Aquaculture 428–429 (2014) 104–110

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

The major lipid changes of some important diet microalgae during the entire growth phase

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ARTICLE INFO

Article history:
Received 9 November 2013
Received in revised form 24 February 2014
Accepted 25 February 2014
Available online 11 March 2014

Keywords:
Diet microalgae
Major lipid change
Growth phase
UPLC-Q-TOF-MS

ABSTRACT

In the present study, we investigated the major lipid changes of six important diet microalgae (*Chlorella* sp., *Nannochloropsis oculata*, *Nannochloropsis* sp., *Isochrysis galbana*, *Phaeodactylum tricornutum* Bohlin and *Chaetoceros calcitrans*) during the entire growth phase using UPLC-Q-TOF MS system. Our results suggested that EPA/AA-rich lipids were largely detected in both *Nannochloropsis* sp. and *N. oculata* as a component of diacylglyceryl-N,N,N-trimethylhomoserine (DGTS). Moreover, docosahexaenoic acids (DHAs) were detected only in *I. galbana* mainly as a component of monogalactosyldiacylglycerol (MGDG) and sulphoquinovosyldiacylglycerol (SQDG). Besides, the growth phase of microalgae could be a useful variable for the content enrichment of EFA-rich lipids. A higher content EPA/AA-rich DGTS could be obtained at the end of stationary phase for *Nannochloropsis* sp. and *N. oculata*, and a higher content of DHA-rich lipids could be obtained at the end of stationary phase for *I. galbana*. However, EPA/AA-rich lipids were mainly detected in both *P. tricornutum* and *C. calcitrans* mainly as a component of TAG and MGDG, respectively. In addition, a higher content of EPA-rich TAG could be obtained at the end of the stationary phase for *P. tricornutum*. A higher content of EPA-rich MGDG could be obtained at the end of stationary phase for *C. calcitrans*. Taken together, considering the high EFA requirement for artificial rearing of marine organisms, *Nannochloropsis* sp., *N. oculata*, *P. tricornutum*, *C. calcitrans* and *I. galbana* would be highly recommended to be harvested at the end of the stationary phase. The distribution characteristics of EFA-rich lipids related to 6 microalgae are described in detail.

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1. Introduction

As diets of juvenile fish and crab, the intensive rearing of shellfish, rotifer and cladoceran mainly relies on the massive production of unicellular algae (Brown et al., 1997; Chebil and Yamasaki, 1998; Coutteau and Sorgeloos, 1992; Lubzens et al., 1995). The composition study of diet microalgae has broad commercial and ecological significance. Due to different biochemical compositions, marine microalgae can vary in their nutritional value compared with a variety of planktivorous animals (Liu et al., 2009; Milke et al., 2004; Xu et al., 2012).

In previous studies, nutritional differences of microalgae species have been ascribed only to qualitative factors, such as digestibility or toxicity (Walne, 1974). Since certain long-chain polyunsaturated fatty acids (PUFAs) are essential fatty acids (EFAs) for many marine animals, especially in larval stages, an increasing number of researchers have investigated the fatty acid composition of microalgae for their nutritional importance (Delaunay et al., 1993; Soudant et al., 1996a, 1996b; Thompson et al., 1996). Some studies showed that lipid, carbohydrate and protein of diet microalgae play a significant role for many marine shellfishes, especially EFAs containing docosahexaenoic acids (DHAs) and eicosapentaenoic acids (EPAs) (Rivero-Rodríguez et al., 2007). In recent years, the nutritional effect of unialgal diets on the artificial rearing has been mainly focused on the profile of fatty acids and sterols in microalgae (Napolitano et al., 1993; Soudant et al., 1996a, 1996b; Volkman, 2003; Xu et al., 2012).

