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Degradation of Phospholipids of Yeast after Freeze-Thawing

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Abstract

As an index of freeze-injury of yeast, the leakage of intracellular substances from yeast cells after freeze-thawing was investigated. It was found that much more ultraviolet-absorbing substances leaked out from non-freeze tolerant yeast(NFTY) than from freeze-tolerant yeast(FTY), and that the amount of leakage tended to increase by prolonged frozen-storage period in the former yeast. Furthermore, the rate of leakage of cellular substances form NFTY during incubation exceeded that of FTY, indicating that NFTY is more susceptible to freeze-injury than FTY during frozen-storage. An apparent degradation of phospholipid was observed during incubation of prefermented frozen-cells of NFTY, while little change of phospholipid occurred in FTY. These results suggested that the difference in the sensitivity for freezing of yeast might be due to the strength of cell membrane in terms of the degradation of phospholipid by enzymes, phospholipases, attached to cell membranes.

Key words: freeze-tolerant yeast, phospholipid, phospholipase, freeze-thawing, frozen-dough

INTRODUCTION

A major problem of frozen-dough process for bread-making is the cellular injury of baker's yeast during freeze-storage of doughs. Freeze-injured yeast cells are known to have poorer fermentative ability, and intracellular substances having an absorption maximum at 260nm leak out from cells by freeze-thawing. Tanaka (1) reported that fermented frozen-doughs prepared by baker's yeast had a weak dough structure and fermentative ability after thawing and decreased volume of bread by baking. This weakening of dough structure is related to glutathione, a low molecular weight sulfhydryl compound, which is released from freeze-injured cells after thawing. It is demonstrated that the addition of the same amount of glutathione as is concerned in baker's yeast to dough results in weakening of dough elasticity due to the reduction of -S-S- linkage in gluten structure. Therefore, in the case of baker's yeast, cell membranes are damaged under freezing conditions, and intracellular glutathione leaks out from yeast cells, which gives a serious influence on rheological and baking properties of doughs.

Liderberg and Lode(2) reported that ultraviolet(UV)-absorbing materials appeared in the extracellular fluid of the frozen-thawed suspensions of *Escherichia coli* in amounts proportional to the loss in viability. Mazur(3) observed the same phenomenon in yeast as was observed in bacteria; UV-absorbing materials were found in extracellular fluid of yeast as a result of depression of the freezing point, and the increase in extracellular materials seemed roughly proportional to the loss in viability when yeast cells were frozen and thawed.

On the other hand, electron-microscopic evidence was shown for the injury of cell membrane of frozen-cell(4,5). There is another report that freeze-thawing of yeast cells caused damage to cell membrane, resulting in the degradation of phospholipid in cell membranes.

Recently, it was found that a FTY, D_{2-4}, classified as *Tolularspora delblueckii*, which was isolated from nature(6,7), showed a small amount of UV-absorbing substances leaking out of the cell after freeze-thawing than a NFTY, which belongs to *Saccharomyces cerevisiae*. It was assumed that the difference in the degradation of phospholipid in cellular membrane of FTY and NFTY might be concerned with the difference in the amount of leakage from frozen-damaged cells of each yeast strain. This report deals with the difference in the amount of intracellular substances which leaks out of the cells from FTY and NFTY after freeze-thawing in a synthetic fermentation medium. The degradation of lipid and phospholipid components of both cells was also investigated during incubation in the medium before and after freezing in relation to cellular injury.

MATERIALS AND METHODS

Microorganism

S. cerevisiae 2001 which was isolated from commercial baker's yeast (Oriental Yeast Co., Ltd., Japan) was used as NFTY, FTY, D_{2-4}, which was isolated from nature, and a commercial FTY(CFY, Oriental Yeast Co., Ltd.) which belong to *S. cerevisiae*(8) were used throughout this work.

Chemicals

Phospholipids(phosphatidylcholine; PC, phosphatidylethanolamine; PE, phosphatidylserine; PS, phosphatidylinositol; PI, phosphatidic acid; PA, lysophosphatidylcholine; LPC, lysophosphatidylethanolamine; LPE, cardiolipin; CA), ergosterine, ergosterol oleate, 1,2- and 1,3-dipalmitic acid, monoolein, triolein, oleic acid were purchased from Sigma Chemical Company (Sl. Lrnis, MO, USA). Other chemicals were pure analytical grade from Nakarai Tesque(Osaka, Japan).

Culture conditions of yeast

The culture medium used in this work was CM medium(9), which contained 20g of glucose, 3.5g of polypeptone, 2.0g of KH_2PO_4, 1.0g of $MgSO_4 \cdot 7H_2O$, 1.0g of yeast extract, 10mg of adenine, 0.2mg of thiamine. 0.2 mg of pyridoxine, 0.2mg of niacin, 0.2mg of Ca-pantothenated, 2μg of biotin in 1 liter of distilled water.

