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Property Characterization and Lipid-Compositional Analysis of Lipid Granules Isolated from an Oleaginous Yeast *Rhodotorula glutinis*

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Abstract

Preparative isolation of lipid granules from *Rhodotorula glutinis*, which has been studied for long time to produce edible lipids, was carried out by flotation method in Ficoll-linear density gradient. When the isolated lipid granules were suspended in a series of solutions containing varying concentration of osmotic stabilizer (sorbitol and mannitol) ranging from 0.8 M to 0 M, the lipid granules appeared to be disrupted at a concentration between 0.8 M and 0.7 M, and again at a concentration below 0.1 M, suggesting that lipid granules have a membraneous structure and that at least two types of lipid granules are present. Compositional analysis of lipids from lipid granules revealed that lipids are composed mainly of neutral lipids (87.8% of total lipids), predominantly as triacylglycerols (71.8%). Marked differences were observed in phospholipids between lipids of lipid granules and those of whole cells. The major components of phospholipids in lipid granules and in whole cells are phosphatidylcholine (38.6%) and phosphatidylserine (42.8%), respectively. In addition, significant differences were also observed in the fatty acid composition of phospholipids. As phospholipids are important structural components of membranes, these differences lead to the suggestion that the membrane of lipid granules may be distinct functionally and structurally from other membranes of yeast cells. The major fatty acid components of neutral lipids of whole cells and lipid granules are palmitic, oleic, and linoleic acid. However, degree of fatty acid unsaturation of neutral lipids of lipid granules was much lower than that of neutral lipids of whole cells.

Key words: lipid granule, *Rhodotorula glutinis*, oleaginous yeast, fatty acid composition

INTRODUCTION

Production of edible oils from microorganisms such as yeasts or fungi has been studied for long time (1,2). The microorganism has also been investigated to produce high value commodities such as polyunsaturated fatty acids of n-3 and n-6 series, some eicosanoids, or unusual lipids (1,3). The living organisms that are used for the production of lipids contain lipid granules in their cells as an organelle to store lipids (4-6). The lipid granules have been studied most extensively in plants, in which there is no doubt that lipid granules is the major site of lipid metabolism (7). In contrast, lipid granules of yeast have been insufficiently studied and most of the available data were obtained from respiratory-deficient yeast such as *Saccharomyces* sp., in which lipid content is about or less than 10% (4,8). In the lipid granules isolated from *S. cerevisiae*, lipids consist mainly of triacylglycerols (approximately 47%) and sterolesters (approximately 44%) (8). However, in an oleaginous yeast *Lipomyces starkeyi*, the lipid granules contain a large amount of triacylglycerol (~85%) (9), which is quite different from the lipid composition of the lipid granules isolated from the non-oleaginous yeast. This leads to the speculation that there may be a fundamental difference in the lipid metabolism between two types of yeast. To the investigators of microbial oil production, it has long been questioned as to why the oleaginous yeast can accumulate so much lipid (in some case, 70% of cell-dry weight). Some investigators have been interested in the enzyme-regulation mechanism of oleaginous yeasts (10,11). Another way to approach to answer the question may be understanding the role of lipid granules in the oleaginous yeasts that may be an important organelle in the lipid metabolism and the difference between the lipid granules of oleaginous yeasts and those of non-oleaginous yeasts. Unfortunately, the lipid granules of oleaginous yeasts have been rarely studied.

In this study, we have isolated the lipid granules from an oleaginous yeast *Rhodotorula glutinis* that was grown in their lipogenic state (nitrogen limited condition), and characterized properties and lipid composition of lipid granules.

MATERIALS AND METHODS

Microorganism and cultivation

The strain of *Rh. glutinis* NRRL Y-1091 used in this study was obtained from Northern Regional Research Laboratory of USDA, Peoria, USA. The yeast was maintained and cultivated as described elsewhere (12). Cultivation was carried out in the two liter Erlenmeyer flask containing 400 ml nitrogen-limited medium (12) at 30°C and 300 rpm for 60

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hours (early stationary phase) on controlled environment shaker (New Brunswick Scientific Co., NJ, USA). Lipid content reached to its maximum value at 60-hour cultivation.

