Improvement of Freezing and Oxidative Stress Tolerance in *Saccharomyces cerevisiae* by Taurine

Ken-ichi Honjoh1, Takeshi Machida2, Koutarou Nishi2, Kanae Matsura2, Kevin Webby Soli1, Takatoshi Sakai3, Hiroya Ishikawa4, Kiyoshi Matsumoto3, Takahisa Miyamoto4 and Masayoshi Iio1

1 Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka 812-8581, Japan
2 Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Fukuoka 812-8581, Japan

Received November 10, 2006; Accepted February 17, 2007

The effect of taurine on the survival of *Saccharomyces cerevisiae* after freezing and oxidative stress was investigated. Proline and NaCl were used in comparison. The accumulation of taurine in yeast cells seemed to lead to the enhancement of tolerance to both freezing and oxidative stress in yeast. Although taurine appeared to be less effective than proline in the development of freezing tolerance, when based on intracellular amounts taurine protected cells more effectively than proline. In order to clarify the effect of taurine on stress tolerance, the expression patterns of stress-responsive genes were observed using RT-PCR. In addition, the contents of glycerol and trehalose as well as the redox states of glutathione in the yeast cells were investigated. Our results suggest that taurine, as well as proline, may function as a cryoprotectant and/or an antioxidant in yeast.

Keywords: cryoprotectant, freezing tolerance, oxidative-stress tolerance, *Saccharomyces cerevisiae*, taurine

Abbreviations: DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid), PBS: phosphate buffered saline, PITC: phenylisothiocyanate, RT-PCR: reverse transcription polymerase chain reaction

Introduction

Improvement of the freezing tolerance of plants is important for viable food preservation at low temperatures, and can be widely applied in agriculture and food technology. Freezing causes severe stress on living plant cells and induces injury leading to a loss of viability. It involves not only temperature stress, but also dehydration and oxidative stress (Mazur, 1970; Kendall and McKersie, 1989).

In order to improve the freezing tolerance of plants, we focused our study on taurine and its utilization as a cryoprotectant. Taurine, a β-amino acid with a sulfonic group instead of a carboxyl group, is synthesized in the liver and some parts of the brain in mammals (Hayes, 1985; Dominy et al., 2004), and is suggested to function as an osmoprotectant, antioxidant, and membrane stabilizer (Schaffer et al., 2003). Taurine has also been reported to exist in fish and shellfish, probably as an osmoprotectant (Takeuchi et al., 2000). Furthermore, taurine is used effectively in the frozen storage of bull sperm, presumably due to its protection against the oxidative stress induced by freezing (Chen et al., 1993). The mechanisms of the antistress functions of taurine are yet to be fully elucidated, but taurine accumulated in cells may prevent the osmotic dehydration of cells, a damaging factor caused by extracellular freezing, thereby providing an antifreezing effect. The antistress functions of taurine in animal cells have prompted its utilization as an antifreezing and antioxidative agent for the preservation of animal cells.

In plants or yeast, taurine is not considered a stress protectant, as it is generally absent, or only present in minimal amounts; however, there are a few exceptions (Huxtable, 1992). Taurine has been identified in the fruit juice of opuntia, a type of cacti (Stintzing et al., 1999; 2001), and in both lentil seeds and seedlings (Rozan et al., 2001). Cacti are found in deserts under drought conditions, which are considered to cause severe stress. The finding that taurine is present in cacti suggests that it could play a role in protecting plant cells against stressful conditions.

We have studied the development of freezing tolerance using *Chlorella vulgaris* (Joh et al., 1995), and the enhancement of freezing tolerance using *Saccharomyces cerevisiae* (Honjoh et al., 1999) and *Nicotiana tabacum* (Honjoh et al., 2001). In the latter study, the degree of freezing tolerance in tobacco plants was increased by the expression of a candidate cryoprotectant, a late embryogenesis abundant protein, but was still insufficient for agricultural production. Thus, the accumulation of multiple cryoprotectants is required to enhance the freezing tolerance of agricultural products.

In plants, proline, glycine betaine, mannitol, and trehalose are also known to function as compatible solutes (Nanjo et al., 1999; Hayashi et al., 1997; Tarczynski et al., 1993; Bhandal et al., 1985). Proline and trehalose are reported

* To whom correspondence should be addressed.
E-mail: honjoh@agr.kyushu-u.ac.jp
to be effective in protecting against freezing stress in not only plant cells (Nanjo et al., 1999; Bhandal et al., 1985), but also yeast cells (Takagi et al., 1997; Coutinho et al., 1988). In addition, glycerol has been reported as an effective cryoprotectant for yeast cells (Izawa et al., 2004). Considering taurine as an osmoprotectant in animal cells (Huxtable, 1992; Dawson et al., 2002), if it functions as a cryoprotectant in yeast cells, then it may have the same function in plant cells.

