Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 335-341

FUNGAL MYCELIA AS A NOVEL SOURCE OF EICOSAPENTAENOIC ACID

Activation of Enzyme(s) Involved in Eicosapentaenoic Acid Production at Low Temperature

Sakayu Shimizu*, Yoshifumi Shinmen[1], Hiroshi Kawashima, Kengo Akimoto[2] and Hideaki Yamada

Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

Received November 30, 1987

Several filamentous fungi belonging to the genus Mortierella were found to produce large amounts of 5,8,11,14,17-cis-eicosapentaenoic acid (EPA) in their mycelia only when grown at low temperature (12°C), i.e., not at physiological growth temperature (20-28°C). The results of experiments with cell-free extracts suggested that this unique phenomenon is due to activation of enzyme(s) involved in EPA formation at low temperature. Mortierella alpina 1S-4 produced 0.3 g/l of EPA (27 mg/g dry mycelia). This high productivity show the practical significance of these novel EPA producers.

5,8,11,14,17-cis-Eicosapentaenoic acid (EPA) is a C-20 polyunsaturated fatty acid (PUFA) with five double bonds and has been shwon to exhibit several unique biological activities (1,2). In addition, it is a natural precursor of a large family of structurally related C-20 compounds, such as the prostaglandine$_3$, thromboxane$_3$ and leucotriene$_5$ groups. This fatty acid is known to be produced and/or to occur as a component of cellular lipids in protozoal, algal, and animal cells (3-5). However, very little attention has been paid so far as to the formation of EPA by lower classes of microorganisms (6), whereas there have been many reports of the formation of PUFAs of the n-6 series, such as γ-linolenic acid and arachidonic acid (7-9).

In the previous paper (9), we reported that several fungal microorganisms accumulate large amounts of arachidonic acid when grown in usual media

* Corresponding author. [1] Present address: Laboratory of Microbial Science, Institute for Fundamental Research, Suntory Ltd., Mishima-gun, Osaka 618, Japan. [2] On leave from Suntory Ltd.

0006-291X/88 $1.50

containing glucose as a carbon source. We have now found that these arachidonic acid producers produce specifically large amounts of EPA, an n-3 PUFA, in their mycelia during growth, but only at low temperature. Although an increase in the unsaturation degree of membrane fatty acids on cold-adaptation has been shown to be a general mechanism for maintaining the proper membrane fluidity (10), these fungi are unique in that they produce a further unsaturated fatty acid (i.e., EPA), as a new component of their lipids. This paper reports that this unique phenomenon is due to activation of the enzyme(s) involved in EPA synthesis at low temperature. The practical significance of these fungi as rich sources of EPA is also shown.

MATERIALS AND METHODS

Chemicals. Fatty acid methylesters were purchased from Funakoshi Chemicals, Tokyo. All other reagents used in this work were of analytical grade and commercially available.

Microorganisms, media and cultivations. The Moltierella strains listed in Table 1 were used. Each fungus was inoculated into 100 ml shaking flasks containing 20 ml of a medium composed of 2% glucose and 1% yeast extract, pH 6.0, and then incubated at various temperatures for 6 days with reciprocal shaking (120 strokes/min). The fungal mycelia were harvested by filtration, washed with water and then used for the following experiments.

Preparation of fungal lipids. Washed mycelia (50 g as wet weight) of Mortierella alpina 1S-4 grown at 28°C were ground in a mortar with 300 ml of n-hexane for 15 min and then filtered. The residue was treated with another 300 ml of n-hexane. To the combined n-hexane extracts was added 3 g of Na_2SO_4. The n-hexane layer, after filtration, was evaporated under reduced pressure at 40°C to give an oily lipid mixture. The resultant lipid contained 20.7 and 74.1% (by weight) of phospholipids and triglycerides fraction, respectively. The fatty acid composition of the lipid was as follows: palmitic acid (10.9%, by weight), stearic acid (5.2), oleic acid (6.0), linoleic acid (5.8), γ-linolenic acid (5.7), dihomo-γ-linolenic acid (5.3) and arachidonic acid (58.1). This was used as a substrate for the enzyme reactions described below.

