CHARACTERIZATION OF A PHOSPHATASE SPECIFIC FOR 2-DEOXYGLUCOSE-6-PHOSPHATE IN A YEAST MUTANT+

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1. Introduction

Yeast growth and fermentation are strongly inhibited by 2-deoxyglucose, an unnatural glucose analogue [1,2]. Previous work in this laboratory showed that the inhibition is a direct consequence of the intracellular accumulation of 2-deoxyglucose-6-phosphate [3]. A yeast mutant was isolated which is resistant to the inhibitory effect of 2-deoxyglucose [4]. The resistance is due to the presence of a phosphatase which prevents the intracellular accumulation of 2-deoxyglucose-6-phosphate [4]. We now report on the partial purification and characterization of this phosphatase. The results show that the enzyme has a narrow substrate specificity. Of a series of sugar phosphates tested as presumptive substrates, only 2-deoxyglucose-6-phosphate and to a less extent fructose-1-phosphate are hydrolyzed by the enzyme. Other characteristics of the enzyme are also reported.

2. Materials and methods

2.1. *Reagents*

All the sugar phosphates, α and β-glycerophosphate and Alumina Cγ were purchased from Sigma Chemical Co. The glucose oxidase reagent was from Worthington.

2.2. *Growth of the yeast cells*

The yeast used throughout this work was *Saccharomyces cerevisiae* strain PM-II [4]. The cells were grown in a culture medium containing per liter: 20 g glucose, 3 g yeast extract (Difco) and 20 mM potassium phosphate, added for repressing the synthesis of the external acid phosphatase [5].

The cultures were incubated with shaking at 30°C in 2 liter erlenmeyer flasks containing 1.5 liter culture medium. Growth was followed turbidimetrically in a Klett–Summerson photocolorimeter at 420 nm. When the cultures reached about 300 Klett units, they were cooled by addition of ice and the cells were collected by centrifugation. After washing the cells three times with cold distilled water, the cake was put on a porous plate to remove the water and stored frozen at −10°C until use.

2.3. *Preparation of yeast extracts*

One part of yeast cells was ground in a cold mortar with three parts of Alumina (Alcoa 305) and extracted with two volumes of 5 mM EDTA. The suspension was centrifuged in the cold at 10 000 × *g* for 10 min. The precipitate was discarded and the supernatant was again centrifuged as described above. The supernatant after this centrifugation is referred to as crude extract.

2.4. *Enzymatic assays*

The 2-deoxyglucose-6-phosphatase activity was measured in reaction mixtures (0.1 ml) containing: 50 mM imidazol pH 6, 10 mM $MgCl_2$, 50 mM 2-deoxyglucose-6-phosphate adjusted to pH 6 and appropriate amounts of enzyme. The reaction mixtures were

+ Dedicated to Professor A. Sols on the occasion of his 60th birthday. This paper completes a series on metabolic studies with 2-deoxyglucose [3,4]

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incubated at 30°C and the 2-deoxyglucose liberated was measured using the glucose oxidase reagent [6]. In other cases the reaction was followed by measuring the liberation of inorganic phosphate. When this method was used, the reaction was stopped by addition of 0.9 ml 5% trichloroacetic acid, and after centrifugation at 5000 × *g* for 10 min the inorganic phosphate was determined by the method of Fiske and Subbarow [7] in appropriate aliquots of the supernatant.

The α-glycerophosphatase activity was measured in reaction mixtures containing the components described above except that the 2-deoxyglucose-6-phosphate was replaced by 50 mM α-glycerophosphate. The reaction was followed by determining the inorganic phosphate liberated as described above. For the assay of the acid phosphatase essentially the same procedure was followed except that 50 mM glucose-6-phosphate was used as substrate and the pH of the reaction mixture was maintained at 4 with 50 mM sodium acetate. The glucose released was determined using the glucose oxidase reagent [6].

One unit of activity is defined as the amount of enzyme which hydrolyzes 1 μmol substrate/min under our assay conditions. Proteins were determined by the method of Lowry et al. [8].

