Biochemistry (Moscow), Vol. 67, No. 5, 2002, pp. 592-596. Translated from Biokhimiya, Vol. 67, No. 5, 2002, pp. 711-716.

The Content and Chain Length of Polyphosphates from Vacuoles of *Saccharomyces cerevisiae* VKM Y-1173

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Received May 14, 2001

Abstract—The content of inorganic polyphosphates (polyP) in vacuoles of the yeast *Saccharomyces cerevisiae* is ~15% of the total cellular polyP. Over 80% of the vacuole polyP are in an acid-soluble fraction. It was first established by ^{31}P-NMR spectroscopy that a polymeric degree (*n*) of two subfractions obtained by precipitation with Ba^{2+} in succession at pH 4.5 and 8.2 was approximately 20 ± 5 and 5 ± 2 residues of ortho-phosphoric acid, respectively. Under a deficit of phosphate (P_i) in the cultivation medium, the polyP content in vacuoles decreased ~7-fold with the same drastic reduction of their content in the cell. Unlike intact yeast cells, where polyP overcompensation is observed after their transfer from phosphate-free to phosphate-containing medium, the vacuoles do not show this effect. The data indicate the occurrence of special regulatory mechanisms of polyP synthesis in vacuoles differing from those in the whole cell.

Key words: yeast, protoplasts, vacuoles, overcompensation, polyphosphates, exopolyphosphatase

Polyphosphates (polyP) are linear polymers of orthophosphate linked by an energy-rich phosphoanhydride bond, serving simultaneously as phosphate and energy reserves of the cell. They have been found in almost all living beings [1]. PolyP may contain as much as 40% of the total cellular phosphate [1]. These compounds are highly mobile cell components [2]. In certain extreme conditions, polyP are able to be involved in metabolic processes [3, 4] and, on the other hand, to be accumulated in an amount exceeding several times that in usual conditions (overcompensation) [1, 3]. Extraction with various reagents can divide polyP into separate fractions differing both in polymeric degree and localization in the cell [1, 5]. PolyP were revealed in nuclei [6, 7], mitochondria [8], cytoplasm [1, 7], plasma membrane [9], and vacuoles [7, 10, 11] of yeast. There is still no consensus in the literature concerning the polyP content in vacuoles. According to the data of some researchers, this compartment contains only a part of the total cell pool of these compounds [5, 7, 10, 12]. Other authors state that vacuoles harbor most of the cell polyP [11]. They consider that inability to accumulate polyP by mutants having no vacuoles as indicative of this hypothesis [13].

It is known from the literature that polyP may play quite a number of roles in microbial cells. In addition to supplying cells with phosphate and energy, they can function as counter-ions under accumulation of cations, basic amino acids, and other positively charged compounds in cells [2, 14, 15]. PolyP hydrolysis is suggested to be one of the protective mechanisms of a cell under alkaline [4] and osmotic [16, 17] stresses. Nevertheless, in spite of the numerous works devoted to the polyP study, many functions of these compounds still remain unclear, including their specific role in separate cell compartments [7, 18, 19].

The goal of this work was to study the content and structure of vacuolar polyP and their metabolism under conditions of P_i extremes in the cultivation medium.

MATERIALS AND METHODS

The subject of investigation was a culture of the yeast *Saccharomyces cerevisiae* VKM Y-1173. The yeast growth and preparation of biomass were as described earlier [3]. To study the polyP content under overcompensation, the yeast cells were first grown for 4 h in a medium with the usual P_i level (9 mM), then for 7 h in P_i-depleted medium, and thereafter again in the initial medium for the next 2 h. To isolate vacuoles, cells were harvested in the mid-logarithmic growth phase or at the time points corresponding to overcompensation conditions in the yeast [3].

Modified methods used at our laboratory were applied to isolate protoplasts and vacuoles [20]. The yeast cells washed from the cultivation medium with 0.8 M mannitol in 0.2 M citrate-phosphate buffer, pH 6.7, were suspended in the same solution. One gram of wet biomass was supple-

Abbreviations: polyP) polyphosphates; polyPase) exopolyphosphate phosphohydrolase.

