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Partial Purification and Characterization of Nuclear Exopolyphosphatase from *Saccharomyces cerevisiae* Strain with Inactivated *PPX1* Gene Encoding a Major Yeast Exopolyphosphatase

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Abstract—Inactivation of *PPX1* encoding the major cytosolic exopolyphosphatase PPX1 in *Saccharomyces cerevisiae* did not alter exopolyphosphatase activity of the isolated nuclei compared with that in the parent strain. The nuclear exopolyphosphatase of the *S. cerevisiae* strain deficient in the *PPX1* gene was purified 10-fold. According to gel filtration on Superose 6, this enzyme has a molecular mass of ~200 kD, and it hydrolyzes polyphosphates with an average chain length of 15 and 208 phosphate residues to the same extent. Its activity is much lower with tripolyphosphate. In the presence of 2.5 mM Mg^{2+}, K_m values are 133 and 25 μM in the hydrolysis of polyphosphates with chain lengths of 15 and 208 phosphate residues, respectively. The enzyme activity is stimulated by 2.5 mM Mg^{2+} and 0.1 mM Co^{2+} 15- and 31-fold, respectively. RNA does not alter the nuclear exopolyphosphatase activity, while polylysine increases it 2-fold.

Key words: exopolyphosphatase, polyphosphate, nuclei, *PPX1*-deficient strain, *Saccharomyces cerevisiae*

According to current concepts, polyphosphates and enzymes of their metabolism are involved in the regulation of gene expression in microorganisms [1-3]. There is experimental evidence that the nuclei of eukaryotes possess polyphosphates [1-3], but their metabolism is poorly known. At present, exopolyphosphatase (polyphosphate-phosphohydrolase, EC 3.6.1.11) is the only enzyme of polyphosphate metabolism detected in the yeast nuclei [4, 5]. The presence of various forms of this enzyme differing in their location and properties is characteristic of yeasts. Only the gene *PPX1* encoding exopolyphosphatase is known in the yeast at present [6]. An investigation of exopolyphosphatases localized in various cell compartments in the *PPX1*-deficient mutant is of special interest, since subcellular fractions free of the enzyme PPX1 can be obtained. Under normal cultivation conditions, this enzyme comprises more than 60% of the total exopolyphosphatase activity of the yeast cell [7]. Inactivation of *PPX1* has no effect on the exopolyphosphatase activity of the isolated nuclei compared with the parent strain, i.e., the nuclear exopolyphosphatase is not encoded by *PPX1* [8].

Here we report on the partial purification of the nuclear exopolyphosphatase from a *PPX1*-deficient strain of *S. cerevisiae* and study its biochemical properties.

MATERIALS AND METHODS

Yeast strain and culture conditions. The yeast strain *S. cerevisiae* CRX with inactivated *PPX1* gene was grown in medium with 1% yeast extract, 2% peptone, and 2% glucose as described earlier [6].

Isolation of spheroplasts. The procedure for spheroplast isolation was devised earlier in our laboratory for *S. cerevisiae* VKM Y-1173 [9] and usefully employed in the present work with the CRX strain. To isolate spheroplasts, the yeast was collected at the end of exponential growth phase.

Preparation of nuclei. The pellet of spheroplasts obtained from 50 g of yeast cells was suspended at room temperature in 100 ml of 18% Ficoll, 0.5 mM $MgCl_2$,

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1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM Hepes-KOH, pH 6.45 (buffer A). The spheroplast homogenate was quickly transferred into a pre-cooled homogenizer with a Teflon pestle and lysed until clumped material was no longer visible (~12 strokes of the pestle). It was diluted with 100 ml of ice-cold 2.4 mM sorbitol in buffer A without Ficoll. Lysis of the spheroplasts was checked with a phase-contrast microscope. Unbroken cells and cell-wall debris were removed from the homogenate by low-speed centrifugation at 3000*g* for 10 min. The supernatant fraction was spun once more at the same speed. To yield a crude nuclear pellet, the supernatant was centrifuged at 15,000*g* for 25 min. The pellet was resuspended in 30 ml of a stabilizing medium containing 1 M sorbitol, 7% Ficoll, 10% glycerol, 20 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM PMSF, and 20 mM Hepes-KOH, pH 6.45 (buffer B) and centrifuged at 3000*g* for 5 min. The nuclei were sedimented at 15,000*g* for 20 min. Nuclear purity was checked with a fluorescence microscope with 5 μg/ml Hoechst 33258 (Sigma, USA), a specific dye for DNA, and with biochemical methods described in detail earlier [4, 5].

