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EXPERIMENTAL ARTICLES

Inorganic Polyphosphates and Sensitivity of *Saccharomyces cerevisiae* Cells to Membrane-Damaging Agents

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Abstract—The leakage of ATP and potassium ions from the cells of *Saccharomyces cerevisiae* with different levels of inorganic polyphosphate was studied under the action of two detergents (natural cellobiose lipid 16-[6-O-acetyl-2'-O-(3-hydroxyhexanoyl)-β-cellobiosyloxy)-2,15-dihydroxyhexadecanoic acid and sodium dodecyl sulfate) and silver cations. Cellobiose lipid had practically the same membrane-damaging activity against the cells grown in phosphate-containing medium, under phosphate starvation, and under polyphosphate hypercompensation. The cells grown under the latter conditions were less sensitive to sodium dodecyl sulfate and silver cations. The possible protective action of polyphosphates against the membrane-damaging agents under study is discussed.

Keywords: yeast, *Saccharomyces cerevisiae,* inorganic polyphosphates, cytoplasmic membrane, fungicide, cellobiose lipid, sodium dodecyl sulfate, silver ions, potassium ions, ATP.

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In microorganisms, inorganic polyphosphates (polyP), linear polymers of orthophosphoric acid, perform a number of functions providing cell adaptation to unfavorable impacts. Abundant experimental data concerning the involvement of polyP in survival of bacteria under different unfavorable environmental conditions have been accumulated [1–3]. In the case of yeasts, the interrelationship between polyP metabolism and resistance of the cells to damaging effects needs special investigation. It should also be taken into account that polyP metabolism in these eukaryotic microorganisms substantially differs from that in prokaryotes, both in the enzymes involved and in polyP structure and localization [3]. These biopolymers are present, in particular, in the yeast cell envelope, as has been demonstrated by different methods [4–9]. The goal of the present work was therefore to study the interrelation between polyP content in the cells of *Saccharomyces cerevisiae* and the sensitivity of the latter to surfactants and membrane-damaging compounds. These compounds were the natural cellobiose lipid with fungicidal and detergent properties, 16-[6-O-acetyl-2'-O-(3-hydroxyhexanoyl)-cellobiosyloxy)-2,15-dihydroxyhexadecanoic acid (CL) [10, 11], a strong anionic detergent sodium dodecyl sulfate (SDS), and the silver salt $AgNO_3$ commonly used as a fungicide [12-15].

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MATERIALS AND METHODS

The yeast *Saccharomyces cerevisiae* VKM Y-1173 was grown at 29°C on the Reader medium with 2% glucose for 12.5 h. Then the cells were subjected to phosphate starvation for 7 h and hypercompensation for 30 min as described [16]. The obtained cells were precipitated at 5000 *g* for 15 min. The cells were twice washed with distilled water.

PolyP was extracted from the cells and assayed by the content of labile phosphorus as described [16]. The following polyP fractions were obtained: acid-soluble fraction polyP1 (extracted with 0.5 N $HClO_4$ at 0°C); salt-soluble fraction polyP2 (extracted with saturated $HClO_4$ solution at 0°C); alkali-soluble fraction polyP3 (extracted with NaOH solution, pH 9–10, at 0°C); and alkali-soluble fraction polyP4 (extracted with 0.05 N NaOH at 0°C, pH 12). The polyP5 fraction was a hot perchlorate extract (0.5 N $HClO_4$ at 90°C), where the polyP content was assayed by the amount of P_i released after treatment of residual biomass with water for 20 min twice.

CL, the natural fungicide produced by the yeast *Pseudozyma fusiformata* VKM Y-2821 from the All-Russian Collection of Microorganisms (VKM), was obtained from the culture liquid of the producer [10].

ATP leakage from yeast cells was detected as follows: samples containing 0.5 ml of 0.04 M citrate–phosphate buffer, pH 4.0 (for CL and SDS) or deion-

The content of polyP of different fractions in the cells of *Saccharomyces cerevisiae* VKM Y-1173 cultivated on the media with different amounts of phosphate

Cultivation conditions	PolyP content, mmol P/g dry biomass					
	Σ polyP	polyP1	polyP2	polyP3	polyP4	polyP5
Cells after 12.5 h of growth on the medium with phosphate, 20 mM, mid-log phase (N)	508	168	110	153	50	27
Cells after 12.5 h of growth and 7 h of phosphate starvation (–P)	65.3	18	9.7	24	6.4	7.2
Cells after 12.5 h of growth, 7 h of phosphate starvation, and 30 min of hypercompensation (+P) (20 mM phosphate)	1085.7	285	96	468	51.7	185

ized water (for Ag^+) and 0.05 ml of cell suspension (A_{600} = 0.6–1.0) were incubated for 15 min at room temperature with different concentrations of damaging agents. Then 0.01–0.05 ml of each sample were taken and the content of ATP was assayed by the luciferin–luciferase method on a 1250 luminometer (LKB, Sweden) as described in [17].

