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Accumulation of Polyphosphates and Expression of High Molecular Weight Exopolyphosphatase in the Yeast *Saccharomyces cerevisiae*

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Abstract—The effect of cultivation time and concentration of inorganic phosphate (P_i) in the culture medium on the accumulation of polyphosphates (polyP) and the activity of two cytosolic exopolyphosphatases of the yeast *Saccharomyces cerevisiae* was studied: an exopolyphosphatase of 40 kD encoded by *PPX1* and a high molecular weight exopolyphosphatase encoded by another gene. Depletion of polyP in the cells on P_i starvation is a signal factor for the accumulation of polyP after the subsequent addition of 5-20 mM P_i and glucose to the cells or spheroplasts. A high activity of both exopolyphosphatases does not prevent the accumulation of polyP. The expression of the high molecular weight exopolyphosphatase is due to the acceleration of metabolism in cells that have reached the stage of growth deceleration on the addition of P_i and glucose or complete culture medium. This process may occur independently from the accumulation of polyP. The activity of exopolyphosphatase PPX1 depends less on the mentioned factors, decreasing 10-fold only under conditions of phosphate surplus at the stationary growth stage.

Key words: polyphosphates, exopolyphosphatase, cytosol, growth stage, phosphate, phosphate surplus, yeast, *Saccharomyces cerevisiae*

Yeast cells have an abundance of polyphosphates (polyP) located in different compartments. They can be a source of energy and also play a regulatory role [1-3]. These biopolymers are important for homeostasis of P_i in the cell. On deficiency in P_i in the culture medium, polyP is split yielding P_i. Under phosphate surplus, when cells after phosphate starvation are transferred to complete medium, a several-fold increase in polyP is observed. This phenomenon is called effect of phosphate surplus [1, 4]. The most important enzymes involved in metabolism of polyP in yeast are exopolyphosphatases (polyphosphate-phosphohydrolase, EC 3.6.1.11) located in different cell compartments [5]. The cytosol of the yeast cell contains no less than 60% of the total exopolyphosphatase activity. In most cases, this activity is due to a 40-kD enzyme [6]. Under conditions of phosphate surplus, a high molecular weight exopolyphosphatase is synthesized in the cytosol *de novo*. This enzyme differs from the known yeast exopolyphosphatases in a number of physicochemical properties including the substrate specificity [5-8]. The 40-kD exopolyphosphatase and the high molecular weight exopolyphosphatase are encoded by different genes [5, 9]. To reveal the possible relationship between the accumulation of polyP and changes in the activity of the two different cytosolic exopolyphosphatases in *Saccharomyces cerevisiae*, we investigated the effect of cultivation time and concentration of P_i in the culture medium on the indicated processes.

MATERIALS AND METHODS

In this work we used the yeast *Saccharomyces cerevisiae* BKM Y-1173 grown at 30°C in Rider medium containing 2% glucose, yeast extract, and mineral compounds [6]. We used medium (+P) containing 18.3 mM P_i and medium deficient in phosphate (−P) containing 1.3 mM P_i as indicated in the legends to the figures and tables.

The cells at different growth stages (Fig. 1) were separated by centrifugation (10 min at 5000*g*), washed with

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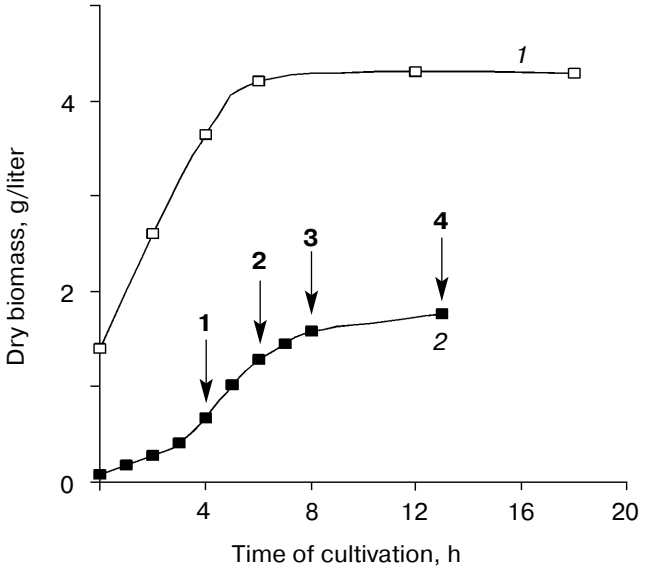