In the present paper, in order to investigate whether taurine functions as a cryoprotectant in the model organism Saccharomyces cerevisiae, we studied the effects of high concentrations of exogenously provided taurine on freezing- and oxidative stress tolerance. To compare these effects, proline, a compatible solute, and NaCl, an osmotic stress inducer, were also investigated. Furthermore, during the accumulation of the three solutes in yeast cells, we also investigated the intracellular levels of other stress protectants (glycerol and trehalose), the expression patterns of several stress-responsive genes of S. cerevisiae, and the intracellular redox states. At present, we plan to express genes, specifically, encoding enzymes related in taurine biosynthesis from the common carp, in S. cerevisiae using an expression vector with a galactose-inducible promoter. Thus, in the present paper, raffinose was used as a carbon source to obtain basic data for the functions of taurine as a stress protectant.

Materials and Methods

Strains and culture The yeast strain used was Saccharomyces cerevisiae YPH500 (Mata, ura3-52, lys2-801ampr, ade2-101his3, trpl-Δ63, his3Δ200, leu2-Δ1; Stratagene, La Jolla, CA, U.S.A.). Yeast cells were cultured in synthetic raffinose (SR) medium, which contained 2% raffinose, 0.67% yeast nitrogen base (Difco, Detroit, MI, USA), 0.2% dropout mix, or YPD agar that contained 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar (Burke et al., 2000). To investigate stress tolerance, taurine, proline, or NaCl was then added to the SR medium.

Freezing tolerance test Cells of S. cerevisiae were precultured in 3 mL of SR medium in a test tube with shaking at 120 rpm for 48 h at 30°C. Thirty μL of the precultured cells were inoculated to 3 mL of SR medium containing taurine, proline, or NaCl. The cells were incubated with shaking at 30°C for 24 h until the stationary phase. In the stationary phase, the cell density in each medium, except for 0.6 M NaCl-containing medium, was approximately 1.5×10⁸ cells/mL, while the cell density in 0.6 M NaCl-containing medium was approximately 5×10⁷ cells/mL. Apart from cells cultured in 0.6 M NaCl-containing medium, 1 mL of the cell culture was centrifuged at 500×g for 5 min, and the harvested yeast cells were washed twice with 1 mL of 0.5% NaCl solution and suspended in 500 μL of the same solution, at approximately 3×10⁸ cells/mL. The cells cultured in 0.6 M NaCl-containing medium were suspended in 200 μL of the same solution, at approximately 2.5×10⁸ cells/mL. For ice formation, the cell suspensions (100 μL), contained in 1.5 mL eppendorf tubes, were cooled in a deep freezer at −80°C for 2 min. After visually confirming that samples were frozen, they were transferred to another freezer and kept at −20°C for 24 h. The frozen samples were thawed in a water bath kept at 25°C for 5 min, and then serially diluted with 0.9% NaCl solution. Diluted cell suspensions (50 μL) were spread on YPD agar plates. After 2 days of growth at 30°C, the individual colonies were counted. The relative survival rate was determined by comparing frozen-thawed cells to unfrozen cells; where the number of colonies after freezing and thawing was expressed as a percentage of the number of colonies before freezing. Cell numbers described above were determined by using numbers of colonies before freezing.

Oxidative-stress tolerance test As described above, 3 mL of yeast cells were cultured, and 1 mL of the cells were harvested by centrifugation at 500×g for 5 min. The cells were washed twice with 1 mL of 100 mM potassium phosphate buffer (pH 7.5), and then suspended in 1 mL of the same buffer. The concentration of the cell suspension was then adjusted to an OD₆₀₀ of 0.1 (approximately 3×10⁸ cells/mL) in a test tube by dilution with buffer, to obtain a final volume of 4 mL of the cell suspension. H₂O₂ solution was added to the test tube at a final concentration of 0.05%. The samples were incubated at 30°C with shaking at 120 rpm. Aliquots of the sample were serially diluted with the buffer, and 50μL of appropriate diluents were spread onto YPD agar plates at the appropriate times. After 2 days of growth at 30°C, the individual colonies were counted. The relative survival rate was determined by comparing stressed cells to unstressed control cells, which were plated prior to the H₂O₂ treatment.

Extraction of amino acids For the analysis of free amino acids, yeast cells were harvested from 3 mL of culture in proline- or taurine-containing medium. The cells were then washed three times with water and lyophilized. The lyophilized samples were suspended in 200 μL of 0.5 μmol/mL of methionine sulfone, which was used as an internal standard. The cell suspension was homogenized with an equal volume of 0.5 mm glass beads by vortexing at maximum speed for 1 min, and then cooling on ice for 1 min. This procedure was repeated 10 times. After disruption with the vortex mixer, the sample concentration was adjusted to 2.5 mg dry weight of yeast cells per 100 μL by adding methionine sulfone solution. The samples were then boiled for 10 min and centrifuged at 20,000×g for 10 min. The supernatants were lyophilized for derivatization of amino acids with phenylisothiocyanate (PITC).

Derivatization of amino acids with PITC Derivatization of the free amino acids with PITC was performed according to the method described by Bidlingmeyer et al. (1984). The lyophilized samples or amino acid standards were treated with 70 μL of a mixture of ethanol-triethylamine-water (1:1:1, v/v/v), and then vacuum dried. The dried samples were dissolved in the derivatization reagent, which consisted of 70 μL of a mixture of ethanol-triethylamine-water (7:1:1, v/v/v) and 5 μL of PITC, vortexed for 30 sec, and allowed to react for 10 min at
room temperature. Excess reagent, triethylamine, and other volatile products were evaporated under vacuum. Derivatized samples were finally dissolved in a mixture of 5 mM sodium phosphate buffer (pH 7.5)-acetonitrile (95: 7, v/v), based on the cell concentrations used for preparation of amino acid analysis.

