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EXPERIMENTAL ARTICLES

Effects of Cellobiose Lipid B on *Saccharomyces cerevisiae* Cells: K^+ Leakage and Inhibition of Polyphosphate Accumulation

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Abstract—Cellobiose lipid B, a natural fungicide produced by the yeast *Pseudozyma fusiformata*, induces the leakage of K^+ and ATP from cells of *Saccharomyces cerevisiae*. The presence of glucose decreases the effective concentration of cellobiose lipid B. The concentration of cellobiose lipid B was selected that results in a high rate of K^+ leakage and a five- to sevenfold decrease in the intracellular ATP content, while the accumulation of acid-soluble polyphosphates decreased only by half. These results indicate the possibility of synthesis of these polymers which is independent of the ATP level and of the ion gradient on plasma membranes.

Key words: cellobiose lipid, fungicide, yeast, *Pseudozyma fusiformata, Saccharomyces cerevisiae*, potassium ions, polyphosphates, cytoplasmic membrane.

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Basidiomycetous yeast *Pseudozyma fusiformata* secretes cellobiose lipid B (CL), which is a (2-O-3-hydroxyhexanoyl-β-D-glucopyranosyl-(1⟶4)-(6-O-acetyl-β-D-glucopyranosyl-(1⟶16)-2,15,16-trihydroxyhexadecanic acid [1]. This compound has a fungicidal activity against various species of fungi and yeasts, including some pathogenic for humans and animals; the mechanism of its action is therefore of interest [2, 3].

This compound causes ATP leakage from yeast cells. The cells can be stained with the nonpenetrating dye bromocresol purple; this is an indication that the fungicidal action is induced by disturbance of the plasma membrane integrity [4].

One of the methods of assessing the state of the plasma membrane in yeasts is determination of K^+ leakage from the cells, especially taking into consideration that the K^+ gradient across the membrane is one of the energy sources for transport processes [5]. Previously, we have used inhibitory analysis to demonstrate that the synthesis of some part of inorganic polyphosphates in *S. cerevisiae* is associated with the electrochemical gradient across the plasma membrane [6].

With the purpose of specifying the mechanism of action of CL, a novel promising fungicide, its effect on the leakage of K^+ ions from *S. cerevisiae* cells was studied in this work. Besides this, the effect of CL on polyphosphate accumulation in yeast cells was studied in order to elucidate the possibilities of interrelation of this process with ion gradients on the plasma membrane and with the ATP level.

MATERIALS AND METHODS

Cellobiose lipid B (CL) was obtained from the yeast *Pseudozyma fusiformata* VKM Y-2821 from the All-Russian Collection of Microorganisms (VKM). Cultivation and CL purification from the culture liquid were performed as described previously [1]. Experiments were made with the preparation stored as a methanol solution at 5°C.

The yeast *S. cerevisiae* VKM Y-1173 grown to the stationary growth phase was used as a test culture [6, 7]. The survival rate of the yeast after the action of CL was determined by inoculation on an agarized medium [3].

Leakage of K^+ ions from the yeast cells was registered by a K^+ selective electrode (Orion, United States) which was via an amplifier of a pH-340 pH-meter connected to a recording potentiometer (BD 12, LKB, the Netherlands). The electrode was calibrated by fractional additions of 0.01 M KCl. EVL-1MZ was used as an auxiliary electrode.

Prior to the experiments, the cells were washed twice with distilled water and resuspended in distilled water to a concentration of 1.16×10^{10} cells ml^{-1}. The

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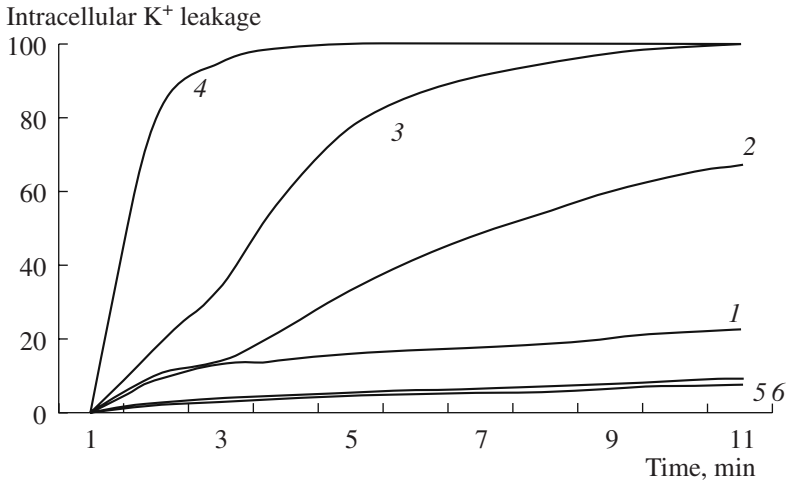

