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FULL PAPER

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Effect of cyanocobalamin and *p*-toluic acid on the fatty acid composition of *Schizochytrium limacinum* (Thraustochytriaceae, Labyrinthulomycota)

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Abstract Changes in the fatty acid composition of docosahexaenoic acid (DHA)-producing *Schizochytrium limacinum* SR21 were investigated. The addition of cyanocobalamin, which is an active component of vitamin B_{12}, decreased the content of odd-chain fatty acids such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0). Cyanocobalamin may upregulate the cobalamin-dependent methylmalonyl-CoA mutase, which converts propionic acid to succinic acid, thereby decreasing the content of odd-chain fatty acids. The addition of *p*-toluic acid resulted in a decrease in docosapentaenoic acid (DPA, 22:5n-6) content and an increase in eicosapentaenoic acid (EPA, 20:5n-3) content in a dose-dependent manner. Two additional peaks of fatty acids, characterized as Δ4,7,10,14-eicosatetraenoic acid (20:4n-7) and Δ4,7,10,14-docosatetraenoic acid (22:4n-9), were detected.

Key words Cyanocobalamin · Docosahexaenoic acid · Methylmalonyl CoA mutase · *p*-Toluic acid · *Schizochytrium limacinum* SR21

Introduction

Long-chain polyunsaturated fatty acids (LCPUFAs) are important dietary constituents. Their beneficial effects on human health are widely accepted, and this realization has led to extensive nutritional and clinical studies on their effects on human physiology. Among the LCPUFAs, Δ4,7,10,13,16,19-docosahexaenoic acid (DHA, 22:6n-3), which is one of the most abundant components of structural lipids in the brain, has recently attracted a great deal of attention because of its specific function in the brain (O'Brien and Sampson 1965; Uauy and Andraca 1995) and retina (Nuringer et al. 1984; Bazan et al. 1986; Uauy et al. 1990).

The largest commercial source of DHA is fish and fish oil; however, fish oil has an undesirable smell. Recently, the production of LCPUFAs by microorganisms has been well investigated, and the microbial production of DHA was industrialized as a new source of DHA (Bajpai et al. 1991; Singh and Ward 1996; Iida et al. 1996). Among them, single cell oils from marine thraustochytrids, which are widely distributed in the oceans around the world, are a satisfactory alternative to fish oil as a DHA source (Bowles et al. 1999; Lewis et al. 1999).

The characteristics of thraustochytrids have been based on their morphological character. However, their evolutionary relationships (Anthony et al. 1988) and taxonomy (Bahnweg and Jäckle 1986) were not well understood until molecular analysis of their 18S rRNA genes showed conclusively that thraustochytrids were deeply divergent from oomycetes and close to labyrinthulids (Cavalier-Smith et al. 1994; Leipe et al. 1994). Thraustochytrids, including *Thraustochytrium aureum, T. roseum, T. aggregatum*, and *Schizochytrium limacinum*, have been reported to produce the significant amount of DHA, and Huang et al. (2003) reported that the C20-22 polyunsaturated fatty acid (PUFA) profile could be applicable as an effective characteristic for grouping thraustochytrids. *Schizochytrium limacinum* SR21, which was isolated from seawater of a coral reef area, has been reported to be a potent producer of a large amount of DHA and DPA (Δ4,7,10,13,16-docosapentaenoic acid, 22:5n-6) (Nakahara et al. 1996). Some studies have reported the suitable conditions required for production of DHA by this strain (Yaguchi et al. 1997). Although the conditions for the production of DHA by *limacinum* SR21 are well defined, the metabolic specificities of PUFAs are still unclear. Some food and spice constituents, such as sesamin (Shimizu et al. 1991), curcumin (Shimizu et al. 1992; Nakano et al. 2000), and capsaicin (Nakano et al. 2001), were reported to inhibit

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fatty acid desaturation. Addition of these compounds resulted in remarkable changes in the fatty acid compositions; these changes in fatty acid composition provided very useful information for elucidating fatty acid biosynthesis. In the present study, we searched for compounds that affected fatty acid composition. The structural analysis of newly detected fatty acids when an inhibitor is added and the putative biosynthetic pathway of fatty acid in *Schizochytrium limacinum* SR21 are discussed.