As the important part of lipids, it is well accepted that fatty acid is mainly found as fatty acyl instead of non-esterified fatty acid in microalgae (Watson, 2006; Xu et al., 2010; Yan et al., 2010). Typically, fatty acids usually exist in total lipid through esterification, such as glycerolipids, glyceryl phosphatide, glycosphingolipids and storage lipid triacylglycerols (TAGs). Studies indicate that free fatty acids, particular free PUFAs, share some similar characteristic features with some diet microalgae (Volkman et al., 1998), but have different nutritional effects (Liu et al., 2009; Xu et al., 2012). Different nutritional effects may be induced by different contents and different positional distributions of the fatty acyl chains in different types of lipids. Therefore, it is

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http://dx.doi.org/10.1016/j.aquaculture.2014.02.032
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important to explore major lipid molecular species in different marine microalgae and the distribution of EFAs in the major lipid class.

Moreover, it has been documented that variations in culture stages of microalgae affect their lipidomic profiles (Su et al., 2013), which in turn can affect their nutritional and energetic values as a food source for planktivorous organism. Therefore, we aimed to investigate the major lipid changes of six important diet microalgae (*Chlorella* sp., *N. oculata*, *Nannochloropsis* sp., *I. galbana*, *P. tricornutum* and *C. calcitrans*) during the entire growth phase. Our data provided a useful lipid database for the screening of appropriate diet microalgae in artificial rearing of marine organism.

2. Material and methods

2.1. Chemicals and lipid standards

Chemicals, HPLC-grade solvents (acetonitrile, isopropanol, tetrahydrofuran, and formic acid), leucine-enkephalin, lithium acetate, sodium formate and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled water (18.2 MΧ) was prepared using the Milli-Q system (Milli-pore, MA, USA). Phosphatidylglycerol (PG) standards and internal standard were provided by Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Glycolipid standards, including monogalactosyldiacylglycerol (MGDG), monogalactosyldiacylglycerol (MGDG) and sulphoquinovosyldiacylglycerol (SQDG), were obtained from Lipid Products, UK. In order to confirm the relative position of fatty acyl and the content of glycolipid standards, *sn*-2 acyl lysoglycolipids were synthesized using the known enzyme lipaseXI (from the fungus *Rhizopus arrhizus*) and analyzed by LC–MS (Xu et al., 2009). Non-polar lipid standard TAGs and internal standard (glycerol 3-^{13}C) (>99%) were supplied from Larodan, Sweden.

2.2. Algal culture and harvest

Microalgal species, including *Chlorella* sp., *Nannochloropsis oculata*, *Nannochloropsis* sp., *Isochrysis galbana*, *Phaeodactylum tricornutum* and *Chaetoceros calcitrans*, were selected from the Microalgal Culture Laboratory, Ningbo University. The seawater (pH 8.30, 28‰ salinity) was used for the microalgal culture in our present study. Briefly, seawater was filtered by 0.45-μm cellulose acetate membranes, sterilized and then supplemented with nutrients as follows: 75 mg L^{-1} $NaNO_3$, 5 mg L^{-1} NaH_2PO_4, 3.15 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 4.36 mg L^{-1} EDTA-Na_2, 10 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 22 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 10 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 18 mg L^{-1} $MnCl_2 \cdot 4H_2O$, 6.3 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 1 mg L^{-1} VB_{12}, 1 mg L^{-1} Biotin and 200 mg L^{-1} VB_1. In addition, 20 mg L^{-1} Na_2SiO_3 was added to culture medium for the growth of *P. tricornutum* and *C. calcitrans* (Lewin and Guilard, 1963). Microalgae were maintained in 5000-mL conical flask at 20 ± 2 °C under a 12/12-h white fluorescent light cycle. Cultures were gently mixed prior to sampling, and cell counting was performed with a blood counting chamber under an optical microscope (each day). Microalgae were respectively harvested at three selected time points (exponential phase, onset of stationary phase and end of stationary phase) (Figs. S1–S6) by centrifugation at 5000 rpm and then freeze-dried under −50 °C. All experiments were performed in triplicate, and data were expressed as average ± standard deviation (SD).

2.3. Total lipid extraction

Briefly, 50 mg of microalgae (freeze-dried) was accurately extracted using chloroform/methanol/water (1:2:0.8, v/v/v) solution containing 0.05% BHT by a Bligh and Dyer method with minor modifications, by which the total lipid was isolated through successive ultrasonication (15 min) and centrifugation (8–10 min) (Bligh and Dyer, 1959). The obtained extract was evaporated on a rotary evaporator, and the residue was stored at −20 °C prior to further analysis.

