Mycoscience (2005) 46:329–333
DOI 10.1007/s10267-005-0255-7
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FULL PAPER

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The characteristics of desaturation of *Trichoderma* sp. AM076 and formation of 9,12,15-hexadecatrienoic acid (16:3ω1) through Δ15 desaturation of 9,12-hexadecadienoic acid

Received: June 6, 2005 / Accepted: July 25, 2005

Abstract We investigated the characteristics of desaturation in *Trichoderma* sp. AM076. Although 6,9,12-octadecatrienoic acid (18:3ω6) was detected when *Trichoderma* sp. AM076 was cultivated in the presence of 6,9-octadecadienoic acid (18:2ω9), the desaturation products of 6,9,12-octadecatrienoic acid (18:3ω6) and 6-octadecenoic acid (18:1Δ6) were not detected. These results suggest that the double bonds at the Δ6 position of 18:3ω6 and 18:1Δ6 disturb their Δ15 and Δ9 desaturation, respectively. This fungus also introduced a double bond at the Δ15 position of 9,12-hexadecadienoic acid (16:2ω4), thereby yielding a novel C16 polyunsaturated fatty acid (PUFA) identified as 9,12,15-hexadecatrienoic acid (16:3ω1). Further investigations revealed that the mutant having enhanced accumulation of linolenic aid (18:3ω3) accumulates 16:3ω1 as one of the major PUFAs, together with 9,12-octadecadienoic acid (16:2ω4), when grown with palmitoleic acid (16:1ω7). These results suggest that, in this strain, the reaction that catalyzes the conversion of linoleic acid to linolenic acid, similar to the conversion of 16:2ω4 to 16:3ω1, is not ω3 desaturation but Δ15 desaturation.

Key words Desaturase · 9,12,15-Hexadecatrienoic acid · Palmitoleic acid · *Trichoderma* fungus

Introduction

Previously, we reported that several polyunsaturated fatty acid (PUFA)-producing fungi possess the ability to convert fatty substrates such as odd-chain fatty acids, odd-chain alkanes, or 1-alkenes to the corresponding odd-chain or ω1 PUFAs (Shimizu et al. 1991a,b; Shirasaka et al. 1995). Our recent studies revealed that when *Trichoderma* sp. AM076 was grown with palmitoleic acid (16:1ω7), it accumulated a rarely occurring C16 PUFA, namely, 9,12-hexadecadienoic acid (16:2ω4) (Shirasaka et al. 1998). Although the mycelial content of α-linolenic acid (18:3ω3) reached approximately 10% when the fungus was grown in a medium without 16:1ω7, a scarce amount of a fatty acid with three double bonds, for example, 9,12,15-hexadecatrienoic acid (16:3ω1), was detected in the mycelial lipid when the fungus was grown with 16:1ω7. This observation suggests that, in this strain, it is more difficult to introduce further double bonds for the conversion of 16:2 to 16:3 when compared with that of linoleic acid (18:2ω6) to 18:3ω3.

In the present study, we investigated the characteristics of fatty acid desaturation in *Trichoderma* sp. AM076. In addition, we report the isolation of the mutant having enhanced accumulation of α-linolenic acid and formation of 16:3ω1 when the mutant was grown in a medium supplemented with palmitoleic acid methyl ester.

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Materials and methods

Chemicals

9,12-Hexadecadienoic acid (16:2ω4) was prepared from the mycelial lipid of *Trichoderma* sp. AM076 grown with palmitoleic acid. All other fatty acids used in this study were purchased from Funakoshi (Tokyo, Japan). All other reagents were of analytical grade.

Microorganism and cultivation

Trichoderma sp. AM076 (AKU3999-15, Faculty of Agriculture, Kyoto University) was used in the present study. The fungus was inoculated into a 20-ml Erlenmeyer flask containing 4 ml GY medium [1% glucose, 0.5% yeast extract

(Oriental), pH 6.0] or PGY medium (GY medium supplemented with 0.5% methyl palmitoleate) and subsequently incubated with reciprocal shaking (120 strokes/min) at 28°C for 7 days, unless otherwise noted.

