Production of $^{13}$C-Labeled Docosahexaenoic Acid by a Thraustochytrid

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Abstract: A $^{13}$C-labeled docosahexaenoic acid (DHA) highly enriched with $^{13}$C atoms was prepared using the DHA-producing Thraustochytrid, Schizochytrium sp. strain N1-27. Following 115 h-cultivation with sodium acetate-$1,2^{13}$C$_2$ as the sole labeled carbon source, 2.53 g/L total lipids were obtained from the cells. The $^{13}$C-labeled DHA ethyl ester was prepared from total lipids and fractionated to almost 100% purity with 53% recovery on HPLC. The $^{13}$C atom ratio was estimated at more than 97% from GC-MS. The productivity of labeled DHA was 0.29 g/L. Other $^{13}$C-labeled fatty acids such as docosapentaenoic acid (DPA), eicosapentaenoic acid, and palmitic acid, were obtained as by-products. The present Thraustochytrid is thus shown useful for producing labeled DHA and DPA.

Key words: $^{13}$C-labeled docosahexaenoic acid, $^{13}$C-labeled docosapentaenoic acid, Schizochytrium sp., stable isotope, Thraustochytrid

1 Introduction

Docosahexaenoic acid (22:6(n-3), DHA) has been shown to be essential for development and the maintenance of life\(^9\). Though the physiological functions of DHA have gradually been elucidated\(^6\)–\(^7\), the mechanisms remain unclear. Experiments of the metabolic pathway involving labeled DHA are an efficient way of clarifying the mechanisms. Schlenk et al. first examined the metabolic pathway for DHA using a radioisotope-, $^{14}$C-, labeled DHA\(^8\). While radioisotopes are excellent for quantitative studies, a stable isotope, $^{13}$C, would also be useful owing to its safety, especially for a human study. A high-sensitive analytical technique has been developed for the metabolic study of $^{13}$C-labeled DHA\(^9\). However, labeled DHA, exogenously taken in, is certainly diluted with the endogenous non-labeled form in living animals. Therefore, it is desirable to enrich labeled DHA with $^{13}$C atoms as much as possible. We tried to prepare a $^{13}$C-labeled DHA, specifically DHA molecules highly enriched with $^{13}$C atoms, using a DHA-producing Thraustochytrid, Schizochytrium sp. strain N1-27.

2 Experimental Procedures

2-1 Microorganism

A DHA-producing Thraustochytrid, strain N1-27, was isolated from surface sea water around Nakaminato Bay, Ibaraki, Japan, using the pine pollen baiting method of Gaertner\(^20\). Microphotographs of the strain were taken and assimilation of the strain was examined for some carbon sources using BIOLOG system (Biolog Inc., Hyward CA, USA)\(^10\).

2-2 Cultivation

Three kinds of media, G, S and labeled S, were used. Medium G consisted of 3% glucose, 0.5% yeast extract in half strength artificial sea water (Tropic Marin, Aquarientechnik, Germany). Medium S contained 3% sodium acetate instead of
glucose, and was adjusted to pH 6.0 before steam sterilization. Labeled medium S consisted of sodium acetate-1,2-13C2 (13C%; 99%; Nippon Sanso Co., Tokyo, Japan) instead of non-labeled sodium acetate in medium S. Strain N1-27 was cultivated in these media at 25°C with shaking on a rotary shaker. The cells were harvested by centrifugation at 1500 x g at 4°C for 15 min and then washed with distilled water.

2·3 Purification of 13C-labeled DHA
After 115 h-cultivation in labeled medium S, lipids were extracted from the cells according to Bligh and Dyer’s method12), and then treated with 0.5N sodium ethoxide in ethanol for 30 min at 50°C. The resultant fatty acid ethyl esters were extracted with n-hexane and then fractionated by silica gel column chromatography with n-hexane/diethyl ether (9:1, v/v). DHA ethyl ester was fractionated from the fatty acid ethyl esters by high performance liquid chromatography (HPLC) (Model System Gold, Beckman) with a UV detector, on a reverse phase column (CAPCELL C18 SG120; 4.6 mm × 250 mm; Shiseido Co., Tokyo, Japan) and acetonitrile/water as the mobile phase. The ratio of acetonitrile to water was changed stepwise from 90 : 10 to 95 : 5, and finally to 100 : 0, by volume.