Preparation of cell-free extracts. Washed mycelia (6 g) of M. alpina 1S-4 were suspended in 5 ml of 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol and 0.1% Tween 20, and ground in a mortar with 2 g of sea sand for 30 min at 5°C. After centrifugation (15,000 x g, 20 min, 5°C), the supernatant was used for the enzyme reaction.

Enzyme reaction. The standard reaction mixture for EPA formation contained 50 mM potassium phosphate buffer, pH 7.0, 10 mM ATP, 1 mM CoA, 0.3 mM NADPH, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Tween 20, fungal lipid (2 mg/ml) and cell-free extract (0.3 mg protein/ml), in a final volume of 3.0 ml. The mixtures were incubated at 12 or 28°C for 5, 10 or 22 h. After freeze-drying of each mixture, the residue was transmethylated and then analyzed for EPA and other fatty acids as described below.

Lipid analyses. For analysis of the fatty acid composition of the total cellular lipid, washed mycelia were treated twice with chloroform-methanol-water according to the procedure of Bligh and Dyer (11). The resultant lipid extract was evaporated to dryness under reduced pressure at 35°C and then used as the sample for transmethylation. For analysis of the fatty acid composition of the triglyceride or phospholipid fraction, the extracted lipid was separated

on a silica gel thin layer plate (Kieselgel $60F_{245}$, 200 x 200 x 0.25 mm; E. Merck, Darmstadt). Solvent systems of petroleum ether-diethyl ether (1:1, by vol.) and chloroform-methanol-water (1:2:0.8, by vol.) were used for triglycerides and phospholipids, respectively. The gel corresponding to the bands of triglycerides and phospholipids stained with 0.02% 2',7'-dichlorofluorescein in ethanol was scraped off. The lipids were then transmethylated.

Transmethylation was usually carried out by incubating the lipids in 20 ml of methylene chloride-10% methanolic HCl (1:1, by vol.) for 3 h at 50°C. As an internal standard, n-heptadecanoic acid was usually included in the methanolysis mixture. After extraction with 10-40 ml of n-hexane, followed by evaporation, the fatty acid methyl esters were dissolved in 0.05-1.0 ml of acetonitrile and then analyzed by gas liquid chromatography as described previously (9).

Other methods. Protein concentrations were determined by the method of Bradford (12). Mass and ^1H NMR spectra were measured with a Hitachi M-80 and a Nicolet NT-360, respectively.

RESULTS

Changes in the fatty acid compositions of Mortierella strains due to the growth temperature. Table 1 shows the fatty acid compositions of five Mortierella strains, which were grown at various temperatures. All stains produced PUFAs such as γ-linolenic acid (18:4), dihomo-γ-linolenic acid (20:3) and arachidonic acid (20:4) in their mycelia when grown at 28°C. At this temperature, however, none of the tested strains produced detectable amounts of EPA (20:5). On the other hand, lowering of the growth temperature to 12°C led to the additional production of EPA by all the strains tested. The EPA content

Table 1. Formation of EPA and changes in cellular fatty acid composition in Mortierella fungi at various growth temperatures

Strain	Growth temp. (°C)	EPA content (mg/g)[a]	Fatty acid composition (%)[b]								
			16:0	18:0	18:1	18:2	18:3	20:3	20:4	20:5	others[c]
M. alpina 1S-4	6	6.43	8.0	2.0	14.0	15.0	10.0	3.3	30.3	14.8	2.6
AKU 3998	12	8.09	8.7	2.2	16.8	14.4	10.1	3.0	28.4	14.9	1.5
	16	1.35	9.6	6.7	14.6	3.9	4.4	4.2	50.2	2.4	4.0
	20	0	12.0	4.5	12.1	5.1	3.4	3.2	56.2	0	3.5
	28	0	15.9	5.9	11.3	9.8	4.1	3.3	47.7	0	2.0
M. hygrophila	12	9.76	16.0	3.8	30.7	8.9	11.3	2.5	13.6	10.4	2.8
IFO 5941	28	0	24.7	2.9	37.4	9.5	5.5	1.6	17.9	0	0.5
M. elongata	12	15.5	13.9	2.5	32.0	7.4	13.1	4.9	12.3	13.9	0
IFO 8570	28	0	25.2	4.3	49.1	3.5	2.5	1.0	13.9	0	0.5
M. exigua	12	5.45	9.8	1.5	22.5	9.9	6.0	3.2	37.5	7.0	2.6
IFO 8571	28	0	22.3	7.2	33.1	10.2	4.7	3.6	18.0	0	0.9
M. parrispora	12	6.97	10.1	4.0	43.3	7.0	8.7	2.7	15.3	5.2	3.7
20-24 AKU 3997	28	0	13.2	3.7	54.6	5.5	5.4	1.7	15.5	0	0.4