Cell harvest and disruption

The 60-hour cultured cells were harvested by centrifugation at 6,000×g for 10 min, and washed twice with pre-cooled 0.1 M potassium phosphate, pH 6.0. The cell pellet was resuspended at 0°C in the isolation medium containing 0.4 M mannitol, 0.4 M sorbitol, and 0.025 M potassium phosphate, pH 6.8, with which the cell concentration was adjusted to 0.4 g/ml. The cells were disrupted within two hours after harvest by a single passage through a pre-cooled French press at 20,000 psig.

Isolation of lipid granules by the rate zonal centrifugation

The method of flotation in a linear density gradient of Ficoll (Type 400-DL, Sigma Chemical Co., MO, USA) was adopted as schematically shown in Fig. 1 for the preparative isolation of lipid granules from the cell lysate of *Rh. glutinis*. Centrifugal force of 8,500×g was selected between 3,000~18,000×g.

Cell lysate solution containing 12% Ficoll (w/v) was prepared by adding 1.2 g Ficoll to 9.3 ml cell lysate. An aliquot (5 ml) of this suspension was placed in the bottom of 50 ml centrifuge tube, and onto this suspension 38 ml Ficoll solution was layered to form a linear Ficoll gradient ranging from 0 to 10% (w/v) using a gradient-forming device. The tube was centrifuged for 10 min at 8,500×g in a swinging bucket type HB-4 rotor, which yielded two compact layers in the top, reddish white creamy and floating red thin layers. The floating red thin layer was identified as aggregated form of crushed lipid granules by light and phase contrast microscopies, and the reddish white creamy layer as intact lipid granules from their staining property and morphology using a microscope. The reddish white creamy layer was collected by aspiration with a curved ("J" shaped) pasteur pipette. It was important to avoid any shock to the tube during this procedure, because the upper layer could be easily dispersed. The collected crude lipid granules were washed with the isolation medium by centrifugation and collected.

Dye uptake and osmotic properties of lipid granules

Suspensions of cells or lipid granules were stained with neutral red, toluidine blue O, Nile blue A, or Sudan black B by adding equal volume of dye and incubating at 4°C for about 30 min. Neutral red (0.05%, w/v) and toluidine blue O (0.1%, w/v) were dissolved in water, and the lipophilic dyes such as Sudan black B (0.5%, w/v) and Nile blue A (0.3%, w/v) were dissolved in 70% (v/v) ethanol.

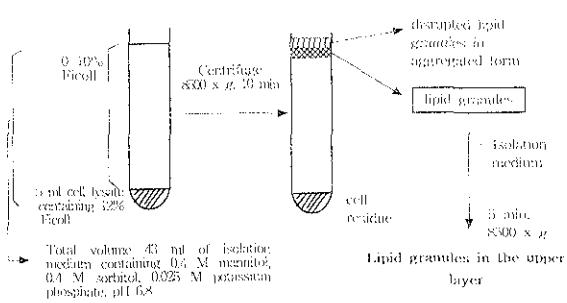

Fig. 1. Schematic diagram of the isolation procedures of lipid granules.

The isolated lipid granules were suspended in the isolation medium containing 0.4 M mannitol, 0.4 M sorbitol, 0.025 M potassium phosphate, pH 6.8, in which mannitol and sorbitol were used as osmotic stabilizers. Aliquots (0.1 ml) of the suspension were diluted with 2.9 ml of series of diluted stabilizer solution. Then absorbances of the diluted suspensions were measured at 660 nm after incubation for 10 min and 30 min at 4°C.

Lipid extraction from whole cells and lipid granules

The cells harvested from about 200 ml cultured broth were washed twice with 0.1 M potassium phosphate, pH 6.0, lyophilized, and slightly ground with a mortar and pestle. The ground cells were used to extract lipids by percolation method as described (13).

