Microbial Conversion of Palmitoleic Acid to 9,12-Hexadecadienoic Acid (16:2ω4) by *Trichoderma* sp. AM076

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ABSTRACT: *Trichoderma* sp. AM076, isolated from a freshwater sample, was found to accumulate 9,12-*cis*-hexadecadienoic acid (16:2ω4), when grown with palmitoleic acid (16:1ω7). Methyl myristate was the best carbon source for the conversion of palmitoleic acid to 16:2ω4. The mycelial 16:2ω4 content reached 17.4 mg/g dry mycelia (443 mg/L) when the fungus was grown in a medium that contained 2.0% methyl myristate, 1.5% yeast extract, and 2.0% methyl palmitoleate, pH 6.0, for 5 d at 28°C with shaking. In both nonpolar and polar lipids from the mycelia, 16:2ω4 was detected as one of the major fatty acids when 16:1ω7 was added. It is probable that 16:1ω7 is converted to 16:2ω4 through the Δ12 desaturation reaction.

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KEY WORDS: 9,12-*cis*-Hexadecadienoic acid, palmitoleic acid, *Trichoderma* sp.

The fatty acids with a 16-carbon chain, such as palmitic acid (16:0) and palmitoleic acid (16:1ω7), can be found as common fatty acids in living organisms. Polyunsaturated fatty acids (PUFA) with a 16-carbon chain have been reported only in some alga (1–5), and not in yeasts, bacteria, and molds. The production of C16 PUFA is always low because of low cell yields and fatty acid accumulation. On the other hand, some microorganisms show the ability to convert fatty substrates to PUFA with the same chainlength. In recent studies, we found that several arachidonic acid (20:4ω6)-producing *Mortierella* fungi and eicosapentaenoic acid (20:5ω3)-producing *Saprolegnia* fungi accumulate large amounts of C17 and C19 PUFA, such as 8,11,14-*cis*-nonadecatrienoic acid (19:3ω5), 5,8,11,14-*cis*-nonadecatetraenoic acid (19:4ω5), and 5,8,11,14,17-*cis*-nonadecapentaenoic acid (19:5ω2), in their mycelia when grown with odd-chain fatty substrates (6,7). Fatty substrates, such as saturated fatty acids or *n*-alkanes were easily converted to PUFA with the same carbon number, and these observations may suggest that it is possible to synthesize C16 PUFA from C16 fatty acids, such as 16:0 or 16:1, through microbial conversion.

Here we report the conversion of palmitoleic acid to 9,12-*cis*-hexadecadienoic acid (16:2ω4) by an isolated filamentous fungus, *Trichoderma* sp. AM076, and the distribution of the acids in individual fungal lipid classes is also described.

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MATERIALS AND METHODS

Chemicals. Palmitoleic acid was purchased from Funakoshi (Tokyo, Japan). All other authentic fatty acids were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Microorganisms. Trichoderma sp. AM076 (AKU3999-15; Faculty of Agriculture, Kyoto University, Kyoto, Japan) and other isolated strains were isolated from freshwater samples, obtained in Mie prefecture by the conventional streaking method on 2% agar plates of PS medium [5.0% potato flour (Difco, Detroit, MI) and 0.5% methyl stearate, pH 6.0]. All other strains were from our culture collection (AKU culture collection).

Media and cultivation. In the screening experiments, each strain was inoculated into 2 mL of PGY medium [1% glucose, 0.5% yeast extract (Oriental, Osaka, Japan), and 0.5% methyl palmitoleate, pH 6.0] in a 10-mL Erlenmeyer flask, and then incubated with reciprocal shaking (120 strokes/min) at 28°C for 5 d.

Optimization of culture conditions for 9,12-hexadecadienoic acid production. These experiments were carried out with the same isolate, *Trichoderma* sp. AM076, throughout. The compositions of the liquid media and the cultivation conditions for 16:2ω4 production are described above or given in the legends to the respective figures.

