Characterization of Acyl-CoA Oxidases from the Lipolytic Yeast *Candida aaseri* SH14

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The lipolytic yeast *Candida aaseri* SH14 contains three Acyl-CoA oxidases (ACOXs) which are encoded by the *CaAOX2*, *CaAOX4*, and *CaAOX5* genes and catalyze the first reaction in the β-oxidation of fatty acids. Here, the respective functions of the three *CaAOX* isozymes were studied by growth analysis of mutant strains constructed by a combination of three *CaAOX* mutations in minimal medium containing fatty acid as the sole carbon source. Substrate specificity of the *CaAOX* isozymes was analyzed using recombinant *C. aaseri* SH14 strains overexpressing the respective genes. *CaAOX2* isozyme showed substrate specificity toward short- and medium-chain fatty acids (C6-C12), while *CaAOX5* isozyme preferred long-chain fatty acid longer than C12. *CaAOX4* isozyme revealed a preference for a broad substrate spectrum from C6-C16. Although the substrate specificity of *CaAOX2* and *CaAOX5* covers medium- and long-chain fatty acids, these two isozymes were insufficient for complete β-oxidation of long-chain fatty acids, and therefore *CaAOX4* was indispensable.

Keywords: Acyl-CoA oxidase, lipolytic yeast, *Candida aaseri*, β-oxidation, substrate specificity

Introduction

The lipolytic yeast *Candida aaseri* SH14, isolated from the compost of oil palm empty fruit bunches, can utilize various fatty acids and long-chain alkanes as a sole carbon source and is resistant to high concentrations of organic acids [1]. The capability to breakdown hydrophobic substrate may explain its isolation from oil palm agricultural waste. Therefore, this strain was considered a microbial factory for the production of bio-based chemicals from plant oils [2]. In mammalian cells, the biodegradation of fatty acids occurs mainly by β-oxidation in mitochondria and peroxisomes, whereas this event is only present in peroxisomes of yeasts [3, 4]. The breakdown of fatty acid is begun with the formation of fatty acyl-CoA by the fatty acyl-CoA ligase, which catalyzes the pre-step reaction for β-oxidation. The β-oxidation of yeast involves the sequential reaction of four enzymes: acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase [4, 5]. ACOX catalyzes the limiting step of peroxisomal β-oxidation by converting acyl-CoA to 2-trans-enoyl-CoA [6].

ACOX has been isolated from various organisms, including microbes [7-12], plants [13-16], and also mammals [17-20]. Most organisms contain several isozymes having different substrate specificities to utilize various carbon chain-length fatty acids as carbon sources. For example, *Saccharomyces cerevisiae* [8] and *Aspergillus nidulans* [21] contain one ACOX, while *Candida tropicalis* [10] and *Yarrowia lipolytica* [22] have five and six isozymes with different substrate specificities.

In a previous study, we identified three genes encoding ACOX (*GaAOX2*, *GaAOX4*, and *GaAOX5*) from the *C. aaseri* SH14 genome on the basis of sequence homology. A β-oxidation mutant completely lacking ACOX activity was directly constructed by simultaneous disruption of six copies of ACOX genes in diploid cells using the CRISPR-Cas9 system; however, the functional differences between these isozymes were not determined [2]. In this study, we have evaluated the ACOX activity of the three isozymes by in vivo study of the respective mutants to understand the utilization of fatty acid in *C. aaseri* SH14.

Materials and Methods

Strains, Chemicals and Medium

*C. aaseri* SH14 (ura3) [2], and their mutant strains were cultured in YPD (1% yeast extract, 2% peptone, and 2% glucose). Selection of URA+ transformants was performed on a synthetic complete medium lacking uracil (SC-ura; 0.67% yeast nitrogen base without amino acids, 0.077% ura dropout supplement, 2% glucose, and 2% agar). For screening of *CaAOX* mutants, YPD supplemented with 20 μg/ml nourseothricin (NPT, Sigma Chemicals Co.,
Construction of CRISPR-Cas9 Vector for Disruption of Acyl-CoA Oxidase Isozyme Genes

