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Fatty Acid Enrichment of Rotifers Using Type-A Gelatin Solution

Yasuhiro ANDO[1)], Tomomi ABE[1)], Yuka OOKUBO[1)] and Saori NAMIKAWA[1)]

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Abstract

Rotifers *Brachionus plicatilis* have been enriched with fish oil emulsions generated in a type-A gelatin solution. In the enrichment periods of 4-24 h, lipid content of the rotifers increased from 11.6% to 15.3-19.7% (dry weight base). This increase accompanied that of triacylglycerols from 20.5% to 34.9-41.9% of total lipids. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) also increased from 0.2 and 0.5% to 4.5-5.7 and 6.4-7.2% of total fatty acids, respectively. Type-A gelatin produced from porcine skin appears to be useful as an emulsifier for fatty acid enrichment of rotifers as well as type-B gelatin from bovine bones and skin.

Key words: *Brachionus plicatilis*, Rotifer, Gelatin, Lipid, Fatty acid, DHA, EPA, Enrichment

Introduction

Rotifers *Brachionus plicatilis* are used in aquaculture as an important live food for marine fish larvae. However, they are deficient in n-3 highly unsaturated fatty acids (HUFA) essential for marine fish (Wilson, 1995; Sargent et al., 1997, 1999, 2002; Izquierdo et al., 2000). In order to ensure successful production of fish larvae, rotifers are enriched with materials rich in n-3 HUFA prior to being fed to fish larvae. Oil-based emulsions prepared from marine oils are widely used for short-term enrichment of rotifers (e.g., Watanabe et al., 1983; Rainuzzo et al., 1989, 1994; Rodríguez et al., 1996).

In recent studies on HUFA enrichment of rotifers and *Artemia* nauplii, gelatin was used as an emulsifier of marine oils (Ando et al., 2004a, b, c). Gelatin is generally grouped into two types. Type-A gelatin is extracted from porcine skin after acidic pretreatment and type-B from bovine bones and skin after basic pretreatment. The isoionic point of type-A is in basic region (pH 8-9), whereas that of type-B is in acidic region (pH 5). In the recent studies above mentioned, only type-B gelatin was used for enrichment.

In the present study, the other type of gelatin has been used. Fish oil was emulsified in a type-A gelatin solution, and then rotifers were reared on this emulsion. The aim of this study is to clarify whether the type-A gelatin is usable as an emulsifier for boosting HUFA content in rotifers. For this purpose, the enriched rotifers were subjected to lipid analysis in order to determine lipid content, lipid class composition, and fatty acid composition.

Materials and Methods

Enrichment of rotifers

Type-A gelatin (from porcine skin, 75-100 bloom; Sigma, St. Louis, MO) was dissolved in water at 50°C to produce a 1% (wt/wt) solution. After the solution was cooled to room temperature, fish oil (cod liver/mackerel oil; 200 mg) was homogenized with 40 ml of this solution for 3 min using a four-blade blender at 15,000 rpm. Resulting emulsions were immediately used for the following enrichment.

All of the enrichments were separately carried out using rotifers *Brachionus plicatilis* that had been continuously cultured on a commercial *Chlorella vulgaris* product. The rotifers were removed by a nylon mesh, washed in distilled water, and distributed at a density of 400 individuals/ml in a 2-l tank containing 2 l of well-aerated 20‰ salinity artificial seawater (25°C). The fish oil emulsions were added to the tank at 100-mg oil/l. At the end of the enrichment (4, 18 and 24 h), the rotifers (about 1.8 l of the middle layer in the tank) were removed by nylon mesh, washed in distilled water and immediately subjected to the lipid analysis. Initial samples (0-h rotifers) were also collected without enrichment.

Lipid analysis

Total lipids (TL) of the rotifers were extracted by a procedure based on the method of Folch et al. (1957) as follows. The rotifers (0.3-0.5 g, wet weight) were

[1)] *Laboratory of Marine Bioresources Chemistry, Graduate School of Fisheries Sciences, Hokkaido University* (北海道大学大学院水産科学研究院生物資源化学分野)

homogenized with a mixture of chloroform (5 ml) and methanol (2.5 ml) for 2 min using Warning blender at 17,000 rpm. The mixture was filtrated, and the residue was again homogenized with a mixture of chloroform (5 ml), methanol (2.5 ml) and water (0.4 ml). After filtration, the combined filtrates were washed once with additional 2.9 ml of water. The solvents of lower layer were evaporated in a stream of nitrogen in the presence of 2 ml of toluene. Lipid contents on the rotifers based on dry weight matter were determined gravimetrically.

