

Effects of Trehalose and Ethanol on Yeast Cytosolic Pyrophosphatase

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Trehalose has been described to protect several enzymes against destabilizing conditions. This sugar is naturally accumulated by yeast as a stress protectant. A common stress condition that yeast is normally submitted is the presence of ethanol, the by-product of fermentation process of several yeast. In this paper we show the effects of trehalose and ethanol, alone or together, on yeast pyrophosphatase, and the effects of these compounds on inhibition and unfolding of pyrophosphatase promoted by urea. We show that both trehalose and ethanol inhibit pyrophosphatase in a dose-dependent manner, and that the presence of ethanol does not modify the inhibition promoted by trehalose as well as the presence of trehalose does not modify the inhibition promoted by ethanol. The effects of trehalose on pyrophosphatase are completely reversible, but the inhibition caused by ethanol was only partially reversible. Incubation of pyrophosphatase with 10% (v/v) ethanol promoted an inhibition of 15%, and the control activity was completely recovered after removal of ethanol. On the other hand, when pyrophosphatase was incubated with 20% (v/v) ethanol an inhibition of 40% of the control activity was observed which persisted after removal of ethanol. Ethanol also potentiates the inhibition of pyrophosphatase promoted by urea, and contributes for an irreversible inactivation and unfolding of pyrophosphatase in the presence of urea. Trehalose, that protects this enzyme against the inhibition and unfolding promoted by the chaotropic compound urea, was inefficient to protect against the effects of ethanol. Trehalose was also efficient to prevent an irreversible inactivation induced by urea.

Introduction

Trehalose is a disaccharide of glucose accumulated by baker's yeast as a stress protectant under distinct conditions, as high temperature or desiccation (Crowe *et al.*, 1984, 1996; De Virgilio *et al.*, 1991, 1994; Wiemken, 1990; Hottiger *et al.*, 1994). It is widely distributed among living systems reaching high concentrations in yeast cells cytosol when these organisms are under stress conditions (Crowe *et al.*, 1984). Such high concentrations can reach 35% of dry weight of the cells (Crowe *et al.*, 1984; Wiemken, 1990), that in hydrated cells reach the molar range. It has been shown that phospholipid bilayers dried in the presence of trehalose preserves its function and structure after re-hydration (Crowe *et al.*, 1984, 1987, 1996). We have

shown that this stabilizer can also preserve function of many enzymes submitted to high temperatures (Sola-Penna and Meyer-Fernandes, 1994, 1998) or the presence of the chaotropic compound guanidinium chloride (Sola-Penna and Meyer-Fernandes, 1996; Sola-Penna *et al.*, 1997).

In *Saccharomyces cerevisiae*, accumulation of trehalose was observed when cells were shifted to temperatures above 28 °C or exposed to ethanol, suggesting that it might correspond to a general response to a physiological stress (Attfield, 1987; Mansure *et al.*, 1994). In this regard the heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap (Piper, 1995). Ethanol is the major product resulting from yeast carbohydrate fermentation, and is produced at relatively high concentrations reaching 10–20% (w/v), being very toxic to yeasts themselves (D'Amore *et al.*, 1990; Mansure *et al.*, 1994). The ability of trehalose to counteract the effects of ethanol on yeast has been studied (Mansure *et al.*,

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1994). It was shown that the presence of trehalose inhibits the effects of ethanol on intact yeast cells as well as in liposomes (Mansure *et al.*, 1994).

The present report examines the effects of trehalose and ethanol on the pyrophosphatase from yeasts. We show that the inhibition promoted by ethanol on pyrophosphatase is not counteracted by trehalose and that ethanol potentiated the inactivation and the unfolding of pyrophosphatase induced by the chaotropic compound urea.

Material and Methods

Yeast inorganic pyrophosphatase (EC 3.6.1.1), was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and exhibit high purity (99.5%). Trehalose, tetrasodium pyrophosphate, urea and Tris (tris[hydroxymethyl]aminomethane) were also purchased from Sigma Chemical Co (St. Louis, MO, USA). Other reagents were of the highest purity available.

Pyrophosphatase activity was determined by measuring the total Pi released at the end of incubation. The Pi concentration was determined as described by Lowry and Lopez (1946). Activity experiments were performed in a medium containing 100 mM Tris-HCl (pH 7.0); 5 mM MgCl₂, 2 mM tetrasodium pyrophosphate and 0.8 µg of purified enzyme per ml of reaction medium. The reactions were stopped after 1 min at 25 °C by addition of 2 vols. of the colorimetric reagent.

Steady-state fluorescence measurements were performed on an Hitachi F4500 spectrofluorimeter. The pyrophosphatase concentration was fixed at 10 µg/ml in 100 mM Tris-HCl (pH 7.0). Appropriate reference spectra were subtracted from the data to correct for background interferences which were always less than 5% of the fluorescence signal. All experiments were performed at 25 °C, with magnetic stirring in the cuvette. The excitation wavelength was set at 280 nm and the emission was scanned at 300–400 nm. Center of mass (average emission wavelength) was calculated using:

$$\text{Center of mass (nm)} = \frac{\sum \lambda I(\lambda)}{\sum I(\lambda)}$$

where λ is the emission wavelength (nm) and $I(\lambda)$ is the fluorescence intensity at a given wavelength.