Mutagenesis and isolation of a mutant with enhanced accumulation of linolenic acid

Trichoderma sp. AM076 was inoculated onto Czapek agar medium (0.2%, w/v, $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 7H_2O$, 3% sucrose, and 2% agar, pH 6.0) and was allowed to sporulate at 28°C for 2 weeks. The spores were collected by pouring 50 ml sterile water containing a drop of Tween 80 into the medium, followed by filtration through four layers of gauze and centrifugation of the filtrate at 3000 rpm for 10 min. The spores thus obtained were suspended in 50 mM-Tris/maleate buffer (pH 7.5) at a concentration of approximately 1×10^6/ml and then exposed to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) (100 mg/ml) at 28°C with reciprocal shaking for 20 min. This step resulted in survival rates of 1%–5%. Three milliliters 10% (w/v) sodium thiosulfate was added, followed by centrifugation at 3000 rpm for 10 min and two washes with sterile distilled water.

The NTG-treated spores were plated onto agar medium composed of 2% glucose, 1% yeast extract, and 1.8% agar, and incubated at 28°C for 3–5 days. Approximately 50 colonies were randomly selected for each mutagenesis, and each colony was grown in liquid culture as described next. Mutants with fatty acid compositions that differed from that of the parent strain were stored on potato dextrose agar medium.

Lipid analysis

The fungal mycelia were harvested by suction filtration and subsequently treated twice with chloroform-methanol-water in accordance with the procedure of Bligh and Dyer (Bligh and Dyer 1959). The resultant lipid extract was evaporated to dryness under reduced pressure at 35°C and then used as the sample for transmethylation. To analyze the fatty acid composition of the triacylglycerol or phospholipid fraction, the extracted lipids were separated on a silica gel thin-layer plate (Kieselgel 60, 200 × 200 × 0.25 mm; Merck, Darmstadt, Germany). *n*-Hexane-diethyl ether-formic acid (80:20:2, by volume) and chloroform-methanol-water (65:25:4, by volume) were used as the solvent systems for triacylglycerols and phospholipids, respectively. The gel that corresponded to the bands of the triacylglycerols and phospholipids was stained with 0.01% primuline in 80% acetone and was scraped off. The lipids were then transmethylated.

The lipids were mostly 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; 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 60°C, and then dissolved in acetonitrile for analysis by capillary gas-liquid chromatography (GLC). The analytical conditions were as follows: apparatus, GC-14B (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) with a split injector; column, ULBON HR-SS-10 (0.25 mm × 50 m; Shinwa, Kyoto, Japan); column temperature, 200°C; injection port temperature, 250°C; carrier gas, N_2 (inlet pressure, 200 kPa); makeup gas, N_2 (60 ml/min); air and H_2, 60 kPa; and split ratio, 25:1.

The purification of fatty acid methyl esters by high performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-5A HPLC system with a Cosmosil 5C18-AR column (4.6 × 250 mm; Nakalai Tesque, Kyoto, Japan) [column temperature, 30°C; detector, SPD-2A (Shimadzu); mobile phase, acetonitrile/water (98:2, by volume); flow rate, 1 ml/min; wavelength, 205 nm]. Five milligrams 16:3ω1 methyl ester was isolated from the mycelia of mutant TU42 (4.9 g dry cells from 500 ml culture) by HPLC under the conditions already described. The methyl ester of 16:3ω1 was eluted at 5.4 min under these conditions. The fractions containing 16:3ω1 were collected and concentrated under reduced pressure.

Picolinyl ester of 16:3ω1 was prepared from 16:3ω1 free acid in accordance with the method of Christie (Christie et al. 1986). Before gas chromatograph-mass spectrometry (GC-MS) analysis, the resultant picolinyl ester was purified by HPLC under the same conditions as previously described except for the wavelength (254 nm).