The episomal CRISPR-Cas9 vector, pAN-Cas9, developed for gene manipulation of diploid yeast C. aasseri SH14, was used for disruption of CaAOX genes [2]. This vector contains an autonomous replicating sequence and NAT1 (NTC acetyl transferase) gene as a selection marker gene against NTC. The sgRNA sequences targeting for each CaAOX gene (CaAOX2: 5′-CTGAGATCAGGAGCTTCCCA-3′, CaAOX4: 5′-GCACATGTTTCTAATGTTCGA-3′, and CaAOX5: 5′-CCAACCCCCAGAAGCTACA-3′) were selected by online CRISPR gRNA design tool, ATUM (www.atum.bio/eCommerse/cas9/input). Target sequences with a low identities against C. aasseri genome database were selected. To construct double mutants by simultaneous disruption of CaAOX genes, sgRNA targeting sequences were selected from the conserved regions containing PAM sequence (NGG) (for CaAOX2/ CaAOX4: 5′-CTCATATTGAGCTTACAAAT-3′, for CaAOX2/CaAOX4: 5′-GGTGCTAAAATGGGTAGAGA-3′) (Fig. S2). Each sgRNA was fused with translation elongation factor (TEF1) promoter, hammerhead ribozyme, and hepatitis delta virus ribozyme by polymerase chain reaction (PCR). The sgRNA expression cassettes were then cloned into the NotI-XbaI sites of pAN-Cas9 to make pAN-Cas9-gAOXn and confirmed by Sanger sequencing. All PCR amplifications were done using high-fidelity Phu DNA polymerase. Primers used in this study were listed in Table S1.

Transformation and Characterization of C. aasseri SH14 Transformants

Transformation of foreign genetic materials into C. aasseri SH14 was done using electroporation as described in previous study [2]. Competent cells were prepared in transformation buffer containing 5 mM lithium acetate, 0.5 M sorbitol, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA, and then 0.01 volume of freshly prepared 1 M dithiothreitol was added. After 1 h incubation at room temperature, cells were washed with 1 M sorbitol three times, and then resuspended in 0.5 ml of 1 M sorbitol for transformation. For each transformation, 0.5 μg of plasmid DNA was mixed with 100 μl of competent cells in a 2 mm electroporation cuvette before being incubated on ice for 10 min. Electroporation was performed using the Gene Pulser II (Bio-Rad, USA) at 2.25 kJ, 50 μF, and 200 Ω. After electroporation, 1 ml of YPD containing 1 M sorbitol was added before incubation at 30°C overnight. All transformants were selected on YPD supplemented with 20 μg/ml NTC as selection media after a 3-day incubation at 30°C. Disruption of AOX genes was confirmed by colony PCR. Randomly selected colonies were suspended in lysis buffer (10 mM sodium phosphate, and 2 mg/ml lyticase) and incubated at 35°C for 30 min. Next, proteinase K solution (2 mg/ml) was added to the mixture, which was then incubated at 50°C for 10 min followed by inactivation of the proteinase K at 80°C for 10 min. Cell lysate (1 μl) was used as a template for PCR analysis. The mutations caused by CRISPR-Cas9 from each transformation were confirmed by direct sequencing of the PCR products by Bionics (Korea) without cloning into a plasmid vector.

Expression and Characterization of AOX Genes in C. aasseri SH14

To use as an auxotrophic selection marker for the expression of AOX genes, CaURA3 expression cassette containing promoter and terminator was amplified from C. aasseri SH14 genomic DNA by PCR, and cloned into the KpnI and XhoI site of pBluescript II vector, and the vector was named as pCaURA3. Open reading frame (ORF) of CaAOX2, CaAOX4, and CaAOX5 gene were fused with 600 bp of C. aasseri glyceraldehyde 3-phosphate dehydrogenase promoter (GAPDHp), and 200 bp of GAPDH terminator (GAPDHub), and then cloned into Xbal, and XhoI site at pCAURA plasmid, respectively. Plasmids were linearized by XbaI and KpnI restriction enzymes to produce a fragment containing CaURA3 and AOX expression cassette. C. aasseri SH14-245 (ura3, aox2, aox4, and aox5 mutant) strain was transformed with the linearized fragments. Integrations of CaAOX expression cassettes into the C. aasseri SH14 genome were confirmed by colony PCR using Gdh-F primer, and each AOXs-R internal primer, located at CaAOX2, CaAOX4, and CaAOX5 ORF. To confirm the CaAOX gene expression, each cell was grown in 50 ml YPD broth for 48 h at 30°C. The cells were harvested by centrifugation at 10,000 x g at 4°C for 30 min, and lysed using TRLZol reagent (Invitrogen) according to manufacturer's instructions. Cell lysates were analyzed by SDS-PAGE using commercial NuPAGE Novex 4–12% Bis-Tris polyacrylamide gels (Invitrogen) and stained with Coomassie blue. Western blot analysis was performed using anti-His antibody (Sigma Chemicals Co.) after 1:1000 dilution. Protein samples were electrophoresed and then transferred to a nitrocellulose membrane using the iBot 2 Dry Blotting System (Thermo Fisher Scientific, USA) following the manufacturer’s instructions. The reacting antibodies were detected with anti-mouse immunoglobulins conjugated to alkaline phosphatase (Sigma Chemicals Co.) Acyl-CoA oxidase activity was determined by using the method describe by Wang et al. [22]. The tested acyl-CoA substrates were butyryl-CoA (C4:0-CoA), hexanoyl-CoA (C6:0-CoA), octanoyl-CoA (C8:0-CoA), decanoyl-CoA (C10:0-CoA), lauroyl-CoA (C12:0-CoA), myristoyl-CoA (C14:0-CoA), and palmitoyl-CoA (C16:0-CoA) (Sigma Chemicals Co.).
Statistical Analyses
All data are presented as the mean value ± SD of three experiments. Statistical comparison of growth and ACOX activity were performed using Student’s t-test with a two-tailed distribution (Microsoft Excel), and compared to the appropriate control strain. Values were considered statistically significant at a p-value of < 0.05.

