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Desaturation of linoleic acid in the marine diatom *Haslea ostrearia* Simonsen (Bacillariophyceae)

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Abstract – The work reported here studied the biosynthesis of polyunsaturated fatty acids in the marine diatom *Haslea ostrearia* Simonsen. Microalgae were incubated with [1-^{14}C] linoleic acid for 4, 8 and 12 hours. Analysis by HPLC showed that linolenic, eicosatetraenoic and eicosapentaenoic n-3 fatty acids were synthesized from radiolabeled linoleic acid. Results showed that this diatom has the enzymatic capacity for the biosynthesis of eicosapolyunsaturated fatty acids through Δ6- and Δ5- desaturation and furthermore has the capacity for synthesis of n-3 polyunsaturated fatty acids from n-6 polyunsaturated fatty acids.

biosynthesis of n-3 polyunsaturated fatty acids / diatom / fatty acid desaturation / *Haslea ostrearia* / linoleic acid /n-3 fatty acids

Résumé – Désaturation de l'acide linoléique dans la diatomée marine *Haslea ostrearia Simonsen* (Bacillariophyceae). Le but de cette expérience a été d'étudier la biosynthèse des acides gras polyinsaturés chez une diatomée marine, *Haslea ostrearia* Simonsen. Les microalgues ont été incubées avec de l'acide linoléique radioactif (^{14}C) pendant 4, 8 et 12 heures. Les analyses par HPLC ont montré que les acides gras de la série n-3, acides linolénique, eicosatétraénoïque et eicosapentaénoïque étaient synthétisés à partir de l'acide linoléique radiomarqué. Cette diatomée a donc la capacité enzymatique pour synthétiser les acides gras polyinsaturés à 20 atomes de carbone par Δ6- et Δ5- désaturation. De plus, elle est capable de synthétiser des acides gras polyinsaturés de la série n-3 à partir d'acides gras polyinsaturés de la série n-6.

acides gras n-3 / acide linoléique / biosynthèse des acides gras n-3 polyinsaturés / désaturation des acides gras / diatomées / *Haslea ostrearia*

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INTRODUCTION

In the marine ecosystem, phytoplankton, such as diatoms, are considered to be the basis of the marine food chain. They make an important contribution to the marine ecosystem by producing high concentrations of eicosapentaenoic acid (EPA), which is generally considered an important fatty acid in marine organisms (Sargent, 1976). Volkman *et al.* (1989) have reviewed the evidence suggesting that diatom lipids are essential for marine animal nutrition. For several years, an important research field in human and animal nutrition has been to develop new n-3 polyunsaturated fatty acid (PUFA) sources. In this context, some microalgae are good examples of a marine source rich in n-3 fatty acids, and especially EPA.

This study has been undertaken because *Haslea ostrearia* Simonsen (Bacillariophyceae) is a microalga used as feed in aquaculture, especially with oysters. The diatom also synthesizes the peculiar pigment marennine, which is ingested by the oysters (Barillé *et al.*, 1994) and induces a blue-green colouring of the gills. While the pigment structure has not yet been well established, a relationship has been shown between its synthesis and an increase in lipid (Groth-Nard, 1994). During pigment synthesis, the lipid content increased from the exponential growth phase to the stationary phase at the same time as pigment content (Robert *et al.*, 1975).

Linoleic acid desaturation is considered to be the limiting step in PUFA synthesis, and is regulated by external factors (Brenner, 1977; Sprecher, 2000). Several studies have reported on aspects of the prokaryotic and eukaryotic pathways of biosynthesis of PUFA. For instance the synthesis of PUFA in higher plants has been reviewed by Browse & Sommerville (1991), who present evidence suggesting both Δ6 and Δ5 desaturation occurs. Nichols & Appleby (1969) suggested that Δ6 desaturase was present in protista and phytoplankton. More recently, metabolic studies showed that, in an eustigmatophyte alga, PUFA were incorporated into several lipid fractions and especially into phosphatidylcholine and phosphatidylethanolamine (Schneider & Roessler, 1994). In other microalgae, including diatoms, it was found through radiolabel incorporation studies that a synthesis of PUFA occurred through desaturation and elongation steps (Arao & Yamada, 1994; Shiran *et al.*, 1996; Khozin *et al.*, 1997). In *H. ostrearia*, however, there is no information to date about fatty acid synthesis. Previous studies (using labelled precursors) have shown fatty acid incorporation by examining fatty acid composition in lipid fractions using gas chromatography (GC). In the present study, labelled precursors are used with living material to provide the fatty acid source for the enzymatic system, used to produce new long chain fatty acids. We have examined the kinetics of this process using a reversed phase HPLC instead of a GC method.

