APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1993, p. 4300–4304
0099-2240/93/124300-05$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 59, No. 12

A Novel Δ5-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4 and Its Dihomo-γ-Linolenic Acid Productivity

SAEREE JAREONKITMONGKOL, EIJI SAKURADANI, AND SAKAYU SHIMIZU*

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Received 6 July 1993/Accepted 27 September 1993

A novel Δ5-desaturase-defective mutant was derived from an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4, after treating the parental spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The mutant produced only a trace (about 1%) amount of arachidonic acid, and the ratio of dihomo-γ-linolenic acid (DGLA) to total fatty acids in each lipid class was markedly high, accounting for as much as 60% in phosphatidylcholine. Under submerged batch culture conditions, the mutant produced 2.4 g of DGLA per liter (43.3% of total fatty acids) when grown at 28°C for 7 days in a 5-liter jar fermentor. The other major (more that 1%) fatty acids were palmitic acid (21.2%), stearic acid (9.6%), oleic acid (14.3%), linoleic acid (4.4%), and γ-linolenic acid (5.8%). About 80 mol% of the DGLA produced was found in triacylglycerol.

Dihomo-γ-linolenic acid [8(*Z*),11(*Z*),14(*Z*)-eicosatrienoic acid] (DGLA), a C_{20}-polyunsaturated fatty acid, is a precursor of physiologically active eicosanoids, including group 1 prostaglandins and thromboxanes. It has attracted a great deal of interest because of its unique biological activities. For example, it can be used in combination with interferons for treatment of virus infections, cancer, and inflammatory diseases (5) and in combination with prostaglandin E_1 for treatment of atopy of the skin and mucosa (11). A small amount of DGLA has been detected as a component of cellular lipids in fungi (15), algae (4), protozoa (6), and animals (10). However, no promising natural source of it was known until our previous work, in which DGLA was produced by growing a soil isolate fungus, *Mortierella alpina* 1S-4, in the presence of an acetone extract of sesame oil (12). The acetone extract of sesame oil was later found to consist of sesamin and some lignan compounds which specifically inhibit the conversion of DGLA to arachidonic acid (AA) (13).

Recently, we reported the production of DGLA by a Δ5-desaturase-defective mutant of *M. alpina* 1S-4. This mutant produced a little more DGLA than on cultivation with inhibitors (7), but the AA concentration in the oil produced was still relatively high. From the standpoint of physiological function, AA is converted to group 2 prostaglandins, leukotrienes, and other eicosanoids, which are considered to be involved in the manifestation of various diseases (9). Thus, we have attempted to eliminate AA completely from the oil.

In this report, we describe the successful derivation of a novel mutant which has almost completely lost Δ5-desaturase activity. The mutant is different from that obtained previously (7) with respect to the content of AA in the mycelia and the fatty acid composition of each lipid class.

MATERIALS AND METHODS

Chemicals. All of the chemicals used are commercially available and were described previously (8).

Microorganism and cultivation. *M. alpina* 1S-4 Mut44 (7) is a mutant defective in Δ5-desaturation derived from *M. alpina* 1S-4 (15, 17). Spores were inoculated into a 10-ml Erlenmeyer flask containing 2 ml of medium GY (2% glucose and 1% yeast extract, pH 6.0) and then incubated with reciprocal shaking (120 strokes per min) at 28°C for 1 week, unless otherwise noted.

Mutagenesis and isolation of a Δ5-desaturase-defective mutant. Mutagenesis and selection of mutants were performed essentially as described previously (8). In brief, *M. alpina* 1S-4 spores were exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and then selection of mutants was performed by analyzing the fatty acids of vegetative mycelia after the growth of these *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated spores for a few days. Mutants with fatty acid compositions different from that of the wild type were picked up and stored on potato dextrose agar medium.

