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Lipid Composition of Freeze-Tolerant Baker's Yeasts

Young-Sook Hahn and Hiroyasu Kawai*

Department of Food and Nutrition, Sungshin Women's University
**Department of Food Science and Nutrition, Nara Women's University, Japan*

냉동내성빵효모의 지질분석

한영숙 · 가와이 히로야스*

성신여자대학교 식품영양학과, *일본 나라여자대학 식물과학강좌

요 약

냉동내성빵효모의 냉동내성기구를 규명하기위한 일환으로 세포의 막 유동성에 관계되는 지질분석을 행하였다. 그 결과, 냉동내성효모 $D_{2\text{-}4}$나 CFY보다 비냉동내성 효모 *Saccharomyces cerevisiae* 2001이 스테롤 : 인지질의 비율이 비교적 높았다. 또 인지질조성이 조사되었는 데, 세효모 모두에 있어 phosphatidylcholine 함량이 가장 높았다. Phosphatidylcholine : phosphatidylethanolamine의 비율은 냉동내성효모가 비내성효모 보다 높게 나타났다. 인지질에 연결된 지방산 함량을 보면, linoleic acid 함량은 $D_{2\text{-}4}$에서 높게 나타났으며 지방산의 불포화도는 $D_{2\text{-}4}$가 CFY나 *S. cerevisiae*보다 높았다. 이 결과에서 yeast 세포막의 유동성은 각 효모마다 다르며, 이것들이 저온에서의 효모의 동결장해에 영향을 주는 것으로 생각된다.

I. Introduction

It is known that the freeze-thawing of living yeast cells brings about freeze-injury and causes death of cells, accompanying the leakage of intracellular substances out of the cells. Recently, it was found that non-freeze-tolerant baker's yeast released a larger amount of intracellular substances out of frozen-thawed cells than freeze-tolerant yeast did[1]. It was considered that the membrane systems which take charge of cell permeability were damaged under the freezing conditions, resulting in the leakage of intracellular substances. Saito *et al.*[2] demonstrated that the ultrastructure of freeze-injured baker's yeast showed structural change in membrane systems, e.g., plasmamembrane and mitochondrial membrane. Komatsu *et al.*[3] obtained electromicroscopic evidence of the damage in yeast nuclear membrane when the cells were treated with liqid-nitrogen. They suggested that a similar mode of mechanism of freeze-injury might be involved in both freeze-tolerant and non-freeze-tolerant yeast, but the cause which brings about the difference in freeze-tolerance yeast is not fully investigated yet. It is reported that phospholipid bilayer of cell membranes should retain a liquid-crystalline phase to maintain the structure and function of membrane at low temperature,and the composition of fatty acid attached to phospholipids has an important role in membrane fluidity[4]. Therefore, the difference in freeze-injury of yeast may be related to the difference of fatty acid compositions in phospholipid, which is a major component of yeast cell membrane. Therefore, the authors investigated the lipid compositions of freeze-tolerant yeast and non-freeze-tolerant yeast to clarify the mechanism of freeze-injury of yeast in relation to lipid related compounds in yeast.

II. METHODS AND MATERIALS

1. Chemicals

Phospholipids(phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine ,phosphatidylinositol, phosphatidic acid, lysophosphatidylcholine, lysophosphatidyl-ethanolamine,cardiolipin) were purchased from Sigma Chemical Company. Cholesterol oleate, cholesterolacetate, and ergosterol were obtained from Nakarai Tesque Co., Ltd. Free fatty acids were obtained from Gasukuro Kogyo Inc.

2. Microorganisms and culture conditions

Saccharomyces cerevisiae 2001 from the laboratory collections which was isolated from commercial baker's yeast(Oriental Yeast Co., Ltd.) was used as non-freeze-tolerent yeast. As freeze-tolerant yeasts, the $D_{2\text{-}4}$ strain which isolated from nature and identified as *Tolulars-*

pora delbrueckii[5], and a commercial freeze-tolerant yeast(CFY, Oriental Yeast Co., Ltd.) which belongs to *S. cerevisiae*[6] were used throughout this work.

