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Quantitative separation of *Trichoderma* lipid classes on a bonded phase column

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ABSTRACT

Bond Elut aminopropyl columns were used to purify the different lipid classes of *Trichoderma harzianum* and *Trichoderma viride*. This methodology permitted good separation of the fungal lipid classes in less time than traditional techniques. The incorporation of [1-^{14}C]linoleic acid into neutral lipids, free fatty acids and phospholipids was quantified for both strains. The fatty acid profile of the different lipid classes of these fungal strains was determined as a function of culture time.

INTRODUCTION

Certain species in the genus *Trichoderma* have been shown to be able to biosynthesize 6-pentyl-α-pyrone, an unsaturated lactone with a characteristic coconut aroma [1–3]. Fungal fatty acid bioconversions into aroma compounds have been reported [4,5]. A better knowledge of the fatty acid composition of fungal cell lipid fractions is required to confirm the lipid origin of such compounds.

Procedures for the purification of different lipid classes from various microorganisms have been reported [6–8]. However, these early procedures were time-consuming, consisting of several steps that limited their application.

Thin-layer chromatographic (TLC) techniques allowed good separation of lipid classes but no quantitative analysis, and oxidation of polyunsaturates often occurred. Recently, an improved purification scheme has been reported for biological fluids using Bond Elut aminopropyl disposable columns [9]. This method allows the separation of neutral lipids, free fatty acids and phospholipid fractions. It also maintains lipid integrity.

This paper describes an application of bonded phase columns for the study of the incorporation of [1-^{14}C]linoleic acid into different lipid classes of two *Trichoderma* strains and for the analysis of the fatty acid composition profile in the different lipid classes during the growth of both microorganisms.

EXPERIMENTAL

Fungal strains and inoculum preparation

Trichoderma viride (strain No. 26), isolated in our laboratory from soil, and *T. harzianum* (IMI 206040) were used. The strains were kept on potato dextrose agar (PDA) medium. The preculture medium (50 ml) consisted of malt extract (20 g/l) and glucose (10 g/l). It was inoculated with five-day-old mycelium (1 cm^2 from conservation medium) and incubated in a 150-ml Erlenmeyer flask at 27°C for 72 h on a rotary shaker at 100 rpm. The mycelium was recovered by centrifugation for 20 min at 8000 *g*. The mycelium sus-

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pension (10 g/l) was homogenized in a Virtis grinder (Products Corporation, Racine, WI, USA) to obtain mycelial fragments 50–200 μm in length.

Culture growth and incorporation of [1-^{14}C]linoleic acid

The mineral basic culture medium was as described by Evans and Ratledge [10]. Glucose was added at a concentration of 30 g/l, and ammonium sulphate at 0.94 g/l (carbon/nitrogen ratio = 60). Cultures were grown in 10 ml of medium in 50-ml Erlenmeyer flasks, inoculated with 0.2 ml of the previously described mycelial suspension and incubated on a shaker at 27°C. For the studies of linoleic acid incorporation in fungal cells, 0.5 μCi of [1-^{14}C]linoleic acid (specific activity 54.6 mCi/mmol, supplied by Amersham, Les Ulis, France) was added to each culture after 48 h of incubation. Incubation was maintained for a further 24 h. Experiments were run in triplicate to allow statistical analysis. Mycelial growth was monitored by dry weight determination. After incubation, control culture mycelium was harvested, washed and dried to constant weight at 105°C.

Chromatographic procedures

The mycelium was broken up by ultrasonication at 250 W, for 10 min, using a Vibra Cell Sonics and Materials apparatus (Danbury, CT, USA) in the presence of a chloroform–methanol mixture (2:1, v/v). The organic phase was recovered after being washed with 0.35 *M* KCl and dried under nitrogen flux. For lipid fractionation, Bond Elut NH_2 aminopropyl columns (500 mg, Prolabo, Paris, France) were placed in a Vac Elut apparatus and equilibrated with two 2-ml volumes of chloroform under vacuum (10 kPa). Then 5–10 mg of total lipids were applied to the column under vacuum and eluted, by consecutive passage of 4 ml of each of the following mixtures: chloroform–propanol (2:1), acetic acid (2%) in diethyl ether, and methanol. The fractions obtained, in order of elution, were neutral lipids, free fatty acids and phospholipids [9]. For the studies of [1-^{14}C]linoleic acid incorporation in fungal cells, an aliquot of each fraction was put into scintillation vials and counted in 4 ml of counting solution in a Tricarb Packard counter. Lipid recovery was calculated from the counts recovered in all the fractions divided by the counts applied to the column. For the studies of fatty acid profiles of fungal lipids, each lipid fraction obtained was dried under nitrogen and methylated at 100°C for 1 h with 1 ml of methanol and five drops of 18 *M* sulphuric acid. The fatty acids in each fraction were analysed in methylated form by gas chromatography (GC).