**Amino acid analysis** Amino acid analysis was performed similarly to the method outlined by Bidlingmeyer et al. (1984). The amino acid standards (type H) were obtained from Wako Pure Chemical Industries, Osaka, Japan, and supplemented with methionine sulfone (Wako), tryptophan, and taurine (Nacalai Tesque, Kyoto, Japan). Free amino acids were separated on a Pico Tag column (3.9 mm i.d. × 300 mm; Waters, Milford, MA, U.S.A.) with a Shimadzu HPLC system (Model LC-9A, Shimadzu, Kyoto, Japan). HPLC analysis was performed according to the manual attached to the Pico-Tag column.

**Assay of glycerol and trehalose** For the analysis of glycerol and trehalose, yeast cells were harvested from 3 mL of culture in proline-, taurine- or NaCl-containing medium. The cells were then washed three times with water and lyophilized. The lyophilized samples were then weighed and suspended in water, for the glycerol assay, or in 0.25 M Na₂CO₃, for the trehalose assay, at a concentration of 20 mg dry cell weight/mL. The suspended cells were boiled for 20 min. After centrifugation at 20,000 × g for 10 min, the supernatant was obtained for further assays. Extraction of trehalose, and hydrolysis of trehalose to glucose with acid trehalase, was performed according to the method described by Zentella et al. (1999), and glucose was measured enzymatically using the glucose assay kit (Roche, Mannheim, Germany). Glycerol was measured enzymatically using the glycerol assay kit (Roche).

**Poly(A)^+ RNA preparation and RT-PCR** According to the method described by Carlson and Botstein (1982), total RNA was isolated from yeast cells cultured in SR containing taurine, proline, or NaCl. Poly(A)^+ RNA was purified from total RNA using Oligotex™-dT30 < Super > mRNA Purification Kit (Takara, Kyoto, Japan), according to the manufacturer’s instructions. The purified poly(A)^+ RNA was dissolved in RNase-free water and the concentration of RNA solution was adjusted to 5 μg/10 μL. A mixture of 10 μL of the RNA solution and 2 μL of 0.5 μg/μL of oligo (dT) primer was heated at 70°C for 3 min, in order to denature the RNA conformation. After cooling on ice, 8 μL of a solution containing 50 mM Tris-HCl (pH 8.8), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT (pH 8.3), 1 mM of each deoxy-nucleotide triphosphate, and 40 U of ReverScript I (Wako Pure Chemical Industries) was added to the mixture. The reaction was started by primer-template annealing at 30°C for 10 min, and primer-extension at 42°C for 60 min. The reaction was terminated by heating at 95°C for 5 min.

The PCR reaction was carried out with a 20 μL mixture containing 1.5 U of Tag DNA polymerase (Sigma), 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 200 μM of each deoxynucleotide triphosphate, 20 pmol of each appropriate primer (Table 1), and 1 μL of the reverse transcription reaction mixture. The mixtures were subjected to altering cycles of PCR amplification, consisting of template DNA denaturation at 94°C for 30 sec, primer-template annealing at 55°C for 30 sec, and primer extension at 72°C for 30 sec. Poly(A)^+ RNA solution was used in place of the reverse transcription reaction mixture, to confirm the absence of genomic DNA in the RNA solution.

**Determination of glutathione levels** Yeast cells, which had been cultured in medium containing taurine, proline, or NaCl for 24 h, were collected, and the concentration of the cell suspension was adjusted with water to an OD₆₀₀ of 1.0 (approximately 1 × 10⁶ cells/mL). Cells from 5 mL of the suspension were harvested by centrifugation at 560 × g for 5 min. The cells were washed twice with phosphate-buffered saline, pH 7.4 (PBS), and resuspended in 2.5 mL of PBS. A hemocytometer was used to determine cell numbers for a part of the cell suspension. Using 0.5 mL of the cell suspension, extraction of intracellular glutathione was performed according to the method described by Grant et al. (1998). The treated yeast cells were harvested, washed twice with PBS, and suspended in 500 μL of ice-cold solution of 8 mM HCl containing 1.3% 5-sulfosalicylic-acid. The cells were broken with glass beads by vortexing for 30 sec at 4°C, followed by incubation on ice for 15 min to precipitate proteins. Unbroken cells, cell debris, and proteins were precipitated by centrifugation at 20,000 × g for 15 min. The supernatant was used for the determination of glutathione levels.

The concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined using a modified version of the method outlined by Griffith (1980).

