Microbial Conversion of an Oil Containing α-Linolenic Acid to an Oil Containing Eicosapentaenoic Acid

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Mycelia of arachidonic acid-producing fungi belonging to the genus *Mortierella* were found to convert an oil containing α-linolenic acid to an oil containing 5,8,11,14,17-*cis*-eicosapentaenoic acid (EPA). This conversion was observed when they were grown in a medium containing the oil, glucose and yeast extract at 28 C. On the screening of various oils, linseed oil, in which α-linolenic acid amounts to about 60% of the total fatty acids, was found to be the most suitable for EPA production. Under the optimal culture conditions, a selected strain, *Mortierella alpina* 20-17, converted 5.1% of the α-linolenic acid in the added oil into EPA, the EPA production reaching 1.35 g/l of culture broth (41.5 mg/g dry mycelia). This value corresponded to 7.1% (by weight) of the total fatty acids in the extracted lipids. The lipid was also found to be rich in arachidonic acid (12.3%). Other major fatty acids in the lipid were palmitic acid (4.4%), stearic acid (3.2%), oleic acid (13.5%), linoleic acid (13.7%), α-linolenic acid (38.5%) and γ-linolenic acid (0.9%).

5,8,11,14,17-*cis*-Eicosapentaenoic acid (EPA) is a C-20 polyunsaturated fatty acid (PUFA) of potential pharmaceutical value. It has been shown to be effective in preventing or curing thrombosis (1-3).

EPA occurs as a component of cellular lipids in protozoal, algal, bacterial and animal cells (4-7). Several marine fish oils have become available recently as sources of EPA, but are not satisfactory for practical purposes because of their low EPA contents, variability of the EPA content between catches and the presence of other fatty acids with less desirable properties.

In recent studies, we have found that arachidonic acid-producing fungi belonging to the genus *Mortierella* accumulate EPA in their mycelia when grown in conventional media containing glucose as the major carbon source at low temperature (6-20 C), and that the resultant mycelia are rich in EPA and arachidonic acid (8-13). We have also suggested that this production may be due to activation of the enzyme(s) involved in EPA formation, probably in the methyl-end directed desaturation of arachidonic acid to EPA, at low temperature (11) (Fig. 1).

In a mammalian system, on the other hand, it has been demonstrated that dietary α-linolenic acid is converted to EPA through the n-3 route, as shown in Figure 1 (14). If the same route occurs in the *Mortierella* fungi, it may be a very promising route for the practical production of EPA, because there are various kinds of natural oils containing α-linolenic acid that are easily available, and these oils may be expected to be converted to EPA-containing oils on incubation with these fungi. The present study was carried out to examine the potential of such natural oils as precursors of EPA. The data presented here show that several arachidonic acid-producing *Mortierella* fungi effectively convert α-linolenic acid-containing oils, such as linseed oil, with the accumulation of EPA-containing oils in the mycelia.

MATERIALS AND METHODS

Chemicals. Linseed oil was purchased from Wako Pure Chemicals, Osaka, Japan. The oil contained palmitic acid (10.0%, by weight), stearic acid (3.7%), oleic acid (10.7%), linoleic acid (17.5%) and α-linolenic acid (58.1%). Fungal oil was prepared from mycelia of *Mortierella alpina* 1S-4 as described (11). Perilla oil, fish oil and the other oils listed in Table 2 were obtained from Yamakei Sangyo, Osaka, Japan, Toyo Jozo Co., Tokyo, Japan, and Sigma Chemical Co., St. Louis, Missouri. The fatty acid compositions, by weight, of these oils are given in the footnote to Table 2. Methyl α-linolenate was purchased from Funakoshi Chemicals, Tokyo, Japan. All other chemicals used in this work were as described previously (12).

Microorganisms, media and cultivations. All fungal strains used were from our stock cultures (AKU Culture Collection, Faculty of Agriculture, Kyoto University). Each fungus was inoculated into a 50-ml shaking flask containing 10 ml of medium GY (13) containing 1% oil, followed by incubation at 28 C for six days with reciprocal shaking (120 strokes/min), unless otherwise stated.