Fig. 1. Growth curves of *S. cerevisiae* at high (*1*) and low (*2*) initial density of the culture. Arrows (1-4) indicate the time of reseeding into fresh medium.

distilled water, and reseeded into the same media at similar initial density of the culture, this yielding mainly a synchronized culture. Growth curves did not depend on concentration of phosphate in the medium and on the growth stage at which they were reseeded (Fig. 1). After 2, 4, 6, and 18 h of growth, the cells were separated by centrifugation and washed with distilled water.

For cell permeabilization, the cells were quickly frozen at –70°C in 1% solution of Triton X-100, and then thawed at 5°C.

Spheroplasts were isolated as described earlier [6] and incubated in the cultivation medium or solution containing 2% glucose and 18.3 mM P_i in the presence of 0.8 M mannitol in both cases as the osmotic stabilizer.

PolyP fractions were isolated from the cells and spheroplasts as described previously [4, 7]. The content of polyP in the acid-soluble (polyP1), salt-soluble (polyP2), and two alkali-soluble fractions (polyP3 and polyP4) was determined by measuring the labile phosphorus [7]. The content of polyP in fraction polyP5 was determined by the formation of P_i after hydrolysis of the biomass (1 M $HClO_4$, 90°C, 20 min) remaining after the preceding extractions.

Preparations of cytosol were obtained from spheroplasts [6]. To separate exopolyphosphatases, the cytosolic fraction was gel-filtered through a Sephacryl S-300 column (Pharmacia, Sweden) in 20 mM Tris-HCl, pH 7.2, containing 2 mM $MgSO_4$, 100 mM NaCl, and 0.1% Triton X-100 [6]. Fractions corresponding to proteins of ~40 kD and above 500 kD and exhibiting exopolyphosphatase activity were collected and used for determination of the total activity of each exopolyphosphatase.

The exopolyphosphatase activity was determined by measuring the rate of accumulation of P_i [6]. The reaction medium contained 50 mM Tris-HCl, pH 7.2, 2.5 mM $MgSO_4$, and 0.005 mM polyP of the average length of 208 phosphate residues. PolyP (Monsanto, USA) was purified from P_i and PP_i [10]. The unit of the activity (U) was determined as the amount of the enzyme producing 1 μmol P_i per min.

RESULTS

Accumulation of different polyP fractions and changes in the activity of exopolyphosphatases under conditions of phosphate surplus. The *S. cerevisiae* cells grown until the late logarithmic stage in (–P) medium (Fig. 1, curve *2*, point 3) contained 6-fold less polyP and 3-fold less P_i compared to the cells of the same growth stage in (+P) medium (Table 1). Four hours after reseeding to (+P) medium, the content of polyP increased almost 14-fold (Table 1). The content of P_i increased 3-fold, reaching the level observed in the cells grown in (+P) medium. Thus, the effect of phosphate surplus was observed. The dynamics of the accumulation of separate fractions of polyP

Table 1. Effect of growth stage and conditions of reseeding on the content of polyP and P_i in cells (μmol P_i per g dry biomass) and on the contribution of the high molecular weight exopolyphosphatase into the total activity of cytosol in *S. cerevisiae* (values corresponding to 0 and 4 h of cultivation after reseeding are given, respectively)

Growth stage before reseeding	Conditions of reseeding	polyP	P_i	High molecular weight exopolyphosphatase, %
Early logarithmic	(–P) → (+P)	400/530	100/110	15/16
Late logarithmic	(–P) → (+P)	54/780	47/150	6/53
– " –	(–P) → (–P)	54/80	47/48	6/14
– " –	(+P) → (+P)	320/330	150/140	7/50

