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Finding of Endopolyphosphatase Activity in the Yeast *Saccharomyces cerevisiae*

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Abstract—Endopolyphosphatase activity has been revealed in cytosol preparations of the yeast *Saccharomyces cerevisiae* with inactivated *PPX1* and *PPN1* genes encoding exopolyphosphatases. The enzyme cleaves inorganic polyphosphates with chain length of 15 to 208 phosphate residues to shorter chains without the release of orthophosphate (P_i). The long chain polyphosphates are cleaved with preference over the short ones. Heparin, a known inhibitor of exopolyphosphatases, represses this activity. The endopolyphosphatase activity is not stimulated by Mg^{2+} or Co^{2+}, in contrast to exopolyphosphatases. This activity along with a pyrophosphatase is supposed to be responsible for polyphosphate utilization as a phosphate reserve in a mutant devoid of exopolyphosphatases.

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Key words: inorganic polyphosphates, endopolyphosphatase, exopolyphosphatase, cytosol, *PPX1* and *PPN1* mutant, *Saccharomyces cerevisiae*

Interest in enzymes participating in the metabolism of inorganic polyphosphates (polyP) is now increasing due to the finding of some new functions of these polymers, such as their involvement in the regulation of gene activity, cytokinesis, and mitochondrial functioning [1-3].

The metabolism of most polymers existing in cells involves hydrolases, which split off monomeric residues from the chain end, and depolymerases splitting long polymer chains into shorter ones. The enzyme catalyzing the reaction $polyP_n + H_2O \rightarrow$ oligophosphates is described in databases (Swiss Prot: http://kr.expasy.org/enzyme; http://www.brenda-enzymes.info/).

A viscometric method with low sensitivity and specificity has been used [4, 5] to identify endopolyphosphatase activity; based on these works, this enzyme (EC 3.6.1.10) was included in the international nomenclature (http://www.chem.gmul.ac.uk/iubmb/enzyme/EC3/). The method of activity assay by the terminal polymer groups, which is commonly used in the case of depolymerases, still has not to be developed for polyphosphates. Thus, all methods of endopolyphosphatase activity assay are rather complex and ineffective [6].

In the yeast (a traditional model for studying polyphosphate metabolism), the set of polyP-dependent enzymes identified to date includes two exopolyphosphatases, Ppx1 and Ppn1 (EC 3.6.1.11), cleaving P_i from the end of the polymeric chain. The enzyme Ppn1 was first regarded as an endopolyphosphatase ([7, 8] and Swiss Prot: http://kr.expasy.org.enzyme). However, the enzyme was shown to form P_i in the course of polyphosphate hydrolysis [9-11]. Such release of P_i in the beginning of the reaction is typical of exopolyphosphatase [11].

We recently found that a mutant *Saccharomyces cerevisiae* with inactivated *PPX1* and *PPN1* genes encoding exopolyphosphatases is able to utilize polyphosphate reserves for growth in P_i-deficient medium [12]. In this connection, the objective of the present work was to find the enzyme activity responsible for this process.

MATERIALS AND METHODS

The yeast *S. cerevisiae* CNX with inactivated *PPX1* and *PPN1* genes from A. Kornberg's laboratory (Stanford University, USA) was the object of our research. The yeast was grown aerobically in a shaker at 30°C in a standard YPD medium [8] to the stationary growth phase (24 h). Preparation of spheroplasts and cytosol fractions, polyphosphate assay, and electrophoresis has been

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described earlier in detail [12, 13]. Polyphosphate hydrolysis was performed in incubation mixture containing 20 mM Tris-HCl, pH 7.2, 10 mM polyphosphate (as labile phosphate) (Sigma and Monsanto, USA), and a cytosol preparation (12-25 mg protein per ml). Samples were incubated at 30°C for different periods of time. The reaction was stopped by addition of an equal volume of 2 N $HClO_4$. The control sample was supplemented with 2 N $HClO_4$ prior to addition of the cytosol preparation. The samples were left to stand for 10 min at 0°C, supplemented with Norit A charcoal (Sigma), and again left to stand for 10 min at 0°C. Then the samples were centrifuged at 5000*g* for 20 min. The supernatant was used for determination of P_i and labile phosphate [12, 13]. The change in polyphosphate chain lengths was shown by PAGE after polyphosphate precipitation from the supernatants with $Ba(NO_3)_2$ as described earlier [13].