Fatty acid analysis and other methods. Fungal mycelia were harvested by suction filtration, washed with 50 ml of ether acidified with 0.5 ml of 2 N HCl and then with 50 ml of water. The filtered mycelia were dried at

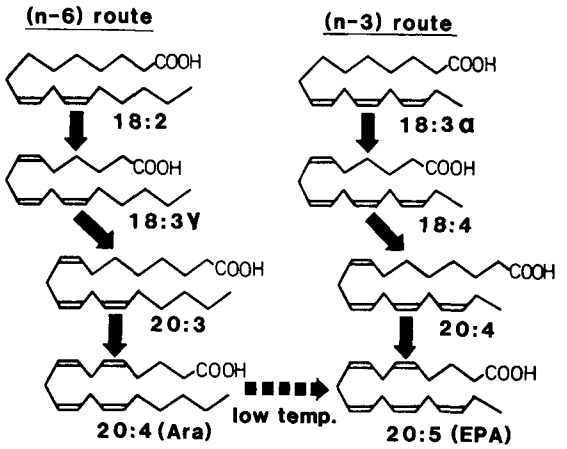

FIG. 1. Pathways for the biosynthesis of arachidonic acid and EPA. 18:2, linoleic acid; 18:3α, α-linolenic acid; 18:3γ, γ-linolenic acid; 18:4, 6,9,12,15-octadecatetraenoic acid; 20:3, dihomo-γ-linolenic acid; 20:4, 8,11,14,17-eicosatetraenoic acid; 20:4 (Ara), arachidonic acid; 20:5 (EPA), eicosapentaenoic acid.

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TABLE 1

EPA Production by *M. alpina* 20-17 on Incubation on a Glucose-Yeast Extract Medium with α-Linolenate and/or Olive Oil at 28 C[a]

Supplement	Mycelial yield (mg)[b]	Total FA[c] (mg)[b]	EPA content (mg/g dry mycelia)	EPA yield (μg)[d]
Exp. I				
None	3.5	0.19	0.	0.
Methyl-α-linolenate (0.5%)	5.1	0.53	1.8	8.9
Olive oil (1.5%)	7.0	2.28	0.	0.
Olive oil (1.5%) + methyl-α-linolenate (0.2%)	10.3	3.20	1.2	12.4
Olive oil (1.5%) + methyl-α-linolenate (0.5%)	10.5	3.47	3.4	35.3
Olive oil (1.5%) + methyl-α-linolenate (1.0%)	15.1	4.75	3.5	52.3
Exp. II				
None	10.4	1.52	0.	0.
Olive oil (1.5%)	16.4	4.31	0.	0.
Olive oil (1.5%) + methyl-α-linolenate (0.2%)	19.0	5.09	3.3	63.1

[a]In Exp. I, *M. alpina* 20-17 was grown on 2% agar plates containing medium GY (2% glucose and 1% yeast extract, pH 6.0) and the supplements indicated for 15 days. In Exp. II, the organism was grown in medium GY with the supplements indicated for 9 days with shaking.
[b]Values are given in mg obtained from 1 g (Exp. I) or 1 ml (Exp. II) of culture broth.
[c]FA, fatty acid.
[d]Values are given in μg found in 1 g (Exp. I) or 1 ml (Exp. II) of culture broth.

TABLE 2

EPA Production by *M. alpina* 20-17 in the Presence of Several Natural Oils[a]

Oil added[b]	Productivity: Mycelial mass (mg/ml of culture broth)	Productivity: EPA (mg/ml of culture broth)	Productivity: Ara[d] (mg/ml of culture broth)	Fatty acid composition (%)[c]: 16:0	18:0	18:1	18:2	18:3α	18:3γ	20:3	20:4	20:5	others
Linseed	15.9	0.25	1.63	6.1	3.3	9.2	9.4	27.8	2.1	3.0	33.8	5.1	0.2
Soybean	19.7	0.02	1.32	8.4	3.8	21.0	40.8	5.3	2.8	2.4	14.8	0.3	0.4
Perilla	15.5	0.18	1.32	8.9	3.6	14.2	11.0	26.6	2.0	2.1	27.6	3.8	2.2
Olive	17.0	0.	1.36	9.5	3.1	49.3	14.2	0.	3.0	3.0	16.2	0.	1.7
Evening primrose	15.3	0.	1.36	9.3	2.4	10.1	45.6	0.	4.9	5.0	21.4	0.	1.3
Peanut	18.1	0.	1.00	8.1	5.1	35.1	27.7	0.	3.8	5.2	12.4	0.	2.6
Mortierella	19.2	0.	2.14	12.8	6.1	37.7	10.6	0.	3.3	5.2	22.7	0.	1.6
Fish	14.4	0.26	0.91	16.0	3.2	20.6	8.1	tr[e]	5.4	tr	27.3	7.9	11.5
None	10.5	0.	0.63	15.9	8.1	21.0	14.3	0.	5.5	4.7	28.4	0.	2.1