Analysis of fatty acids and lipids. Fungal cells were harvested by suction filtration and then dried at 100°C overnight. The drying process did not result in remarkable oxidation of mycelial fatty acids, as the fatty acid amounts obtained were comparable to those obtained on lyophilization of the fungal cells. The dried cells were directly transmethylated with 10% methanolic HCl, and the resultant fatty

TABLE 1. Comparison of the fatty acid compositions of fatty acid desaturation-defective mutants of *M. alpina* 1S-4[a]

Fatty acid	Fatty acid composition (wt %)		
	Wild type	Mut44[b]	S14[c]
16:0	11.1	10.8	16.9
18:0	5.3	7.8	7.5
18:1ω9	13.7	11.1	10.7
18:2ω6	7.4	5.6	2.9
18:3ω6/20:0[d]	4.6	9.6	6.7
20:1ω9	1.1	1.1	0.6
20:2ω6	0.6	1.0	0.5
DGLA/22:0[d]	6.5	32.8	48.8
AA	44.4	11.7	0.9
24:0	5.2	8.5	4.2

[a] All strains were grown in medium GY at 28°C for 7 days.
[b] Mutant defective in Δ5-desaturation as described previously (7).
[c] First described in this report.
[d] Both were found in all strains. The first fatty acid is the major one (more than 95%).

* Corresponding author.

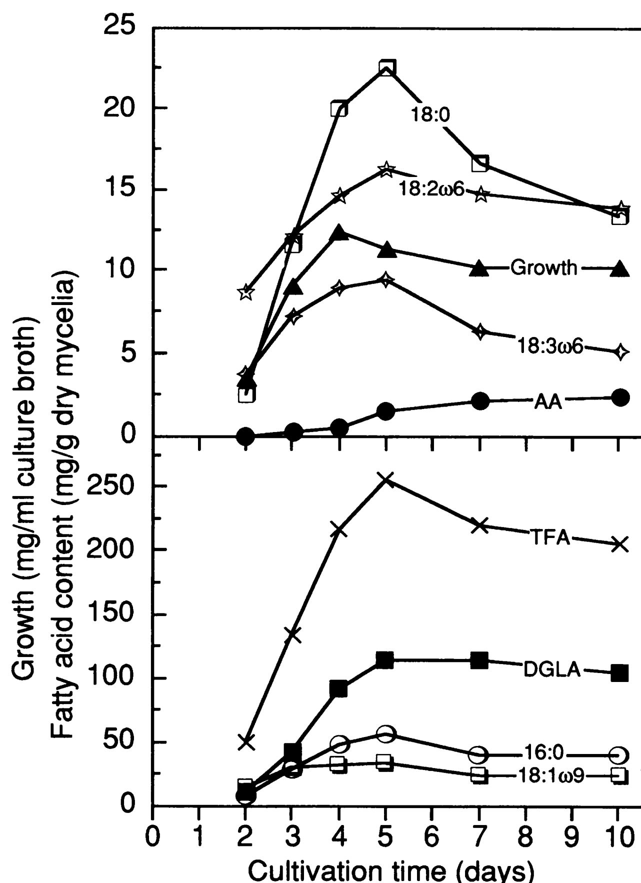

FIG. 1. Time courses of changes in the contents of mycelial fatty acids in *M. alpina* 1S-4 S14. The fungus was grown in medium GY at 28°C for the times indicated. TFA, total fatty acids. A small amount of 22:0 (less than 5%) was included in DGLA.

