Biochimica et Biophysica Acta, 522 (1978) 113—121

BBA 68325

PROPERTIES OF ALKALINE PHOSPHATASE OF THE HALOTOLERANT YEAST *DEBARYOMYCES HANSENII*

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(Received June 13th, 1977)

Summary

The molecular weight of a partially purified alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) from the halotolerant yeast *Debaryomyces hansenii* was estimated to 110 000 by gel filtration. The isoelectric point determined by electrofocusing was at approximately pH 4.4. The enzyme had a broad specificity against phosphomonoesters and also attacked some acid anhydrides. Arsenate, molybdate, and orthophosphate acted as competitive inhibitors. Various metal-binding agents inhibited enzyme activity. A zinc addition almost completely reversed the EDTA inhibition.

Magnesium stimulated enzyme activity and was required for maintenance of activity at high concentrations of Na^+. Increasing glycerol concentration increased the value of the Michaelis constant (K_m) and decreased the maximum velocity (V). Solutions equimolar in KCl and NaCl stimulated enzyme activity by increasing V, whereas the K_m was almost unaffected by salt concentration. Enzyme extracted from cells cultured at low salinity was indistinguishable from that of cells grown in the presence of 2.7 M NaCl with respect to several criteria.

Introduction

Enzymes from extremely halophilic bacteria require high concentrations of K^+ or Na^+ for full enzymatic activity and are structurally stabilized by the high concentration of KCl which normally occur in their cells [1,2].

The basis for tolerance of low water activity (a_w) of enzymes from halophilic algae and the so-called osmophilic yeasts appears to be different [2]. Accumulation of intracellular polyhydric alcohols to high concentrations have been reported for these yeasts [3,4] and algae of the genus *Dunaliella* [5,6], growing in media with low a_w. According to Brown [2] an important physiological role of the polyols is that of a compatible solute, i.e. a solute which,

even at high concentrations does not markedly inhibit enzymes. The compatible role for glycerol in the osmophilic yeast *Saccharomyces rouxii* has been demonstrated for an NADP-specific isocitrate dehydrogenase [2,7].

Marine occurring yeasts differing in halotolerance have been isolated by Norkrans [8]. One of these, *Debaryomyces hansenii*, was strongly halotolerant and capable of growing in the presence of 4 M NaCl. Its intracellular salt content is regulated by extrusion of Na^+ and uptake of K^+. Under strongly saline conditions the internal salt concentration is kept below that of the environment [9], whereas glycerol accumulates intracellularly in amounts that increase with the salt concentration of the growth medium [10].

The purification of alkaline phosphatase (orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) from *D. hansenii*, was previously described [11]. The present study deals with some general properties of the enzyme and compares phosphatase prepared from cells cultured at two different salinities. Salt and glycerol effects on the enzyme are also reported.

Materials and Methods

Growth conditions and preparation of alkaline phosphatase. *D. hansenii* (Zopf) van Rij strain 26, was grown in the following media (quantities are specified per l of distilled water): Medium BC: 5.0 g Difco yeast extract, 1.0 g urea, 10 g glucose (or sucrose). The medium contained 6 mM NaCl as determined by atomic absorption spectrophotometry. Medium BCS: same composition as medium BC, but supplemented with 157 g of NaCl (2.7 M).

Cells grown in media BC and BCS were harvested at a cell density of 7–9 and 3–5 mg dry weight cells/ml, respectively. Growth conditions and the preparation of enzyme were as already described [11]. Experiments were run with partially (500–900-fold) purified enzyme. Dilute enzyme solutions were concentrated by ultrafiltration or in dialysis tubing placed in solid polyethyleneglycol 20 000. *Saccharomyces cerevisiae*, strain 12 [8] was grown at 25°C on a rotatory shaker in 2.5-l Fernbach flasks, each containing 500 ml medium BC. The crude extract supernatant, prepared as described for *D. hansenii* [11], was used for enzyme activity studies. *Halobacterium salinarium*, strain 1 [12] was cultivated in Fernbach flasks containing 500 ml medium [13] at 35°C on a rotatory shaker. Cell extract was prepared by grinding a pellet of whole cells in a chilled mortar with a glass tube filled with ice. The highly viscous cell paste was suspended in five volumes of 0.02 M Tris · HCl, pH 7.2, containing 20 mM $MgCl_2$ and 4.3 M NaCl and was dialysed against the same buffer for several days at 4°C. Centrifuged dialysate (15 000 × *g* for 10 min) was used for activity studies.