Results and Discussion

Fig. 1 shows the effects of trehalose on pyrophosphatase. It can be seen that trehalose inhibit the pyrophosphatase activity in a dose-dependent manner (Fig. 1, open circles). The presence of 10% (v/v) ethanol did not modify this pattern and the enzyme was equally inhibited by trehalose (Fig. 1, filled circles). On the other hand, the chaotropic compound urea at 1.5 M protected pyrophosphatase from the inhibition promoted by trehalose (Fig. 1, open squares).

Ethanol also inhibited pyrophosphatase in a dose-dependent manner (Fig. 2, filled circles). The presence of 1 M trehalose did not protect pyrophosphatase from the inhibition promoted by ethanol (Fig. 2, open circles), and the curves of ethanol in the absence and in the presence of tre-

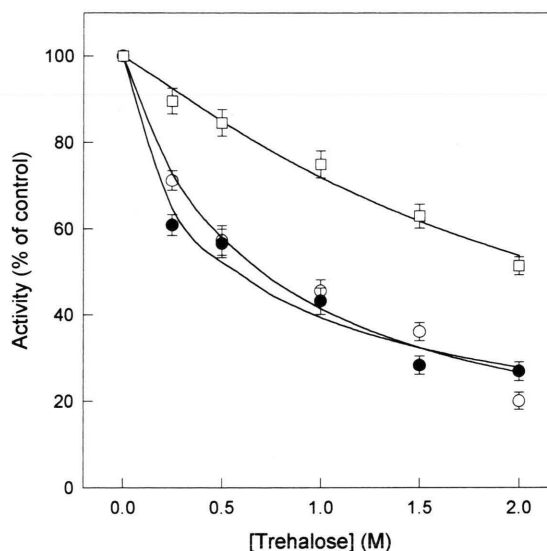


Fig. 1. Dose response of trehalose on pyrophosphatase activity. Pyrophosphatase activity was determined as described under Material and Methods, in the presence of the trehalose concentrations indicated on abscissa. (○) control, (●) in the presence of 10% (vol./vol.) ethanol and (□) in the presence of 1.5 M urea. The absolute activity in the absence of additions was $1.10 \pm 0.1 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ (mean \pm standard error) for control experiments, $0.72 \pm 0.05 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ in the presence of 10% (vol./vol.) ethanol and $0.54 \pm 0.03 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ in the presence of 1.5 M urea. Data correspond to the mean of the four experiments with different enzyme samples \pm standard errors ($n = 4$). The standard errors were calculated from the absolute activity values and converted to percentage of the control value.

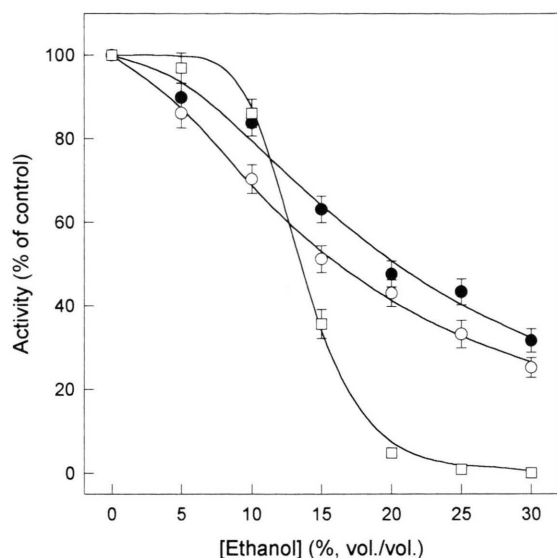


Fig. 2. Dose response of ethanol on pyrophosphatase activity. Pyrophosphatase activity was determined as described under Material and Methods, in the presence of the ethanol concentrations indicated on abscissa. (●) control, (○) in the presence of 1 M trehalose and (□) in the presence of 1.5 M urea. The absolute activity in the absence of additions was $1.07 \pm 0.08 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ (mean \pm standard error) for control experiments, $0.47 \pm 0.02 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ in the presence of 1 M trehalose and $0.58 \pm 0.04 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ in the presence of 1.5 M urea. Data correspond to the mean of the four experiments with different enzyme samples \pm standard errors ($n = 4$). The standard errors were calculated from the absolute activity values and converted to percentage of the control value.

halose had no significant difference. However, the presence of 1.5 M urea potentiates the effects of ethanol at concentrations above 15% (v/v) on pyrophosphatase that becomes totally inhibited with 20% (v/v) ethanol (Fig. 2, open squares). Taken together the data shown in Figs 1 and 2 indicate that the mechanisms involved in the inhibition of yeast pyrophosphatase by ethanol and trehalose may be different.