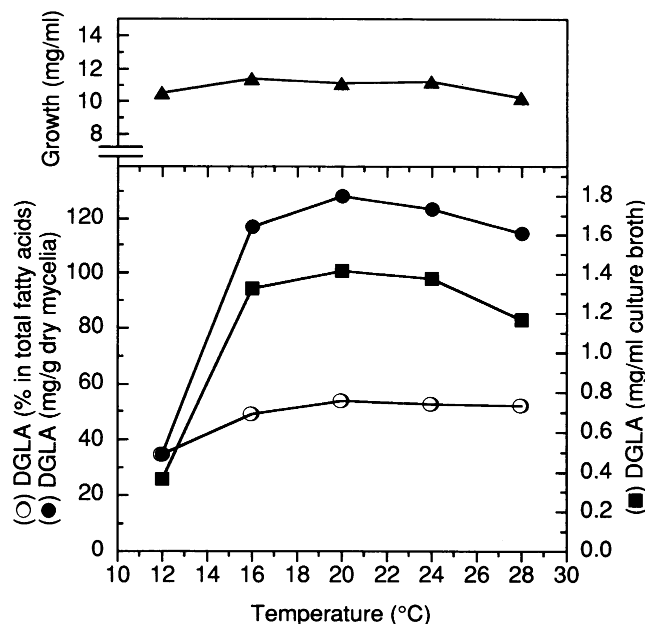

FIG. 2. Effect of growth temperature on DGLA production by *M. alpina* 1S-4 S14. The fungus was grown at 28°C for 1 day before being shifted to the temperatures indicated and grown for a further 6 days.

acid methyl esters were extracted with *n*-hexane and then analyzed by gas-liquid chromatography. A glass column (3 mm by 3 m) packed with Advanced DS (Nishio Kogyo, Tokyo, Japan) was used. The gas-liquid chromatograph was operated at a constant temperature of 195°C, and the other conditions for apparatuses and carrier gas were as given in detail previously (14). Fungal lipids were extracted with $CHCl_3$-CH_3OH (2:1, vol/vol) as described by Folch et al. (3). The lipids were separated into individual lipid classes by thin-layer chromatography, and the fatty acid composition of each lipid class was analyzed by gas-liquid chromatography as described previously (14). Lipid compositions were calculated from the amounts of total fatty acids of individual lipid classes as recommended by Christie et al. (2). Heptadecanoate was usually added as an internal standard before transmethylation.

Determination of the positional distribution of fatty acids in phospholipids. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were isolated from the total lipids by thin-layer chromatography as described above, extracted with $CHCl_3$-CH_3OH (2:1, vol/vol), and then concentrated with a rotary evaporator. The extracted PC and PE were hydrolyzed with snake (*Ophiophagus hannah*) venom which contains phospholipase A_2, and free fatty acids liberated from the *sn*-2 position of phospholipids were isolated by thin-layer chromatography and analyzed for fatty acid composition by gas-liquid chromatography (1).

Other methods. DGLA accumulated in mutant strain S14 was isolated by high-performance liquid chromatography and identified by comparing its mass and proton nuclear magnetic resonance spectra with those of an authentic standard. The analytical data (not shown) were the same as those reported previously (7). Fungal growth was measured by determining the mycelial weight after drying at 100°C overnight. The glucose concentration of the culture medium was measured with a commercial kit (Blood Sugar-GOD-Period-Test, Boehringer GmbH, Mannheim, Germany), based on glucose oxidase essentially as described by Werner et al. (16). All of the values shown in the figures and tables are means of two independent determinations. The differences between pairs of values were less than 5% of the means.

RESULTS

Isolation and characterization of a Δ5-desaturase-defective mutant. Through analysis of the fatty acid compositions of the vegetative mycelia obtained from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-treated spores, we obtained several Δ5-desaturase-defective mutants, but all of them, except for the one designated S14, were leaky mutants producing more than 10% AA. Strain S14, which produced only a minute amount of AA, was not distinguishable from the wild type or strain Mut44 in morphology, but its fatty acid profile was apparently different (Table 1). The fatty acids of S14 included an about 10-fold lower level of AA than those of Mut44, and the DGLA ratio was higher, accounting for as much as about 50% of the total mycelial fatty acids. As in Mut44, the mycelial fatty acids of S14 exhibited a higher γ-linolenic (18:3ω6) acid-to-linoleic acid (18:2ω6) ratio. However, the proportion of palmitic acid (16:0) in S14 was a little