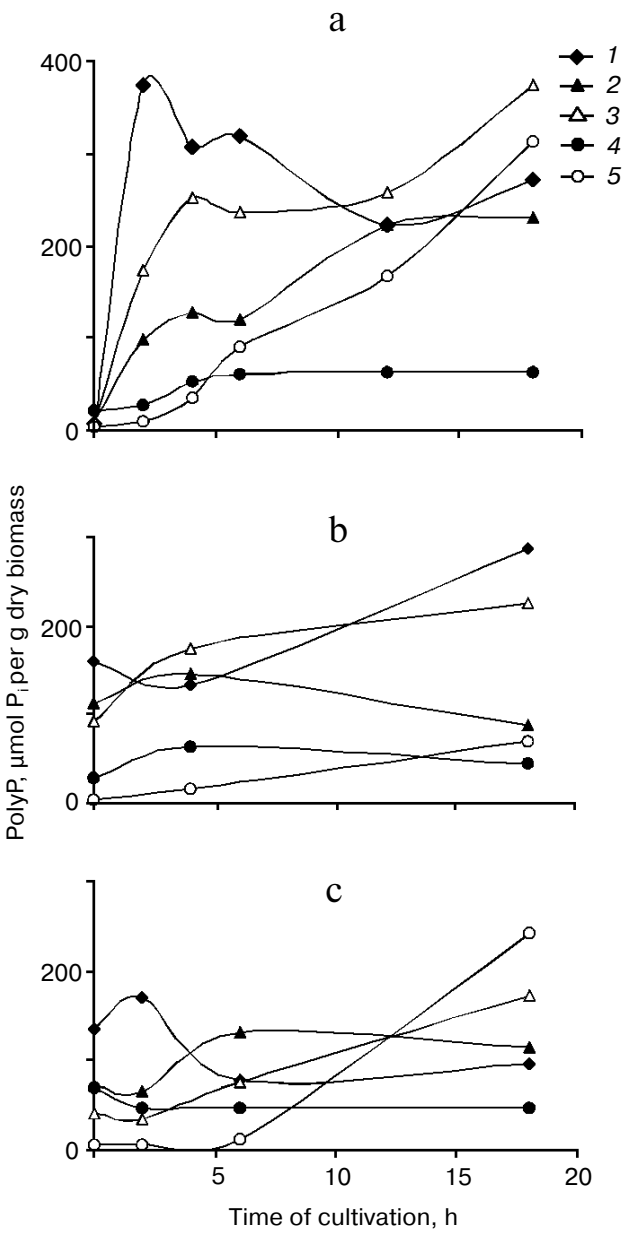

Fig. 2. Dynamics of the accumulation of different polyP fractions in *S. cerevisiae*: a) reseeding of the cells in the late logarithmic growth phase from (–P) medium to (+P) medium; b) reseeding of the cells in the early logarithmic growth phase from (–P) medium to (+P) medium; c) reseeding of the cells in the late logarithmic growth phase from (+P) medium to (+P) medium. *1-5*) PolyP1, polyP2, polyP3, polyP4, and polyP5, respectively.

during the cultivation were different (Fig. 2a). The content of polyP1, polyP2, and polyP3 increased mainly during the first 2 h. On reaching the stage of growth deceleration (Fig. 1, curve *2*), redistribution of polyP between the fractions was observed. The content of polyP1 decreased, and the content of polyP2, polyP3, and polyP5 increased (Fig. 2a). Fraction PolyP4 was not affected by the phosphate surplus: its level was restored to that characteristic for (+P) medium after 4 h and remained constant during further cultivation (Fig. 2, a and b). Such behavior of the polyP4 fraction is in agreement with the idea that it is involved in biosynthesis of the cell wall, but not in the maintenance of homeostasis of P_i in the cell [4]. A complex dynamics of separate polyP fractions under conditions of phosphate surplus indicates their different role in the development of the yeast culture from active growth to the stationary growth phase.

