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Purification and properties of exopolyphosphatase isolated from *Saccharomyces cerevisiae* vacuoles

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Abstract An exopolyphosphatase (polyPase) with a specific activity of 60 U/mg protein has been purified from the vacuolar sap of *Saccharomyces cerevisiae*. The molecular mass of the intact enzyme was found to be 245 kDa. It is highly specific towards high-molecular polyphosphates (polyP). The activity with polyP$_9$ is 24% of that with polyP$_{208}$. The apparent K_m for polyP$_{15}$ and polyP$_{208}$ hydrolysis is 93 and 2.4 μM, respectively. The enzyme is slightly active with polyP$_3$ and adenosine-5′-tetraphosphate, but does not hydrolyze pyrophosphate, ATP, GTP and *p*-nitrophenylphosphate. It is stimulated by divalent metal cations. Co^{2+}, the best activator, stimulates it 6-fold. Antibodies that inhibit the cell envelope and cytosol polyPases of *S. cerevisiae* have no effect on the vacuolar polyPase. The vacuolar polyPase differs from other yeast polyPases in molecular mass, substrate specificity and effects of activators.

Key words: Vacuole; Vacuolar sap; Exopolyphosphatase; Purification; (*Saccharomyces cerevisiae*)

1. Introduction

It has become evident that inorganic polyphosphates (polyP$_n$, where *n* is the average number of phosphate residues in the polymer chain) are not only a phosphate reserve but perform various functions in the cell [1–3]. One of the enzymes participating in polyP metabolism is exopolyphosphatase (polyPase) (EC 3.6.1.11). This enzyme releases orthophosphate (P$_i$) from the end of the polyP polymer chain. We have observed multiple polyPases in different yeast cell compartments [4].

The yeast vacuoles possess a polyP pool [5,6] which functions as a phosphate reserve and a chelator of different cations that accumulate in vacuoles [6,7]. The polyPase activity detected in these organelles differs in a number of properties from the analogous activities found in other cell compartments [4,8]. Endopolyphosphatase that releases intermediate-size polyP chains during polyP hydrolysis is suggested to be localized in vacuoles as well [9].

The aim of this work was to purify and characterize the vacuolar polyPase of *S. cerevisiae*.

2. Materials and methods

2.1. Strain and growth conditions

The strain *S. cerevisiae* VKM Y-1173 was cultivated, spheroplasts and vacuoles were obtained by the methods described elsewhere [8].

2.2. Enzyme purification

The vacuolar fraction free of other cell organelles [8] was frozen at −20°C in 10 mM Tris-HCl, pH 7.2, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride and after thawing treated with ultrasound (MSE, USA) for 10 s. Vacuolar membranes were sedimented at 15 000×*g* for 90 min. The supernatant was applied to a Q-Sepharose column (1.6×5 cm) equilibrated with 25 mM Tris-acetate, pH 7.2, containing 0.1% Triton X-100 as enzyme stabilizer. After washing with the same buffer, extraneous proteins were eluted with the same buffer, containing 0.3 M KCl. PolyPase was eluted with 0.7 M KCl in the same buffer. Removal of KCl and substitution of the buffer with 25 mM Tris-acetate, pH 6.0, was performed in an Amicon system (PM-10 membrane). Thereafter, the preparation was applied to an S-Sepharose column (1.6×5 cm) equilibrated with 25 mM Tris-acetate, pH 6.0, containing 0.1% Triton X-100. Extraneous proteins were eluted with the same buffer, containing 0.2 M KCl. PolyPase was eluted with increasing concentrations of KCl (0.2–1.2 M) in the same buffer. The enzyme preparation was stored at −20°C.

2.3. Enzyme and protein assay

Non-specific phosphatase activity was assayed with *p*-nitrophenylphosphate as the substrate [10].

Other phosphohydrolase activities were estimated from the rates of P$_i$ formation during a 10–40-min incubation in 1 ml of reaction mixture at 30°C, as described previously [11]. For polyPase assay, the reaction mixture contained 0.167 mM polyP$_{15}$ (the concentration cited refers to polymer), 0.05 mM Co^{2+} and 100 mM ammonium acetate in 50 mM Tris-HCl, pH 7.2. Preliminary experiments showed that Co^{2+} and ammonium acetate activate the vacuolar polyPase. All modifications in the reaction media are given in the tables and legends to figures.

The enzyme amount liberating 1 μmol of P$_i$ per 1 min (for polyPase) or hydrolyzing 1 μmol of substrate per 1 min (for other enzymes) was taken as a unit of enzyme activity (U).

The dependence of polyPase activity on pH was measured with 100 mM Tris-acetate as buffer.

The effects of different reagents were estimated by preincubating polyPase with them for 5 min at 20°C in the buffer used for the determination of the enzyme activity. The reaction was started with substrate.

