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Purification and Properties of Exopolyphosphatase from the Cytosol of *Saccharomyces cerevisiae* Not Encoded by the *PPX1* Gene

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Abstract—A novel exopolyphosphatase has been isolated from the cytosol of *Saccharomyces cerevisiae* grown to the stationary phase after its transfer from phosphate-deficient to complete medium. The *PPX1* gene responsible for 40-kD exopolyphosphatase of the cytosol does not encode it. Specific activity of the preparation is 150 U/mg, purification degree is 319, and the yield is 16.9%. The minimal molecular mass of the active but unstable enzyme complex is ~125 kD. A stable enzyme complex with a molecular mass of ~500 kD is composed of two polypeptides of ~32 and 35 kD and apparently polyphosphates (polyP). Unlike the enzyme encoded by *PPX1*, the high-molecular-mass exopolyphosphatase is slightly active with $polyP_3$, not inhibited by antibodies suppressing the activity of 40-kD exopolyphosphatase, inhibited by EDTA, and stimulated by divalent cations to a lesser extent. The high-molecular-mass exopolyphosphatase hydrolyzes polyP with an average chain length of 208 to 15 phosphate residues to the same extent, but is inactive with ATP, PP_i, and *p*-nitrophenyl phosphate. The activity with $polyP_3$ is 13% of that with $polyP_{208}$. The K_m values for $polyP_{208}$, $polyP_{15}$, and $polyP_3$ hydrolysis are 3.5, 75, and 1100 μM, respectively. The enzyme is most active at pH ~7. Co^{2+} at the optimal concentration of 0.1 mM stimulates the activity 6-fold, while Mg^{2+} at the optimal concentration of 1 mM enhances it 2-fold. The enzyme under study is similar in some properties to an exopolyphosphatase purified earlier from yeast vacuoles.

Key words: polyphosphate, exopolyphosphatase, cytosol, cations, inhibitors, yeast, *Saccharomyces cerevisiae*

Current increased interest in polyP biochemistry is motivated by its significant regulatory role and the multitude of functions performed by polyP in cells of microorganisms [1-3]. Exopolyphosphatase (polyphosphate phosphohydrolase, EC 3.6.1.11) is the most active enzyme of polyP metabolism in yeasts. This enzyme releases P_i from the end of the polyP chain. The yeast *S. cerevisiae* is characterized by the presence of several exopolyphosphatases differing in their location, properties, and genetic determinants [4]. An exopolyphosphatase with a molecular mass of ~40 kD, which is the most active with respect to $polyP_3$ and nucleoside tetraphosphates, was purified from cell cytosol of *S. cerevisiae* [4, 5]. This enzyme is encoded by the *PPX1* gene [6, 7] and is the major exopolyphosphatase of the cytosol and the cell as a whole under the usual cultivation conditions [8]. However, the transfer of the yeast cells from P_i-deficient to complete medium causes a predominance of a high-molecular-mass exopolyphosphatase (~830 kD) in the cytosol. It differs from the 40-kD enzyme not only in molecular mass but also in substrate specificity and sensitivity to various effectors. The synthesis of this enzyme, not encoded by the *PPX1* gene [4], occurs *de novo* under the above-mentioned conditions [9]. The aim of this work was to purify and to study the properties of the high-molecular-mass exopolyphosphatase of *S. cerevisiae*.

Abbreviations: $polyP_n$) inorganic polyphosphates, where *n* is an average number of phosphate residues in chain.

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MATERIALS AND METHODS

Yeast strain and culture conditions. *Saccharomyces cerevisiae* VKM Y-1173 (IBPM-366) was grown on a shaker in flasks with 200 ml of medium at 30°C [8]. The yeast culture was grown on Reader medium with 1.3 mM P_i to the late logarithmic growth phase ($A_{600} = 5$). Biomass was separated by centrifugation at 5000*g* for 10 min, washed with distilled water, and placed on fresh Reader medium with 18.3 mM P_i so that the initial density remained the same. After cultivation for 18 h, the cells were centrifuged, washed with distilled water as above, and used for the cytosol preparation.

