ORIGINAL

Biochemical Properties of Cerebrosides from Hydrocarbon-Assimilating Yeasts

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Hydrocarbon-assimilating yeasts, *Candida rugosa* JF-101 and *Saccharomycopsis lipolytica* MIL 4040, gave respectively 7.6-fold more Candicin (galactosylceramide) and 3.4-fold more Candicin-like cerebroside (CLC) from *n*-hexadecane than from glucose. Each Candicin and CLC were present in significant amounts in the intracellular membrane fraction containing mitochondria and microbodies. In both yeasts, the amount of cerebroside in the microbodies fraction was about 2.5 times that in the mitochondria fraction. Candicin was noted to induce the fruiting of *Schizophyllum commune*.

1 Introduction

Sphingolipids are mainly located in cell membrane where they may have important functions expressed both in the polar head group and two hydrophobic hydrocarbon chains. The cerebrosides from yeasts of *Candida utilis* [1], *C. rugosa* [2], *C. albicans* [3] and *Hansenula ciferri* [4] have been isolated and characterized. For fungi except for yeasts, the structures of cerebrosides from *Schizophyllum commune* [5)~7)], *Penicillium funiculosum* [8] and *Fusicoccum amygdali* [9] have been discussed in relation to their stimulating activities for the fruiting body formation in *Sch. commune* that is often used as a model system of cell differentiation. In yeasts and fungi, however, no specific endogenous function has been attributed to these molecules as yet.

In the previous paper [2], we reported the structure of Candicin (1-β-galactopyranosyl-2-(α-hydroxyhexadecanoyl)-2-amino-1,3-dihydroxy-*trans*-4, *cis*-12-nonadecadiene) from *n*-alkane-assimilating yeast of *C. rugosa* JF-101, and that approximately sixfold more Candicin was produced from *n*-hexadecane than from glucose. We more previously reported that abundant microbodies surrounded by single unit membrane were found in *n*-hexadecane-grown cells of *C. rugosa* JF-101, whereas glucose-grown cells contained few microbodies [10]. Recently, we found that another hydrocarbon-assimilating yeast of *Saccharomycopsis lipolytica* MIL 4040[11], which was newly isolated one of three yeasts from jet fuel, produced Candicin-like cerebroside (CLC) from glucose and *n*-hexadecane.

In this paper, we describe the production of Candicin and CLC from glucose and *n*-hexadecane, subcellular localization of each cerebroside in *C. rugosa* JF-101 and *S. lipolytica* MIL 4040, and fruiting-inducing activity of Candicin with *Sch. commune*.

2 Experimental

2·1 Materials

C. rugosa JF-101 and *S. lipolytica* MIL 4040 were used in this study. *n*-Alkanes (*n*-decane, *n*-C_{10}~*n*-octadecane, *n*-C_{18}) and Zymolyase 100 T were purchased from Tokyo Chemical Industry Co. and Kirin Brewery Co., respectively. Cytochrome *c* (Type Ⅲ) was purchased from Sigma Co. Ficoll and Percoll were from Pharmacia LKB Biotechnology. Other chemicals were the usual commercial products of analytical grade and used without further purification.

2·2 Cultivation

Each of *C. rugosa* JF-101 and *S. lipolytica* MIL 4040 was grown on a medium composed of 1% *n*-alkane (or 1% glucose), 0.2% $(NH_2)_2CO$, 0.22% $(NH_4)_2SO_4$, 0.3% $Na_2HPO_4 \cdot 12H_2O$, 0.15% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.001% $FeCl_3 \cdot 6H_2O$ and 0.02% yeast

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extract, pH 7.2. Cultivation was done at 27℃ in a 500 mL Erlenmeyer flask[12] with 80 mL of medium or in a 7 L jar fermenter[2] with 4.5 L of medium.

2·3 Measurement of oxygen uptake

Oxygen uptake of the cells for n-C_{16} was measured by the Warburg manometric technique and represented as μL/h/mg of dry-cells as described previously[13].

