Essential fatty acids for oleaginous fungus *Mortierella alpina*

Tomohiro Fujita^a, Hiroshi Kawashima^b, Eiji Sakuradani^{c,*}, Takaiku Sakamoto^c, Akinori Ando^d, Jun Ogawa^d, Sakayu Shimizu^a

^aDepartment of Bioscience and Biotechnology, Faculty of Bioenvironmental Science, Kyoto Gakuen University, 1-1 Nanjo, Sogabe, Kameoka 621-8555, Japan

^bInstitute for Health Care Science, Suntory Wellness Ltd., Osaka 618-8503, Japan

^cGraduate School of Bioscience and Bioindustry, Tokushima University, Tokushima 770-8513, Japan

^dGraduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Correspondence to: * Eiji Sakuradani, Ph.D.

Graduate School of Bioscience and Bioindustry, Tokushima University,

2-1 Minami-josanjima, Tokushima 770-8513, Japan

Phone: +81-88-656-7528

FAX: +81-88-656-9074

e-mail: sakuradani.eiji@tokushima-u.ac.jp

Keywords: *Mortierella alpina*; polyunsaturated fatty acid; inhibitor

Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1 ω 7, vaccenic acid; 18:1 ω 9, oleic acid; 18:2 ω 6, linoleic acid; 18:2 ω 9, 6Z,9Z-octadecadienoic acid; 18:3 ω 6, γ -linolenic acid; 20:0, arachidic acid; 20:1 ω 9, eicosenoic acid; 20:2 ω 9, 8Z,11Z-eicosadecadienoic acid; 20:3 ω 6, dihomo- γ -linolenic acid; 20:3 ω 9, Mead acid; 20:4 ω 6, arachidonic acid; 22:0, behenic acid.

Abstract

Oleaginous fungus *Mortierella alpina* 1S-4 accumulates arachidonic acid (20:4 ω 6) as a major polyunsaturated fatty acid. However, 20:4 ω 6 is not essential for the growth of *M. alpina* 1S-4, because various mutants that produce no 20:4 ω 6 were isolated. *M. alpina* JT-180, a Δ 12 desaturation-defective mutant that was derived from *M. alpina* 1S-4 by treatment with a chemical mutagen, accumulates Mead acid (20:3 ω 9) instead of 20:4 ω 6. *p*-Anisidine was found to be a Δ 6 desaturation inhibitor in this study. The concentration of 0.1 mg/ml of *p*-anisidine had an inhibitory effect on the growth of *M. alpina* JT-180. The addition of *p*-anisidine to the medium caused *M. alpina* JT-180 to produce only monoenoic acids such as oleic acid (18:1 ω 9) and eicosenoic acid (20:1 ω 9) as unsaturated fatty acids. The effects of exogenous fatty acids were investigated when *M. alpina* JT-180 was cultivated in medium containing *p*-anisidine. The addition of linoleic acid (18:2 ω 6) and 6Z,9Z-octadecadienoic acid (18:2 ω 9) restored the growth of *M. alpina* JT-180 cultivated on the medium containing *p*-anisidine, but palmitic acid (16:0), 18:1 ω 9, and vaccenic acid (18:1 ω 7) had no effect. For the growth of oleaginous fungus *M. alpina*, 18-carbon length fatty acids with more than two double bonds are considered to be essential.

1. Introduction

Filamentous fungus *Mortierella alpina* 1S-4 is an industrial strain for production of arachidonic acid (20:4 ω 6), which is the most abundant C20 polyunsaturated fatty acid (PUFA) in humans (Yamada et al., 1987; Sakuradani et al., 2013), and not only exhibits various regulation effects and physiological activities but also plays important roles in infant nutrition (Carlson et al., 1993; Gill and Valivety, 1997). However, the physiological function of 20:4 ω 6 in this fungus remains unclear. Thus far, we have derived various mutants from *M. alpina* 1S-4 by means of chemical mutagenesis, which exhibited different fatty acid compositions from the wild strain *M. alpina* 1S-4 (Jareonkitmongkol et al., 1992a). *M. alpina* JT-180 and *M. alpina* S14 are Δ 12 fatty acid desaturation-defective and Δ 5 fatty acid desaturation-defective mutants that accumulate Mead acid (20:3 ω 9) and

dihomo- γ -linolenic acid (20:3 ω 6), respectively (Jareonkitmongkol et al., 1993a; Sakuradani et al., 2003). Neither mutant produces 20:4 ω 6, which means that 20:4 ω 6 is non-essential for the growth of *M. alpina* strains.