Materials and methods

Chemicals

All the authentic fatty acid methyl esters were obtained from Funakoshi (Tokyo, Japan). All the other reagents were of analytical grade.

Microorganism

Schizochytrium limacinum SR21 (IFO 32693) (Nakahara et al. 1996) was used in the present study. All the other strains were obtained from the American Type Culture Collection (ATCC).

The cultures were grown in a medium (10 ml, pH 5.5) containing 2% glucose, and 1% yeast extract in a half-concentration of artificial seawater in a 50-ml flask for 4–6 days at 28°C with reciprocal shaking (120 strokes/min). When the reagents were tested for their effects on fatty acid composition, 0.05% of each test reagent was added and the strain was inoculated and grown as described above.

Lipid analysis

The culture broth was harvested during the stationary growth phase, and the cells were washed with water by centrifugation. The washed cells were dried at 105°C for 3 h and weighed to obtain the dry cell weight (DCW). The dried cells were then resuspended in 5 ml dichrolomethane/10% methanolic HCl (1:1, v/v) for 3 h at 50°C. After extraction with 10 ml *n*-hexane, followed by evaporation, the fatty acid methyl esters were dissolved in 0.5–1 ml acetonitrile and then subjected to gas-liquid chromatography (GLC). The analytical conditions were as follows: apparatus, GC-14B (Shimadzu, Kyoto, Japan), equipped with flame ionization detector (FID) with a split injector; column, ULBON HR-SS-10 capillary column (0.25 mm × 50 m; Shimadzu), column temperature, 200°C; injection port temperature, 250°C; carrier gas, He (inlet pressure 200 kPa); makeup gas, N_2 (60 ml/min); air and H_2, 60 kPa; and split ratio, 25:1. The purification of fatty acid methyl esters by HPLC was performed using a Shimadzu LC-5A system with a Cosmosil 5C18-AR column (20 × 250 mm; Nacalai Tesque, Kyoto, Japan) [wavelength, 205 nm; mobile phase, acetonitrile:water (90:10, v/v); flow rate, 7 ml/min; column temperature, 30°C]. The fractions were collected in the eluted order and subjected to GLC analysis. The fraction containing UK1 and UK2 was collected and concentrated under reduced pressure.

The picolinyl esters of UK1 and UK2 were prepared from their free acids by using the method of Christie et al. (1986). Before mass spectrometry (MS) analysis, the resultant picolinyl ester was purified by HPLC under the conditions already described, except for the wavelength of 254 nm. Mass spectra were performed with a Shimadzu GCMS-9100MK (ionization potential, 70eV).

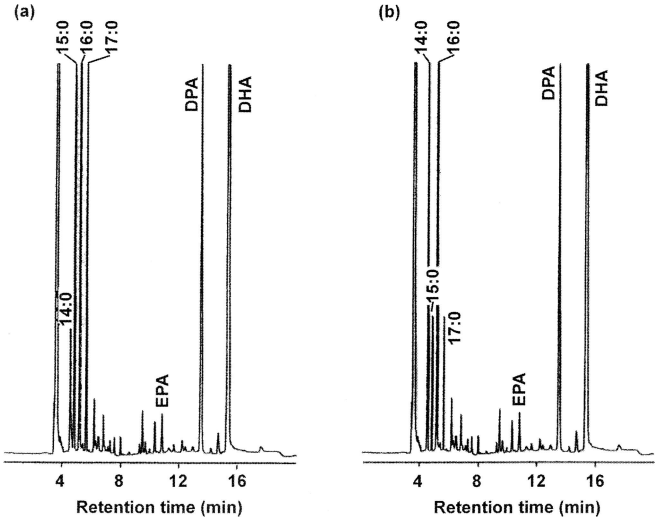

Fig. 1. Gas-liquid chromatography (GLC) chromatograms of fatty acid methyl esters obtained from *Schizochytrium limacinum* SR21 grown without (**a**) or with (**b**) cyanocobalamin. *14:0*, tetradecanoic acid; *15:0*, pentadecanoic acid; *16:0*, palmitic acid; *17:0*, heptadecanoic acid; *18:0*, stearic acid; *EPA*, 5,8,11,14,17-*cis*-eicosapentaenoic acid; *DPA*, 4,7,10,13,16-*cis*-docosapentaenoic acid; *DHA*, 4,7,10,13,16,19-*cis*-docosahexaenoic acid