3. Results and discussion

3.1. *Purification of the enzyme*

In a typical run 25 ml of crude yeast extract (16 mg protein/ml) were mixed with Alumina Cγ (0.5 mg of Alumina Cγ gel per mg of protein). After stirring at 4°C for 10 min, the mixture was centrifuged in the cold at 10 000 × *g* for 5 min. After this treatment, a substantial amount (80%) of the α-glycerophosphatase present in the crude extracts is retained in the gel while practically all the 2-deoxyglucose-6-phosphatase activity remains in the supernatant. The supernatant after this centrifugation (25 ml) was concentrated by mixing with dry gel of Sephadex G-25 followed by centrifugation at 10 000 × *g* for 5 min. The resultant supernatant (5 ml) was filtered through a Sephadex G-200 column (42 × 2 cm) equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. Fractions of 2 ml were collected at a flow rate of 0.2 ml/min. The fractions (25 to 35) containing the enzymatic activity were pooled and the pool was concentrated by treatment with dry gel of Sephadex G-25 as described above. The concentrated pool (3 ml) was chromatographed in a DEAE–Sephadex column (30 × 2 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. After washing the column with 20 ml of the equilibration buffer, a linear gradient (0–1 M) of KCl in the above mentioned buffer (total volume of 200 ml) was applied to the column and fractions of 2 ml were collected at a flow rate of 0.2 ml/min. As shown in fig.1, a single peak of activity emerges at about 0.7 M KCl. Table 1 summarizes the results of the purification. The most purified fractions have no detectable levels of α-glycerophosphatase and acid phosphatase and can be stored frozen at −10°C for several weeks without appreciable loss of enzymatic activity.

3.2. *General characteristics of the enzyme*

A divalent cation is needed for enzymatic activity. Maximal activity is reached with 10 mM $MgCl_2$. Mg^{2+} can be replaced by other cations to different extent. At 10 mM concentrations, the relative activities with several cations are as follows (in per cent of that obtained with Mg^{2+}): Co^{2+} (65), Ni^{2+} (35), Mn^{2+}, Zn^{2+} and Cd^{2+} less than 15%. The enzyme shows maximal activity at pH values between 6 and 7 with a 50% loss at pH values 5 and 8. 20% of the activity is lost after incubation of the enzyme at 35°C for 5 min at pH 7,

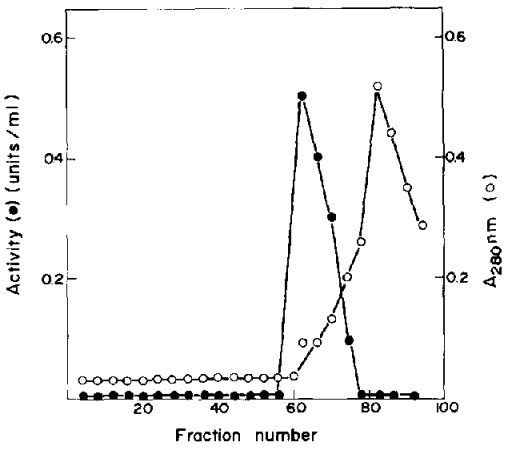

Fig.1. Elution profile of the enzyme after chromatography in DEAE–Sephadex. The experimental conditions are described in the text.

Table 1
Purification of the enzyme[a]

Step	Volume (ml)	Protein (mg)	Activity (total units)	Specific activity	Yield
1. Crude extract	25	325	56	0.17	100
2. Supernatant of Alumina C_γ	25	150	50	0.33	90
3. Concentration with Sephadex G-25	5	100	37	0.37	66
4. Filtration on Sephadex G-200	20	16	32	2	57
5. Concentration with Sephadex G-25	3	7	12	1.7	21
6. DEAE–Sephadex chromatography	30	2	9.2	4.6	16

[a] The experimental procedure is described in the text

and practically the whole activity is lost after 5 min incubation at 40°C and pH 7.

3.3. *Substrate specificity*

A series of related sugar phosphates were tested as presumptive substrates or inhibitors of the enzyme. The results in table 2 show that 2-deoxyglucose-6-phosphate is hydrolyzed with the highest efficiency. Besides it, fructose-1-phosphate is the only compound so far found to be hydrolyzed by the enzyme. From the data in figs 2 and 3 a K_m value for 2-deoxyglucose

Table 2
Substrate specificity[a]

Compound	Relative rates
2-Deoxyglucose-6-phosphate	100
Fructose-1-phosphate	80
Fructose-6-phosphate Fructose-1,6-diphosphate Glucose-1-phosphate Glucose-6-phosphate Mannose-1-phosphate Mannose-6-phosphate Galactose-1-phosphate α-Glycerophosphate β-Glycerophosphate	< 2