We evaluated the effects of trehalose, ethanol and urea on the structure of pyrophosphatase as well as the reversibility of these effects. Fig. 3 shows the effects of those compounds on the center of mass of intrinsic fluorescence spectra of pyrophosphatase. Fig. 3 summarizes these results. The blank bar (bar 1) represents the center of mass of spectra of pyrophosphatase measured with no additions. The set of bars 2 are in the pres-

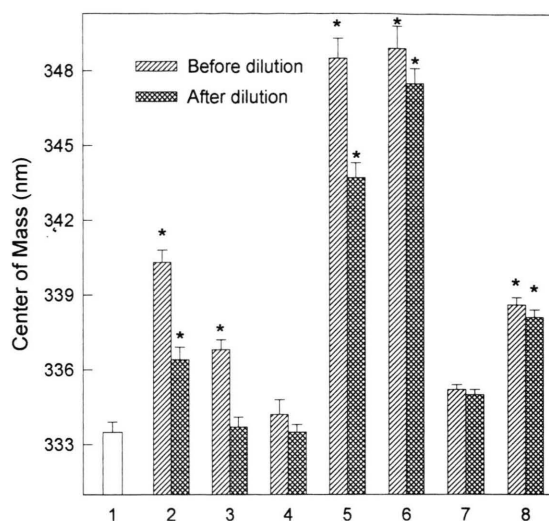


Fig. 3. Reversibility of the effects of trehalose, ethanol and urea on pyrophosphatase structure. Center of mass of the intrinsic fluorescence spectra of pyrophosphatase was determined as described under Material and Methods. Bar 1 is the Center of mass of pyrophosphatase without additions; Bars 2 were measured in the presence of 1.5 M urea and after dilution of enzyme and urea, as indicated; Bars 3 were in the presence of 1.5 M urea and 1 M trehalose; Bars 4 were in the presence of 1.5 M urea and 2 M trehalose; Bars 5 were in the presence of 1.5 M urea and 10% (vol./vol.) ethanol; Bars 6 were in the presence of 1.5 M urea and 20% (vol./vol.) ethanol; Bars 7 were in the presence of 10% (vol./vol.) ethanol; Bars 8 were in the presence of 20% (vol./vol.) ethanol. * represents values that were statistically different from group 1 in the absence of additions ($p < 0.001$).

ence of 1.5 M urea and after dilution. The red-shift promoted by urea indicates protein unfolding, and it partially persistent after dilution. This red-shift promoted by urea is attenuated by the presence of 1 M and 2 M trehalose (bars 3 and 4, respectively). And in both cases, after dilution, the enzyme recovered the original value for center of mass. The simultaneous presence of urea and ethanol promoted a large red-shift in the center of mass, that was practically irreversible (bars 5 and 6), supporting the idea that the mechanism involved in the inhibition of the pyrophosphatase by ethanol and trehalose may be different. The presence of 10% and 20% (v/v) ethanol only slightly shifted the center of mass (bars 7 and 8), indicating that unfolding promoted by ethanol is only significant in the presence of urea. Trehalose did not significantly shift the center of mass of

pyrophosphatase in any concentration tested (data not shown).

In the last years we have investigated the effects of several osmolytes, and specially sugars and polyols, on different enzyme systems (Vieyra *et al.*, 1989, 1991; Chini *et al.*, 1991; Sola-Penna and Meyer-Fernandes, 1994, 1996; 1998; Sola-Penna *et al.*, 1994, 1995 a, 1995 b, 1997). The aim of those studies was to search for the modulation that naturally occurring osmolytes exert on enzymes (Vieyra *et al.*, 1989, 1990; Chini *et al.*, 1991; Sola-Penna and Meyer-Fernandes, 1994; 1998; Sola-Penna *et al.*, 1994, 1995 b), and to understand some aspects of the mechanisms by which these class of compounds stabilize or destabilize enzyme structure and function (Timasheff, 1993; Sola-Penna and Meyer-Fernandes, 1998; Sola-Penna *et al.*, 1995 a; 1997). The studies with sugars and polyols suggested that the ability to modulate or stabilize enzyme function and structure by poly-hydroxylated compounds depends on several factors, including the number of hydroxyl groups of the molecules (Chini *et al.*, 1991; Sola-Penna and Meyer-Fernandes, 1994, 1996; Sola-Penna *et al.*, 1997).

Ethanol is produced in relatively high amount by these organisms reaching 10–20% (w/v) (D'Amore *et al.*, 1990; Mansure *et al.*, 1994). Here we tested the effects of ethanol on yeast pyrophosphatase, compared and combined with the effects of trehalose, a stabilizer of this enzyme (Sola-

Penna and Meyer-Fernandes, 1994, 1996; 1998; Sola-Penna *et al.*, 1997), and urea, a common destabilizing compound (Robinson and Jencks, 1965; Sola-Penna *et al.*, 1995 a).

Results presented here show that ethanol inactivated pyrophosphatase, and this inactivation was followed by a red-shift of the center of mass of the intrinsic fluorescence spectra, indicative of protein unfolding (Lackowicz, 1983). The inactivation and unfolding promoted by ethanol was not reversible with concentrations higher than 20% (v/v). Mansure *et al.* (1994) showed that trehalose protect yeasts membranes against the effects of ethanol. The natural yeast protectant trehalose was not able to protect pyrophosphatase against ethanol effects. The mechanism of protection of membranes by this sugar may be different from the protection of cytosolic enzymes.

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