Lipid analysis. The fungal mycelia were harvested by suction filtration, and then treated twice with chloroform/methanol/water according to the procedure of Bligh and Dyer (8). 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 (PL) fraction, the ex-

tracted lipids were separated on a silica gel thin-layer plate (Kieselgel 60, 200 × 200 × 0.25 mm; E. Merck, Darmstadt, Germany). Solvent systems of *n*-hexane/diethyl ether/formic acid (80:20:2, vol/vol/vol) and chloroform/methanol/water (65:25:4, vol/vol/vol) were used for triglycerides and PL, respectively. The gels corresponded to the bands of triglycerides and PL, stained with 0.01% primuline in 80% acetone, were scraped off. The lipids were then transmethylated.

The lipids were usually treated with 10% methanolic HCl for transmethylation. As an internal standard, *n*-pentadecanoic acid (0.2 mg) was usually included in the methanolysis mixture. Fatty acid methyl esters were extracted with 4 mL *n*-hexane, and then the extracts were concentrated in a centrifugal evaporator at 40°C.

The fatty acid methyl esters were extracted with *n*-hexane, concentrated in a centrifugal evaporator at 70°C, and then dissolved in acetonitrile for analysis by capillary gas–liquid chromatography (GLC) or high-performance liquid chromatography (HPLC). The analytical conditions were as follows: capillary GLC: apparatus, GC-14B (Shimadzu, Kyoto, Japan), equipped with a flame-ionization detector with a split injector; column, ULBON HR-SS-10 (0.25 mm × 50 m; Shinwa Chemical Industries, Tokyo, Japan); column temperature, 200°C; injection port temperature, 250°C; carrier gas, N_2 (inlet pressure, 200 kPa); make-up gas, N_2 (60 mL/min); air and H_2, 60 kPa; and split ratio, 25:1. HPLC: pump, LC-5A (Shimadzu); column, Inertsil ODS-2 (4.6 × 250 mm; GL Science, Tokyo, Japan); detector, SPD-2A (Shimadzu); wavelength, 205 nm; mobile phase, acetonitrile/water (95:5, vol/vol); flow rate, 1 mL/min; and column temperature, 30°C.

16:2ω4 methyl ester (10 mg) was isolated from mycelia of *Trichoderma* sp. AM076 (2.5 g dry cells from 300 mL culture) by HPLC under the conditions described above. The methyl ester of 16:2ω4 was eluted at 7.1 min under these conditions. The fractions that contained 16:2ω4 were collected and concentrated under reduced pressure.

The picolinyl ester of 16:2ω4 was prepared from 16:2ω4 free acid by the method of Christie *et al.* (9). Prior to gas chromatography–mass spectrometry (GC–MS) analysis, the resultant picolinyl ester was purified by HPLC under the same conditions as described above except for the wavelength (254 nm).

Other method. Mass spectra were measured with a Shimadzu GCMS-9100MK (ionization potential, 70 eV).

RESULTS

Isolation of microorganisms that accumulate 16:2ω4. The intercellular accumulation of 16:2ω4 was examined in about 750 culture strains, comprising 180 strains of yeasts, 120 strains of molds, and 450 isolated fungi from soil or freshwater samples. Through this screening, 11 strains of molds from stock cultures and 20 mold isolates showed 16:2ω4 production. Among these strains, one soil isolate, AM076, showed the maximal 16:2ω4 accumulation when grown in PGY medium (Table 1).

Identification of the isolate. The isolated fungal strain grew rapidly on 2% malt extract agar at 25°C in the dark. Its colonies reached over 9 cm in diameter in 5 d. The colonies were white at first but became greenish with age. The conidia were borne at the apex of flask-shaped phialides. They were one-celled, subglobose or globose, and greenish.

These taxonomic characteristics placed the isolated fungal strain in the hyphomycetous genus, *Trichoderma,* as judged by Domsch *et al.* (10). So, this strain was labelled as *Trichoderma* sp. AM076.

