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Miyamoto, Takahisa
Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

Kawabata, Ko-ichi
Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

Honjoh, Ken-ichi
Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

Hatano, Shoji
Laboratory of Food Hygienic Chemistry, Faculty of Agriculture, Kyushu University

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Effects of Trehalose on Freeze Tolerance of Baker's Yeast

Takahisa Miyamoto, Ko-ichi Kawabata, Ken-ichi Honjoh and Shoji Hatano

Laboratory of Food Hygienic Chemistry, Faculty of Agriculture,
Kyushu University, Fukuoka 812-81, Japan.
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A conventional baker's yeast strain D incorporated trehalose into its cells from a YPG medium supplemented with trehalose. Cells cultured in a medium containing 3 to 5% trehalose increased to nearly 5 times the trehalose content of cells cultured in the absence of trehalose. After 1 day of frozen storage at −20 ℃, cells cultured in 3% trehalose medium experienced a lesser decrease in both viability and CO, productivity than cells cultured in the absence of trehalose. Even after 10 days frozen storage, the strain D cultured in the 3% trehalose medium retained to nearly 50% of the viability and CO, productivity of the unfrozen cells. Although the freeze-tolerant yeast strains DFT and S. *cerevisiae* MAFF lo-03056 showed, during freezing, smaller decreases in viability than strain D, the large decreases in CO_2 productivity were comparable among all three strains. The CO_2 productivity in both freeze-tolerant strains cultured in the presence of 3% trehalose was about 60% of that in the unfrozen cells, even after 10 days of frozen storage. The CO_2 productivity and actin content of the cell-free extracts prepared from the strain D cultured in YPG medium decreased significantly to about 15% and 30% of those of the unfrozen cells, respectively, after 7 days of frozen storage. When the cells cultured in the presence of 3% trehalose were frozen-stored, the CO_2 productivity of the cell-free extracts prepared from 7-days frozen-stored cells decreased to 50% of that from the unfrozen cells. The actin content, however, did not decrease after the same frozen storage. In eukaryotic cells, the activities of some glycolytic enzymes are increased by association with actin. It seems that the native structure of actin is necessary for yeast CO, productivity after frozen storage.

INTRODUCTION

The practice of freezing prepared dough has been gaining acceptance in the baking industry (Hsu et al., 1979 a, b). However, because of the injury to yeast cells induced by freezing, frozen dough yields a poorer quality bread than unfrozen dough (Wolt and D'appolonia, 1984 a, b; Morris *et al.*,1988). It has been reported that the bread-making potential of frozen dough decreases substantially with increasing duration of frozen storage (Hsu *et al.*, 1979 a). The most important problem in the frozen-dough method is determining how to maintain the viability and gassing power of frozen yeast. Freeze-tolerant yeast strains have been discovered in order to overcome these difficulties, (Hino *et al.*, 1987; Oda *et al.*, 1986). These yeasts, however, remain less than completely satisfactory for use in frozen-dough baking. It has also been reported, though, that the content of trehalose in freeze-tolerant yeast strains was higher than that in conventional yeast strains (Hino *et al., 1990).* The trehalose content of commercial baker's yeast is widely believed to be a critical parameter for its stress resistance, and this relation has received a lot of attention, particularly with respect to the production of instant dry yeast. Over the years, the culture conditions for commercial baker's yeast have been optimized

in order to obtain a high trehalose content. At present, trehalose levels of 15 to 20% of the dry weight are common, with 10% considered a critical threshold. For the preservation of yeast viability in frozen doughs, a high trehalose content is also crucial. Yeast cells growing on a rapidly fermented sugar, such as glucose, have little stress resistance and also a very low trehalose level (Dijck et al., 1990). On the other hand, stationary phase cells and cells grown on nonfermentable carbon sources have both high stress resistance and high trehalose content (Kotyk and Michaljanicova, 1979; Panek, 1975). Whereas the stress resistance of commercial yeast cells is very high before contact with nutrients, mixing of yeast cells with flour results in rapid mobilization of trehalose and rapid loss of stress resistance (Dijck et *al.,* 1990). In recent years, much attention has been drawn to the possible function of trehalose as a stress protectant, attention based mainly on the remarkable stress-protection properties of trehalose in vitro and the strong correlation between trehalose content and stress resistance *in vivo* (Eleutherio, 1993; De Vergilio et al., 1994). The protective effect of trehalose, however, is not well understood at a molecular level.