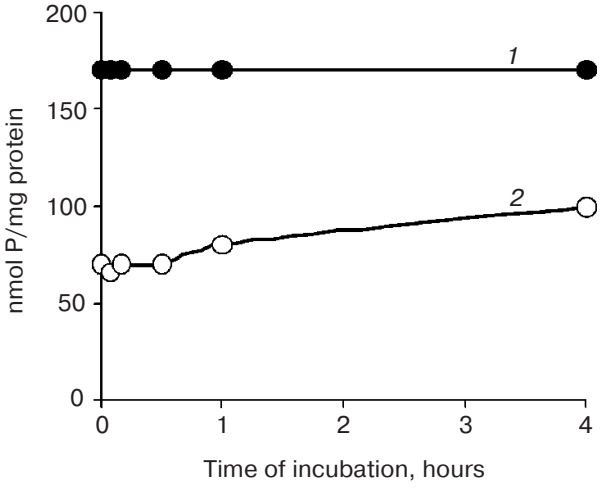

Fig. 1. Changes in polyphosphate (*1*) and P_i (*2*) contents under incubation of 10 mM $polyP_{208}$ with cytosol preparations of CNX strain of *S. cerevisiae*.

RESULTS

It was previously shown that incubation of spheroplast homogenate of the yeast *S. cerevisiae* with inactivated *PPX1* and *PPN1* genes in 20 mM Tris-HCl, pH 7.2, 30°C, results in a decrease in the average polyphosphate chain lengths from ~45 to ~15 phosphate residues within 4 h (data not shown). The content of labile phosphate was practically unchanged and the P_i content increased by no more than 10%, suggesting the occurrence of depolymerase activity. The same effect was found in the cytosol preparations of this strain, and we suggested that polyphosphate degradation was performed by endopolyphosphatase without P_i release. Note that exopolyphosphatase activity was absent from cytosol preparations of the double mutant CNX [12].

Exogenous $polyP_{208}$ is used for the assay of endopolyphosphatase activity in cytosol preparations. Polyphosphate and P_i contents are practically unchanged during a 4-h incubation (Fig. 1), and the average polyphosphate chain lengths decrease from ~208 to ~10 phosphate residues (Fig. 2, lanes *1-7*). Polyphosphate chain lengths are unchanged in the absence of the cytosol preparations (not illustrated). Relatively long-chain polyphosphates disappear in the first 5 min of incubation (Fig. 2), which points to a non-processive character of this reaction. The findings let us evaluate the endopolyphosphatase activity. Judging from PAGE electrophoresis (Fig. 2), $polyP_{208}$ was totally degraded to $polyP_{15}$ in 10 min with unchanged labile phosphate content. Since 10 mM $polyP_{208}$ (labile phosphate) corresponds to 0.05 mM of the polymer, the specific endopolyphosphatase activity was evaluated as ~0.4 nmol polyP/min per mg.

Thus, fragmentation of long-chain polyphosphates without P_i release is observed in the cytosol preparations of *S. cerevisiae* with inactivated *PPX1* and *PPN1* genes, i.e. endopolyphosphatase activity is revealed. The substrate specificity of this activity is estimated. The polyphosphate and P_i contents in the incubation mixture are practically unchanged during 90 min incubation with 10 mM of $polyP_{75}$, $polyP_{45}$, $polyP_{25}$, $polyP_{15}$ (labile phosphate) (not illustrated), whereas the average chain lengths of polyphosphates decreased to ~10 phosphate residues irrespective of the substrate used (Fig. 2, lanes *8-15*). Polyphosphates with the chain lengths of 208 to 25 phosphate residues were degraded with equal efficiency. $PolyP_{15}$ shortened only slightly, suggesting the low affinity of the endopolyphosphatase to short-chain polyphosphates.

