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PREDICTED PROTEINS THAT CATALYZE POLY UNSATURATED FATTY ACID SYNTHESIS IN INDONESIAN MARINE DIATOM *CHAETOCEROS GRACILIS*

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ABSTRACT

Poly Unsaturated Fatty Acid (PUFA) is valuable lipid due to their benefits for nutrition and health, and diatom is known as an excellent source of PUFA. Mechanism of PUFA synthesis in diatom is controlled by protein catalysts. The diatom was grown in the specific media; the cells were harvested and the extracellular proteins were extracted for two dimentional electrophoresis experiment. The cells were sonicated and the fatty acids content was analysed using GC/MS. The proteins involved in PUFA biosynthesis were predicted based on their molecular weight, pI and the following bioinformatic analysis. A preliminary hypothesis on PUFAs biosynthesis in Indonesian diatom *Chaetoceros gracilis* was suggested based on this analysis In conclusion, we suggested eleven proteins involved in the biosynthesis of PUFAs as : Δ9 DES, Δ6 DES, ELO Δ6, precursor ω-6 DES, precursor ω-3 DES, spingolipid Δ8 DES, Δ12 DES, Δ4 DES, microsomal Δ4 DES, microsomal ω-6 DES and microsomal Δ6 DES.

KEYWORDS: ***Diatom Chaetoceros gracilis, proteins, 2D electrophoresis, PUFA biosynthesis***

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INTRODUCTION

Chaetoceros gracilis one of the marine diatoms abundant in Indonesia, is a unicellular photosynthetic eukaryote within *Bacillariophyceae* family, known for the presence of high omega-3 (ω-3) Docosahexanoic-acid (DHA), one of the PUFA related with nutrition and health [1-4]. PUFA is a long chain fatty acid with a minimum carbon of 20 and > 1 double bond in *cis* conformation . PUFA can be classified into omega-3 (ω-3), omega-6 (ω-6), omega-7 (ω-7), and omega-9 (ω-9). Omega-3 and omega-6 are considered the dominant PUFA in microalgae [3-5]. The average concentration of Eicosapentanoic acid (EPA), another omega-3 PUFA is 0.6 to 40.7% and for DHA, the content can be 0.1-6.6 % of the total fatty acid in diatom [3-5]. Fatty acid synthesis in diatom resemble that in plant, it starts from acetyl CoA produced by photosynthesis in the plastids. Following de novo synthesis of saturated or monounsaturated fatty acid from acetyl CoA, the next step is conversion of this fatty acid to PUFA through a series of desaturation and elongation processes [3-4, 6-8]. In algae and plant, fatty acid synthase is responsible for formation of stearic acid (18:0) which will be used to synthesize monounsaturated fatty acid MUFA and polyunsaturated fatty acid PUFA using several desaturase enzymes. Longer fatty acid chain required elongase enzymes. Detail of biosynthesis pathway of PUFA in microlagae is not as extensively studied as those in animal or plant [8-11], eventhough factors affecting fatty acids synthesis in diatom such as media component, duration and intensity of light, carbon flow have been documented [8,12-13]. Enzymes elongase 1, elongase 2, Δ12-desaturase, and Δ6-desaturase involved in PUFA biosynthesis in diatom *Phaeodactylum tricornutum* have been identified [9-10]. However, these enzymes in other diatoms are not as well documented. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a form of gel electrophoresis in which total proteins are separated based on their charge and molecular weight. The resulting protein spots can be identified, quantified and further analyzed. 2D-PAGE has been used to study different expression of proteins at different physiological conditions, or specific protein for medical target, and analysis of proteins involved in spesific metabolic pathway including in microalgae [14]. In this study, we applied 2D electrophoresis to predict the protein catalyst involved in biosynthesis of PUFA in marine diatom *Chaetoceros gracilis.* The proposed biosynthesis model based on these data and data on fatty acids during growth is presently at preliminary stage. Nevertheless, the information generated is still useful to pave the way of finding the key enzymes and their kinetic reactions in the future.