[a]*M. alpina* 20-17 was grown in medium GY under the conditions in Materials and Methods with the addition of each oil (1.0%), as indicated.
[b]The fatty acid compositions of the oils, in wt %, were as follows: soybean oil, 16:0, 10.0; 18:0, 3.7; 18:1, 23.1; 18:2, 53.8; 18:3, 8.8; others, 0.6; perilla oil, 16:0, 7.1; 18:0, 2.0; 18:1, 14.1; 18:2, 14.5; 18:3α, 62.3; olive oil, 16:0, 9.9; 18:0 + 18:1, 82.2; 18:2, 6.4; 18:3α, 1.6; evening primrose oil, 16:0, 6.7; 18:0, 1.9, 18:1, 10.2; 18:2, 68.9; 18:3γ, 11.3; others, 1.0; peanut oil, 16:0, 14.4; 18:0, 2.5; 18:1, 50.8; 18:2, 32.3; *Mortierella* oil, 16:0, 19.4; 18:0, 5.3, 18:1, 42.8; 18:2, 9.3; 18:3γ, 2.5; 20:3, 2.6; 20:4, 15.4; others, 2.7; fish oil, 16:0, 13.8; 16:1, 8.5; 18:0, 2.5; 18:1, 16.8; 18:2, 1.9; 20:5, 16.1; 22:6, 20.0; others, 20.4. For linseed oil, see the text.
[c]16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3α, α-linolenic acid; 18:3γ, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 20:5, EPA.
[d]Ara, arachidonic acid.
[e]tr, trace.

100 C overnight. Transmethylation of the sample in methylene chloride-10% methanolic HCl and then extraction of the fatty acid methyl esters with *n*-hexane were carried out with *n*-heptadecanoic acid as an internal standard as described previously (12,13). The resultant esters were analyzed by gas liquid chromatography (GLC) (12,13). The analysis well resolved all PUFAs of the n-3 and n-6 families. Mycelial fatty acid composition values are given in weight %. Each PUFA of the n-3 family found in mycelia was usually less than 1% of total fatty acids except for α-linolenic acid and EPA. Compositions of these minor PUFAs are summed up and given as "others" in each table.

Productivity of EPA usually was represented by using two indexes, mg of EPA/g of dry mycelia and mg of EPA/ml of culture broth, which, respectively, indicate mycelial content of EPA and total EPA produced in one ml of culture broth.

The methods used for fungal growth measurement and mass and ^1H NMR spectral analyses were described

TABLE 3

Comparison of EPA Productivities and Mycelial Fatty Acid Compositions in *Mortierella* Fungi Grown with Linseed Oil[a]