Results and Discussions
Construction of ACOX Isozyme Mutants
Three acyl-CoA oxidase genes found in C. aaseri SH14 genome were named as CaAOX2, CaAOX4, and CaAOX5 based on the result of a homology search against the NCBI database using BLASTP program. The amino acid sequence of CaAOX2 (55.17%) and CaAOX4 (66.43%) showed the highest homology with ACOX2 and ACOX4 from Candida tropicalis. The amino acid sequence of CaAOX5 showed the highest homology (57.37%) with ACOX5 from Candida albicans. Although we named the CaAOX genes according to homology analysis, identification of the functional differences among ACOXs using homology study is very difficult because there is no information on the residues that determine the substrate specificity, and the catalytic core regions are almost conserved (Fig. S1).

To identify the functional difference between the three C. aaseri SH14 ACOX isozymes, single, double, and triple mutant strains were constructed. Respective genes were inactivated by the CRISPR-Cas9 system that was developed for genome engineering of diploid yeast C. aaseri SH14. The Cas9 gene and sgRNA were expressed under the control of constitutive TEF1 and GAPDH promoters, respectively, in an episomal vector, pANCas9sgRNA. The sgRNA target sequences for each isozyme were designed using the CRISPR gRNA design tool, ATUM (www.atum.bio/eCommerce/cas9/input). The highest scores from ATUM with fewer than 15 identities from BlastN analysis against C. aaseri genome database were selected as sgRNA target sequences. For single disruption, sgRNA specific to each isozyme was designed as sgAOX2, sgAOX4 and sgAOX5 (Fig. S2). The pANCas9 plasmids carrying different sgRNAs were transformed into the C. aaseri SH14 strain to create an indel mutation at the target sites. Mutations were confirmed by direct sequencing of the target locus amplified by PCR with ACOX2, ACOX4 and ACOX5 primers (Fig. S2). The SH14-245 was constructed by sequential disruption of ACOX2 and ACOX5 because there was no available sgRNA target sequence specific to CaAOX2 and CaAOX5 (Fig. S2). Mutation efficiencies in each case were higher than 40%. The triple mutant, SH14-245, was constructed in a previous study [2]. In this study, we created 6 mutants and their genotypes are listed in Table 1.

Characterization of Mutant Strains
The involvement of acyl-CoA oxidase isozymes in peroxisomal β-oxidation was investigated by the evaluation of the growth of mutants on minimal media containing oleic acid as the sole carbon source. Serially diluted cells were dotted on YPD, and SMO plates (Fig. 1). There was no significant difference in growth between wild-type strain, SH14-2, SH14-5, and SH14-25 strain. Slightly lower growth of SH14-2 in SMO medium than wild-type strain was a result of a low number of dotted cells compared to other strains (Fig. 1A). On the other hand, none of the strains containing the AOX4 mutation (SH14-4, SH14-24, SH14-45, and SH14-245) could grow on SMO medium (Fig. 1B). Moreover, similar results were observed in liquid culture (p < 0.05) (Fig. 1C).

