NOTE

Conversion of 18-carbon Fatty Acids to Long Chain Polyunsaturated Fatty Acids in Some Thraustochytrids

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Abstract : Four strains that produce docosahexaenoic acid (DHA), two *Schizochytrium*s (SR21/N1-27) and two *Thraustochytrium*s (F3-1/W20), were cultured using oleic acid (OA), linoleic acid (LA), and α -linolenic acid (ALA) as carbon sources. These fatty acids in the medium were emulsified with Tween 80 using an ultrasonicator. Though SR21 grew faster than the other strains did, the fatty acid profile of SR21 did not change throughout the culture period and the DHA content was not affected by the three fatty acids. The fatty acid profiles of the other three strains were affected by the fatty acids, i.e., ALA resulted in a higher DHA content and LA in a lower DHA content. *Schizochytrium limacinum* SR21 seems to have a different fatty acid synthetic pathway from the other strains and none of OA, LA and ALA seem to become precursors of DHA in the strain.

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Key words : thraustochytrid, DHA, *Schizochytrium*, *Thraustochytrium*

1 Introduction

The long chain polyunsaturated fatty acids (PUFA), especially n-3 PUFAs, have attracted increasing attention owing to their medicinal and physiological advantages to reduce cardiovascular disorders such as atherosclerosis and myocardial infarct (1). Among PUFAs, docosahexaenoic acid (DHA, 22:6n-3), especially, has attracted the greatest attention with its functions related to brain (2) and retina (3). Fish oil as the main DHA source has the disadvantage of having a low DHA content with similar PUFAs resulting in a difficult purification process. This is why microbial DHA production has been an important area in the research on single cell oils (4). Thraustochytrids have recently been recognized as the most promising microbial source of DHA (5). Fatty acid profiles of

thraustochytrids was characterized by the coexistence of both n-3 DHA and n-6 docosapentaenoic acid (DPA, 22:5n-6) as major PUFAs. The synthetic pathway to DHA in thraustochytrids is not known yet and whether n-6 DPA is the direct precursor of n-3 DHA or not is to be made clear. In animals, n-3 and n-6 PUFAs are not interchangeable with each other and DHA is proposed to be synthesized from icosapentaenoic acid (EPA, 20:5n-3) via 24:6n-3 in rat hepatocyte (6). In some microbes, though, n-3 EPA is synthesized from arachidonic acid, 20:4n-6 (7). In the present research, culture of some thraustochytrids was performed using precursor fatty acids as a sole carbon source in order to clarify the synthetic pathway to DHA. Three precursor fatty acids, oleic acid (OA, 18:1n-9), linoleic acid (LA, 18:2n-6) and linolenic acid (ALA, 18:3n-3), belonging to the n-9, n-6 and n-3 series, respectively, were chosen in this experiment. The purpose of this experiment was also to increase the future availability of some plant oils as a microbial

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DHA source.

2 Experimental

2.1 Microorganisms, Media and Cultivation :

Four strains used in this study were SR21, N1-27, W20, and F3-1. The strain SR21 (8-11) (*Schizochytrium limacinum*) was isolated from the seawater of Yap Island (Federation States of Micronesia) using a filter method; and N1-27, W20, and F3-1 were isolated from the seawaters of Nakaminato Bay (Japan), Iriomote Islands (Japan), and Fiji Islands (Republic of Fiji), respectively, using the pine pollen baiting method (12). These strains certainly belong to thraustochytrids with their ovoid vegetative cells at 10 to 40 μm diameters and with heterokont zoospores. Among these strains, N1-27 (13) seems to be in the genus *Schizochytrium* due to their successive binary divisions of the vegetative cell (Fig. 1). On the other hand, W20 and F3-1 are thought to belong to the genus *Thraustochytrium* without binary division of their vegetative cells (Fig. 2 and 3) (14). All of these strains accumulated DHA and n-6 DPA as their main PUFAs.