In this study the PUFA synthesis in *H. ostrearia* was examined. Particular emphasis has been placed on studying the desaturation steps in fatty acid biosynthesis through incorporation experiments, using ^{14}C-labelled linoleic acid as precursor.

MATERIAL AND METHODS

Cultivation

Cells of *H. ostrearia* (an axenic strain isolated from greening-ponds of the Bouin district, Vendée, France) were grown under axenic conditions in 500 mL Erlenmeyer flasks on Provasoli 1/3 medium as modified by Robert (1983). The flasks were placed in a culture room at 16 °C and illumination was provided by cool-white fluorescent tubes in a 14/10 light/dark cycle at 100 μmol photon m^{-2} s^{-1} to the culture vessel surface. The cells used for the assay were taken from batch culture maintained in exponential growth phase.

Chemicals, incubation and preparation of samples

[1-^{14}C] Linoleic acid (98.2 % radiochemical purity) was purchased from NEN Research Products (Dupont de Nemours, Belgium). The radiopurity of the substrate was that indicated by the manufacturer.

The incubation procedure was mainly after Moreno *et al.* (1979) as follows; [1-^{14}C] linoleic acid (in ethanolic solution) was incubated in flasks with Provasoli medium containing 75 000 cells mL^{-1}, with a total incubation volume of 10 mL. Flasks were incubated on a shaking plate at 16 °C for 4, 8 or 12 h on the light cycle described above. After incubations, samples were centrifuged at 200 g for 10 min. After removing the supernatant, containing radiolabeled substrate, the cell pellets were rinsed with fresh medium and centrifuged. After repeating this operation twice, pellets were resuspended with 1.5 mL of distilled water to produce a hypo-osmotic stress. Then, cells were put in liquid nitrogen (thermical stress). These two steps were shown to disrupt the plasma membranes of the cells. During these two steps, microscopic observations were made to observe the cell integrity. At the last step, we obtained cell "ghosts" with a green colouration of the external medium (corresponding to broken cell membranes). Fifteen mL of a chloroform/methanol/water (2/1/0.8, v/v/v) solution were added. After vigorous homogenization, samples were stored at 4 °C for 24 h.

Extraction of lipids and separation of fatty acids

Lipids from the samples above were saponified and methylated according to the method of Slover & Lanza (1979). The method to obtain the lipids is described by Slover & Lanza (1979). After adding the chloroform / methanol / water solution, the organic phase was taken up and dried using a rotary evaporator. After drying with ethanol, samples were dissolved in 1 mL of chloroform and transferred to methylation vials. After evaporation under nitrogen, 1 mL of NaOH (0.5 N) in methanol solution was added. Samples were closed under nitrogen and incubated for 20 min at 80 °C. The use of nitrogen is to prevent any oxidation phenomena. This first step corresponds to the saponification step.

The second step is the methylation step: 2 mL of boron trifluoride methanol (BF3-MeOH 14 %) were added to the samples and these were incubated as described above. The last step is the extraction phase: fatty acid methyl esters were extracted with 1 mL of iso-octane (trimethyl-pentane) and dried on an anhydric sodium sulfate column. This operation was conducted twice.

The distribution of radioactivity between substrate (linoleic acid) and desaturation and elongation products was determined by a reversed phase high

performance liquid chromatography (HPLC) method described by Narce *et al.* (1988), using a Waters chromatograph (600 Controller equipped with a 410 Differential Refractometer, Waters, Milford, USA) and a Lichrocart column (Superspher RP 18, 250 mm × 4 mm i.d., Merck-Clévenot S.A., Nogent-sur-Marne, France). After separation by HPLC, each fatty methyl ester was collected at the detector outlet and its radioactivity was directly measured in the solvent by liquid scintillation counting (Kontron Betamatic 1, Saint Quentin en Yvelines, France). The fatty acid fractions were identified using available standard fatty acids provided by Sigma-Aldrich chemicals (Saint Quentin Fallavier, France).