Culture conditions for the production of 16:2ω4 by Trichoderma *sp. AM076*. *Effect of culture time*. The mycelial yield in PGY medium reached the maximum after 5-d cultivation and then decreased gradually. The amount of 16:2ω4 increased markedly from 3 to 5 d of cultivation, and reached 98 mg/L culture broth (8 mg/g dry mycelia) after 6 d.

Effect of methyl palmitoleate concentration. The highest 16:2ω4 yield was obtained at 0.5% (wt/vol) methyl palmitoleate (8 mg/g dry mycelia, 98 mg/L culture medium), and further increased in methyl palmitoleate causing higher cellular yields, but the 16:2ω4 yield did not increase.

TABLE 1
Hexadecadienoic Acid Production by the Isolated Fungus and Fungal Type Cultures

Strain[a]	Mycelial yield (mg/mL of culture medium)	Total FA (mg/g dry mycelia)	16:2[b] yield (mg/mL of culture medium)	16:2 content (mg/g dry mycelia)
Trichoderma sp. AM076	15.4	0.11	0.133	8.6
T. longibrachiatum IFO4847	11.0	0.16	0.050	4.5
T. viride IFO 5720	13.1	0.11	0.039	3.0
T. polysporum IFO 9322	10.0	0.17	0.061	6.1
T. pseudokoningii IFO 30545	10.2	0.11	0.028	2.7
T. hamatum IFO 31291	7.8	0.15	0.055	7.0
T. reesei IFO 31326	12.4	0.11	0.088	7.1
T. aureoviride IFO 31694	9.9	0.16	0.054	5.5
Mortierella nana IFO 8190	3.1	0.13	0.012	3.8
Cladosporium cladosporioides IFO 30314	8.4	0.09	0.018	2.1
C. sphaerospermum IFO 6377	5.9	0.07	0.025	4.2
C. berbarium IAM 5059	5.1	0.06	0.016	3.1

[a]All strains were grown in PGY medium at 28°C for 5 d under the conditions given in the Materials and Methods section.
[b]Abbreviations: 16:2, 9,12-*cis*-hexadecadienoic acid; FA, fatty acid.

Effects of additional carbon and nitrogen sources. The carbon compounds listed in Figure 1 were tested as carbon sources for 16:2ω4 production. As shown in Figure 1, when myristic acid methyl ester (14:0ME) was added, growth, 16:2ω4 accumulation, and mycelial content reached 18.5 g/L culture broth, 247 mg/L culture medium, and 10.9 mg/g dry mycelia, respectively. These values were 1.1, 1.8, and 1.6 times higher than those for glucose addition. As shown in Figure 2A, the optimal 14:0ME concentration was 2%, and the production of 16:2ω4 reached 247 mgL medium (13.5 mg/g dry mycelia), but a further increase in the 14:0ME concentration did not increase the mycelial yield, and resulted in a decrease in 16:2ω4 production.

The accumulation of 16:2ω4 increased in parallel with the palmitoleic acid concentration with 14:0ME as the carbon source, and 2.0% palmitoleic acid gave the maximal 16:2ω4 productivity (430 mg/L culture medium, 16.5 mg/g dry mycelia).

Addition of a nitrogen source to the basal medium generally resulted in decreased production of 16:2ω4, but mycelial growth was rapid and dense (data not shown). As shown in Figure 2B, the optimal yeast extract concentration was in the range of 1–1.5%. Further increases in the yeast extract concentration resulted in increases in mycelial mass produced but in decreases in the 16:2ω4 yield. The maximal production of 16:2ω4 (443 mg/L, 17.4 mg/dry mycelia) was observed under the conditions of 2% methyl myristate, 2% methyl palmitate, and 1.5% yeast extract.