The fatty acid methyl esters were analysed by GC with a Packard chromatograph, Model 627A (Chrompack, Middelburg, Netherlands), equipped with a flame ionization detector and an SE-30 capillary column (0.32 mm I.D., 0.25 μm film thickness; Spiral, Dijon, France). The oven temperature was adjusted from 150 to 230°C at 1°C/min. The injector and detector temperatures were 250 and 315°C, respectively. Nitrogen was used as carrier gas. The internal standard was heptadecanoic acid.

RESULTS AND DISCUSSION

Incorporation of [1-^{14}C]linoleic acid in fungal cells

Incorporation of [1-^{14}C]linoleic acid by *T. harzianum* and *T. viride* was monitored for 24 h. Table I shows the lipid recovery yields, after fractionation by the bonded phase column, obtained with the different samples from incorporation kinetics. Good lipid recoveries were obtained. Total lipids were separated with more than 90% yield, and a lipid assimilation of *ca.* 50% was observed at the end of the culture time. Purified lipid fractions are shown in Fig. 1.

[1-^{14}C]Linoleic acid incorporation into different lipid classes is reported in Table II. The radiolabel was principally assimilated into phospholipids in both strains. *T. viride* incorporated more than 70% of the label into phospholipids; for *T. harzianum* cultures this value varied between 54 and 66%. Divergences in fatty acid assimilation in both strains were probably due to differences in structural membrane composition. However,

TABLE I

RECOVERY OF LABELLED LINOLEIC ACID AT DIFFERENT CULTURE TIMES

Total counts represent the amount of labelled linoleic acid applied to the Bond Elut column. Recovered counts were evaluated by addition of individual countings of each lipid fraction after column separation.

Time (days)	*Trichoderma harzianum*			*Trichoderma viride*		
	Total counts (dpm)	Recovered counts (dpm)	Yield (%)	Total counts (dpm)	Recovered counts (dpm)	Yield (%)
4	107 440	98 556	92	27 200	25 315	93
8	155 150	139 080	90	64 640	59 786	92
12	283 740	258 204	91	151 090	140 084	93
24	251 090	230 294	92	234 750	224 969	96

the greater linoleic acid consumption in *T. harzianum* cells should also be considered.

Neutral lipids were the second most abundant group in which [1-^{14}C]linoleic acid was incorporated. Triacylglycerols, which constitute the cell energy reserve, are the major components of this group (see Fig. 1). It has been reported that certain microorganisms are able to accumulate lipids when the culture medium is deficient in nitrogen [11–13].

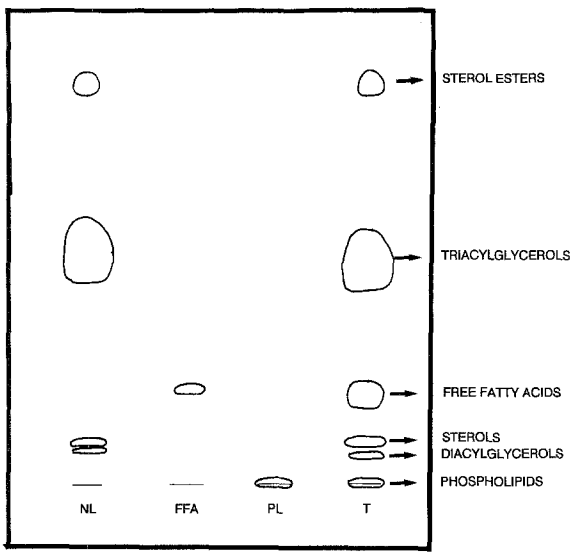

Fig. 1. Purity verification by TLC of fractionated lipids of *Trichoderma harzianum*. After separation of lipid classes on a Bond Elut aminopropyl column, the recovered fractions were applied to silica plates with a solvent system of hexane–diethyl ether–acetic acid (80:20:1, v/v/v). NL = neutral lipids; FFA = free fatty acids; PL = phospholipids; T = total lipids.

TABLE II

INCORPORATION OF LABELLED LINOLEIC ACID BY *T. HARZIANUM* AND *T. VIRIDE*

Values represent the percentage of recovered radioactivity (dpm) found in each lipid fraction: NL = neutral lipids; FFA = free fatty acids; PL = phospholipids.

Time (h)	*Trichoderma harzianum*			*Trichoderma viride*		
	NL	FFA	PL	NL	FFA	PL
4	44.14	1.12	54.74	22.31	0.92	76.76
8	38.37	1.19	60.44	19.93	0.76	79.31
12	45.13	1.34	53.53	27.04	0.96	72.00
24	32.71	1.09	66.20	27.27	0.90	71.83

The labelled free fatty acid content in both fungal strains remained very low: accumulation in free fatty acids fungal cells should be a transient stage before incorporation into the other lipid classes.

