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Properties of Partially Purified Endopolyphosphatase of the Yeast *Saccharomyces cerevisiae*

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Abstract—Partially purified endopolyphosphatase from cytosol of the yeast *Saccharomyces cerevisiae* with inactivated genes *PPX1* and *PPN1* encoding exopolyphosphatases was obtained with ion-exchange and affinity chromatography. The enzyme activity was estimated by decrease of polyphosphate chain length determined by PAGE. The enzyme cleaved inorganic polyphosphate without the release of orthophosphate (P_i) and was inhibited by heparin and insensitive to fluoride. Mg^{2+}, Mn^{2+}, and Co^{2+} (1.5 mM) stimulated the activity, and Ca^{2+} was ineffective. The molecular mass of the endopolyphosphatase determined by gel filtration was of ~20 kDa.

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Inorganic polyphosphates (polyP) are biopolymers that perform a great number of regulatory functions in cells [1]. PolyP and enzymes involved in their metabolism are directly concerned with pathogenic processes caused by bacteria [2] and protozoa [3]. Recently, it has become evident that these biopolymers are involved in energy metabolism in animal mitochondria [4] and in the process of blood coagulation [5]. In this connection, the necessity to study the enzymes of polyP metabolism has increased in recent years. Yeast, the most investigated microorganism with fully sequenced genome, continues to be the most suitable model to study polyP metabolism in eukaryotes. Exopolyphosphatases Ppx1 and Ppn1 (EC 3.6.1.11) cleaving P_i from the end of the polymeric chain are localized in several cell compartments ([6-9], Swiss Prot: http://kr.expasy.org/enzyme). High activity of these enzymes presents a considerable problem for identification of new enzymes of polyP metabolism. The use of a double mutant with inactivated genes encoding both exopolyphosphatases [8, 9] and analysis of activity by defining the average polyP chain length by PAGE make it possible to reveal fragmentation of the long-chain polyP to shorter ones without the release of P_i in cytosol preparations of *Saccharomyces cerevisiae* [10]. Detection of endopolyphosphatase activity explains polyP use by the double mutant lacking exopolyphosphatases on cultivation in P_i-deficient medium [11] and parallel polyphosphate synthesis and depolymerization seen in this yeast [12].

The objective of the present work was to purify the enzyme and study some of its properties.

MATERIALS AND METHODS

Strain, growth conditions, and preparation of spheroplasts and cytosol. The yeast *S. cerevisiae* CNX with inactivated *PPX1* and *PPN1* genes from A. Kornberg's laboratory (Stanford University, USA) [8, 9] was the object of our research. The yeast was grown aerobically in a shaker at 30°C in a standard YPD medium [10, 13] to the stationary growth phase (24 h). Preparation of spheroplasts has been described earlier [11-14]. The cytosol fractions were prepared by disruption of spheroplasts in 20 mM Tris-HCl, pH 7.2, containing 0.1 M sorbitol, 1 mM phenylmethylsulfonyl fluoride, and 5% glycerol. The spheroplast homogenate was layered with 20 mM Tris-HCl, pH 7.2, and spun at 5000*g* for 60 min. This procedure let lipid granules float in the buffer layer. The cytosol fraction was removed with a syringe and centrifuged once more at 12,000*g* for 60 min.