Protein concentration was measured by a modified method of Lowry et al. [12], using bovine serum albumin as a standard.

2.4. PolyP purification

PolyP with an average chain length of 9, 15, 208 (Monsanto, USA) and 45 (Sigma, USA) orthophosphate residues was freed of P$_i$ and pyrophosphate by gel filtration on a Sephadex G-10 column as described previously [10].

2.5. Immunoserum

Polyclonal antiserum against the purified cell envelope polyPase of *S. cerevisiae* was raised in rabbit as described earlier [13].

2.6. Determination of the molecular mass

The molecular mass of the polyPase was estimated by gel filtration on a Sephacryl S-300 column (1.6×80 cm) equilibrated with 25 mM Tris-HCl, pH 7.2, containing 0.1% Triton X-100 and 0.1 M NaCl. The following marker proteins were used: ferritin (440 kDa), catalase (232 kDa), β-amylase (200 kDa), aldolase (158 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) (Pharmacia, Sweden and Serva,

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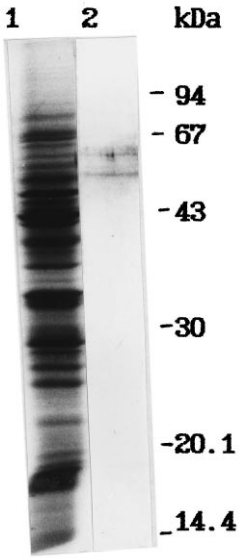

Fig. 1. SDS-PAGE of vacuolar polyPase preparations at different purification steps: 1, the vacuolar sap; 2, the S-Sepharose-purified enzyme.

Germany). The column void volume was determined with dextran blue (2000 kDa).

2.7. Electrophoresis

Electrophoresis in 12.5% SDS-PAGE was performed according to Laemmli [14]. Proteins were detected by silver staining [15].

3. Results and discussion

A preparation of polyPase with specific activity of 60 U/mg, 13.3-fold purification (relative to vacuolar sap) and 4.1% yield was obtained (Table 1). We could not evaluate the purification degree in relation to cell homogenate because the latter contains large amounts of other polyPases [4].

Gel filtration on Sephacryl S-300 revealed a molecular mass of 245 ± 30 kDa for the vacuolar polyPase. On SDS-PAGE, one major polypeptide band of 55 kDa was detected (Fig. 1). The minor bands (60 and 62 kDa) might be artifacts as observed earlier [16]. It is suggested that this enzyme is a tetramer of 55-kDa subunits. Previously isolated yeast polyPases with molecular masses of 40 kDa [10,17,18] and 28 kDa [19] are monomeric, whereas polyPase from *E. coli* is a dimer of 58 kDa [20].

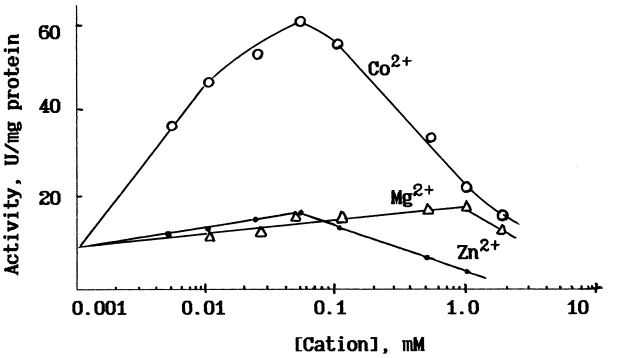

Fig. 2. The effect of Co^{2+}, Mg^{2+} and Zn^{2+} on the vacuolar polyPase activity.

As for other yeast polyPases, the pH optimum of the vacuolar polyPase activity is about 7.

The isolated polyPase was stimulated by divalent metal cations (Fig. 2). Co^{2+} proved to be the best stimulator. At their optimal concentration (0.05 mM) in the reaction medium, the activity increased 6-fold. Mg^{2+} and Zn^{2+} stimulated the activity much less (2-fold). Unlike the vacuolar polyPase, the purified cell envelope and cytosol polyPases of *S. cerevisiae* were much more activated by Mg^{2+} and Co^{2+} (14-fold) [10,17].

The polyPase isolated did not hydrolyze ATP, GTP, pyrophosphate, and *p*-nitrophenylphosphate in the presence of 0.05 mM Co^{2+} or 2.5 mM Mg^{2+}. However, it hydrolyzed $polyP_3$ and adenosine-5′-tetraphosphate at a rate up to 7% of that with $polyP_{208}$ (Table 2). The rate of polyP hydrolysis dropped to 24% with decreasing chain length from $polyP_{208}$ to $polyP_9$ (Table 2). Unlike the polyPases of the cell envelope and cytosol [10,17] (which should be more reasonably named tripolyPases, according to their substrate specificity), the purified vacuolar polyPase was much more active with high-molecular polyP. In this respect, it shows similarity with polyPases of *E. coli* [20] and *Acinetobacter johnsonii* [21].