Results and discussion

Effect of cyanocobalamin on fatty acid composition of *S. limacinum* SR21

A total of 155 compounds, such as inhibitors of fatty acid desaturation of *Mortierella* fungi or rat liver microsomes, and their structural analogues were tested. It was observed that the contents of odd-chain fatty acids, particularly pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), were remarkably decreased when the strain was cultivated in a medium supplemented with cyanocobalamin (Fig. 1). Cyanocobalamin is effective in reducing the contents of odd-chain fatty acids in all the other DHA-producing strains listed in Table 1. Although the addition of cyanocobalamin resulted in a decrease in the ratio of C15:0 and C17:0 to palmitic acid (C16:0), remarkable changes in the cell growth and total content of polyunsaturated fatty acid were not observed. The effect of cyanocobalamin was not observed in a dose-dependent manner (data not shown). Cyanocobalamin is the active component of

Table 1. Effect of cyanocobalamin on the fatty acid compositions of the docosahexaenoic acid (DHA)-producing strains[a]

Strain	CC[b]	Fatty acid composition (/16:0)[c]				
		14:0	15:0	16:0	17:0	18:0
Schizochytrium limacinum SR21	–	0	0.14	1	0.12	0.04
	+	0.03	0.02	1	0.02	0.04
Schizochytrium sp. ATCC 20889	–	0	0.32	1	0.42	0.04
	+	0.07	0.03	1	0.03	0.07
Thraustochytrium sp. ATCC 26185	–	0.04	0.39	1	0.29	0.04
	+	0.07	0.03	1	0.02	0.03
Schizochytrium aggregatum ATCC 28209	–	0	0.37	1	0.44	0.04
	+	0.12	0.04	1	0.02	0.04
Thraustochytrium aureum ATCC 34304	–	0.05	0.29	1	0.13	0.04
	+	0.04	0.03	1	0.02	0.03

[a] Each strain was grown in medium supplemented with or without cyanocobalamin, and culture conditions were as described in Materials and methods
[b] The concentrations of cyanocobalamin were 0 (–) or 0.05 mg/l (+)
[c] Data were presented as ratio to 16:0, and values are mean of two determinations; see Fig.1 for abbreviations

vitamin B_{12} that upregulates methylmalonyl-CoA mutase (Fenton et al. 1982). Generally, cellular propionic acid is generated from amino acids and subsequently incorporated into odd-chain fatty acid generation or metabolized to succinic acid by methylmalonyl-CoA mutase. It is suggested that activation of methylmalonyl-CoA mutase results in the acceleration of propionic acid metabolism by the methylmalonate pathway and decrease in the propionic acid concentration. Galli et al. (1985) reported that the incorporation of propionic acid into fatty acids increases in *Euglena gracilis* when the concentration of vitamin B_{12} is decreased. Based on the result obtained in the present study, it is suggested that the low activity of methylmalonyl-CoA mutase leads to the accumulation of odd-chain saturated fatty acids in *S. limacinum* SR21. The supplementation of cyanocobalamin may activate the enzyme and result in a decrease of the contents of C15:0 and C17:0.

