Characterization of S-Acyltransferase Gene Fragment from an Isolate of Tropical Marine Microalgae *Chlorella vulgaris* CBI

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Abstract. S-Acyltransferase gene (pat) encodes a protein acyltransferase (PAT) that acts as an enzyme catalyzing an acyl chain transfer to the molecular acceptor, mono- or di-acylglycerol, in the biosynthesis of triacylglycerol (TAG). To date, pat from tropical marine microalgae has yet to be explored. Here, we identified a marine microalgae isolate (CBI) based on its physical morphology and 18S rRNA gene, then we isolated the pat fragment by reverse transcription (RT) PCR, followed by ligation into pGemT vector and cloning in *E. coli* TOP10. The pat fragment was characterized using several DNA analysis softwares. Our results showed that the isolate CBI obtained from Maluku waters was green-colored and spherical in shape with a cell diameter of 2–10 μm. A 1644-bp of 18S rRNA gene was obtained and showed a 99% nucleotide sequence similarity with 18S rRNA gene of *Chlorella vulgaris* (GenBank no. KF574391.1). The microalgae isolate was identified as *Chlorella vulgaris* CBI. Reverse transcription produced a 600-bp cDNA, in which the first 300-bp nucleotide was unidentified, however starting from nucleotide no. 301 to the end, it showed a 51% identity with the pat fragment of *Chlorella variabilis* (GenBank no. XP_005849764.1). A 77-residue peptide chain deduced was identified as the first N-terminus of PAT (domain transporter protein). This pat fragment of *Chlorella vulgaris* CBI was useful as a starting point to isolate and characterize the whole pat sequence.

Keywords: marine, microalgae, *Chlorella vulgaris*, S-acyltransferase (pat).

1. Introduction

Marine microalgae are potential biota that produce biomass as alternative sources of renewable energy. The biomass from microalgae contains lipids that can be processed into biodiesel. Nevertheless, high production costs become an obstacle in the biodiesel production [1]. Therefore, we need to look for a method to reduce the production cost of biofuel from microalgae. An alternative solution for this obstacle is increasing the microalgae capability to synthesize lipids (TAG droplet) as biodiesel feedstocks. Efforts to increase the lipid production from microalgae have been conducted in various ways, such as by cultivating microalgae in a large scale [1], conditioning microalgae cultivation in a
limited medium (reduction of nitrogen, carbon, sulfur, zinc, phosphorus etc.) [2], and limiting or inhibiting cell metabolism on carbohydrates production [3]. However, these efforts often resulted in a lower quality of microalgae life cycle and the metabolism products, including the quality of lipids, as well as a lower quantity of total biomass [3]. The other alternative to increase cellular lipid biosynthesis in microalgae is by genetic engineering. Genetic engineering efforts to transform microalgae to possess certain desirable traits, such as an excessive increase in cellular lipid biosynthesis, have not been widely reported. Similarly, there is only a limited amount of studies on lipid biosynthesis and molecular genetics in microalgae [4] [5].

To genetically transform microalgae into a lipid overproducing cell, a complete information on the genes encoding the enzymes involved in the lipid biosynthesis pathway is required. Based on this information, genetic engineering or reconstruction of available gene fragments shall be carried out. Protein S-acyltransferase (PAT) is one of enzymes that play a major role in the lipid (TAG) biosynthesis pathway. This enzyme catalyzes the transfer of an acyl chain to its acceptor molecule, either mono- or diacylglycerol [6]. Hence, without PAT, the lipid biosynthesis pathway will result in the formation of fatty acids only, instead of TAG. Published studies on PAT encoding genes (pat) are carried out, in majority, on green microalgae such as Chlamydomonas [7] and Chlorella [8], with the observation and discussion specifically focused on acyltransferases type of DGAT (diacylglycerol acyltransferase). This is because the genomic information of green microalgae is rather extensive and complete compared to that of other types of microalgae [5]. On the other hand, while various species of green microalgae can be easily found in the territorial waters of Indonesia [9], their genomic information is largely incomplete. In order to provide an insight into a chosen genetic trait of Indonesian microalgae, we aimed to obtain the gene fragment encoding the S-acyltransferase (pat) from a new isolate of green microalgae originating from Indonesian ocean, and to characterize the pat fragment including the deduced amino acid sequence. The species identity of microalgae isolate was assessed based on cell morphology and 18S rRNA nucleotide sequence.

