Saccharides That Protect Yeast against Hydrostatic Pressure Stress Correlated to the Mean Number of Equatorial OH Groups

Shinsuke Fujii,*,** Kaoru Obuchi,* Hitoshi Iwashashi,* Takaaki Fujii,** and Yasuhiko Komatsu*,*

*National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, 1–1 Higashi, Tsukuba, Ibaraki 305, Japan
**Department of Bioproduction Science, Chiba University, 648 Matsudo, Matsudo-shi 271, Japan

Received October 23, 1995

Several saccharides were found to be significantly effective in providing protection against hydrostatic pressure and high temperature damage in the yeast Saccharomyces cerevisiae. The extent of barotolerance and thermotolerance with seven different sugars showed a linear relationship to their mean number of equatorial OH groups. The same linear relationship is seen when sugars protect protein molecules against elevated temperatures in vitro. Some sugars were more effective in providing protection against hydrostatic pressure nearly a hundred times than high temperature. Pre-heat shock treatment on yeast cells induce various stress tolerances. In this report, pre-heat shocked cells showed potent protection against elevated temperature, but these cells showed faint protection against elevated pressure.

These results suggest that sugars may protect cells against hydrostatic pressure and high temperature in a similar manner, probably by stabilizing the macromolecule(s), and such type of protection may be suited for pressure stress.

Key words: hydrostatic pressure; barotolerance; thermotolerance; trehalose

The transient exposure of non-adapted organisms and cells to high temperature or hydrostatic pressure often results in physiological changes1 that lead to death. Protein thermodenaturation is thought to be one of the main causes of death in living cells exposed to lethal high temperatures. It has also been known that protein is denatured with increasing hydrostatic pressure.2 When hydrostatic pressure increases, thermodynamic and kinetic changes occur in the properties of aqueous solutions, such as the relative and absolute viscosity,3 hydrogen bonding,4 and the overall free energy change in the biochemical processes.2 Furthermore, the absolute and relative viscosity of pure water decrease with increases in temperature or hydrostatic pressure until approximately 200 MPa at ambient temperature.3

Sugars, especially trehalose, and polysaccharides are known as protein and membrane stabilizers.5–8 It is thought that trehalose is not only a reserve carbohydrate but also one of the agents that are important as macromolecular protectants against some stresses in yeast cells.9–10 Ueda et al. show that several mono- and disaccharides have the ability to prevent thermal denaturation of proteins, which can be correlated to their mean number of equatorial OH groups in vitro. They postulated that the OH groups of additives, especially equatorial OH groups in sugar molecules, can array water molecules and improve the structurization of water.11,12 Based on this discussion, we have reported that the exposure of yeast cells to dimethylsulfoxide (known as a water structure improver)13 and deuterium oxide (known to form stronger hydrogen bonds than H2O)14 confer protection against hydrostatic pressure stress.15

These reports raise the possibility that the equatorial OH groups of sugars, as macromolecular stabilizers, would protect cells against elevated temperature and hydrostatic pressure conditions as lethal stresses, through arrayed water molecules and the improved structurization of water. However, there has been no evidence that the equatorial OH groups of sugars stabilize membranes or proteins in vivo. In this report, we demonstrate that the in vitro observation11,12 could be applied to elevating temperature and hydrostatic pressure conditions in vivo.

Materials and Methods

Yeast strain and culture conditions. S. cerevisiae, Hansen IFO-0224 was grown in YPD medium (glucose, 2%v; yeast extract, 1%v; peptone, 2%v). Cells were grown at 30°C overnight (cell density, 8 × 107 cells/ml). The culture in the logarithmic phase of growth was samples and divided into 2 tubes. Cells in one tube were collected at 3000 rpm for 30 s, washed 2 times with distilled water and resuspended in distilled water containing ultrasonic, fructose, mannose, galactose, glucose, sucrose, or trehalose. Cells in another tube were given the pre-heat shock treatment. Thus, cells were incubated at 45°C for 1 h in new YPD medium. Cells were collected at 3000 rpm for 30 s, washed 2 times with distilled water, and resuspended in distilled water.

These were then immediately stressed by elevated pressure or temperature.

