and air (400 ml/min). The injection volume was 1 µl with a split ratio of 10:1. The injection temperature was 250°C. The column temperature was held at 100°C for 1 min, and then increased to 180°C at 20°C/min. After the column had been held at 180°C for 1 min, the temperature was increased to 220°C at 20°C/min and held for 2 min. Using 1-octanol as an internal standard, which had been subjected to the same extraction procedure as the samples, terpene concentrations were calculated. The product was identified by co-elution with a citronellal standard and mass spectral analysis.

Results and Discussion

The OYE Homologues of *G. oxydans*

Two OYE homologues were identified during a BLAST search of the *G. oxydans* genome using the amino acid sequence of *S. carlsbergensis* OYE isoform 1 (OYE1; NCBI accession number Q02899) as a probe. The two proteins were designated as GYE1 and GYE2, consistent with the nomenclature used for *Kluyveromyces lactis* (KYE1) [14] and *Hansenula polymorpha* (HYE1, HYE2 and HYE3) [15]. GYE1 (Genbank accession number YP_190936) and GYE2 (Genbank accession number PC_190404) are both annotated as putative oxidoreductase and belong to NADH: flavin oxidoreductase/NADH oxidase family [11]. Sequence analysis revealed that GYE1 and GYE2 shared 30% identity (43.5% similarity) and 31% identity (44% similarity) with OYE1, respectively. The *gye1* gene was localized in chromosome, while *gye2* was positioned in plasmid.

Database Comparisons and Sequences Alignments

The deduced amino acid sequences of the two GYEs were compared with sequence information available in the nr database using the NCBI blastp and tblastn programs, respectively. The two proteins showed high sequence similarities to a wide variety of members belonging to the OYE family of α, β-barrel flavoprotein oxidoreductases. For GYE1, the highest percentages of identity and similarity were found with *Pseudomonas syringae pv. glycinea* NAD(P)H-dependent 2-cyclohexen-1-one reductase (NCBI accession number AF093246; 75.1% similarity and 64.9% identity) [16].

The amino acid sequence alignment of GYE1 and GYE2 showed that the two proteins shared 73.3% sequence identity. It is not clear why two OYE homologues with

high level of similarity are simultaneously present in a single bacterial species.

Functional Overexpression and Purification of the Recombinant GYEs

The two *gye* genes were inserted in the pET28a vector downstream of the 6 × His coding sequence and transformed into *E. coli* BL21(DE3). When the cultures were grown initially at 37°C, each of the fusion proteins was mainly present in inclusion bodies. Therefore, the incubation temperature as well as the IPTG concentration was lowered. In case of culture temperature of 30°C and 0.5 mM IPTG used, large amount of soluble 6 × His-GYEs could be detected. The SDS-PAGE analysis of the soluble fractions gave an evident protein band with a molecular mass of about 43 kDa, which was consistent with the calculated molecular mass based on the deduced amino acid sequence (Fig. 2). However, it was observed that the soluble proteins were colourless and no enzymatic activities could be detected. The phenomenon could be associated with the insufficiency of FMN (flavin mononucleotide) in the medium, and the overexpressed proteins were therefore present in form of colourless apoproteins. Consequently, 0.1% riboflavin served as the precursor of FMN was added in the culture medium when IPTG was added. Under the effect of flavokinase, riboflavin was converted into FMN which was a cofactor bound to GYE proteins. The result showed that the soluble fractions displayed a bright yellow colour, which implied the presence of a flavin prosthetic group in target proteins.

The GYEs fusion proteins were purified via an affinity chromatographic step for further analysis. The final sample showed a single band on SDS-PAGE (Fig. 2 lane 8). The yield of each of the two proteins was about 45 mg/l. The elution volume of the both GYE proteins on a Superdex 200 column corresponded to a molecular mass of approximately 80 kDa, indicating that the native protein could form dimmers in solution.

