In vivo conversion of triacylglycerol to docosahexaenoic acid-containing phospholipids in a thraustochytrid-like microorganism, strain 12B

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Key Words Bioconversion · Docosahexaenoic acid (DHA) · DHA-containing phospholipid · Glucose starvation · Thraustochytrid

Abstract

The thraustochytrid-like microorganism, strain 12B, cultivated in peptone, yeast extract, and 8% (w/v) glucose in 50% (v/v) seawater, accumulated docosahexaenoic acid (DHA)-rich triacylglycerol (TAG) at 67% of total lipid. When these TAG-accumulated cells were cultivated in glucose-deficient medium, dry cell weight (3 mg) per ml culture increased approximately threefold relative to baseline and TAG/total lipid decreased to 5%. At the same time, the amount of phospholipid (5 mg) per whole culture increased threefold. Hence, phospholipid/total lipid increased from 13% to 67%. High levels of DHA (more than 50% of total) were maintained in phosphatidylcholine.

Introduction

Docosahexaenoic acid (DHA; 22:6, *n*-3) has various physiological functions in the brain, the nervous system, the cardiovascular system and the retina. Its structural and functional roles in humans and other animals have been well reviewed (Gibson et al. 1996; Holub and Holub 2004; Das 2006). In mammals, a small amount of DHA can be synthesized from the shorter chain *n*-3 fatty acid, α-linolenic acid (18:3, *n*-3), by a combination of chain elongation and desaturation (Shaefer et al. 2006). Accordingly, DHA must be taken in either in the diet or in dietary supplements.

At present, fish oil is the most important source of DHA (as well as eicosapentaenoic acid, EPA; 22:5, *n*-3), which is consumed in the diet and in dietary supplements exclusively in the form of triacylglycerol (TAG). Another potential form of DHA is phospholipids, although DHA-containing phospholipids (DHA-containing PL) have rarely been considered commercially as a source of DHA.

Various physiological functions specific to DHA-containing PL were identified by *in vitro* experiments using cultured animal cells (Hossain et al. 2006). DHA-containing phosphatidylcholine (PC) was more toxic to murine leukemia cells than PC containing other *n*-3 or *n*-6 fatty acids (Kafrawy et al. 1998). *In vivo* studies showed that DHA-containing PL was more effective than DHA-containing TAG in the development of fish larvae (Gisbert et al. 2005). The most abundant fatty acid in the brain and the eye is DHA, where it is found exclusively in the form of PL. Schaefer et al. (2006) showed that increased levels of DHA-containing PL in plasma were associated with a significant reduction (47%) in the risk of developing dementia.

Although there is interest in elucidating the specific function(s) of DHA-containing PL, we have not yet identified an appropriate source of the compound. Eggs of fish such as herring and salmon are known to have high contents of DHA-containing PL, however the proportion of DHA in lipids from fish eggs was 30% or less. Eggs from hens that were fed fish oil could be used as a source of DHA-containing PL, and squid meal and skin are generally rich in DHA- containing PL (Inoue and Kaneda 2002). However, the lipid contents of these materials were low. In addition, materials of marine origin face other problems such as the contamination of fish due to pollution, unstable fish catches and consistent and worldwide decreases in fishery resources. These factors indicate a need to find alternative sources of DHA (Qi et al. 2004; Orikasa et al. 2007).

Some eukaryotic marine or estuarine microorganisms, such as thraustochytrids and dinoflagellates, accumulate extremely high levels of DHA-rich TAG. Thus, these organisms have been utilized as source materials of DHA-containing TAG. However, to our knowledge, such microorganisms have never been considered as a source of DHA-containing PL (see Ratledge 2005). In this study, we demonstrate a fermentation technology for enriching DHA-containing PL using the thraustochytrid-like microorganism strain 12B.

Materials and methods

Strains and cultivation

The thraustochytrid-like strain 12B (Perveen et al. 2006) was used throughout this study. Strain 12B was normally cultivated at 30 °C in F medium consisting of 1% (w/v) peptone, 1% (w/v) yeast extract and 8% (w/v) glucose in 50% (v/v) seawater. Cells of strain 12B were collected using a sterile loop (equivalent to approximately 500 μg cell

dry weight) from an agar plate containing 0.1% peptone, 0.1% yeast extract, 0.5% glucose and 1% agar in 50% seawater. The cells were inoculated into 10 ml F medium in a 50 ml flask. After incubating the culture at 30 °C for 72 h with shaking at 180 rpm, 4 ml of this culture was transferred to 25 ml of Z×1 medium, which consisted of 1% peptone and 1% yeast extract in 50% seawater. The culture was then incubated for up to 48 h under the same conditions. When necessary, two modified Z media containing either 2% peptone and 2% yeast extract or 4% peptone and 4% yeast extract, named Z×2 and Z×4, were used. The optical density of the cultures was measured at 600 nm (OD_{600}), after they had been diluted 10 to 20 times in cultivation medium. Cells were harvested, washed twice with 1% NaCl and rinsed once with distilled water by centrifugation as described by Perveen et al. (2006). Cells were freeze-dried, weighed and stored at –30 °C until use.