Other methods

Mass spectrum was measured using a Shimadzu GCMS-9100MK (ionization potential, 70 eV), and proton nuclear magnetic resonance (^{1}H-NMR) was performed using a JOEL NMR-270EX apparatus (270 MHz) with tetramethylsilane as the internal standard.

Results

Specificity of double-bond position on fatty acid desaturation

The effects of fatty acid addition on the fatty acid compositions of *Trichoderma* sp. AM076 were investigated. Although the supplemented fatty acids were detected in cellular lipids, additional peaks were detected on GLC only when 6,9-octadecadienoic acid (18:2ω9) or 9,12-hexadecadienoic acid (16:2ω4) was supplemented to the medium (Fig. 1). An additional peak corresponding to 6,9,12-octadecatrienoic acid (γ-linolenic acid; 18:3ω6) was observed on GLC when 18:2ω9 was added to the medium. An unusual fatty acid that was considered to be 16:3ω1 was also detected when the fungus was grown in the presence of 16:2ω4. Supplementation of 6-octadecenoic acid (18:1Δ6)

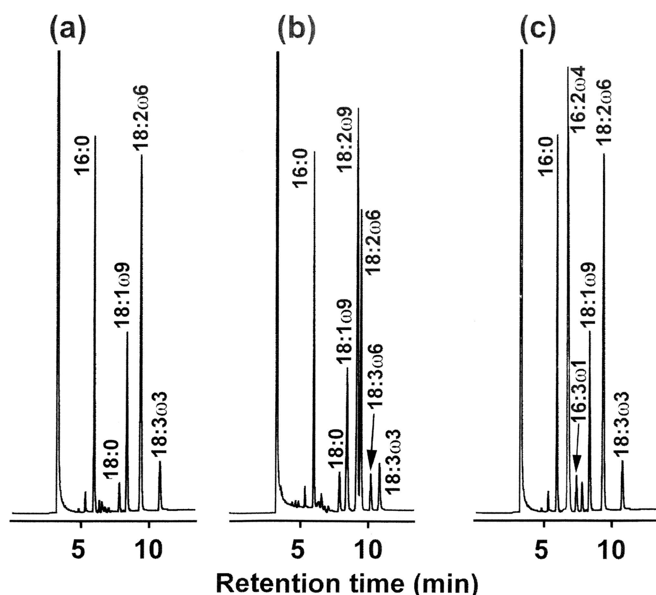

Fig. 1. Gas-liquid chromatograhy (GLC) chromatograms of fatty acid methyl esters obtained from mycelia of *Trichoderma* sp. AM076 grown in medium supplemented without 18:2ω9 and 16:2ω4 (**a**), with 18:2ω9 (**b**), and with 16:2ω4 (**c**). The cultures were carried out in triplicate in a medium containing 0.5% glucose and 0.5% yeast extract (pH 6.0) at 28°C for 1 day. Subsequently, the fatty acids were added in the form of an albumin complex, which was prepared by sonicating 10 mg fatty acid with 1 ml sterile 5% bovine serum albumin (BSA) for 20 min, into the culture medium at a rate of 0.5 mg/ml medium. Incubation was carried out for an additional 6 days. Cells grown without the addition of fatty acids were considered as the controls

or 18:3ω6 did not produce any peaks that could be considered as desaturation products on GLC analysis.

Characterization of 16:3ω1

The mass spectrum of the picolinyl ester of the isolated unknown fatty acid showed fragment ion peaks at m/z 303 (M^+; relative intensity, 52%), 274, 260, 234, 220, 164, and 92 (base peak) (Fig. 2). The ^{1}H-NMR spectrum of the methyl ester in $CDCl_3$ showed absorption peaks at $\delta = 5.02$ and 5.82, thereby indicating an ω-1 double bond (Fig. 2). These results showed that the isolated fatty acid was 9,12,15-hexadecatrienoic acid (16:3ω1).