Separation of lipid fractions

The fractionation of neutral lipids (NL), glycolipids (GL) and phospholipids (PL) were performed on a column consisting of 2 parts (weight) silicic acid (Fluka AG, 100 mesh) and 1 part (weight) of celite 545 (Prolabo), and with a diameter of 1.5 cm (1.5 g of the mixture was used per 30 mg of lipids). The columns were washed over-night with a dichlorometane/methanol (2/1, v/v) solution. NL were eluted twice with 100 mL chloroform, GL with 100 mL acetone and PL with 100 mL methanol. The lipid classes were identified by TLC: after separation on the column, fractions were concentrated and a thin layer chromatography was made to verify the purity of each fractions. Identifications of fractions were made with the use of standards and comparison of migration distances The NL fractions comprised chlorophyll (*a* and *c*), carotenoids, sterols, triacylglycerols, and diacylglycerols. The composition of GL fraction was monogalactosyldiacylglycerol, sulfoquinovosyldiacylglycerol, and digalactosyldiacylglycerol, and the composition of the PL fraction was phosphatidylglycerol and phosphatidylethanolamine (Groth-Nard & Robert, 1993).

Results were presented as the mean of two incubations. Results from the time-course experiment were expressed in dpm (quench was corrected using external standard ratio method), and the fatty acid composition of total lipid and lipid fractions as the relative percentage of total fatty acids.

RESULTS

Incubations with cells were conducted with two concentrations of [1-^{14}C] linoleic acid (0.3 and 0.5 µmol L^{-1}) over 4, 8 and 12 h. Fig. 1 shows the distribution of radioactivity in linoleic acid (substrate), and the products: α-linolenic (18:3n-3), eicosatetraenoic (20:4) and eicosapentaenoic acids (20:5n-3) during the incubations with 0.3 µmol L^{-1} substrate. There was an increase in radiolabelling of C20 products (20:4 and 20:5) with time (expressed in dpm), and the major increase for the 20:4 was observed within 4 h of incubation and for 20:5 was observed after 12 h incubation. From these results it can be seen that desaturase activity (expressed by 18:3, 20:4 and 20:5 radioactivity counts in dpm) increased steadily with time using 0.3 µmol substrate. When 0.5 µmol substrate was used the conversion to desaturase products was reduced compared to 0.3 µmol substrate (not shown). At the same time as the increase in labelling of products was observed, there was also a decrease in labelling of the substrate linoleic acid (Fig. 1). When using 0.3 µmol L^{-1} a plateau was reached after 4 h of incubation, while a constant decrease was observed with 0.5 µmol L^{-1} (not shown).