The amount of intracellular ATP leaking into the incubation medium was assessed as follows: equal volumes of dimethyl sulfoxide were added to cell aliquots, and the ATP extracted from the cells was measured. This amount was taken as 100%.

K^+ leakage from the cells was registered by a K^+ selective electrode (Orion, United States). Measurements were performed in a thermostatically controlled 2.5-ml cell at 25°C under stirring. The measurement medium contained 0.01 M citrate–phosphate buffer, pH 4.0, or deionized water (in the experiments with Ag^+). The cells were introduced to a final suspension concentration of 6–6.5 × 10^8 cells per 1 ml. The SDS detergent interfered with the work of the electrode, and, therefore, it was not used in these experiments. The maximum quantity of K^+ found in the medium was taken as 100%.

The average results of three measurements are presented.

RESULTS AND DISCUSSION

The model of phosphate starvation followed by polyP accumulation (hypercompensation) is a convenient method of obtaining *S. cerevisiae* cells with different content of polyP [16]. The table shows that the cells grown in the presence of phosphate (N) contain at least 1.5 times less polyP compared to the cells grown under hypercompensation (+P), whereas under phosphate starvation (–P) the content of polyP decreased several times. It should be noted that the negative charge of the cell envelope increases in a series of (–P) – (N) – (+P) cells, as has been demonstrated previously using the starvation–hypercompensation model [8, 9]. The obtained cell variants were used for the measurement of effective concentrations of selected compounds damaging the cytoplasmic membrane.

The cytoplasmic membrane damage was tested by measurement of ATP and K^+ release from the cells, because this method allowed quick determination of acting concentrations.

The ability of CL to cause leakage of ATP and K^+ ions from yeast cells has been demonstrated previously [10, 11, 18]. Figure 1a shows that the cells with different polyP content did not differ significantly in their sensitivity to this membrane-damaging agent. CL concentration causing practically complete ATP release from the control cells (N) was the same as in our previous experiments [10]. The experiments on the effect of different CL concentrations on K^+ leakage from the cells with different polyP content confirmed the equal efficiency of this fungicide for the variants used (Fig. 2, curves *1–3*). This was an expected result, because CL is a weak anionic detergent, and the cell envelope charge seemed to have no substantial effect on the interaction between CL and the cells.

As far as SDS is concerned, this detergent had a less pronounced membrane-damaging activity than CL (Fig. 1b). The treatment of the control (N) cells resulted in ATP leakage; however, even relatively high SDS concentration (2 mM) caused release of less than 50% of the ATP contained in the biomass. Although cell sensitivity to SDS decreased under phosphate starvation, almost the same ATP leakage could be obtained at a concentration of 2 mM. (+P) cells exhibited low sensitivity to SDS treatment in the used range of concentrations. This is probably due to the fact that increased negative charge of the cell envelope due to polyP [8, 9] counteracts the penetration of SDS, a strong anionic detergent, through the cell envelope. It is in agreement with the notion developed in [8, 9] that the sensitivity of *S. cerevisiae* cells to the cationic detergent cetyl trimethyl ammonium bromide (CTAB) increases at enhanced polyP content.

In comparison with CL and SDS compounds, silver cations had much lower effective concentrations for cytoplasmic membrane damage. The control (N) cells proved to be most sensitive to Ag^+ ions (Fig. 1c). The concentration of 0.02 mM was sufficient for complete ATP leakage. Complete ATP leakage from (–P)