METHODOLOGY

Culture conditions

C. *gracilis* was obtained from Research Center of Oceanography, Indonesian Institute of Science. The diatom was cultured with modified Guilard f/2 media at pH 8 and salinity 28 ‰ . Diatom at 10^{6} ml^{-1} was innoculated and grown at 16-18 ºC, with continues aeration and kept in light 4000-5000 lux for 12 hours/day [15-17] The cell growth was monitored using *Neubauer haemocytometer*. Cells were harvested by centrifuged at 6 000 g, 4 °C, 30 minutes and freezed at -20 °C untill used

Protein analysis

Protein was extracted following Rousch et al (2004) [18] The cells separated through centrifugation was resuspended at buffer solution (100 mM Tris-HCl, pH 6.8, 4% SDS) and washed 2 times, boiled for 5 minutes, centrifuged 14 000 g, 30 minutes at 4 °C to seperate the undissolved materials.

Analysis of lipid and Fatty Acid

Diatom cells were sonnicated for 3x3 minutes at 20 KHz (Soniprep 150 MSE) in 5 ml $CHCl_3$-MeOH-H_2O (5:10:4) solution and mixed with $CHCl_3$: H_2O (1:1) solution to achieve the ratio of Cl_3-MeOH-H_2O to 10:10:9 and form 2 phases. The chloroform phase was removed using N_2 gas, the resulting solid material was weighed as total lipid [5]. The lipid was furthur saponified and esterified for lipid identification. Furthur steps were addition of 15 ml NaCl 20% and mixing the solution throughly to form 2 phases. The upper phase (isooctane and lipid) was removed, dissolved in 25 ml of petroleum benzene (40-60 °C) and filtered. The FAME obtained was dissolved in hexane for fatty acid identification with GC/MS. As much as 1 μl of FAME in hexane was injected into DB-17 column (30 m height, 0.25 mm diameter) in GC/MS QP-5000.

2 D electrophoresis.

Cell pellet was resuspended in sonnication buffer consisted of 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 25 mM KCl, 1 mM EDTA, 0.5 mM PMSF, 0.1 mM Triton X-100, 0.1% mercaptoethanol [8]. Sonnication was performed for 3x3 minutes at 20 KHz, (Soniprep 150 MSE), centrifuged at 6 000 g, 4°C for 30 minutes. The filtrate was precipitated with 10% TCA in acetone containing 0.07% mercaptoethanol, kept overnight at -20°C. The precipitate was washed 3x with acetone containing 0.07% mercaptoethanol [14], then vacuum dried and kept -20°C until furthur used. The first step for 2D electrophoresis analysis was IEF. The protein samples were dissolved in rehydration containing 9.5 M urea, 2% Triton X-100, 5% mercaptoethanol and 5% of ampholine pH 3-10. IPG strip 7 cm pH 7-10 was used. The gel was run in the PROTEAN IEF (Isoelectric Focusing) at 250 V (linear) 20 minutes , 400 V (linear) 6 hours, followed by 800 V (rapid) for 2 hours 10 °C [14]. For the first dimention, the gel was melted at room temperature and soaked in equilibration buffer I containing 6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glyserol, 130 mM DTT for 10-15 minutes, followed by soaking in equilibration buffer II containing 6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8,8, 20% glyserol and 135 mM iodoacetamide for 1- 3 hours. The gel was placed in the SDS-PAGE and run at 150 V, 80 minutes, then stained with coomasie blue. Determination of pI and molecular weights of the proteins was based on the protein standard curve. Prediction and quantification of the protein spots were

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conducted with Melanie 7.0. and TagIdent in the *expasy proteomic tools* http://www.expasy

RESULTS AND DISCUSSION

The strategy for studying PUFA biosynthesis mechanism in this diatom was through analysis of fatty acids which serve as substrate and products of the protein enzymes predicted through 2D electrophoresis. Table 1 describes the percentage of fatty acid in *C. Gracilis* during growth. We observed increasing concentrations of PUFA until day 10 and continuous reduction of MUFA (as precursor of PUFA) by longer time of incubation. By day 10, Eicosadienoate (omega 6 fatty acid) achieved the highest percentage (21 %) and linoleate (omega 6 fatty acid) as its precursor was lowest at the same time (0.59 %).