A different way to change the fatty acid composition in *M. alpina* is to use inhibitors of fatty acid desaturation. We have reported three types of inhibitors, i.e., lignan compounds (Shimizu et al., 1991), alkyl gallate derivatives (Kawashima et al., 1996a), and curcumin derivatives (Kawashima et al., 1996b). The lignan compounds in sesame seeds and oil are specific inhibitors of Δ 5 desaturase, which catalyzes the conversion of 20:3 ω 6 to 20:4 ω 6 (Shimizu et al., 1991). Alkyl gallate derivatives, which are known to be antioxidants, show inhibitory effects on Δ 6 desaturase as well as Δ 5 desaturase (Kawashima et al., 1996a). The curcumin derivatives, which include the main component of the yellow spice turmeric, show different inhibitory effect on Δ 5 desaturase and a weak one on Δ 6 desaturase (Kawashima et al., 1996b). In addition, 2,2-diphenyl-5-(4-[(1*E*)-pyridin-3-yl-methylidene]amino}piperazin-1-yl)pentanenitrile (SC-26196) inhibits Δ 6 desaturation in isolated rat liver microsomes (Harmon et al., 2003). Treatment of the microalga *Porphyridium cruentum* with salicylhydroxamic acid (SHAM) inhibits growth and affects the fatty acid composition due to Δ 6 fatty acid desaturation (Khozin-Goldberg et al., 1999).

In this research, we found a new Δ 6 desaturation inhibitor, 4-methoxyaniline (*p*-anisidine), for *M. alpina* strains (Fig. 1). We suggest essential fatty acid candidates for *M. alpina* strains based on comparison of growth and fatty acid composition between wild strain *M. alpina* 1S-4 and Δ 12 desaturation-defective mutant JT-180 cultivated in medium containing *p*-anisidine.

2. Materials and methods

2.1. Chemicals

p-Anisidine was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Fatty acid methyl esters were purchased from Funakoshi (Tokyo, Japan). 6*Z*,9*Z*-octadecadienoic acid (18:2 ω 9) methyl ester was purified from fatty acid methyl esters prepared from microbial oil of Δ 12

desaturation-defective mutant *M. alpina* JT-180 (Sakuradani et al., 2003). All other reagents were of analytical grade.

2.2. Microorganisms and cultivation

M. alpina 1S-4 (wild strain) and its derivative mutant JT-180 were cultivated on a medium containing 2% glucose and 1% yeast extract (pH 6.0) with *p*-anisidine, as indicated, at 28°C with reciprocal shaking at 120 rpm for 7 d.

2.3. Fatty acid analysis

Mycelial cells were harvested by suction filtration, washed with water and then dried at 100°C for 2 h for subsequent fatty acid analysis by gas-liquid chromatography after transmethylation with methanolic HCl as described previously (Shimizu et al., 1988). Desaturation activity was expressed as the 'desaturation index', which is the ratio of the amount of the substrate of the desaturase to that of the product and further metabolites. For example, $\Delta 6$ desaturation activity was expressed as the $\Delta 6$ desaturation index, i.e., the ratio of linoleic acid (18:2 ω 6) to γ -linolenic acid (18:3 ω 6) + 20:3 ω 6 + 20:4 ω 6.