2·4 Fatty Acid Analysis
Cells cultivated in medium S and G were lyophilized and then treated with an 8% methanolic HCl solution to prepare methyl esters. The methyl esters or ethyl esters mentioned above were analyzed by gas-liquid chromatography (GLC), according to a previous report13). The amounts of DHA was determined using heneicosa-noic acid, 21:0, as an internal standard.

2·5 Estimation of the 13C Atom Ratio in Labeled DHA
Gas chromatography-mass spectrometry analysis of the labeled DHA ethyl ester fractionated by HPLC was conducted as reported elsewhere14). The 13C atom ratio in the labeled DHA ethyl ester was estimated from the m/zs of the molecular ions and the intensity ratio of them. To confirm the molecular ion, secondary ion mass spectrometry (SIMS) analysis was also conducted using Hitachi mass spectrometer M-80A. The sample was mixed with p-nitrobenzyl alcohol as a matrix and NaCl on a probe tip, and was subjected to bombardment with xenon ions.

3 Results and Discussion
A microphotograph of vegetative cells of strain N1-27 shows the successive binary cell division which has been observed in Schizochytrium sp19). (Fig. 1). The carbon assimilation by strain N1-27 is shown in Table 1.

As labeled carbon sources for the organism, 13C-labeled sodium acetate and 13C-labeled glucose are commercially available. Strain N1-27 was preliminarily cultivated in medium S and G. Sodium acetate and glucose gave similar growth until 50 h-culture time, but the former gave higher growth in the later growth phase (Fig. 2). Glucose was not adequate as a carbon source for strain N1-27 as described above. The DHA productivity from sodium acetate was 7 times as high as that from glucose (Fig. 3). These results indicated that sodium acetate-13C2 is preferable as a labeled carbon source for the production of labeled-DHA.

![A Microphotograph of Vegetative Cells of Strain N1-27.](image)

**Fig. 1** A Microphotograph of Vegetative Cells of Strain N1-27.

**Table 1** Assimilation of Some Carbon Sources by Strain N1-27.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Growth</th>
</tr>
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<tbody>
<tr>
<td>L-glutamic acid</td>
<td>- *</td>
</tr>
<tr>
<td>α-keto-glutaric acid</td>
<td>+</td>
</tr>
<tr>
<td>2-keto-D-gluconic acid</td>
<td>+</td>
</tr>
<tr>
<td>cellbiose</td>
<td>+</td>
</tr>
<tr>
<td>gentiobiose</td>
<td>-</td>
</tr>
<tr>
<td>α-D-glucose</td>
<td>+</td>
</tr>
<tr>
<td>D-galactose</td>
<td>+ +</td>
</tr>
<tr>
<td>glycerol</td>
<td>+ +</td>
</tr>
<tr>
<td>D-xylene</td>
<td>+</td>
</tr>
<tr>
<td>β-methyl-D-glucoside</td>
<td>+**</td>
</tr>
</tbody>
</table>

* not grow
** trace
Fig. 2 Growth of Schizochytrium sp. Strain N1-27 in Medium S and G. Medium S (○) and G (●) contained sodium acetate and glucose as carbon sources, respectively. The cultivation temperature was 25°C.

