Nutritional Enrichment and Cultivation of Rotifers by Feeding of Docosahexaenoic Acid-enriched *Chlorella vulgaris* K-22

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**Abstract:** The cultivation of rotifers using docosahexaenoic acid (DHA)-enriched *Chlorella vulgaris* K-22 as a food was investigated. The exogenous DHA was incorporated into neutral lipids in DHA-enriched *C. vulgaris* cells. The highest percentage of DHA in the total fatty acids of cellular lipids reached 82.9%. During the nutritional enrichment of rotifers with DHA-enriched *C. vulgaris*, the amount of DHA in the rotifers rapidly increased to 3.3% over 6 hours. During the course of the 3-day cultivation of the rotifers, those provided with DHA-enriched *C. vulgaris* showed higher population growth, and maintained higher stable levels of DHA, than rotifers that were given non-enriched *C. vulgaris*. Thus, cultivation by providing rotifers with DHA-enriched *C. vulgaris* resulted in rotifers possessing high DHA content without the use of additional nutritional enrichment procedures.

**Key words:** *Chlorella vulgaris*; *Brachionus rotundiformis*; Rotifer; Docosahexaenoic acid

The importance of docosahexaenoic acid (DHA) as an essential fatty acid for marine fish has been widely recognized (Izquierdo et al. 1989; Watanabe et al. 1989; Takeuchi et al. 1996; Furuita et al. 1996). For the production of marine fish larvae, rotifers typically have their nutritional value enhanced with commercial feed containing DHA before they are given to larvae (Hayashi et al. 1993b; Takeuchi et al. 1994; Barclay and Zeller 1996). Recently, an ultra-high-density culture system for rotifers has been developed in Japan (Yoshimura et al. 1996a, b, 1997). In this system, a large number of *Chlorella* cells are used as feed for the rotifers. The provision of *Chlorella* as feed greatly improves the productivity of rotifers in hatcheries. Therefore, the consumption of commercial *Chlorella* cells in Japan is increasing year by year. *Chlorella* cells, however, do not naturally contain DHA. Thus, rotifers that are fed *Chlorella* cells also do not contain DHA. Although utilization of *Chlorella* as feed for rotifers has greatly improved the productivity of the rotifers, nutritional enhancement of rotifers by feeding them DHA-containing feed is an essential procedure for the successful production of fish larvae (Maruyama et al. 1988).

We previously reported the uptake of fatty acids in the hydrolysate of fish oil by *Chlorella vulgaris* (Hayashi et al. 2001). It was shown that exogenous DHA in the fatty acid mixture from fish oil was taken up into the *C. vulgaris* cells. In the present studies, we prepared *C. vulgaris* containing DHA in their cells as feed...
for the cultivation and nutritional enrichment of rotifers. Since Chlorella is apparently a profitable feed for rotifers, preparation of DHA-enriched C. vulgaris would provide progressive fish larvae production without additional nutritional enrichment procedures for the rotifers. The results of these studies suggest a labor-saving method of increasing the dietary value of rotifers used as feed for marine fish larvae.

Materials and Methods

Cultivation of Chlorella vulgaris K-22

In all experiments, C. vulgaris strain K-22, isolated from a pond in Saga prefecture, was used. C. vulgaris strain K-22 was grown at 28°C in a medium composed of 2% glucose, 0.15% urea, 0.15% KH₂PO₄, 0.06% MgSO₄·7H₂O, 5 mg/liter EDTA-Na-Fe, and 2 ml/liter A5 mineral solution for 72 h in the dark. The initial pH of the medium was 7.0. After the addition of 0 – 0.5% of DHA into the culture, the cultivation was continued for an additional 24 h. Cells were harvested by centrifugation, and washed three times with distilled water. For nutritional enrichment and cultivation of rotifers, C. vulgaris cells supplemented with 0.5% of DHA in the culture were suspended in tap water. For the analysis of the cells, samples were kept at -80°C until analysis. DHA was provided by Bizen Chemical Co., Ltd. and contained 96.8% of DHA in the total fatty acids. The DHA was supplemented into the culture of C. vulgaris with 0.02% of α-tocopherol and 0.01% of Tween80 as an emulsifier.

Lipid analysis

Extraction of total lipid was carried out by the method of Murakami et al. (1997). The total lipids (TL) was fractionated using silica cartridge (Sep-pack silica, Waters Co., Ltd) into neutral lipids (NL), glycolipids (GL), and phospholipids (PL) (Juaneda and Rocquelin 1985). The fatty acid compositions of TL and each lipid class were analyzed by gas liquid chromatography after methanolysis with 10% HCl-methanol solution (Hayashi et al. 1993a). DHA was also quantified by gas liquid chromatography using nonadecanoic acid as an internal standard.