---

**Table 1. Primers used for RT-PCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR1</td>
<td>TTR1-For</td>
<td>5'-ITCAGTGGCGCAAAAGGAAG-3'</td>
</tr>
<tr>
<td>TTR1</td>
<td>TTR1-Rev</td>
<td>5'-GGCCGCGGAGTCTCTTTCAA-3'</td>
</tr>
<tr>
<td>TPS1</td>
<td>TPS1-For</td>
<td>5'-TCTTTAGCCGAGAAGATGG-3'</td>
</tr>
<tr>
<td>TPS1</td>
<td>TPS1-Rev</td>
<td>5'-AGCCAGGACGACCCTTATCT-3'</td>
</tr>
<tr>
<td>CTT1</td>
<td>CTT1-For</td>
<td>5'-AGCCTGGAAAGAAGACAGA-3'</td>
</tr>
<tr>
<td>CTT1</td>
<td>CTT1-Rev</td>
<td>5'-CTTCAAGTTGATCAGACT-3'</td>
</tr>
<tr>
<td>HSP12</td>
<td>HSP12-For</td>
<td>5'-CTTGGTGAGCTTCTTCACC-3'</td>
</tr>
<tr>
<td>HSP12</td>
<td>HSP12-Rev</td>
<td>5'-CGAACCAACCAAAGAAAAC-3'</td>
</tr>
<tr>
<td>HSP42</td>
<td>HSP42-For</td>
<td>5'-AACGCTTAGTTCAGACCT-3'</td>
</tr>
<tr>
<td>HSP78</td>
<td>HSP78-For</td>
<td>5'-GCTGTCACAGAAAGACGCTTA-3'</td>
</tr>
<tr>
<td>HSP78</td>
<td>HSP78-Rev</td>
<td>5'-CTTACCACATCTACCAACCTA-3'</td>
</tr>
<tr>
<td>GPX1</td>
<td>GPX1-For</td>
<td>5'-ATTCATCCCTCTTCAACCTC-3'</td>
</tr>
<tr>
<td>GPX1</td>
<td>GPX1-Rev</td>
<td>5'-CACCCTCCATTTCCGTTCA-3'</td>
</tr>
<tr>
<td>GPX2</td>
<td>GPX2-For</td>
<td>5'-CGGAATTTGCGATGTTAAT-3'</td>
</tr>
<tr>
<td>GPX2</td>
<td>GPX2-Rev</td>
<td>5'-CAGGAAGATGATGTTTGT-3'</td>
</tr>
<tr>
<td>GPX3</td>
<td>GPX3-For</td>
<td>5'-CGCGGTGACCCGTTTTA-3'</td>
</tr>
<tr>
<td>GPX3</td>
<td>GPX3-Rev</td>
<td>5'-GGTITCCGACACAAAGACG-3'</td>
</tr>
<tr>
<td>CCP1</td>
<td>CCP1-For</td>
<td>5'-GCTGTTTTGTCCTCACCCTA-3'</td>
</tr>
<tr>
<td>CCP1</td>
<td>CCP1-Rev</td>
<td>5'-TGAGGTGCGCAACAGAGAC-3'</td>
</tr>
<tr>
<td>TSA1</td>
<td>TSA1-For</td>
<td>5'-GCGATCGAGAGCCGTGTTTA-3'</td>
</tr>
<tr>
<td>TSA1</td>
<td>TSA1-Rev</td>
<td>5'-AGTCAGTGCGAGGGAAAAGA-3'</td>
</tr>
<tr>
<td>TSA2</td>
<td>TSA2-For</td>
<td>5'-GAAGAAGCCGGCGTAGTCG-3'</td>
</tr>
<tr>
<td>TSA2</td>
<td>TSA2-Rev</td>
<td>5'-ACGGCTTCCTGCGGGAGATG-3'</td>
</tr>
<tr>
<td>DOTS</td>
<td>DOTS-For</td>
<td>5'-CACCAGGAATGCTTAAGAAA-3'</td>
</tr>
<tr>
<td>DOTS</td>
<td>DOTS-Rev</td>
<td>5'-TCAGTCCAAAGACGACGCA-3'</td>
</tr>
<tr>
<td>ACT1</td>
<td>ACT1-For</td>
<td>5'-GUCCTGCATGTCGTTCCATCA-3'</td>
</tr>
<tr>
<td>ACT1</td>
<td>ACT1-Rev</td>
<td>5'-GGCAGAAGGATGCTCTAAA-3'</td>
</tr>
</tbody>
</table>
The amount of total glutathione was determined using the sample solution prepared above. For GSSG only, the sample solution was treated with a final concentration of 2% 2-vinylpyridine for 1 h at 25°C to remove GSH. Samples were assayed in 3 mL of reaction mixture containing 100 mM sodium phosphate (pH 7.5), 5 mM EDTA, 0.2 mM NADPH, 0.6 mM DTNB, 0.5 units glutathione reductase, and sample solution (0.05 mL). The reaction was started by the addition of glutathione reductase. The amount of 5-mercapto-2-nitrobenzoic acid produced by the reduction of DTNB was monitored for 0.5 min at 412 nm in a recording spectrophotometer (UV-160; Shimadzu, Kyoto, Japan). The GSH content of the assayed samples was determined by comparison of the observed rate with a standard curve generated from known amounts of GSH. The amount of GSH was calculated by subtracting the amount of GSSG from the amount of total GSH. Glutathione levels were expressed as nanomoles of GSH per 1.0 × 10⁷ cells.