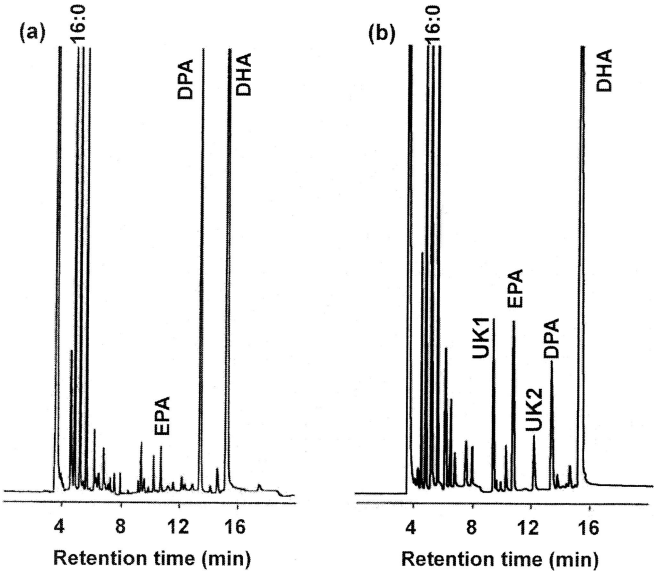

Fig. 2. GLC chromatograms of fatty acid methyl esters obtained from *Schizochytrium limacinum* SR21 grown without (**a**) or with (**b**) *p*-toluic acid

Effect of *p*-toluic acid on fatty acid composition of *S. limacinum* SR21

Among the tested compounds, addition of *p*-toluic acid to the medium resulted in a remarkable difference in the fatty acid composition of *S. limacinum* SR21. The addition of *p*-toluic acid to the culture medium resulted in a remarkable decrease in Δ4,7,10,13,16-docosapentaenoic acid (DPA, 20:5n-6) content, and several additional peaks were observed (Fig. 2). Among the additional peaks, two major peaks were designated as UK1 and UK2. A similar effect of *p*-toluic acid on the fatty acid composition in other DHA-producing strains was also observed (Table 2). The addition of *p*-toluic acid resulted in an increase in the EPA/DPA ratio, decrease in the DPA/DHA ratio, and increase in the contents of UK1 and UK2. A remarkable increase in the C16:0 content and a decrease of DHA/C16:0 ratio were also observed. Although the addition of *p*-toluic acid suppressed cell growth, the changes in fatty acid composition occurred in a dose-dependent manner (Fig. 3). UK1 and UK2 were purified by high-performance liquid chromatography, and the double-bond positions of UK1 and UK2

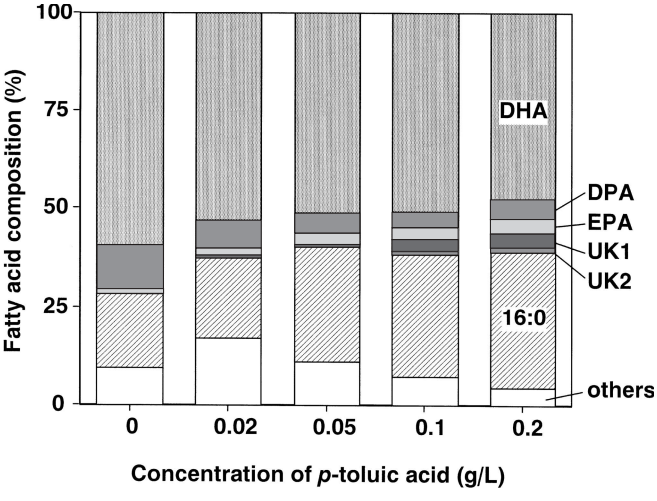

Fig. 3. Effect of *p*-toluic acid on the fatty acid composition of *Schizochytrium limacinum* SR21

Table 2. Effect of *p*-toluic acid on fatty acid compositions of DHA-producing strains[a]

