Algal Research 31 (2018) 225–231

Contents lists available at ScienceDirect

Algal Research

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

Characterization of 3-ketoacyl-coA synthase in a nervonic acid producing oleaginous microalgae *Mychonastes afer*

Yong Fan[a], Cheng Yuan[b], Yi Jin[a], Guang-Rong Hu[b], Fu-Li Li[a,*]

[a] Key Laboratory of Biofuels, Shandong Provincial Key Laboratory of Energy Genetics, Qingdao Engineering Laboratory of Single Cell Oil, Chinese Academy of Sciences, Qingdao 266101, PR China

[b] Shandong Provincial Key Laboratory of Synthetic Biology, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266101, PR China

ARTICLE INFO

Keywords:
Nervonic acid
3-ketoacyl-coA synthase
Mychonastes afer
Saccharomyces cerevisiae

ABSTRACT

Nervonic acid (C24:1, Δ15), a monounsaturated long chain fatty acid, is a major component of mammalian neural tissue. Lack of nervonic acid in human usually leads to neurological disorder. Recently, production of nervonic acid from plant for pharmaceutical, nutraceutical, and functional food received increasing attention. We have previously isolated a microalgal strain *Mychonastes afer* HSO-3-1, which can accumulate approximately 6% of nervonic acid and < 0.5% of erucic acid in triacylglycerol. In this study, the full length of 3-ketoacyl-coA synthase gene *MaKCS*, encoding the first component of fatty acid elongation complex, was cloned and expressed in *Saccharomyces cerevisiae* BY4741 under the control of GAL1 promoter. *S. cerevisiae* harboring pYC230-Gal-MaKCS resulted in the production of 1.5% nervonic acid in total lipid. The expression of *KCS* gene in *M. afer* was found to be induced under stress conditions such as nitrogen deficiency and high light, which was revealed by real-time PCR analysis. MaKCS was demonstrated to be able to extend very long chain monounsaturated fatty acids including nervonic acid, which provides insights into how nervonic acid is synthesized in microalgae.

1. Introduction

Nervonic acid, C24:1 Δ15 (*cis*-tetracos-15-enoic acid) ω-9, is a major very long-chain monounsaturated fatty acid (VLCMFA) distributed widely in human nervous system. Nervonic acid is bound via amide bond to a sphingosine base [1,2], while the nervonyl sphingolipids are main component of the white matter of brains and myelin sheath of nerve fibers [3]. It can regenerate the damaged protective sheath and induce the growth of nerve fibers. Therefore, supplement of nervonic acid has become a means to treat the symptoms of many neurological disorders, including multiple sclerosis symptoms [4]. In addition, nervonic acid has been reported as one of the essential long-chain fatty acids for infant, particularly premature infants' nerve cells development [5,6]. Bettger and coworkers have shown that dietary nervonic acid significantly elevates the nervonic acid content of sphingomyelin in liver, heart, skeletal muscle and adipose tissue in developing rats [7,8]. Therefore, nervonic acid is regarded as a bioactive supplement, similar to docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and conjugated linoleic acid, for human and animal health [6,9].

Previously, nervonic acid has been prepared mainly from marine organisms. Since many restrictions on capture of marine animal have been gradually established, natural nervonic acid is limited and can't meet people's health needs. Though *cis*-13-docosenyl methyl ester could be used as materials to synthesize nervonic acid by chemical process [10], it is more suitable and accessible to find new alternative natural sources to extract nervonic acid for pharmaceutical and food industry. In addition to animals, nervonic acid has also been found in seed oils of some plants, mainly including *Malania oleifera* [11], *Cardamine graeca* [12], *Lunaria annua* [13], *Tropaeolum speciosum* [14], *Acer mono*, *Acer palmatum*, *Thlaspi arvense*, and *Macaranga henricorum* [15,16]. Some of the plants, for example *L. annua* and *A. truncatum*, have been grown for the production of nervonic acid. However, they all face the problem of seed harvest since the seeds drop to the ground easily. More recently, a filamentous fungal strain *Mortierella capitata* RD000969 has been selected and can accumulate 6.94% of nervonic acid in total fatty acids [17].