For the extraction of lipids from lipid granules, one volume of the isolated lipid granules were mixed with two volumes of chloroform-methanol (2 : 1, v/v) and lipids were extracted by stirring for 20 min at 30°C. Then the extract was washed twice with 0.2 volume of 3 mM $MgCl_2$. The chloroform layer was dehydrated with anhydrous Na_2SO_4, filtered through a Toyo No. 2 filter paper, and then evaporated by a rotary evaporator.

Lipid analysis

Lipids extracted from whole cells or lipid granules were separated into neutral lipids, glycolipids, and phospholipids by silicic acid column (1.5×8 cm) chromatography as described previously (13). Each separated lipid fraction was further fractionated into its components by thin layer chromatography (TLC) using commercially available TLC plates pre-coated with silicagel 60 (Merck, Darmstadt, Germany) as described (13). The TLC plates after separation were visualized by charring with saturated potassium dichromate in 70% sulfuric acid (v/v) followed by heating for 25 min at 100°C. The quantitative analysis of visualized lipid classes was carried out using Fiber Optic Scanner (Kontes Model 800, Kontes Co., NJ, USA) as described (13).

Compositional analysis of fatty acids in the fractionated lipids

Fatty acid composition of neutral lipids, glycolipids, and phospholipids was analyzed by high performance liquid chromatography using μ Bondapak C_{18} reverse phase column (Waters Co., MA, USA) as described (13). In the preparation of sample, neutral lipids or glycolipids were saponified by alcoholic alkali hydrolysis, while phospholipids were safonified by acid hydrolysis (13).

RESULTS AND DISCUSSION

Microscopic observation

Rh. glutinis cultured in nitrogen-limited condition (lip-

ogenic state, about 31% lipid content in dry weight basis) contains several large lipid granules (Fig. 2), while the yeast grown in carbon-limited condition (non-lipogenic state, about 10% lipid content) has several relatively small lipid granules (data not shown). The lipid granules in the cells, which could be stained by dyes such as Sudan black and Nile blue A, were clearly visible without staining in the form of spherical particles that strongly refract light when examined with a phase contrast microscope. In Fig. 2, it could be observed that many of lipid granules are arranged closely to the plasma membrane of the cell, which may be related to the suggestion that lipid granules are involved in the plasma membrane biogenesis in addition to the role to store lipids (4).

The lipid granules of yeast cells were stained redish blue with neutral red or toluidine blue O (data not shown), indicating accumulation of these dyes in lipid granules of intact cells. However, these dyes stained faintly lipid granules isolated from the cell lysate. This observation suggests that lipid granules may have vacuolar properties because vacuoles accumulate compounds such as cationic dyes (for example, neutral red, toluidine blue O, etc.). Faint staining of the isolated lipid granules may be because the isolated lipid granules may not function fully. In contrast, lipophilic dyes such as Sudan black or Nile blue A stained well both lipid granules of intact yeast cells and lipid granules isolated from the cell lysate (data not shown).

Osmotic property of lipid granules

Isolated lipid granules were suspended in a series of osmotic stabilizer solutions containing various concentration of stabilizer ranging from 0 to 0.8 M and the absorbances of the suspensions after 10 or 30 min incubation were determined (Fig. 3). The absorbance decreased sharply when the stabilizer concentration decreased from 0.8 to 0.7 M, suggesting rupturing of the granules. However, the absorbance increased in the suspensions containing stabilizer below 0.7 M until stabilizer concentration reached 0.1 M, suggesting