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0006-2979/02/6705-0592$27.00 ©2002 MAIK "Nauka/Interperiodica"

mented with 6 ml of the above medium, 6 mg of dithiothreitol, and 50 mg of a "snail gut enzyme" (lyophilized gut sap of the snail *Helix pomatia*). The suspension was incubated for 70-75 min at 29°C. Protoplasts were separated from the medium and washed by laying their suspension on 1 M sucrose in 10 mM citrate buffer, pH 6.8, and centrifuged at 1800*g* for 15 min. This centrifugation and the following procedure of vacuole isolation were carried out at 0-4°C. Protoplasts were suspended in 1 M sorbitol and disintegrated by a Potter homogenizer. Under microscopic control, the percentage of disintegrated protoplasts accounted for 65-85%. The protoplast homogenate was diluted with a solution of 1 M sucrose and 1 M sorbitol in the ratio of 1.5 : 1. Unbroken protoplasts were precipitated at 1800*g* for 15 min. The supernatant was supplemented with 1 M sorbitol to obtain a molar ratio of sucrose/sorbitol in the solution equal to 1 : 3. Then it was centrifuged at 3600*g* for 50 min. The resulting vacuolar pellet was washed with 1 M sorbitol followed by centrifugation at 3600*g* for 30 min. All solutions used for vacuole isolation contained 10 mM citrate buffer, pH 6.8.

The yeast growth in conditions of sharp P_i changes in the medium, the treatment of polyP fractions for ^{31}P-NMR spectroscopy, and spectroscopic analysis as such were as described earlier [3]. PolyP extraction from cells, protoplasts, and vacuoles and determination of the polyP content in fractions obtained were carried out according to the methods described [3].

The activities of ATPase [21], α-mannosidase [22], α-glucosidase [23], and exopolyphosphatase [20] were estimated in protoplasts and vacuoles by the known methods. To reveal the presence of other cellular organelles in the vacuolar preparation, the contents of the corresponding marker enzymes were estimated in it: ATPase of the cytoplasmic membranes (an inhibitor, sodium orthovanadate [21]), mitochondrial ATPase (an inhibitor, sodium azide [21]), and cytosol α-glucosidase. Activities of the vacuolar ATPase were determined in the preparations of protoplasts and vacuoles in the presence of 50 μM sodium orthovanadate and 5 mM sodium azide. One activity unit (U) was defined as the enzyme amount sufficient to catalyze the formation of 1 μmole P_i or *p*-nitrophenol in 1 min. Protein concentration was determined by the method of Lowry et al. in Peterson's modification [24].

All the data presented in the tables are the mean values of three or more individual experiments.

Dowex AG 50W×8 and sorbitol were from Serva (Germany), dithiothreitol and polyP with the average chain length $\tilde{n} = 45$ from Sigma (USA). All other chemicals of analytical grade were from Reakhim (Russia).

RESULTS AND DISCUSSION

Isolation of vacuoles from intact yeast cells was associated with certain difficulties. The isolation procedure depended on yeast strain, growth phase, and cultivation conditions. In vacuoles isolated by the hypotonic method [25] successfully employed in our laboratory for studying vacuolar enzymes [20], no detectable amount of polyP was found. This may be due to the conditions of vacuole isolation. An increase in the organelle size followed by extension of the vacuolar membrane was observed in this case [26]. This resulted in a disruption of the normal operation in the organelle followed by possible polyP depletion. The isotonic method used in the current work allowed us to obtain vacuoles with a size comparable to that in the intact cells under phase-contrast microscopic examination. Besides, these vacuoles contained polyP in an appreciable amount. Examination of the preparation with a phase-contrast microscope showed that it lacked unbroken cells and protoplasts. The enzyme analysis revealed the presence of cytoplasmic membranes and mitochondria in the preparation (Table 1). It contained only 3% of the cell plasma membrane (judging from the ATPase activity sensitive to VO_4^{2-}) and only 2% of mitochondria (ATPase activity sensitive to N_3^-) (Table 1). It follows from the analysis performed that the obtained preparation represented a rather purified vacuolar fraction. Contaminating structures were present in minor amounts, allowing investigation of polyP in this cell compartment.