Strain	TA[b]	Fatty acid composition (%)						Fatty acid ratio	
		16:0	EPA	DPA	DHA	UK1	UK2	EPA/DPA	DPA/DHA
Schizochytrium limacinum SR21	–	16.1	2.74	13.0	59.3	tr.	tr.	0.21	0.22
	+	26.2	3.05	6.66	51.1	0.57	0.25	0.46	0.13
Schizochytrium sp. ATCC 20889	–	10.2	6.84	18.4	49.4	tr.	0.72	0.37	0.37
	+	21.8	4.27	11.2	40.8	0.33	2.13	0.38	0.28
Thraustochytrium sp. ATCC 26185	–	11.4	4.52	15.1	44.1	0.28	0.77	0.30	0.34
	+	19.8	3.44	7.60	33.8	0.46	2.20	0.45	0.22
Schizochytrium aggregatum ATCC 28209	–	11.0	4.99	17.5	51.6	tr.	1.60	0.29	0.34
	+	14.4	6.51	13.7	50.3	tr.	2.29	0.47	0.27
Thraustochytrium aureum ATCC 34304	–	24.2	1.09	10.9	46.5	0.48	0.50	0.10	0.23
	+	16.7	2.51	9.56	53.1	0.82	0.90	0.26	0.18

Values are mean of two determinations

[a] Each strain was grown in medium supplemented with or without *p*-toluic acid, and culture conditions were as described in Materials and methods

[b] The concentrations of *p*-toluic acid were 0 (–) or 0.05 mg/l (+)