**Results**

*Taurine content in yeast cells*  The amount of taurine or proline incorporated into yeast cells was determined by amino acid analysis (Fig. 1). After 24 h, the cultures of *S. cerevisiae* in SR medium containing 0.2, 0.4, or 0.6 M taurine led to the accumulation of 66.7±6.4, 101±7, or 126±16 μmol/g dry cell weight of taurine, respectively. In contrast, the 24-h culture of *S. cerevisiae* in the medium containing 0.2, 0.4, or 0.6 M proline led to the accumulation of 347±42, 371±32, or 424±26 μmol/g dry cell weight of proline, respectively. Furthermore, in order to compare the effects of similar intracellular amounts of taurine and proline on freezing tolerance, cells cultured in the medium containing 0.025–0.15 M proline were also studied. In particular, yeast cells cultured in the medium containing 0.025 or 0.05 M proline led to the accumulation of 78±14 or 146±27 μmol/g dry cell weight, respectively. These levels were close to the accumulated taurine levels in cells that were cultured in the medium containing 0.4 or 0.6 M tau-

Fig. 1. Intracellular contents of taurine (A) and proline (B) in *S. cerevisiae*. Detailed conditions for the experiments are described in Materials and Methods. Results represent the mean±SD from three independent experiments.

Fig. 2. Effect of taurine on freezing tolerance of *S. cerevisiae*. Detailed conditions for the experiments are described in Materials and Methods. Results represent the mean±SD from three independent experiments.
Improvement of Stress Tolerance in Yeast by Taurine

Relationship between taurine and freezing tolerance
Freezing tolerance of yeast cells grown in SR medium containing taurine, proline, or NaCl was investigated. Proline and NaCl were used for comparison with taurine. As shown in Fig. 2, the accumulation of taurine led to the enhancement of the yeast cells protection against damage induced by freezing and thawing in a dose-dependent manner. The viabilities of yeast cells cultured in the medium containing 0.2, 0.4, or 0.6 M taurine were 22±5, 37±7, or 50±8%, respectively. In contrast, the addition of proline to the medium led to a higher tolerance for yeast cells than taurine at the same molar concentrations. However, the accumulated taurine levels were much lower than the proline levels when the same concentration of each solute was added to the medium (Fig. 1). Based on the intracellular amounts of the solutes (taurine and proline), taurine appeared to have a greater effect on freezing tolerance than proline (Fig. 2). The addition of NaCl to the medium also led to an improvement in the freezing tolerance of yeast. However, the effect of NaCl on the freezing tolerance was lower than that of taurine.

Relationship between taurine and oxidative-stress tolerance
For estimation of the effect of taurine on the alleviation of oxidative stress, viable cell counts of *S. cerevisiae*, which had been cultured in SR medium containing taurine, proline, or NaCl, followed by exposure to 0.05% H₂O₂, were determined (Fig. 3). Yeast cell cultures containing taurine led to the enhancement of oxidative stress tolerance. In particular, the tolerance of yeast cells cultured in the taurine-containing medium increased concomitant with an increase in the concentration of the solute. Even after 3-h incubation with H₂O₂, the viability of yeast cells cultured in 0.6 M taurine was approximately 20%. Yeast cell cultures containing proline or NaCl also led to an enhancement of oxidative-stress tolerance. The tolerance of cells cultured in the medium containing 0.2 M or 0.4 M proline was almost equal. Yeast cells cultured in the medium containing 0.6 M proline led to a decrease in tolerance. Furthermore, the tolerance of cells cultured in NaCl-containing medium decreased gradually with increasing concentrations of NaCl. Thus, out of the three solutes, taurine was the most effective in enhancing tolerance against oxidative stress.

Intracellular contents of glycerol and trehalose
In order to investigate effects of other stress protectants, in particular, glycerol and trehalose, on stress tolerance, the intracellular levels of the two substances were studied (Fig. 4A). The levels of glycerol were increased in yeast cells cultured in the taurine- or NaCl-containing medium, and these levels were correlated with the concentration of taurine or NaCl. However, the levels of glycerol in yeast cells cultured in taurine-containing medium varied from 1.9±0.5 to 8.4±4.0 μmol/g dry cell weight, which were lower than those in yeast cells cultured in NaCl-containing medium (from 3.2±0.8 to 13.5±5.0 μmol/g dry cell weight). On the other hand, no significant increase in glycerol levels was found in yeast cells cultured in proline-containing medium.

The intracellular contents of trehalose were increased in yeast cells cultured in 0.2 or 0.4 M solute-containing medium (Fig. 4B). Conversely, culturing in 0.6 M solute-containing medium led to a decrease in the levels of trehalose. When cultured in taurine-containing medium, 0.2 or 0.4 M taurine led to accumulation of 109±10 or 116±10 μmol/g dry cell weight of trehalose, respectively. However, the trehalose content in cells in 0.6 M taurine-containing medium was 31.6±9.6 μmol/g dry cell weight, lower than that of the control cells (63.2±5.6 μmol/g dry cell weight of trehalose).