As seen from Table 1, the activity of vacuolar ATPase (a specific vacuolar enzyme defined at pH 7 in the presence of Mg^{2+}) in the vacuolar preparation obtained was 18-fold higher than that in the protoplasts. The level of another marker enzyme usually used to evaluate the puri-

Table 1. Activity of some enzymes in protoplasts and vacuolar preparation of the yeast *Saccharomyces cerevisiae*

Enzyme	Activity, mU per mg protein		Activity ratio (vacuoles to protoplasts)
	protoplasts	vacuoles	
VO_4^{3-}-sensitive ATPase (plasma membrane)	56	127	2.3
N_3^--sensitive ATPase (mitochondria)	13	18	1.4
N_3^-, VO_4^{3-}-insensitive ATPase (vacuoles)	11	201	18.3
α-Mannosidase	0.3	1.45	4.8
α-Glucosidase	1.4	< 0.01	< 0.01
Protein, mg per g wet biomass	167	2.4	0.014

Table 2. P_i and polyP content (μg P_i per g dry cell weight) in intact cells, protoplasts, and vacuoles* at a standard P_i level (9 mM) in the cultivation medium

Fraction	P_i	PolyP
Cells	3430	16 850
Protoplasts	4890	12 270
Vacuoles	103	2280

* Calculation of the polyP content in vacuoles per g dry biomass was performed taking into consideration the losses of vacuoles during isolation which were taken as proportional to the losses of vacuolar ATPase.

ty of vacuolar preparations, α-mannosidase, was 5-fold higher in the preparation than in protoplasts. The difference in the enrichment of ATPase and α-mannosidase in the course of vacuole purification was probably due to the fact that the latter enzyme was not rigorously the vacuolar one, as shown earlier [27].

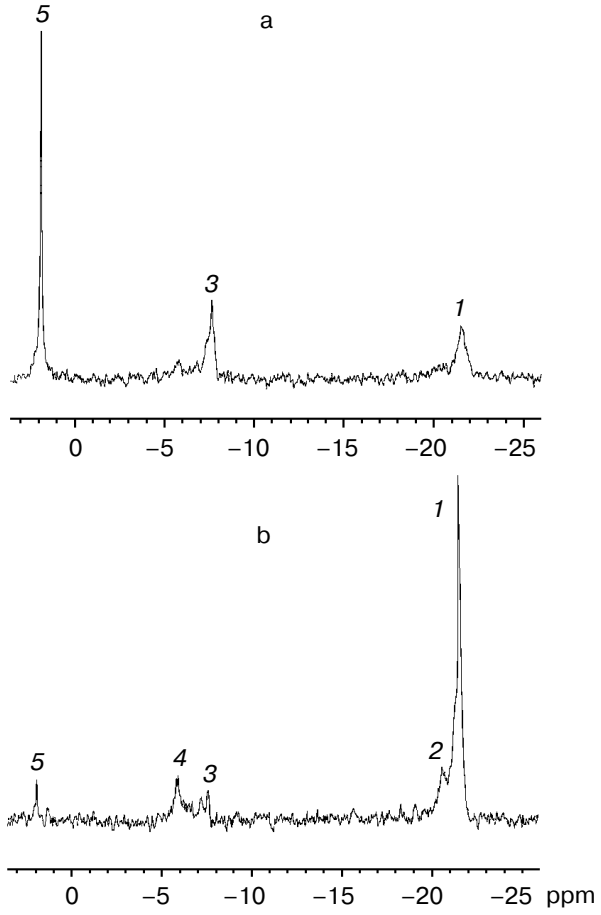
Analysis of the polyP content in cells and isolated protoplasts indicated that isolation of the yeast protoplasts might result in a loss of some part of cellular high-molecular-weight polyP. In the protoplasts, the polyP content was 75% of the total amount of cell polyP (Table 2), and this was supported by literature data [28]. In this case, the P_i level increased in the protoplasts as compared with the intact cells (Table 2), which might be due to a partial hydrolysis of polyP in the course of protoplast isolation. As Table 2 shows, the vacuoles contained ~15% of the cellular polyP. The rest of the polyP was localized in the cytoplasm and other cellular organelles. The given experimental data are in agreement with the data obtained from the study of vacuolar polyP in the yeast *Saccharomyces carlsbergensis* [10]. Similar data were obtained for *Candida utilis*, where only a part of cellular polyP was detected in vacuoles independent of the culture growth rate [7, 12]. Along with the above, there are literature data indicating that most of these compounds are localized in vacuoles of some strains of *S. cerevisiae* and *Neurospora crassa* [14, 29]. In our opinion, this discrepancy resulted from changes in the polyP amount in different compartments, associated with special features of the biological subject under investigation, growth medium composition, phase of the yeast development, as well as from the methods of vacuole isolation and detection of localization of these compounds in the cell.