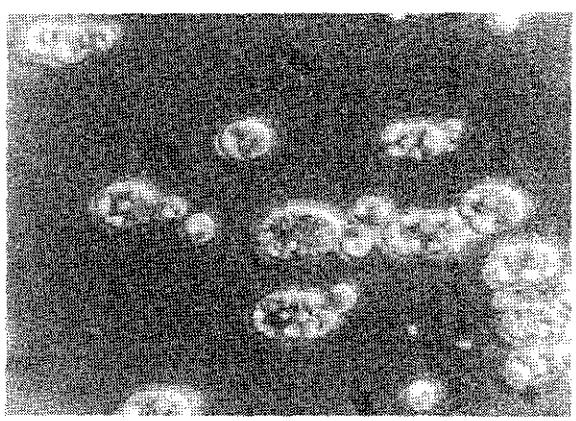

Fig. 2. Phase contrast microgram of *Rh. glutinis* in the early stationary phase of growth that contains several large lipid granules. The lipid granules were preliminarily identified by staining with a lipophilic dye, Nile blue A.

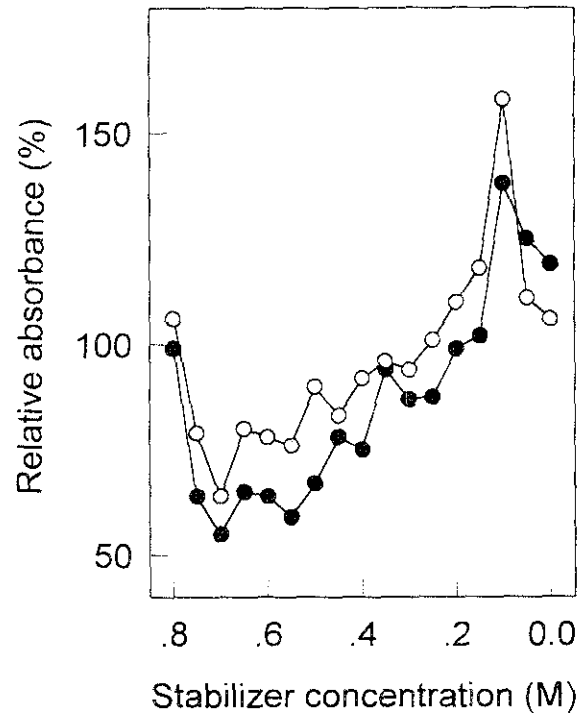

Fig. 3. Effect of stabilizer concentrations on the stability of lipid granules isolated from *Rh. glutinis*. Aliquots (0.1 ml) of the isolated lipid-granule suspension were diluted with 2.9 ml of a series of osmotic stabilizer solutions containing varying concentration of stabilizers (sorbitol+mannitol) ranging from 0 to 0.8 M. After 10 min (●) or 30 min (○) incubation at 4°C, absorbances of the suspensions were measured at 660 nm.

that the lipid granules increased in volume. In the suspensions containing stabilizer below 0.1 M, the absorbance decreased again. These observations lead to two suggestions. First, the lipid granules are enveloped by a membraneous structure. This suggestion is consistent with the results of many other investigators (7,9,14), but inconsistent with the suggestion by a group that at least one of two classes of lipid granules from *S. cerevisiae* is more or less solid lumps of lipids containing proteins (15). Second, at least presence of two classes of lipid granules that are different from each other in the osmotic property is suggested. Presence of more than two classes of lipid granules in yeast have been suggested by other investigators (14,16).

Lipid analysis

The lipids from whole cells and lipid granules were fractionated into three classes of lipids: neutral lipids, glycolipids, and phospholipids (Table 1). The lipids of lipid granules were composed mainly of neutral lipid (87.8%), predominantly as triacylglycerols (71.8% of neutral lipids) as shown in Table 2. In contrast, triacylglycerol content of neutral lipids in whole cells is very low (28.8%), although neutral lipid content of whole cells (82%) is a little lower than that in lipid granules. This result indicates that triacylglycerols are preferentially accumulated in lipid granules, suggesting that lipid granules are an organelle that accumulates lipids as a re-

Table 1. *Lipid classes of whole cells and lipid granules*

Lipid class	Contents of lipid classes (%, w/w)	
	Whole cells	Lipid granules
Neutral lipids	82.0	87.8
Glycolipids	14.1	4.2
Phospholipids	3.8	8.0