The culture medium of yeast used in this work was a YPG medium which contained 5 g of yeast extract, 5 g of polypeptone, 20 g of glucose and 1 liter of distilled water.

The cells were grown at 30℃ for 24 hr under reciprocal shaking with 100 ml of the above medium in a 500 ml shaking flask inoculated with 5% precultured cells. The cells were harvested by centrifugation, washed three times with distilled water and filtered with a membrane filter(pore size 1.2 um).

3. Extraction of lipids from yeast

Harvested yeast cells(100 mg as dry weight) were suspended into 3~4 ml of distilled water,heated for 10 min in a boiling water bath, and sonicated at 0~5℃ for 10 min with glass beads(0.4 mm in diameter) using a 20 Kc oscillator(Kaijo Denki). Lipid were extracted from the disintegrated cells with chloroform-methanol (1 : 2, by volume)by the method of Bligh and Dyer[7].

4. Sterol and sterol ester analysis

Appropriate amounts of extracted lipids were subjected to analysis by thin-layer-chromatography(Merk, silica gel 60), and developed with a solvent system consisted with petrolium ether : diethyl ether : acetic acid (80 : 30 : 1, by volume)[8]. Spots of sterol and sterol ester were detected in a desiccator with iodine vapor. Each spot was scrapped off, extracted with chloroform and concentrated by evaporation with nitrogen gas. Obtained sterol and sterol esters were dissolved in tetrahydrofuran, and subjected to analysis[9] with a Shimazu 14A gas chromatgraphy equipped with a flame ionization detector. A capillary column(0.2 mm×25 m) coated with methyl silicon(Shimazu capillary column CBP1-M25-025) was used. Column temperature was maintained at 300℃ and the sample was injected at 320℃.

5. Phspholipid analysis

Phospholipid was seperated from nutral lipids by the use of a Sep-pak silica cartridge(Milipore) and determined by thin-layer chromatography with chloroform : acetone : methanol : acetic acid : water(100 : 100 : 50 : 4 : 10, by volume). After development, the plate was dried in vacuo, and was re-developed with a solvent system, chloroform : methanol : acetic acid : water(120 : 100 : 20 : 6.7, by volume). Each spot of phospholipids developed on the plates was scraped off, and phosphorus content was determined by the method of Fiske-Subbarow[10] after hydrolysis of the sample in 10N sulfuric acid at 150℃ for 5 hr.

6. Analysis of fatty acid in phospholipid

A portion of phospholipid sample was esterified with methanolic sodium methoxide at 60℃, and the fatty acid methyl esters were analyzed by a Shimadzu 9A gas chromatograph with a glass column filled 10% Silar 10C at 160°~240℃. Individual fatty acid methyl esters were identified by comparing their retention times with those of standard fatty acid methyl esters.

III. RESULTS AND DISCUSSION

1. Lipid composition of freeze-tolerant and non-freeze-tolerant yeasts

Table 1 shows the total lipid content and the contents of phospholipid, sterol in freeze-tolerant and non-freeze-tolerant yeasts. The amount of phospholipid extracted from freeze-tolerant yeasts was found to be less than that of non-freeze-tolerant yeast. Gas chromatography have shown that there is one major sterol component in all yeasts with the same retention time as that of the authentic ergosterol. The molar ratios of sterol/phospholipid for *S. cerevisiae* 2001, $D_{2\text{-}4}$, and CFY were 0.44, 0.35, 0.33, respectively. It was reported that the effect of cholesterol contained into lipid bilayer membranes is to attenuate the thermal motion of hydrocarbon chains of phospholipids above the gel liqid-crystalline phase transition temperature[11]. Therefore, it is expected that the presence of sterol in the phospholipid bilayer results in the reduction of membrane fluidity above the phase transition temperature, and that the ratio of sterol to phospholipid in cell membranes is an important factor which determines the membrane fluidity. As shown in Table 1, the ratio of sterol to phospholipid of freeze-tolerant yeasts was lower than that of *S. cerevisiae*, which suggests that relatively