Procedures for pressure or heat stress treatment. For pressure stress treatment, 0.4 ml cell suspensions with or without sugars were packed into a 1 ml sterilized syringe (Terumo, Japan) and pressurized.14 Also, another cell suspension was pressurized after pre-heat shock treatment. Samples were pressurized under 150 MPa at 4°C for 1 h in the absence of any air bubbles. The low temperature (4°C) had been selected under the expectation that cells do not take up any sugars. The subsequent reduction of pressure to ambient levels took place over a 2-min period. After decompression, adequately diluted cell suspensions were plated out on YPD agar medium and incubated at 30°C for 48 h until colony counting became possible.

For heat stress treatment, 0.3 ml cell suspensions with or without sugars were packed into 1 ml sterilized Eppendorf tubes and heated. Also, another cell suspension was heated after pre-heat shock treatment. Samples were heated in a water bath at 51°C for 10 min, followed by immediate cooling...
in ice water. After cooling, adequately diluted cell suspensions were plated out on YPD agar medium and incubated at 30 C for 48h until colony counting became possible.

Estimation of barotolerance and therotolerance values. Barotolerance or therotolerance values were measured by comparing the colony forming units (cfu) of pressure or heat stressed samples. The toxic effect of the sugars themselves was measured by comparing the cfu of samples incubated with 0.5, 1, or 2 mol liter of sugars for 1.5h at 4 C versus samples incubated without sugars for 1.5h at 4 C.

Results
At first, we estimated the toxic effects of the sugar solutions on the cells. As shown in the Table, 0.5 or 1 mol liter sugar resulted in a slightly toxic effect to yeast cells, but some sugars showed a strong toxic effect (less than 50% viability) on cells at the concentration of 2 mol liter. Barotolerance values endowed by four sugars (fructose, galactose, glucose, and sucrose) are shown in Fig. 1. Cell survival after a pressure stress of 150 MPa at 4 C for 1h without sugars was 0.01%. Barotolerance values with sugars increased dose-dependently. Sugars at the concentration of 1 and 2 mol/liter were significantly effective (over hundred times compared to that without sugars) in providing protection against pressure stress.

The barotolerance values for 1 mol/liter of sugars were calibrated in terms of the mean number of equatorial OH groups of the various sugars. The barotolerance values of each sugar were 0.12%, altrose; 2.0% ± 0.8, fructose; 0.45% ± 0.06, mannose; 3.3% ± 1.8, galactose; 12.3% ± 3.3, glucose; 27% ± 9.0, sucrose; and 35% ± 2.8, trehalose. A linear relationship could be traced when the barotolerance values were plotted versus the mean number of the equatorial OH groups of these sugars (Fig. 2, curve fitting:

<table>
<thead>
<tr>
<th>Sugars</th>
<th>Concentration (mol/liter)</th>
<th>Percent viability and standard deviation with different sugar concentrations</th>
<th>The mean number of equatorial OH groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D-Altrrose</td>
<td>86 ± 4.4</td>
<td>75 ± 1.7</td>
<td>41 ± 2.7</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>86 ± 4.4</td>
<td>75 ± 1.7</td>
<td>41 ± 2.7</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>96 ± 5.3</td>
<td>86 ± 6.0</td>
<td>3.0</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>101 ± 1.4</td>
<td>91 ± 5.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>102 ± 2.6</td>
<td>92 ± 5.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>102 ± 2.6</td>
<td>92 ± 5.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Trehalose</td>
<td>102 ± 2.6</td>
<td>92 ± 5.4</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* Number of experiments for D-altrrose is 2 but for other sugars it is 3.

Table 4. The Mean Number of Equatorial OH Groups and Toxic Effect of Sugar Solutions at 4 C for 1.5h

Fig. 1. Concentration Effects of Sugars on Barotolerance Values. Cells were pressurized under 150 MPa at 4 C for 1h with 0.5, 1, or 2 mol/liter of each sugar. Bars, solid, fructose; open, galactose; stripes, glucose; dots, sucrose. Error bars indicate standard deviation from three independent experiments.

Fig. 2. The Barotolerance Values versus the Mean Number of the Equatorial OH Groups of the Sugars. Cells were pressurized under 150 MPa at 4 C for 1h with 1 mol/liter of each sugar. Barotolerance values are plotted versus the mean number of the equatorial OH groups of seven sugars. Symbols 1, with altrose; 2, fructose; 3, mannose; 4, galactose; 5, glucose; 6, sucrose; 7, trehalose. Curve fitting, y = 0.0x + 23, R² = 0.98. Error bars indicate standard deviation from three (altrose was two) independent experiments.