Table 2. Composition of neutral lipids of whole cells and lipid granules

Lipid	Contents of neutral lipid classes (%)	
	Whole cells	Lipid granules
Sterol ester	19.7	19.2
Triacylglycerol	28.8	71.8
Diacylglycerol	tr[1]	tr
Monoacylglycerol	1.1	1.5
Free fatty acid	25.1	2.0
Sterol	3.3	5.5
Unknown	22.0	ND[2]

[1]Trace (less than 0.5%)
[2]Not detected.

serve material. However, it is difficult to determine what proportion of lipids in whole cells is in the lipid granules, because it was not easy to quantitatively determine the yield of cell disruption and recovery of lipid granules in the isolation procedures.

Lipid granules have higher content of phospholipids (8.0 %) than that in whole cells (3.8%) (Table 1), suggesting that lipid granules have a membraneous structure and the membraneous structure is more concentrated than whole cells. However, the relative amount of polar lipids (glycolipids + phospholipids) is higher in whole cells, which is consistent with the results reported before (9).

Lipids fractionated into neutral lipids, glycolipids, and phospholipids were further separated by TLC and the quantities of separated lipid components on TLC plates were determined by the densitometer. As discussed above, the major lipid of neutral lipids of lipid granules and of whole cells was triacylglycerols (Table 2). The free fatty acid content of neutral lipids in whole cells (25.1%) was much higher than that in lipid granules (2%) (Table 2). This relatively high value of free fatty acids in whole cells may be due to the high temperature extraction of lipids by percolation method. Compositions of other neutral lipids of whole cells and lipid granules were quantitatively and qualitatively similar (Table 2).

In the compositional analysis of glycolipids of whole cells and lipid granules, the patterns of the spots separated by TLC were similar between the glycolipids of whole cells and those of lipid granules (data not shown), although there were differences in the contents of monogalactosyldiacylglycerol (4.8% in whole cells vs trace in lipid granules) and of cerebroside (4.8% in whole cells vs 12.4% in lipid granules) (Table 3).

Phospholipids are considered to be a major structural component of yeast membranes. While the major phospholipid in the lipid granules was phosphatidylcholine (38.6%), in the whole cells the content of this phospholipid was only 14.7% and instead phosphatidylserine was the major phospholipid (42.8%) (Table 4). There were also differences in the contents of phosphatidylethanolamine and phosphtidylinositol of phospholipids in the whole cells and in the lipid granules. These results lead to a speculation that membranes of lipid granules are structurally and furthermore functionally distinct from the membranes of whole cells, in which the cytoplasmic membrane is probably the major component of membraneous structures. It was observed that the whole cells contained much greater amount of lysophosphatidylcholine (22.6%) than the lipid granules (4.4%) (Table 4). High content of lysophosphatides was observed in the lipids extracted from the baker's yeast, which was attributed to the action of phospholipase A on the phosphatidylcholine during the extraction of lipids. A similar explanation could be applied to the high content of lysophosphatidylcholine in the phospholipids of whole cells. The relatively low content of lysophosphatidylcholine (4.4%) in the lipid granules may be due to the inactivation or removal of phospholipase A during the isolation of lipid granules.

Table 3. *Composition of glycolipids of whole cells and lipid granules*

Lipid	Contents of glycolipid classes (%)	
	Whole cells	Lipid granules
Monogalactosyl-diacylglycerol	4.8	tr
Cerebroside	4.8	12.4
Sterylglucoside	1.8	1.6
Digalactosyl-diacylglycerol	tr	1.2
Unknown	88.6	84.8