The mechanism of yeast cell injury caused by freezing and thawing, and the mechanism of freeze tolerance are poorly clarified. A number of studies have been made on the metabolic impairment or changes in cellular constituents of yeast after freeze-thawing. The glycolytic system is essential for gassing power in baking yeast. Impairment of the glycolytic system decreases the gassing power of yeast cells. Clarke and Masters (1975) have reported that, in skeletal muscle, some of the glycolytic enzymes are associated with actin, one of the constituents of the cytoskeleton of eukaryotic cells. The activities of some glycolytic enzymes are increased by association with actin (Arnold and Pette, 1970). We have shown that the capacity of restoration of actin and the glycolytic system from the injury induced by frozen storage and thawing might contribute to the freeze tolerance of yeast cells (Hatano et *al.,* 1996).

This study was undertaken to investigate the mechanism of the protective effect of trehalose against freezing injury in baker's yeast. Trehalose was incorporated into yeast cells, and CO_2 productivity and actin of the cell-free extract were compared between normal cells and the cells incorporated with trehalose after frozen storage for 7 days.

MATERIALS AND METHODS

Strains and culture

Daiya Yeast (abbreviated D, a conventional baker's yeast) and Daiya Yeast FT (abbreviated DFT, a freeze-tolerant yeast used for frozen-dough baking), both strains of *Saccharomyces cerevisiae,* were purchased from Kyowa Hakko Co., Tokyo, Japan. *Saccharomyces cerevisiae* MAFF lo-03056 (a freeze-tolerant yeast) was obtained from the National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry, and Fisheries, Tsukuba, Ibaraki, Japan. Yeast cells were cultured in a YPG medium containing 0.4% yeast extract, 0.5% polypeptone, 0.5% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$ and 5% glucose at 30 °C unless otherwise stated. For frozen storage, cells were suspended in ASF medium (Hatano et *al.,* 1996) containing 8.2 mM phosphate buffer (pH6.0), 0.01 mM thiamine, 0.02 mM pyridoxine, 0.4 mM nicotinic acid, 9.3 mM $MgSO_4 \cdot 7H_2O$, 22 mM $(NH_4)_2SO_4$, 95 mM urea, 24 mM glucose, and 119 mM maltose.

Cell-counting and measurement of viability

The number of yeast cells in suspension was measured with a Coulter particle counter (Coulter Electronics Ltd., Luton, Beds, U.K.). The viability of yeast cells was measured using an agar-plating method with YPG agar. After incubation of plates at 30 ˚C for 24 hr, yeast colonies were counted. The viability was calculated by dividing the cell-counts of the frozen sample by those of an unfrozen sample.

Preparation of cell-free extract

Yeast cells (about 5.0×10^{10}cells) in the stationary phase of growth were harvested by centrifugation at $2000\times g$ for 5 min. The cells were washed with an actin extraction buffer (abbreviated AEB; 0.1 M imidazole-HCl buffer, pH 7.5, containing 0.1 mM $CaCl_2$, 0.5 mM ATP, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonylfluoride, 0.75 mM 2-mercaptoethanol, and 5 mM KCl) (Zechel, 1980) and then resuspended in 20 ml of the same buffer. The suspension was homogenized with glass beads of 0.3 mm in diameter in a reciprocal shaker (Vibrogen-Zellmuhle, Edmund Buhler Co., Tubingen, F. R. G.) operated for 20 min at 4500 rpm and 4°C. The homogenate was centrifuged at $2000\times g$ for 5 min at 4 ˚C, and the resultant supernatant was further centrifuged at 60,000 $\times g$ for 20 min at 4 “C. The secondary supernatant was used as a cell-free extract.