Enzyme assay. Enzyme activity measurements and the definition of an enzyme unit were as described elsewhere [11]. With substrates other than *p*-nitrophenyl phosphate the amount of released orthophosphate was followed in 0.1 M Tris · HCl, pH 8.5 (assay buffer) containing 10 mM $MgCl_2$ at 25°C. To avoid acid hydrolysis of labile substrates a modification [14] of the method of Peel and Loughman [15] was used. All substrates were obtained from Sigma Chem. Co. (U.S.A.). Unless otherwise stated all activity measurements were run in duplicate.

Gel filtration. Estimation of molecular weight with Sephadex G-200 (Pharmacia Fine Chemicals, Sweden) was performed on a column 1.5 × 90 cm equilibrated with 0.05 M Tris · HCl, pH 7.5, containing 0.1 M KCl. The column, operated at a flow rate of 7 ml/h by means of a peristaltic pump (Vario Perpex 2000, LKB, Sweden), was calibrated by use of: ovalbumin (Sigma), M_r 45 000; bovine serum albumin (Sigma), M_r 67 000 (monomer) and M_r 134 000 (dimer); *Escherichia coli* alkaline phosphatase (kindly supplied by Dr. H. Csopak), M_r 80 000; calf intestinal alkaline phosphatase (Sigma), M_r 100 000. Fractions of 2.5 ml were collected.

Isoelectric focusing. Electrofocusing in a 110 ml column (LKB) was run for 34 h at 9°C followed by 5 h at 23°C with 1% ampholine of pH range 3—10 as carrier ampholytes. The procedure used was as recommended in the LKB manual. Ten units of enzyme dialysed against 0.05 M potassium phosphate, pH 7.1, were applied to the column. Prior to determination of enzyme activity, the individual fractions (3 ml) were dialysed at 4°C for 3 days against 20 mM Tris · HCl, pH 7.2, containing 10 mM $MgCl_2$, 75 mM NaCl, and 0.01 mM $ZnCl_2$ [16].

Incubation with metal-binding agents. The enzyme (0.06 units/ml) was dialysed for 48 h against several changes of 0.1 M Tris · HCl, pH 8.0. Samples were preincubated with the appropriate concentration of inhibitors (EDTA, L-cysteine, Na_2S, or NaCN) for 1 h at 25°C before the reaction was started by the addition of *p*-nitrophenyl phosphate. All glassware used was kept overnight in 1 M HCl and rinsed thoroughly in double-distilled water to avoid metal contamination.

Reactivation of EDTA-treated alkaline phosphatase. The enzyme was extensively dialysed against 0.01 M Tris ·HCl, pH 7.2, containing 0.1 M KCl and incubated (diluted 300 times) in assay buffer with 10^{-5} M EDTA at 25°C. After incubation samples containing 0.07 unit of enzyme/ml were assayed by adding *p*-nitrophenyl phosphate and then $ZnCl_2$, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $NiCl_2$, or $CoCl_2$ immediately afterwards to give a final metal concentration of $2 \cdot 10^{-5}$ M. Reactivation values are given as percent of the activity of the dialysed enzyme assayed under standard conditions with Mg^{2+} omitted.

Heat stability. The enzyme was incubated (0.014 units/ml) at 60°C in assay buffer containing 10 mM $MgCl_2$ and 6 mg/ml of bovine serum albumin. At intervals samples were removed, placed on ice, and assayed under standard conditions.