The effect of some compounds involved in phosphate metabolism, divalent cations, and heparin (an inhibitor of the known exopolyphosphatases) on endopolyphosphatase activity was studied (Fig. 3). The addition of 3 mM Mg^{2+} and 3 mM Co^{2+} has no effect on the cytosol endopolyphosphatase activity. Conceivably, the yeast cytosol contains a sufficient amount of these divalent cations to achieve maximum activity. P_i, PP, ADP, ATP (5 mM each), and 1% glucose had no effect on the endopolyphosphatase reaction, in contrast to heparin, which repressed this reaction at a concentration of 0.26 mg/ml (Fig. 3).

The endopolyphosphatase activity is probably responsible for the dynamics of polyphosphate chain-length changes in the cytosol on cultivation of the mutant lacking Ppx1 and Ppn1 exopolyphosphatases on the P_i-deficient medium [12]. In accordance with the data obtained, this enzyme does not release P_i. However, incubation of the cytosol preparations for 24 h with $polyP_{208}$ was followed by a decrease in polyphosphate content more than 2-fold and the parallel increase in P_i to the

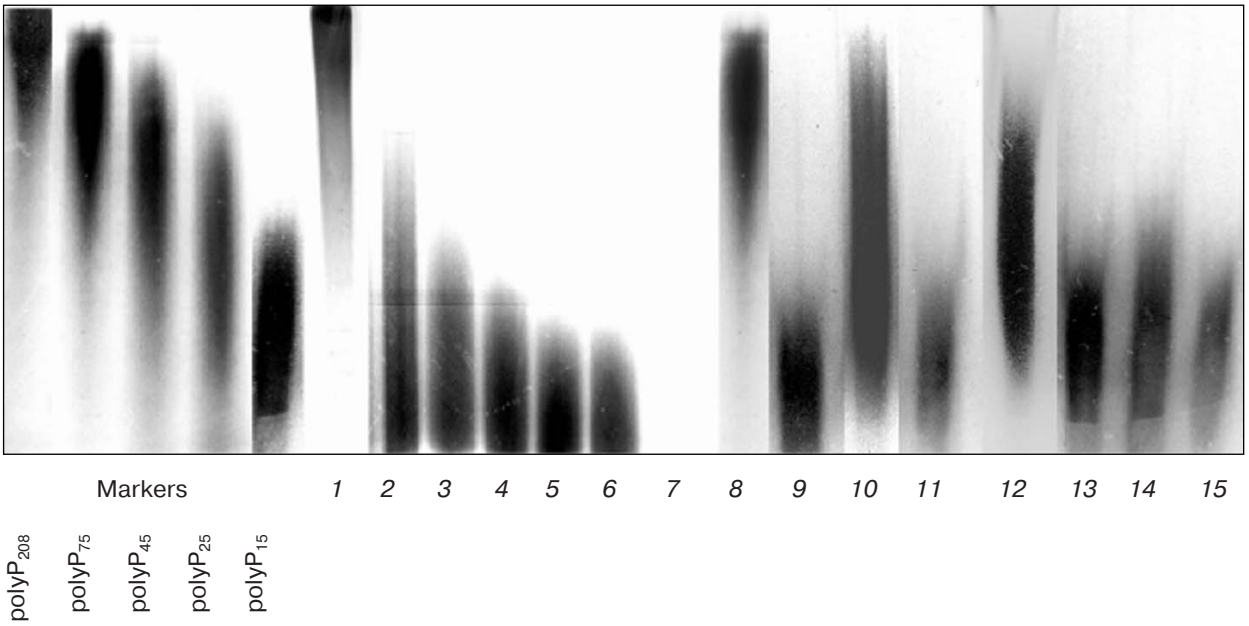

Fig. 2. Effect of incubation time and substrate chain length on polyphosphate shortening under incubation with cytosol preparations. Chain lengths of polyP markers are indicated on the left. Polyphosphates were added to 10 mM concentration. Lanes *1-7*, $polyP_{208}$ chain length depending on the incubation time: 0, 5, 10, 30, 60 min, 4, and 24 h, respectively. Lanes *8-15*, endopolyphosphatase activity with different substrates: *8*) $polyP_{75}$, 0 min; *9*) $polyP_{75}$, 90 min; *10*) $polyP_{45}$, 0 min; *11*) $polyP_{45}$, 90 min; *12*) $polyP_{25}$, 0 min; *13*) $polyP_{25}$, 90 min; *14*) $polyP_{15}$, 0 min; *15*) $polyP_{15}$, 90 min.