Preparation of nuclear extract. The nuclei obtained were subjected to osmotic lysis in buffer B containing 1% Triton X-100 instead of Ficoll and sorbitol (buffer C). Nuclear pellet was suspended in ~10 ml of ice-cold buffer C and incubated at 0°C for 10 min with constant stirring. The nuclear lysate was spun at 15,000*g* for 25 min. The pellet was discarded and the supernatant was used for ion-exchange chromatography and gel filtration.

Ion-exchange chromatography. Preparation of nuclear extract was applied onto a column (1.6 × 25 cm) with DEAE-Toyopearl 650 M (Toson, Japan) equilibrated with 20 mM Tris-HCl, pH 7.2, containing 10% glycerol and 4 mM $MgSO_4$ (buffer D). The column was washed with 100 ml of buffer D and then eluted with the same buffer containing increasing concentrations of KCl (0-0.4 M) at 30 ml/h. The eluent volume was 200 ml. Fractions with exopolyphosphatase activity were pooled and subjected to ultrafiltration with the use of an Amicon system (Amicon, USA; membrane PM 10) with three volumes of buffer D. Then the preparation (1-2 ml) was applied to a Mono-Q HR 5/5 column (Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl, pH 7.2 (buffer E), and chromatographed with the use of FPLC. The preparation was first washed with buffer E and then with 0.2 M KCl. Exopolyphosphatase was eluted with the same buffer containing increasing KCl concentrations (0.2-0.4 M) at 0.5 ml/min. Eluent volume was 15 ml. The protein peaks were pooled and analyzed for exopolyphosphatase activity.

Gel filtration. To determine the molecular mass of the nuclear exopolyphosphatase, both the nuclear extract and the preparation after DEAE-Toyopearl chromatography were used. Each preparation was applied onto a column (0.75 × 30 cm) with Superose 6 (Pharmacia) equilibrated with buffer E containing 100 mM KCl and chromatographed with FPLC. Exopolyphosphatase was eluted with the mentioned buffer at 0.5 ml/min. The fraction volume was 0.5 ml. The following marker proteins were used: ferritin, β-amylase, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase, and cytochrome *c* (Pharmacia, Sweden; Sigma, USA) with molecular masses of 440, 200, 150, 66, 29, and 12.4 kD, respectively.

All the other methods, including phosphohydrolase assays and SDS-PAGE, were described in our recent papers [9-11].

RESULTS AND DISCUSSION

Characterization of nuclear preparation. The purity and intactness of the nuclear fraction isolated from the *PPX1*-deficient strain of *S. cerevisiae* was rather satisfactory as determined by examination using the phase-contrast and fluorescence microscopes. The nuclear purity was also characterized biochemically by the absence of marker enzymes of other compartments: α-mannosidase, a marker of vacuoles, succinate dehydrogenase, a marker of mitochondria, and glucose-6-phosphate dehydrogenase, a marker of cytosol. The protein-to-DNA ratio of the purified nuclei was ~25, which is close to that obtained previously for *S. cerevisiae* [4].

Purification of nuclear exopolyphosphatase. The extract of nuclei resulting from their osmotic lysis and concurrent treatment with 1% Triton X-100 (~15 ml) was applied onto a column with DEAE-Toyopearl 650 M and chromatographed as indicated in "Materials and Methods". The results of standard chromatographic resolution of proteins are depicted in Fig. 1. About 70-90% of pyrophosphatase and 10-19% of exopolyphosphatase activities were not bound to the carrier. Further separation of the pyrophosphatase activity from exopolyphos-

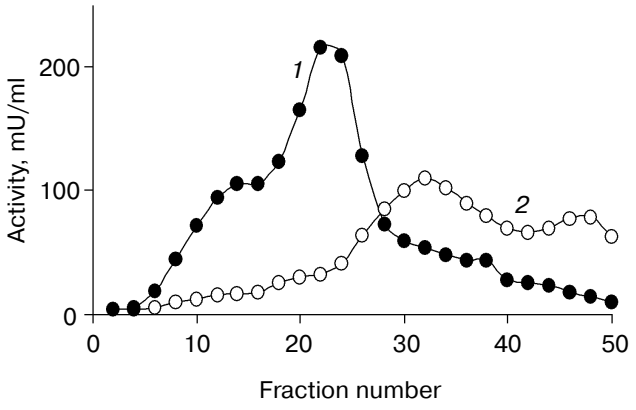

Fig. 1. Chromatography of nuclear extract on DEAE-Toyopearl 650 M: pyrophosphatase (*1*) and exopolyphosphatase (*2*) activities. Elution with a KCl gradient.