2. Method

2.1. Sampling and cultivation of microalgae isolate CBI
An isolate CBI was sampled from Maluku waters. The seawater characteristics were salinity of 35.0 ppt, temperature of 29–30 °C and pH of 7.84–7.90. A single colony of CBI was picked up from cultured agar medium, which consist of Walne medium and bacto agar 2% (w/v), and grown initially in 2 mL of Walne medium [10], which was later scaled up until 50 mL volume of culture. Growth conditions were as follows: medium salinity of 27–28 ppt; pH of the medium ranging from 8.20–8.70; growing temperatures ranging from 21–25 °C; lighting at 10,000 lux with a photoperiod (light:dark) of 12h:12h and growing time of 14d. The cultures were maintained as laboratory collections and regenerated in a new medium every 8d.

2.2. Identification of CBI cell morphology
Morphology of CBI cells was observed under a light microscope at 400x and 1000x magnification. The cells were prepared on a glass preparation or a hemocytometer. The grid pattern inside the hemocytometer can be used as a comparison scale to estimate the cell size.

2.3. Isolation of CBI total DNA and amplification of 18S rRNA gene
CBI total DNA was isolated from a cell culture in log phase using Qiagen® Dneasy Plant Mini Kit (Qiagen Inc.). Amplification of 18S rRNA Gene was proceed by PCR using KAPA Taq Extra Hot Start DNA Polymerase KK3504 (KAPA Biosystems). Primers used for PCR amplification were 5'-CCAACCTGGTTGATCCTGCCAGTA-3' and 5'-CCTTGTTACGACTTCACCTTCCTCT-3' as forward and reverse primer, respectively [11]. The PCR profile was 95 °C initial denaturation for 5 min, 30 cycles of 95 °C for 30s, 55 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 10 min. The total DNA of CBI and amplified product of 18S rRNA gene were visualized by electrophoresis in 1.0% agarose gel containing ethidium bromide [12].
2.4. Sequencing of CBI 18S rRNA gene and analysis of the sequence
CBI 18S rRNA gene sequencing reaction followed the Sanger method [13]. The primers used for sequencing were the same forward and reverse primers as described for PCR (Method 2.3). Nucleotide sequences were analyzed using several software such as: DNA Baser Assembler V4 for analysis of the nucleotide chromatogram quality and for assembly of the DNA contig sequence, BLAST (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information) for determining the highest similarity of the nucleotide sequence with the GenBank database, Bioedit version 7.0.1 [14], and MEGA 6 [15] software for analysis of the phylogenetic tree. Nucleotide sequence of the CBI 18S rRNA was submitted to GenBank (NCBI).

2.5. Isolation of CBI total RNA, reverse transcription and amplification of pat fragment
CBI total RNA was isolated from a cell culture in the log phase using The Aurum Total RNA Mini Kit 7326820 (Biorad). cDNA first strands were synthesized by RT-PCR method using the RevertAid RT cDNA Kit #K1691 (Thermo scientific) and primer oligo-(dT)n. The cDNA first strands were used as a template to amplify the CBI pat fragment. Amplification was carried out by PCR using the KAPA Taq Extra Hot Start DNA Polymerase KK3504 (KAPA Biosystems), and primers of ZcoF3 (5’-AGGCCAGGTGKTSTACAT-3’) as forward and ZcoR2a (5’-GTGATGATGAAGTCGYSR-3’) as reverse primer. The PCR profile was 95 °C initial denaturation for 3 min, 30 cycles of 95 °C for 30s, 48 °C for 1 min, 72 °C for 1 min, and a final extension of 72 °C for 12 min. CBI total RNA, cDNA first strands and amplified pat fragment were visualized by electrophoresis in 1.0% agarose gel containing ethidium bromide [12].