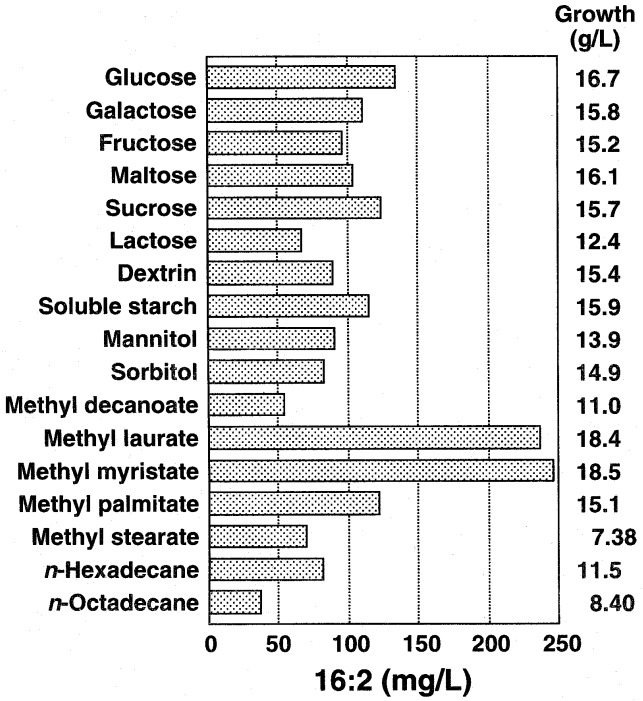


FIG. 1. Effects of carbon sources on the production of 9,12-hexadecadienoic acid (16:2ω4) by *Trichoderma* sp. AM076. The fungus was cultivated in a medium composed of 0.5% methyl palmitoleate, 1.0% yeast extract, and 2% of each carbon source, as indicated, for 5 d under the conditions given in the Materials and Methods section. All values are the means of three determinations, and the standard deviation is less than 7%.

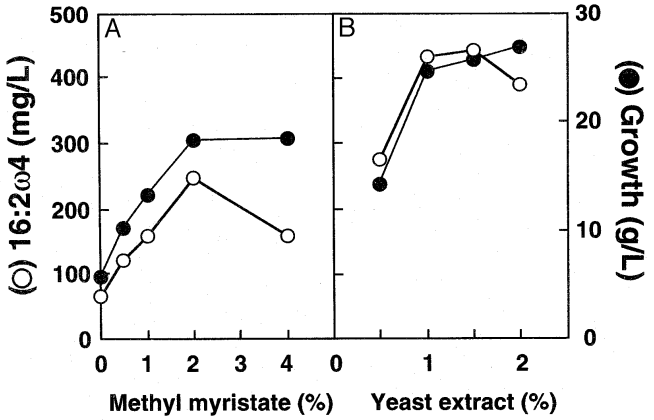
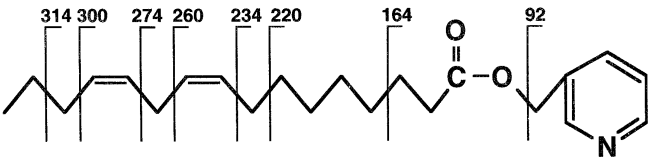


FIG. 2. Effects of the concentrations of methyl myristate and yeast extract on 16:2ω4 production. The fungus was grown at 28°C for 5 d in a medium that contained 0.5% methyl palmitoleate, 1.0% yeast extract, and the indicated concentrations of methyl myristate (A), or in a medium that contained 2% methyl palmitoleate, 2% methyl myristate, and the indicated concentrations of yeast extract (B). All values are the means of three determinations, and the standard deviation is less than 7%.