Table 1

Relative percentage of various fatty acid to the total fatty acids during growth of C. gracilis. Diatom was grown in f2 media. Samples were taken at the indicated times and analysed with Gas Chromatography as mentioned in the method.

Fatty acid		% of total fatty acid Days of incubation				
		3	7	10	14	17
16:0	Palmitate	35.75	32.83	24.03	31.50	40.35
18:0	Stearate	-	2.18	3.3	1.3	2.12
18:1Δ9	Oleate	20.94	31.05	17.42	23.04	19.66
18:2Δ9,12(ω6)	Linoleate	3.19	1.18	0.59	1.14	1.19
18:3Δ6,9,12(ω6)	γ-linolenate	-	-	-	0.35	0.44
18:3Δ9,12,15(ω3)	α-linolenate	-	2.24	1.26	2.33	2.15
20:2Δ11,14(ω6)	Eicosadienoate	-	1.91	**21.46**	9.97	-
20:3Δ11,14,17(ω3)	Eicosatrienoate	-	3.12	0.96	0.46	0.75
20:3Δ8,11,14(ω6)	Dihomo-γ linolenate	-	0.64	-	-	-
20:4Δ5,8,11,14(ω6)	Arachidonate (AA)	-	-	0.43	1.05	1.39
22:6Δ4,7,10,13,16,19(ω3)	Docosahexaenoate (DHA)	-	-	-	-	**4.56**

Theoritically, as many as 9 families of desaturases and elongases are involved in biosynthesis of PUFA up to DHA. We attempted to apply 2 D electrophoresis to search for these proteins. Base on 2 D electrophoresis and bioinformatic analysis which revealed data on molecular weight and pI and in reference to the previous report (Table 2), we detected proteins spots related to the enzymes for formation of PUFA started from oleic acid (18:1Δ9-ω9) which act as substrate for the long chain DHA. These protein enzymes include : desaturase delta 9 (Δ9 DES), Δ6 DES, ELO Δ6, precursor ω6 DES), desaturase precursor omega 3 (precursor ω3 DES), desaturase delta 8 (Δ8 DES), desaturase delta 12 (Δ12 DES), desaturase delta 4 (Δ4 DES), desaturase microsomal delta 4 (microsomal Δ4 DES), desaturase microsomal omega 6 (microsomal ω6 DES), and desaturase microsomal delta Δ6 (microsomal Δ6 DES). We also detected spots related with protein responsible for fatty acid transport. This protein prediction was based on the reference in Table 2 Among the 10 enzymes required in the pathway for PUFA and DHA biosynthesis [8], 9 plastids enzymes and 3 microsomal enzymes were spotted (Figure 1). Part of fatty acid biosynthesis such as that for oleic acid (18:1Δ9), linoleate (18:2Δ9,12), α-linoleate 18:3Δ9,12,15), octadecatetraenoate (18:4Δ6,9,12,15), docosatetraenoate (20:4Δ8,11,14,17) and EPA (20:5Δ5,8,11,14,17) may have occured in the diatom microsomes. The protein responsible for fatty acid *transport* implied in this experiment may have important role in poviding the necessary fatty acid substrates for enzymatic reactions in both plastide and microsome. The molecular weight of 29.42 and pI 7.92 of this protein resemble that of fatty acid transport protein of the diatom *Phaeodactylum tricornutum* CCAP 1055 (B7G6E8 http://www.uniProt.org).