2·4 Preparation and analysis of cerebroside

Candicin and CLC from cells of each yeast were extracted with $CHCl_3/CH_3OH$ (2 : 1, vol/vol), purified and analyzed by the methods described previously with silica gel TLC and secondary ion mass spectrometry (SI-MS, Hitachi M-80)[2]. The quantitative analysis of cerebrosides from two yeasts used was performed as follows. The total lipid extracted with $CHCl_3/CH_3OH$ was applied on silicagel 60 plate (Art. 5721, E. Merck, Darmstadt) and developed with the solvent system of $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, by vol.). After the plate was colorified with anthrone/H_2SO_4[14], the cerebroside was quantified by chromatoscanner (Shimazu CS-910) with the purified Candicin as the standard.

2·5 Preparation of protoplasts and particulate fraction

Protoplasts from 20 g (wet weight) of cells, harvested at early stationary growth phase, were prepared according to the method of Kamiryo *et al.*[15] with Zymolyase 100 T. All subsequent operations, including subcellular fractionations, were carried out at 0 to 4℃. The resulting protoplasts were suspended in 60 mL of 2.5 mM potassium 3-(N-morpholino)propanesulfonate (K-MOPS), pH 7.2, containing 5% (wt/vol) Ficoll 400, 0.6 M sorbitol and 1 mM EDTA (F buffer), homogenized in ice bath with Potter-Elvehjem (Teflon-glass) homogenizer, and centrifuged 1,500×g for 15 min to separate heavy particles (P-1) and supernatant (S-1). The S-1 fraction was centrifuged at 20,000×g for 20 min to obtain particulate fraction (P-2)[15]. The remaining supernatant (S-2) was further centrifuged at 183,000×g for 2h to yield the pellet (P-3) and the soluble fraction.

Sucrose density gradient centrifugation : P-2 from each yeast was gently suspended in 50 mM phosphate buffer (pH 7.2) containing 20% sucrose, 10 mM EDTA and 10 mM KCl to be 3~5 mg-protein/mL. Six mL of the suspension was layed over a discontinuous sucrose density gradient consisting each 6 mL of 30, 40, 41.3, 42.5 and 50% (wt/vol) sucrose solution in 50 mM phosphate buffer, pH 7.2. After centrifugation was carried out at 49,600×g for 6h with RPS 27-2 rotor in a Hitachi ultracentrifuge model 65 P-7, the gradient was fractionated from the top of the tube into six portions.

Percoll density gradient centrifugation : P-2 from *S. lipolytica* MIL 4040 was suspended in F buffer to be 3~5 mg-protein/mL and was subjected to Percoll density gradient centrifugation. Namely, 6 mL of the suspension was applied onto 30 mL of the Percoll solution (ρ=1.070 g/mL) in Hitachi 40 PC tube. The Percoll solution was consisted of 1 vol. of 25 mM K-MOPS buffer, pH 7.2, containing 5 M sorbitol and 5 mM EDTA and 9 vol. of Percoll (ρ=1.15 g/mL), and appropriate vol. of F buffer (ρ=1.05 g/mL) to be above density. Centrifugation was conducted at 87,700×g for 100 min with RPS 27-2 roter in a Hitachi ultracentrifuge model 65 P-7. The gradient was fractionated from the bottom into 12 fractions by 3 mL.

2·6 Assay of enzymes

Catalase activity was assayed at 25℃ by the method of Teranishi *et al.*[16] based on the titanium color reaction for hydrogen peroxide[17]. Cytochrome *c* oxidase was assayed at 35℃ according to the method of Wharton and Tzagoloff[18]. One unit of the enzyme is defined as the amount catalyzing the conversion of 1 μmol of substrate per min.

2·7 Determination of Protein

Protein was determined by the method of Bradford[19] or that of Lowry *et al.*[20] using bovine serum albumin as a standard.