Lipid class composition of the rotifers was analyzed by thin-layer chromatography (TLC) and scanning densitometry based on the method of Olsen and Henderson (1989). After a plate of silica gel 60 (high-performance TLC plate pre-coated with concentrating zone, 10×10 cm; Merck, Darmstadt, Germany) was pre-developed by chloroform to remove impurities, chloroform solutions of the rotifers TL (1 mg/ml, 2 μl) were applied to the plate. Lipid standards (0.5-1.5 μg of each lipid class) were also applied to the same plate for construction of calibration curves. The plate was developed by methyl acetate/2-propanol/chloroform/methanol/water (25 : 25 : 25 : 10 : 9, by volume) to a distance of 3.7 cm from origin. Following dryness *in vacuo* for 2 min, the plate was developed by hexane/diethyl ether/acetic acid (80 : 20 : 2, by volume) to 7 cm from origin. Lipid components were detected by spraying the plate with 3% cupric acetate in 8% phosphoric acid and by charring at 170°C for 15 min. Quantification was performed by a Scion Image software (Scion Co., Frederic, MD) for image processing and analysis program.

Fatty acid composition of the rotifers TL was determined by gas-liquid chromatography (GLC) as their methyl esters. The TL (1 mg) were converted to fatty acid methyl esters in a mixture of 7% (wt/wt) BF_3-methanol (2 ml) and benzene (1 ml) by heating at 100°C for 1 h. After purification by column chromatography on silica gel 60, fatty acid methyl esters were analyzed by GLC with Shimadzu GC-17A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and an Omegawax 250 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA). Column temperature was isothermal at 200°C, and injector and detector temperatures were 250 and 260°C, respectively. The carrier gas was helium (250 kPa). Peak area percentages were measured with a Shimadzu C-R3A integrator.

Results

Lipid content and lipid class composition

Lipid content and lipid class composition of the rotifers are shown in Table 1 together with those of the fish oil fed to the rotifers. The lipid content in the rotifers increased from 11.6% (0 h) to 15.3% (4 h), 19.7% (18 h) and 16.7% (24 h) during the enrichment. The lipid contents in the enriched rotifers were higher than that in the initial rotifers. The highest content was observed for the 18-h enriched rotifers.

The fish oil fed to the rotifers was predominant in triacylglycerols (TAG) (Table 1). In the initial rotifers (0 h), TAG was found at 20.5% of TL, whereas phosphatidylethanolamines (PE) were the most abundant lipid component (36.2%). In contrast, the enriched rotifers were rich in TAG. The TAG in the 4, 18 and 24-h rotifers were 34.9, 41.9 and 37.4%, respectively. The highest concentration was observed for the 18-h enriched rotifers. In all of the enriched rotifers, the TAG concentration was higher than that in the initial rotifers, and phospholipids including PE, phos-

Table 1 Lipid Content and Lipid Class Composition of the Enriched Rotifers

		Enriched rotifers			
	Fish oil[a] $n=1$	0 h $n=1$	4 h $n=2$[b]	18 h $n=3$[b]	24 h $n=2$[b]
Lipid content (wt%, dry-base)	—	11.6	15.3	19.7	16.7
Lipid class composition (wt%)					
Sterol esters (SE)	3.1	3.6	3.7	3.5	3.3
Triacylglycerols (TAG)	90.9	20.5	34.9	41.9	37.4
Free fatty acids (FFA)	2.7	6.1	6.3	3.4	1.2
Sterols (ST)	3.2	7.3	6.5	3.4	4.3
Phosphatidylethanolamines (PE)	0.0	36.2	25.3	27.0	28.8
Phosphatidylinositols (PI)	0.0	14.1	11.2	9.3	10.2
Phosphatidylcholines (PC)	0.0	12.2	9.7	9.8	15.5

[a] Cod liver/mackerel oil used for enrichment of rotifers.
[b] Data are expressed as mean value of the duplicate or triplicate enrichments.

phatidylinositols (PI) and phosphatidylcholines (PC) were lower than those in the initial.