Hyphothesis on PUFA biosynthesis in diatom Chaetoceros gracilis

Domergue *et al.* [9-10] proposed PUFA biosynthesis pathway based on his analysis on fatty acid composition in diatom *Phaeodactylum tricornutum* in combination with detection of gene encoded for the enzymes involved in the biosynthesis, while metabolic pathway in diatom *Thalassiosira pseudonana* was analysed based on total protein expressed in the *mid-exponential* growth phase [19,20] . From the 12 proteins related to PUFA synthesis implied in this research, we proposed specific enzymatic reactions involved in desaturation and elongation of fatty acids, in which the enzyme, substrates and products are shown in Figure 2. The proposed desaturation reaction in microalgae, is the ability to change omega 9 to omega 6 fatty acid by precursor ω6 (pre ω6 DES) desaturase enzyme and conversion of omega 6 to omega 3 fatty acid by precursor omega 3 (pre ω3 DES) desaturase which are not present in animal or human [8]. Oleic acid formed by delta 9 (Δ9 DES) desaturase from stearic acid is further used as a substrate to make linoleic acid (18:2Δ9,12-ω6) by Δ12 DES enzyme. Linoleate or linoleic acid (18:2Δ9,12-ω6) is used as the substrate for α-linolenate (ω3) formation by pre ω3 DES enzyme. In general, formation of α-linolenate (18:3Δ6,12,15-ω3) is catalyzed by Δ15 DES desaturase enzyme, namely the enzyme which specifically catalyzes the formation of double bond on C15, and thus forming α-linolenate (18:3Δ9,12,15-ω3). However, this enzyme was not detected by our 2 D data. Instead we suggested the pre ω3 DES enzyme which is known capable of

catalyzing the formation of omega 3 fatty acid substrate from omega 6 fatty acid. Through formation of oleate, linoleate and α-linolenate, the diatom has necessary precursors to build omega 9, 6 and 3 families by Δ6 DES desaturase family. Desaturase Δ6 catalysed the formation of γ-linolenate (18:3Δ6,9,12-ω6) from linoleate (18:2Δ9,12-ω6). This enzyme can also form octadecadienoate (18:2Δ6,9-ω9) from oleate (ω9) and further to octadecatetraenoate (18:4Δ6,9,12,15-ω3). In this pathway, the octadecadienoate (ω9) acts as a substrate for synthesis of γ-linolenate by pre ω6 DES desaturase, while octadecatetraenoate (18:4Δ6,9,12,15-ω3) acts as further substrate for formation of eicosatetraenoate (20:4Δ8,11,14,17-ω3) by ELO Δ6 enzyme. This enzyme will also synthesize dihomo γ-linolenate (20:3Δ8,11,14-ω6) from γ-linolenate (20:3Δ8,11,14-ω6) which has been previously synthesized by the activity of Δ6 DES enzyme. Dihomo γ-linolenate (ω6) will be further converted to arachidonic acid/AA (20:4Δ5,8,11,14-ω6) catalyzed by the putative Δ5 DES enzyme. In this reaction, the substrates and products were detected but the Δ5 DES enzyme was not shown on 2 D. We remain suggesting this mechanism because of the nature of the substrate conversion which requires catalysis by this particular enzyme resulting in expected known products as observed in this study.Another type of omega 6 PUFA found in our experiment was eicosadienoate (20:2Δ11,14-ω6) which was produced from linoleate (18:2Δ6,12-ω6) through activity of the putative ELO Δ9. The omega 3 family is synthesized from the precursor α-linolenate (18:3Δ6,12,15-ω3) which acts as a substrate for eicosatrienoate (ω3) synthesis. Eicosatrienoate (20:3Δ8,11,14-ω3) will be further acts as a substrate for eicosatetraenoate (20:4Δ5,8,11,14-ω3) synthesis by Δ8 DES desaturase enzyme. In this case the enzyme was detected on 2 D, but not the fatty acid product which might be instantly transformed by the following enzyme in the pathway [6] below (Figure 1)