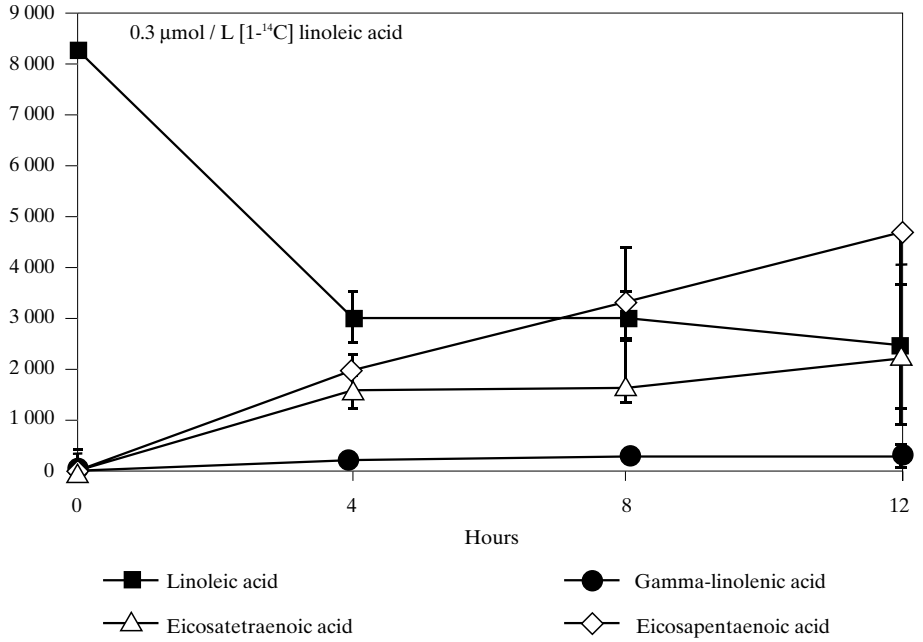

Fig. 1. Time course of desaturation of [1-^{14}C] linoleic acid in *Haslea ostrearia*. Distribution of radioactivity using 0.3 mmol/L [1-^{14}C] linoleic acid. Results are expressed in dpm.
The radioactivity at time 0 corresponds to the medium radioactivity before starting incubation and the errors bars show the range of the two replicates.

Analysis of the fatty acid composition of lipid fractions in the cells (Tab. 1) revealed that PUFA (in %) were mainly recovered in the PL and GL fractions and especially in 20:5n-3, representing around 22 % of the total fatty acids. Monounsaturated fatty acids were mainly found in the NL fraction, the major fatty acid being palmitoleic acid. Saturated fatty acids were mainly found in the NL and PL fractions, and the major fatty acid was palmitic acid. For the PUFA in the PL and GL fractions, it was shown that the majority of the fatty acids were of the n-3 fatty acid family (with 22.7 and 21.6 % of EPA, and 7.9 and 6.3 % of DHA, respectively). High concentrations of hexadecatrienoic acid (16:3) were found in the GL fraction as also observed by Groth-Nard & Robert (1993).

DISCUSSION

Incubation of the diatom *H. ostrearia* with ^{14}C-linoleic acid showed that this micro-organism was able to metabolize 70-80 % of the substrate to desaturation and elongation products as 18:3, 20:4 and 20:5n-3 fatty acids (Fig. 1). However, other fatty acids were also identified and radioactivity was detected in these fractions as well. Less than 3 % of radioactivity was recovered on shorter and less-unsaturated fatty acids (unpublished data). The results also showed that only a minor retroconversion of oxidation products was observed. This means that even

Table 1. Fatty acid composition of total lipid (TL), neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions in *Haslea ostrearia*. Results are expressed as percentage of total fatty acids in each fraction. The samples consisted of 16 % dry material, of which NL amounted to 57 %, GL to 26 % and PL to 17 %. SFA; saturated fatty acids, MUFA; monounaturated fatty acids, PUFA; polyunsaturated fatty acids.

Fatty acids	*TL*	*NL*	*GL*	*PL*
14:0	8.9	9.1	3.6	15.7
16:0	21.3	26.1	7.8	16.8
18:0	0.2	0.3	0.5	0.8
22:0	0.4	0.2	-	–
16:1n-7	29.9	36.1	11.6	12.1
18:1n-9	0.4	0.4	1.1	2.4
18:1n-7	0.2	0.2	0.2	0.8
16:2	5.7	4.3	8.2	4.7
16:3	8.8	4.3	28.9	5.0
16:4	0.6	0.5	1.4	1.2
18:2n-6	0.6	0.2	0.5	0.8
22:5n-6	0.3	0.4	-	–
18:3n-3	0.8	0.4	1.4	2.4
18:4n-3	1.7	1.2	4.9	3.0
20:4n-3	1.8	2.1	1.9	1.6
20:5n-3	14.5	12.3	21.6	22.7
22:5n-3	0.4	-	-	0.7
22:6n-3	2.1	0.6	6.3	7.9
Total SFA	30.8	35.7	11.9	33.3
Total MUFA	30.5	36.7	12.9	15.3
Total PUFA	37.3	26.3	75.1	60.0
n-6	0.9	0.6	0.5	0.8
n-3	21.3	16.6	36.1	38.3

if such reactions appeared during the 4 h incubation, the major fraction of the linoleic acid radioactivity was recovered as desaturation and elongation products.