Yeasts were grown at 30°C for 24hr under reciprocal shaking with 100ml of CM medium. The cells were harvested by centrifugation, washed 3 times with distilled water and filtered by membrane filter(Millipore. pore size 1.2μm).

Freezing conditions of yeast

Harvested yeast cells(100mg as dry weight) were suspended in 25ml of a ASF medium(10) containing 5% sucrose and prefermented at 30°C for 2hr, followed by storage at -20°C for 24hr. In another experiment, the yeast suspension was frozen immediately at -20°C without prefermentation. After 24hr storage, the frozen yeast suspension was thawed for 10min in a water bath at 30°C, and centrifuged at 5000rpm for 10min, and the supernatant was used for UV-absorbancy measurement at 260nm with a Hitachi spectrophotometer(Model 100-40)

Extraction and analysis of lipids from yeast

Fermented or non-fermented yeast suspensions as described above(100mg as dry weight in 25ml of ASF medium) were heated for 10min in boiling water, and sonicated at 0~10°C in an ice bath for 10min with glass beads using a 20Kc Kaijo Denki oscillator. Then, lipid was extracted from the disintegrated cells with chloroform-methanol by the method of Bligh and Dyer (11). Extracted lipids were separated into two fractions by a Sep-pak silica cartridge(Millipore). Separated neutral lipid fractions were analyzed by Iatroscan(TLC/FID analyzer IATROSCAN Laboratories. Inc) and phospholipids were analyzed by thin layer chromatography (TLC).

Appropriate amounts of extracted lipids were spotted on a TLC-rod with a solvent system consisted with toluene : chloroform : formic acid(50 : 20 : 1, by volume). After development, TLC-rods were scanned by a Iatroscan(Model MK-5). Identification of lipid components was performed by comparing the retention times of samples with those of standard compounds, i.e., triglyceride, sterol ester, 1,2- and 1,3-diglyceride, sterol, monoglyceride. Phospholipid separated by Sep-pak was applied to TLC using silica gel plates(Merck, silicagel 60 with a concentrating zone) with chloroform : acetone : methanol : acetic acid : water(120 : 100 : 20 : 6.67)(12). Spots of phospholipid on TLC plates were detected by putting them in a desiccator containing iodine. Each spot developed on the plates corresponding to authentic phospholipids was scrapped off, and phosphorus content was determined by the method of Fiske-Subbarow(13) after hydrolysis of the sample in 10*N* sulfuric acid at 150°C for 5hr.

RESULTS AND DISCUSSION

Leakage of UV-absorbing substances from yeast

The UV-absorbing spectrum of supernatant solutions from FTY, D_{2-4} and CFY, and a NFTY(*S. cerevisiae* 2001) was measured after freeze-thawing of the suspensions in a synthetic fermentation medium. As shown in Fig. 1, substances with a maximum absorption at 260nm were released from three strains of yeast, but the amount of leakage of cellular substances from *S. cerevisiae* was increased by prolongation of frozen storage days. This tendency seemed to be rather weak in D_{2-4} strain and CFY. These results indicate that *S.*

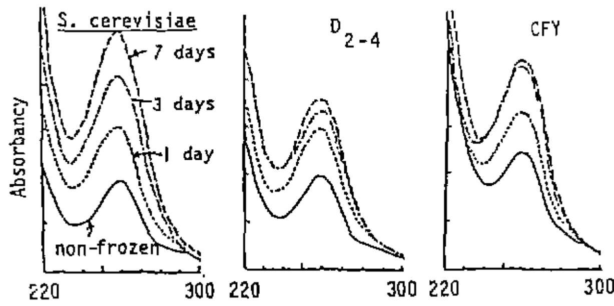

Fig. 1. Leakage of UV-absorbing substances of yeast during frozen storage.
Frozen-storage period: ········; 1day, - · - · -; 3 day, -----; 7day, ———; non-frozen cells

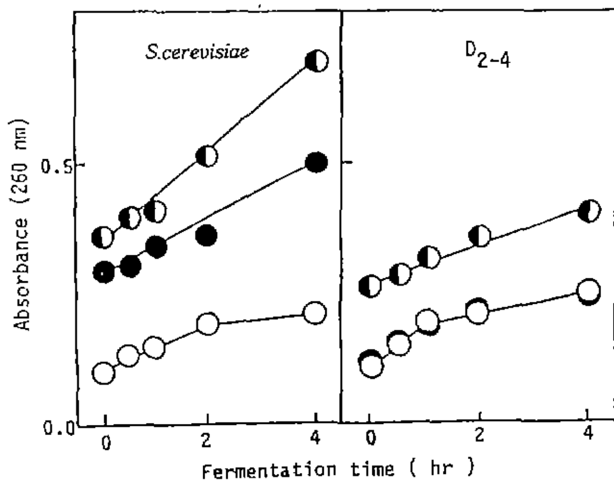

Fig. 2. Effect of freezing of prefermentation on leakage of UV-absorving substances from yeast cells.
◐: 2-hr prefermented frozen cell.
●: frozen cell, ○: non-frozen cell

cerevisiae is more suspectible to freeze-injury than D_{2-4} or CFY during frozen storage in liquid fermentation medium.