Table 1. Lipid composition of freeze or Non-freeze-tolerant yeasts (mg/100 mg dry weight cells)

Lipids	*S. cerevisiae*	$D_{2\text{-}4}$	CFY
Total lipid	2.8	2.2	2.5
Phospholipid(p)	1.67(59.6%)	1.14(51.8%)	1.42(56.8%)
Sterol(S)	0.38(13.6%)	0.21(9.5%)	0.24(9.6%)
S/P molar ratio*	0.44	0.35	0.33

*The molar ratio of S/P was calculated using the following molecular weight: phospholipid(775), sterol(397)

higher amount of sterol to phospholipid may serve to decrease the membrane lipid fluidity in *S. cerevisiae*, resulting in a low freeze-tolerance rate as described previously[12].

2. Phospholipid composition of freeze-or non-freeze-tolerant yeasts

Phospholipids are recognized as the main constituents of cellmembrane in eucaryotic microorganisms[13]. The phospholipid composition of the freeze-or non-freeze-tolerant yeast cells were investigated. As shown in Table 2, phosphatidyl-choline(PC), phosphatidylethanolamine(PE), phosphatidylinositol(PI), phosphatidylserine(PS), cardiolipin(CL) were detected as major phospholipids, and small amounts of phosphatidic acid (PA), lysophosphatidylcholine(LPC), lysophosphatidylethanolamine(LPE) were also detected in all yeast strains. It is known that, among various phospholipids, phase transition temperature of PC is lower than that of PE[14], and, therefore, the higher ratio of PC to PE implies that the yeast lipid may have lower transition temperature than the yeast lipid with lower ratio of PC/PE. The results obtained in Table 2 show that freeze-tolerant yeasts have higher ratios of PC/PE than non-freeze-tolerant yeast, *S. cerevisiae* 2001. Therefore, it is assumed that the phase transition temperature of phospholipids in cell membrane of freeze-tolerant yeasts may be lower than that of baker's yeast, which probably be related in part to the freeze-tolerance of these yeasts.

3. Fatty acid components in phospholipids of yeasts

Kinds of fatty acid attached to phospholipids extracted from freeze-or non-freeze-tolerant yeasts were determined(Table 3). Major fatty acids were oleic(18 : 1), palmitic(16 : 0), palmitoleic(16 : 1), linoleic(18 : 2)acid in both yeast strains. Higher proportion of 18 : 2 acid was observed in $D_{2\text{-}4}$ strain than that in *S. cerevisiae* and CFY, which may only be due to the difference of yeast speices[15]. Moreover, the degree of unsaturation of fatty acids of freeze-tolerant yeasts, $D_{2\text{-}4}$ and CFY,was higher than that of *S. cerevisiae*. Since the increase of unsaturated fatty acid content serve to lower the melting points of lipids, the higher proportion of unsaturated fatty acid in the phospholipid of freeze-tolerant yeasts may serve to maintain the optimal membrane fruidity for the cellular activities at lower temperatures. Fatty acid compositions of PC and PE in phospholipid fractions extracted from freeze-and non-freeze-tolerant yeasts were investigated(Table 4), as these fatty acids attached to phospholipid may play an important role for membrane fluidity at low temperature[16]. A large part of fatty acid in both PC and PE from non-freeze-tolerant *S. cerevisiae* consisted of two unsaturated acids, 16 : 1 and 18 : 1, no 18 : 2 acid being detected. On the other hand, it was of characteristics that 18 : 2 acid in amounts of 18% to 24% in total fatty acid was detected in PC and PE fractions from freeze-tolerant $D_{2\text{-}4}$ strain. The degree of unsaturation of fatty acid attached to PC and PE extracted from freeze-tolerant yeasts was also found to be slightly higher than that of non-freeze-tolerant yeast,these results suggesting that $D_{2\text{-}4}$ strain is more stable and tolerant at low temperatures than *S. cerevisiae* in relation to the fluidity of cell membrane.