Distribution of fatty acids among major mycelial lipids. Upon cultivation in PGY medium at 28°C, the major fungal lipids were triacylglycerol (TG), diacylglycerol (DG), monoacylglycerol (MG), and PL fractions (Table 2). In all fractions, palmitoleic acid was the most abundant fatty acid. However, the percentages of dienoic fatty acids were relatively high in the MG and PL fractions (about 13% of total fatty acids found in these fractions), compared with the TG or DG fraction. A similar distribution was found for 16:2ω4. About 20% of 16:2ω4 produced was found in the PL fraction (12.7% of total 16:2ω4 found in this fraction) and the MG fraction (7.4%).

Identification of 16:2ω4 from Trichoderma *sp. AM076*. The chemical structure of 16:2ω4 was determined from its mass spectrum. The mass spectrum of the methyl ester showed fragment ion peaks at *m/z* 266 (M^+; relative intensity, 9%), 234, 164, 135, 123, 110, 95, 81 (base peak), 80, 67, and 55. This suggests that the isolated fatty acid methyl ester was hexadecadienoic acid.

The double-bond position was determined from the mass fragment pattern of the picolinyl ester of the fatty acid. The mass spectrum of the picolinyl ester showed characteristic fragment ion peaks at *m/z* 343 (M^+; relative intensity, 40%), 314, 300, 274, 260, 234, 220, 164 and 92 (base peak) (Scheme 1). This demonstrated that double bonds existed at



SCHEME 1

TABLE 2
Fatty Acid Compositions of the Major Lipids in *Trichoderma* sp. AM076

Lipid fraction[a]	Lipid comp. (%)[b]	Fatty acid composition (%)							
		16:0	16:1	16:2ω4	18:0	18:1	18:2ω6	18:3ω3	Others
TG	80.2	2.4	81.4	4.4	2.3	5.7	1.3	0.9	1.6
DG	7.0	8.9	81.6	1.4	0.8	4.8	0.6	trace	1.9
MG	4.8	7.8	74.6	7.1	0.9	5.0	2.8	0.9	0.9
PL	7.0	7.4	69.6	7.2	1.0	5.8	2.9	0.8	5.3

[a]Abbreviations: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; PL, phospholipid; 16:0, palmitic acid; 16:1, palmitoleic acid; 16:2ω4, 9,12-hexadecadienoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2ω6, linoleic acid; 18:3ω3, α-linolenic acid.
[b]Lipid composition. The indicated percentages of fatty acids were distributed in these fractions. Other minor lipids, such as sterols, sterol esters and free fatty acids, were not included in the calculation.

the Δ9 and Δ12 positions, and that the isolated fatty acid methyl ester was that of 9,12-*cis*-hexadecadienoic acid (16:2ω4).

DISCUSSION

The present report shows that 16:2ω4 accumulates in the mycelium of *Trichoderma* and some other filamentous fungi at room temperature, and palmitoleic acid (16:1ω7) is proposed to be converted to 16:2ω4 through the Δ12 desaturation reaction. The major fatty acid composition of *Trichoderma* sp. AM076, grown in GY medium (1% glucose, 0.5% yeast extract, pH 6.0), was 16:0 (20.3%), 18:0 (5.1%), 18:1 (16.3%), 18:2ω6 (42.6%), and 18:3ω3 (13.9%), and unsaturated fatty acids with a 16-carbon chain were detected in trace amounts. The accumulation of 16:2ω4 in *Trichoderma* sp. AM076 was observed when 16:1ω7 was added to the culture medium.

When *Trichoderma* sp. AM076 was grown in media with natural oils as carbon sources, the resultant mycelial fatty acid compositions were similar to those of the oils added. When some 16:1ω7-containing natural oils, such as lard (4.3% of total fatty acid), horse tallow (9.1%), beef tallow (2.8%), whale oil (11.1%) and macadamia nut oil (22.3%), were added to the medium as sources of both carbon for growth and palmitoleic acid, only a small amount of 16:2ω4 was observed (data not shown). This suggests that 16:1ω7 was only converted to 16:2wω4 when the mycelial levels of other unsaturated acids, such as 18:2 and 18:3, were low.

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