2·8 Assay of fruiting-inducing activity

Fruiting-inducing activity of Candicin was assayed according to the method of Kawai and Ikeda[5]. Candicin was dissolved in $CHCl_3/CH_3OH$ (2 : 1, vol/vol) and applied onto a paper disc and the solvent was removed under reduced pressure. *Schizophylum commune* IFO 6502 was used for fruiting strain.

3 Results

3·1 *n*-Alkane-assimilating activity of the yeasts

S. lipolytica MIL 4040 assimilated n-alkanes

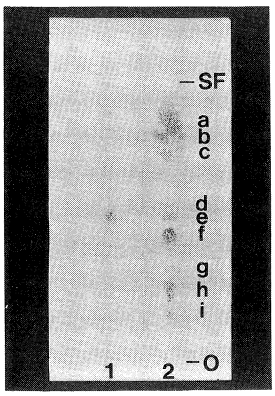
from n-C_{10} to n-C_{18} well. When n-C_{16} was used as a oxidation substrate, the respiratory activity of glucose-grown cells of *S. lipolytica* MIL 4040 (103 μL/h/mg of dry cells) was 1.5-and 7.5-fold higher than those of *C. rugosa* JF-101 (70 μL/h/mg of dry cells)[13] and *S. lipolytica* No. 6-20 (14 μL/h/mg of dry cells)[13], respectively. Jet fuel was also assimilated well by *S. lipolytica* MIL 4040 as well as done by *C. rugosa* JF-101[21].

3·2 Cerebrosides from two yeasts

Candicin, galactocerebroside produced by *C. rugosa* JF-101, has been found in 1984 and the structure and some properties were reported [2]. We found that *S. lipolytica* MIL. 4040 also produced CLC in the subsequent study. This cerebroside showed positive reaction(purple)for anthrone reagent, and gave the same R_f value(R_f =0.52)and similar fragmentation pattern as those of canditin [2] in TLC(**Fig. -1**)and SI-MS(data not shown), respective analyses. Futher analyses of CLC are under investigation and its structure will be described elsewhere.

3·3 Effect of growth substrate on the productivity of cerebrosides

In the previous paper [2], we reported that the production of Candicin in *C. rugosa* JF-101 reached its maximum in the early stationary growth phase, and more amount of Canditin was Contained in n-C_{16}-grown cells(1.45 mg/g-dry cells)than in glucose-grown cells(0.19 mg/g-dry cells). It was also with *S. lipolytica* MIL 4040 that n-C_{16} was bettercarbon source for production of CLC(3.4 mg/g-dry cells)than glucose(1.0 mg /g-dry cells). Namely, 2.3-fold production of the cerebroside occurred in n-C_{16}-grown cells of *S. lipolytica* MIL 4040 than in those of *C. rugosa* JF-101.



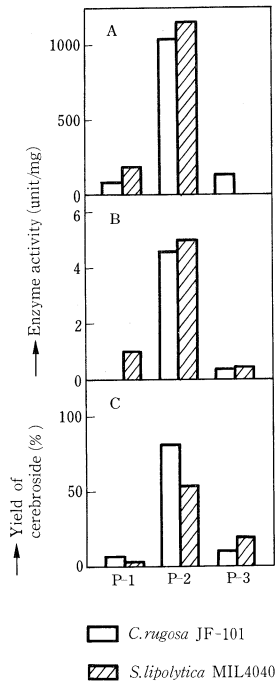
The lipid was applied on silicagel 60 plate and developed with the solvent system of chloroform/methanol/water(65 : 25 : 4, by vol.). The plate was colorified with anthrone/sulfuric acid.

1 : Candicin from *C. rugosa* JF-101. 2 : total lipids from *S. lipolytica* MIL 4040. SF : solventfront, a : triglyceride and sterolester, c : fatty acid, d : unknown lipid, e : Candicine-like cerebroside(CLC), f : phosphatidylethanolamine, g : unknown lipid, h, i : phospholipid, o : origin.

Fig.-1 Thin-layer chromatography of total lipids from *S. lipolytica* MIL 4040 and Candicine.