Nervonic acid is produced by three consecutive steps of carbon chain elongation from oleic oil (C18:1 ω-9). In general, very long chain fatty acids (VLCFAs) are synthesized by fatty acid elongation system on the endoplasmic reticulum membrane. This process involves four reactions: firstly, malonyl-CoA and long-chain acyl-CoA are condensed by a 3-ketoacyl-CoA synthase (KCS), then the 3-ketoacyl-CoA is reduced by

* Corresponding author at: Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Science, 189 Songling Road, Qingdao, PR China.
E-mail address: lifl@qibebt.ac.cn (F.-L. Li).

https://doi.org/10.1016/j.algal.2018.02.017
Received 7 June 2017; Received in revised form 12 February 2018; Accepted 14 February 2018
2211-9264/ © 2018 Elsevier B.V. All rights reserved.

the 3-ketoacyl-CoA reductase (KCR) to generate a 3-hydroxyacyl-CoA. The 3-hydroxyacyl-CoA is dehydrated by 3-hydroxacyl-CoA dehydratase (HCD) and subsequently reduced by trans-2,3-enoyl-CoA reductase (ECR) to form the elongated acyl-CoA. In fatty acid elongation, the membrane-bound fatty acid elongase, KCS, is the initial and rate-limiting enzyme. More importantly, the substrate specificities of fatty acid elongation are determined by KCS, whereas, in VLCFA biosynthesis, the three other enzymes usually have a broad substrate specificity and are shared by all tissues [18,19]. The enzyme KCS was identified and characterized from *Arabidopsis thaliana* firstly [19,20]. FAE1 enzymes from plant are specific for monounsaturated and saturated fatty acids, and they show no activity towards polyunsaturated fatty acids (PUFA) [21]. KCS enzymes anchor to the endoplasmic reticulum via one or two transmembrane helices at the N-terminus, and have three key catalytic residues, i.e. Cys, His, and Asn or Cys, His, and His [22–24]. Over the past decade, numerous KCS proteins for VLCFA biosynthesis have been identified from plants [22,25]. Many evidences have shown that the chain length of VLCFAs depends on the substrate specificity of the KCS [19,20,23,25–29].

Mychonastes afer, which can accumulate 53.9% total lipid of the dry cell weight, has been isolated and characterized [30]. More than 6% of nervonic acid has been detected in TAG, while $< 0.5\%$ of erucic acid (C22:1 Δ13) presented, showing great potential for the production of nervonic acid. Earlier studies have shown that diets rich in erucic acid appears to have toxic effects on the heart, so it is undesirable for human consumption [27]. Till now, there is no KCS reported in microalgae responding for the accumulation of nervonic acid. In this study, a *KCS* gene from *M. afer* was cloned, and the role of *MaKCS* in the elongation of fatty acid to generate VLCMFA, nervonic acid, was investigated by heterologous expression in *Saccharomyces cerevisiae* and *Phaeodactylum tricornutum*, respectively.

2. Materials and methods

2.1. Strain and culture condition

M. afer HSO-3-1 was stored at the China General Microbiological Collection Center (CGMCC 464, Beijing, China). Microalgal cells were incubated in a column bioreactor (3.2 cm in diameter, 60 cm in height) containing 300 mL BG-11 medium [31] bubbled with CO_2-enriched air (1.5% CO_2), and maintained at 24 °C under continuous illumination provided by fluorescent at 80 μmol photons·m^{-2}·s^{-1}. Stress treatments were applied by increasing the light intensity to 180 μmol photons·m^{-2}·s^{-1} or resuspending cells to nitrogen free BG11 medium when the optical density at 680 nm of microalgal cells reached 4.

2.2. Clone of KCS gene from M. afer

According to the conserved sequences in *KCS* genes, including those from *A. thaliana* (gi: 881614), *Chlamydomonas reinhardtii* (gi: 159478236), *P. tricornutum* (gi: 217409269), *Dunaliella salina* (gi: 12963438), *Volvox carteri f Nagariensis* (gi: 302828998), and *Chlorella variabilis* (gi: 552824028), two pairs of primers were designed for the multiplex PCR. The cDNA of *M. afer* was used as template, and a fragment about 600 bp was obtained and verified by DNA sequencing. The method of rapid amplification of cDNA ends (RACE) was used to get the nucleotide sequences of 5′ and 3′ ends [32]. The primers were listed in Supplemental Table 1.

Gene sequence similarity analysis was conducted by using BLASTN. The amino acid sequences of the KCS enzymes from different species were aligned with ClustalW. Prediction of transmembrane region was performed using the TMHMM Server v 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). MEGA 6.0 was used for phylogenetic reconstruction [33]. The I-TASSER online (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was served for homology modeling and structure prediction.