To use long-chain fatty acid as the carbon source by β-oxidation, sequential reaction by the long chain-, medium chain-, and short chain-specific ACOX or an enzyme with broad substrate specificity is required. The CaAOX2, CaAOX5 single mutants (SH14-2 and SH14-5), and double mutant (SH14-25) grew similarly to wild type in SMO medium due to the remaining CaAOX4 isozyme (Fig. 1). On the other hand, despite CaAOX2 and CaAOX5 isozymes working normally, CaAOX4 mutant (SH14-4) did not grow in SMO medium. These results imply that CaAOX4 is an essential ACOX with broad substrate specificity for the utilization of long-chain fatty acids.

Expression of ACOX Isozyme in C. aaseri SH14-245
To study the substrate specificity of acyl-CoA oxidase isozymes, each isozyme was expressed in C. aaseri triple mutant (SH14-245). Since the transcription of AOX genes in yeast is repressed in glucose [23], for strong and stable expression of AOX genes, the ORFs of CaAOX2, CaAOX4, and CaAOX5 were fused with constitutive GAPDH promoter and integrated into the genome of C. aaseri SH14-245 using CaURA3 as selection marker.

Table 1. C. aaseri SH14 AOX mutant strains constructed in this study.

| Type             | Strain | Genotype | Growth in SMO |
|------------------|--------|----------|---------------|
| Wild type        | SH14   | ura3     | Normal        |
| Single mutant    | SH14-2 | ura3, aox2 | Normal        |
|                  | SH14-4 | ura3, aox4 | None          |
|                  | SH14-5 | ura3, aox5 | Normal        |
| Double mutant    | SH14-24| ura3, aox2, aox4 | None          |
|                  | SH14-45| ura3, aox4, aox5 | None          |
|                  | SH14-25| ura3, aox2, aox5 | Normal        |
| Triple mutant    | SH14-245| ura3, aox2, aox4, aox5 | None          |
Transformants were verified by colony PCR and expression of ACOX was confirmed by western blot analysis of cell extract using an anti-His antibody. The expected sizes of \( \text{CaAOX2} \), \( \text{CaAOX4} \), and \( \text{CaAOX5} \) isozymes were 79.1 kDa, 79.6 kDa, and 80.2 kDa, respectively. In the western blot analysis, the expected size protein bands, with several smaller protein bands, were detected only in the transformants, indicating successful expression of the recombinant proteins (Fig. 2A). In addition, the smaller bands seem to be proteins degraded by endoprotease.

**Characterization of Recombinant ACOX Isozyme Produced in* C. aaseri* SH14**

The activity of ACOX isozymes against fatty acids with chain lengths between 4 and 16 carbons was analyzed using the cell extract of transformants. As the SH14-245 strain cannot use long-chain fatty acid as a carbon source (Fig. 1), no ACOX activity was measured in the SH14-245 strain grown in a glucose medium. The absence of detectable ACOX activity in the SH14-245 strain means that there are no functional ACOX genes except \( \text{CaAOX2} \), \( \text{CaAOX4} \), and \( \text{CaAOX5} \). \( \text{CaAOX5} \) isozyme showed substrate specificity toward long-chain fatty acid longer than \( \text{C12} \). Meanwhile, \( \text{CaAOX2} \) isozyme preferred short- and medium-chain (\( \text{C6-C12} \)) fatty acids. \( \text{CaAOX4} \) isozyme showed broad–spectrum substrate specificity from \( \text{C6-C16} \) (\( p < 0.05 \)) (Fig. 3). Considering the result that \( \text{CaAOX4} \) mutant did not grow in SMO medium despite \( \text{CaAOX2} \) and \( \text{CaAOX5} \) isozyme working normally (Fig. 1), \( \text{CaAOX2} \) and \( \text{CaAOX5} \) isozymes were shown to be insufficient for complete \( \beta \)-oxidation of long-chain fatty acid, and \( \text{CaAOX4} \) isozyme was indispensable. Since the \( \text{CaAOX5} \) isozyme has substrate specificity toward long-chain fatty acid longer than \( \text{C12} \), it is indispensable in the complete \( \beta \)-oxidation of long-chain fatty acid in this strain.
Fig. 3. Characterization of substrate specificity of recombinant CaACOX. ACOX isozyme activity was measured independently using C6-C16 fatty acyl-CoA. Protein concentrations were standardized.

fatty acid, the SH14-24 strain (CaAOX2 and CaAOX4 double mutant) could be used for bioconversion of long-chain fatty acid to medium-, and short-chain dicarboxylic acids such as sebacic acid (C10), suberic acid (C8), and adipic acid (C6) for polymer-based industries.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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