[a] All the compounds were tested at a concentration of 50 mM

Activity on 2-deoxyglucose-6-phosphate, glucose-6-phosphate and glucose-1-phosphate was determined with the glucose oxidase assay method. The activity on all the other compounds was measured by determining the inorganic phosphate liberated. The enzyme preparation used was from step 6 (table 1)

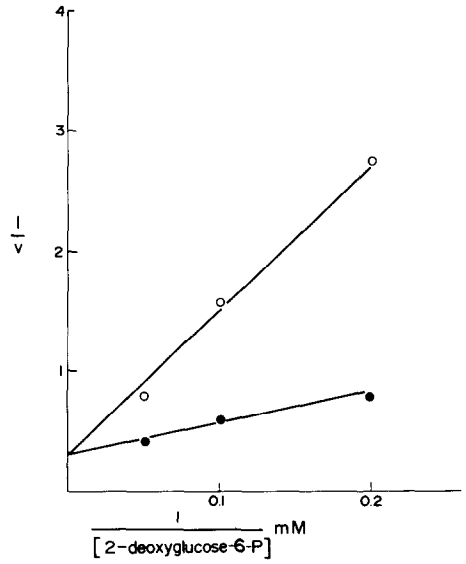

Fig.2. Inhibition of the hydrolysis of 2-deoxyglucose-6-phosphate by fructose-1-phosphate. Assay conditions as described in Methods at the substrate concentrations indicated in the figure, with (○) and without (●) 30 mM fructose-1-phosphate. The reaction was followed by the glucose oxidase method. The enzyme preparation used was from step 6 (table 1).

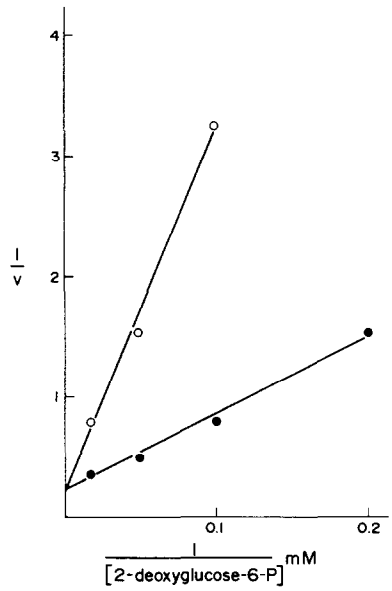

Fig.3. Inhibition of the hydrolysis of 2-deoxyglucose-6-phosphate by inorganic phosphate. Assay conditions as described in Methods, using the glucose oxidase procedure, with (○) and without (●) 10 mM inorganic phosphate. The enzyme preparation used was from step 6 (table 1).

6-phosphate of around 15 mM can be calculated. At saturating concentrations, the activity of the purified enzyme preparations on mixtures of both substrates is not additive, indicating that the hydrolysis of these two compounds is due to the same enzymatic activity. The results in fig.2 show that fructose-1-phosphate acts as competitive inhibitor of the hydrolysis of 2-deoxyglucose-6-phosphate; from these results a K_i value around 8 mM for fructose-1-phosphate can be calculated. None of the compounds listed in table 2 that were not substrates of the enzyme, were inhibitors of the hydrolysis of 2-deoxyglucose-6-phosphate when tested at concentrations (50 mM) 2.5-times higher than that of 2-deoxyglucose-6-phosphate (10 mM). Of the two products of the reaction, inorganic phosphate acts as a competitive inhibitor with a K_i value of 5 mM (fig.3). Neither 2-deoxyglucose or fructose inhibited the enzyme at 100 mM concentrations with 10 mM substrate.

We have found that the phosphatase described here is also present in wild type yeast, although its levels are ten to twenty times lower than those found in the yeast mutant. It seems then reasonable to assume that the enzyme has a general physiological significance, presumably by acting on a substrate(s) as yet not identified. To our knowledge, no enzyme has been described so far having the substrate specificity found for the phosphatase here reported. From the kinetic data and the apparent role played by this enzyme in the yeast mutant [4] we propose to name this enzyme tentatively as 2-deoxyglucose-6-phosphatase or 2-deoxyglucose-6-phosphate phosphohydrolase.

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