Each strain was stored on slant cultures with 2 g-glucose, 1 g-peptone, 0.5 g-yeast extract, and 15 g-agar in a 1 L-half salt concentration of artificial seawater (ASW) (Tropic Marin, Aquarientechnik, Germany). These strains were inoculated into a 300-mL flask containing 100 mL-GY medium (glucose 20 g/L and yeast extract 10 g/L) in a half salt concentration of ASW from slant cultures followed by incubation at 25°C at 120 rpm in a rotary shaker. After

a 4 day-cultivation, 10 mL of seed cultures were inoculated into 500-mL baffled flasks with 200 mL medium including precursor fatty acids of 18 carbons, oleic acid (OA), linoleic acid (LA), or α -linolenic acid (ALA) (Wako Pure Chem. Inc., Tokyo), as sole carbon sources. To make it easy for microorganisms to contact the hydrophobic precursor fatty acids, surfactants were used as the emulsifier. Seven surfactants including polyoxyethylene sorbitan (Tween 80) (Nacalai Tesque, Kyoto) were examined. The medium included 2 g-fatty acid, 1 g-yeast extract, 2 g- KH_2PO_4 , 1 g- $\text{CH}_3\text{COONH}_3$ and 0.2 g-surfactant in 1 L-ASW at half salt concentration. Although an increase in dry cell weight of Thraustochytrids by the method was observed, the usability of the fatty acids seemed to be very low. In the subsequent experiments, therefore, a fatty acid emulsion was prepared by ultrasonication as follows : 2.5 g-precursor fatty acids in 50 mL of 1%

Fig. 1 Vegetative Cells of N1-27. Successive binary divisions of the vegetative cell are observed.

Fig. 2 Vegetative Cells of W20.

Fig. 3 Vegetative Cells of F3-1.

Tween 80 aqueous solutions were sterilized and ultrasonicated to form an emulsion aseptically by a rod-type ultrasonicator (US-50, Nissei, Tokyo) for 3 min. Aliquots (10 mL) of the emulsion, as well as 10 mL of seed cultures, were added to 500-mL baffled flasks including 200 mL medium of 5 g/L yeast extract in ASW at half salt concentration, and then incubated as above. The resultant medium included 2.5 g/L fatty acid, 5 g/L yeast extract and 0.5 g/L Tween 80 in ACW at half salt concentration.

2.2 Cell Growth and Lipid Analysis

Culture broths were sampled at appropriate intervals to analyze dry cell weight, lipid content and fatty acid composition. Grown cells in the broths were harvested by centrifugation at 2,500 rpm for 5 min followed by washing with water and centrifuging again. The washed cells were then dried at 105°C for 3 h and weighed to obtain the dry cell weight (DCW, g/L). Methylation of fatty acids in the dried cells was performed with 10% methanolic HCl at 60°C for 3 h (15,16). The methanolic HCl was prepared from acetyl chloride methanol according to Christie (17). The resultant fatty acid methyl esters were extracted into n-hexane and analyzed by a gas liquid chromatograph (GC-17A Shimadzu, Kyoto) equipped with a capillary column (TC-70, 0.25 mm inner diameter, GL Science, Tokyo) and a flame ionization detector. The injection port and detector temperatures were adjusted to 240°C. The oven temperature was increased from 180°C to 220°C at a 4°C/min increment. Each analysis was duplicated and the mean values were plotted.

3 Results and Discussion

The effect of various kinds of surfactants on the growth of N1-27 strain was examined using ALA as the carbon source. Dry cell weights increased to 0.3–1.3 g/L at 3 d and continued to increase until 8 d indicating that the fatty acids were apparently utilizable as a carbon source of N1-27. But the DCW levels with surfactants were almost the same as that without surfactant, and the fatty acid profiles of the cell were hardly changed throughout the culture period, indicating also that ALA did not seem to be effectively utilized as a carbon source. This medium with the addition of any surfactant was ineffective for the organism to utilize fatty acid as a carbon source even in the baffled flasks.