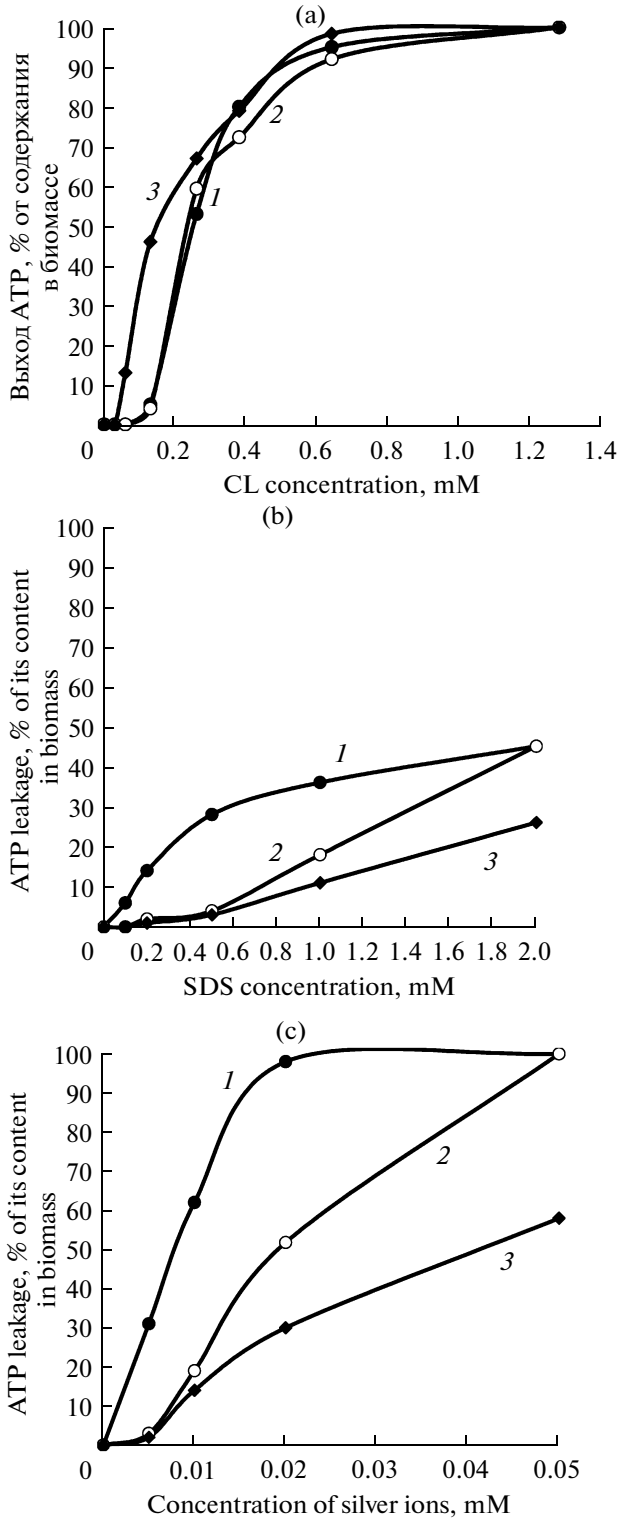

Fig. 1. Leakage of ATP from the cells of *Saccharomyces cerevisiae* VKM Y-1173 with different polyP content under the action of CL, SDS, and $AgNO_3$: control cells N (*1*), (-P) cells (*2*), (+P) cells (*3*). CL (a), SDS (b), and $AgNO_3$ (c). The time of incubation with the reagents was 15 min.