Fig. 1. K^+ leakage from *S. cerevisiae* cells under incubation with cellobiose lipid B at concentrations of 0.14 (*1*), 0.22 (*2*), and 0.29 (*3*) mg ml^{-1} or with 20 μM Ag^+ (*4*) or 2% methanol (*5*) or ethanol (*6*).

cells were stored at 4°C for the three to four hours of the experiment.

Measurements were made in a 2.5-ml thermostat cell at 25°C under stirring. The measurement medium containing 0.01 M citrate–phosphate buffer with the required pH was supplemented with 50 μl of cell suspension to a final cell concentration of $6–6.5 \times 10^8$ ml^{-1}. The total level of intracellular K^+ in the yeast was determined after the addition of 20 μM Ag^+ to the suspension or after the thermal treatment of cells at 70°C for 15 min in a water bath. The K^+ levels determined by the above two kinds of treatment were practically the same.

ATP leakage from yeast cells under the influence of CL was determined by the luciferin–luciferase method in a 1250 luminometer (LKB, Sweden) [4]. Cells with the added respective amount of methanol were used as a control.

The accumulation of orthophosphate and polyphosphates was determined in *S. cerevisiae* cells grown in the Reader medium with 1 mM phosphate to the stationary phase. They were incubated in the solution containing 2% glucose and 20 mM KH_2PO_4. The pH of the solution was brought to 4.0 with citric acid; biomass concentration in these experiments was 0.04–0.06 g of wet biomass per 1 ml. The incubation was performed at 30°C for 60 min under stirring in the presence of different CL concentrations or with the addition of respective amounts of methanol. After incubation, the cells were precipitated at 5000 *g* and washed with water. Then, the cells were extracted with 0.5 N $HClO_4$ on ice for 10 min under intensive stirring. The supernatant containing acid-soluble polyphosphates was separated by centrifugation at 4000 *g* for 15 min. The obtained extracts were used for detection of orthophosphate and labile phosphorus, the content of which was used to assess the amount of polyphosphates [6, 7].

The mean values of three experiments are given in the figures and tables.

RESULTS AND DISCUSSION

K^+ leakage under the influence of cellobiose lipid. The K^+ gradient across the plasma membrane is a source of energy for transport processes [5]. The leakage of these ions can be measured directly during the action of the fungicide.

When affecting *S. cerevisiae* cells, CL induced K^+ leakage, the rate of which depended on fungicide concentration (Fig. 1). At higher fungicide concentrations, K^+ leakage increased to the same value as when Ag^+ was applied [8, 9]. The rate of K^+ leakage in the presence of CL was higher at pH 4 than at pH 5 (Fig. 2). Thus, the effects of pH and glycolipid concentration on K^+ leakage from the yeast cells exposed to CL were

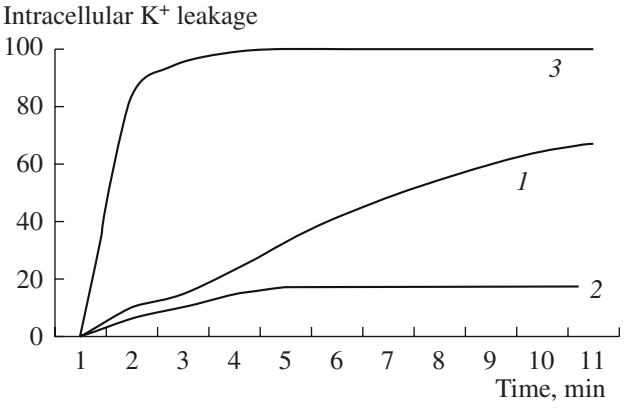

Fig. 2. K^+ leakage from *S. cerevisiae* cells under incubation with cellobiose lipid B (0.24 mg ml^{-1}) at pH 4.0 (*1*) and 5.0 (*2*) and under the effect of 20 μM Ag^+ (*3*).