Cultivation of rotifers

Brachionus rotundiformis, so-called s type rotifers, were cultivated in a 1L-flask at 28°C. Oxygen gas of high purity (93.9 – 96.2% O₂) was supplied into the flask using an air stone. The cultivation was carried out for 3 days without water renewal. Feeding rates of C. vulgaris cells were 10,000 – 20,000 cells/ml for the nutritional enrichment and 40,000 cells/ml/day for the cultivation, respectively. The initial density of rotifers was adjusted at 840 ind/ml. During the cultivation, water temperature, dissolved oxygen, and density of rotifers were measured daily, and salinity and pH were measured on the final day of the cultivation.

Results

Preparation of DHA-enriched C. vulgaris

The addition of DHA into the culture resulted in great increases in TL in the cells (Table 1). Increased supplementation with DHA resulted in increases in TL content in the range of 0 – 0.5% of the supplementation. Particularly, cellular NL increased in parallel with the content of TL. On the other hand, the contents of GL and PL were not changed by the addition of DHA, being 3.1 – 3.8% in the dry cells.

As for the fatty acid compositions, the cells without DHA supplementation did not contain DHA in the total fatty acids of TL (Table 2). However, the percentages of DHA in the total fatty acids of the cells were greatly increased by DHA supplementation in the range of 0 – 0.5%.

Table 1. Effect of the DHA supplementation on the contents of total lipids and lipid classes in the Chlorella cells

<table>
<thead>
<tr>
<th>Amount of DHA supplementation (%)</th>
<th>0</th>
<th>0.10</th>
<th>0.25</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>12.0</td>
<td>18.7</td>
<td>27.7</td>
<td>42.1</td>
</tr>
<tr>
<td>NL</td>
<td>5.6</td>
<td>11.9</td>
<td>20.6</td>
<td>35.2</td>
</tr>
<tr>
<td>GL</td>
<td>3.3</td>
<td>3.6</td>
<td>3.7</td>
<td>3.8</td>
</tr>
<tr>
<td>PL</td>
<td>3.1</td>
<td>3.2</td>
<td>3.4</td>
<td>3.1</td>
</tr>
</tbody>
</table>

%, in the dry cells.
In the TL of the cells supplemented with 0.5% of DHA, the percentage of DHA reached 82.9% in the total fatty acids. The percentages of DHA in NL, GL, and PL also increased depending on the amount of supplemented DHA (Fig. 1). The DHA in NL showed a larger increase than those in GL and PL. Although the contents of GL and PL in the dry cells were not changed by DHA supplementation, some of DHA was incorporated into GL and PL.

These results show that DHA-enriched C. vulgaris could be easily prepared through DHA supplementation into the culture. Furthermore, the DHA in the DHA-enriched C. vulgaris was mainly accumulated in the NL, with some DHA incorporated into the GL and PL molecules as well.

Table 2. Effect of the DHA supplementation on the fatty acid compositions in the total fatty acids of Chlorella

<table>
<thead>
<tr>
<th>Amount of DHA supplementation (%)</th>
<th>0 %</th>
<th>0.1 %</th>
<th>0.25 %</th>
<th>0.5 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>19.3</td>
<td>11.7</td>
<td>5.0</td>
<td>3.5</td>
</tr>
<tr>
<td>C16:1</td>
<td>3.8</td>
<td>2.4</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>C16:2</td>
<td>16.8</td>
<td>8.4</td>
<td>3.7</td>
<td>2.7</td>
</tr>
<tr>
<td>C16:3</td>
<td>5.1</td>
<td>2.5</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.3</td>
<td>0.8</td>
<td>0.4</td>
<td>Tr.</td>
</tr>
<tr>
<td>C18:1</td>
<td>8.1</td>
<td>6.1</td>
<td>3.1</td>
<td>1.5</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>33.5</td>
<td>15.6</td>
<td>7.2</td>
<td>5.6</td>
</tr>
<tr>
<td>C18:3 n-3</td>
<td>8.2</td>
<td>3.6</td>
<td>2.0</td>
<td>1.1</td>
</tr>
<tr>
<td>C20:5 n-3</td>
<td>ND</td>
<td>2.5</td>
<td>1.6</td>
<td>Tr.</td>
</tr>
<tr>
<td>C22:6 n-3</td>
<td>ND</td>
<td>1.7</td>
<td>0.7</td>
<td>Tr.</td>
</tr>
</tbody>
</table>
| ND, not detected. Tr., less than 0.1%.

**Fig. 1.** Effects of DHA-supplementation on the contents of DHA in lipid classes of DHA-enriched C. vulgaris. ○: neutral lipids, ●: phospholipids, □: glycolipids.

**Fig. 2.** Changes of DHA content in the rotifers fed on DHA-enriched C. vulgaris for 18 hours. ○: 10,000 cells/ml, ●: 20,000 cells/ml.

**Fig. 3.** Population growth of rotifers fed on either non-enriched or DHA-enriched C. vulgaris during a 3-day cultivation. ○: DHA-enriched C. vulgaris, ●: normal C. vulgaris.