We also investigated the changes in the spectrum of cytosolic exopolyphosphatases in cells grown until the late logarithmic stage after their reseeding from (–P) to (+P) medium. During the first 4 h after the reseeding, the activity of the high molecular weight exopolyphosphatase increased almost 10-fold, this level remaining constant during further cultivation (Fig. 3a, curve *2*). The activity

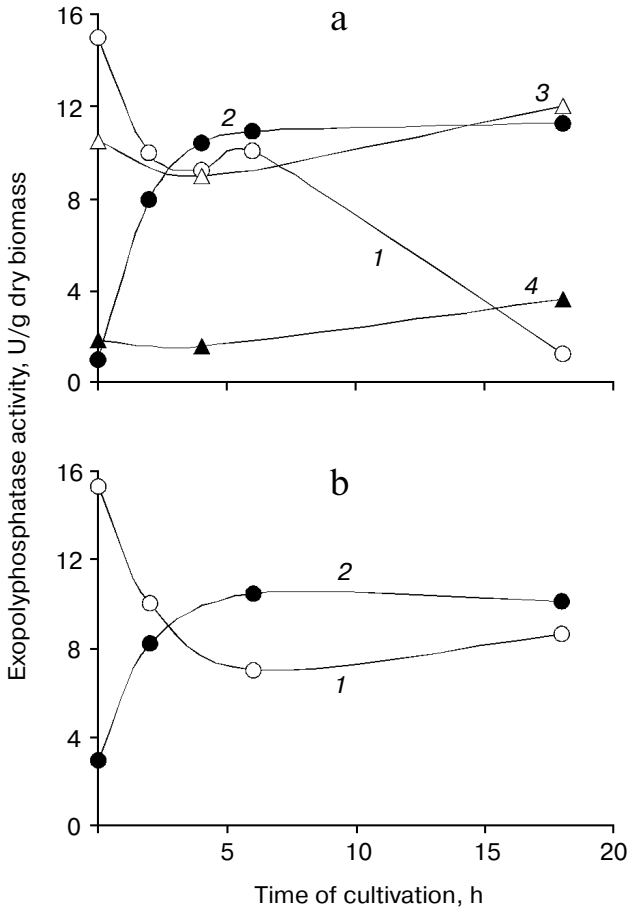

Fig. 3. Dynamics of the change in the activity of the 40-kD and the high molecular weight exopolyphosphatases in cytosolic fraction of *S. cerevisiae* after reseeding: a) from (–P) to (+P) medium; b) from (+P) to (+P) medium. *1*) 40-kD exopolyphosphatase, cells in the late logarithmic stage; *2*) high molecular weight exopolyphosphatase, cells in the late logarithmic stage; *3*) 40-kD exopolyphosphatase, cells in the early logarithmic stage; *4*) high molecular weight exopolyphosphatase, cells in the early logarithmic stage.

of the 40-kD exopolyphosphatase changed little during active growth, but decreased significantly in the stationary phase (Fig. 3a, curve *1*). As a result, the contribution of the high molecular weight exopolyphosphatase to the total activity of cytosol increased from 6 to 90%. The dynamics of the activity of the two exopolyphosphatases had opposite character, but the changes were independent (Fig. 3a). It is important that during the intensive accumulation of polyP in the active growth stage, the total exopolyphosphatase activity increased by 50-70% compared to that of the reseeding point (Figs. 2a and 3a). The comparison of the dynamics of the accumulation of polyP and the changes in the activity of two cytosolic exopolyphosphatases indicates that high exopolyphosphatase activity does not prevent polyP accumulation.

Effect of growth stage of the culture used for reseeding. The effect of the growth stage of the cells on the subsequent dynamics of the accumulation of polyP and exopolyphosphatases was determined after reseeding from (−P) medium to (+P) medium. The cells reaching the stationary growth phase (Fig. 1, curve *2*, point 4) demonstrated the same effects as the cells of the late logarithmic phase (data not shown). On reseeding the cells of the early logarithmic growth stage (Fig. 1, curve *2*, points 1 and 2), neither effect of phosphate surplus nor change in the spectrum of exopolyphosphatases was observed (Table 1, Fig. 2b, Fig. 3a (curves *3* and *4*)). At the reseeding point, the level of polyP in the cells grown on (−P) medium was almost the same as that observed in (+P) medium (Table 1, Fig. 2, b and c, point 0). This explains the absence of the effect of phosphate surplus that develops after the preliminary phosphorus starvation and on the depletion of polyP [4]. The cells reaching the middle of the logarithmic growth stage (Fig. 1, curve *2*, point 2) exhibited neither the effect of phosphate surplus nor change in the spectrum of exopolyphosphatases (data not shown).