Table 1. Purification of nuclear exopolyphosphatase from *S. cerevisiae* deficient in the *PPX1* gene

Purification step	Protein, mg	Specific activity, mU/mg protein	Yield, %
Isolated nuclei	92	30	100
Nuclear extract	59	44	94
DEAE-Toyopearl	12	83	36
Mono-Q	1	300	11

Note: $PolyP_{208}$ (2 mM) was used as a substrate in assays of exopolyphosphatase activities.

Table 2. Effect of divalent metal cations on the activity of partially purified exopolyphosphatase of nuclei from *S. cerevisiae*

Cation concentration, mM	Activity, %	
	Mg^{2+}	Co^{2+}
0.002	n.d.	2400
0.1	170	3230
1.0	1515	2055
2.5	1370	n.d.

Note: Activity in the absence of divalent metals taken as 100% was 8 mU/mg protein. $PolyP_{208}$ was used as a substrate in assays of exopolyphosphatase activity; n.d., not determined.

Table 3. Effect of some reagents on the activity of partially purified exopolyphosphatase of nuclei from the yeast *S. cerevisiae*

Reagent	Activity, %
No addition	100
Heparin, 20 μg/ml	15
Sodium fluoride, 1 mM	100
RNA, 20 μg/ml	95
Polylysine	
20 μg/ml	115
100 μg/ml	190
NH_4^+ (100 mM)	135
K^+ (100 mM)	130

Note: Activity in the presence of 0.1 mM Co^{2+} (300 mU/mg protein) taken as 100%.

phatase activity was made with a KCl gradient. However, we failed to separate these two activities completely (Fig. 1).

Fractions with exopolyphosphatase activity (28-42) were pooled, desalted, and subjected to FPLC on Mono-Q. When chromatographed on Mono-Q, exopolyphosphatase activity was eluted in a wide range of KCl concentrations (0.25-0.5 M) (not illustrated). The pyrophosphatase activity was present in the same fractions, though to a lesser extent. The resulting partially purified preparation of exopolyphosphatase from the yeast nuclei had a specific activity of 0.3 U/mg protein and 11% yield relative to isolated nuclei (Table 1). The enzyme substantially lost its activity during purification steps (Table 1).

Molecular mass. A standard column for FPLC with Superose 6 was used to determine the molecular mass of the native enzyme. When using both the initial nuclear extract and the enzyme preparation partially purified on DEAE-Toyopearl 650 M, the same value of the molecular mass was obtained: ~200 kD. These data were inconsistent with the data that we had obtained previously using Sephacryl S-200: 57 kD [8]. There are two possible explanations for this discrepancy. First, underestimation of the molecular mass might be due to unspecific exopolyphosphatase interaction with Sephacryl S-200, which is responsible for underrating of the mass. Second, overestimation of the molecular mass of nuclear exopolyphosphatase might result from the formation of complexes of the native enzyme with some other proteins and/or polyphosphates due to Superose 6. This problem could be solved by the use of electrophoresis if we had a purified enzyme. However, according to the preliminary data, electrophoresis of the partially purified exopolyphosphatase of nuclei after Mono-Q in denaturing conditions revealed no less than 10 proteins, and hence the question about the molecular mass of the nuclear exopolyphosphatase remains unsolved.

Exopolyphosphatases isolated from different compartments of *S. cerevisiae* VKM Y-1173 substantially differ in their molecular masses: 40 kD for enzymes isolated from cell envelope, cytosol, and mitochondrial matrix, 245 kD from vacuoles, 120 and 76 kD from mitochondrial membranes [7]. The molecular masses of exopolyphosphatases from cell homogenate of *Endomyces magnusii* [12] and *Neurospora crassa* [13] were estimated as 48 and 50 kD, respectively. Two exopolyphosphatases of *Escherichia coli* were found to be dimers with molecular masses of 100 and 120 kD [14, 15]. By molecular mass (~200 kD), the nuclear exopolyphosphatase under study was close to the vacuolar enzyme (~245 kD) purified from the yeast *S. cerevisiae* VKM Y-1173 [16].