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Endopolyphosphatase purification. All steps of the enzyme purification were carried out at 4°C. The cytosol preparation (~15 ml) in 20 mM Tris-HCl, pH 7.2, was loaded onto a column (1.6 × 7 cm) with DEAE-Toyopearl 650M (Toson, Japan) that had been equilibrated with 20 mM Tris-HCl, pH 7.2. The column was washed with 50 ml of 0.1 M KCl in the same buffer and then eluted at flow rate 30 ml/h with increasing KCl concentration (0.1-0.4 M) in the same buffer. The gradient volume was 50 ml. Fractions with endopolyphosphatase activity were pooled and applied to a column (1.6 × 7 cm) with heparin-agarose (Sigma, USA) without preliminary desalting. The enzyme was eluted at flow rate 15 ml/h with a step KCl gradient in 20 mM Tris-HCl, pH 7.2, using 10 ml of each salt concentration (0.5, 0.6, 0.7, and 0.8 M). Fractions with endopolyphosphatase activity were pooled and subjected to ultrafiltration using an Amicon system (Amicon, USA; membrane YM-10) with two volumes of initial buffer. Then the preparation (2-4 ml) was applied to a Mono-Q HR 5/5 column (Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl, pH 7.2, and chromatographed by FPLC. Elution was carried out at 0.5 ml/min with step KCl gradient in 20 mM Tris-HCl, pH 7.2 (0.1, 0.15, 0.2, and 0.25 M). Fraction volume was 2-5 ml.

Gel filtration. To determine the molecular mass of the enzyme, gel filtration on FPLC Superose 6 and Superose 12 columns (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.2, containing 0.1 M KCl was used. Elution rate was 0.5 ml/min. The following marker proteins (Pharmacia) were used: thyroglobulin (669 kDa), ferritin (440 kDa), β-amylase (200 kDa), aldolase (160 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (13 kDa).

Enzyme assay. Endopolyphosphatase activities in the cytosol and fractions obtained after DEAE-Toyopearl chromatography were determined in 1.4 ml of an incubation mixture containing 20 mM Tris-HCl (pH 7.2), 3 mM $MgSO_4$, and 10 mM polyP (as labile phosphate; Reakhim, Russia) at 30°C for 60 min. The reaction was stopped by addition of an equal volume of 1 N $HClO_4$. Further operations including polyP precipitation with $Ba(NO_3)_2$ and electrophoresis in 20% polyacrylamide gel to determine changes in polyP chain lengths were described earlier [10]. Endopolyphosphatase activities in the fractions after affinity chromatography on heparin-agarose and FPLC were determined in 50 μl of incubation mixture containing 20 mM Tris-HCl (pH 7.2), 4 mM $MgSO_4$, and 2 mM $polyP_{208}$ (as labile phosphate; average chain length of 208 phosphate residues; Monsanto, USA) at 30°C for 60 min. Samples were subjected to electrophoresis after the incubation.

To evaluate the endopolyphosphatase activity, such sample quantity was selected that resulted in total degradation of high-length polyP to $polyP_{15}$ in 60 min. The enzyme amount changing 1 mM $polyP_{208}$ (as labile phosphate) to $polyP_{15}$ per minute was taken as a unit of enzyme activity.

Pyrophosphatase activity was estimated from the rate of P_i formation during 10-15 min incubation in 1 ml of a reaction mixture containing 20 mM Tris-HCl, pH 7.2, 1.25 mM $MgSO_4$, and 1.25 mM PP_i at 30°C. Protein concentration was measured with BSA as a standard [15], and P_i was measured as described earlier [16].

RESULTS AND DISCUSSION

Partial purification of endopolyphosphatase. In the present work, a partial purification of the enzyme with endopolyphosphatase activity from the cytosol of the yeast *S. cerevisiae* was carried out (table). Endopolyphosphatase activity was eluted from DEAE-Toyopearl in a wide range of KCl concentrations (0.25-0.35 M) (Fig. 1). Under affinity chromatography on heparin-agarose, the activity was revealed in the fractions with KCl concentrations of 0.6 and 0.7 M (Fig. 2), and after subsequent chromatography on Mono-Q it appeared in fractions with 0.1 M KCl (Fig. 3).

The cytosol preparation contained a great quantity of pyrophosphatase activity, the main part of which was eluted by 0.1 M KCl from DEAE-Toyopearl. At the last stage of purification, the endopolyphosphatase preparation was free from pyrophosphatase activity (table). Electrophoresis of the endopolyphosphatase preparation in 15% polyacrylamide gel with SDS revealed several protein bands and therefore identification of the enzyme requires further investigations. Endopolyphosphatase activity was lacking after chromatography of the preparation on a Mono-S column after Mono-Q, indicating either the inactivation of the enzyme or elimination of a certain component required for manifestation of activity at this stage of purification.