The change of optical density at 260nm of supernatants of cell suspensions for baker's yeast and D_{2-4} during incubation in ASF medium at 30°C is shown in Fig. 2. An apparent increase of leakage of UV-absorbing substances from *S. cerevisiae* was observed after freeze-thawing. Especially, a rapid increase of the leakage of intracellular substances occurred in 2hr prefermented cells of baker's yeast by incubation, while no such a remarkable leakage was observed for D_{2-4} strains. The amount of leachate from 2hr prefermented frozen-thawed cells of D_{2-4} was larger than non-prefermented frozen cells before incubation, but the rate of leakage of intracellular substances was lower as compared to the prefermented cells of baker's yeast.

It is reported that the fermentation of dough before freezing is detrimental to the viability of yeast, mainly due to ethanol as a major fermentation product by yeast. Ichimasa(14), Souze(15), Harrison and Trevelyan (16) reported that the primary cell injury was recognized by the leakage of UV-absorbing substance from yeast cells caused by the breakdown of cell membranes which consist of lipid and protein.

Degradation of lipid by yeast cells during incubation

Fig. 3 shows the change of various lipid components of frozen and non-frozen cells of *S. cerevisiae*, D_{2-4}

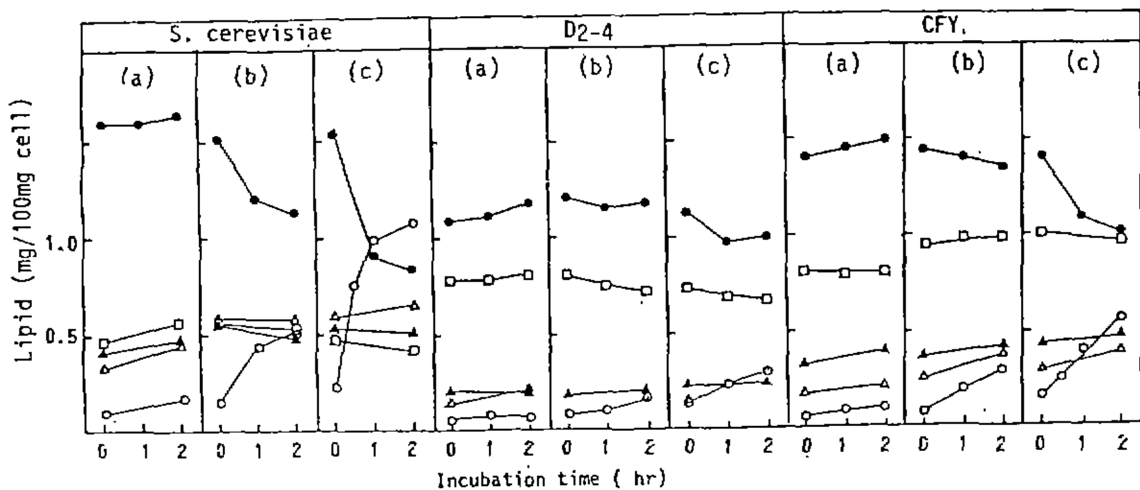

Fig. 3. Change of lipid components of yeast cells during incubation at 30°C.
(a); non-frozen cells (b); frozen cells (c); 2hr-prefermented frozen cells
●; Phospholipid, ○; Free fatty acid □; Triglyceride ▲; Sterol, △; Sterol ester

and CFY during fermentation in ASF medium. It was found that, in non-frozen cells of each yeast, little change in phospholipid component occurred during incubation. In frozen cells, however, free fatty acid was increased gradually during early stage of incubation with decrease of phospholipid, although no significant change of triglyceride, sterol and sterol ester was observed. This tendency was more remarkable in prefermented *S. cerevisiae,* where phospholipid was decreased rapidly during an early 30min incubation with the formation of equivalent amount of free fatty acids. These results indicate that, when *S. cerevisiae* was frozen-thawed in ASF synthetic medium, phospholipid components of yeast cell membrane partially deacylated to form fatty acid and deacylated phospholipids.

An appreciable amount of free fatty acid was formed in *S. cerevisiae* during an early stage of incubation which was frozen-thawed after prefermentation for 2 hr before freezing. The results indicate that the deacylation of phospholipid might be occurred by the action of phospholipase A+lysophospholipase.

