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EXPERIMENTAL ARTICLES

Concentration Dependence of the Effect of *Bacillus intermedius* Ribonuclease on the Yeast *Saccharomyces cerevisiae*

A. I. Kolpakov, F. G. Kupriyanova-Ashina, and I. B. Leshchinskaya

Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Tatarstan, Russia

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Abstract—*Bacillus intermedius* RNase added at a low concentration (0.001 μg/ml) stimulated yeast growth, while a high RNase concentration (1500 μg/ml) was inhibitory to yeast growth. The inhibitory effect of RNase was transient and correlated with the increase in the trehalose pool of yeast cells. The number of unbudded cells in the yeast population tended to decrease under the action of low concentrations of bacillar RNase and to increase under the action of high concentrations of this enzyme.

Key words: ribonuclease, trehalose, stress.

It is known that low concentrations (0.001–01 μg/ ml) of the exogenously added *Bacillus intermedius* RNase stimulate yeast budding [1, 2] and promote bacterial growth [3, 4]. On the other hand, high concentrations of RNase (1–10 mg/ml) exhibit antiviral and antitumor activities, exert mutagenic effects on both pro- and eukaryotic cells, and display antibacterial activity against gram-negative and gram-positive bacteria [5, 6].

The present work was undertaken to study the effect of low and high concentrations of *B. intermedius* RNase on the growth of the yeast *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Experiments were performed with *Saccharomyces cerevisiae*, race 823, obtained from the Collection of Microorganisms of the All-Russia Research Institute of Food Biotechnology.

The extracellular alkaline RNase of *B. intermedius* (binase) purified to an apparent homogeneity had a molecular weight of 12300 Da and a specific activity of 1 million units/mg protein. The catalytic, physical, and chemical characteristics of this enzyme were described in detail by Balaban *et al.* [7].

S. cerevisiae was grown in a medium with malt wort (6% of dry matter according to Balling) on a thermal shaker (185 rpm; 28°C). The initial concentration of cells in the growth medium was 10 million cells/ml. Cell cycle–related changes in the yeast population were analyzed as described earlier [8].

The effect of exogenous RNase on the yeast culture was studied by adding the enzyme at different growth phases. For this, the enzyme preparation was dissolved in distilled water and added, in an amount of 100 μl, to 10 ml of the yeast culture. The respective amount of distilled water was added to control cultures instead of the enzyme.

The activity of RNase was estimated by measuring the amount of the acid-soluble products of RNA hydrolysis [9].

The concentration of exogenous RNase in the yeast culture was determined by measuring the RNase activity of the culture liquid.

The trehalose pool of yeast cells was estimated by high-performance liquid chromatography. For this purpose, the yeast culture was rapidly cooled in an ice bath, and cells were harvested by centrifugation at 6000 *g* for 15 min at 4°C. The precipitated cells were washed thrice with distilled water at 4°C, suspended in three volumes of 0.5 M trichloroacetic acid (TCA), and extracted thrice at room temperature for 40–60 min. The three extracts were pooled and analyzed for the content of trehalose on a Separone NH_4 column with acetonitrile as the eluant [10].

All experiments were conducted in five replicates. The difference between control and experimental measurements was considered significant if the calculated Student's *t*-test values exceeded the respective tabular values at a 95% confidence level.

RESULTS AND DISCUSSION

As shown earlier [1, 2], the growth-stimulating effect of low RNase concentrations was especially pronounced on exponential-phase yeast cells. In view of this, the effects of low (0.001 μg/l) and high (1500 μg/ml) concentrations of *B. intermedius* RNase