Table 2. Phospholipid composition of freeze or non-freeze-tolerant yeasts (ug phosphorus/100 mg dry weight of cell)

Phospho-Lipids	*S. cerevisiae*	$D_{2\text{-}4}$	CFY
Unknown	2.00(3.8%)	1.89(5.3%)	2.00(4.5%)
LPE	2.44(4.7%)	1.91(5.4%)	0.13(0.3%)
LPC	1.14(2.2%)	0.66(1.9%)	2.00(4.5%)
PA	0.77(1.5%)	1.56(4.4%)	3.01(6.8%)
PS	1.44(2.8%)	4.48(12.7%)	4.01(9.1%)
PI	3.60(7.0%)	4.71(13.3%)	6.01(13.6%)
CL	4.73(9.2%)	2.61(7.4%)	3.51(8.0%)
PE	11.24(21.7%)	5.31(14.5%)	4.01(9.1%)
PC	24.23(46.9%)	12.22(34.5%)	19.55(44.3%)
Total	51.75(100%)	35.40(100%)	44.10(100%)
PC/PE	2.16	2.40	4.89

LPE: lysophosphatidylethanolamine, LPC: lysophosphatidyl-choline, PA: phosphatidic acid, PS: phosphatidylserine, PI: Phosphatidylinositol, CL: cardiolipin, PE: phosphatidyl-ethanolamine, PC: phosphatidylcholine

Table 3. Fatty acid composition of total phospholipids (%)

Fatty acid	*S. cerevisiae*	$D_{2\text{-}4}$	CFY
14 : 0	0.98	–	–
14 : 1	0.69	–	–
16 : 0	7.12	9.13	9.21
16 : 1	54.77	33.73	57.84
18 : 0	3.54	3.77	1.48
18 : 1	31.23	33.75	27.00
18 : 2	1.67	19.62	4.46
unsaturated fatty acid	88.36	87.10	89.30
△/mol*	0.90	1.07	0.94

*Degree of unsaturation(△/mol) was calculated as (% monoene)+2(% diene)

Table 4. Fatty acid composition of PC and PE (%)

	S. cerevisia		D_{2-4}		CFY	
Fatty acid	PC	PE	PC	PE	PC	PE
14 : 0	0.94	0.38	0.46	–	–	0.32
14 : 1	0.85	0.24	1.07	–	–	0.24
16 : 0	5.06	2.94	4.65	2.89	6.13	10.17
16 : 1	60.31	67.07	41.12	40.79	63.16	50.14
18 : 0	3.76	–	5.07	3.26	3.52	0.73
18 : 1	29.08	29.37	29.72	28.79	23.72	37.64
18 : 2	–	–	18.56	24.26	2.65	0.75
unsaturated fatty acid	90.24	96.68	90.47	93.84	89.92	88.77
△mol*	0.90	0.97	1.09	1.18	0.93	0.90

* see Table 3.

IV. SUMMARY

The molar ratio of sterol to phospholipid differed from yeast strains, and the ratio was relatively higher in non-freeze-tolerant yeast strain, *S. cerevisiae* than freeze-tolerant yeast strains, D_{2-4} or CFY. Phospholipid composition of these yeast were also investigated. Phosphatidylcholine content was larger among phospholipids in all yeasts. Higher ratio of PC/PE was found in freeze-tolerant yeast than non-freeze-tolerant yeast. Higher proportion of linolein acid(18 : 2) against total fatty acid attached to phospholipid was observed in D_{2-4} than *S. cerevisiae* or CFY, and the degree of unsaturation of fatty acid was higher in D_{2-4} and CFY than in *S. cerevisiae*. These results suggested that the fluidity of yeast cell membrane was different in yeast strains, which might result in the difference in freeze-injury of yeast at low temperatures.

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