Properties of the partially purified endopolyphosphatase. The resulting preparation was used for the study of properties of the endopolyphosphatase. The preparation free from pyrophosphatase degraded polyP without

Purification of endopolyphosphatase from *S. cerevisiae* deficient in the *PPX1* and *PPN1* genes

Purification step	Endopolyphosphatase activity, mU/mg protein	Pyrophosphatase activity, mU/mg protein
Cytosol fraction	0.05	2500
DEAE-Toyopearl 650M	0.45	70
Heparin-agarose	0.8	50
Mono-Q	15.3	< 7

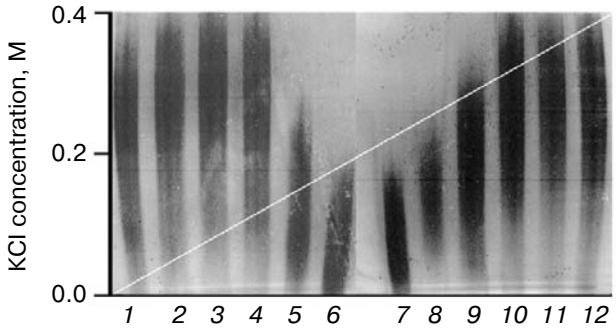

Fig. 1. Endopolyphosphatase activity in fractions after DEAE-Toyopearl chromatography. PolyP_{208} (Reakhim, Russia) (here and in Fig. 2) was used as a substrate. Electrophoresis was carried out after polyP precipitation from the samples with $Ba(NO_3)_2$ as described in "Materials and Methods".

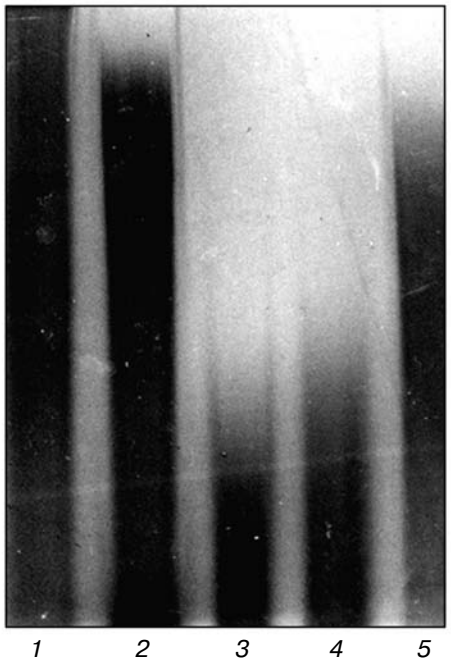

Fig. 2. Endopolyphosphatase activity in fractions after heparin-agarose chromatography. PolyP_{208} was used as a substrate. *1-5*) KCl concentrations (0.3, 0.5, 0.6, 0.7, and 0.8 M, respectively).

the release of P_i even after long incubation, up to 24 h. This confirms our suggestion that polyP hydrolysis to P_i in the double mutant free from exopolyphosphatase genes was performed by combined action of endopolyphosphatase and pyrophosphatase [10]. It is probable that the cytosol pyrophosphatase uses as a substrate not only PP_i but short-chain polyP as well, at least tripolyphosphate being a substrate for many pyrophosphatases [17].