Compositional analysis of fatty acids of the fractionated lipids

Neutral lipids, glycolipids, and phospholipids fractionated by silicic acid column chromatography from lipids of whole cells and of lipid granules were hydrolyzed and the fatty acid compositions were analyzed by HPLC (Table 5). The neutral lipids of both lipid granules and whole cells consisted mainly of palmitic, oleic, and linoleic acid. In glycolipids, a big difference between lipid granules and whole cells was in the oleic acid content (10.9% in the lipid granules versus trace in the whole cells). The most marked difference in the

Table 4. Composition of phospholipids of whole cells and lipid granules

Lipid	Contents of phospholipid classes (%)	
	Whole cells	Lipid granules
Cardiolipin	tr	tr
Phosphatidylethanolamine	11.2	4.6
Phosphatidylinositol	2.4	14.6
Phosphatidylserine	42.3	33.3
Phosphatidylcholine	14.7	38.6
Lysophosphatidylcholine	22.6	4.4
Phosphatic acid	tr	tr
Unknown	6.0	3.7

Table 5. Fatty acid compositions of the fractionated lipids of whole cells and lipid granules

Fatty acid	Fatty acid composition (%, w/w)					
	Neutral lipids		Glycolipids		Phospholipids	
	WC[1]	LG[2]	WC	LG	WC	LG
12 : 0[3]	–[4]	–	9.1	6.4	tr[5]	14.8
16 : 0	28.7	33.2	27.5	23.0	15.6	7.2
18 : 0	7.0	10.4	tr	10.9	2.9	tr
18 : 1	30.5	32.6	27.6	22.4	22.6	23.1
18 : 2	25.6	19.8	25.0	29.8	43.2	39.9
18 : 3	8.2	4.0	10.7	9.1	15.0	15.0
Unsaturation, Δ/mol[6]	1.06	0.84	1.1	1.09	1.54	1.48

[1]Whole cells.
[2]Lipid granules.
[3]The fatty acids are designated x:y, where x is the number of carbon atoms and y is the number of double bonds/molecule.
[4]Fatty acid not detected.
[5]Trace (less than 1%).
[6]Calculated from the following formula (13): Δ/mol=1.0×(% monoene/ 100)+2.0×(% diene/100)+3.0×(% triene/100).

fatty acid composition was in the phospholipids. Lauric acid was detected as trace in the phospholipids of whole cells, but 14.8% in the lipid granules (Table 5). In addition, arachidonic acid was detected only in the phospholipids of lipid granules (data not shown). These results suggest that a marked difference is shown in the membrane structures between lipid granules and whole cells, when we consider that phospholipids are a major structural component of the yeast membranes and that the fatty acids are an important variable in determining the membrane morphology.

Generally storage lipids have higher content of saturated fatty acids than the total lipids (17). Degree of unsaturation of fatty acids (Δ/mol) in the neutral lipids from lipid granules and whole cells were 0.84 and 1.06, respectively (Table 5), indicating that fatty acids of the neutral lipids in lipid granules are more saturated than those in whole cells. This result suggests again that lipid granules accumulate storage lipids. In glycolipids and phospholipids, which are not considered as storage lipids, differences of the degree of unsaturation between whole cells and lipid granules were negligible (Table 5).

It has been known that most phospholipids have a high level of unsaturated fatty acids (17). It turned out to be true in *Rh. glutinis* as the degree of unsaturation of fatty acids from phospholipids (Δ/mol≒1.5) was greater than those from other lipid fractions (Table 5).

From the results described in this article, it is evident that lipid granules of *Rh. glutinis* are an organelle that accumulates storage lipids and that is enveloped by a membraneous structure rather than just solid-lipid lumps. It is still long way to go to answer the questions how the oleaginous yeast accumulates greater amount of lipids in the cell than the non-oleaginous yeasts do and how the lipid granules are involved in the lipid metabolism of the oleaginous yeasts. It will be essential to understand enzymes and other proteins (for example, proteins like oleosin in plant-lipid granules) that are associated with lipid granules. By understanding more about lipid granules, we will have more knowledges of the lipid metabolism in the oleaginous yeasts and it will be easier to manipulate the oleaginous yeasts to produce edible oils we require.

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