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Preparation of cytosol. The cytosol was obtained by disintegration of spheroplasts in 20 mM Tris-HCl buffer, pH 7.2, with 0.1 M sorbitol followed by centrifugation at 15,000*g* for 60 min, as described earlier [8]. Under centrifugation, the precipitation of organelle fractions and membranes was observed.

Enzyme assays. Exopolyphosphatase activity was determined by the amount of P_i formed during 10-30-min incubation at 30°C in 1 ml of reaction mixture containing 50 mM Tris-HCl buffer, pH 7.2, 0.1 mM $CoSO_4$, 200 mM NH_4Cl, and 0.01 mM polyP$_{208}$. The changes in incubation media are specified in the table and figure legends. The reaction was initiated by addition of the enzyme. P_i formed during the reaction was determined with ascorbic acid and SDS [10]. To determine the pH optimum for activity, 100 mM Tris-acetate buffer, pH 5-9, was used. To study the effect of reagents, the sample was preincubated with them in the above-mentioned incubation mixture for 5 min at 20°C. The reaction was initiated by addition of the substrate. Nonspecific phosphatase activity was determined using 0.1% *p*-nitrophenyl phosphate as described earlier [11].

One activity unit (U) was defined as the enzyme quantity sufficient to catalyze the formation of 1 μmol P_i in 1 min.

Exopolyphosphatase purification. All steps of enzyme purification were carried out at 4°C. The cytosol preparation was supplemented with ammonium sulfate to 50% saturation. After 1 h, the precipitate formed was removed by centrifugation at 12,000*g* for 20 min. Ammonium sulfate was added to the supernatant to 75% saturation. After 1 h the precipitate was collected by centrifugation under the same conditions, and the pellet was dissolved in 25 mM Tris-acetate buffer, pH 7.2, with 0.1% Triton X-100. Ammonium sulfate was removed with five cycles of concentration/dilution using an Amicon system (YM-10 membrane, Amicon, USA). Then it was loaded onto a column (1.5 × 7.5 cm) with DEAE-Toyopearl 650 M (Toson, Japan) equilibrated with the same buffer. The column was washed with 100 ml of the starting buffer and then with 150 ml of the same buffer with 0.1 M KCl. The exopolyphosphatase was eluted at a flow rate of 30 ml/h with increasing KCl concentrations (0.1-0.7 M) in the same buffer (the gradient volume was 200 ml). Fractions with exopolyphosphatase activity were pooled and incubated with heparin-agarose (Sigma, USA) at 4°C for 1 h. The resin was equilibrated with the starting buffer containing 0.2 M KCl. The material not sorbed was removed and the heparin-agarose was washed with the same buffer containing 0.5 M KCl. The exopolyphosphatase was desorbed from the heparin-agarose with 1 M KCl or 10 mM polyP in the same buffer. Concentration of polyP (Reakhim, Russia) used under chromatography is indicated by labile phosphorus. According to the data of electrophoresis in polyacrylamide gel (not illustrated), the given polyP preparation contained largely polyP with chain length similar to that in the preparation of polyP$_{208}$ (Monsanto, USA).

Gel filtration. To determine the molecular mass, the exopolyphosphatase preparation desorbed from heparin-agarose was loaded onto a 0.75 × 30 cm Superose 6 column (Pharmacia, Sweden) equilibrated with 25 mM Tris-HCl buffer, pH 7.2, containing 0.1 M KCl and chromatographed with the use of an FPLC system (Pharmacia). The exopolyphosphatase was eluted with the same buffer at a flow rate of 0.5 ml/min. The following marker proteins were used: ferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome *c* (Pharmacia and Sigma) with molecular masses of 440, 200, 150, 66, 29, and 12.4 kD, respectively.

Other methods. PolyP (Monsanto and Sigma) was separated from P_i and PP_i by gel filtration on Sephadex G-10 as described earlier [11]. $PolyP_3$ (Sigma) and PP_i (Koch-Light, UK) were used without further purification.