Expression of several stress-responsive genes
For comparison of the stress tolerance achieved by the addition of high concentrations of solute (taurine or proline), the effects of stress responsive genes on stress tolerance could

![Fig. 3. Effects of taurine (A), proline (B), and NaCl (C) on oxidative-stress tolerance of *S. cerevisiae*.](image)

Detailed conditions for the experiments are described in Materials and Methods. The closed-square and the closed-circular symbols represent concentrations of 0.2 M, the closed-triangular symbols represent concentrations of 0.4 M, and the closed-circular symbols represent concentrations of 0.6 M. The open-circular symbols represent control. Results represent the mean±SD from three independent experiments.
not be ignored. In order to investigate the expression of genes that have a stress-response element (STRE) in the promoter regions (Martínez-Pastor et al., 1996) or that encode peroxidase, RT-PCR analysis was performed (Fig. 5). The investigated STRE genes included TTR (glutathione reductase), TPSI (trehalose synthase subunit), CTT (catalase), HSP12 (unknown), HSP42 (heat shock protein), HSP78 (chaperone), and UBC5 (ubiquitin conjugating enzyme). The investigated peroxidase genes included GPX1, GPX2, GPX3 (glutathione peroxidases), CCP1 (cytochrome c peroxidase), TSA1, TSA2 (thioredoxin peroxidases), and DOT5 (thioredoxin peroxidase). ACT1 was used as an internal control. As shown in Fig. 5, expression of CTT1 was induced with an increase in the concentration of the three solutes. The expression pattern of CTT1 was relatively correlated with the patterns of freezing tolerance of solute-accumulating yeast cells (Fig. 2) but not with the patterns of oxidative-stress tolerance (Fig. 5). Thus, we could not conclude that the difference in the type of solute influenced the expression of the CTT1 gene. Furthermore, although differences in the expression patterns of four other genes (HSP42, GPX1, GPX2, and TSA2) were observed, they were not correlated with the tolerance of freezing or oxidative stress.

**Effect of taurine on the intracellular redox state** To investigate the effect of taurine on the redox states of glutathione in yeast cells, the intracellular levels of reduced and oxidized glutathione were investigated (Fig. 6). The levels of reduced (GSH), oxidized (GSSG) glutathione as well as the glutathione redox ratio (GSH: GSSG) were determined. Results represent the mean±SD from three independent experiments.

Fig. 4. Intracellular contents of glycerol (A) and trehalose (B) in S. cerevisiae.

Detailed conditions for the experiments are described in Materials and Methods. Results represent the mean±SD from three independent experiments.

![Graph A](image)

**Graph A**

- **A**
  - Intracellular contents of glycerol (A) and trehalose (B) in S. cerevisiae.
  - The bars represent the mean±SD from three independent experiments.
  - The x-axis shows the concentration of each solute in the SR medium (M).
  - The y-axis shows the content (mmol/g dry cell weight).

**Graph B**

- **B**
  - The levels of reduced (GSH), oxidized (GSSG) glutathione as well as the glutathione redox ratio (GSH: GSSG) were determined.

![Graph B](image)

**Graph B**

- The bars represent the mean±SD from three independent experiments.

Fig. 5. Transcript expression profiles of stress-responsive genes.

Semi-quantitative RT-PCR was performed with primers shown in Table 1. PCR cycles are indicated in parentheses on the right. ACT1 gene was used as a control.

![Image](image)

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>Taurine</th>
<th>Proline</th>
<th>NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR</td>
<td>(30)</td>
<td>(35)</td>
<td>(35)</td>
<td>(35)</td>
</tr>
<tr>
<td>TPSI</td>
<td>(35)</td>
<td>(35)</td>
<td>(25)</td>
<td>(25)</td>
</tr>
<tr>
<td>CTT</td>
<td>(25)</td>
<td>(25)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>HSP12</td>
<td>(25)</td>
<td>(25)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>HSP42</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>GPX1</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>GPX2</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>GPX3</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>CCP1</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>TSA1</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>TSA2</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>DOT5</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
<tr>
<td>ACT1</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
<td>(30)</td>
</tr>
</tbody>
</table>

Fig. 6. GSH levels and redox state of GSH.

The levels of reduced (GSH), oxidized (GSSG) glutathione as well as the glutathione redox ratio (GSH: GSSG) were determined. Results represent the mean±SD from three independent experiments.
The levels of total glutathione (GSH and GSSG) were slightly increased in yeast cells cultured in taurine- or proline-containing medium, in comparison to those in cells cultured in control or NaCl-containing medium. However, no significant differences in the levels of reduced glutathione to oxidized glutathione (GSH/GSSG) were found among the investigated samples.

Discussion

For confirmation of the incorporation of taurine or proline into S. cerevisiae, free amino acid analysis was performed (Fig. 1). The incorporation levels of proline were found to be higher than those of taurine. In S. cerevisiae, four kinds of amino acid permeases are reported to be involved in the incorporation of proline into the cells (Andréasson et al., 2004). For example, Put4 has a high affinity to proline (Jauniaux et al., 1987). Thus, amino acid permeases such as Put4 were assumed to play an important role in the uptake and accumulation of proline in yeast cells. On the other hand, a taurine-specific amino acid permease has not yet been reported. As taurine is not an essential amino acid for yeast, the cells likely lack permeases highly specific to taurine. In the present study, taurine might be incorporated into yeast cells by low affinity permeases, leading to the different accumulation levels of the two amino acids, proline and taurine, in yeast. In this way, the incorporation levels of the amino acids may be related to differences such as the existence of specific permeases.