[a] Values are given in mg/mg dry mycelia.
[b] Values are given in weight %. 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 20:5, EPA. α-Linolenic acid and other n-3 PUFAs were not detected.
[c] mainly myristic acid.

of M. alpina 1S-4 reached about 15% of the total extractable fatty acids.

In all cases, cultivation at low temperature significantly increased the mycelial phospholipid content. For example, the mycelial lipids extracted from M. alpina 1S-4 grown at 12°C contained 47.6% (by weight) of phospholipids and 35.7% of triglycerides. More than 60% of the EPA accumulated in the mycelia was found in the phospholipid fraction. On the other hand, the lipid from the mycelia grown at 28°C contained only 21.6% of phospholipids, the triglyceride fraction comprising 73.5% of the total extractable lipid.

Formation of EPA by a cell-free extract of M. alpina under a low temperature. In order to clarify whether growth under low temperature is necessary for EPA formation or not, EPA formation at 12°C was compared with that at 28°C using a cell-free extract of the mycelia of M. alpina 1S-4 grown at 28°C as the enzyme and the lipid extracted from the same mycelia as the substrate. The data in Table 2 show that (i) the cell-free extract contained the enzyme(s) necessary for the formation of EPA, (ii) it catalyzed the EPA formation at 12°C but apparently not at 28°C, (iii) the addition of the lipid as the substrate was necessary for the formation of EPA, and (iv) the addition of CoA, ATP, NADPH and $MgCl_2$ accelerated the reaction significantly. These facts suggest that the enzyme(s) that catalyzes the formation of EPA was produced even when the organism was grown at 28°C, but that the reaction(s) yielding EPA did not take place at this temperature. Further evidence supporting this assumption is that

Table 2. Enzymatic formation of EPA by a cell-free extract of M. alpina 1S-4 grown at 28°C[a]

Omission from reaction mixture	Reaction time (h)	EPA found (nmol/ml) on incubation at 12°C	EPA found (nmol/ml) on incubation at 28°C
None	0	0 (2)[b]	0 (2)
	5	18 (23)	0 (4)
	10	38 (49)	0 (3)
	22	48 (67)	0 (4)
Lipid	10	5 (8)	0 (3)
Cofactors[c]	10	8	0
Lipid, cofactors	10	1	0
Enzyme	10	0	0

[a] The reaction conditions are given in the text.
[b] Values obtained with the cell-free extract prepared from the mycelia grown at 12°C are given in parentheses.
[c] ATP, CoA, NADPH and $MgCl_2$.

neither preincubation of the cell-free extract at 28°C for 4 h in 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol and 0.1% Tween 20 nor addition of cycloheximide (1.0 mM) to the reaction mixture affected the EPA formation at 12°C (data not shown). When the cell-free extract of 12°C grown mycelia was incubated at 12°C instead of that from 28°C grown mycelia, the amount of EPA in the reaction mixture increased gradually and reached 67 nmol/ml after 22 h. On the other hand, at 28°C, the final amount of EPA found was only 4 nmol/ml. This value was almost the same as that at the time of initiation of the reaction, again suggesting that the reaction takes place only at 12°C. The possibility that EPA was formed but then metabolized rapidly at 28°C was easily ruled out because the lipid from the mycelia grown at 12°C or EPA itself incubated with the cell-free extract showed no change throughout the reaction at 28°C (data not shown).