	Productivity			Fatty acid composition (%)[b]									
	Mycelial mass (mg/ml of culture broth)	EPA	Ara[b]	16:0	18:0	18:1	18:2	18:3α	18:3γ	20:3	20:4	20:5	others
M. alpina 20-17 AKU 3996	15.1	0.24	1.12	10.6	4.9	11.2	13.0	22.3	2.8	3.0	22.9	4.9	4.4
M. alpina 1S-4 AKU 3998	29.2	0.11	0.82	9.0	3.8	19.7	16.9	42.0	tr[c]	1.2	6.1	0.8	0.5
M. alpina 1-83 AKU 3995	26.8	0.14	0.71	12.4	4.6	23.9	14.5	30.9	1.2	1.6	8.2	1.7	1.0
M. alpina CBS 250.53	10.3	0.01	0.21	10.5	5.1	23.2	15.2	35.0	2.1	1.5	6.8	0.3	0.3
M. elongata 1S-5 AKU 3999	14.5	0.05	0.33	10.4	6.9	21.7	12.5	37.2	2.2	1.9	5.6	0.9	0.7
M. elongata IFO 8570	13.2	0.05	0.34	8.4	6.5	20.5	13.5	38.5	2.0	1.5	7.5	1.2	0.4
M. elongata NRRL 5513	14.9	0.33	0.28	11.1	6.4	23.3	13.6	35.3	2.2	1.3	5.5	0.6	0.7
M. parvispora AKU 3994	11.0	0.02	0.21	7.2	5.1	23.7	14.0	40.6	1.8	1.2	5.3	0.4	0.7
M. beljakovae CBS 601.68	14.7	0.01	0.11	7.7	3.8	19.0	17.2	48.4	tr	0.7	2.3	0.2	0.7
M. epigama CBS 489.70	13.5	0.01	0.10	11.0	5.6	23.0	14.8	38.2	1.7	0.8	3.8	0.3	0.8
M. verticillate IFO 8575	12.9	0.02	0.22	12.4	5.7	25.1	13.5	31.6	2.6	1.7	5.7	0.6	1.1
M. hygrophila IFO 5941	8.7	0.02	0.15	13.0	6.1	25.6	12.1	30.1	3.3	2.0	6.2	0.8	0.8
M. kuhlmanii CBS 157.71	15.5	0.03	0.28	11.4	7.5	20.9	13.3	37.8	1.3	1.3	4.8	0.5	1.2
M. zychae CBS316.52	14.9	0.03	0.22	9.1	5.1	19.1	16.0	45.5	0.7	0.8	3.0	0.4	0.3
M. bainieri IFO 8569	8.9	0.03	0.18	14.9	6.9	24.6	11.5	27.2	2.7	2.8	7.8	1.2	0.4
M. minutissima IFO 8573	13.0	0.01	0.15	12.0	6.4	22.8	13.8	30.9	2.9	1.7	4.7	0.4	4.4
M. bisporalis NRRL 2493	5.5	0.02	0.05	7.3	2.8	16.7	18.4	47.3	1.6	0.5	3.0	1.0	1.4
M. schmuckeri NRRL 2761	14.4	0.01	0.23	13.3	8.1	27.3	12.7	29.5	1.9	1.9	4.4	0.2	0.7
M. hyalina NRRL 6427	11.5	0.03	0.19	11.5	7.0	23.1	13.4	35.5	2.3	1.4	4.5	0.7	0.6
M. sp. NRRL 1458	9.8	0.01	0.12	14.3	6.5	26.1	13.1	28.6	3.6	1.5	5.8	0.3	0.2

[a]Each strain was grown under the conditions in Materials and Methods.
[b]Abbreviations for fatty acids are given in the footnotes to Table 2.
[c]tr, trace.

previously (12). Glucose concentrations were determined with a commercially available kit (Blood Sugar-GOD-Perid-Test, Boehringer, Mannheim, Germany), essentially according to the method of Werner et al. (15).

RESULTS

EPA production by M. alpina *20-17 during growth on medium supplemented with α-linolenic acid.* As described previously, most *Mortierella* strains capable of producing arachidonic acid produce EPA when grown at low temperature (6-16 C), but not at 28 C (8-12). Because these fungi cannot produce α-linolenic acid (11-13), we examined whether or not added α-linolenate is converted to EPA at this temperature. Under all the tested conditions when α-linolenate was present, detectable amounts of EPA were found in the mycelia of *M. alpina* 20-17, suggesting that the addition of α-linolenate is indispensable for EPA production at 28 C (Table 1).

Screening of suitable oils as starting substrates for EPA production. Because α-linolenic acid itself is a rather expensive reagent as a practical precursor of EPA, we examined several easily available natural oils as sources of α-linolenic acid. The results in Table 2 show that *M. alpina* 20-17 accumulated a detectable amount of EPA in its mycelia on incubation with linseed oil, perilla oil or soybean oil, all of which contain α-linolenic acid as a major fatty acid. EPA was also found in the mycelia grown with fish oil which contains EPA as a major fatty acid. However, the amount found after the cultivation was only about 50% of that initially added to the medium. EPA was not detected in any myelia obtained with the other oils tested, which contain no or only very small amounts of α-linolenic acid (less than 3% of the total fatty acids). On the other hand, high amounts of arachidonic acid were found after the cultivation, regardless of the type of oil. These results again indicate that the presence of α-linolenic acid can result in EPA production. The α-linolenic acid probably was converted to EPA during the growth of the fungi. The highest accumulation of EPA was found in the mycelia obtained with linseed oil. The EPA content reached 15.7 mg/g dry mycelia (0.25 mg/ml). This value corresponded to a molar conversion ratio of added α-linolenic acid to EPA of about 4%.