Table 3. Distribution of vacuolar polyP in fractions and their polymer degree (total polyP amount in vacuoles is taken as 100%)

Fraction	PolyP content, %	Polymer degree, $\tilde{n}$
Acid-soluble fraction		
pH 4.5	44.2	20 ± 5
pH 8.2	39.4	5 ± 2
Alkaline-soluble fraction	12	—
Remainder	4.4	—
Sum of vacuolar polyP	100	

The vacuolar polyP were divided by the extraction into two fractions, acid- and alkaline-soluble. As seen from Table 3, the acid-soluble fraction comprised more then 80% of vacuolar polyP. In turn, it consisted of two subfractions precipitated with Ba^{2+} at different pH values. It was found with ^{31}P-NMR spectroscopy analysis (figure) that the average chain length of the polyP fraction precipitated with Ba^{2+} was 20 ± 5 and 5 ± 2 orthophosphate residues at pH 4.5 and 8.2, respectively. A minor amount of the vacuolar polyP was detected in the alkaline-soluble fraction. We were not able to measure the polyP chain length in this fraction because of its small amount. The above results correlated well with the values of the polymeric degree of vacuolar polyP evaluated by gel filtration technique for *S. cerevisiae* [14]. In yeast cells, polyP with the chain length of 100 and more orthophosphate residues were determined [1, 30], and polyP having the average polymeric degree of $\tilde{n} = 20$ comprised ~35% of their total amount in the cell [30]. The presence in vacuoles mainly of the polyP fraction with a low molecular mass, revealed in the current work, showed once again that this compartment contained only a part of the cellular polyphosphate pool. It should be mentioned, however, that the vacuoles might contain endopolyphosphatase—an enzyme splitting the high-molecular-weight polyP to low-molecular-weight fragments [31]. Hence, possible hydrolysis of high- to low-molecular-weight polyP in vacuoles under protoplast isolation must not be completely ruled out. In this case, detection of a small amount (~12%) of the alkaline-soluble high-molecular-weight polyP in vacuoles seems quite justified. On the other hand, it is possible that the presence of the alkaline-soluble polyP fraction was due to contamination of the vacuolar preparation with other cell fragments containing this polymer fraction.

The overcompensation phenomenon has been known for a long time [32]. The principle of this process is that cells grown under short supply of phosphate, once placed in a fresh complete medium with P_i, synthesized several times more polyP in a short period of time than those growing under standard conditions. The study of quantitative and qualitative changes in the vacuolar polyP in this case is of a special interest to reveal the role of these compounds specif-



^{31}P-NMR spectra of the acid-soluble polyP extracted from vacuoles of the yeast *S. cerevisiae*: a) polyP precipitated from the extract with Ba^{2+} at pH 8.2; b) polyP precipitated from the extract with Ba^{2+} at pH 4.5. *1*) Core phosphate groups; *2*) preterminal phosphate groups; *3*) terminal phosphate groups; *4*) γ-phosphate groups of nucleoside triphosphates; *5*) P_i

ically in the vacuoles functioning under extreme conditions, caused, e.g., by P_i depletion in the medium.