Protein concentration was determined using bovine serum albumin as the standard [12]. Polyclonal rabbit antiserum against the purified cell-envelope exopolyphosphatase of *S. cerevisiae* [13] was used in 1 : 400 dilution.

Electrophoresis in 12.5% polyacrylamide gel with SDS was performed by the method of Laemmli [14]. Proteins were detected by gel staining with Coomassie blue R-250 [11] and with silver [15].

RESULTS AND DISCUSSION

Purification of the high-molecular-mass exopolyphosphatase. In the cytosol preparation obtained in the course of growing of the yeast *S. cerevisiae*, only a minor amount of 40-kD exopolyphosphatase was detected. This was confirmed by insensitivity of exopolyphosphatase activity in the preparation to the antibodies suppressing this enzyme [13]. In the course of purification, the 40-kD enzyme was entirely eluted from DEAE-Toyopearl with 0.1 M KCl, while the high-molecular-mass exopolyphosphatase was eluted as a broad peak at ~0.2-0.3 M KCl.

After desorption from heparin-agarose with 1 M KCl, the preparation of high-molecular-mass exopolyphosphatase with a specific activity of 150 U/mg, 319-fold purification, and 16.9% yield was obtained (Table 1). The specific activity of the resulting preparation was close to that of the purified preparations of 40-kD exopolyphosphatases from the cytosol [5] and cell envelope [11] of the same yeast strain.

The exopolyphosphatase activity was also completely desorbed from heparin-agarose with 10 mM polyP. The specific activities were similar when either desorption method was applied.

Table 1. Purification of the high-molecular-mass exopolyphosphatase from the cytosol of *S. cerevisiae* (from 21 g wet cells)

Purification step	Protein, mg	Specific activity, U/mg*	Yield, %
Cytosol	216	0.47	100
Ammonium sulfate precipitation	76.3	1.11	85
DEAE-Toyopearl 650M	3.72	5.0	18.2
Heparin-agarose, desorption with 1 M KCl	0.115	150	16.9

* All measurements were performed with $polyP_{208}$ in the presence of 0.1 mM Co^{2+} and 200 mM NH_4^+.

Table 2. Effect of monovalent cations on the activity of the high-molecular-mass exopolyphosphatase of *S. cerevisiae*

Salt	Concentration, mM	Activity, %*
Without salts	0	100
KCl	50	139
	200	138
NaCl	50	146
	200	142
NH_4Cl	50	142
	200	167

* Specific activity of the enzyme corresponding to 100% was 5 U/mg. Measurements were carried out with $polyP_{208}$ in the presence of 0.1 mM Co^{2+}.

Stability of the enzyme during storage. Preliminary experiments on the storage of partially purified exopolyphosphatase preparation after DEAE-Toyopearl chromatography showed that 0.1% Triton X-100 had a stabilizing effect on the activity. During storage at 4°C for 5 days, 92% of the preparation activity was retained in the presence of 0.1% Triton X-100, while only 34% in its absence. Triton X-100 was also essential to stabilize the activity of the 40-kD exopolyphosphatase from cell envelope [11] and cytosol [5] and exopolyphosphatase from vacuoles [16].

Addition of 0.05 mM $CoSO_4$ did not increase the enzyme stability independent of the presence of Triton X-100. Thereafter, all buffer solutions were supplemented with 0.1% Triton X-100 during purification.

The exopolyphosphatase preparation desorbed from heparin-agarose with 1 M KCl and 0.1% Triton X-100 was entirely stable for at least 4 days when stored at –4°C, while the preparation obtained by desorption with polyP was unstable and the enzyme became entirely inactive under the same storage conditions. It lost ~30% activity even when stored at 4°C for 2 h. It should be mentioned that polyP hydrolysis and P_i accumulation occurred in the preparation. Inactivation of the enzyme was not due to this accumulation, since P_i removal by dialysis failed to restore the activity. Therefore, all the enzyme properties were studied in the preparations obtained by desorption with 1 M KCl.

pH optimum. The purified exopolyphosphatase was most active at pH values of ~7 (Fig. 1) and in this respect is similar to other yeast exopolyphosphatases [4]. The pH dependence curve of the enzyme was similar to that of the exopolyphosphatase activity in vacuoles of the same yeast [17] and distinguished from the 40-kD exopolyphosphatase by having a less pronounced pH optimum [18].