3. Results and discussion

3.1. Inhibition of fatty acid desaturase in *M. alpina* by *p*-anisidine

$\Delta 6$ desaturase activity was defined as a desaturase index. i.e., the ratio of 18:2 ω 6 to 18:3 ω 6 + 20:3 ω 6 + 20:4 ω 6 in total mycelial fatty acids, since the desaturation reaction is not reversible and 20:4 ω 6 is not further metabolized in the fungus (Yamada et al., 1987; Sakuradani et al., 2013). Therefore, the desaturation index increases when the desaturase activity is reduced and the substrate 18:2 ω 6 for $\Delta 6$ desaturase is not consumed through $\Delta 6$ desaturation. Table 1 shows that *p*-anisidine supplementation caused increases in stearic acid (18:0), oleic acid (18:1 ω 9), and 18:2 ω 6, and decreases in 20:3 ω 6 and 20:4 ω 6. Addition of *p*-anisidine to the culture medium is considered to cause $\Delta 6$ desaturation inhibition through an increase in the $\Delta 6$

desaturase index. Similarly, the supplementation of methyl *p*-coumarate and propyl gallate caused inhibition of the $\Delta 6$ desaturase activity (Kawashima et al., 1996a; Kawashima et al., 1996b). On the other hand, the supplementation of (+)-sesamine, which is a specific inhibitor of $\Delta 5$ desaturase, hardly influenced the fatty acid composition, except for the levels of 20:3 ω 6 and 20:4 ω 6, and caused a remarkable decrease in only the $\Delta 5$ desaturase index (Shimizu et al., 1991).

3.2. Inhibitory effects of *p*-anisidine on growth of *M. alpina* mutants

The inhibitory effects of *p*-anisidine on the growth of various *M. alpina* mutants on agar medium were investigated. No inhibitory effect of *p*-anisidine was observed on the cultivation of $\Delta 6$ desaturation-defective mutant Mut49 (Jareonkitmongkol et al., 1993b), $\Delta 5$ desaturation-defective mutant S14 (Jareonkitmongkol et al., 1993a), or $\Delta 9$ desaturation-defective mutant T4 (Jareonkitmongkol et al., 2002), which accumulated 18:2 ω 6, 20:3 ω 6, and 20:4 ω 6, respectively. On the other hand, the growth of $\Delta 12$ desaturation-defective mutants Mut48 and JT-180 was definitely inhibited on the agar medium containing *p*-anisidine (Jareonkitmongkol et al., 1992b; Sakuradani et al., 2003). *p*-Anisidine (0.1 mg/ml) showed a low inhibitory effect on the growth of the wild strain (*M. alpina* 1S-4), but a significant inhibitory effect on the growth of JT-180. Strain JT-180 biosynthesizes several PUFAs such as 18:2 ω 9, 8Z,11Z-eicosadecadienoic acid (20:2 ω 9), and 20:3 ω 9 on cultivation in medium without *p*-anisidine. However, the addition of $\Delta 6$ desaturation-inhibitor *p*-anisidine prevented JT-180 from biosynthesizing such PUFAs (Fig. 2). Strain JT-180 accumulated 18:1 ω 9 and eicosenoic acid (20:1 ω 9) as unsaturated fatty acids on cultivation in the medium containing *p*-anisidine through the route shown in Fig. 3. Other $\Delta 6$ desaturation-inhibitors such as propyl gallate, sufrole, and methyl *p*-coumarate similarly prevented the growth of JT-180 (data not shown).

3.3. Effect of supplementation of various fatty acids on growth of the *M. alpina* JT-180 mutant

The effects of addition of a variety of fatty acids on the growth of JT-180 on the agar medium containing *p*-anisidine were investigated, as shown in