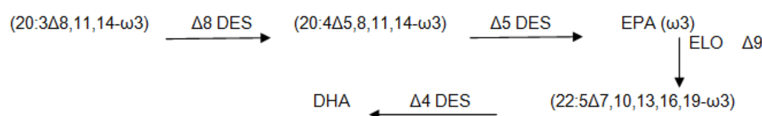

Figure 1
Conversion of (20:3Δ8,11,14-ω3) to EPA and DHA

DHA and the Δ4 DES enzymes were both observed by the 2 D experiment, but Δ5 DES and ELO Δ9 were not detected which may be replaced by pre ω3 DES enzyme. This enzyme is known to catalyze the conversion of omega 6 fatty acid substrate to omega 3 fatty acid [8,9]. Therefore we suggest that, the synthesis of eicosatrienoate (20:3Δ11,14,17-ω3) from eicosadienoate (20:2Δ11,14-ω6); eicosatetraenoate (20:4Δ8,11,14,17-ω3) from dihomo γ-linolenate (ω6); and EPA (20:5Δ5,8,11,14,17-ω3) from AA (ω6) were catalysed by pre ω3 DES enzymes Domergue *et al.* [9,10] reported that pre ω3 DES enzymes possess activity similar to Δ17 desaturase which catalyzes EPA from AA substrate and similar to linoleate catalysis to α-liolenate by Δ15 desaturase. The consecutive reactions involving putative enzyme desaturase Δ5 and elongase Δ5 for synthesis of eicosapentaenoate (20:5Δ5,8,11,14,17-ω3) down to synthesis of DHA are reported in our hypothesis. In order to detect and identify some of the proposed enzymes and fatty acid intermediates, the optimization of samping time is still required. Nevertheless, DHA catalysed by various desaturation and elongation processes will require activities of these two enzymes. Infact in the formation of DHA from eicosatetraenoate (20:4Δ8,11,14,17-ω3) substrate , the pathway involving these two enzymes is the only known mechanism. Our hypothesis stating the synthesis of eicosatrienoate ω3 and the presence of desaturase Δ8 DES enzyme is quite different from those reported earlier. This pathway, rarely found in diatom was only reported in *T. Pseudonana* [7,19,20] Even though this study is still preliminary and requires further confirmation with other techniques, the information generated is still useful anyhow for further exploration of the key enzymes involved in the natural production of fatty acids which is needed for molecular biologist to clone or modify the related gene and further improving their expression. In addition, cloning these genes into other more suitable and economical host is another promising way for industrial production of the healthy fatty acids.

Table 2
Reported molecular weight and pI (in brackets) of enzymes involved in PUFA biosynthesis in several Diatom (http://www.uniProt.org)

Δ 9 DES	37.97 (7.73)	*Phaeodactylum tricornutum* CCAP 1055/1	B7G313
Δ 12 DES	49.04 (6.76)	*Phaeodactylum tricornutum* CCAP 1055/1.	B5Y580
	49.09 (6.76)	*Phaeodactylum tricornutum*	Q84KI9
	54.32 (6.74)	*Phaeodactylum tricornutum*	Q84KI8
Precursor ω-6 DES	54.38 (6.74)	*Phaeodactylum tricornutum* CCAP 1055/1	B7G730
Microsomal ω-6 DES	52.12 (5.97)	*Thalassiosira pseudonana* CCMP1335	B8BWX9
Precursor ω-3 DES	49.92 (6.98)	*Phaeodactylum tricornutum* CCAP 1055/1	B7GEK2
Δ 8 DES	56.02 (7.41)	*Thalassiosira pseudonana*	Q4G2T2
Δ 6 DES	53.45 (7.13)	*Phaeodactylum tricornutum*	Q8RXB0
	25.51 (8.67)	*Phaeodactylum tricornutum* CCAP 1055/1	B7G6E0
	53.45 (7.13)	*Phaeodactylum tricornutum* CCAP 1055/1	B7G6R1
	55.45 (7.34)	*Thalassiosira pseudonana*	Q4G2T1
Microsomal Δ6 DES	55.44 (7.34)	*Thalassiosira pseudonana* CCMP1335	B8C684
Microsomal Δ 5 DES	53.77 (8.77)	*Thalassiosira pseudonana* CCMP1335.	B5YMH5
Δ 4 DES	61.94 (6.52)	*Thalassiosira pseudonana*	Q4G2T0

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Figure 2
Propose mechanism of PUFA biosynthesis in Indonesian Marine Diatom Chaetoceros gracilis based on detection of fatty acids and prediction of proteins involved

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CONFLICT OF INTEREST

Conflict of interest declared none.

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