Effect of cations. The effect of some monovalent cations on the enzyme activity was studied in the presence of 0.1 mM Co^{2+} in preliminary experiments with the enzyme preparation after DEAE-Toyopearl chromatography and ultrafiltration. The highest activity was observed with 200 mM NH_4^+ (Table 2); therefore, all further measurements of the activity were carried out in the presence of this cation. The effect of monovalent cations on the tested exopolyphosphatase was similar to that of the 40-kD enzymes of the cytosol and cell envelope [5, 11].

The effect of divalent cations on the purified high-molecular-mass exopolyphosphatase was studied, since all currently known exopolyphosphatases of *S. cerevisiae* required them for their activity, except for the one from

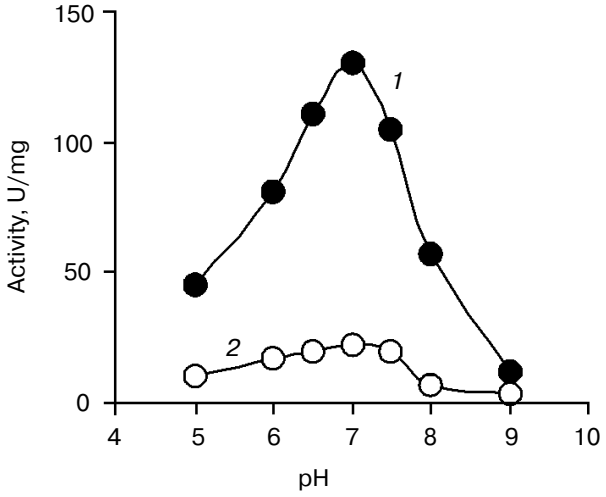

Fig. 1. Effect of pH on the activity of purified high-molecular-mass exopolyphosphatase from the cytosol of *S. cerevisiae*: *1*) with $polyP_{208}$; *2*) with $polyP_3$.

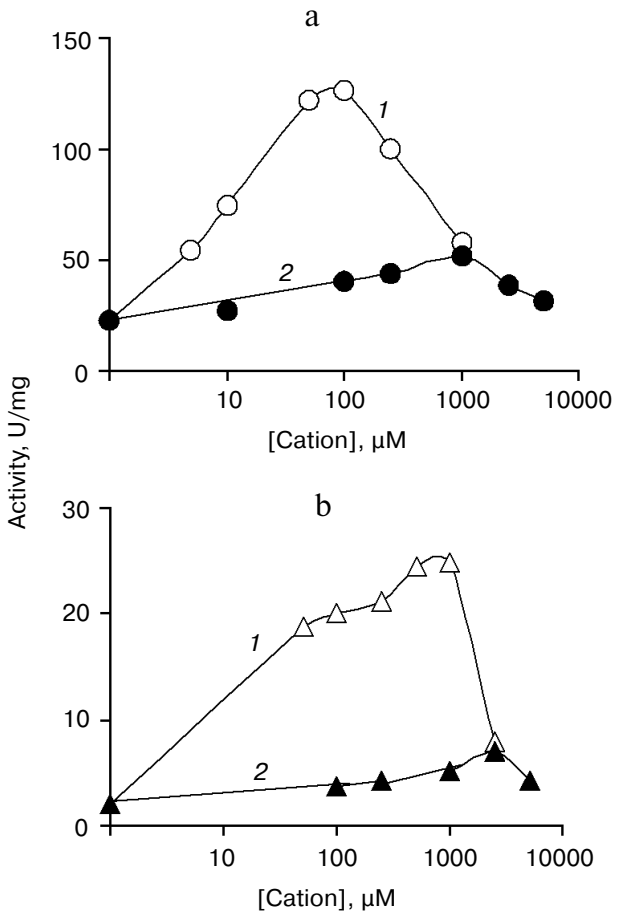

Fig. 2. Dependence of the activity of purified high-molecular-mass exopolyphosphatase from the cytosol of *S. cerevisiae* on the concentrations of Co^{2+} (*1*) and Mg^{2+} (*2*): a) with $polyP_{208}$; b) with $polyP_3$.