When using gel filtration, endopolyphosphatase activity was eluted in volumes corresponded to molecular mass of ~20 kDa. The enzyme was inhibited by heparin

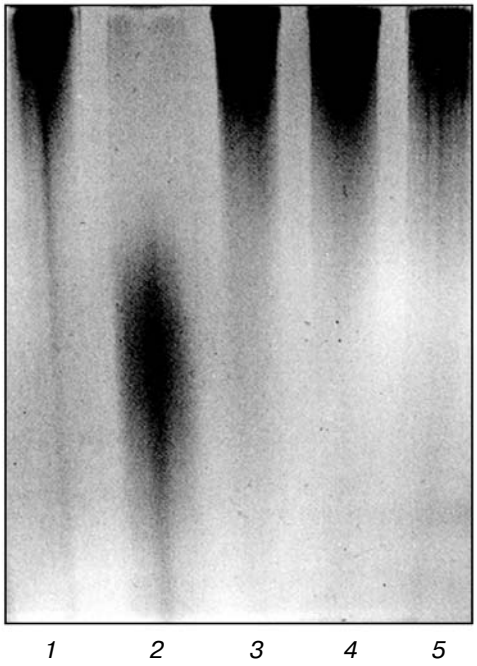

Fig. 3. Endopolyphosphatase activity in fractions after Mono-Q chromatography. PolyP_{208} (Monsanto, USA) was used as a substrate. Electrophoresis was carried out without polyP precipitation as described in "Materials and Methods". *1-5*) KCl concentrations (0, 0.1, 0.125, 0.14, and 0.15 M, respectively).

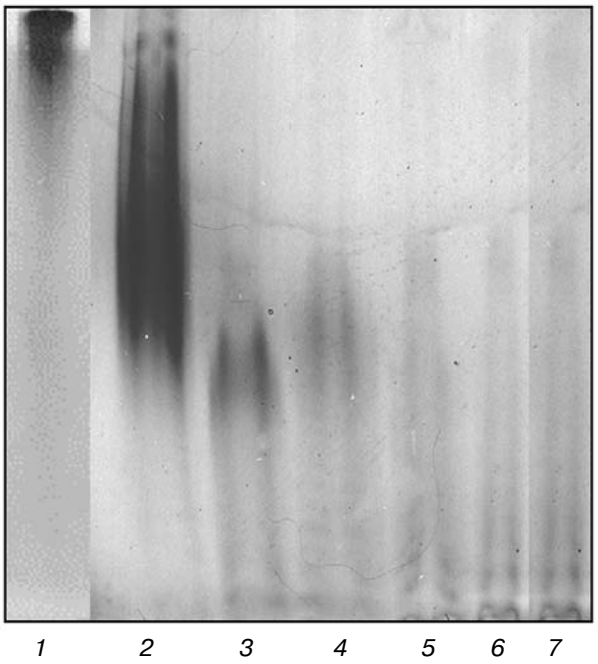

Fig. 4. Effect of various Mg^{2+} concentrations on endopolyphosphatase activity (preparation after purification on heparin-agarose). 11 mM polyP_{208} (Monsanto, USA) was used as a substrate. *1*) Control; incubation medium without the enzyme preparation; *2-7*) Mg^{2+} concentrations (0, 0.3, 0.6, 1.5, 3, and 6 mM, respectively).

(0.26 mg/ml), a known inhibitor of exopolyphosphatases [10], and was insensitive to 5 mM fluoride.

The effect of different Mg^{2+} concentrations on endopolyphosphatase activity was studied (Fig. 4). The enzyme was most active at 1.5 mM concentration of this cation. Possible stimulation of endopolyphosphatase by other divalent cations was examined. It was found that Mn^{2+} and Co^{2+} taken at the same concentration as Mg^{2+}stimulated the activity as much as Mg^{2+}, whereas Ca^{2+} failed to stimulate it (not illustrated).

The data reported here support the presence in *S. cerevisiae* of a special enzyme fragmenting long-chain polyP to shorter chains and exhibiting neither exopolyphosphatase nor pyrophosphatase activity. Further purification of the enzyme and identification of the encoding gene may be of interest for understanding polyP metabolism in yeast and will be a subject of our future investigations.

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