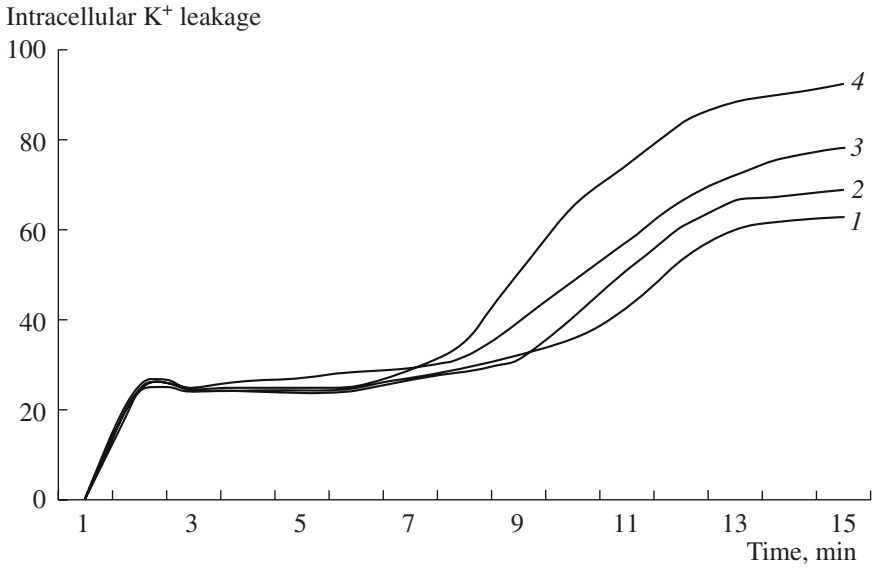

Fig. 3. K^+ leakage from *S. cerevisiae* cells under incubation with cellobiose lipid B (0.29 mg ml^{-1}) at pH 4.0 in media of different composition. *1*, 0.01 M citrate–phosphate buffer; *2*, 0.01 M citrate–phosphate buffer, 0.1 M mannitol; *3*, 0.01 M citrate–phosphate buffer, 2% ethanol; *4*, 0.01 M citrate–phosphate buffer, 0.1M glucose. Ethanol and glucose were introduced into the cell suspension 4 min before the introduction of cellobiose lipid B.

similar to the effects observed at the action of CL on the survival rate of target cells and ATP leakage [1–4]. K^+ leakage starts in the first minutes of CL action, which reflects its ability to disturb membrane integrity at the initial stage of the process.

The effect of glucose. One of the poorly studied effects of glucose on *S. cerevisiae* cells is its complex influence on metabolism and membrane processes [10]. The yeast is characterized by catabolite repression: valid mitochondria are not developed in the presence of glucose [11]. Glucose is known to activate the ATPase of the plasma membrane; the mechanism of this activation is still unknown [12]. We have observed an unexpected effect of glucose under CL action. In the presence of glucose, K^+ leakage was observed at a lower CL concentration than in the control and its rate was higher (Fig. 3). Ethanol, which can be utilized by *S. cerevisiae*, and mannitol, which is not metabolized by this organism, were used in the control experiments. They did not change the acting concentration of CL (Fig. 3).

Table 1. Effect of glucose on ATP leakage from *S. cerevisiae* cells under treatment with different concentrations of cellobiose lipid B; pH 4.5, 20°C, incubation time of 15 min

Cellobiose lipid B, mg ml^{-1}	ATP leakage from cells, μmol g^{-1} wet biomass	
	Incubation without glucose	Incubation with 2% glucose
0.03	0	0.09
0.06	0	0.29
0.14	0.09	2.41
0.29	0.57	0.8
0.58	1.42	0.4

The increase of sensitivity of *S. cerevisiae* cells to CL in the presence of glucose was also observed by the ATP leakage assay (Table 1). In the presence of glucose, the amount of ATP in the incubation medium upon increasing CL concentration first increased and then dropped. ATP remaining in the cells after CL action was extracted by dimethyl sulfoxide. The amount of ATP in the cells did not increase with increasing CL concentration. The decrease in the amount of ATP leaking into the medium at high CL concentrations may be due to the fact that ATPase of the plasma membrane hydrolyzes ATP more intensively under these conditions. Such an effect is known to take place at locations of damage to the plasma membrane [13, 14].