3·4 Subcellular localization of cerebrosides

Catalase was used as the maker enzyme of microbodies, and cytochrome *c* oxidase was used for mitochondria [22]. Protoplasts of n-C_{16}-grown cells were disrupted and applied differential centrifugation. As shown in **Fig. -2**, cytochrome *c*

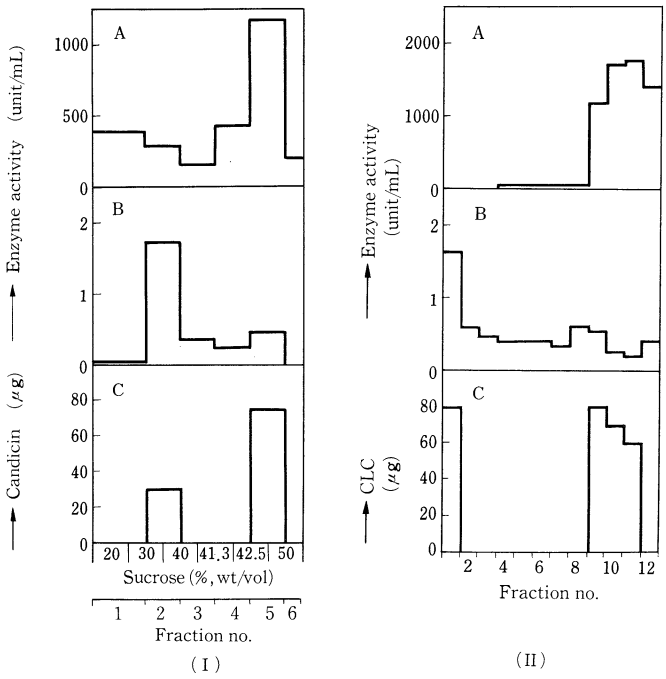


Protoplasts of n-hexadecane-grown cells were homogenated and centrifuged. Catalase and cyt. *c* oxidase were used as the marker enzyme of microbodies and mitochondria, respectively. Cerebrosides of Candicin and CLC were produced by *C. rugosa* JF-101 and *S. lipolytica* MIL 4040, respectively. A, catalase; B, cytochome *c* oxidase; C, cerebroside(Candicine or CLC); P-1, 1,500 × g fraction; P-2, 20,000 × g fraction; P-3, 183,000×g fraction

Fig.-2 Fractionation by differential centrifugation of the homogenate derived form n-C_{16}-grown cells.

oxidase and catalase were exclusively recovered in particulate fraction (P-2) in both of *C. rugosa* JF-101 and *S. lipolytica* MIL 4040. And the recovery of each cerebroside was significantly high in P-2. Namely, 80 and more percent of Candicin and 54% of CLC were recovered in each P-2. In *S. lipolytica* MIL 4040, however, 19% of the cerebroside was found in microsome fraction (P-3), this would be ascribed to degradation of a part of particles during the preparation steps. Catalase contained in degraded particles would be in the supernatant solution because its activity was not found in P-3. A few cerebroside was found in cell membrane fraction (P-1) in each yeast.

To get more information, P-2 was subjected to sucrose or Percoll density gradient centrifugation. The activity of cytochrome *c* oxidase in the early particulate fraction (fraction 2 in *C. rugosa* and fraction 1 in *S. lipolytica* MIL 4040) was remarkably higher than those in other fractions as shown in **Fig. -3**. On the other hand, the catalase activity was significantly higher in the late particulate fraction (fraction 5 in the former yeast and fractions 9~11 in the latter). From these results, fraction 2 in *C. rugosa* and fraction 1 in *S. lipolytica* MIL 4040 mainly contained mitochondria, and fraction 5 in *C. rugosa* and fractions 9~11 in *S. lipolytica* MIL 4040 mainly microbodies. Candicin and CLC exclusively distributed at the respective fractions of mitochondria and microbodies, and the amount of them in the micro bodies fraction was about 2.5 times more than that in the mitochondria fraction in each yeast. From electron mic-



(I) SDGC of P-2 from *C. rugosa* JF-101. A. catalase; B, cyt. *c* oxidase; C, Candicine. Fractionation was performed from the top of the tube (volume of each fraction : 1, 9 mL; 2-5, 6 mL; 6, 3 mL). (II) PDGC of P-2 from *S. lipolytica* MIL 4040. A, catalase; B, cyt. *c* oxidase; C, CLC. Fractionation was performed from the bottom of the tube (3 mL/fraction). While microbodies (catalase) exhibited higher density than mitochondria (cyt. *c* oxidase) in SDGC, the density of microbodies was lower than that of mitochondria in Percoll solution.