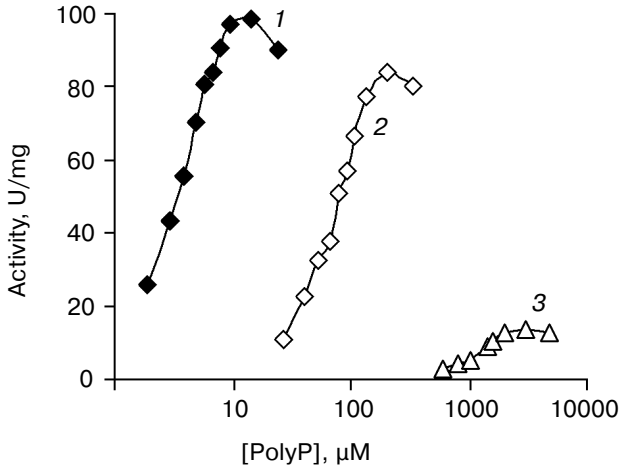

Fig. 3. Dependence of the activity of purified high-molecular-mass exopolyphosphatase from the cytosol of *S. cerevisiae* on polyP concentrations: *1*) $polyP_{208}$; *2*) $polyP_{15}$; *3*) $polyP_3$. Measurements with different substrates were carried out in the presence of 0.1 mM Co^{2+} (*1*, *2*) and in the presence of 0.5 mM Co^{2+} (*3*).

mitochondrial membranes [4]. Figure 2 demonstrates the dependence of the enzyme activity in the preparation after purification on heparin-agarose on the concentrations of $MgSO_4$ and $CoSO_4$ both with $polyP_{208}$ and with $polyP_3$. At the optimal 0.1 mM Co^{2+} concentration, the enzyme activity with $polyP_{208}$ increased 6-fold, while at the optimal 1 mM concentration of Mg^{2+} it increased only 2-fold. Zn^{2+} (0.1 mM) increased this activity 1.5-fold (not illustrated).

The activity with $polyP_3$ at all used concentrations of Co^{2+} and Mg^{2+} was substantially lower, and the optimal concentrations of these cations were 1 and 2.5 mM, respectively.

The stimulating effect of divalent cations for the exopolyphosphatase under study was similar to that for the enzyme of vacuoles of *S. cerevisiae* [16]. The 40-kD exopolyphosphatase showed a considerably higher level of stimulation by divalent cations and smaller difference in the stimulating effect between Co^{2+} and Mg^{2+} [5, 11].

Substrate specificity. The purified high-molecular-mass exopolyphosphatase of the cytosol hydrolyzed polyP with different chain length. The activity decreased only slightly as the average chain length decreased from 208 to 15 phosphate residues (Table 3). With $polyP_3$, however, it was only 13% compared with the activity with $polyP_{208}$. The enzyme was inactive with ATP, PP_i, and *p*-nitrophenyl phosphate (Table 3). By substrate specificity, the exopolyphosphatase under study is similar to the vacuolar enzyme and differs greatly from the 40-kD exopolyphosphatase, which was most active with $polyP_3$ [5, 11].

The dependence of the enzyme activity on the concentrations of $polyP_{208}$, $polyP_{15}$, and $polyP_3$ in the presence of Co^{2+} was not in agreement with the Michaelis–Menten kinetics (Fig. 3). The substrate concentrations, which resulted in half-maximal reaction rates ($K_{m\ app}$), were 3.5, 75, and 1100 μM for $polyP_{208}$, $polyP_{15}$, and $polyP_3$, respectively. They were similar to those for the vacuolar exopolyphosphatase [16]. The high-molecular-mass exopolyphosphatase was characterized by higher enzyme–substrate affinity along with the increase in the polyphosphate chain length, just as with other yeast exopolyphosphatases [4].