Fig. 4. Control strain *M. alpina* 1S-4 formed colonies on the agar medium containing *p*-anisidine, which were similar to the colonies on the medium without *p*-anisidine (Fig. 4). The growth of JT-180 was restored by the addition of 18:2 ω 6 or 18:2 ω 9. No effect of the addition of 16:0, 18:1 ω 9 or 18:1 ω 7 was observed on the growth of JT-180 on the agar medium containing *p*-anisidine. The total fatty acid compositions of strains that were cultivated under the conditions of the addition of fatty acids to the agar medium were analyzed. Wild strain *M. alpina* 1S-4 accumulated 20:4 ω 6 as a major PUFA regardless of added fatty acid. The level of 20:4 ω 6 in the total fatty acids from *M. alpina* 1S-4 under the condition of the addition of 18:1 ω 7 was lowest among the tested strains, which might mean that the addition of 18:1 ω 7 caused slight inhibition of the growth of the wild strain. The addition of 18:2 ω 6 or 18:2 ω 9 to the medium induced JT-180 to accumulate 20:4 ω 6 or 20:3 ω 9, respectively. The melting points of 18:1 ω 7 and 18:1 ω 9 are 14°C and 13°C, respectively. On the other hand, the melting points of 18:2 ω 6, 18:3 ω 6, and 20:4 ω 6 are -5°C, -11°C, and -50°C, respectively. *M. alpina* strains grow well at between 4 to 28°C. PUFAs that have more than two double bonds in their structures are considered to be essential for the growth of *M. alpina*.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work was supported in part by the Advanced Low Carbon Technology Research and Development Program (ALCA) of the Japan Science and Technology Agency (JST).

References

- Carlson, S.E., Werkman, S.H., Peeples, J.M., Cooke, R.J., Tolley, E.A., 1993. Arachidonic acid status correlates with first year growth in preterm infants. *Proc. Natl. Acad. Sci. U. S. A.* 90, 1073-1077.

- Gill, I., Valivety, R., 1997. Polyunsaturated fatty acids, part 1: occurrence, biological activities and applications. *Trends Biotechnol.* 15, 401-409.
- Harmon, S.D., Kaduce, T.L., Manuel, T.D., Spector, A.A., 2003. Effect of the $\Delta 6$ -desaturase inhibitor SC-26196 on PUFA metabolism in human cells. *Lipids* 38, 469-476.
- Jareonkitmongkol, S., Shimizu, S., Yamada, H., 1992a. Fatty acid desaturation-defective mutants of an arachidonic-acid-producing fungus, *Mortierella alpina* 1S-4. *J. Gen. Microbiol.* 138, 997-1002.
- Jareonkitmongkol, S., Kawashima, H., Shimizu, S., Yamada, H., 1992b. Production of 5,8,11-cis-eicosatrienoic acid by a $\Delta 12$ -desaturase-defective mutant of *Mortierella alpina* 1S-4. *J. Am. Oil Chem. Soc.* 69, 939-944.
- Jareonkitmongkol, S., Sakuradani, E., Shimizu, S., 1993a. A novel $\Delta 5$ -desaturase-defective mutant of *Mortierella alpina* 1S-4 and its dihomog γ -linolenic acid productivity. *Appl. Environ. Microbiol.* 59, 4300-4304.
- Jareonkitmongkol, S., Shimizu, S., Yamada, H., 1993b. Occurrence of two nonmethylene-interrupted Δ^5 polyunsaturated fatty acids in a Δ^6 -desaturase-defective mutant of the fungus *Mortierella alpina* 1S-4. *Biochim. Biophys. Acta* 1167, 137-141.
- Jareonkitmongkol, S., Sakuradani, E., Shimizu, S., 2002. Isolation and characterization of a $\Delta 9$ -desaturation-defective mutant of an arachidonic acid-producing fungus, *Mortierella alpina* 1S-4. *J. Am. Oil Chem. Soc.* 79, 1021-1026.
- Kawashima, H., Akimoto, K., Shirasaka, N., Shimizu, S., 1996a. Inhibitory effects of alkyl gallate and its derivatives on fatty acid desaturation. *Biochim. Biophys. Acta* 1299, 34-38.
- Kawashima, H., Akimoto, K., Jareonkitmongkol, S., Shirasaka, N., Shimizu, S., 1996b. Inhibition of rat liver microsomal desaturases by curcumin and related compounds. *Biosci. Biotechnol. Biochem.* 60, 108-110.
- Khozin-Goldberg, I., Bigogno, C., Cohen, Z., 1999. Salicylhydroxamic acid inhibits $\Delta 6$ desaturation in the microalga *Porphyridium cruentum*. *Biochim. Biophys. Acta* 1439, 384-394.
- Sakuradani, E., Kamada, N., Hirano, Y., Nishihara, M., Kawashima, H., Akimoto, K., Higashiyama, K., Ogawa, J., Shimizu, S., 2003. Production of 5,8,11-eicosatrienoic acid by a $\Delta 5$ and $\Delta 6$ desaturation activity-enhanced mutant derived from a $\Delta 12$ desaturation activity-defective mutant of