During accumulation of solutes in yeast cells, the addition of solutes (taurine, proline, or NaCl) to the medium at high concentrations might induce changes in the levels of other amino acids within the yeast cells, leading to the enhancement of stress tolerance. Although the cellular levels of several amino acids were slightly altered by the addition of high concentrations of the solutes, no significant differences were observed in the levels of the investigated amino acids, except for two amino acids, taurine and proline (data not shown).

As shown in Fig. 2, the addition of taurine or proline to the medium enhanced the freezing tolerance of yeast. When yeast cells were cultured in the same concentration of proline or taurine, it was found that the intracellular concentration of proline was higher than taurine, which led to a greater degree of freezing tolerance. However, at the same intracellular concentration, taurine showed a greater degree of freezing tolerance than proline (Fig. 1 and 2). These results suggest that taurine protects yeast cells against freezing and thawing more effectively than proline.

Taurine has been proposed as a cryoprotectant in an intertidal bivalve (Loomis et al., 1988), and is likely to work as a cryoprotectant in combination with 10% dimethyl sulfoxide in the cryopreservation of human hematopoietic stem cells (Limaye and Kale, 2001). However, only few reports investigating the effect of taurine on the viability of organisms after freezing and thawing are available (Limaye and Kale, 2001; Chen et al., 1993). Furthermore, there are no reports regarding the effects of taurine on the viability of microorganisms after freezing and thawing. Thus, our report is the first to show that intracellular accumulation of taurine enhanced the viability of a microorganism after freezing and thawing.

Oxidative stress is known to be attendant with freezing and thawing. In order to investigate whether taurine alleviates the oxidative damage to yeast cells during freezing and thawing, an oxidative stress tolerance test was performed. As reported by Terao et al. (2003), the intracellular proline in yeast plays a role in alleviating the oxidative stress induced during freezing and thawing, or during exposure to H$_2$O$_2$. Therefore, we investigated whether taurine could protect yeast cells under conditions of oxidative stress. In the present paper, yeast cells were exposed to 0.05% (14.7 mM) H$_2$O$_2$. In preliminary experiments, lower concentrations (1-4 mM) of H$_2$O$_2$, which had been used by other researchers (Garrido and Grant, 2002; Nomura and Takagi, 2004), were imposed on yeast cells. However, the viability of yeast cells were kept at high levels, and no significant differences in the viability of cells cultured in the medium containing either of three solutes (taurine, proline or NaCl) were observed (data not shown). Although yeast cells in the stationary phase were used in the present paper, the strain used seemed to be relatively resistive to oxidative stress in comparison to strains used by other researchers (Garrido and Grant, 2002; Nomura and Takagi, 2004). As shown in Fig. 3, taurine showed enhanced protection against oxidative stress in yeast cells. The viability of yeast cells cultured in taurine-containing medium was correlated to the concentration of taurine. On the other hand, yeast cells cultured in proline- or NaCl-containing medium also led to the enhancement of viability; however, this effect was not correlated with the administered concentration for either solute.

During the culturing of yeast cells in a high concentration of each solute (taurine, proline, or NaCl), there may have been an accumulation of other stress protectants in the cells. Thus, the intracellular contents of glycerol and trehalose were investigated (Fig. 4). Glycerol and trehalose are well known as stress protectants and have been shown to accumulate under osmotic stress conditions (González-Hernández et al., 2005; Shen et al., 1999). In the present paper, although glycerol content in yeast cells increased with an increase in taurine or NaCl concentration, the glycerol levels in yeast cells in taurine-containing media were lower than those in NaCl-containing media. By comparing glycerol levels (Fig. 4A) with taurine and trehalose levels (Fig. 1 and 4B), the direct effect of glycerol on the stress tolerance of yeast cells cultured in taurine-containing media seems to be low.

On the other hand, the levels of trehalose were increased in yeast cells cultured in 0.2 or 0.4 M solute-containing media (Fig. 4B). However, intracellular trehalose levels in yeast cells cultured in 0.6 M solute-containing media were found to be lower than control yeast cells. In S. cerevisiae, NTH1 encoding neutral trehalose has been shown to be up-regulated in 0.5 M NaCl-containing medium (Zähringer et al., 2000). Thus, decreases of trehalose in...
cells cultured in 0.6 M solute-containing media are likely
due to the up-regulation of \( NTHI \). In the present paper,
the fact that yeast cells cultured in 0.6 M taurine-containing
medium led to the enhancement of stress tolerance
suggested that taurine functions as a stress protectant.