**Nutritional enrichment of rotifers with DHA-enriched C. vulgaris**

The content of DHA in rotifers fed on DHA-enriched C. vulgaris increased rapidly depending on the feeding rate (Fig. 2). DHA content reached a maximum level 6 hours after the start of the enrichment, and then gradually decreased. At 20,000 cells/ml feeding, the rotifers contained 3.3% of DHA in the dry matter after 6 hours enrichment.

**Cultivation of rotifers with DHA-enriched C. vulgaris**

The population growth of rotifers fed on DHA-enriched C. vulgaris was slightly better than those that were fed non-enriched C. vulgaris (Fig. 3). During the cultivation, no
Fig. 4. Changes in DHA contents of rotifers fed on either non-enriched or DHA-enriched C. vulgaris during a 3-day cultivation. ◯: DHA-enriched C. vulgaris, ●: normal C. vulgaris.

Differences in the water temperature (27.5 – 27.7°C) and dissolved oxygen (19.1 – 22.6 mg/l) were found between experimental groups. Moreover, the salinity (28.4 – 28.7‰) and pH of the water in the samples on the final day of the cultivation revealed no significant differences.

As for the DHA content of the rotifers, rotifers fed on non-enriched C. vulgaris did not contain DHA at any point during the cultivation (Fig. 4). On the other hand, rotifers fed on DHA-enriched C. vulgaris maintained a constant 2.5 – 2.6% DHA content throughout the cultivation.

Discussion

For the rotifer cultivation, several types of feed such as Nannochloropsis, Chlorella, baker’s yeast, and Tetrasselmis have been introduced for hatcheries (Hirayama et al. 1979; Hirayama and Funamoto 1983; Fukusho et al. 1984, 1985; Maruyama et al. 1988; Abe et al. 2001). However, none of these contain DHA in the cells. Therefore, DHA enrichment procedures for rotifers by commercial feeds were essential prior to the provision of rotifers to the fish larvae. On the other hand, many diets for nutritional enrichment of rotifers have been developed. Yeast and Euglena, enriched with fish oil or DHA, are widely used in Japan (Imada et al. 1979; Hayashi et al. 1993a, b).

It was reported that dried shark eggs and Schizochytrium contain large amounts of DHA (Barclay and Zeller 1996; Hayashi et al. 2002). However, reports that rotifers were successfully cultivated on a diet rich in DHA are not available.

The results of the present study show that rotifers fed on DHA-enriched C. vulgaris contained significant amounts of DHA. Therefore, by using DHA-enriched C. vulgaris for the cultivation of rotifers, it becomes unnecessary to nutritionally enrich the rotifers with commercial supplements. Furthermore, DHA-enriched C. vulgaris might improve the nutritional value and productivity of rotifers used in a hatchery.

In the cases of both rotifers cultivated for 3 days and rotifers enriched for several hours, rotifers fed on DHA-enriched C. vulgaris were themselves found to be rich in DHA. The content of DHA in the rotifers was sufficient to satisfy the DHA requirements for many kinds of marine fish larvae. These results suggest that DHA-enriched C. vulgaris may be a profitable feed for both rotifer cultivation and the nutritional enrichment of rotifers.

It was reported that rotifers fed on vitamin E-fortified C. vulgaris surpassed the growth of a non-fortified group. In the present study, high population growth of rotifers fed on DHA-enriched C. vulgaris might be due to the high lipid or tocopherol contents of the cells. However, further investigation is required for the evaluation of improvements in the productivity for rotifers as a result of a diet of DHA-enriched C. vulgaris.

The results of the current study should contribute to improvements in the productivity and nutritional value of rotifers used in hatcheries by recommending the use of DHA-enriched C. vulgaris as rotifer feed.

References


Barclay, W. and S. Zeller (1996) Nutritional enhancement...


DHA強化クロレラ Chlorella vulgaris K-22 による
ワムシ培養と栄養強化

雪野健代・林 雅弘・吉松隆夫・丸山 功・村田 寿

ドコサヘキサエン酸（DHA）を強化したクロレラ Chlorella vulgaris K-22によるワムシ培養の効果を検討した。各種濃度でDHAを強化したクロレラ細胞には主として中性脂質にDHAが取り込まれており、総脂質中のDHA含有率は最高82.9%に達した。DHA強化クロレラを給餌したワムシ中に
は6時間で乾物中3.3%のDHAが蓄積し、短時間でワムシの栄養強化が可能であった。また、3日間によるワムシ培養では通常のクロレラ給餌より高い増殖が得られた。培養期間中、ワムシ中のDHA含有量は安定して高い値を示し、DHA強化クロレラ給餌によるワムシ培養で、栄養強化処理の必要なレベルのDHAを含有するワムシを安定して得ることができた。