Effect of inhibitors. Table 4 demonstrates the effect of some reagents on the activity of the purified high-molecular-mass exopolyphosphatase of the cytosol. Heparin was an effective inhibitor of this enzyme, as it is of other yeast exopolyphosphatases [4]. Zn^{2+}, as distinguished from Mg^{2+}, suppressed the activity of the enzyme in the presence of Co^{2+}, which was known for other yeast exopolyphosphatases [5]. The high-molecular-mass exopolyphosphatase was completely inhibited by EDTA at a concentration similar to the cation concentration (Table 4). This distinguished the enzyme under study from the 40-kD exopolyphosphatase, which was stimulated by EDTA over a wide range of concentration [5].

Table 3. Activity of the high-molecular-mass exopolyphosphatase of the cytosol with different substrates

Substrate	Concentration, mM*	Measurement conditions	Specific activity, U/mg
$PolyP_{208}$	0.01	0.1 mM Co^{2+}, 200 mM NH_4^+,	135
$PolyP_{45}$	0.044	pH 7.2	133
$PolyP_{25}$	0.08		130
$PolyP_{15}$	0.133		114
$PolyP_3$	2.0	0.5 mM Co^{2+}, 200 mM NH_4^+, pH 7.2	17.3
PP_i	2.0	5 mM Mg^{2+}, pH 7.2	0
		0.1 mM Co^{2+}, pH 7.2	0
ATP	2.0	2 mM Mg^{2+}, pH 7.2	0
p-Nitrophenyl phosphate	3.8	2.5 mM Mg^{2+}, pH 7.2	0
		0.1 mM Co^{2+}, pH 7.2	0
		2.5 mM Mg^{2+}, pH 9.0	0

* $PolyP_{15}$-$polyP_{208}$ concentrations corresponded to the same concentrations of labile phosphate equal to 2 mM.

Similar to exopolyphosphatases of vacuoles, nuclei, and mitochondria [4], the enzyme under study was not inhibited by antibodies against the purified cell-envelope exopolyphosphatase, which effectively suppressed the 40-kD enzyme. The high-molecular-mass exopolyphosphatase was almost insensitive to P_i, the reaction product, and less sensitive to PP_i and $polyP_3$ as compared with the vacuolar exopolyphosphatase [16].

SDS-PAGE. Figure 4 demonstrates electrophoregrams of the exopolyphosphatase preparations at different purification stages. In the preparation desorbed from heparin-agarose with 10 mM polyP, the major protein band of ~32 kD was observed. In the preparation desorbed with 1 M KCl, an additional band of ~35 kD was seen. In the cytosol preparation, this exopolyphosphatase exhibited molecular mass of ~830 kD as determined under gel filtration on Sephacryl S-300 [9]. One can suggest that the enzyme consists of several subunits; moreover, the ~35-kD polypeptide found in the preparation desorbed with 1 mM KCl relates to the enzyme–complex stability.

Gel filtration of different enzyme preparations. Gel filtration of preparations of the high-molecular-mass exopolyphosphatase was carried out at different purification stages on a column with Superose 6 using FPLC. We failed to establish the exopolyphosphatase molecular mass after its partial purification on DEAE-Toyopearl, since the activity was removed from the column as a wide peak whose onset was close to the free column volume. This suggests that at the given purification stage the exopolyphosphatase exists in the form of a large complex, found earlier in the initial cytosol preparation [8]. Using the same technique, the exopolyphosphatase activity was not revealed in the fractions during gel filtration of the enzyme preparation desorbed from heparin-agarose with 1 M KCl. On addition of both 0.1% Triton X-100 and 5 mM polyP to the fractions, no activity was registered. However, the addition of 5 mM polyP to the eluent solution resulted in retention of almost complete exopolyphosphatase activity. This activity appeared in the form of two broad peaks with molecular masses of ~500

Table 4. Effect of some reagents on the activity of purified high-molecular-mass exopolyphosphatase from the cytosol of *S. cerevisiae*

Reagent	Concentration, mM	Activity, %*
Control (no addition)		100
Iodoacetamide	10	74
NaF	10	91
Dithiothreitol	0.1	87
EDTA	0.1	0
$MgSO_4$	0.1	95
$ZnSO_4$	0.1	24
P_i	0.2	106
PP_i	1.0	76
$PolyP_3$	1.0	96
Heparin (1 μg/ml)		35
Heparin (10 μg/ml)		5
Antibodies against the 40-kD exopolyphosphatase (1 : 400)		135

* Activity was measured in the presence of 0.1 mM $CoSO_4$ and 200 mM NH_4Cl with 0.01 mM $polyP_{208}$. Specific activity taken as 100% was 100 U/mg.