2.6. Cloning of pGEM-pat in E. coli TOP10
The 600-bp CBI pat fragment was purified from the gel agarose using the Qiaquick gel extraction kit (QIAGEN). The pure pat fragment was ligated to the pGEMT easy vector (Promega). Competent cells of E.coli TOP 10 bacteria were prepared using the CaCl2 method. The DNA recombinant of pGEM-pat was inserted into the competent cells by heat-shock method [10]. The cells were finally grown in LB-agar media containing 1mg of X-gal, 1μmol of IPTG, 0.1% (v/v) ampicillin and 0.1% (v/v) tetracycline at 37 °C for 16 – 18h [12]. Successful cloning was proved by isolation of the pGEM-pat recombinant plasmids from the cell clones using the Plasmid purification Kit (Termossalient) and by cutting the plasmids using EcoRI restriction enzyme (Thermossicient). The plasmids were visualized by electrophoresis in 1.0% agarose gel containing ethidium bromide [12].

2.7. Sequencing and Analysis of the pat fragment
Sequencing reaction of the pat fragment followed the Sanger method [13]. The primers used for sequencing were M13Fwd as forward and M13Rev as reverse primers (Promega). Nucleotide sequences were analyzed using the same software as used for 18S rRNA analysis, such as: DNA Baser Assembler V4, BLAST from NCBI and Bioedit version 7.0.1 [14]. In addition, analysis using ExPASy was carried out in order to deduce the amino acid sequence. Further analysis using SAS-Pro (EMBL-EBI) and Swiss-Model software were performed to predict the 2- and 3-dimensional structure of the PAT fragment.

3. Result and Discussion
A microalgae CBI sample originating from Maluku waters, Indonesia, was investigated based on its cell morphology and genetics. Figure 1a shows the microalgae colonies of CBI in the form of green spots separating from each other and Figure 1b shows the CBI cells under a light microscope. CBI cells were green in color and spherical in shape with a cell diameter of 2–10 μm. The CBI sample represented characteristics of green microalgae morphology.
To further identify the species, the gene of 18S rRNA of CBI sample was analyzed. Total DNA isolated from CBI cells was prepared and used as template for PCR amplification of the 18S rRNA gene (Figure 2a). By using primers designed by Yu et al. (2007) [11], a 1700-bp band of PCR product was observed (Figure 2b). This was a 1644-bp nucleotide of 18S rRNA which had been deposited at GenBank (NCBI) under accession number of MH125175.1. The sequence of CBI 18S rRNA showed a 99% similarity with that of *Chlorella vulgaris* (Genbank No. KF574391.1). This indicates that the CBI isolate is closely related to *Chlorella vulgaris*. The phylogenetic tree of CBI 18S rRNA and other algal 18S rRNAs with a maximum likelihood of 1000 bootstraps showed that CBI isolate belonged to *Chlorella vulgaris*. Thus, it was identified as *Chlorella vulgaris* CBI.

In order to obtain the *pat* fragment, total RNA was isolated from the cells of *Chlorella vulgaris* CBI and used as a template for reverse transcription using oligo-(dT)$_{18}$ primer to produce cDNA first strands. Afterwards, the cDNA first strands were used as templates for PCR amplification of the *pat* cDNA fragment using our self-designed primers ZCoF3 and ZChCoR2a. Figure 3a shows bands of 1500-bp and 1000-bp nucleotide representing 28S and 18S ribosomal RNA, respectively. Figure 3b shows a band of 600-bp nucleotide of the *pat* cDNA fragment resulted from RT-PCR. After purification, the *pat* cDNA fragment was cloned in *E. coli* TOP 10 bacteria using pGEMT plasmid as vector. Successful cloning was confirmed by isolating the recombinant plasmid of pGEM-*pat* from *E. coli* transformants, cutting the plasmid using *EcoR*I restriction enzyme, and analyzing the resulting DNA fragments by electrophoresis on agarose gel. The insertion of *pat* gene in the plasmid was proven based on gel electrophoresis comparison between empty pGEM plasmid and recombinant pGEM-*pat* plasmid. Electrophorogram for empty pGEM plasmid shows a 3000-bp nucleotide of linear pGEM plasmid after cutting (Figure 3c). Meanwhile, the recombinant pGEM-*pat* plasmid appears as two bands of DNA fragments upon cutting (Figure 3d), i.e. the 3000-bp and 600-bp nucleotide bands, each representing linear pGEM plasmid and inset *pat* cDNA fragment, respectively. This result hence confirms a successful cloning.
In order to characterize the pat fragment, cDNA (Figure 3b) was sequenced, and the nucleotide A 600-bp nucleotide of cDNA sequence was analyzed using ExPASy and BLAST, in which the first 300-bp nucleotide was unidentified, whereas nucleotide number 301 towards the end resembled the N-terminus part of the protein S-acyltransferase (PAT) from Chlorella variabilis (GenBank no. XP_005849764.1). Figure 4 shows the 77 amino acid residues deduced from pat gene fragment of Chlorella vulgaris CBI, which is identical to the N-terminus part of PAT from Chlorella variabilis (GenBank no. XP_005849764.1) with 51% identity.