A certain increase of the fungicidal effect of CL in the presence of glucose can be observed also at determination of survival rate of the cells (Table 2). The increase of sensitivity of *S. cerevisiae* cells to the damaging effect of CL in the presence of glucose probably indicates that glucose causes reorganization in the plasma membrane of the yeast, which makes it more susceptible to the action of this membranotropic agent.

Table 2. Effect of glucose on survival of *S. cerevisiae* cells treated with different concentrations of cellobiose lipid B

Cellobiose lipid B, mg ml^{-1}	Cell survival rate in medium without glucose, %	Cell survival rate in the presence of 2% glucose, %
0	100	100
0.03	100	100
0.05	52	42
0.08	42	30
0.13	1	0

Thus, CL may be used as an agent that disturbs the integrity of the plasma membrane and serves for indirect assessment of its state under different physiological conditions.

The effect of CL on accumulation of inorganic polyphosphates. If *S. cerevisiae* cells are cultivated under phosphate limitation and then transferred into the medium with glucose and phosphate, they accumulate polyphosphates. A fraction of acid-soluble polyphosphates was analyzed, because its content increases to the utmost upon cultivation of phosphate-starving yeast on complete medium during the first hours of cultivation [6, 7]. Under these conditions, the quantity of acid-soluble polyphosphates in the cells increased fivefold (Table 3). ATP leakage into the medium, the drop of the level of intracellular ATP, and the decrease of accumulation of polyphosphates were observed upon incubation in the presence of CL (Table 3). The effect of cellobiose lipid at 0.03–0.06 mg ml^{-1} is the most interesting: the level of intracellular ATP decreased about sevenfold, whereas the accumulation of polyphosphates decreased only by half. Further increase of the fungicide concentration resulted in the loss of orthophosphate by the cells, which probably caused the arrest of polyphosphate accumulation.

Thus, we have determined the conditions when the cells lose K^+ and ATP, while polyphosphate accumulation persists at least at the level of 50% of the control. It follows from the above that these polyphosphates are synthesized independently of the ion gradients across the plasma membrane and of the ATP content. It may be supposed that they are formed directly in the course of glycolysis due to 1,3-diphosphoglycerate polyphosphate kinase found in microorganisms of different taxonomic affiliation [15]. In this case, the integrity of the plasma membrane is not necessary.

Thus, the work has demonstrated the ability of cellobiose lipid to cause rapid damage to the cytoplasmic membrane of yeast cells resulting in K^+ leakage; the data obtained favor the possibility of synthesis of some part of acid-soluble polyphosphates in *S. cerevisiae* independent of the state of the cytoplasmic membrane and ATP level in a cell. The results define more exactly the effect of the new, little-studied natural fungicide and allow it to be used as a membranotropic agent for yeast and fungal cells.

Table 3. Accumulation of acid-soluble polyphosphates in cells of *S. cerevisiae* VKM Y-1173 and the effect of cellobiose lipid B on this process. Incubation time, 60 min; temperature, 30°C; pH 4.0

Incubation medium composition	Content, μmol g^{-1} wet biomass, of			
	P_i	Acid-soluble polyphosphates	ATP in cells	ATP in the incubation medium
Control	10.8	2.0	8.6	0
2% glucose + 20 mM KH_2PO_4	13.2	10.0	5.8	0.08
2% glucose + 20 mM KH_2PO_4 + 0.03 mg ml^{-1} cellobiose lipid B	9.7	4.8	0.76	13.0
2% glucose + 20 mM KH_2PO_4 + 0.14 mg ml^{-1}cellobiose lipid B	0	0	0.2	4.4

Note: Citrate buffer, pH 4.0, was used in the control conditions; phosphate–citrate buffer, pH 4.0, was used in other cases (see Materials and Methods).

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