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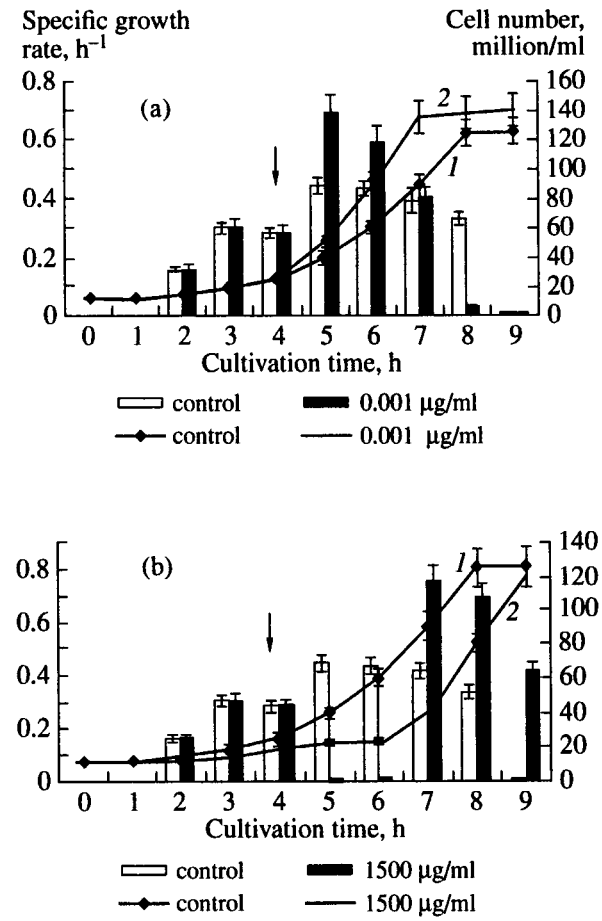

Fig. 1. The effect of *B. intermedius* RNase added at (a) a low (0.001 μg/ml) and (b) a high (1500 μg/ml) concentration on the growth of *S. cerevisiae*: (*1* and *2*) number of cells in the absence and presence of RNase, respectively; open and black bars illustrate the specific growth rates of the yeast culture in the absence and presence of RNase, respectively. The arrows indicate the time of enzyme addition.

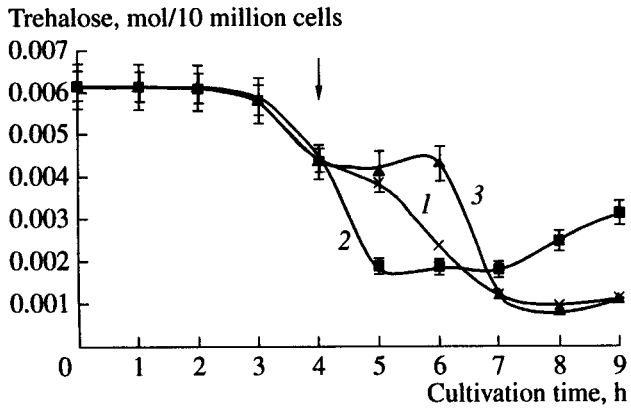

Fig. 2. RNase-induced changes in the trehalose content of *S. cerevisiae* cells: (*1*) control; (*2*) 0.001 μg/ml RNase; and (*3*) 1500 μg/ml RNase. The arrow indicates the time of enzyme addition.

on the yeast *S. cerevisiae* were studied by adding the enzyme to the midexponential yeast culture (4 h of cultivation). As is evident from Fig. 1a, a low RNase concentration stimulated the growth of the yeast culture, so that it reached the stationary growth phase one hour earlier than the control culture and, after 6 h of growth, accumulated 50% more biomass than the control culture.

The addition of RNase to the exponential-phase yeast culture at a concentration of 1500 μg/ml (i.e., at a concentration more than six orders of magnitude greater than that exerting the growth-stimulating effect) inhibited yeast budding. As can be seen from Fig. 1b, as soon as one hour after the addition of RNase at this concentration, the number of cells in the yeast culture decreased by 30% as compared to the control.