Fig. 3 DHA Productivity of Schizochytrium sp. Strain N1-27 in Medium S and G. Details the same as in Fig. 2.

using Schizochytrium sp. strain N1-27. While there is little information about the biosynthesis pathway for DHA in Thraustochytrids, it is certain that the carbon chain is elongated by condensation of the C2 units, malonyl-CoA, like in the fatty acid synthesis pathway in other organisms\[^{16}\]. In general, sodium acetate is converted to acetyl-CoA just after its incorporation into a cell, and then further converted to malonyl-CoA. Glucose, on the other hand, has to be incorporated into glycolytic pathway for it to be catabolized to acetyl-CoA. Therefore, sodium acetate seems to be more favorable with respect to efficiency of fatty acid synthesis.

After cultivation in labeled medium S, total lipids extracted from the cells amounted to 2.53 g/L. The amount of the ethyl ester mixture recovered on silica gel column chromatography was 1.31 g/L, which represented 4.4 wt% of the sodium acetate-1,2-\(^{13}\)C\(_2\) added to the medium. The fatty acid composition of the ethyl esters is shown in Table 2.

The ratio of DHA to total fatty acids of strain N1-27 was about ten times larger than those of some Schizochytrium strains\[^{17,18}\] except S. limacinum SR-21\[^{19}\] at 25°C. The ratio of total polyunsaturated fatty acids to total fatty acids of strain N1-27 was larger than that of S. limacinum SR-21, while the ratio of DHA in the former was similar to that in the later\[^{19}\]. The productivity of DHA ethyl ester was calculated to be 0.55 g/L from the ratio to that of the total ethyl esters, 42 wt%, on GLC. After fractionation by HPLC, the productivity and purity of the labeled DHA ethyl ester were 0.29 g/L and almost 100% on GLC, respectively. The recovery of the product on HPLC was 53%. Mass spectrum of non-labeled DHA ethyl ester was shown in Fig. 4. The molecular ion was shown at m/z 356 (Fig. 4). The relative intensity was presented as the intensity of ion at m/z 356 to be 100.

The ions at m/zs 358 and 357 were also the molecular ions containing \(^{13}\)C atoms in natural abundance. The ions at m/zs 355 and 354 seemed to be fragments eliminated one and two hydrogen atoms from the molecular ion, respectively. The

| Table 2 Fatty Acid Composition of Fractionated Ethyl Esters Prepared from Schizochytrium sp. Strain N1-27 Cultivated in Labeled Medium S. |
|-----------------|--------|
| Fatty acids     | wt%    |
| 14:0            | 7.8    |
| 15:0            | 5.4    |
| 16:0            | 23.6   |
| 16:1(n-7)       | 1.9    |
| 17:0            | 1.2    |
| 18:0            | 0.7    |
| 18:1(n-9)       | 0.9    |
| 20:5(n-3)       | 2.1    |
| 22:5(n-6)       | 11.8   |
| 22:6(n-3)       | 43.2   |
| others          | 1.5    |
| total           | 100.0  |
ion at m/z 341 seemed to be a fragment eliminated a methyl group of the acyl chain from the molecular ion. The mass spectrum of the labeled DHA is shown in Fig. 5. Since it was not able to separate 13C-labeled and non-labeled DHA ethyl esters by HPLC in this study, the spectrum in Fig. 5 comprised all the molecular ions labeled with different 13C atom ratios. The relative intensity was presented as the intensity of ion at m/z 378 to be 100. Molecular ions were also confirmed by SIMS of free acid of DHA which was prepared from the ethyl ester.

In a mass spectrum of SIMS of 13C-labeled DHA in the presence of NaCl, an ion at m/z 373 was detected and was assigned to [13C22H32O2+Na]+ (Fig. 6a). In that of non-labeled DHA, an ion at m/z 351 was detected and was assigned to [C22H32O2+Na]+ (Fig. 6b).