Fig. 3 demonstrates the dependence of enzyme activity on the concentration of $polyP_{15}$ and $polyP_{208}$. The apparent K_m is 93 and 2.4 μM for $polyP_{15}$ and $polyP_{208}$, respectively. Thus, the catalytic efficiency of polyPase, as characterized by the catalytic constant and the Michaelis constant, increases with

Table 1
PolyPase purification from the vacuolar sap of *S. cerevisiae*

Purification step	Protein (mg)	Specific activity (U/mg)	Purification degree	Yield (%)
Vacuolar sap	2.28	4.5	1	100
Q-Sepharose	0.104	28	6.1	28
S-Sepharose	0.007	60	13.3	4.1

Table 2
Substrate specificity of the purified vacuolar polyPase of *S. cerevisiae*

Substrate	Concentration (mM)	Specific activity (%)
$PolyP_{208}$	0.012	100
$PolyP_{45}$	0.056	100
$PolyP_{15}$	0.167	85
$PolyP_9$	0.278	24
$PolyP_3$	2.5	7.6
Adenosine-5′-tetraphosphate	1.0	7.3

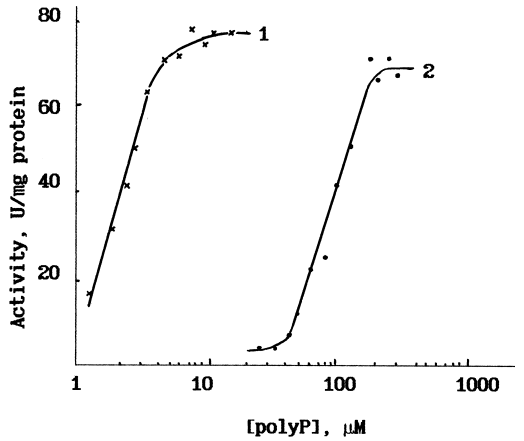

Fig. 3. The dependence of the vacuolar polyPase activity on the concentration of polyP with an average chain length of 208 (1) and 15 (2) phosphate residues.

Table 3
Effect of different reagents on the purified vacuolar polyPase of *S. cerevisiae*

Reagent	Concentration (mM)	Activity (%)
None	–	100
Ammonium acetate	100	95
Arginine	100	200
Iodoacetamide	10	78
Sodium fluoride	10	80
Pyrophosphate	1	52
Tripolyphosphate	1	38
Zn^{2+}	0.05	29
Mg^{2+}	0.05	70
EDTA	0.025	85
	0.05	16
Heparin	1 μg/ml	10

increasing polyP chain length. By its substrate specificity, the vacuolar polyPase differs from any of the described yeast polyPases [10,17–19].

Table 3 shows the action of some effectors on the activity of the vacuolar polyPase. The polyPase activity is inhibited by heparin. Addition of other divalent metal ions to the reaction mixture containing Co^{2+} at its optimal concentration appreciably decreases the enzyme activity. A similar effect of divalent metal cations and heparin is also characteristic of *S. cerevisiae* cell envelope and cytosol polyPases [10,17].

In contrast to the polyPases of the cell envelope and cytosol [10,17], the vacuolar polyPase is inhibited by EDTA. It is not inhibited with polyclonal antibodies against the cell envelope polyPase, which are efficient inhibitors of the cell envelope and cytosol polyPases [4]. $PolyP_3$ and pyrophosphate inhibit the activity of polyPase. Arginine (100 mM) activated the purified vacuolar polyPase 2-fold, in contrast to the cytosol polyPase [11]. Stimulation of the vacuolar polyPase activity by arginine was also observed in *Neurospora crassa* [22]. Since vacuoles are considered to be the major yeast cell compartment for arginine [7], this fact may be of physiological significance. The NH_4^+ ions have no effect on the purified enzyme (Table 3), yet increase the polyPase activity of the vacuolar sap 1.5-fold, possibly due to the presence of other polyP-hydrolyzing enzymes, stimulated with NH_4^+, in the vacuolar sap.

To summarize, the vacuolar polyPase differs from the cell envelope and cytosol polyPases of *S. cerevisiae* in molecular mass, substrate specificity, stimulation by divalent metal cations, and sensitivity to EDTA, arginine and antibodies against cel envelope polyPase [4,10,17]. It also differs from other yeast polyPases [18,19]. We conclude that the vacuolar polyPase is a novel enzyme participating in polyP metabolism of the yeast cell.

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