Selection of Mortierella *strains showing high EPA productivity.* Arachiodinic acid-producing *Mortierella* fungi (20 strains) obtained in the previous studies were compared as to their EPA productivity in medium GY supplemented with 1% linseed oil at 28 C. All were confirmed not to produce EPA at 28 C in the unsupplemented medium, although they produce it at a low growth temperature (6-16 C) (11,12,16). The data in Table 3 show that all the tested strains produced detectable amounts of EPA on incubation with linseed oil at 28 C. In particular, several *Mortierella alpina* strains were found to accumulate EPA at more than 3.0 mg/g dry mycelia. These *M. alpina* strains were also excellent at production of arachidonic acid in the medium either with or without linseed oil [for the data without linseed oil, see (12) and (13)]. On the other hand, some *Mortierella* strains (21 strains) only produced fatty acids with 18 or less carbon atoms (i.e., palmitic acid, stearic acid, oleic acid, linoleic acid and γ-linolenic acid), that is, not C-20 PUFAs such as dihomo-γ-linolenic acid or arachidonic acid, on incubation with linseed oil (data not shown). Through this test, we selected three strains of *M. alpina* (i.e., 20-17, 1S-4 and 1-83) for the following studies, on the basis of their high EPA productivities at 28 C.

Factors affecting EPA production. (i) Time course

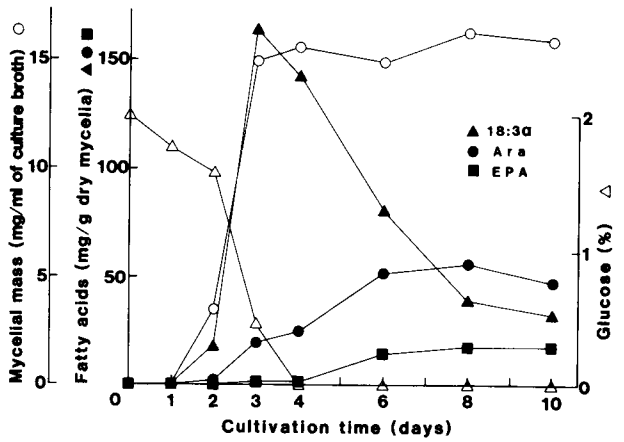

FIG. 2. Effect of the culture time on the production of EPA. *M. alpina* 20-17 was grown under the conditions in Materials and Methods except for the cultivation time as indicated. 18:3α, α-linolenic acid; Ara, arachidonic acid.

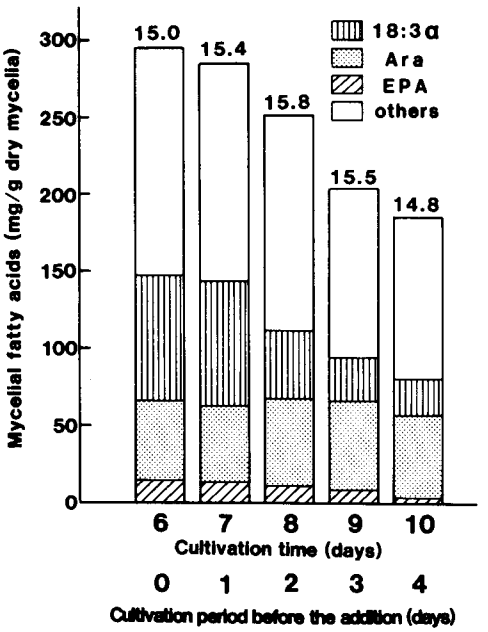

FIG. 3. Effect of the time of linseed oil addition on the production of EPA. *M. alpina* 20-17 was grown under the conditions in Meterials and Methods except for the time of addition of the oil (1%) and the cultivation time, as indicated. The values given on the top of each bar indicate mycelial mass (mg/ml) after cultivation. 18:3α, α-linolenic acid; Ara, arachidonic acid.