Gel electrophoresis. Vertical polyacrylamide gradient gel electrophoresis was performed in a Gradipore 3 cell apparatus (Universal Scientific Ltd., England) using preformed gel slabs (Gradipore gels) with a concave gradient of acrylamide ranging from 4 to 26%. The electrophoresis was run at 4°C for 48 h at 150 V in 0.05 M Tris/acetic acid, pH 8.0. All gels were prerun for 2 h before application of 10 µl of enzyme solutions. Zones of enzyme activity were located as described by Hubby and Lewontin [17].

Results

Properties of alkaline phosphatase from D. hansenii grown in low NaCl medium

The pH optimum of alkaline phosphatase acting on *p*-nitrophenyl phosphate was dependent on substrate concentration (cf. ref. 18), but not affected by the

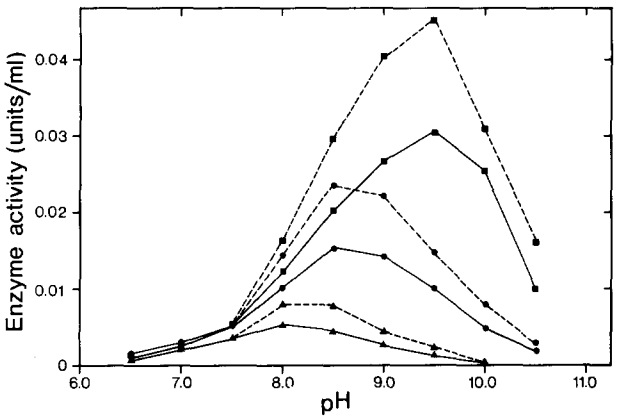

Fig. 1. Effect of pH on the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase. Incubations were carried out in 0.05 M maleate/Tris/2-amino-2-methyl-1-propanol buffer containing 10 mM $MgCl_2$ at three different concentrations of substrate: 0.1 mM (▲); 1 mM (●) and 6 mM (■). At pH below 8.0, absorbance readings were taken after addition of an equal amount of 0.5 M NaOH to the assay mixture. Dashed lines indicate addition of NaCl to a final concentration of 3 M.

addition of 3 M NaCl (Fig. 1), indicating that ionic strength does not much affect the p*K* values of the ionizable groups that are important for activity. The stimulation of enzyme activity by NaCl decreased at lower pH and was lost below pH 7.5. This is consistent with results reported for the *E. coli* phosphatase [19].

Electrofocusing in the pH range 3—10 revealed one peak of enzyme activity between pH 4.2 and pH 5.0 with pI at approx. 4.4. The recovered activity was low (approx. 10%), probably due to enzyme instability at pH below neutrality, and the chelating properties of the carrier ampholytes [16].

Table I lists the relative rates of hydrolysis of 20 different substrates by the enzyme. All phosphomonoesters tested were hydrolyzed. Inorganic phoshate was also formed from the acid anhydrides. The diesters served as poor substrates.

Divalent cations are essential for alkaline phosphatase from various sources [18,20—24]. The enzyme of *D. hansenii* was treated with four metal binding agents by the method used by Plocke et al. [25]. The concentration required to produce 50% inhibition of the enzyme activity was $1 \cdot 10^{-6}$ M with EDTA, $5 \cdot 10^{-5}$ M with L-cysteine, $1 \cdot 10^{-4}$ M with Na_2S, and $3 \cdot 10^{-4}$ M with NaCN. The values are very similar to those given for the inhibition of the *E. coli* enzyme [25]. The *D. hansenii* enzyme was completely inactivated within 3 min after the addition of 10^{-5} M EDTA. This inhibition was reversed by the addition of various divalent metal chlorides. After incubation with EDTA for 1 h, only Zn^{2+} gave an almost complete reversal of the inhibition (89%). A small reactivation (19%) was achieved by Mg^{2+} whereas Ca^{2+}, Mn^{2+}, Fe^{2+}, Ni^{2+}, and Co^{2+} were ineffective.