Previous metabolic studies have reported on the incorporation of unlabeled fatty acids in the red microalga *Porphyridium cruentum* Bory (Rhodophyceae), (Shiran *et al.*, 1996). These studies showed that after incorporation of unlabeled linoleic acid, the intermediates of the n-6 pathway were increased in NL and GL. In the PL fractions some of the intermediates were increased, while others were decreased. Concerning the n-3 fatty acids, results showed that the levels of 18:3 and of 20:4 were increased in NL and PL fractions. When using unlabeled substrate, the exogenous fatty acid is mixed with the intracellular fatty acid pool, which makes it difficult to quantify each metabolic pathway. Moreover, previous studies on rats (Ulmann *et al.*, 1994) have shown that there is not necessarily a relationship between the measured fatty acid composition and desaturation activity.

In a continuation of this work the same conclusion was reached using radiolabeled precursors (Khozin *et al.*, 1997). The microalga (*P. cruentum*) was pulse-labelled for 30 min and cultivated as previously described (Cohen *et al.*, 1988). These authors confirmed that long chain fatty acids were incorporated into several lipid fractions. From these incorporation studies, they suggested two possible metabolic pathways; one pathway leading from linoleic acid to longer n-6 PUFA with a Δ15 desaturation transfer from n-6 to n-3 fatty acids by 20:4n-6 to 20:5n-3, and another pathway with a Δ17 desaturation transfer from n-6 to n-3 fatty acids by linoleic acid to α-linolenic acid that is desaturated/elongated to longer chain n-3 PUFA such as EPA (Khozin *et al.*, 1997). During these reported studies, however, no quantification was made for incorporation rate or metabolic rate. Our present study has quantified the rate of conversion (substrate / product ratio) and established a relationship between the n-6 and n-3 metabolic pathways, confirming the hypothesis put forward by Shiran *et al.* (1996) and Khozin *et al.* (1997). In the diatom *H. ostrearia* we found an increased labelling of 20:5n-3 from 18:2, corresponding to its biosynthesis by desaturation and elongation steps from the n-6 substrate, and also that there was a desaturation of the n-6 fatty acids to n-3 fatty acids.

These conclusions are supported by a previously reported hypothesis (Browse & Sommerville, 1991; Shiran *et al.*, 1996) concerning the Δ6- and Δ5-linoleic desaturation and the conversion from the n-6 to the n-3 PUFA series. The high desaturation rates found in *H. ostrearia* suggest that linoleic acid in this diatom is preferentially desaturated and elongated into EPA and DHA. This hypothesis is confirmed by the data in Tab. 1. In effect, the results showed a very low percentage of linoleic acid (less than 1 %) and a high percentage of n-3 PUFA, especially 20:5 and 22:6. Moreover, the total n-6 PUFA percentage is twenty fold less than for the n-3 PUFA. Thus, in *H. ostrearia*, a relationship between the fatty acid composition and an active PUFA synthesis through desaturation steps is observed.

From this study it is not possible to distinguish between the two pathways of linoleic acid (n-6) to EPA (n-3) ; the one where linoleic acid is desaturated and elongated to arachidonic acid (n-6) which is finally Δ17 desaturated to EPA, and the other pathway where linoleic acid is Δ15 desaturated to α-linolenic acid (n-3) which is further desaturated and elongated to EPA.

The present work should be followed to establish the balance between the two pathways from linoleic acid to EPA and to study the relationship found between the synthesis of lipids and the blue pigment marennine involved in oyster greening (Groth-Nard, 1994).

To identify the two pathways, the use of labelled substrates of the first linoleic conversion step, ie gamma-linolenic (n-6) and alpha-linolenic (n-3) acids, is necessary. By using this method and examining the conversion rate, we could establish which pathway is preferentially followed. To identify the other steps, the use of dihomo-gamma-linolenic (20:3) will be necessary. By labelling this molecule, we could establish that linoleic acid is converted into 20:3n-6, precursor of the 20:4n-6. As there is an EPA synthesis, the last pathway would be a delta-17 desaturation. In the case of no 20:3 labelling, this pathway would not exist, thus a delta-15 desaturation would be suspected.

REFERENCES

ARAO T. & YAMADA M., 1994 – Biosynthesis of polyunsaturated fatty acids in the marine diatom, *Phaeodactylum tricornutum*. *Phytochemistry* 35 (5): 1177-1181.