Isolation of α-linolenic acid highly accumulating mutant and conversion of palmitoleic acid to 9,12,15-hexadecatrienoic acid by mutant

Tests were carried out on approximately 1300 colonies, and 10 mutants with enhanced accumulation of linolenic acid (20%–30% in mutants, 8% in the parent strain) were obtained. These mutants possessed the ability to utilize glucose as a substrate for growth; however, they were unable to grow on *n*-alkane and fatty acid methyl esters (data not shown). Although the mycelial yield was decreased in the mutants, increase in the total fatty acid content prevented changes in the total fatty acid productivities of the mutants (data not shown). When the parent strain AM076 and the mutant TU42 were grown in a medium supplemented with palmitoleic acid (16:1ω7), an apparent additional peak on GLC chromatograms was observed in the mycelial lipid of mutant TU42 when compared with those of the parent

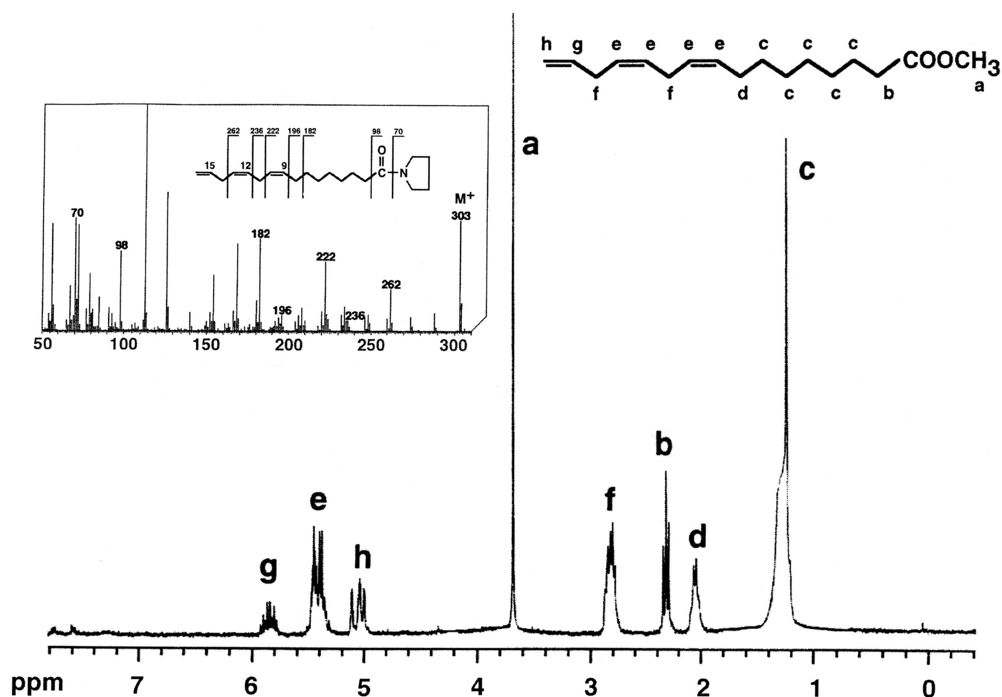

Fig. 2. Characterization of 9,12,15-hexadecadienoic acid from mutant TU42, a *Trichoderma* sp. AM076 mutant, by proton nuclear magnetic resonance (^{1}H-NMR) and mass spectrometry (MS) spectra