2.3. Expression profiling of the MaKCS gene under stress conditions

The high intensity of illumination and nitrogen deficiency were introduced to induce lipid accumulation when the optical density at 680 nm of microalgal cells reached 4. The samples were withdrawn at regular intervals for measuring cell growth and lipid composition. The expression levels of *MaKCS* gene was determined under stress conditions at different time points. The RNAs of microalgal cells were extracted using the EasyPure Plant RNA Kit (TransGen Biotech, Beijing, China). DNA fragment analysis of PCR with 18S rDNA primer was conducted to detect the contamination of DNA in the total RNA samples. Then RNAs were converted into complementary DNAs (cDNAs) by reverse transcriptase with Reverse Transcriptase M-MLV (RNase H-) following the manufacturer's protocols (Takara, Dalian, China). Quantitative real-time (q-RT) PCR was performed using the KCS-5RP and KCS-3RP primers (Supplemental Table 1). DNA fragment of β-actin, *rbcL*, and α-tubulin in *M. afer* was amplified and evaluated as an internal control (primers shown in Supplemental Table 1). Among them, *rbcL* gene showed the most stable expression level and Ct value and was selected as the internal control. For quantitative analysis of RNA transcript levels, the Light Cycler® R480 II (Roche Diagnostics, Mannheim, Germany) was used according to manufacturer's instructions. The qRT-PCR reaction mixtures were incubated for 5 min at 95 °C, followed by 40 amplification cycles of 10 s at 95 °C, 10 s at 59 °C and 15 s at 72 °C. All reactions were performed in 96-well plates in triplicate. We calculated the relative levels of transcript abundance of the genes with $2^{-\Delta\Delta CT}$ method [34].

2.4. Lipid extraction and fatty acid analysis

Total lipids were extracted using a chloroform/methanol (2:1, v/v) mixture based on a modified procedure [35]. Briefly, the cells were centrifuged at 4000 $\times g$ for 10 min and washed 3 times by deionized water, then freeze-dried for weighing and lipid extraction. Lyophilized algal pellets were suspended in chloroform/methanol and homogenized by vortex mixer for 10 min; in the meantime, freezing-thawing was taken twice by liquid nitrogen. After centrifugation at 4000 $\times g$ for 10 min, the supernatant was collected and the precipitate was extracted once more. Potassium chloride solution (0.9%) was added at a proportion of 1:3 (v/v) of lipid extract supernatant. The mixture was then shaken vigorously for 1 min and centrifuged at 4000 $\times g$ for 5 min. The chloroform layer was collected and dried under nitrogen stream to constant weight and then quantified gravimetrically.

Fatty acid methyl esters (FAMEs) were prepared by incubating the lipid extracted with 2% H_2SO_4-methanol solution at 85 °C for 3 h. The composition of FAMEs was analyzed by using a gas chromatography–mass spectrometry (GC–MS, 7890A-5975C, Agilent technologies, Santa Clara, CA) equipped with a fused column (HP-INNOWAX, 30 m × 0.25 mm × 0.25 μm; for the regiospecific analysis of nervonic acid in TAG: HP-5, 30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas. The injector temperature was set at 250 °C. The column temperature was maintain at 100 °C for 1 min and elevated to 240 °C at a rate of 10 °C·min^{-1}, and then maintained for 10 min. The composition of FAMEs was analyzed by comparing the obtained mass spectra with Wiley libraries. The internal standard C17:0 added in each sample was used for calculating the recovery of FAMEs.

2.5. Regiospecific analysis of nervonic acid in TAG

Polar and non-polar lipids in *M. afer* were separated by using solid phase extraction (SPE) column (Bond Elut SI, 500 mg/3 mL, Agilent, Santa Clare, CA). The 3 mL silica column was equilibrated by using 10 mL of chloroform:acetic acid (100:1, V/V) and lipid extracted from *M. afer* was added into the column. Neutral lipids were eluted with 5 mL chloroform:acetic acid (100:1, V/V) and 5 mL chloroform:acetone (80:20, V/V). Glycolipids and phospholipids were eluted with 10 mL of

Download English Version:

https://daneshyari.com/en/article/8086172

Download Persian Version:

https://daneshyari.com/article/8086172

Daneshyari.com