Lipid extraction and analysis

Lipids were extracted from freeze-dried cells, as described previously (Okuyama et al. 1984). Extracted lipids were dissolved in chloroform/methanol (2:1, v/v) containing 0.1% butylated hydroxytoluene at 50 mg ml^{-1} and then stored at –30 °C.

The lipids were separated into polar and nonpolar (neutral) fractions by one-dimensional thin-layer chromatography (TLC) on silica gel plates (Silica gel G60, Merck) using hexane/diethyl ether/acetic acid (50:50:1, by vol.). To separate individual polar lipids, two-dimensional TLC was carried out on the same silica gel plate using chloroform/methanol/water (65:25:4, by vol.) and chloroform/acetone/methanol/acetic acid/water (80:40:40:10:1, by vol.) as solvents for the first and second dimensions, respectively.

To identify TAG and free fatty acids, their mobilities in TLC were compared with those of authentic standards (Sigma). The amount of each lipid class was determined by estimating the content of fatty acid (see below). To identify PL, the reactivity of each spot against various spray reagents was examined after two-dimensional TLC (Istokovics et al. 1998). Lipids, identified tentatively as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI), were scraped off the plate and extracted with chloroform/methanol/water (1:1:0.9, by vol.). Each PL was identified by comparing its R_f with those of its authentic standards (Sigma) by one-dimensional TLC using three different solvent systems simultaneously.

Analysis of phosphorus and fatty acid

The amount of phosphorus in total lipids and in individual PL classes separated by TLC was quantified using the method described previously (Okuyama et al. 1984). Fatty acids from the total lipids and from the individual lipid classes were analyzed as their methyl esters by gas-liquid chromatography, as described previously (Perveen et al. 2006).

All data in this study are means for three or more independent experiments.

Results and discussion

Effects of depleted levels of glucose on the growth and lipid content of strain 12B

The OD_{600} of the strain 12B culture grown in F medium at 30 °C for 72 h was approximately 37. The dry cell weight (DCW) per 1 ml culture and the weight of total cellular lipid per DCW are shown in Table 1. The cells were notably rich in TAG,

whereas PL made up only 13% of total lipids (Table 1). A 4 ml aliquot of the TAG-rich strain 12B culture in F medium was transferred to 25 ml of Z media that lacked glucose.

The baseline DCW of strain 12B in 4 ml F medium was calculated to be 3 mg ml^{-1}. After being transferred to Z×1 medium, DCW showed a transient threefold increase at 24 h and then a slight decrease to 8 mg ml^{-1} at 48 h. The total lipid/DCW value of 43% decreased to 9% over 48 h, mainly because of a significant decrease in the content of TAG (Table 1). On the other hand, the calculated baseline amount of PL (5 mg) per total volume of culture increased to 15 mg after cultivation for 48 h in Z×1 medium. Hence, PL/total lipid increased from 13% to 67% (Table 1).

Table 1

When TAG-rich strain 12B cells that had been cultivated in F medium were transferred to Z×2 and Z×4 media and grown for 48 h, total lipid/DCW was much higher than in Z×1 medium (Table 1). However, PL/total lipid (67%) was highest for cells grown in Z×1 medium. The increase in total lipid/DCW in cells grown in Z×2 and Z×4 media was mainly due to the increase in the TAG content. The increase in DCW in Z media was not reflected in the OD_{600} of the cultures, because the cells formed small aggregates for an unknown reason.

Composition of phospholipids and their DHA content

In one-dimensional TLC of total extractable lipids, lipids that showed no movement over the plate were regarded as polar lipids. The proportions (as weight % of the amount of fatty acid) of TAG, other neutral lipids and polar lipids of strain 12B cells grown in F medium were 88%, 4%, and 8%, respectively. No free fatty acid was detected. Cultivation of the cells in Z×1 medium resulted in TAG decreasing to 13% and polar lipids increasing to 73% after 48 h. The transfer of TAG-rich cells grown in F medium

Table 2

to Z media led to only a slight increase in the levels of free fatty acid (9%), suggesting the preferential consumption for carbon and energy of fatty acids released from TAG.

In strain 12B cells grown in Z×1 medium for 48 h the major PL was PC, PE, and PI (Table 2). Interestingly, PC and PE each formed two distinct spots on the two-dimensional TLC plate (not shown). PC1 and PE1 moved faster than PC2 and PE2, respectively. The ratios of PC1 to PC2 and PE1 to PE2 were approximately 1:1.17 and 1:1.14, respectively (Table 2).