same extent (not illustrated). In this case, polyphosphates were not detected by electrophoresis (Fig. 2, lane *7*). Their chain length probably becomes less than 10 phosphate residues, and such short-chain polymers are not stained with toluidine blue [14]. The short-chain polyphosphates accumulated in the yeast cytosol could be hydrolyzed by pyrophosphatase to P_i as shown earlier [15]. According to our data, high pyrophosphatase activity was detected in the CNX cytosol.

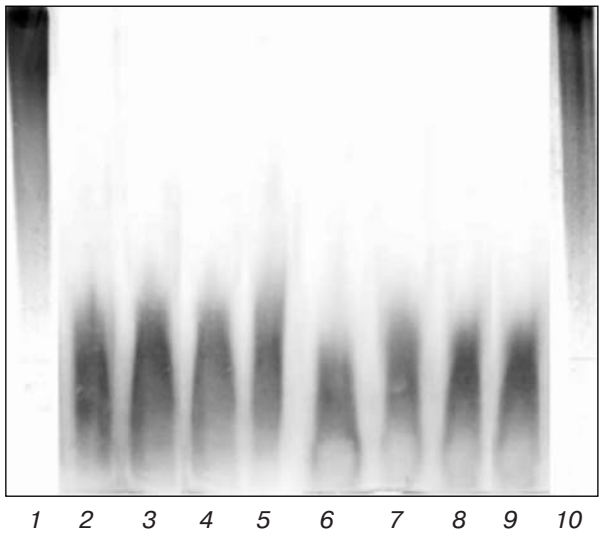

Fig. 3. Effect of some reagents on decrease in polyphosphate chain lengths under incubation with cytosol preparations. Electrophoresis of polyphosphates in 20% polyacrylamide gel. $PolyP_{208}$ (10 mM) was used as a substrate: *1*) control, 0 min incubation; *2-10*) 90 min incubation: *2*) without effectors; *3*) 3 mM Mg^{2+}; *4*) 3 mM Co^{2+}; *5*) 1% glucose; *6*) 5 mM P_i; *7*) 5 mM PP_i; *8*) 5 mM ADP; *9*) 5 mM ATP; *10*) heparin (0.26 mg/ml).

DISCUSSION

In this work, strong evidence of the presence of endopolyphosphatase activity in the yeast has been obtained for the first time. This activity is detected only in the strain CNX devoid of both exopolyphosphatases, Ppx1 and Ppn1. Detection of such activity in the strains possessing exopolyphosphatases is difficult because of the very high activity of these enzymes in spheroplast homogenates and cytosol preparations accompanied by P_i release from polyphosphates at the beginning of incubation [12]. The endopolyphosphatase activity is localized in the yeast cytosol. The data on the presence of endopolyphosphatase activity in nuclei, microsomes, and cell envelope of *Neurospora crassa* [1] need careful checking by the modern methods of isolation of subcellular fractions and the analysis of this activity. It is difficult to answer the question concerning the presence of this activity in vacuoles characterized by intensive polyphosphate metabolism because even in the strain CNX this compartment possesses an exopolyphosphatase not encoded by the *PPX1* and *PPN1* genes [16].

In the phase of active growth of *S. cerevisiae*, the synthesis and depolymerization of polyphosphates are known to occur in parallel [17]. The chain length does not increase in the first stage of polyphosphate accumulation under their "overcompensation" but increases later [18]. The analysis of works on polyphosphate "overcompensation" in the yeast [1, 14, 18] suggests that the accumulation of polyphosphates and the change in their degree of polymerization are separated in time and seem to be variously regulated. It is probable that endopolyphosphatase activity could take part both in polyphosphate depolymerization and in the reverse formation of longer chains from shorter ones. Purification of this enzyme and proper estimation of its role in the cell will be the subject of our future investigations.

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