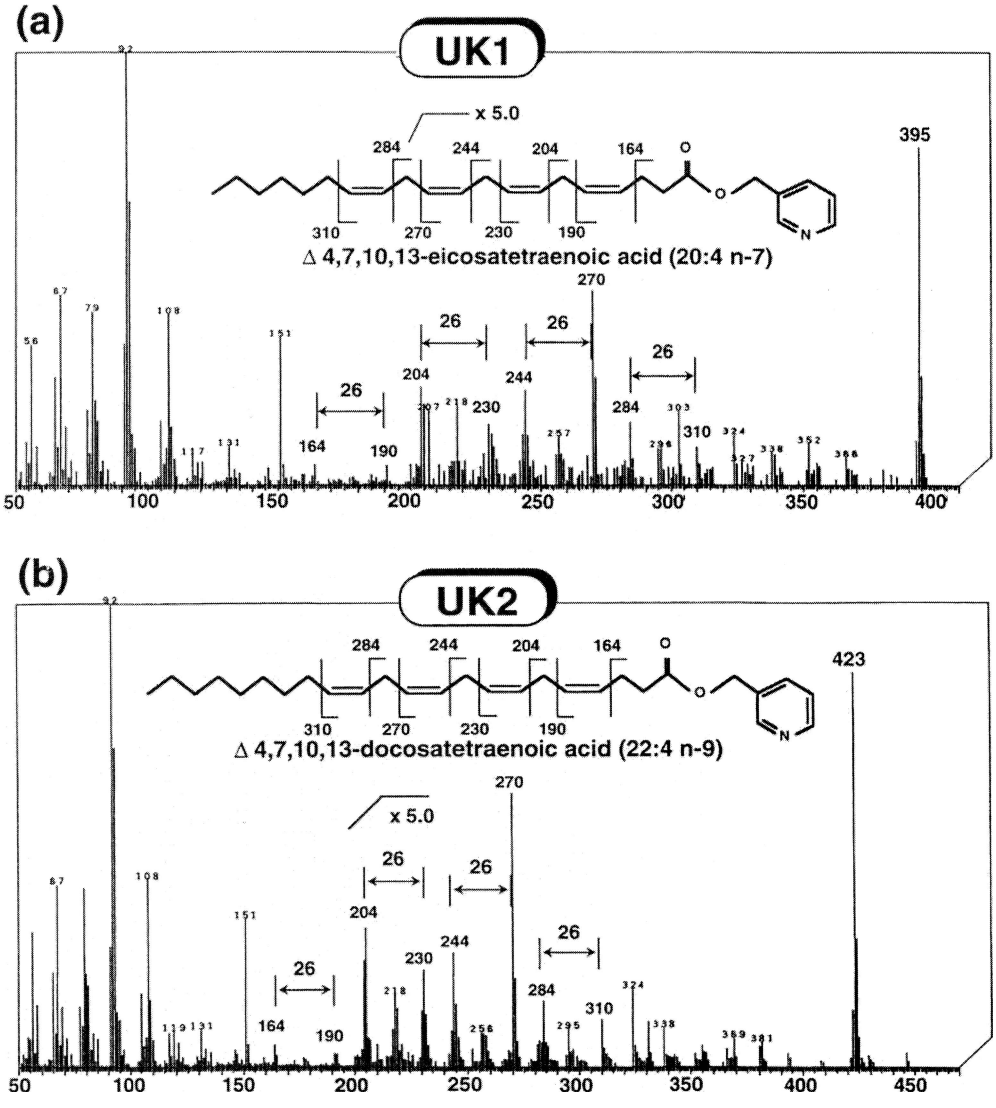

Fig. 4. Mass chromatograms of picolinyl esters of UK1 and UK2

were determined from the mass spectra of their picolinyl esters.

The mass spectrum of UK1 picolinyl ester showed fragment ion peaks at m/z 395 (M^+, relative intensity, 16%), 310, 284, 270, 244, 230, 204, 190, 164, and 92 (base peak) (Fig. 4A). This result demonstrated that double bonds existed at the Δ4, Δ7, Δ10, and Δ13 positions, and UK1 was Δ4,7,10,13-eicosatetraenoic acid (20:4n-7).

The mass spectrum of UK2 picolinyl ester showed fragment ion peaks at m/z 423 (M^+, relative intensity, 18%), 310, 284, 270, 244, 230, 204, 190, 164, and 92 (base peak) (Fig. 4B). This result demonstrated that double bonds existed at the Δ4, Δ7, Δ10, and Δ13 positions, and UK2 was Δ4,7,10,13-docosatetraenoic acid (22:4n-9).

The characteristic of the fatty acid composition of *S. limacinum* SR21 is different from that of arachidonic acid-producing *Mortierella* fungi. Most of the intermediate fatty acids that occur during the biosynthesis of arachidonic acid by *Mortierella* fungi have been detected, and the fatty acid biosynthetic pathway of *Mortierella* fungi has been determined based on these fatty acid compositions (Yamada et al. 1987). On the other hand, the most abundant fatty acid of *S. limacinum* SR21 is DHA, and the content of other unsaturated fatty acids, except for DHA and DPA, is very low. These characteristics of fatty acid composition are very similar to EPA-producing marine bacterium *Shewanella* sp. SCRC-2738 (Watanabe et al. 1997). The proposed pathway for the production of EPA in the EPA-producing marine bacterium *Shewanella* sp. SCRC-2738 was hypothesized as follows: chain extension to eicosanoic acid (C20:0), followed by generation of unsaturated fatty acids by subsequent desaturation. Aki and Suzuki (2001) also hypothesized a similar biosynthetic pathway in *S. limacinum* SR21. They also reported that the addition of erucic acid (Δ13-docosenoic acid, 22:1n-9) resulted in an increase of DHA content. Thus, it was considered that erucic acid is the precursor of DHA biosynthesis. In the present study, it is suggested that the addition of *p*-toluic acid caused the suppression of cell growth and the chain extention to C22 fatty acids (DHA, DPA, and 22:4n-9), and then the content of C20 fatty acids (EPA and 20:4n-7) increased. A remarkable decrease of DPA and increase of 22:4n-9 and 20:4n-7 were also observed in the presence of *p*-toluic acid. The double-bond positions of 22:4n-9 and 20:4n-7 are both at Δ4, Δ7, Δ10, and Δ13. It is suggested that methyl-directed desaturations from the Δ13-position were suppressed by *p*-toluic acid. *p*-Toluic acid is detected as an intermediate of biodegradation of aromatic hydrocarbons such as toluene and xylene. However, notable physiological activity of this compound has not been reported. Therefore, it was not possible to analyze the mechanism of the changes in fatty acid composition of this strain caused by *p*-toluic acid. In the present study, although the critical information required for the analysis of the DHA biosynthesis could not be obtained, it was shown that the analysis of fatty acid metabolism in *S. limacinum* SR21 by using an inhibitor is effective to some extent.

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