The involvement of membrane-mediated mechanisms in the growth-stimulating effect of low concentrations of *B. intermedius* RNase on the yeast *C. tropicalis* was shown in our earlier work [11]. Information on the effect of high concentrations of *B. intermedius* RNase on microorganisms is limited. Thus, Ilinskaya *et al.* [6] reported the weak mutagenic effect of this enzyme at a concentration of 1 mg/ml on bacterial cultures. The genotoxic effect of *B. intermedius* ribonuclease on human lymphocytes in vitro, manifested as a twofold increase in the repair DNA synthesis, was reported by Ivanchenko *et al.* [12]. High concentrations of *B. intermedius* RNase (3 mg/ml per million cells) were also found to inhibit the growth of the epithelial cells of human pulmonary carcinoma A549. It was noted that actively dividing cells are more susceptible to the toxic influence of RNase [13], which agrees with our data [1].

An investigation of the effect of RNase at a concentration of 1500 μ g/ml on the growth of *S. cerevisiae* showed that the inhibitory effect of this enzyme is not related to its cytotoxic effect, since the number of dead (stained with methylene blue) cells in the experimental culture did not exceed the number of cells dying spontaneously in the control culture (Table 1).

It should be noted that the inhibitory effect of high concentrations of RNase on the growth of *S. cerevisiae* lasted for about two hours (from the 4th to 6th h of growth) and was followed by an increase in the cell budding rate, so that the control and experimental cell populations became equal by the time they reached the stationary growth phase (Fig. 1b).

One of the reasons for the above phenomenon could be the decrease in the concentration of RNase in the medium due to its sorption on yeast cells. However, the data presented in Table 2 suggest that the RNase activity in the culture liquid of *S. cerevisiae* virtually did not change within a 5-h incubation of yeast cells with the enzyme. Therefore, the amount of the enzyme sorbed by yeast cells from the medium is insignificant.

The resumed yeast budding after 2 h of growth in the presence of a high concentration of RNase can also be explained by some changes in the physiological

properties of cells. An analysis of the cell cycle–related changes in the *S. cerevisiae* population grown in a medium without RNase showed that 35.5% of the original cell population (0 h of growth) represented unbudded cells (the so-called G1 stage). In the exponential-phase yeast culture (4–5 h of growth), the number of unbudded cells decreased, indicating active budding. In the phase of growth retardation and in the early stationary phase (8–9 h of growth), the number of unbudded cells increased again.

In the presence of small concentrations of RNase (Table 3, column O1) in the medium, the number of cells occurring in budding phase I increased after 5–6 h of culture growth. This fact can explain the increase in the total number of cells (both budded and unbudded) under the action of bacterial RNase.

The 2-h suspension of yeast growth (Fig. 1b) observed after the addition of high concentrations of RNase (Table 3, column O2) correlated with the increase in the number of unbudded cells in the culture. Therefore, unlike low concentrations of *B. intermedius* RNase, high concentrations of this enzyme caused a retardation of yeast budding. Taking into account that the cell cycle duration is mainly determined by the duration of the phase preceding cell budding [8], it can be suggested that bacillar RNase exerts its stimulating (or inhibiting) effect on yeast cells occurring at the G1 stage.

It is known that if the organism is exposed to unfavorable factors, it tries to maintain the constancy of its internal environment (homeostasis) and the functional activity of cells (enantiostasis) [14]. It should be noted that one of the primary responses of a cell to a stress is the synthesis of protective compounds (amino acids, proteins, carbohydrates, etc.) [15]. For example, fungi respond to unfavorable environmental conditions by increasing the cellular content of trehalose, whose accumulation in cells is closely associated with the cessation of fungal growth [16]. These data allow the suggestion that the observed changes in the activity of reproductive processes in *S. cerevisiae* under the action of bacillar RNase might be related to some changes in the trehalose pool of yeast cells.

The investigation of the dynamics of trehalose in *S. cerevisiae* cells grown in the medium without bacillar RNase showed that the trehalose pool was maximum in the lag phase and minimum in the phase of active growth (this observation is consistent with the relevant data available in the literature [15]). As the yeast growth slowed down, the trehalose pool somewhat increased (Fig. 2).