Citral Biotransformation Profile

Although asymmetric hydrogenation of citral to produce chiral citronellal by bacteria, yeasts and filamentous cells has been reported previously [17], the enzyme involved in the reaction has not yet been identified. Figure 3 showed the profiles of citral biotransformation by the purified GYE1 in the aqueous/acetonitrile two-phase system. The concentration of citronellal increased rather linearly over the 3-h period. For the substrate citral *cis*-isomer 'neral' and citral *trans*-isomer 'geranial', however, only the concentration of neral was observed to decrease over time. The amount of neral decreased significantly relative to internal standard, while the amount of geranial did not decrease. The biocatalysis functions of GYE2 to citral is similar to those of GYE1 (data not shown). These results indicated that the neral was reduced stereo-selectively by GYEs to produce citronellal. This may be due to a steric hindrance to hydride transfer [7].

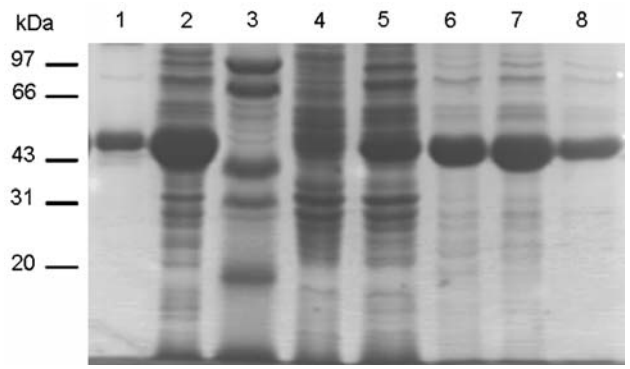

Fig. 2 Expression of GYEs in *E.coli* and purification of the recombinant enzymes. Lane1, purified recombinant GYE1 after affinity chromatography; lane 2, supernatant of cell-free extracts of recombinant BL21/ pET28a-*gye*1 before affinity purification; lane 3, molecular mass standard; lane 4, supernatant of cell-free extracts of recombinant E.coli BL21 (DE3) containing pET28a; lane 5, total cell-free extracts of recombinant BL21/ pET28a-*gye*2 after induction by IPTG; lane 6, supernatant of cell-free extracts of BL21/ pET28a-*gye*2; lane 7, purified GYE2 after affinity chromatography; lane 8, final sample of GYE2 after desalted

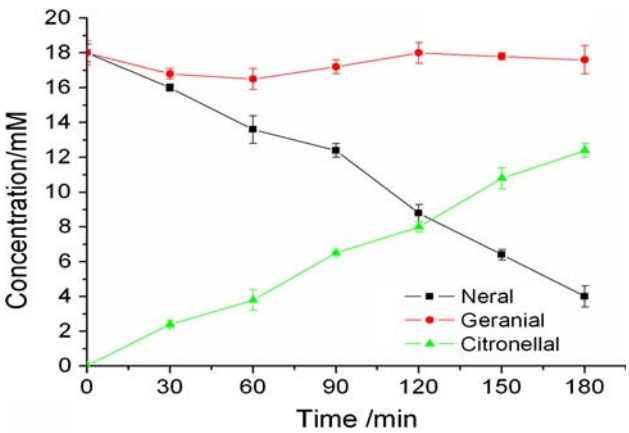

Fig. 3 Citral biotransformation by GYE in the aqueous/acetonitrile two-phase system. The biocatalysis assay was carried out in a two-phase reaction system contained 50 mM sodium phosphate, pH 7.0, 0.15 mg/ml purified enzyme, 100 mM NADH or NADPH and 40 mM citral. Average values from three replicates with error bars are given

Conclusion

As demonstrated by the data, the old yellow enzyme homologues GYEs from *G. oxydans* are novel NADH dependent FMN-oxidoreductases, which can stereo-selectively reduce the carbon double bond of α, β-unsaturated carbonyls of citral. It is worth mentioning that the citral *cis*-isomer 'neral' can be stereoselectively reduced to produce citronellal by purified OYE homologue, GYEs.

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