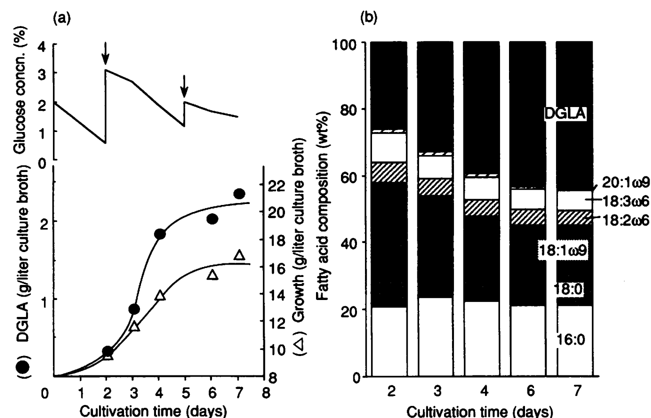

FIG. 3. Production of DGLA by *M. alpina* 1S-4 S14 under submerged batch culture conditions. The fungus was precultured at 28°C for 3 days in 40 ml of medium GY, and then the resultant culture was inoculated into 2 liters of medium GY containing 0.05% Adekanol (Asahi Denka Industries, Tokyo, Japan), in a 5-liter jar fermentor (Able Ltd., Tokyo, Japan). Cultivation was performed at 28°C, with aeration at 1 vol/vol/min and agitation at 300 rpm. Glucose was added at the times indicated by the arrows (a). Changes in mycelial fatty acid composition during growth are shown in panel b.

higher than in the wild type and Mut44. The increase in the DGLA/AA ratio and simultaneous accumulation of 18:3ω6 suggested a defect in Δ5-desaturase.

Time courses of changes in mycelial fatty acid contents. Representative time courses of the changes in the contents of mycelial fatty acids in strain S14 are presented in Fig. 1. Fungal growth in terms of dry mycelial mass reached a plateau after 4 days of cultivation. Except for that of AA, which continued increasing up to day 10 of cultivation, the amounts of 16:0, stearic acid (18:0), 18:2ω6, 18:3ω6, and total fatty acids reached the respective maximal values after 5 days and then decreased gradually. DGLA also reached the maximal value after 5 days but remained relatively constant thereafter. On cultivation for 7 days, the mycelial DGLA content reached 114 mg/g of dry mycelia and AA content amounted to 2 mg/g of dry mycelia.

Effect of growth temperature on DGLA production. Production of fungal mycelia did not change markedly with growth temperature, but the mycelial DGLA content, its production per milliliter of culture broth, and its ratio to total fatty acids decreased markedly when the growth temperature was lowered to 12°C (Fig. 2). In the growth temperature range of 16 to 28°C, production of DGLA did not change markedly; the percentage of DGLA in the total fatty acids was about 50%. It was found that 8(*Z*),11(*Z*),14(*Z*),17(*Z*)-eicosatetraenoic acid was formed as an additional fatty acid when the growth temperature was 24°C or lower, and the percentage ranged from 2.7% on growth at 24°C to as much as 9% on growth at 16°C. The AA ratio increased with increasing growth temperatures, reaching 1% on growth at 28°C (data not shown).

Production of DGLA under submerged culture conditions. Bench scale production of DGLA by strain S14 was performed under the conditions given in the legend to Fig. 3a. Glucose was added to the culture medium to maintain its concentration at about 2%, as this is necessary for bench scale fermentor production. The mycelial DGLA content increased markedly from 2 to 4 days of cultivation, reaching 140 mg/g of dry mycelia (2.4 g/liter of culture broth) after 7 days of cultivation. The percentage of DGLA in the total mycelial fatty acids increased markedly, from 25.4% on day 2 to 43.3% on day 6, at the expense of 18:1ω9, which was the dominant fatty acid on day 2 (Fig. 3b). This shift from 18:1ω9 dominance to DGLA dominance may have been due to increases in the activities of enzymes involved in the conversion of 18:1ω9 to DGLA, such as Δ12-desaturase. The

