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Essential fatty acids for oleaginous fungus *Mortierella alpina*

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ABSTRACT

Oleaginous fungus *Mortierella alpina* 1S-4 accumulates arachidonic acid (20:4ω6) as a major polyunsaturated fatty acid. However, 20:4ω6 is not essential for the growth of *M. alpina* 1S-4, because various mutants that produce no 20:4ω6 were isolated. *M. alpina* JT-180, a Δ12 desaturation-defective mutant that was derived from *M. alpina* 1S-4 by treatment with a chemical mutagen, accumulates Mead acid (20:3ω9) instead of 20:4ω6. *p*-Anisidine was found to be a Δ6 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 (18:1ω9) and eicosenoic acid (20:1ω9) 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 (18:2ω6) and 6*Z*,9*Z*-octadecadienoic acid (18:2ω9) restored the growth of *M. alpina* JT-180 cultivated on the medium containing *p*-anisidine, but palmitic acid (16:0), 18:1ω9, and vaccenic acid (18:1ω7) 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 (20:4ω6), 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 20:4ω6 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 Δ12 fatty acid desaturation-defective and Δ5 fatty acid desaturation-defective mutants that accumulate Mead acid (20:3ω9) and dihomo-γ-linolenic acid (20:3ω6), respectively (Jareonkitmongkol et al., 1993a; Sakuradani et al., 2003). Neither mutant produces 20:4ω6, which means that 20:4ω6 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 Δ5 desaturase, which catalyzes the conversion of 20:3ω6 to 20:4ω6 (Shimizu et al., 1991). Alkyl gallate derivatives, which are known to be antioxidants, show inhibitory effects on Δ6 desaturase as well as Δ5 desaturase (Kawashima et al., 1996a). The curcumin derivatives, which include the main component of the yellow spice turmeric, show different inhibitory effect on Δ5 desaturase and a weak one on Δ6 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 Δ6 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 Δ6 fatty acid desaturation (Khozin-Goldberg et al., 1999).

In this research, we found a new Δ6 desaturation inhibitor, 4-methoxyaniline (*p*-anisidine), for *M. alpina* strains (Fig. 1). We

Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1ω7, vaccenic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:2ω9, 6*Z*,9*Z*-octadecadienoic acid; 18:3ω6, γ-linolenic acid;; 20:0, arachidic acid; 20:1ω9, eicosenoic acid; 20:2ω9, 8*Z*,11*Z*-eicosadecadienoic acid; 20:3ω6, dihomo-γ-linolenic acid; 20:3ω9, Mead acid; 20:4ω6, arachidonic acid; 22:0, behenic acid

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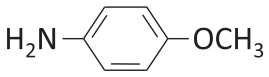

Fig. 1. Chemical structural formula of *p*-anisidine.

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 Δ12 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 (18:2ω9) methyl ester was purified from fatty acid methyl esters prepared from microbial oil of Δ12 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, Δ6 desaturation activity was expressed as the Δ6 desaturation index, i.e., the ratio of linoleic acid (18:2ω6) to γ-linolenic acid (18:3ω6)+20:3ω6+20:4ω6.