The effect of NaCl on the activity of alkaline phosphatase from *S. cerevisiae*, the halotolerant *D. hansenii* and the extreme halophile *H. salinarium* is shown in Fig. 2. The inhibitory effect of salt on the *S. cerevisiae* phosphatase contrasted markedly with the stimulation exhibited by salt on the other preparations.

D. hansenii [10], like some other microorganims thriving in concentrated

TABLE I

RELATIVE ACTIVITY OF ALKALINE PHOSPHATASE ACTING ON VARIOUS SUBSTRATES

Assays were performed using 0.035 unit of enzyme/ml. Substrate concentrations were 10 mM except for RNA where 7 mg/ml was used. Samples of 0.5 ml were withdrawn for phosphate determinations at intervals.

Substrate	Relative reaction velocity
Monoesters	
5′-AMP	1.0
5′-GMP	1.1
5′-CMP	1.0
5′-UMP	1.0
p-Nitrophenyl phosphate	1.0
α-Glycerophosphate	0.6
α-Naphthyl phosphate	0.6
Ribose 5-phosphate	0.6
Fructose 1,6-diphosphate	0.6
Glucose 1-phosphate	0.6
Phospho-DL-serine	0.6
Phospho*enol*pyruvate	0.8
Ethanolamine phosphate	0.8
Phosphocholine	0.7
Pyridoxal 5′-phosphate	0.3
Diesters	
RNA	No hydrolysis
Diphenyl phosphate	Trace
Acid anhydrides	
ADP	0.3
ATP	0.1
Pyrophosphate	0.1

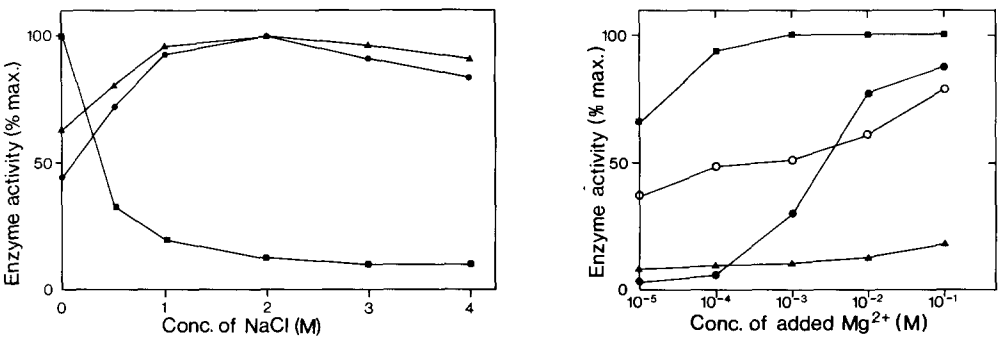

Fig. 2. Effect of NaCl on alkaline phosphatase extracted from *Halobacterium salinarium* (●); *Debaryomyces hansenii* (▲) and *Saccharomyces cerevisiae* (■). Enzyme solutions were diluted to give an increase in absorbance of 0.05/min under standard conditions. Reactions were followed for 15 min. The maximum activity of each preparation is set = 100%.

Fig. 3. Effect of Mg^{2+} on the activity of alkaline phosphatase. The enzyme (0.005 unit/ml) was incubated in assay buffer (○); assay buffer containing 3 M NaCl (●); 3.2 M KCl (■) or 4.4 M glycerol (▲). All activities were recorded 10—15 min after the reaction was started as the enzyme was progressively inactived during the first 10 min at low concentrations of Mg^{2+} in the presence of 3 M NaCl. The Mg^{2+} content of the pure solutions of KCl and NaCl was determined by atomic absorption spectrophotometry to be <0.01 mM in each solution.