As expected, with P_i deficit in the medium the yeast cells mobilized internal polyP stores. In this case, their level decreased 6-8-fold in the structures under study: intact cells, protoplasts, and vacuoles (Table 4). Transfer of the culture to a phosphate-containing medium resulted in accumulation of polyP in both cells and protoplasts in a short time (during one generation) [3] twice as many as in cells growing under usual conditions. At this time, the polyP content in vacuoles, on the contrary, increased slightly and reached as much as 25% of their content in this yeast compartment under normal growth conditions. In all variants under study, only a small part of the cell polyP (2-14%) was revealed in vacuoles (Table 4). Their content was especially low in the vacuoles after overcompensation and comprised only 2% of the polyP content in intact cells.

Thus, the studies have shown that vacuoles of the yeast under investigation contained only a part of the cellular polyP. They were represented largely by the acid-soluble fraction. The polymer is not oversynthesized in vacuoles as distinct from other cell structures, when the yeast is grown under overcompensation conditions. This indicates that different cell compartments possess different regulatory mechanisms of polyP biosynthesis.

In case of P_i-depleted medium, cells used the internal stores of this compound in the polyP form as demonstrated in Table 4. One way for the involvement of polyP in metabolic processes of the cell was polymer hydrolysis to P_i by exopolyphosphatases (polyPase). Evaluation of the enzyme activity in protoplasts and vacuoles experimentally showed that the latter really increased by several times in vacuoles under P_i starvation (Table 5). At the same time, it has been shown earlier that the vacuolar polyP pool was used to maintain the level of P_i in the medium under its deficit [33]. By correlating these two facts, one can suggest that the vacuolar polyPase is involved in maintenance of cell vitality in the conditions of a P_i-free medium. A quite different picture is observed with the changes in the total polyPase activity in the protoplast homogenate, which

Table 4. PolyP content (μg P_i per g dry weight) in cells, protoplasts, and vacuoles of the yeast *S. cerevisiae*. The yeast was grown for 4 h in a medium containing 9 mM P_i (+P), then for 7 h in a phosphate-free medium (−P), and finally for 2 h in a medium with the standard 9 mM P_i level (+P, overcompensation)

Cultivation conditions	Cells	Protoplasts	Vacuoles
+P	16 850	12 270	2290
−P	2010	1810	280
+P, overcompensation	38 320	27 210	650

Table 5. Exopolyphosphatase activity in protoplasts and vacuoles of the yeast *S. cerevisiae* (experimental scheme is the same as in Table 4; synthetic polyP with $\tilde{n} = 45$ was used as a substrate)

Cultivation conditions	Protoplasts		Vacuoles	
	mU per mg protein	mU per $4 \cdot 10^{11}$ protoplasts	mU per mg protein	mU per $4 \cdot 10^{11}$ vacuoles
+P	29	23 082	20	692
−P	21	17 131	93	2861
+P, overcompensation	27	22 275	12	548

includes the enzyme activities of cytosol and other cell compartments [34]. As is seen from Table 5, the polyPase level under conditions of phosphate starvation changed insignificantly. These data suggest that the main polyP pool of the cell under these growth conditions is mobilized apparently with the involvement of other enzymes of polyphosphate metabolism.

In conditions of polyP oversynthesis, when cells were grown in the medium with P_i, the polyPase activity in vacuoles decreased more than 5-fold (Table 5) to the activity level in original cells. Therefore, the vacuolar polyPase was probably regulated by exogenous P_i. It should be emphasized that bacteria have a similar system for regulation of phosphohydrolase synthesis including polyPase [35, 36]. On the other hand, Table 5 shows that in the yeast strain under study the synthesis of polyPases localized exterior to vacuoles was not derepressed by P_i deficit in the medium. The same was true for *N. crassa* [37]. The regulation of polyPases localized in different compartments of a yeast cell will require, however, a more detailed analysis.

The authors would like to thank L. A. Sibeldina and I. N. Shchipanova for their help in ^{31}P-NMR spectroscopy studies and fruitful discussion of the results obtained.

This work was supported by the grant INCO-Copernicus (PL971185), Russian Foundation for Basic Research (99-04-48246 and 01-04-97021), and by the grant of the Leading Scientific Schools of Russia (00-15-97851).

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