Freezing and thawing

In general, both cooling rate and period of frozen storage affect the viability of microorganisms (Mazur, 1965 and 1970). To diminish the effects of cooling in this study, yeast cells were cooled at a rate of 0.5 ˚C/min. Yeast cells were harvested and washed once with sterile water and suspended in ASF medium at a density of 5×10^6 cells/ml. Five ml of the suspension was placed in a test tube (18 x 180 mm) set at a slant to decrease supercooling, was cooled at a rate of 0.5 ˚C/min in an air-blast freezer at -20 ˚C and kept at -20 “C. The frozen specimen was thawed at a rate of 20 ˚C/min in a bath kept at 30 ˚C. In the frozen-storage test of the cell-free extract, 5 ml of the cell-free extract was treated in the same manner.

Measurement of CO, productivity

To measure the CO_2 productivity of yeast, the suspension of yeast cells in ASF medium (10^6cells/ml) was placed in a rubber-stopped plastic syringe to avoid contact with air, and incubated at 30 “C. Twenty ml of the suspension was withdrawn at the start of the incubation and again after 2 hr. Two ml of 1 M citrate buffer (pH 4.5) was added to the suspension, and the CO_2 concentration of the mixture was measured with an ion meter (Model SA720, Orion Research Co., Ltd., Boston, MA, U.S.A.) with CO_2 electrode (Model 95-02, Orion Research Co., Ltd., Boston, MA, U.S.A.). In this assay system, CO_2 concentration increased with increasing incubation period until 4 hr. CO_2 productivity was expressed as an increment of CO, concentration after a 2-hr incubation, and was calculated by dividing the CO, productivity of the frozen sample by that of the unfrozen sample.

To measure the CO, productivity of cell-free extract, the extract was mixed with 0.6 M glucose, 1 mM ATP, 1 mM ADP, 1 mM NAD, and 1 mM NADH, and incubated at 30°C. After 1 ml of the cell-free extract was withdrawn and mixed with 19 ml of deionized water

and 2 ml of 0.1 M citrate buffer (pH 4.5), the CO_2 concentration of the mixture was measured. The CO_2 productivity was expressed as μ mole of CO_2 produced per mg protein per 2 hr.

Trehalose determination

Yeast cells were harvested and washed once with sterile water. Trehalose was extracted from yeast cells with 10% trichloroacetic acid and determined by the anthrone method (Arnold and Mclellan, 1975). To confirm the presence of trehalose in the yeast cells, the trichloroacetic acid extract was analyzed by thin-layer chromatography on a Silicagel 60 plate (Merck, Darmstadt, F. R. G.) with developing solvent (60% (v/v) ethyl acetate, 15% (v/v) acetic acid, 15% (v/v) methanol, and 10 % (v/v) distilled water). After development, the plate was air-dried and spots were detected by spraying with 20% H_2SO_4.

Measurement of actin content

The contents of total actin in the cell-free extracts of yeast cells were measured by a DNase I inhibition assay (Blikstad et *al.*, 1978) using rabbit muscle actin (>90% G-actin, Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard.

RESULTS AND DISCUSSION

Effects of trehalose on viability and CO_2 productivity

Figure 1 shows changes in viability and CO_2 productivity of baker's yeast D during