environments [2—6], produce glycerol in amounts that increase with external salinity. The activity of alkaline phosphatase determined in assay buffer and in the same agent containing either 3 M NaCl, 3.2 M KCl or 4.4 M glycerol (approximately isotonic concentrations [26]) showed a dependence on Mg^{2+} concentration (Fig. 3). Contrary to KCl, NaCl markedly inhibited the enzyme activity at low concentrations of Mg^{2+}. This inactivation could be reversed by the addition of Mg^{2+}. Glycerol clearly reduced enzyme activity and in this solute the stimulating effect of Mg^{2+} was weak. The effect of various concentrations of salt and glycerol on enzyme kinetics is shown in Fig. 4. To study the kinetic parameters at solute concentrations that may prevail within cells grown under salty conditions, the enzyme was also assayed in various combinations of a glycerol and a salt solution of equimolar amounts of KCl and NaCl. The cellular molar ratio of K^+ to Na^+ of *D. hansenii* grown in a strongly saline medium generally lies close to 1 [9]. In the glycerol solution an increase of the solute concentration caused a decrease of enzyme activity by increasing the value of K_m and decreasing the maximum velocity (V). In the salt solution the enzyme activity was stimulated due to an increase of V with salt concentration, whereas K_m was almost independent of salinity. In the glycerol/salt mixture the enzyme activity was relatively unaffected by glycerol concentration at low glycerol to salt ratios, while

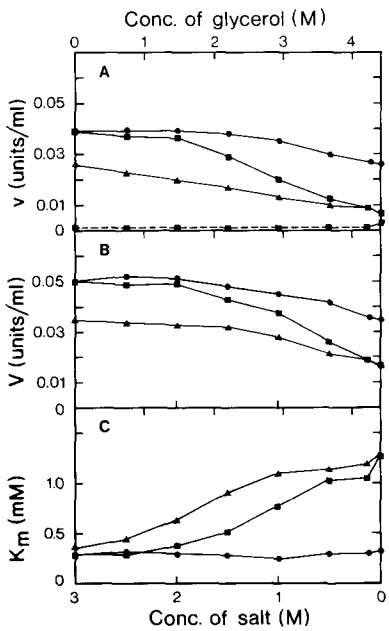

Fig. 4. Effect of salt and glycerol on kinetics of alkaline phosphatase. The enzyme (0.026 unit/ml) was assayed in glycerol (▲); an equimolar mixture of KCl and NaCl (●); or various combinations of a 4.4 M glycerol solution and an equimolar (3 M) mixture of KCl and NaCl (■). Enzyme activities (A) refer to standard concentration of substrate (1 mM *p*-nitrophenyl phosphate). Dashed line indicates that the enzyme was assayed in the absence of added $MgCl_2$. Estimations of V (B) and K_m (C) were conducted by plotting s/v against s [27] for substrate concentrations in the range 0.3—2 mM and a straight line was fitted to experimental data by least-squares linear regression.

TABLE II

K_m AND K_i VALUES OF ALKALINE PHOSPHATASE GROWN AT TWO DIFFERENT CONCENTRATIONS OF NaCl

K_m estimations were conducted as described in Fig. 4. K_i was estimated by plotting $1/v$ against inhibitor concentration for three different *p*-nitrophenyl phosphate concentrations [27]. Incubations were carried out with 0.01—0.03 unit of enzyme/ml in assay buffer containing 10 mM Mg^{2+}. Each K_m value is the mean ± S.D. of at least three replicates; K_i values refer to one determination.

Additions to assay mixture	Enzyme from cells grown in medium BC		Enzyme from cells grown in medium BCS	
	K_m (mM)	K_i (mM)	K_m (mM)	K_i (mM)
No	0.33 ± 0.03		0.32 ± 0.07	
3 M NaCl	0.35 ± 0.07		0.35 + 0.06	
3 M KCl	0.43 ± 0.09		0.45 ± 0.05	
3 M CsCl	0.51 ± 0.06		0.47 ± 0.09	
3 M glycerol	0.98 ± 0.15		1.00 ± 0.12	
Na_2HPO_4		0.75		0.80
Na_2MoO_4		0.066		0.075
Na_2HAsO_4		0.005		0.004

at higher ratios a decrease of activity took place. In the absence of Mg^{2+}, the inactivation of the enzyme was almost complete at all glycerol to salt ratios tested.