Many researchers have demonstrated that osmotic stress
induces the expression of stress responsive genes (Martinez-
Pastor et al., 1996; Gasch et al., 2000; Hohmann, 2002).
Culturing yeast cells in a medium containing high con-
centrations of taurine, proline, or NaCl was presumed to
lead to the expression of osmotic stress-responsive genes.
Thus, the expression of several stress responsive genes,
which have STRE in the promoter regions (Martinez-
Pastor et al., 1996), and of genes coding for peroxidase
were investigated (Fig. 5). The results of RT-PCR showed
that \( CTTI \) gene expression was concentration dependent.
Expression patterns of \( HSP42, GPXI, GPX2 \), and TSA2
were changed under several conditions. \( CTTI \) is induced
by heat shock (Wieser et al., 1991), and \( GPXI, GPX2 \), and
TSA2 are induced by oxidative stress (Inoue et al., 1999;
Wong et al., 2002). Ctt1 (catalase), Gpx1, Gpx2 (gluta-
thione peroxidase), and Tsa2 (thioredoxin peroxidase)
scavenge \( \text{H}_2\text{O}_2 \), leading to the enhancement of the oxida-
tive stress tolerance of yeast cells. Out of the four genes,
the expression pattern of \( CTTI \) was moderately correlated
with the pattern of freezing tolerance in yeast cells (Fig.
2). However, differences in the expression patterns of the
four genes were not correlated with differences in the
oxidative stress tolerance of yeast cells (Fig. 3). Although
we could not completely exclude the possibility that \( CTTI \)
contributed to the enhancement of freezing tolerance, the
fact that taurine was the most effective in the enhance-
ment of oxidative stress tolerance (Fig. 3) suggests that
the solute type is the main influence on stress tolerance,
rather than \( CTTI \). Thus, in the present paper, high con-
centrations of taurine in medium led to the improvement
of stress tolerance, suggesting that taurine can function
as a cryoprotectant and/or an antioxidant, as does pro-
line.

Glutathione is a well-known antioxidant that is usually
the most abundant low-molecular-weight-thiol present in
organisms, and is suggested to be the primary antioxi-
dant for protection against hydrogen peroxide (Grant et al.,
1998). As taurine improved the oxidative stress toler-
ance of yeast (Fig. 3), we considered the possibility that
taurine had a specific influence on the redox states of
 glutathione in yeast cells. Therefore, the redox states of
 glutathione in yeast cells cultured in taurine-containing
 media were compared with those in proline- or NaCl-
 containing media (Fig. 6). However, no correlation was
 observed between the redox states and the stress toler-
 ance. These results coincided with the finding that there
 were no significant differences in the expression patterns
 of stress responsive genes in yeast cells cultured in re-
spective solute-containing media (Fig. 5). Thus, the free-
zing tolerance of yeast cells cultured in taurine- or pro-
line-containing medium and the oxidative-stress toler-
ance of yeast cells cultured in taurine-containing medium
would mainly be due to the type of solute.

Several possible functions have been suggested for taur-
inine in protecting cells against various stresses (Huxtable,
1992). Although taurine can not directly scavenge reac-
tive oxygen species (Schaffer et al., 2003), it seems to work
as a good protectant of cellular components, including
DNA, against oxidative stress (Messina and Dawson, 2000).
In the present paper, taurine showed enhancement of the
oxidative stress tolerance of yeast. Thus, because oxida-
tive stress induces damage to macromolecules such as
DNA and proteins, as well as membranes, taurine might
play a role in the alleviation of such damage.

Carpenter and Crowe (1988) investigated the cryoprotec-
tion of several amino acids, including taurine, for cold-
labile lactate dehydrogenase, against freeze-inactivation.
While proline showed cryoprotection for this enzyme,
taurine did not (Carpenter and Crowe, 1988). It was
suggested that preferential exclusion of proline from the
surface of proteins leads to the prevention of freeze-
inactivation. Thus it would be interesting to see if our
observed enhancement of the freezing tolerance with taur-
ine was due to an action different from that of proline.

For the development of freezing tolerance, the protec-
tion of membranes is one of the most important issues.
Limaye and Kale (2001) suggested that taurine could work
as a membrane stabilizer or a cryoprotectant during the
freezing and thawing process. Loomis et al. (1988) also
suggested that taurine protects phospholipid vesicles
against freeze-induced fusion and leakage. They also
proposed the hypothesis that taurine might interact with
phospholipids due to its structural similarity to the polar
head groups of phospholipids. Furthermore, taurine seems
to bind to membranes through ionic interactions (Schaffer
et al., 2003). Thus, the hypothesis of membrane protec-
tion by taurine in the freezing tolerance of yeast cells
should be investigated in the future.

In the present paper, we showed that the accumulation
of taurine in yeast led to the enhancement of freezing
tolerance and oxidative stress tolerance, suggesting that
taurine functions as a cryoprotectant or antioxidant. No
significant differences in the expression patterns of stress-
responsive genes were observed among the three solutes
(taurine, proline, and NaCl). However, we could not com-
pletely exclude the effect of the up-regulation of \( CTTI \) on
stress tolerance, nor the possibility that the high concen-
tration of taurine in the medium might induce the expres-
sion of other stress tolerance genes. We will address
these and other possibilities by investigating the stress
tolerance of a ‘modified yeast’ strain, which is currently
being genetically engineered in our laboratory to express
genes for taurine biosynthesis enzymes from the common
carp.

Acknowledgements We are grateful to Dr. Abu Sayed for helpful
comments, to Mr. Hsu-Ming Wen for the preparation of this
manuscript, and to Ms. Akiko Sato for technical assistance.

References
permeases import proline and the toxic analogue azetidine-2-
carboxylate into yeast. Yeast, 21, 193–199.