Fatty acid profile in lipid fractions

T. harzianum and *T. viride* were cultured for six days, and the fatty acid profiles of neutral lipids, free fatty acids and phospholipids were obtained (Table III).

It can be seen that, in *T. harzianum* the neutral lipids represent the more important class of intracellular lipids in all of the samples. A slow increase in this lipid class was observed during fungal growth. Phospholipids and free fatty acids remained nearly constant in the same conditions.

TABLE III

FATTY ACID DISTRIBUTION IN DIFFERENT LIPID FRACTIONS OF *T. HARZIANUM* AND *T. VIRIDE* AT DIFFERENT CULTURE TIMES

Analyses were done by gas chromatography (see Experimental). Values are expressed as % (w/w) of total fatty acids. Values in parentheses represent the standard deviation calculated from three replicates. The values of mg FA/100 mg dry weight are the total amounts of fatty acids in each lipid fraction.

Fatty acid	Neutral lipids			Free fatty acids			Phospholipids		
	Two days	Four days	Six days	Two days	Four days	Six days	Two days	Four days	Six days
Trichoderma harzianum									
16:0	21.75 (1.25)	39.25 (1.08)	23.06 (1.18)	24.00 (1.37)	22.72 (1.48)	25.00 (0.75)	15.43 (0.68)	19.26 (1.12)	14.18 (1.03)
18:2	33.11 (1.42)	25.23 (1.31)	37.44 (1.22)	44.00 (1.27)	40.91 (1.21)	37.50 (0.87)	58.28 (1.36)	61.48 (1.67)	69.40 (1.36)
18:1	38.96 (1.15)	26.63 (1.38)	34.47 (1.22)	20.00 (0.93)	27.27 (1.20)	25.00 (0.72)	25.71 (1.31)	16.29 (0.89)	16.41 (0.71)
18:0	6.17 (1.09)	8.82 (0.97)	5.02 (0.75)	12.00 (0.68)	9.09 (0.64)	12.50 (0.83)	0.57 (0.18)	2.96 (0.18)	–
mg FA/100 mg dry weight	3.08	2.74	4.38	0.25	0.22	0.24	1.75	1.35	1.34
Trichoderma viride									
16:0	15.13 (0.95)	16.00 (0.74)	15.63 (0.82)	22.80 (1.24)	18.05 (1.48)	19.56 (1.71)	11.34 (0.91)	11.70 (0.86)	11.70 (1.03)
18:2	37.50 (1.15)	29.06 (0.97)	26.68 (1.04)	42.85 (1.33)	29.86 (1.02)	18.69 (0.79)	56.41 (1.42)	45.28 (1.09)	35.67 (1.17)
18:1	40.13 (1.23)	48.26 (1.14)	52.56 (1.48)	25.71 (1.52)	48.61 (1.21)	54.34 (1.39)	31.64 (1.81)	42.64 (1.45)	52.04 (1.33)
18:0	7.23 (0.35)	6.66 (0.79)	5.12 (0.84)	8.57 (0.78)	3.47 (0.65)	6.52 (0.43)	0.90 (0.28)	0.37 (0.17)	0.59 (0.16)
mg FA/100 mg dry weight	1.52	3.75	3.71	0.35	1.44	0.46	3.35	2.65	1.71

Neutral lipid progression was faster in *T. viride* cultures than in *T. harzianum* cultures (1.52–3.75 mg fatty acids per 100 mg dry weight from two to four days), whereas the phospholipid fraction decreased. The high content in phospholipid fatty acids (3.35–2.65 fatty acids per 100 mg dry weight from two to four days) should explain the greater [1-^{14}C]linoleic acid assimilation into *T. viride* phospholipid fraction than in *T. harzianum*. The free fatty acids fraction did not vary significantly.

Unsaturated fatty acids were, in almost cases, the principal constituents of lipids in both strains, although some differences were found. In *T. viride* cultures, the oleic acid (18:1) content increased in all the fractions and represented the principal fatty acid. In contrast, linoleic acid (18:2) was the most important fatty acid present in lipids from *T. harzianum* cultures, even though its level fluctuated during growth.

CONCLUSION

The bonded phase column method was successfully applied to the separation of lipids from fungal cells. This methodology allowed accurate separation of small amounts of lipids in less time and required smaller amounts of solvents than traditional techniques, such as TLC.

The incorporation of a labelled fatty acid into the different fungal lipid classes was evaluated after their fractionation on the bonded phase columns. The membrane structure of these microorganisms appears to have an effect on the assimilation profile of exogenous fatty acids. Unsaturated fatty acids were predominant in all the lipid fractions from fungal extracts. We plan to use this methodology in further investigations to study fatty acid bioconversions into aroma compounds by *T. harzianum* and *T. viride*.

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