Effects of some metal ions. Exopolyphosphatase of the yeast nuclei exhibited low activity in the absence of divalent cations (Me^{2+}): 8 mU/mg protein. Addition of Me^{2+} to the enzyme preparation resulted in considerable stimulation of the activity that depended on the cation

and its concentration (Table 2). The best activator was Co^{2+}, so all studies were carried out in the presence of 0.1 mM Co^{2+}.

Similar dependence on Me^{2+} was observed for exopolyphosphatase activities of nuclear preparations from the yeast *S. cerevisiae* VKM Y-1173 [5] and CRX [8]. However, the degree of stimulation for the enzyme under study was 10-fold higher. This suggests a probably loss of divalent cations, presumably included in the protein structure, in the course of enzyme purification. By the effect of Mg^{2+} and Co^{2+}, the greatest similarity was found between the nuclear and vacuolar exopolyphosphatases of the same strain CRX (unpublished) and purified vacuolar exopolyphosphatase from the yeast *S. cerevisiae* VKM Y-1173 [16].

Monovalent metal cations stimulated the nuclear exopolyphosphatase by no more than 30-35% (Table 3).

Effect of some specific reagents. The partially purified exopolyphosphatase of the yeast nuclei was almost insensitive to 10 mM sodium azide, 1 mM ammonium molybdate, 1 mM sodium orthovanadate, antibodies against the purified cell-envelope exopolyphosphatase, and 1 mM sodium fluoride (Table 3). Heparin, an effective inhibitor of exopolyphosphatases from all yeast-cell compartments studied earlier, suppressed the partially purified exopolyphosphatase of nuclei by 85% (Table 3).

The study is focused on the effect of RNA and polylysine on exopolyphosphatase activity of the nuclei. RNA is known to be possibly bound to polyphosphates [1], which may reflect on the enzyme activity. However, RNA in the used concentration of 20 μg/ml did not significantly change the exopolyphosphatase activity of the partially purified nuclear enzyme (Table 3). There are some data on the stimulation of exopolyphosphatase activity by arginine [11, 17]. Polylysine in the concentration of 100 μg/ml increased the nuclear exopolyphosphatase activity by 90% (Table 3). The mechanism of enzyme stimulation by arginine and polylysine remains unknown. It is likely that this enzyme is bound to histones of the yeast nuclei.

Table 4. Influence of polyphosphate chain length on hydrolysis by partially purified exopolyphosphatase of nuclei

Substrate	Activity, mU/mg protein		
	2.5 mM Mg^{2+}	1 mM Co^{2+}	0.1 mM Co^{2+}
PP_i	40	0	0
$PolyP_3$	50	0	0
$PolyP_{15}$	100	210	230
$PolyP_{208}$	95	280	300

Note: PP_i and $polyP_3$ concentrations were 1.25 mM each; $polyP_{15}$ and $polyP_{208}$ concentrations were 130 and 10 μM, respectively.

Substrate specificity. The partially purified exopolyphosphatase of the yeast nuclei hydrolyzed polyphosphates with different chain lengths (Table 4). Exopolyphosphatase activities with $polyP_{15}$ and $polyP_{208}$ were nearly the same as in the initial nuclear preparation [8] and depended on the divalent cation and its concentration. In the presence of 2.5 mM Mg^{2+}, they were essentially similar for these two substrates. In the presence of 0.1 mM Co^{2+}, the exopolyphosphatase activity with $polyP_{208}$ increased 1.3-fold compared with that with $polyP_{15}$ (Table 4). Pyro- and tripolyphosphatase activities were absent in the preparation in the presence of 0.1 and 1.0 mM Co^{2+}, while with 2.5 mM Mg^{2+} these activities were twice lower than with $polyP_{15}$ and $polyP_{208}$ (Table 4). Thus, the conditions for manifestation of the maximal activity with pyrophosphate and tripolyphosphate differ significantly from those for the activity with long-chain polyphosphates.