Protein structure analysis has been carried out in order to further characterize the PAT fragments of Chlorella vulgaris CBI. The SAS-Pro and Swiss-Model software were used to predict the 2D and 3D structure of PAT. Predicted 2D structure of the PAT fragment from Chlorella vulgaris CBI was compared to the PAT structure of Chlorella variabilis. Both had a similar pattern of an α-helix followed by a β-sheet structure at the N-terminus (Figure 5). The 28-first amino acid residues of both PAT fragments had same structure (Figure 5a and 5b). However, different structures were found at positions of 28-38 and 53-65, where β-sheets took shape in the PAT of Chlorella vulgaris CBI instead of helical structures seen in the PAT of Chlorella variabilis.

Figure 3. CBI total RNA (a) pat cDNA fragment (b) cut pGEM (c) and cut pGEM-pat (d) GR = GeneRuler™ 1-kb DNA Ladder.

Figure 4. PAT fragment of Chlorella vulgaris CBI.
A 3D-structural analysis based on amino acid sequence was performed using the SWISS-Model, a software for fully automated protein structure homology-modeling. The results of the analysis grouped the two PAT fragments, both from *Chlorella vulgaris* CBI and from *Chlorella variabilis*, into the same function as a protein transporter. According to Linder et al. (2007), the topology of S-acyltransferase membrane protein (PAT) has a part that functions as a transporter with its α-helix-shaped secondary structure [16]. Figure 6 shows the similarity of 3D structure of both PAT fragments from *Chlorella vulgaris* CBI (a) and *Chlorella variabilis* (b).

Acyttransferase is a key enzyme that catalyzes the transfer process of activated acyl chains to its acceptor molecules in the TAG biosynthetic pathway [6]. PAT or also called Palmitoyl transferase was identified for the first time in the microorganism of *Saccharomyces cerevisiae* [17]. PAT is a eukaryotic specific protein with the enzyme number EC.2.3.1.225 [16]. Recent proteomic studies indicated that there are many types of PAT found in eukaryotic organisms, such as at least about 50 in yeast [18] and more than 500 in *Arabidopsis* [19]. The number of PAT in microalgae, however, is little-known. According to Boyle et al. (2012), there are 5 acyltransferases with the type of DGAT (diacylglycerol acyltransferase) found in *Chlamydomonas reinhardtii* at different locations such as at endoplasmic reticulum, chloroplasts and other cellular membranes [7]. The pat fragment of *Chlorella vulgaris* CBI reported here would play a role as a useful starting point to find the complete pat sequence from Chlorella that has not been reported to date, specifically from Indonesian local strains of Chlorella.
the availability of the complete PAT sequence, the structure and function of PAT enzyme in microalgal lipid biosynthesis can be further identified and characterized.

4. Conclusions

The morphology and genetic properties of a tropical marine microalgae sample CBI isolated from Maluku waters, Indonesia, were found to be *Chlorella vulgaris*. A 77-amino acid residues of cDNA of *Chlorella vulgaris* CBI was identified as the N-terminus of PAT. This fragment of *Chlorella vulgaris* CBI was an important sequence to design primers to obtain whole *pat* gene.

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