Thus, the change in the spectrum of exopolyphosphatases and accumulation of polyP after reseeding from (−P) to (+P) medium was characteristic only for cells reaching the stage of growth deceleration by the time of reseeding. Thus, all the experiments described below were performed on cells reaching the late logarithmic growth stage (Fig. 1, curve *2*, point 3).

Effect of P_i. After reseeding from (−P) to (−P) medium, accumulation of polyP was not observed (Table 1). The total level of polyP increased after 4 h of incubation only 1.5-fold, this being due to the accumulation of polyP4 (data not shown). The activity and spectrum of cytosolic exopolyphosphatases changed little (Table 1). Consequently, transition from the growth deceleration stage to the active growth stage is not the only condition for the change in these characteristics.

After reseeding from (+P) to (+P) medium, the content of polyP did not change during 4-6 h of growth, increasing only in the stationary growth phase due to the accumulation of polyP3 and polyP5 (Fig. 2c, Table 1).

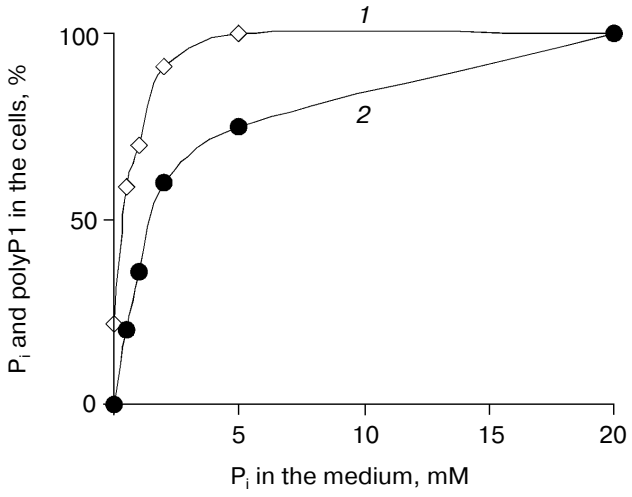

Fig. 4. Accumulation of P_i (*1*) and polyP1 (*2*) by *S. cerevisiae* in the presence of 2% glucose and P_i. The cells were grown in (−P) medium until the late logarithmic growth phase.

The content of P_i in this experiment was constant (Table 1). The absence of the effect of phosphate surplus can be explained by an originally high content of polyP in the cells grown in (+P) medium (Fig. 2c, Table 1). An unexpected result was that the dynamics of the growth of the activity of the high molecular weight exopolyphosphatase was virtually the same as under conditions of phosphate surplus (Fig. 3b). At the same time, the activity of 40-kD exopolyphosphatase decreased only 2.5-fold (Fig. 3b). As a result, after 18 h of cultivation the contribution of the high molecular weight enzyme to the total exopolyphosphatase activity was less than 60%. Thus, conditions were found when the high molecular weight exopolyphosphatase was expressed without parallel accumulation of polyP.

The influence of P_i concentration on the effect of phosphate surplus was investigated on the polyP1 fraction, whose content increased to a greater degree during the first 2 h (Fig. 2a). The accumulation of polyP1 was observed not only in complete medium, but also when the cells grown in (−P) medium were then incubated in the presence of 2% glucose and 18.3 mM P_i. After 2 h of incubation under these conditions, the level of polyP1 was 60% of that obtained in the Rider's (+P) medium. Figure 4 demonstrates the effect of phosphate surplus observed for the polyP1 fraction as the dependence on P_i concentration. At low concentrations of P_i when the high-affinity phosphate transporter is active [11], no accumulation of polyP1 was observed.

Permeabilized cells and spheroplasts under conditions of phosphate surplus. Yeast cells grown in (−P) medium until the late logarithmic phase were subjected to permeabilization. After subsequent incubation of the cells in (+P) medium for 2-4 h, no accumulation of polyP was

Table 2. Content of polyP1 and contribution of the high molecular weight exopolyphosphatase to the total activity of cytosol on incubation of spheroplasts* under phosphate surplus

Time of incubation, h	Conditions of incubation	PolyP1, μmol P per g dry biomass	High molecular weight exopolyphosphatase, %
0		5	6
2	(+P) medium, 0.8 M mannitol	190	40
2	2% glucose, 18.3 mM P_i, 0.8 M mannitol	70	60

* Spheroplasts were obtained from the cells grown until the late logarithmic growth stage in (−P) medium.

observed. Consequently, continuity of the membrane is necessary for polyP accumulation, this being in agreement with the data on the inhibitory effect of the protonophore FCCP on this process [7].