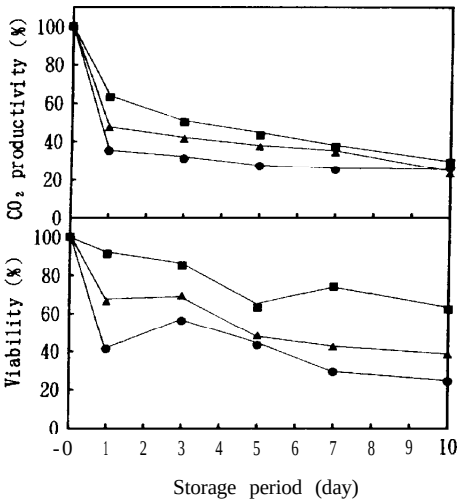

Fig. 1. Changes in viability and CO, productivity of baker's yeast D during frozen storage at -20 ℃ in ASF medium containing trehalose. Symbols: ●, stored in ASF medium; A, stored in ASF medium containing 1% trehalose; ■, stored in ASF medium containing 3% trehalose.

frozen storage at -20 ˚C in ASF medium containing trehalose. After 1 day of storage, the CO_2 productivity and viability of strain D in ASF medium decreased to 38% and 40%, respectively, of those of the unfrozen cells. In the presence of 3% trehalose, although the CO_2 productivity decreased to 40% of that of the unfrozen cells after storage for 7 days, the viability decreased only slightly, retaining 80% of the viability of the unfrozen cells after the same storage period. These results indicate that the addition of trehalose to a freezing medium is effective in minimizing the decreased viability of baker's yeast during frozen storage, but not so for the maintenance of CO, productivity.

Incorporation of trehalose into yeast cells

Thin-layer chromatography of the trichloroacetic acid extracts of yeast cells cultured in the presence of trehalose showed that the yeast cells had incorporated trehalose from the culture medium (data not shown). Table 1 shows trehalose content of baker's yeast D cultured in YPG medium containing trehalose. The trehalose content of yeast cells increased after cultivation, but the cell concentration of yeast cells decreased with increasing concentration of trehalose in YPG medium. When the strain D was cultured in the medium containing 3 to 5% trehalose, trehalose content was nearly 5 times that of the strain cultured in an absence of trehalose. These trehalose contents were at the same level as those of the stress-tolerant yeasts reported previously (Hino et al., 1990; Dijck *et al.*, 1995).

Table 1. Trehalose contents in baker's yeast D cultured in YPG medium containing trehalose.

	Trehalose cone. (%) / Glucose comc.(%) in medium					
	0 / 5	1 / 4	2 / 3	3 / 2	4 / 1	5 / 0
Trehalose content (mg/10^9 cells)	1.35	3.40	4.35	4.98	6.43	5.77
(% dry weight)	2.1	5.3	6.8	7.8	10.0	9.0
Final cell concentration ($\times 10^9$ cells/ml)	2.90	2.02	2.34	2.10	1.80	1.77

Yeast was cultured at 30 ˚C for 48hr with shaking.

Freeze tolerance of yeast cells incorporating trehalose

Figure 2 shows the changes in viability and CO_2 productivity in yeast cells that were cultured in a YPG medium containing 3% trehalose and stored frozen at -20 "C. After 1 day of storage, the rates of viability and CO, productivity in the cells cultured in YPG medium containing trehalose had decreased less than the rates of cells cultured in YPG

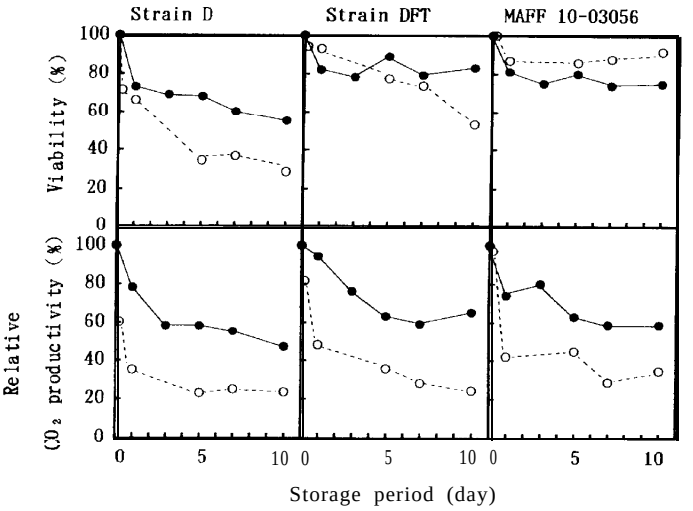

Fig. 2. Changes in viability and CO, productivity of yeast cells cultured in YPG medium containing 3 % trehalose during frozen storage at -20 °C. Values are the means of two separate experiments.
Symbols: **0,** cells cultured in YPG medium; ●, cells cultured in YPG medium containing 3 % trehalose.