Comparison of alkaline phosphatase from cells cultured in high and low NaCl media

The molecular weight of alkaline phosphatase grown in medium BC and in the same medium containing 2.7 M NaCl (medium BCS) was estimated by gel filtration on a Sephadex column. The two enzyme preparations eluted identically to each other in a volume indicating a molecular weight of 110 000. Furthermore, the estimation of K_m under various conditions, and the inhibitor constant (K_i) for three competitive inhibitors (orthophosphate, molybdate, and arsenate) revealed no significant differences between the two preparations (Table II). Similarly, enzyme from media BC- and BCS-grown cells showed indistinguishable heat stabilities: the thermal half-life at 60°C, pH 8.5 was 1.7 min and, in the presence of 3 M NaCl, 3.2 min. Identical electrophoretic patterns were also obtained with enzyme from the two cultures on polyacrylamide gradient gel electrophoresis.

Discussion

The results presented indicate that alkaline phosphatase of *D. hansenii* is a metalloenzyme with a molecular weight around 110 000. With regard to a number of general properties it behaves as a typical alkaline phosphomonoesterase (cf. refs. 18 and 20).

Various divalent metals reversed the enzyme inactivation produced by a few minutes of EDTA treatment. After treatment for 1 h, only Zn^{2+} markedly reestablished the activity (to 89%), suggesting a primary formation of a metal chelate complex on the enzyme while, after prolonged incubation a metal free apoenzyme is formed. The high stability constant of the Zn-EDTA system [28]

makes a removal of the metal from the enzyme plausible. Like many alkaline phosphatases from mammalian sources [18,24] the *D. hansenii* enzyme was stimulated by Mg^{2+} (Fig. 3). The marked inhibition, provoked by NaCl but not by KCl at low Mg^{2+} concentrations, was reversed by addition of Mg^{2+}, indicating an antagonistic relationship between Mg^{2+} and Na^+. The differences between K^+ and Na^+ in the ability to interact with Mg^{2+} may be due to the different charge per unit surface area, and the distinctly different types of hydration shells of the two monovalent ions [29].

The alkaline phosphatase *D. hansenii* seems to be an intracellular enzyme [11]. An increase of the external salt concentration leads to an increased intracellular accumulation of glycerol [10], whereas the internal salt level (i.e. NaCl + KCl) remains relatively constant [9]. An approximate constancy of internal ionic strength in media with widely varied salt concentration may be of critical physiological importance to the organism. However, above a certain concentration of Mg^{2+}, the *D. hansenii* enzyme exhibits a high tolerance to variations of both ionic strength and glycerol concentration (Fig. 4). In the presence of 10 mM Mg^{2+}, the stimulating effect on enzyme activity by salt was counteracted by glycerol. The effect of glycerol on enzyme activity was due to combined effects on the K_m and V; an increasing concentration of glycerol increased the K_m value whereas V decreased. The effect on the K_m by NaCl and KCl, on the other hand, was insignificant or slight. A salt effect on the transphosphorylation of Tris and glycerol by the *E. coli* phosphatase has been demonstrated by Wilson et al. [19]; the activity of the acceptors relative to water is increased by 1 M NaCl.

As highly saline conditions may lead to production of a modified enzyme some properties of enzyme derived from high NaCl (2.7 M) and low NaCl (6 mM) cultures were compared. The two preparations seemed indistinguishable in respect of thermal stability, molecular size indicated by elution pattern from Sephadex G-200 and electrophoretic pattern obtained on run under "limiting pore size" conditions. Other points of similarity include values of K_i and K_m (Table II); the latter responded almost identically to strong solutions of glycerol and salt. This suggests that no salinity-dependent modification of the enzyme exists.

Acknowledgements

I wish to thank Dr. B. Norkrans for her stimulating advice and encouragement in this study. I am also indebted to Mrs. K. Rydell for valuable technical assistance, to Dr. S. Olofsson for kindly performing the isoelectric focusing and to Dr. H. Egnéus for constructive criticism of the manuscript.

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