Degradation of phospholipid by yeast cells during incubation

The changes of each component of yeast phospholipid before and after incubation of prefermented frozen cells were examined. As shown in Fig. 4, PC, a major component of phospholipid in yeasts, was decreased to half of the initial amounts in *S. cerevisiae,* while the decrease of PC in FTY was relatively small. The

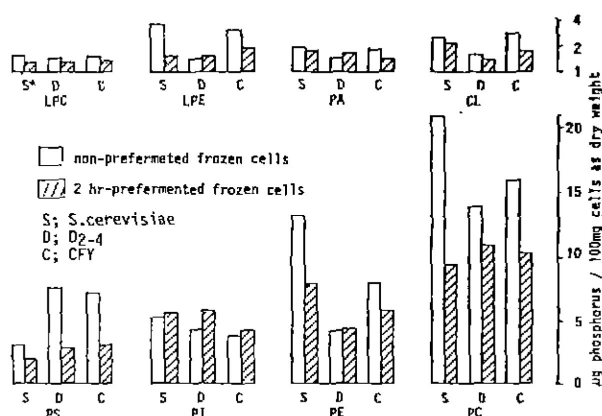

Fig. 4. Changes of phospholipid components of frozen yeast cells.
LPC; lisophosphatidylcholine, LPE; lisophosphatidylethanolethanolamine, PA; phosphatidic acid, CL; cardiolipin, PS; phosphatidyl serine, PI; phosphatidyl mositol, PE; phosphatidylethanolamine, PC; phosphatidylcholine.

amount of PE in *S. cerevisiae* also decreased by incubation, but no significant change in PI, CA, PA was observed. These results indicate that phospholipase of yeast cells may be activated by freezing, resulting in the formation of deacylated phospholipids and free fatty acids.

REFERENCES

1. Tanaka, Y. : Freezing damage of yeast in frozen dough. *Jpn. J. Freez. Dry.,* **28**, 83(1982)
2. Liderberg, G. and Lode, A. : Release of ultraviolet-absorbing material from *Esherichia coli* at subzero temperatures. *Can. J. Microbiol.,* **9**, 523(1963)
3. Mazur, P. : Studies on rapidly frozen suspensions of yeast cells by differential thermal analysis and conductometry. *Biophys. J.,* **3**, 323(1963)
4. Komatsu, Y., Saito, M. and Osumi, M. : Biochemical and electron-microscopic evidence for membrane injury in yeast cells quickly frozen with liquid nitrogen. *J. Ferment. Technol.,* **65**, 127(1987)
5. Saito, H., Shimada, S., Nakatomi, Y., Nagashima, A. and Tanaka, Y : The mechanism of tolerance to freeze-thaw injury in *Saccharomyces* species. *Tech. Rep. Jpn. Yeast Ind. Assoc.,* **52**, 33(1982)
6. Hahn, Y. S. and Kawai, H. : Screening of freeze-tolerant yeasts and their bread dough fermentative properties. *J. Home Econ. Jpn.,* **41**, 115(1990)
7. Hahn, Y. S. and Kawai, H. : Isolation and characterization of freeze-tolerant yeasts from nature available for the frozen dough method. *Agri. Biol. Chem.,* **54**, 829(1990)
8. Nakatomi, Y., Saito, H., Nagashima, A. and Umeda, F. : *Saccharomyces* species FD 612 and the utilization. *Jpn. patent* S58-201978, 389(1983)
9. Iguti, S. : Effects of mannitol on protoplasts of *Saccharomyces cerevisiae. Plant Cell Physiol.,* **9**, 573(1968)
10. Atkin, L., Achults, A. A. and Frey, C. N. : Influence of dough constituents on fermentation. *Cereal Chem.,* **22**, 321(1945)
11. Bligh, E. G. and Dyer, W. J. : A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.,* **37**, 911(1959)
12. Hayashi, Y., Urade, R. and Kito, M. : Distribution of phospholipid molecular species containing arachidonic acid and cholesterol in V79-UF cells. *Biochem. Biophys. Acta,* **918**, 267(1987)
13. Fiske, C. H. and Subbarow, Y. : The colorimetric determination of phosphorus. *J. Biol. Chem.,* **66**, 375(1925)
14. Ichimasa, M. : Degradation of lipids in yeast at the early phase of organic solvent-induced autolysis. *Agri. Biol. Chem.,* **42**, 247(1978)
15. Souzu, H. : The phospholipid degradation and cellular death caused by freeze-thawing or freeze-drying of yeast. *Cryobiology.,* **10**, 427(1973)
16. Harrison, J. S. and Trevelyan, W. E. : Phospholipid breakdown in Baker's yeast during drying. *Nature.,* **200**, 1189 (1963)

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