medium alone. Even after 10 days, the strain cultured in the presence of 3% trehalose had retained about 50% of the CO, productivity and viability of the unfrozen cells. These values were nearly double those of the yeast cells cultured in YPG medium alone. The decrease in viability of the freeze-tolerant yeast DFT and *S. cerevisiae* MAFF lo-03056 were smaller than that of the strain D after frozen storage. The CO, productivity of both DFT and MAFF lo-03056 largely decreased after 1 day of storage, while productivity was at the same level as for strain D after frozen storage of 10 days. The CO_2 productivity of both DFT and MAFF lo-03056 cultured in the presence of trehalose were about 60% of those of the unfrozen cells even after 10 days of storage.

CO_2 productivity and actin content of cell-free extracts

Table 2 shows the CO, productivity and actin content of cell-free extracts prepared from strain D cultured in the presence and absence of trehalose. CO, productivity of cell-free extracts prepared after 7 days of storage from cells cultured in the absence of trehalose decreased to about 15% of that of the unfrozen cells. The actin content of cell-free extracts also decreased to about 30% after the same period of storage. When the cells cultured in the presence of 3% trehalose were stored frozen, the CO_2 productivity of the cell-free extracts prepared from cells stored frozen for 7-days decreased to 50% of the productivity in unfrozen cells. Actin content, however, did not decrease after the same period of frozen storage. Hitchcock et *al.* (1976) have reported that the native structure

Table 2. CO_2 productivity and actin content of yeast D cultured in YPG medium containing trehalose.

Cell-free extract	CO_2 productivity (μ mole $CO_2/10^{10}$cells/2h)	Actin content (mg/10^{10} cells)
Cultured in YPG medium		
Unfrozen cells	113.0	3.65
Frozen-stored cells	17.9	1.04
Cultured in YPG medium containing 3% trehalose		
Unfrozen cells	118.0	3.01
Frozen-stored cells	61.8	3.33

Values are the means of two separate experiments.

of actin is important for the binding and inhibition of DNase I. The decrease in actin content, as measured by the DNase I inhibition assay, was indicative of conformational changes in actin which yields a form no longer capable of inhibiting DNase I. We have shown that freezing injury of actin is greater in freeze-sensitive yeast than in freeze-tolerant yeast (Hatano et *al.*, 1996). These results and data indicate that trehalose incorporated into yeast cells functions as a freeze-protectant on actin in yeast cells. In eukaryotic cells, some of the glycolytic enzymes bind to actin, which is known to increase the activities of some of these enzymes (Arnold and Pette, 1970). The rates of decrease in CO_2 productivity and viability were almost the same in each of the yeast strains cultured in the medium containing trehalose (Fig. 2). It seems that the native structure of actin is necessary for CO_2 productivity in yeast following frozen storage. Further investigations on the interaction of the glycolytic system and actin might clarify the mechanism of the protective effect of trehalose on the gassing power of yeast cells.

Since trehalose is a by-product in L-glutamic acid fermentation by *Brevibacterium lactofermentum* with sucrose used as source of sugar (Yoshii et al., 1993), it is readily available for the commercial production of baker's yeast. Although the effects of prefermentation before freezing on the CO_2 production of frozen dough made with trehalose-incorporated yeast cells will need to be examined, media containing trehalose appear suitable for production of baker's yeast for use in the frozen dough method.

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