TABLE 2. Fatty acid compositions of the major lipids in *M. alpina* 1S-4 and Δ5-desaturase-defective mutants on growth at 28°C

Strain and fraction[a]	Lipid composition (mol%)[b]	Fatty acid composition (mol%)									
		16:0	18:0	18:1ω9	18:2ω6	18:3ω6	20:1ω9	20:2ω6	DGLA	AA	24:0
Wild type											
TG	83.8	23.7	8.7	18.3	8.5	3.5	1.2	0.6	5.8	28.0	1.7
DG	3.6	15.8	6.4	31.1	10.1	11.8	0.6	0.7	3.4	20.2	—[c]
PE	4.0	20.2	5.6	22.8	12.1	12.4	1.5	1.3	1.6	22.5	—
PC	6.3	17.9	2.5	7.3	16.9	5.4	0.5	5.2	Tr[d]	44.4	—
PS	2.3	34.2	6.7	21.0	17.5	4.3	0.4	1.5	2.6	11.8	—
Mut44[e]											
TG	84.4	21.6	10.7	34.6	6.4	5.9	1.8	0.5	13.1	3.4	2.0
DG	2.4	17.2	8.0	31.8	11.5	21.8	1.3	—	4.1	4.2	—
PE	5.0	16.0	4.8	26.9	12.9	23.7	2.1	0.3	2.9	10.3	—
PC	5.8	16.6	2.9	8.7	13.4	46.6	1.5	0.5	0.8	8.6	0.5
PS	2.4	30.1	6.0	24.5	17.0	8.1	0.6	—	7.9	5.7	—
S14											
TG	82.0	28.8	8.7	17.3	3.7	3.9	0.8	0.4	31.8	0.6	4.0
DG	2.6	19.6	8.1	28.2	6.2	13.3	—	—	24.5	—	—
PE	4.5	24.4	5.4	18.1	3.5	9.2	1.2	—	37.7	0.5	—
PC	8.5	22.3	2.3	4.2	1.7	5.5	0.5	0.5	61.8	1.2	—
PS	2.4	31.5	6.3	19.5	3.6	6.3	0.5	—	32.3	—	—

[a] PS, phosphatidylserine.
[b] Other, minor lipids, such as free fatty acids, sterols, sterol esters, and glycolipids, were not included in the calculation.
[c] —, undetectable.
[d] Tr, trace.
[e] Mutant defective in Δ5-desaturation as described previously (7).

TABLE 3. Positional distribution of fatty acids in the phospholipids of *M. alpina* 1S-4 and Δ5-desaturase-defective mutants[a]

Phospholipid and strain	Position	Fatty acid composition (mol%)									
		16:0	18:0	18:1ω9	18:2ω6	18:3ω6	20:1ω9	20:2ω6	DGLA	AA	24:0
PC											
1S-4	*sn*-1	35.7	5.6	7.1	5.2	2.9	0.8	—[b]	—	42.7	—
	sn-2	6.2	4.1	8.6	27.7	3.9	—	0.3	3.1	46.2	—
Mut44[c]	*sn*-1	31.5	8.8	10.6	7.2	30.5	2.6	0.6	1.0	7.1	—
	sn-2	8.1	5.3	11.4	17.3	52.1	—	—	0.6	5.3	—
S14	*sn*-1	30.0	6.2	14.5	3.7	8.8	1.0	0.4	33.8	1.7	—
	sn-2	6.2	3.0	5.5	1.8	3.3	0.2	0.8	78.6	0.7	—
PE											
1S-4	*sn*-1	34.1	7.6	40.0	7.6	2.6	2.5	0.2	1.0	4.4	—
	sn-2	6.1	4.7	13.4	20.7	11.3	—	—	2.2	41.8	—
Mut44	*sn*-1	25.7	6.7	46.2	9.2	7.9	2.3	0.4	1.2	0.4	—
	sn-2	9.4	5.8	20.0	21.1	35.4	—	—	1.7	6.5	—
S14	*sn*-1	44.1	9.1	28.3	4.3	4.3	3.3	—	6.6	—	—
	sn-2	10.7	5.3	13.7	3.0	14.0	—	—	52.6	0.8	—