of EPA formation. First, the changes in the concentrations of glucose and α-linolenic acid in the linseed oil in the growth medium, and those in the mycelial contents of α-linolenic acid, arachidonic acid and EPA during the growth of *M. alpina* 20-17 in medium GY supplemented with 1% linseed oil were monitored (Fig. 2). The α-linolenic acid and other fatty acids in the linseed oil in the culture filtrate were undetectable when analyzed on the third day of cultivation, suggesting that they were almost completely consumed by the fungus during the first three days. During the same period, 75% of the added glucose was consumed, the mycelial yield being 15.0 mg/ml of culture broth. Eighty percent of the α-linolenic acid was found in the mycelia at this point. But the EPA content of the mycelia was only 1.6 mg/g dry mycelia. This value corresponds to only 0.5% of the total extracted fatty acids. Other

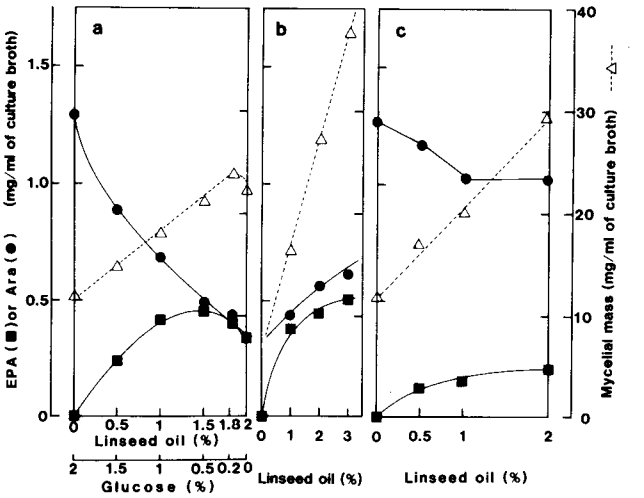

FIG. 4. Effect of the linseed oil concentration on the production of EPA. *M. alpina* 20-17 was grown for 6 days at 28 C. The concentrations of glucose and linseed oil in the medium were varied as shown. In b and c, initial glucose concentrations were 0.5% and 2.0%, respectively. In all cases, the medium contained 1% yeast extract. Other conditions are given in Materials and Methods. Ara, arachidonic acid.

major fatty acids found in the mycelia were palmitic acid (9.9%), stearic acid (5.7%), oleic acid (18.1%), linoleic acid (15.1%), α-linolenic acid (42.9%) and arachidonic acid (5.2%). After eight days, mycelia containing 17.9 mg EPA/g dry mycelia were obtained, the mycelial yield being 16.1 mg/ml of culture broth. The predominant fatty acids in the extracted lipids were as follows: palmitic acid (10.9%), stearic acid (5.7%), oleic acid (12.8%), linoleic acid (11.5%), α-linolenic acid (17.5%), arachidonic acid (25.1%) and EPA (7.5%).

(ii) Time of the addition of linseed oil. The mycelial EPA content varied markedly, depending on the growth phase at which the oil was added. The data in Figure 3 indicate that addition at an early phase (i.e., 0 to 3 days after inoculation) is effective for high EPA production. The level of mycelial α-linolenic acid was only 23.4 mg/g dry mycelia, and about 30% of the added α-linolenic acid remained in the culture filtrate when the oil was added on the fourth day of cultivation and analysis was performed after the mycelia had been cultivated for a further six days. These results suggest that the low EPA productivity on delayed addition of linseed oil is due to the low efficiency of the mycelia to incorporate the added oil.

Relationship between the linseed oil and glucose concentrations. The EPA production at various concentrations of linseed oil was compared, as shown in Figure 4. The presence of glucose in the medium is not essential for EPA production, because *M. alpina* 20-17 used the added linseed oil as either a carbon source for growth or a precursor of EPA, and produced enough mycelia rich in EPA (Fig. 4a). The amount of EPA/ml of culture broth increased with increasing linseed oil concentration (Fig. 4b and c). Under the conditions of 3% linseed oil, 0.5% glucose and 1% yeast extract, the amount of EPA accumulated reached 0.52 mg/ml of culture broth. It should be noted that a high concentration of glucose (2.0%) repressed the EPA production; the amount of EPA accumulated with 2% linseed oil

TABLE 4

Changes in Mycelial EPA Content during Aging of Harvested Mycelia[a]