- Mortierella alpina* 1S-4. Appl. Microbiol. Biotechnol. 60, 281-287.
- Sakuradani, E., Ando, A., Shimizu, S., Ogawa, J., 2013. Metabolic engineering for the production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4. J. Biosci. Bioeng. 116, 417-422.
- Shimizu, S., Shinmen, Y., Kawashima, H., Akimoto, K., Yamada, H., 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.
- Shimizu, S., Akimoto, K., Kawashima, H., Sugano, M., Yamada, H., 1991. Sesamin is a potent and specific inhibitor of $\Delta 5$ desaturase in polyunsaturated fatty acid biosynthesis. Lipids 26, 512-516.
- Yamada, H., Shimizu, S., Shinmen, Y., 1987. Production of arachidonic acid by *Mortierella elongata* 1S-5. Agric. Biol. Chem. 51, 785-790.

Figure legends

Fig. 1. Chemical structural formula of *p*-anisidine.

Fig. 2. GC chromatograms of the fatty acid methyl esters obtained from *M. alpina* JT-180 cultivated in a medium without (A) or with (B) *p*-anisidine. Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1 ω 9, oleic acid; 18:2 ω 6, linoleic acid; 20:0, arachidic acid; 20:1 ω 9, eicosenoic acid; 20:2 ω 9, 8Z,11Z-eicosadecadienoic acid; 20:3 ω 9, Mead acid; 22:0, behenic acid.

Fig. 3. Saturated and unsaturated fatty acid biosynthetic pathways in *M. alpina* JT-180. *p*-Anisidine inhibited Δ 6 desaturation in these pathways.

Fig. 4. Comparison of growth of *M. alpina* JT-180 (A) and wild strain *M. alpina* 1S-4 (B). The strains were cultivated in agar medium containing 2% glucose, 1% yeast extract, and 0.01% *p*-anisidine (pH 6.0).

Fig. 1

Fig. 2

Fig. 3

(A) *M. alpina* JT-180 mutant

(B) *M. alpina* 1S-4 (wild strain)

Fig. 4

Table 1

Effect of *p*-anisidine on $\Delta 6$ desaturase activity of *M. alpina* 1S-4 ^a.

Inhibitor	Fatty acid composition (%) ^b								$\Delta 6$ Desaturase index ^c
	16:0	18:0	18:1 ω 9	18:2 ω 6	18:3 ω 6	20:3 ω 6	20:4 ω 6	Others	
None	16.7	8.5	20.6	11.1	7.1	5.6	20.3	10.3	0.34
<i>p</i> -Anisidine	9.5	15.7	23.5	16.5	8.0	2.3	11.1	13.4	0.77

^a *M. alpina* 1S-4 was grown in a medium containing 2% glucose and 1% yeast extract (pH 6.0) supplemented with *p*-anisidine (0.1 mg/ml).

^b Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1 ω 9, oleic acid; 18:2 ω 6, linoleic acid; 18:3 ω 6, γ -linolenic acid; 20:3 ω 6, dihomog γ -linolenic acid; 20:4 ω 6, arachidonic acid.

^c $\Delta 6$ Desaturase index, ratio of 18:2 ω 6/(18:3 ω 6 + 20:3 ω 6 + 20:4 ω 6) in total mycelial fatty acids.