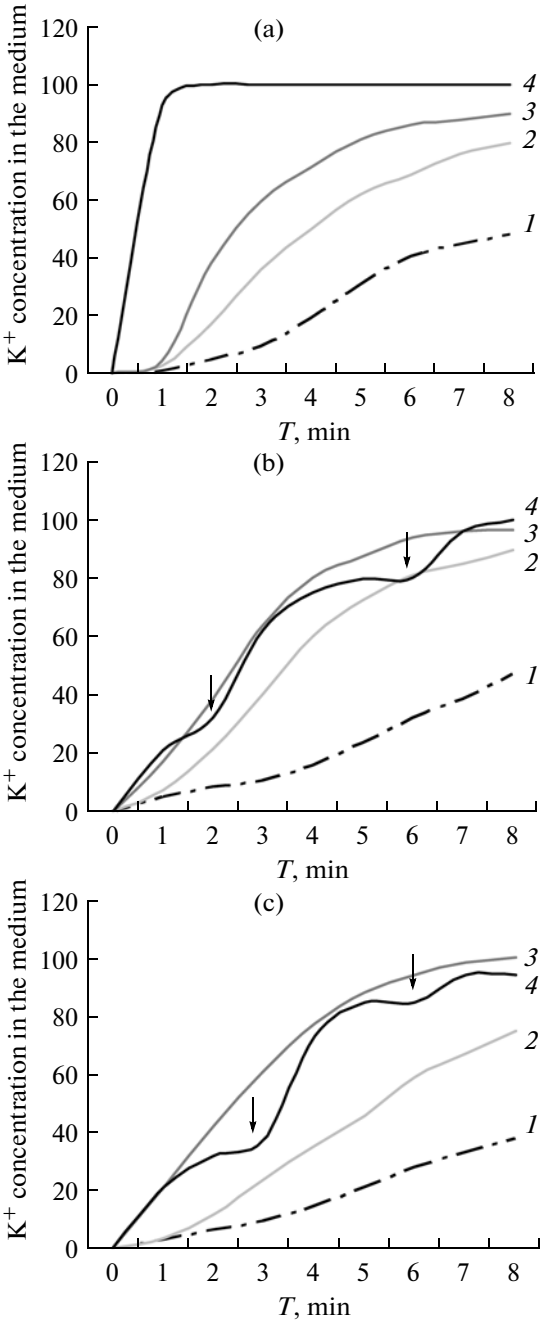

Fig. 2. Leakage of K^+ ions from the cells of *Saccharomyces cerevisiae* VKM Y-1173 with different polyP content under the action of CL and $AgNO_3$. (N) cells (a), (-P) cells (b), and (+P) cells (c). Concentrations of reagents: CL, 0.13 mM (*1*); 0.26 mM (*2*); 0.38 mM (*3*); $AgNO_3$, 0.02 mM (*4*). Arrows indicate the addition of $AgNO_3$ up to 0.04 and 0.06 mM, respectively.

cells was observed only with 0.05 mM Ag^+. (+P) cells were the least sensitive to this agent.

Similar results were obtained in experiments on the effect of Ag^+ ions on K^+ leakage (Fig. 2, curve *4*). It should be taken into consideration that (–P) cells, due to the absence of phosphate in the medium, are in a

state close to the stationary phase [16]. The properties of the cell envelope vary depending on the growth phase [19]. As regards (+P) cells, their sensitivity to Ag^+ by the indices of ATP and K^+ leakage proved to be the lowest of the three variants used. (+P) cells actively grow, but their lower sensitivity to Ag^+ compared to (N) cells could be determined by the much higher content of polyP, especially of the fractions polyP1, polyP2, and polyP5 (table), including the PolyP of the cell envelope [8, 9], and by the possible protective effect of these biopolymers. It has been demonstrated for *Candida utilis* that yeast cells adsorb and retain Ag^+ ions on their surface and the quantity of bound (adsorbed) silver increases together with the increase in the phosphorus content in the cells [15]. Thus, a protective effect of negatively charged polyP during the treatment of yeast cells with silver cations seems possible.

The question of how the supposed protective role of polyP is realized may be considered in the context of involvement of these compounds in formation of the negative charge of yeast cell envelope [8]. The reagents used in the present work are different in their mechanism of action. CL, being a very weakly charged compound, probably reacts directly with the cytoplasmic membrane. This assumption is favored by its practically identical effects on the cells and spheroplasts [18]. On incorporation into the membrane, cellobiose lipids induce the formation of pores for the leakage of low-molecular substances [20]. Thus, the differences in the cell wall (envelope) charge observed in cells with different polyP contents [8, 9] are not essential for the effect of this compound.

As concerns SDS, it is a known detergent used for solubilization of membranes and proteins. This reagent is able to affect *S. cerevisiae* cells only in rather higher concentration than CL. One of the reasons may be the shorter fatty chain of SDS compared to CL. It is known that ionic detergents with longer hydrocarbon chains more efficiently influence the yeast cytoplasmic membrane [21]. It is probable that polyP of the cell envelope prevents penetration of SDS (as molecules with the same charge) into the cytoplasmic membrane. This fact is confirmed by the lower SDS sensitivity of cells most enriched in polyP.

The yeast cell wall is a complex and dynamical molecular ensemble; not only polysaccharides, but also specific structural proteins, enzymes [22], and polyP [23, 24], play a significant role in its functioning. Silver ions, being cations, interact primarily with proteins of the cell wall (envelope), and only after penetration into the latter do they interact with the cytoplasmic membrane proteins and other cell components. This assumption is favored by the fact that our experiments revealed no leakage of potassium ions from the spheroplasts of *S. cerevisiae* under the action of silver ions [18]. At the same time, it seems quite possible that polyP as negatively charged polyanions may absorb silver cations, preventing their influence on the proteins.

The results show the presence of an interrelationship between the sensitivity of *S. cerevisiae* cells to SDS and silver ions and the content of polyP. It suggests a possible protective role of polyP and requires a study of the effect of the structural and functional peculiarities of cell envelope associated with the growth conditions of yeasts on their sensitivity to different chemical fungicides.

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