The effects of phosphate surplus and changes in the spectrum of exopolyphosphatases were also exhibited by spheroplasts in the case when they were isolated from cells grown in (−P) medium until the late logarithmic stage and then incubated in (+P) medium or in the presence of glucose and P_i together with the osmotic stabilizer (Table 2). The effect of phosphate surplus was studied on the polyP1 fraction, since it contained 85% of the total content of polyP in spheroplasts. After 2 h of incubation, spheroplasts were effectively lysed in 0.1 M sorbitol while preparing the cytosolic fraction. Light microscopy revealed no budding under these conditions. Consequently, neither cell wall regeneration nor cell division occurred. Thus, the presence of the cell wall and the process of cell division are not necessary conditions for accumulation of polyP1 and expression of the high molecular weight exopolyphosphatase.

If the spheroplasts, which had accumulated polyP, were lysed in 0.1 M sorbitol, the polyP was completely hydrolyzed to P_i during 30 min of incubation in the presence or in the absence of P_i and glucose. Presumably, the continuity of the cytoplasmic membrane is important for the accumulation of polyP, its destruction increasing the accessibility of polyP to exopolyphosphatases.

DISCUSSION

The results indicate that the depletion of polyP in *S. cerevisiae* is a necessary condition for the effect of phosphate surplus. The subsequent rapid accumulation of polyP starts on the addition of glucose and high concentration of P_i to the cells or spheroplasts. The enzyme systems involved in the accumulation of polyP are probably expressed during the phosphorus starvation, since cycloheximide did not affect the accumulation of polyP under conditions of phosphate surplus [7].

Conversely, the high molecular weight exopolyphosphatase was not expressed under the phosphorus starvation. Its activity increased only on the transition from the stage of growth deceleration to the activation of metabolism after the addition of glucose and P_i or complete medium. Blocking of this process by cycloheximide indicates that the high molecular weight exopolyphosphatase is synthesized *de novo* [7]. Unlike polyP accumulation, the depletion of polyP was not a necessary factor for the synthesis of the high molecular weight exopolyphosphatase, since its activity increased without accumulation of polyP after reseeding from (+P) to (+P) medium.

In spite of the fact that a high concentration of P_i is a common necessary factor for the accumulation of polyP and change in the spectrum of exopolyphosphatases, these two effects can arise independently, as was shown above. It should be noted that in the absence of glucose none of these effects was observed.

Expression of the high molecular weight exopolyphosphatase is probably connected with the *PHO*-regulon containing many genes whose functioning depends on P_i concentration and is little studied [11]. As for the 40-kD exopolyphosphatase encoded by gene *PPX1*, its activity little depends on P_i concentration in the medium. It is considered that this enzyme is not controlled by the *PHO*-regulon [9, 11]. Nevertheless, under some changes in phosphorus metabolism of *S. cerevisiae*, namely on the stationary growth phase under conditions of phosphate surplus, we observed a significant decrease in the activity of PPX1. This suggests a relationship between the activity of PPX1 and phosphorus metabolism under certain conditions.

Interestingly, high activity of both exopolyphosphatases does not prevent the accumulation of polyP. Destruction of the continuity of the cytoplasmic membrane prevents the accumulation of polyP, as in the case of the cell permeabilization, or results in their hydrolysis, as in the case of the osmotic lysis of spheroplasts. Destruction of the continuity of the membranes presumably increases the accessibility of polyP to exopolyphosphatases.

The data obtained in the present work indicate a close relationship between the spectrum of exopolyphosphatases in cytosol, the stage of development of yeast culture, and concentration of P_i in the medium. The results also demonstrate the independence of the processes of polyP accumulation and the expression of the high molecular weight exopolyphosphatase.

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