In the initial nuclear preparation, the pyro- and tripolyphosphatase activities exceeded the exopolyphosphatase activity [8]. It should be mentioned that

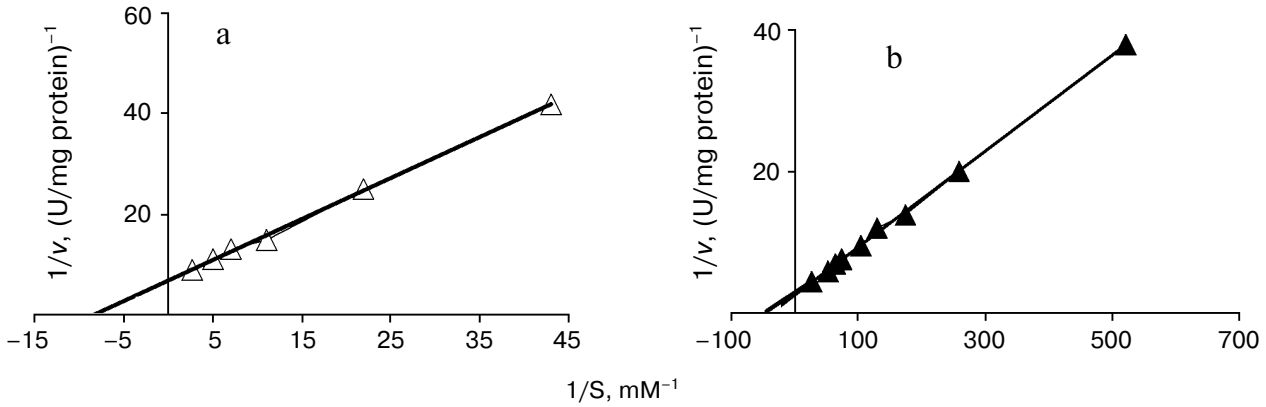

Fig. 2. Dependence of the exopolyphosphatase reaction rate on polyphosphates with an average chain length of 15 (a) and 208 (b) in Lineweaver–Burk coordinates.

pyrophosphatases exhibit tripolyphosphatase activity [18]. During purification, the preparation became free from pyrophosphatase (Fig. 1), and this resulted in a considerable decrease in tripolyphosphatase activity as well. Conceivably, the nuclear exopolyphosphatase might exhibit no pyrophosphatase activity, and tripolyphosphatase activity is as small as in the case with the vacuolar exopolyphosphatase of the yeast [16]. In the experiments where pyrophosphatase activity was 10-fold lower than exopolyphosphatase activity, tripolyphosphatase activity in the preparation of partially purified exopolyphosphatase of nuclei was not measured even with 2.5 mM Mg^{2+} (not illustrated). Therefore, tripolyphosphatase activity observed in the preparation of partially purified exopolyphosphatase of nuclei seems to be a result of the action of the two enzymes: pyrophosphatase and exopolyphosphatase.

The dependence of hydrolysis rates of polyphosphates on their concentrations in the presence of 2.5 mM Mg^{2+} was in agreement with Michaelis kinetics (Fig. 2). The K_m values were 133 and 25 μM for hydrolyzing polyphosphates with average chain length of 15 and 208 phosphate residues, respectively (Fig. 2). The K_m values significantly decreased during the enzyme purification and corresponded to minimal values found for the yeast exopolyphosphatases. They were nearest the K_m values obtained for the enzyme from vacuoles [16]. Previously we have reported that crude nuclear exopolyphosphatase had the following K_m values: 25 and 1.5 μM for polyphosphates with average chain length of 15 and 208, respectively [8]. A similar effect of a drop in affinity to polyphosphates in the course of purification was also detected for a soluble mitochondrial exopolyphosphatase [10]. This may be due to removal of some components required for display of exopolyphosphatase activity during purification.

Thus, the present work deals with the properties of the nuclear exopolyphosphatase of the yeast *S. cerevisiae* deficient in *PPX1* and is an extension of works published earlier [4, 5, 8]. The above mentioned mutant allowed us to obtain a preparation of the nuclear exopolyphosphatase free from PPX1. The nuclear exopolyphosphatase differs from those obtained from other compartments in its overall physicochemical properties. A certain similarity in these properties is observed with the exopolyphosphatase purified from yeast vacuoles [16].

During purification of the nuclear exopolyphosphatase, a change in the properties of this enzyme is observed. In particular, the activating effect of Mg^{2+} and Co^{2+} on the enzyme increased and the affinity to high-molecular-weight polyphosphates decreased during purification. Unfortunately, further purification of the nuclear exopolyphosphatase is a particular problem due to inactivation of the enzyme unless ways of stabilization of the enzyme are found. In this respect, activation of the nuclear enzyme by polylysine may be of interest.

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