Strain	Mycelial mass (mg/ml of culture broth)	PUFA content EPA (mg/g dry mycelia)	PUFA content Ara[b] (mg/g dry mycelia)	Fatty acid composition (%)[b] 16:0	18:0	18:1	18:2	18:3α	18:3γ	20:3	20:4	20:5	others
M. alpina 20-17													
Condition I													
Before aging	32.8	26.5	49.6	4.7	3.8	13.9	14.8	45.5	1.0	1.1	8.0	4.3	2.9
After aging	32.4[c]	41.5	71.5	4.4	3.2	13.5	13.7	38.5	0.9	1.4	12.3	7.1	5.0
Condition II													
Before aging	33.0	25.3	50.8	4.5	3.8	14.2	15.1	45.0	1.3	1.2	7.5	3.7	3.7
After aging	32.1	30.5	60.9	7.1	2.4	13.8	14.7	37.1	1.6	1.5	11.0	5.5	5.3
M. alpina 1S-4													
Condition I													
Before aging	21.8	15.8	33.3	7.7	3.6	14.9	14.3	44.7	1.3	1.3	6.7	3.2	2.3
After aging	23.1[c]	36.3	56.8	6.8	3.1	13.7	13.0	35.0	1.5	1.6	13.3	8.5	3.5
Condition II													
Before aging	22.5	16.3	30.0	7.6	3.3	14.9	14.8	45.4	1.0	1.0	7.0	3.8	1.4
After aging	23.4	20.1	39.3	6.8	2.3	13.4	13.8	37.2	1.3	1.4	12.9	6.6	4.3
M. alpina 1-83													
Condition I													
Before aging	21.2	23.4	57.5	7.1	5.0	13.4	12.5	35.9	1.4	2.6	14.0	5.4	2.7
After aging	22.2[c]	39.9	78.4	5.0	3.6	12.9	12.3	31.6	1.3	2.1	18.5	9.4	3.3
Condition II													
Before aging	22.8	20.4	61.0	7.5	3.5	15.0	15.0	41.7	1.2	1.4	10.0	3.3	1.4
After aging	22.3	25.3	70.1	7.0	2.7	14.7	14.4	37.5	1.0	1.4	11.9	4.1	5.3

[a]Each strain was cultivated for 6 days under the conditions in Materials and Methods except for the glucose concentration (4%) in the case of *M. alpina* 20-17. In Condition I the mycelia, after removal of the medium by suction filtration, were divided into two portions, one of which was analyzed for fatty acids and the other allowed to stand for a further 7 days at 28 C to age before being analyzed. In Condition II, the mycelia after removal of the medium were divided into two portions of equal weight, one of which was analyzed for fatty acids and the other incubated in 5 ml of culture filtrate for a further 7 days at 28 C. All operations were carried out under sterile conditions.

[b]Abbreviations for fatty acids are given in the footnotes to Table 2.

[c]Mycelial weight corresponding to that obtained from 1 ml of culture broth.

was only 0.21 mg/ml of culture medium. This value is about one-half of that attained under the conditions of a low glucose concentration (0.5%) with the same linseed oil concentration. On the other hand, arachidonic acid production was markedly enhanced with a high glucose concentration; for example, the amount of arachidonic acid (1.03 mg/ml) obtained under the conditions of 2% glucose and 2% linseed oil was about two-fold higher than that obtained under the conditions of 0.5% glucose and 2% linseed oil (Fig. 4b and c).

(iv) Growth temperature. The EPA production and mycelial yield in medium GY containing 1% linseed oil with various growth temperatures were investigated. The maximum EPA production (0.22 mg/ml) by *M. alpina* 20-17 was attained at 28 C on six days cultivation. This was suggested to be due mainly to the rapid growth of the fungus at this temperature (mycelial mass, 16.2 mg/ml), because the mycelial EPA content did not vary significantly at any temperature tested. To obtain the same mycelial yield, a further three or two days was required at 20 or 30 C, respectively (data not shown). At 35 C, the fungus could not grow under the conditions tested.