Fatty acid composition

Table 2 shows the fatty acid compositions of the rotifers and fish oil. In the fish oil, concentrations of docosahexaenoic acid (DHA; 22 : 6n-3) and eicosapentaenoic acid (EPA; 20 : 5n-3) were 8.0 and 9.6% of total fatty acids, respectively. Other major fatty acids found at more than 10% were 16 : 0 (10.8%), 18 : 1 n-9 (11.0%), 20 : 1 isomers (12.6%) and 22 : 1 isomers (14.3%).

In the initial rotifers (0 h), 18 : 2n-6 was the predominant fatty acid (38.9%) followed by 16 : 0 (13.7%). Compared with the fish oil, the initial rotifers were generally higher in n-6 series polyunsaturated fatty acids, i.e., 16 : 2n-6 (8.7% including anteiso-17 : 0), 18 : 2 n-6 (38.9%), 20 : 2n-6 (3.8%), 20 : 3n-6 (2.3%), and 20 : 4 n-6 (1.3%). Monounsaturated fatty acid reported to be characteristic of rotifers, 18 : 1n-13 (Ando et al., 1999), was found at 2.9%. In contrast, the initial rotifers were low in DHA (0.2%), EPA (0.5%), 20 : 1 isomers (1.9%), and 22 : 1 isomers (0.3%).

In the enriched rotifers, DHA and EPA were found at 4.5-5.7 and 6.4-7.2%, respectively. These n-3 HUFA increased in the enrichment periods of 4-24 h. Other

Table 2 Fatty Acid Composition of Total Lipids Extracted from the Enriched Rotifers (wt%)

		Enriched rotifers			
Fatty acid	Fish oil[a] $n=1$	0 h $n=1$	4 h $n=2$[b]	18 h $n=3$[b]	24 h $n=2$[b]
14 : 0	4.9	1.7	5.1	4.1	3.3
iso-15 : 0	0.2	0.3	0.5	0.5	0.4
15 : 0	0.3	0.5	0.6	0.6	0.5
16 : 0	10.8	13.7	13.0	12.5	11.8
16 : 1n-7+16 : 1n-9	6.0	2.0	6.4	7.4	7.5
16 : 1n-5	0.3	0.2	0.7	0.6	0.5
iso-17 : 0	0.2	0.7	0.7	0.6	0.5
16 : 2n-6+anteiso-17 : 0	0.2	8.7	2.0	3.0	1.1
16 : 2n-4+phytanic	0.9	0.2	1.0	0.6	0.7
16 : 3n-4	0.5	0.2	0.5	0.4	0.5
16 : 4n-3	0.1	2.1	1.1	0.8	1.3
18 : 0	1.8	3.6	2.8	2.6	2.5
18 : 1n-13	0.0	2.9	2.0	1.6	1.8
18 : 1n-9	11.0	1.2	7.9	8.2	9.8
18 : 1n-7	3.0	0.6	2.3	3.5	3.9
18 : 2n-6	1.1	38.9	14.3	13.8	9.6
18 : 3n-3	0.8	3.1	2.0	1.9	1.4
18 : 4n-3	2.9	0.1	2.1	1.6	1.6
20 : 1n-9+20 : 1n-11+20 : 1n-13	12.6	1.9	6.4	6.5	7.4
20 : 1n-7	0.5	0.2	0.3	0.6	0.7
20 : 2n-6	0.2	3.8	1.1	1.3	0.8
20 : 3n-6	0.1	2.3	0.8	0.8	0.7
20 : 4n-6	0.4	1.3	1.1	0.8	0.9
20 : 4n-3	0.8	0.8	1.1	1.4	1.7
20 : 5n-3	9.6	0.5	6.5	6.4	7.2
22 : 1n-11+22 : 1n-13	14.3	0.3	4.3	3.4	3.7
22 : 1n-9	1.8	0.3	0.7	0.7	0.9
22 : 5n-3	1.2	0.0	0.9	1.2	1.7
22 : 6n-3	8.0	0.2	4.5	4.8	5.7
24 : 1n-9	0.8	0.1	0.2	0.4	0.5
Others[c]	5.0	7.6	7.4	7.7	9.3