Much more DHA was found in PC2 and PE2 than in PC1 and PE1 (Table 2). The calculated proportion of DHA in total PC was approximately 54%. This was much higher than the proportion of DHA in PC prepared from squid (42.6%) (Hossain et al. 2006). However, the content of DHA in each PL class was unexpectedly low. Only 20–30% of fatty acids in PE and PI were DHA. Considering the high content of PL (67%) and DHA (57%) in the respective totals (Table 1), the DHA content of each PL class, including PC, would be expected to be underestimated. Such an underestimation would be caused by the preferential oxidative degradation of DHA during extraction, TLC and subsequent GC analysis.

In this study, we showed that a DHA-rich, TAG-accumulating, eukaryotic microorganism, a thraustochytrid-like strain 12B, could be used as a source organism of DHA-containing PL (particularly PC). Cultivation of these organisms in media that contain high concentrations of sugars such as glucose would be indispensable for increasing the density of cells containing DHA-rich TAG, because DCW was less than 2 mg ml^{-1} when strain 12B cells were inoculated directly from the agar plate into Z×1 medium and cultivated at 30 °C for 72 h (not shown). However, the TAG/total lipid ratio was higher in strain 12B cells cultured in Z×2 and Z×4 media than in Z×1 medium

(Table 1), suggesting that the oleaginous characteristic of this microorganism would not be lost even under glucose-deficient conditions. On the other hand, no decrease in TAG content was observed when cells of the TAG-rich strain 12B were cultivated in 50% seawater containing no nutrients (not shown). Hence, supplementation with additional nutrients such as peptone and/or yeast extract with appropriate levels of C, N and P to promote cell proliferation, which decreases the content of TAG and increases the content of membrane PL, would be necessary. This fermentation technology could be applied to TAG-accumulating microorganisms, which contain specific fatty acids, as a means of producing PL and probably other types of polar lipid that accumulate these fatty acids.

Acknowledgement

Strain 12B was isolated from samples collected in association with Iriomote Station, Tropical Biosphere Research Center, Joint-use Inter-University Institute, Ryukyu University. The authors gratefully acknowledge A. Shinohara for her valuable assistance in cultivation of microbial cells and analysis of lipids.

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Table 1. Effects of medium composition and cultivation time on cell growth and total lipid, phospholipids, TAG, and DHA contents of strain 12B cells

	Media[a]				
	F	Z×1	Z×1	Z×2	Z×4
Cultivation time[b] (h)	72	24	48	48	48
DCW[c] (mg ml^{-1})	23	9	8	8	12
Total lipid[d] / DCM (%)	43	16	9	12	12
TAG[e] / total lipid (%)	67	49	5	22	35
PL[f] / total lipid (%)	13	29	67	52	52
DHA / total fatty acid (%)	45	53	57	55	55

[a] F medium consists of 1% (w/v) peptone, 1% (w/v) yeast extract, and 8% (w/v) glucose in 50% (v/v) sea water. Z×1, Z×2, and Z×4 media contain either 1% peptone, 1% yeast extract, 2% peptone, 2% yeast extract, or 4% peptone, 4% yeast extract in 50% sea water, respectively.

[b] A 4-ml aliquot of the culture grown at 30 °C in 10 ml F medium was transferred to 25 ml Z media and then grown at 30 °C for the indicated time.

[c] The baseline DCW of strain 12B cells in Z media was calculated to be 3 mg ml^{-1}.

[d] The amount of total lipids from dry cell matter was weighed directly.

[e] TAG and other lipid classes were separated by one-dimensional TLC, and the amount of each lipid class was estimated by measuring the content of fatty acids using GC as described previously (Istokovics et al. 1998).

[f] The amount of phospholipid was estimated by measuring the content of phosphorus in total lipids following the published method of Okuyama et al. (1984). Phosphatidylserine (from bovine brain, Sigma) was used as a standard.

The calculated amount of PL per 1 ml culture at baseline and after 48 h cultivation was 5 mg and 15 mg, respectively.

Table 2. Phospholipid classes and their DHA content in strain 12B cells grown in Z×1 medium for 48 h at 30 °C

	Content in total lipids[a] (wt%)	Content in total PL[b] (mole%)	Relative content of PL subclass[c] (wt%)	DHA (wt%)
Total phospholipid	67			
Total PC		61	(100)	
PC1			46	39
PC2			54	67
Total PE		12	(100)	
PE1			47	23
PE2			53	33
PI		13		21
Others		15		N.D.[d]

[a] Estimated by measuring the content of phosphorus in total lipids using the method of Okuyama et al. (1984). Phosphatidylserine (from bovine brain, Sigma) was used as standard.

[b] Combined amounts of phosphorus from PC1 and PC2 and from PE1 and PE2 after TLC were measured.

[c] Relative contents of PC1 and PC2 and of PE1 and PE2 were estimated by measuring the amount of each fatty acid by GC (Perveen et al. 2006).

[d] Not determined.