BARILLÉ L., BOUGRIER S., GÉAIRON P. & ROBERT J.M., 1994 – Alimentation expérimentale de l'huître *Crassostrea gigas* à l'aide de navicules bleues *Haslea ostrearia* (Simonsen) de différentes tailles. *Oceanologica Acta* 17: 201-210.

BRENNER R.R., 1977 – Regulatory function of delta 6 desaturate key enzyme of polyunsaturated fatty acid synthesis. *Advances in Experimental and Medical Biology* 83: 85-101.

BROWSE J. & SOMMERVILLE C.R., 1991 – Glycerolipid synthesis: Biochemistry and regulation. *Annual Review of Plant Physiology and Plant Molecular Biology* 42: 467-506.

COHEN Z., VONSHAK A. & RICHMOND A., 1988 – Effect of environmental conditions on fatty acid composition of the red alga *Porphyridium cruentum*: correlation with growth rate. *Journal of Phycology* 24: 328-332.

GROTH-NARD C., 1994 – Les lipides des diatomées : exemple d'*Haslea ostrearia* (Simonsen), responsable du verdissement des huîtres. Thesis, University of Nantes. 201 p.

GROTH-NARD C. & ROBERT J.M., 1993 – Les Lipides des diatomées. *Diatom Research* 8: 281-308.

KHOZIN I., ALDERSTEIN D., BIGONGO C., HEIMER Y.M. & COHEN C., 1997 – Elucidation of the biosynthesis of eicosapentaenoic acid in the microalga *Porphyridium cruentum*. II. Studies with radiolabeled fatty acids. *Plant Physiology* 114: 223-230.

MORENO V.J., DE MORENO J.E.A. & BRENNER R.R., 1979 – Biosynthesis of unsaturated fatty acids in the diatom *Phaeodactylum tricornutum*. *Lipids* 14:15-19.

NARCE M., GRESTI J. & BEZARD J., 1988 – A method for evaluating the conversion of radioactive polyunsaturated fatty acids by reverse phase liquid chromatography. *Journal of Chromatography* 448: 250-263.

NICHOLS B.W. & APPLEBY R.S., 1969 – The distribution of arachidonic acid in algae. *Phytochemistry* 8: 1907-1915.

ROBERT J.M., 1983 – Fertilité des eaux de claires ostréicoles et verdissement : utilisation de l'azote par les Diatomées dominantes. Thesis, University of Nantes, 281 p.

ROBERT J.M., PAGES J. & PRAT D., 1975 – Application de la biométrie cytologique à la définition des stades de développement du *Navicula ostrearia* (Bory) ; incidences de l'évolution pigmentaire sur le verdissement des claires à huîtres. *Physiologie Végétale* 13: 225-241.

SARGENT J.R., 1976 – The structure, metabolism and function of lipids in marine organisms. *In*: Malines P.C. & Sargent J.R. (eds.), *Biochemical and biophysical perspectives in marine biology. III*. London Academic Press, pp. 150-212.

SCHNEIDER J.C. & ROESSLER P., 1994 – Radiolabelling studies of lipids and fatty acids in *Nannochloropsis* (Eustigmatophyceae), an oleaginous marine alga. *Journal of Phycology* 30: 594-598.

SHIRAN D., KHOZIN I., HEIMER Y.M. & COHEN Z., 1996 – Biosynthesis of eicosapentaenoic acid in the microalga *Porphyridium cruentum*. I. The use of externally supplied fatty acids. *Lipids* 31: 1277-1282.

SLOVER H.T. & E. LANZA E., 1979 – Quantitative analysis of food fatty acids by gas chromatography. *Journal of American Oil Chemist's Society* 56: 933-943.

SPRECHER H., 2000 – Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochimica et Biophysica Acta* 1486: 219-231.

ULMANN L., BLOND J.P., POISSON J.P. & BEZARD J., 1994 – Incorporation of Δ6 and Δ5 desaturation fatty acids in microsomal lipid classes of obese Zucker rats fed n-6 or n-3 fatty acids. *Biochimica et Biophysica Acta* 1214: 73-78.

VOLKMAN J.K., JEFFREY S.W., NICHOLS P.D. & GARLAND C.D., 1989 – Fatty acid and lipid composition of 10 species of microalgae used in mariculture. *Journal of Experimental Marine Biology and Ecology* 128: 219-240.