Increase in mycelial EPA content during aging of the harvested mycelia. The data in Table 4 show that EPA in the harvested mycelia can be specifically enriched when the mycelia are allowed to stand for a further few days. This phenomenon was observed for every *M. alpina* strain tested. The EPA content of the mycelia of *M. alpina* 20-17 which were collected on the sixth day of cultivation and then allowed to stand for a further seven days at 28 C reached 41.5 mg/g dry mycelia; this value was about 1.6 times higher than that determined at the initiation of this aging. Because no significant change in mycelial weight was observed on the aging, the corresponding value, in mg/ml of culture broth, was calculated to be 1.35, suggesting that 0.48 mg of EPA was newly produced by the mycelia obtained from one ml of culture medium. A similar increase was observed in arachidonic acid. As a result, EPA and arachidonic acid comprised 7.1 and 12.3% of the total mycelial fatty acids, respectively. On continuing cultivation for the same period in the liquid medium without shaking, the increase in the EPA content was only 20-30% of the content before the aging (Table 4).

The EPA methyl ester (16 mg) was isolated from the lipids extracted from 20 g of wet mycelia of *M. alpina* 20-17, which was grown for six days under the conditions given in Table 4 and aged for a further seven days, according to essentially the same procedure as described previously (13). The mass and ^1H NMR spectra of the isolated methyl ester corresponded well to those previously reported (12).

DISCUSSION

We have reported that most arachidonic acid-produc-

ing *Mortierella* fungi can produce EPA when grown at low temperature (6-16 C) (8-12). The n-6 route to arachidonic acid from a common C-18 fatty acid, linoleic acid, followed by the methyl-end directed desaturation of the arachidonic acid has been suggested for the route to EPA (11). The present finding that mycelia of the same fungi can produce EPA on incubation with α-linolenic acid, added as the methyl ester or an oil, at high temperature (20-30 C) suggested that there is also a temperature-independent route to EPA through which the added α-linolenic acid is converted to EPA, in these fungi. The most probable route for this EPA production is the n-3 route, which involves the following three successive reactions; Δ6-desaturation of α-linolenic acid to octadecatetraenoic acid (18:4 n-3), elongation to eicosatetraenoic acid (20:4 n-3) and Δ5-desaturation of the eicosatetraenoic acid to EPA, as shown in Figure 1. It has been reported that both the n-6 and n-3 routes share enzymes concerned in Δ6-desaturation, elongation and Δ5-desaturation in a mammalian system (17). If the same mechanism occurs in the *Mortierella* fungi reported here, the added α-linolenic acid, in place of linoleic acid, could be desaturated and converted to EPA through the n-3 route. The findings reported here that only the *Mortierella* strains producing arachidonic acid can produce EPA on incubation with linseed oil containing α-linolenic acid and that the strains which cannot produce C-20 PUFAs of the n-6 route, i.e., dihomo-γ-linolenic acid and arachidonic acid, cannot produce EPA under the same conditions may be consistent with the above assumption. The results shown in Figure 4, i.e., the elevation of arachidonic acid production with repressed production of EPA on incubation with a high concentration of glucose and the increased production of EPA with repressed production of arachidonic acid on incubation with a high concentration of α-linolenic acid, may be explained by this sharing of enzymes by the two routes. Under the conditions used here, linoleic acid is thought to be produced mainly from glucose, while α-linolenic acid is derived soley from the added linseed oil. Therefore, a high concentration of glucose is probably effective in increasing only the linoleic acid content in the mycelia. As a result, these two fatty acids would compete with each other as the substrate for the enzyme catalyzing Δ6-desaturation.

The ability of the *Mortierella* fungi to convert added α-linolenic acid to EPA is very promising from a biotechnological viewpoint because there are various kinds of easily available natural oils containing α-linolenic acid, and it is expected that they can be converted to oils rich in EPA on incubation with these fungi. Indeed, the incubation of linseed oil with *M. alpina* 20-17 resulted in the production of a novel oil rich in EPA and arachidonic acid. The EPA production obtained here (1.35 mg/ml) is 2.8-fold higher than that obtained under low temperature growth conditions. Another advantage of the present EPA production is that it can be carried out under normal growth temperature conditions (20-30 C). Under such conditions, the fungal growth is rapid and dense, and the energy costs for temperature control may be less than those for cooling.

Increase in mycelial contents of EPA and arachidonic acid by aging of the harvested mycelia as shown in Table 4 indicates that these PUFAs were still synthesized actively after cultivation in the liquid medium had stopped. Because an increase in PUFAs on continuing cultivation in the liquid medium with or without shaking was not so prominent, some environmental change which causes enhancement of PUFA production might be induced by this treatment. For practical purposes, this aging is very useful to enrich EPA content of harvested mycelia.

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