3. Results and discussion

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

Δ6 desaturase activity was defined as a desaturase index. i.e., the ratio of 18:2ω6 to 18:3ω6+20:3ω6+20:4ω6 in total mycelial fatty acids, since the desaturation reaction is not reversible and 20:4ω6 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 18:2ω6 for Δ6 desaturase is not consumed through Δ6 desaturation. Table 1 shows that *p*-anisidine supplementation caused increases in stearic acid (18:0), oleic acid (18:1ω9), and 18:2ω6, and decreases in 20:3ω6 and 20:4ω6. Addition of *p*-anisidine to the culture medium is considered to cause Δ6 desaturation inhibition through an increase in the Δ6 desaturase index. Similarly, the supplementation of methyl *p*-coumarate and propyl gallate caused inhibition of the Δ6 desaturase activity (Kawashima et al., 1996a, 1996b). On the other hand, the supplementation of (+)-sesamine, which is a specific inhibitor of Δ5 desaturase, hardly influenced the fatty acid composition, except for the levels of 20:3ω6 and 20:4ω6, and caused a remarkable decrease in only the Δ5 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 Δ6 desaturation-defective mutant Mut49 (Jareonkitmongkol et al., 1993b), Δ5 desaturation-defective mutant S14 (Jareonkitmongkol et al., 1993a), or Δ9 desaturation-defective mutant T4 (Jareonkitmongkol et al., 2002), which accumulated 18:2ω6, 20:3ω6, and 18:0, respectively. On the other hand, the growth of Δ12 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 18:2ω9, 8*Z*,11*Z*-eicosadecadienoic acid (20:2ω9), and 20:3ω9 on cultivation in medium without *p*-anisidine. However, the addition of Δ6 desaturation-inhibitor *p*-anisidine prevented JT-180 from biosynthesizing such PUFAs (Fig. 2). Strain JT-180 accumulated 18:1ω9 and eicosenoic acid (20:1ω9) as unsaturated fatty acids on cultivation in the medium containing *p*-anisidine through the route shown in Fig. 3. Other Δ6 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 18:2ω6 or 18:2ω9. No effect of the addition of 16:0, 18:1ω9 or 18:1ω7 was observed on the growth of JT-180 on the agar medium containing *p*-anisidine. The total fatty acid compositions of strains that were

Table 1
Effect of *p*-anisidine on Δ6 desaturase activity of *M. alpina* 1S-4 [a].

Inhibitor	Fatty acid composition (%)[b]								Δ6 Desaturase index[c]
	16:0	18:0	18:1ω9	18:2ω6	18:3ω6	20:3ω6	20:4ω6	Others	
None	16.7	8.5	20.6	11.1	7.1	5.6	20.3	10.3	0.34
p-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; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω6, γ-linolenic acid; 20:3ω6, dihomo-γ-linolenic acid; 20:4ω6, arachidonic acid.
[c] Δ6 Desaturase index, ratio of 18:2ω6/(18:3ω6+20:3ω6+20:4ω6) in total mycelial fatty acids.

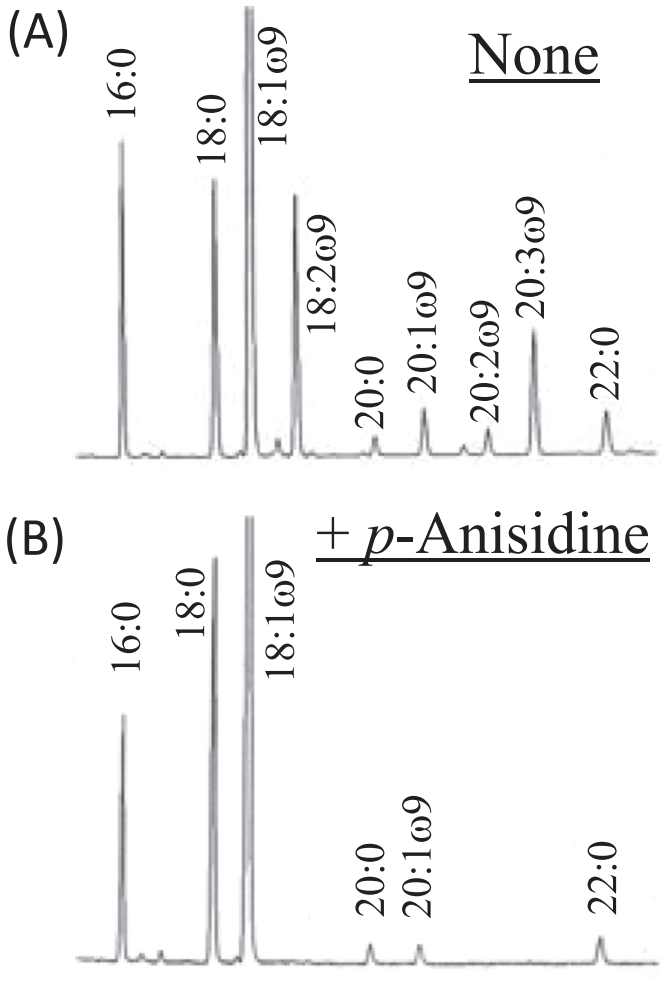

Fig. 2. GC chromatograms of the fatty acid methyl esters obtained from *M. alpina* JT-180 cultivated in a medium without (A) or with (B) *p*-anisidine. Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 20:0, arachidic acid; 20:1ω9, eicosenoic acid; 20:2ω9, 8*Z*,11*Z*-eicosadecadienoic acid; 20:3ω9, Mead acid; 22:0, behenic acid.