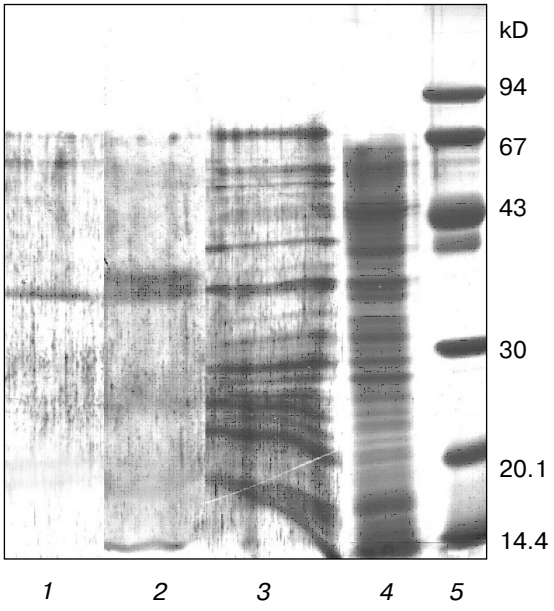

Fig. 4. SDS-PAGE in 12.5% gel: *1*) preparation of purified high-molecular-mass exopolyphosphatase desorbed from heparin-agarose with 10 mM polyP; *2*) preparation of purified high-molecular-mass exopolyphosphatase desorbed from heparin-agarose with 1 M KCl; *3*) partially purified preparation after DEAE-Toyopearl chromatography; *4*) cytosol; *5*) protein standards. Lanes: *1-3*) silver staining; *4*, *5*) Coomassie staining.

and 4800 kD. As a result, the complex molecular mass proved to be close to the one observed in the initial cytosol preparation [8].

Gel filtration of the enzyme preparation desorbed from heparin-agarose with 10 mM polyP was carried out as well. Exopolyphosphatase activity was not detected at gel filtration when polyP was absent from the eluent solution. On addition of 5 mM polyP, the exopolyphosphatase activity corresponded to a protein of ~125 kD and was unstable.

Thus, the polyP exerts a stabilizing effect on the high-molecular-mass exopolyphosphatase under gel filtration. PolyP seems to be a member of the enzyme complex. The total data on electrophoresis and gel filtration of two exopolyphosphatase preparations suggests that both polypeptides with molecular masses of ~32 and 35 kD are required for the formation of an active and stable complex. Under gel filtration in the absence of polyP, there is a possibility of separation of these polypeptides, while the presence of polyP provides formation of active complexes.

Many proteins have been reported to form complexes with polyP [19]. One example is polyphosphate glucokinase, an essential enzyme of polyP metabolism in bacteria, which has numerous forms differing from each other in molecular mass of the native enzyme and isoelectric point [20]. These distinctions are supposed to be due to the presence of firmly bound polyP in the enzyme preparation [20]. The question about molecular organization of the complex of the cytosolic high-molecular-mass exopolyphosphatase calls for further investigation.

Thus, the high-molecular-mass exopolyphosphatase of the cytosol is significantly different in its properties from the 40-kD enzyme of this compartment. It is similar in some properties to the one purified from the vacuoles [16].

The high-molecular-mass exopolyphosphatase becomes the major one in a cell not only under "hypercompensation" with respect to phosphate [8], but also due to inactivation of the gene encoding the 40-kD exopolyphosphatase [7]. It seems that there is co-regulation of the expressions of these two enzymes. The significance of this phenomenon for polyP metabolism in the yeast is still obscure.

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