[a] PC and PE were from fungi grown at 28°C for 7 days.
[b] —, undetectable.
[c] Mutant defective in Δ5-desaturation as described previously (7).

other major fatty acids were 16:0 (21.2%), 18:0 (9.6%), oleic acid (14.3%), 18:2ω6 (4.4%), and 18:3ω6 (5.8%). AA accounted for 1.5% of the total fatty acids.

Distribution of fatty acids in major lipid classes of the mutant. As shown in Table 2, DGLA accumulated in all major lipid classes, accounting for one-fourth or more of the total fatty acids in each lipid class. DGLA was present at about 82 mol% in the triacylglycerol (TG) fraction, and the remainder was in the diacylglycerol (DG), PE, PC, and phosphatidylserine fractions, at concentrations of 1.4, 3.6, 11, and 1.6%, respectively. The highest proportion of DGLA was found in PC, where it accounted for 62% of the total fatty acids. It is notable that the levels of 18:3ω6 in DG, PC, and PE, which accounted for more than 20% of the total fatty acids in Mut44, were much lower for strain S14. The levels of 18:2ω6 in all lipid classes were also much lower than those in Mut44 or the wild type.

Positional distribution of fatty acids in PC and PE. Determination of the fatty acid composition at each position revealed that, especially in PE, saturated fatty acids and monounsaturated fatty acids exist preferentially at the *sn*-1 position, whereas polyunsaturated fatty acids exist at the *sn*-2 position (Table 3). However, in the PC fraction of strain S14, 18:2ω6 and 18:3ω6 were present at the *sn*-1 rather than at the *sn*-2 position. DGLA accounted for as much as 78 mol% at the *sn*-2 position of PC.

DISCUSSION

The present study showed that a novel Δ5-desaturase-defective mutant, S14, of a potent AA-producing fungus, *M. alpina* 1S-4, is also a potent producer of DGLA. Under optimal conditions, it could produce more than 2 g of DGLA per liter of culture broth. This value is comparable to that obtained previously by cultivation in the presence of Δ5-desaturase inhibitors (12), but the mycelial DGLA content (140 mg/g of dry mycelia) was higher than that obtained with the previous method (107 mg/g of dry mycelia). The oil produced by this mutant is characterized by an extremely low level of AA; the level of AA in its mycelia is about 1/10 of that in the oil produced by Mut44, in which the AA content is 19 mg/g of dry mycelia (7), and about 1/30 of that obtained with inhibitor addition.

Studies on the distribution of fatty acids showed that DGLA was present in all of the lipid fractions, including PC and PE, in which the proportion of AA was higher than that of DGLA in strain Mut44. In the previous report on Mut44 (7), we suggested that phospholipids were possible substrates for Δ5-desaturase, on the basis of the finding that the level of AA was much higher than that of DGLA in phospholipids, whereas the level of DGLA was higher in TG. On the basis of the finding of a high DGLA level in phospholipids of strain S14, which is almost completely devoid of Δ5-desaturase activity, we suggest again the possible role of phospholipids as sites for Δ5-desaturation.

However, it is not clear why such a high level of DGLA is present in the PC of strain S14, whereas 18:3ω6 is the most abundant fatty acid in strain Mut44. Further work is required to explain this phenomenon.