Production of EPA by M. alpina. Based on the above results, we optimized the culture conditions for the high yield production of EPA. M. alpina 1S-4, when grown for 2 days at 28°C followed by incubation for a further 8 days at 12°C, produced 0.3 g/l of EPA (27 mg/g dry mycelia). This value accounted for 11% of the total fatty acids in the extracted lipid. Other major fatty acids in the lipid were palmitic acid (6.4%, by weight), stearic acid (4.8), oleic acid (3.2), linoleic acid (3.1), γ-linolenic acid (4.5) and arachidonic acid (63.8). The methyl ester of EPA (1.8 mg) was obtained by means of high performance liquid chromatography from the lipid extracted from 3.9 g wet mycelia of M. alpina 1S-4. Analysis of the isolated methyl ester by ^{1}H NMR and mass spectrometry showed that it was identical with authentic EPA methyl ester.

DISCUSSION

Lowering the growth temperature has been shown to increase the degree of unsaturation of fatty acids in membrane lipids of cells of ranging from microorganisms to animals. Such lipid changes have been suggested to be one of the mechanisms for controlling cellular membrane fluidity (10,13). The Mortierella fungi tested here, as well as other organisms, show an increase in

the PUFA to saturated fatty acid ratio on growth at low temperature. However, the former are very unique in that they newly produce significantly high amounts of a further unsaturated fatty acid (i.e., EPA), which was not found in the mycelia grown at a higher temperature, as a component of their cellular lipids. This unique phenomenon might be explained by the data in Table 2 that suggest that the enzyme(s) or enzyme system involved in the formation of EPA is present in the cells regardless of the growth temperature but is only active at low temperature. Such a temperature-dependent mechanism may be of great benefit to the fungi for maintaining the proper membrane fluidity on cold-adaptation.

Because we used the crude lipid isolated from the mycelia, from which we also prepared the cell-free extract, as the substrate for the enzymatic reactions, we could not determine which fatty acid in the lipid is the precursor to EPA. However, all the arachidonic acid-producing Mortierella strains tested produce detectable amounts of EPA at low temperature without any detectable formation of other n-3 PUFAs (Table 1), but it was undetectable in all the nonproducing strains of the same genus tested (unpublished observation). Furthermore, the methyl-end directed desaturation of the n-6 PUFAs has been suggested in several organisms (3). These facts may substantiate the assumption that one of the C-20 PUFAs of the n-6 series such as arachidonic acid is the precursor of EPA.

The unique characteristic of the Mortierella strains tested here, i.e., the accumulation of large amounts of EPA in their mycelia, would make their use as practical sources of EPA very promising.

REFERENCES

1. Dyerberg, J. (1986) Nutr. Rev. 44, 125-134.
2. Kromhout, D., Bosschieter, E.B., and Coulander, C.D.L. (1985) N. Engl. J. Med. 312, 1205-1209.
3. Hulanicka, D., Erwin, J., and Bloch, K. (1964) J. Biol. Chem. 2778-2787.
4. Chu, F.E., and Dupuy, J.L. (1980) Lipids 15, 356-364.

5. Brockerhoff, H., Hoyle, R.J., and Wolmark, N. (1966) Biochim. Biophys. Acta 116, 67-72.
6. Gellerman, J.L., and Schlenk, H. (1979) Biochim. Biophys. Acta 573, 23-30.
7. Show, R. (1965) Biochim. Biophys. Acta 98, 230-237.
7. Iizuka, H., Ohtomo, T., and Yoshida, K. (1979) Eur. J. Microbiol. Technol. 7, 173-180.
8. Suzuki, O, (1985) Hakko to Kogyo 43, 1024-1031.
9. Yamada, H., Shimizu, S., and Shinmen, Y. (1987) Agric. Biol. Chem. 51, 785-790.
10. Kates, M., and Kuksis, A. (eds.) (1980) Membrane Fluidity, Humana Press, New Jersey.
11. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
12. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
13. Fulco, A.J. (1974) Ann. Rev. Biochem. 43, 215-241.