The ratio of the labeled molecular ions was estimated by comparing the intensities of the molecular ions in Fig. 5, according to Biemann’s method\(^{20}\). Mole ratios of these labeled DHA, \(13C_{22}H_{32}O_2\), \(13C_{21}12CH_{32}O_2\), \(13C_{20}12C_{2}H_{32}O_2\) and \(13C_{19}12C_{3}H_{32}O_2\), were calculated as 0.58, 0.18, 0.20 and 0.05, respectively. The average of 13C atom mole per cent in the total labeled DHA molecules was calculated as 97% from the result. Besides DHA, other 13C-labeled fatty acids, docosapentaenoic acid (22:5(n-6), DPA), eicosapentaenoic acid (20:5(n-3), EPA) and palmitic acid (16:0), were fractionated as by-products. The yields and purities on GLC, and 13C atom ratios of these labeled fatty acids were estimated by the same manner as described for those of DHA, and are shown in Table 3.

While there is also a marine bacterium which produces DHA\(^{14}\), the productivity by the bacterium is lower than those of DHA-producing Thraustochytrids such as *Schizochytrium* sp. strain

### Table 3 Yields, Purities and 13C Atom Ratios of Labeled Fatty Acids in Labeled Medium S.

<table>
<thead>
<tr>
<th>Labeled fatty acid</th>
<th>g/L Purity on GLC(%)</th>
<th>13C atom (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22:6(n-3)</td>
<td>0.29</td>
<td>100</td>
</tr>
<tr>
<td>22:5(n-6)</td>
<td>0.16</td>
<td>55</td>
</tr>
<tr>
<td>20:5(n-3)</td>
<td>0.02</td>
<td>96</td>
</tr>
<tr>
<td>16:0</td>
<td>0.21</td>
<td>100</td>
</tr>
</tbody>
</table>

While there is also a marine bacterium which produces DHA\(^{14}\), the productivity by the bacterium is lower than those of DHA-producing Thraustochytrids such as *Schizochytrium* sp. strain.
N1-27. Such Thraustochytrids are suitable organisms for producing labeled DHA, and DPA as well.

(Received Jan. 31, 2000 ; Accepted Aug. 14, 2000)

References

[ノート] ポリ(α-ヒドロキシアクリル酸ナトリウム)の木綿複合人工汚染布に対する洗浄性能

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ポリ（α-ヒドロキシアクリル酸ナトリウム）；PHA の複合人工汚染布に対する洗浄性能を、PHA 分子量、アニオン界面活性剤（SDS）および PHA と構造の類似している高分子；ポリアクリル酸ナトリウム（PA）、ポリビニルアルコール（PVA）による洗浄性能と比較した。

水の硬度が高い領域では PHA は SDS より高い洗浄性を示し、しかも分子量 10,500 までは、分子量の増加と共に洗浄性は増加したが、それ以上では一定になった。さらに PHA は PA や PVA より高い洗浄性を示した。

（連絡者：駒城素子） Vol.49, No.11, 1433 (2000)

[ノート] スラウストキトリッドによる13C 標識ドコサヘキサエン酸の生産

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ドコサヘキサエン酸産生スラウストキトリッド, Schizochytrium sp. N1-27 株, を用いて, 13C 存在比の高い13C 標識ドコサヘキサエン酸を調製した。[1,2-13C]酢酸ナトリウムを唯一の炭素源とした培地で 115h 培養した後, 菌体から抽出した総脂質は培養液 1L 当たり 2.53g であった。総脂質から脂肪酸エチルエステルを調製し, HPLC で分析した。回収率 53% で, 化学的純度がほぼ 100% の13C 標識ドコサヘキサエン酸を得た。この標識ドコサヘキサエン酸の13C 存在比は GC-MS から 97% 以上と推定した。培養液 1L 当たりの生産量は 0.29g であった。

副生成物として13C 標識ドコサベンタエン酸, エイコサンベンタエン酸及びパルミチン酸を得た。このスラウストキトリッドは13C 標識ドコサヘキサエン酸及びドコサベンタエン酸の生産に適した微生物である。

（連絡者：渡部和郎） Vol.49, No.11, 1437 (2000)