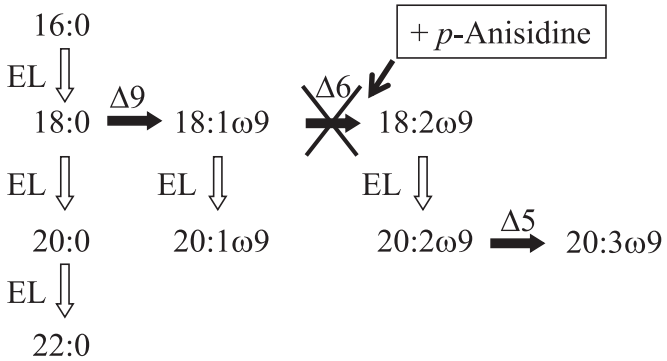

Fig. 3. Saturated and unsaturated fatty acid biosynthetic pathways in *M. alpina* JT-180. *p*-Anisidine inhibited Δ6 desaturation in these pathways.

cultivated under the conditions of the addition of fatty acids to the agar medium were analyzed. Wild strain *M. alpina* 1S-4 accumulated 20:4ω6 as a major PUFA regardless of added fatty acid. The level of 20:4ω6 in the total fatty acids from *M. alpina* 1S-4 under the condition of the addition of 18:1ω7 was lowest among the tested strains, which might mean that the addition of 18:1ω7 caused slight inhibition of the growth of the wild strain. The addition of 18:2ω6 or 18:2ω9 to the medium induced JT-180 to accumulate 20:4ω6 or 20:3ω9, respectively. The melting points of

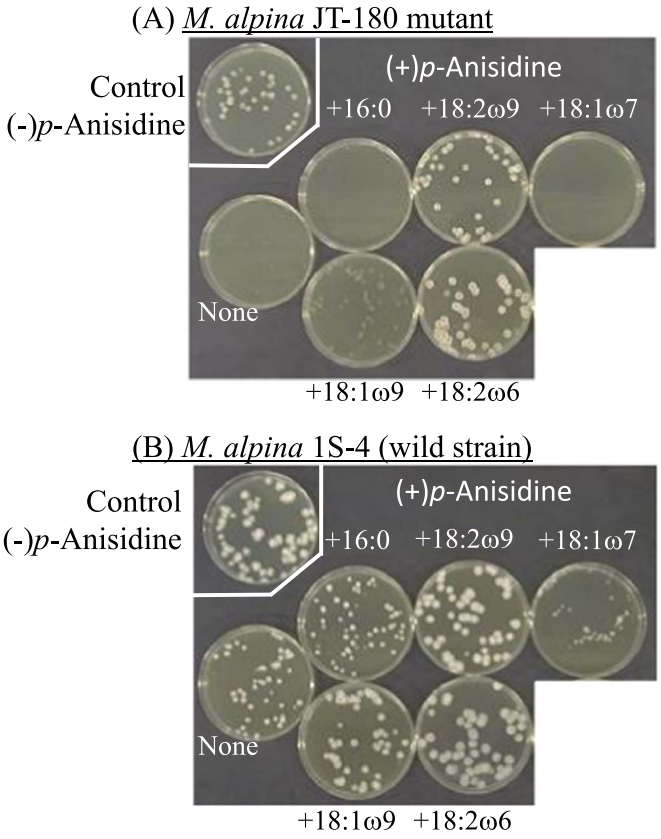

Fig. 4. Comparison of growth of *M. alpina* JT-180 (A) and wild strain *M. alpina* 1S-4 (B). The strains were cultivated in agar medium containing 2% glucose, 1% yeast extract, and 0.01% *p*-anisidine (pH 6.0).

18:1ω7 and 18:1ω9 are 14 °C and 13 °C, respectively. On the other hand, the melting points of 18:2ω6, 18:3ω6, and 20:4ω6 are −5 °C, −11 °C, and −50 °C, respectively. *M. alpina* strains grow well at between 4–28 °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.

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