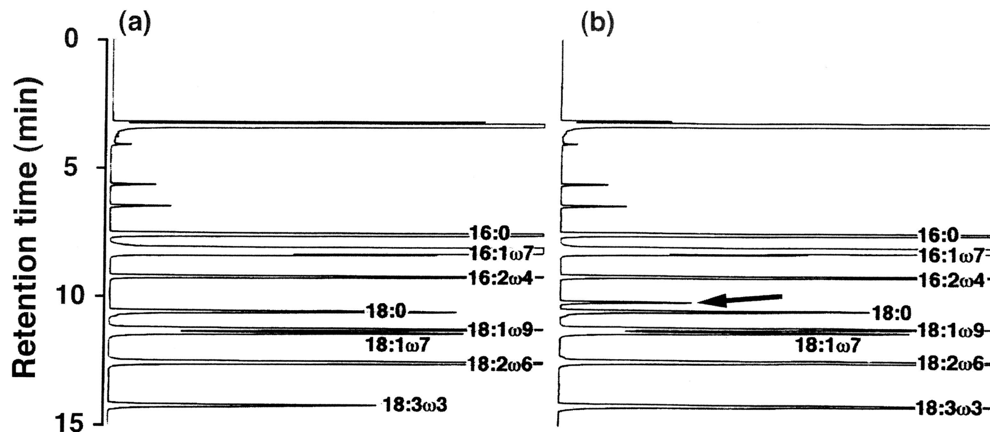

Fig. 3. GLC chromatograms of fatty acid methyl esters obtained from the mycelia of (**a**) *Trichoderma* sp. AM076 and (**b**) mutant TU42 grown with palmitoleic acid methyl ester. Cultivation of the strain and fatty acid analysis were carried out as described in the text. 14:0, myristic acid; 15:0, pentadecanoic acid; 16:0, palmitic acid; 16:1ω7, palmitoleic acid; 16:2ω4, 9,12-hexadecadienoic acid; 18:0, stearic acid; 18:1ω7, vaccenic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω3, α-linolenic acid

Table 1. Comparison of 16:2 and 16:3 productivities of *Trichoderma* sp. AM076 and its mutant

Conditions		Productivity					Mycelial fatty acid composition (weight %)								
Strain	Medium	Growth (mg/ml)	Mycelial PUFA content[a]		PUFA yield[b]										
			16:2	16:3	16:2	16:3	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	Others
AM076	GY[c]	13.1	–	–	–	–	23.9	0.48	–	–	4.81	15.9	46.3	7.47	1.15
	PGY[d]	16.7	8.11	–	135	–	4.55	78.0	5.25	–	1.44	4.31	4.62	0.69	1.14
TU42	GY	9.70	–	–	–	–	24.7	0.66	–	–	2.71	8.41	37.8	24.0	1.43
	PGY	10.5	9.17	1.79	95.9	18.8	6.07	72.6	3.66	0.72	1.56	3.87	5.69	4.39	1.44

PUFA, polyunsaturated fatty acid; –, not detectable
[a] Content in mg/g dry mycelia
[b] Yield in mg/l
[c] Grown for 4 days at 25°C in medium GY containing 2% glucose, 1% yeast extract, pH 6.0
[d] Grown for 4 days at 25°C in medium PGY containing 2% glucose, 1% yeast extract, 0.5% methyl palmitoleate, and 0.01% Tween 80, pH 6.0

Table 2. Fatty acid compositions of the major lipids in *Trichoderma* mutant TU42

Lipid fraction	Lipid composition (%)[a]	Fatty acid composition (%)								
		16:0	16:1	16:2ω4	16:3ω1	18:0	18:1	18:2ω6	18:3ω3	Others
TG	81.5	2.6	81.0	3.2	0.6	2.1	5.0	1.5	2.5	1.5
DG	6.5	8.2	81.2	0.8	0.2	0.6	4.4	1.2	2.1	1.3
MG	4.9	8.2	74.0	5.4	1.4	0.7	4.7	3.2	1.4	1.0
PL	7.1	10.5	68.5	5.3	1.8	0.8	5.2	3.5	1.3	3.1

TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; PL, phospholipid
[a] The indicated percentages of fatty acids were distributed in these fractions

strain (Fig. 3). The same results were observed in all other mutants obtained.