After several trials to raise the effect of surfactant,

Fig. 4 Time Course of Dry Cell Weight of N1-27 with Linoleic Acid as Carbon Source.

we found an ultrasonication method as written above. Figure 4 shows the time course of DCW with LA in the medium with ultrasonication. Aggregation of cell/substrate was observed for four strains after 3 d of culture time. The other fatty acids, ALA and OA, had similar growth curves (data not shown). The strain SR-21 showed the highest growth rate followed by N1-27, and the others (F3-1 and W20) showed the lag phase for 1 d. Dry cell weights of all the strains were highest at 3 d and three cultures except SR21 showed rapid decrease at 4 d. The apparent decrease in DCW seems to be caused by the adherence of grown cells to the flask wall. Culture broth was sampled just after mixing to prevent measurement errors due to aggregation, but the cells that stuck to the wall were not collected because the metabolism of fatty acids is more clearly known when dead/stagnant cells do not stick to the wall. Fatty acids seemed to be taken in more rapidly by SR21 than the others, since the amount of SR21 cells that stuck to the wall was much less than the others.

Figure 5 shows the time course of the main fatty acid composition of SR21 and F3-1. Although SR21 seemed to take in fatty acids faster than the others, the fatty acid profile of SR21 did not change throughout the culture period. Levels of DHA at 7 d continued to be 7 to 9% for OA, LA and ALA. The incorporated precursor-intended 18-carbon fatty acids, OA, LA and ALA, did not seem to work as the precursor of PUFAs in SR21. In F3-1, on the other hand, levels of incorporated 18-carbon fatty acids decreased with the culture time concomitantly with the increase in DHA

Fig. 5 Time Courses of Main Fatty Acid Profile of SR21 and F3-1.

as well as DPA levels indicating that the three fatty acids seemed to be metabolized and converted to DHA in the cell. Moreover, DHA compositions at 7 d were 26.9, 19.8 and 29.3% with OA, LA and ALA, respectively, suggesting that the n-3 precursor fatty acid, ALA, gave a higher DHA level and that the n-6 fatty acid, LA, a lower level. In both W20 and N1-27, levels of incorporated 18-carbon fatty acids decreased with the culture time, though increase in DHA compositions was not so clear as in F3-1. Levels of

DHA at the end of the cultures were 17 to 22% for W20 and 14 to 24% for N1-27 indicating that precursor fatty acids were also metabolized in these sirains.

Figure 6 depicts the time course of the n-3 PUFA/n-6 PUFA ratio of the two *Thraustochytriums*, F3-1 and W20. In both strains, ALA (n-3 precursor fatty acid) gave the higher ratio and LA (n-6 precursor fatty acid) the lower ratio, and OA (n-9 fatty acid) gave a ratio in between ALA and LA. These two strains in *Thraustochytrium* seem to have a similar

Effect of precursor fatty acid in F3-1

Effect of precursor fatty acid in SR21

Effect of precursor fatty acid in W20

Effect of precursor fatty acid in N1-27

Fig. 6 Effect of Precursor Fatty Acid on n-3 PUFA/n-6 PUFA Ratio of F3-1 and W20. n-3 PUFA=DHA+EPA, n-6 PUFA=n-6 DPA+arachidonic acid.

Fig. 7 Effect of Precursor Fatty Acid on n-3 PUFA/n-6 PUFA Ratio of SR21 and N1-27.

fatty acid synthetic pathway to animals.

Figure 7 depicts the time course of the n-3 PUFA/n-6 PUFA ratio of the two *Schizochytrium*s, SR21 and N1-27. As was shown in **Fig. 5**, very little effects of the precursor fatty acids were observed in SR21. None of the 18-carbon unsaturated fatty acids, OA, LA and ALA, seem to become precursors of n-6 DPA and n-3 DHA in the strain. *Schizochytrium limacinum*, SR21, seems to have a completely different fatty acid synthetic pathway from other thraustochytrids. In N1-27, on the other hand, ALA gave a higher n-3 PUFA/n-6 PUFA ratio than LA as in *Thraustochytrium*, but OA resulted in the lowest ratio suggesting a fatty acid synthetic pathway in N1-27 which is also different from the two *Thraustochytrium*s.

riums.

The present results showed the variety of fatty acid synthetic pathways among thraustochytrids, but the pathway may be to some extent genus dependent. The results also show the possibility of changing the n-3 PUFA/n-6 PUFA ratio of the microbes by a precursor fatty acid as a carbon source. Further study is to be performed to clarify the biosynthetic pathway to DHA in thraustochytrids.

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