2.4. Chromatography

Reversed-phase analysis was performed on a Waters ACQUITY Ultra Performance LC system (UPLC) coupled with an ACQUITY UPLC BEH C8 analytical column (i.d. 2.1 × 100 mm, particle size 1.7 μm). Briefly, 0.01% lithium acetate and 0.1% formic acid were added to the mobile phase as the electrolyte. A 1:4 split of the column effluent was used to achieve a flow rate of 50 μL min^{-1} into the electron spraying ionization (ESI) source. To obtain an efficient separation of total lipids, water/acetonitrile (1:2, v/v) and acetonitrile/isopropylalcohol/tetrahydrofuran (1:1:1, v/v/v) were used as mobile phase A and mobile phase B, respectively. The mobile phase B was changed from 0 to 50% in 15 min, then reached 80% in 30 min and held for 2 min, returned to the initial 0 in 2 min and equilibrated for 11 min. The temperature of sample chamber was set at 4 °C, the column temperature was set at 40 °C, and the injection volume was 3 μL for each analysis. Samples were filtered through a 0.22-μm ultrafiltration membrane (Millipore, USA) prior to the injection.

2.5. Mass spectrometry

Mass spectrometry was performed on a Waters Q-TOF Premier mass spectrometer operating in both positive and negative ESI modes. High-purity nitrogen was supplied at a constant flow rate of 400 L h^{-1} as nebulizer and drying gas, and the source temperature was set at 120 °C. The mass ranged from 150 to 1200 with a scan duration of 0.3 s and an interscan delay of 0.02 s. For the ESI^+ mode, the capillary voltage was set at 3.0 kV, and the sampling cone voltage was set at a ramp of 25–40 V. MGDG, DGDG, diacylglyceryl-N,N,N-trimethylhomoserine (DGTS) and TAG were determined in the positive ion mode. For the ESI^- mode, the capillary voltage was set at 2.6 kV, and the sampling cone voltage was set at a ramp of 25–40 V. SQDG and PG were determined in the negative ion mode. MS^2 analysis was performed in a collision energy range of 25–40 V, and the argon was used as the collision gas. The TOF analyzer was used in the V mode and tuned to a maximum resolution (>10,000 resolving power at *m/z* 1000). Prior to the experiment, the instrument was calibrated with sodium formate, and the lock mass spray for precise mass determination was set with leucine enkephalin at a concentration of 0.2 ng μL^{-1}, generating an $[M + H]^+$ ion at 556.2771 Da and an $[M\text{-}H]^-$ ion at 554.2615 Da in the ESI^+ mode and ESI^- mode, respectively. The lock spray frequency was set at 10 s.

2.6. Data processing

The original data from both ESI modes were acquired in the centroid mode by UPLC-Q-TOF-MS system and analyzed by MassLynx 4.1 data processing system (Waters, Milford, MA, USA). For the data collection, parameters were set as follows: retention time range: 5–40 min; mass range: 150–1200 Da; mass tolerance: 0.05 Da; retention time tolerance: 0.2 min; peak width: 5%; peak height: 5.00 s; peak-to-peak baseline noise: 5.00, peak intensity threshold: 30 counts; and noise elimination level: 30. Pareto scaling (scaled to square root of standard deviation) was used in all models to avoid chemical noise and artifacts. The MarkerLynx matrices, including peak numbers (based on the retention time and m/z), sample names and normalized peak intensity, were exported and analyzed by principal component analysis (PCA) using SIMCA-P^+ V12.0 software.

2.7. Identification and semi-quantitative analysis of lipids

The raw LC–MS data were analyzed using MassLynx (v4.1) software (Waters, USA). Identification and semi-quantitative analysis of each lipid were achieved according to our and some others' previous works with minor modifications. Briefly, in the positive mode, the ion $[C_9H_{16}O_6Li]^+$ at *m/z* 227.03 was used as the characteristic fragment ion of MGDG detection, while the ion $[C_{15}H_{26}O_{11}Li]^+$ at *m/z* 389.14

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