REFERENCES

1. **Christie, W. W.** 1982. Structural analysis of lipids by means of enzymatic hydrolysis, p. 162–163. *In* W. W. Christie (ed.), Lipid analysis, 2nd ed. Pergamon Press, Oxford.
2. **Christie, W. W., R. C. Noble, and J. H. Moore.** 1970. Determination of lipid classes by a gas-chromatography procedure. Analyst **95:**940–944.
3. **Folch, J., M. Lees, and G. H. Sloane-Stanley.** 1957. A simple method for isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497–509.
4. **Fu-Lin, E., and J. L. Dupuy.** 1980. The fatty acid compositions of three unicellular algal species used as food sources for larvae of the American oyster. Lipids **15:**356–364.
5. **Horrobin, D. F.** February 1991. Combination of interferons and γ-linolenic acid or other fatty acid enhancers for treating virus infection, cancer and inflammatory diseases. Canadian patent CA 2,022,608.
6. **Hulanicka, D., J. Erwin, and K. Bloch.** 1964. Lipid metabolism of *Euglena gracilis*. J. Biol. Chem. **239:**2778–2787.
7. **Jareonkitmongkol, S., H. Kawashima, N. Shirasaka, S. Shimizu, and H. Yamada.** 1992. Production of dihomo-γ-linolenic acid by a Δ5-desaturase-defective mutant of *Mortierella alpina* 1S-4. Appl. Environ. Microbiol. **58:**2196–2200.
8. **Jareonkitmongkol, S., S. Shimizu, and H. Yamada.** 1992. Fatty acid desaturation-defective mutants of an arachidonic-acid-producing fungus, *Mortierella alpina* 1S-4. J. Gen Microbiol. **138:** 997–1002.
9. **Lands, W. E. M.** 1986. Foods, drugs, and disease mechanisms, p. 15–19. *In* W. E. M. Lands (ed.), Fish and human health.

Academic Press, Inc., London.
10. **Mead, J. F.** 1971. The metabolism of polyunsaturated fatty acids, p. 161–192. *In* R. T. Holman (ed.), Progress in the chemistry of fats and other lipids, vol. IX. Pergamon Press, Inc., Oxford.
11. **Melnik, B., and G. Plewing.** October 1990. Baby food containing γ-linolenic acid, dihomo-γ-linolenic acid, or PGE_1 for atopy prophylaxis. European patent EP 391,218.
12. **Shimizu, S., K. Akimoto, H. Kawashima, Y. Shinmen, and H. Yamada.** 1989. Production of dihomo-γ-linolenic acid by *Mortierella alpina* 1S-4. J. Am. Oil Chem. Soc. **66:**237–241.
13. **Shimizu, S., K. Akimoto, Y. Shinmen, H. Kawashima, M. Sugano, and H. Yamada.** 1991. Sesamin is a potent and specific inhibitor of Δ5-desaturase in polyunsaturated fatty acid biosynthesis. Lipids **26:**512–516.
14. **Shimizu, S., S. Jareonkitmongkol, H. Kawashima, K. Akimoto, and H. Yamada.** 1991. Production of a novel ω1-eicosapentaenoic acid by *Mortierella alpina* 1S-4 grown on 1-hexadecene. Arch. Microbiol. **156:**163–166.
15. **Shimizu, S., Y. Shinmen, H. Kawashima, K. Akimoto, and H. Yamada.** 1988. Fungal mycelia as a novel source of eicosapentaenoic acid: activation of enzyme(s) involved in eicosapentaenoic acid production at low temperature. Biochem. Biophys. Res. Commun. **150:**335–341.
16. **Werner, W., H.-G. Rey, and H. Wielinger.** 1970. Über die Eigenschaften eines neuen Chromogens für die Blutzuckerbestimmung nach der GOD/POD-Methode. Z. Anal. Chem. **252:**224–228.
17. **Yamada, H., S. Shimizu, and Y. Shinmen.** 1987. Production of arachidonic acid by *Mortierella alpina* 1S-5. Agric. Biol. Chem. **51:**785–790.