Fig. 3. Thermotolerance Values versus the Mean Number of the Equatorial OH Groups of the Sugars. Cells were heated at 51 C for 10min with 1 mol/liter of each sugar. Thermotolerance values are plotted versus the mean number of the equatorial OH groups of seven sugars. The numbers in the figure denote the same number as in Fig. 2. Curve fitting, y = 0.13x - 0.31, R² = 0.98. Error bars indicate standard deviation from three (altrose was two) independent experiments.
$y = 8.0x - 23$, $R^2 = 0.98)$. The mean numbers of the equatorial OH groups are shown in the Table. Sugars have a few types of conformations in solution, so that the mean numbers of the equatorial OH groups are not whole numbers. Mannose is an epimer of altrose and glucose, and glucose is an epimer of galactose. In other words, these four α-d-aldoses have the same molecular weight and basic structure but have not the same mean numbers of equatorial OH groups.

The same series of experiments were done to characterize thermostability. Cell survival after heat stress at 51°C for 10 min without sugars was 0.01%. The thermostability values of each sugar were 0.02%, altrose; 0.12% ± 0.03, fructose; 0.07% ± 0.05, mannose; 0.20% ± 0.03, glucose; 0.25% ± 0.06, glucose; 0.51% ± 0.17, sucrose; and 0.63% ± 0.03, trehalose. A linear relationship could be traced when the thermostability values were plotted versus mean number of the equatorial OH groups of the sugars (Fig. 3, curve fitting: $y = 0.13x - 0.31$, $R^2 = 0.98$) similar to Fig. 2. Although a similar linear relationship were observed for barotolerance values and thermostability values with sugars in the same concentration, barotolerance values were at maximum over 50 times higher than the thermostability values with the same sugars.

On the other hand, pre-heat shocked cells showed 0.11% ± 0.06 and 5.4% ± 0.41 as barotolerance and thermostability values, respectively.

**Discussion**

We have succeeded in correlating our in vitro observations with that of the in vitro observations\(^1\) - \(^3\), that is, sugars could protect not only proteins against thermo-denaturation correlated to their mean number of equatorial OH groups but also protect cells against both hyperthermia and hydrostatic pressure stress as well. Hottiger et al. have reported that glucose, sucrose, and trehalose are protein stabilizers against elevated temperatures, and trehalose is superior to the other sugars, polyalcohols, and amino acids.\(^1\) Our results support this report and answer the question why trehalose shows the most potent effect of thermo-protectant by acting as a protein stabilizer. Trehalose has a higher mean number of equatorial OH groups among the well known sugars. Concerning the thermostability studies in yeast, trehalose is thought to play an important role as a thermostable.\(^1\) We have previously reported that lethal hydrostatic pressure stress on yeast cells had an analogous effect on the viability after lethal heat stress.\(^1\)

These results suggest that the cells were injured by hyperthermia and hydrostatic pressure stress on a molecular basis, that is, the injury by hyperthermia and hydrostatic pressure stress can be protected by macromolecular stabilizers by improving water structurization. Furthermore, it raises the possibility that sugars may have the ability to prevent not only thermo-denaturation but also hyperbaric denaturation of proteins or disruption of other macromolecules correlated to their mean number of equatorial OH groups in vitro.

Pre-heat shock treatment on yeast cells induces various stress tolerances that include thermostability\(^2\) and barotolerance.\(^4\) The studies of yeast cells during hyperthermia, heat shock proteins (hsp) induced by pre-heat shock treatment are thought to protect cells by repairing or digesting denatured proteins.\(^5\) In this report, heat shock treatment elevated the barotolerance values only ten times, from 0.01% to 0.11%, but elevated the thermostability values over 500 times, from 0.01% to 5.4%. On the other hand, 1 mol/liter trehalose elevated the barotolerance values over 3000 times, but elevated the thermostability values only 60 times. Low temperature at pressurization would be one of the causes of these differences.

These results suggest that this protection manner of sugars and heat shock treatment may be suited for pressure and heat resistance, respectively. Protection of the former may be done by trehalose and on the latter by hsp in natural yeast cells.

The studies of barotolerance and comparison with thermostability could help to reveal and understand the mechanism of multiple stress tolerance in yeast cells.

**Acknowledgments.** We special thank Drs. Uedaira and Dr. Sunil C. Kaul for their valuable comments and criticism.

**References**