One hour after the addition of the low concentration of bacillar RNase to the exponential-phase yeast culture (4 h of growth), the cellular trehalose pool drastically decreased, which correlated with the enhanced growth of yeast and the increase in the number of stage I budding cells (Fig. 1 and Table 3). In the phase of growth retardation, which began earlier than in the control culture because of the enhanced yeast growth, the cellular trehalose pool increased to a level that was 0.002 M higher than in the control.

Table 1. The effect of *B. intermedius* ribonuclease (1500 μg/ml) on the content of dead cells in the *S. cerevisiae* culture

Cultivation time, h	Content of dead cells, %		Growth inhibition, %
	control	experiment	
5	4.2 ± 0.5	4.2 ± 0.6	30
7	4.5 ± 0.6	4.6 ± 0.5	13
9	5.4 ± 0.8	5.6 ± 0.9	9

Table 2. Changes in the activity of bacillar RNase added to *S. cerevisiae* culture at a concentration of 1500 μg/ml

Incubation time, h	RNase activity, 10^3 U/ml culture liquid	
	control	experiment
0	1470 ± 115	1462 ± 94
1	1455 ± 101	1447 ± 102
2	1402 ± 95	1420 ± 99
3	1485 ± 103	1395 ± 110
4	1468 ± 99	1380 ± 104
5	1470 ± 110	1371 ± 115

Within two hours after the addition of the high concentration of bacillar RNase to the 4-h yeast culture, cells retained their trehalose pool at the original value. But in the phase of active budding (6–7 h of growth), the trehalose pool decreased to the level typical of the control culture. Thus, the suspension of yeast growth by high RNase doses correlates with the increased level of trehalose in yeast cells as compared to the control culture.

It is known that the function of trehalose in cells is manifold. If an actively growing culture is exposed to some stress (in our case, to a high concentration of bacillar RNase), trehalose carries away the energy substrate glucose from metabolism and serves as its depot, which is necessary for the inhibition of growth processes under stress conditions, when the organism tries to maintain its enantiostasis. Furthermore, trehalose may serve as a signal for the termination of growth processes and as a stabilizer of membrane lipids [15, 16].

Thus, high concentrations of bacillar RNase can be considered a stress factor for yeast cultures. Probably, they can resume their growth after the survival of enantiostasis associated with the structural and functional restoration of the cell membrane due to the

Table 3. Cell cycle–related changes in the *S. cerevisiae* population in response to the addition of *B. intermedius* ribonuclease

Cultivation time, h	Cell cycle stage	Cells at a given stage, %		
		control	O1	O2
0	A	35.5	35.5	35.5
	I	–	–	–
	II	10.0	10.0	10.0
	III	54.5	54.5	54.5
4	A	17.8	17.8	17.8
(Enzyme addition)	I	25.2	25.2	25.2
	II	16.8	16.8	16.8
	III	41.6	41.6	41.6
5	A	29.0	23.1	34.2
	I	23.0	33.6	14.8
	II	20.0	20.9	15.5
	III	28.0	22.4	35.5
6	A	32.2	29.0	42.1
	I	15.2	34.8	12.9
	II	27.8	14.8	15.7
	III	24.8	21.4	29.3
7	A	45.1	51.7	33.4
	I	6.2	9.5	22.6
	II	8.9	16.1	13.3
	III	39.8	22.7	30.7
8	A	50.8	52.0	30.1
	I	5.1	8.2	19.5
	II	7.4	14.3	18.4
	III	36.7	25.5	32.0
9	A	56.2	54.5	35.3
	I	4.1	6.8	17.2
	II	6.5	12.5	15.8
	III	31.2	26.3	31.7

Note: O1 = RNase at a concentration of 0.001 μg/ml; O2 = RNase at a concentration of 1500 μg/ml; Control = medium without RNase; A denotes unbudded cells; I, II, and III denote yeast cells of the 1st, 2nd, and 3rd budding stages, respectively.

increase in the cellular content of the protective compound trehalose.

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