Fig.-3 Fractionation of P-2 derived from *n*-hexadecane-grown cells by sucrose or Percoll density gradient centrifugation. (SDGC or PDGC)

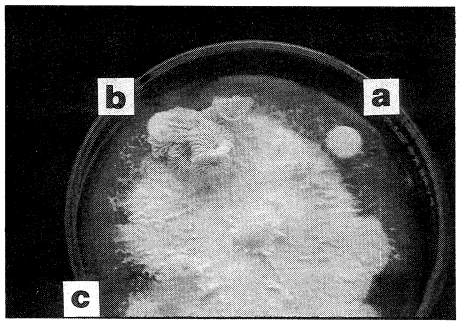
Table-1 Relative contents of cerebroside in each fraction of mitochondria and microbodies.

Fraction	Candicin from *C. rugosa*		CLC[a] from *S. lipolytica*	
	G[b]-grown cells	*n*-C_{16}[c]-grown cells	G-grown cells	*n*-C_{16}-grown cells
Whole cells[d]	0.2	1.5	1.0	3.4
Mitochondria[e]	6.1	9.3	6.1	10.5
Microbodies[e]	7.8	9.8	9.4	12.4

a) Candicin-like cerebroside. b) Glucose. c) *n*-Hexadecane.
d) μg/mg of dry cells. e) μg/mg of dry weight.

roscopic observations, however, these fractions were slightly contaminated with disrupted membrane (data not shown). About one-fifth of each catalase activity of *S. lipolytica* MIL 4040 and *C. rugosa* was detected in the lightest fraction, respectively, suggesting that a part of microbodies was disrupted during centrifugation. In *S. lipolytica* MIL 4040, the activities of catalase and cytochrome *c* oxidase were insufficiently separated by sucrose density gradient. Therefore, Percoll density gradient was used for subcellular fractionation in this strain.

The subcellular fractionation of P-2 derived from glucose-grown cells of each yeast was also performed by the same procedure. **Table-1** shows the relative contents of cerebroside in each fraction with glucose-grown and n-C_{16}-grown cells. Both of Candicin and CLC were found in the fractions of mitochondria and microbodies with significantly high relative contents in both of glucose-grown and n-C_{16}-grown cells. In n-C_{16}-grown cells, relative contents of each cerebroside in these fractions were 1.3~1.7 times higher than those in glucose-grown cells. Higher relative contents of the cerebroside (1.1~1.5 times) occurred in the microbodies fraction than in the mitochondria fraction irrespective of the growth substrate. Moreover, the consistency of cerebroside in the fraction derived from *S. lipolytica* MIL 4040 was higher than corresponding one from *C. rugosa* JF-101 except the mitochondria fractions of glucose-grown cells.



Sch. commune IFO 6502 was inoculated on the center of malt-yeast extract agar medium in a 9 cm Petri dish and incubated three days at 25°C, and then Candicine applied on paper discs (8 mm diameter, 0.7 mm thickness) were placed on a margine of the plate and continued to cultivate for two weeks. Amount of Candicine applied on the discs were, in μ g, 0, a (control); 10.0, b; and 0.5, c.

Fig.-4 Induction of fruiting by Candicin.

3·5 Fruiting-inducing activity of Candicin

As shown in **Fig.-4**, Candicin clearly induced fruiting body in *Sch. commune* IFO 6502 at 10.0 μg/disc and the specific activity of it was calculated as 2,000 units/mg because more than 0.5 μg of Candicin/disc was needed to stimulate the fruiting.