[a] Cod liver/mackerel oil used for enrichment of rotifers.
[b] Data are expressed as mean value of the duplicate or triplicate enrichments.
[c] Including unidentified peak components.

fatty acids high in the dietary fish oil also increased in the rotifers fatty acids. The 20 : 1 and 22 : 1 isomers increased to 6.4-7.4 and 3.4-4.3%, respectively. In contrast, concentrations of n-6 series polyunsaturated fatty acids lowered, e.g., 18 : 2n-6, 9.6-14.3%; 16 : 2n-6, 1.1-3.0%; and 20 : 2n-6, 0.8-1.3%. The 18 : 1n-13 fatty acid also decreased from 2.9% to 1.6-2.0%.

Discussion

The enrichment carried out in this study resulted in increases in the lipid content, TAG concentration, and DHA and EPA concentrations in the rotifers within 24 h. Such rapid increases are typical in short-term enrichment of rotifers with oil-based emulsions, formulated diets, or fish roe powders (Watanabe et al., 1983; Rainuzzo et al., 1994; Dhert et al., 2001). It is apparent that the fish oil emulsions generated in the type-A gelatin solution were successfully ingested by the rotifers and boosted DHA and EPA contents in the rotifers.

In a recent study of Ando et al. (2004a), rotifers were enriched with fish oil TAG emulsified in a type-B gelatin solution. The TAG isolated from the cod liver/mackerel oil were emulsified in a 10% (wt/wt) solution of type-B gelatin originating from bovine skin. The resulting emulsions were poured into the rearing tank of rotifers at the concentration same as that in the present study (100-mg TAG/l). The lipid contents of the rotifers were 10.4% (0 h), 15.6% (4 h), 24.9% (18 h) and 19.0% (24 h) on dry weight base. TAG concentrations in the TL were calculated as 4.8% (0 h), 39.1% (4 h), 41.0% (18 h) and 40.5% (24 h). Compared with these data, the rotifers in the present study showed lower contents of TL for the 18- and 24-h enrichments (19.7 and 16.7%, respectively; Table 1). However, these differences probably resulted from different rearing conditions of rotifers, i.e., temperatures, 25 *vs.* 18°C; salinities, 20 *vs.* 35‰; and tank volumes, 2 *vs.* 10 l. On the other hand, TAG concentrations were not very different between the present and previous studies.

The pervious paper (Ando et al., 2004a) also reported fatty acid composition of the TAG isolated from rotifers TL. DHA was found in the TAG at the concentrations of 0.3% (0 h), 5.6% (4 h), 7.1% (18 h) and 7.4% (24 h). EPA was 0.3, 6.9, 8.7 and 8.0% in the order of the same enrichment periods. DHA and EPA concentrations observed in the present study were 0.4-2.3% less than the previous data (Table 2). Lower percentages in the present study were probably caused by difference in the lipid classes subjected to the fatty acid analysis, i.e., TL in the present study *vs.* TAG in the previous study. It has been pointed out that dietary fatty acids assimilated by rotifers are mainly incorporated into TAG fraction of the rotifers (Fernández-Reiriz et al., 1993; Rainuzzo et al., 1994). Percentages of DHA and EPA observed for TAG are generally higher than those in TL.

A main advantage of the use of gelatin solution is that live food (rotifers and *Artemia* nauplii) can be enriched with marine oils without fatty acid-related emulsifiers. Several kinds of emulsifiers, such as Tween 80 (McEvoy et al., 1995), natural phospholipids (McEvoy et al., 1996), and egg yolk (Watanabe et al., 1983), used for the enrichment contain fatty acid moieties in their molecules. In experiment of lipid metabolism in the live foods, the use of gelatin can avoid interference from fatty acids of emulsifier origin. The present study revealed that DHA and EPA increases in the rotifers enriched with type-A gelatin-emulsifying fish oil. Therefore, type-A gelatin produced from porcine skin seem to be useful for n-3 HUFA enrichment of rotifers as well as type-B gelatin.

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