Productivity of 16:3ω1 and fatty acid distribution among the major mycelial lipids

In all the mutants included in this study, the mycelial yields were reduced to approximately 60% of that of the parent strain when the mutants grown in a medium supplemented with 16:1ω7 (Table 1). Among them, mutant TU42 showed maximum 16:3ω1 productivity (18.8 mg/l culture broth, 1.79 mg/g dry mycelia).

When cultivated in PGY medium at 28°C, the major lipids of mutant TU42 were triacylglycerol (TG), diacylglycerol (DG), monoacylglycerol (MG), and a phospholipid fraction (PL) (Table 2). In all fractions, palmitoleic acid was the most abundant fatty acid, but the percentages of polyenoic fatty acids were relatively high in the MG and PL fractions (approximately 15% of the total fatty acids found in these fractions) when compared with those in the TG or DG fraction. A similar distribution was observed in the case

of 16:3ω1; 18.3% of total 16:3ω1 was found in the PL fraction and 9.8% was in the MG fraction.

Discussion

Previous investigations showed that odd-chain polyunsaturated fatty acids with chain lengths of 17 and 19 carbons were produced when *Mortierella alpina* 1S-4 was grown with *n*-pentadecane (or *n*-heptadecane) (Shimizu et al. 1991b). A similar conversion was also demonstrated in the case of the C16, C18, and C20 ω1-unsaturated fatty acids from 1-hexadecene (or 1-octadecene) by the same fungus (Shimizu et al. 1991a). In these cases, the introduction of double bonds was carried out in the same manner to mimic the ω6 route for the conversion of 18:2ω6 to 20:4ω6 and proceeding irrespective of the ω-terminal structure of the precursor fatty acids. In a recent study, we demonstrated that *Trichoderma* sp. AM076 introduces the double bond at Δ12 position of 16:1ω7 to form 16:2ω4; this is similar to the conversion of oleic acid to linoleic acid. In this study, the addition of 16:2ω4 resulted in the formation of 16:3ω1 (see Fig. 1). This result indicates that *Trichoderma* sp. AM076 is capable of introducing a double bond at the Δ15 position of 16:2ω4 to yield 16:3ω1. On the other hand, in this fungus, desaturation was not observed at the Δ9, Δ12, or Δ15 position of 18:1Δ6 and at the Δ15 position of 18:3ω6. These results indicate that each desaturation reaction may require a specific double-bond configuration.

To evaluate the double-bond introducion at the Δ12 and Δ15 position to C16 PUFA in the *Trichoderma* strain, conversion of 16:1ω7 in mutant TU42 was compared with *Trichederma* sp. AM076. Although a trace amount of 16:3ω1 was detected in mycelial lipid of the parent strain in the presence of 16:1ω7, apparent accumulation of 16:3ω1 was observed in that of mutant TU42. The 16:2ω4 content in mutant TU42 was slightly decreased when compared with that in the parent strain; however, no significant difference was observed in the 16:2ω4 and 16:3ω1 content between both strains. The conversion of 16:2ω4 to 16:3ω1 was enhanced similar to the conversion of linoleic acid to linolenic acid in the mutants. The biosynthetic route to 16:3ω1 was presumed to mimic the route to 18:3ω3.

It is generally believed that the desaturation of C18 fatty acids occurs as follows. Oleic acid is formed from stearic acid through Δ9 desaturation, followed by Δ12 desaturation to linoleic acid. Then, desaturation occurs either at the Δ6 position to yield γ-linolenic acid or at the ω3 position to yield α-linolenic acid. In these reactions, three classes of regioselectivity have been observed: Δ^x desaturases introduce a double bond for x carbons from the carboxyl end; ω-x desaturases dehydrogenate x carbons from the methyl terminus; while desaturases use a pre-existing double bond as a reference point and dehydrogenate x carbons from the nearest olefinic carbon (Meesapyodsuk et al. 2000). In this study, the conversion of 16:2ω4 to 16:3ω1 was not catalyzed by ω-x (ω-3) type desaturase, but by either Δ^x (Δ15) or ν + x (Δ12+3) type desaturase.

Acknowledgment This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

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