4 Discussion

S. lipolytica MIL 4040 newly isolated from jet fuel had higher activity of n-alkane-assimilation than those of *S. lipolytica* No. 6-20 and *C. rugosa* JF-101 which is one of the strongest hydrocarbon-assimilating yeasts[21]. *S. lipolytica* MIL 4040 also produced CLC as Candicin in *C. rugosa*, and 2-or 3-fold more amount of cerebroside occurred from each of glucose and n-C_{16} than in *C. rugosa*. In both yeasts, the production of these cerebrosides from n-C_{16} was significantly higher than those from glucose, *i. e.*, 3.4-fold in *C. rugosa* and 4.7-fold in *S. lipolytica* MIL 4040. Consequently, the production of cerebroside in *S. lipolytica* MIL 4040 was more constitutive than in *C. rugosa*, though it was greatly induced by n-C_{16}.

Candicin and CLC were significantly collected in P-2 fractions by differential centrifugation and mainly distributed in the fractions of mitochondria and microbodies. The relative contents (μg/mg of dry weight) of each cerebroside in respective fractions were higher in n-C_{16}-grown cells than in glucose-grown cells. In each yeast, however, the difference of the relative contents of the subcellular fractions between glucose-grown cells and n-C_{16}-grown cells did not directly reflect the difference of the cerebroside contents of whole cells between glucose-grown cells and n-C_{16}-grown cells. Moreover, in n-C_{16}-grown cells, numerous microbodies were induced[10] and about 2.5 times amount of the cerebroside was contained in the microbodies fraction compared with that in the mitochondria one. These results suggest that the higher contents of Candicin and CLC of n-C_{16}-grown cells than those of glucose-grown cells are not only due to increasing of their consistency in the membrane but also due to increasing of amount of membrane, *i. e.*, mainly due to increasing of microbodies, and these cerebrosides probably participate in the assimila-

tion of n-C_{16} on the intracellular membrane and cell membrane.

On the other hand, Candicin induced the fruiting of *Sch. commune*, though the activity (2,000 units/mg) was weak compared with that of cerebrosides from *Sch. commune*[5], *P. funiculosum*[8] and *F. amygdali*[9] (more than 10,000 units/mg). It is essential for high activity to have double bond at position 8 (Z) or the methyl branch at C-9 in the N-acyl sphingoid[6,23]. The cerebrosides from latter three fungi have such structure, and hydrogenated cerebroside from *Sch. commune*, which has methyl branch at C-9, still has specific activity of 2,000 units/mg[6]. On the other hand, Candicin, which has not such structure but the structurte of (4E, 12Z)-N-2'-hydroxypalmitoyl-1-O-β-galactopyranosyl-2-amino-4,12-nonadecadiene-1,3-diol[2], showed the same activity as that of hydrogenated cerebroside from *Sch. commune*. Since Candicin had such activity relating to cell differentiation, a possibility that Candicin has some participation in inducing the microbodies of the yeast will be conceivable, though no evidence of it has been obtained. The fruiting-inducing activity of CLC is under investigation and the result of it will be described with the structure of CLC elsewhere.

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炭化水素資化性酵母由来のセレブロシドの生化学的性質

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炭化水素資化性酵母である *Candida rugosa* JF-101 と *Saccharomycopsis lipolytica* MIL 4040 は n-ヘキサデカンを炭素源とする時に，グルコースを炭素源とする時の，それぞれ 7.6 倍の Candicin（ガラクトシルセラミド）と 3.4 倍の Candicin 様セレブロシド（CLC）を生産した。Candicin と CLC は，それぞれミトコンドリアとマイクロボディーを含む細胞内膜画分に有意に見いだされた。これらのセレブロシドは，いずれの酵母とも，ミトコンドリア画分よりもマイクロボディー画分の方に約 2.5 倍多く含まれていた。また，Candicin は *Schizophyllum commune* の子実体形成を誘導した。