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CONIDIAL ALKALINE PHOSPHATASE FROM *NEUROSPORA CRASSA*

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Abstract—An alkaline phosphatase was purified from conidia of a *Neurospora crassa* wild type strain. The M_r of the purified native enzyme was estimated as *ca* 145000 and 110000 by gel filtration, in the presence and absence of magnesium ions, respectively. A single polypeptide band of M_r 36000 was detected by SDS–PAGE, suggesting that the native enzyme was a tetramer of apparently identical subunits. Conidial alkaline phosphatase was an acidic protein (p*I* = 4.0 ± 0.1), with 40% carbohydrate content. Optimal pH was affected by substrate concentration and magnesium ions. Low concentrations of calcium ions (0.1 mM) had slight stimulatory effects, but in excess (5 mM) caused protein aggregates with decreased activity. The enzyme specificity against different substrates was compared with those reported for constitutive or Pi-repressible alkaline phosphatases produced by *N. crassa*. The results suggested that the conidial alkaline phosphatase represented a different class among other such enzymes synthesized by this organism.

INTRODUCTION

A number of phosphomonohydrolase activities have been recognized for many years in *Neurospora crassa*. Constitutive intracellular acid and alkaline phosphatases have been described, which are not repressed by high levels of inorganic phosphate in the growth medium and probably accomplish housekeeping metabolic functions [1, 2]. Under phosphate starvation or in phosphate-limited media, *N. crassa* also produces and releases into the culture medium a number of phosphate-metabolizing enzymes [3–9]. These adaptive enzymes are controlled by a complex genetic circuit involving structural and regulatory elements [10, 11]. Among these enzymes, are described Pi-repressible alkaline phosphatases isolated from mycelium [3, 4] or from the culture filtrate [9]. In addition, an alkaline phosphatase activity is present at the cell surface of *N. crassa* conidiospores, among other cell wall-bound hydrolases [12]. The synthesis of this alkaline phosphatase may be under control of conidiogenic developmental programs and it may be interesting to know whether this enzyme is unique to conidiospore cells. While most available information is related to the enzyme localization in conidia [12–14], very little is known about its biochemical properties. Preliminary observations from our laboratory suggested that the alkaline phosphatase solubilized from intact conidia of *N. crassa* had different biochemical properties from those reported for constitutive [1, 2], or Pi-repressible alkaline phosphatases [3, 4, 9]. Here, we describe the purification as well as the biochemical and kinetic characterization of the conidial extracellular alkaline phosphatase from *N. crassa* wild type.

RESULTS AND DISCUSSION

Enzyme purification

Hydrophobic interaction chromatography has been successfully applied for purification of alkaline phosphatases from several mammalian sources [15–18]. With

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Table 1. Purification of conidial alkaline phosphatase from *Neurospora crassa**

Step	Total EU	Total protein (mg)	U mg^{-1}	Yield (%)	Purification
Crude extract	52.7	37.1	1.4	100	1
Peak I	2.1	14.2	0.1	4	0.1
Peak II	29.9	2.1	14.2	57	10

*The activity was determined in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 10 mM PNPP at 37°.

this approach, conidial alkaline phosphatase from a crude extract was purified to homogeneity in a single step by phenyl-Sepharose CL-4B chromatography. The enzyme was purified 10-fold with a yield of 57% and showed a specific activity of 14.2 U mg^{-1} (Table 1). The specific activity of the conidial alkaline phosphatase, determined in crude conidial washes (1.4 U mg^{-1}) was similar to that reported for extracts of phosphate-derepressed cultures [9, 19, 20]. Considering that alkaline phosphatases are useful biochemical reagents [21–23], the one-step purification procedure of the *N. crassa* conidial alkaline phosphatase, described here, may be of interest for eventual practical applications.

Isoelectric point and structural sugars

Isoelectric focusing of the purified conidial alkaline phosphatase yielded a single protein band, coincident with the activity band, with an isoelectric point near pH 4.0 ± 0.1, indicating its acidic character. The conidial alkaline phosphatase was a glycoprotein like other alkaline phosphatases from the same organism, but its carbohydrate content of about 40% (w/w) was much higher than that reported for intracellular and extracellular forms of *N. crassa* alkaline phosphatases [5, 9, 19].

M_r *determinations*

Purified conidial alkaline phosphatase exhibited an apparent M_r of 110 000 by gel filtration in the absence of magnesium ions. In the presence of 2 mM $MgCl_2$ this value was increased to 145 000. On the other hand, under SDS–PAGE the enzyme showed a M_r of *ca* 36 000 regardless of the presence of $MgCl_2$, suggesting that the native enzyme was a tetramer. The higher M_r value obtained by gel filtration in the presence of 2 mM $MgCl_2$ could be due to conformational changes of the tetrameric protein. The M_r of the native conidial alkaline phosphatase was very close to those reported for the Pi-repressible alkaline phosphatase from *N. crassa* [5, 9, 19]. However, the tetrameric structure of the conidial enzyme was in contrast with the dimeric structure reported for constitutive and repressible enzymes [2, 3, 9].

Non-denaturing electrophoresis

Non-denaturing PAGE of the conidial enzyme revealed only a diffuse protein band after silver staining. However, by assaying phosphohydrolytic activity, two distinct bands were observed: a fast-moving band which appeared within 30 min of incubation, and a slow-moving one which appeared after an additional 4 hr period of incubation. The former was apparently the native tetramer, while the latter probably represented enzyme aggregates. When electrophoresis was carried out in the presence of 5 mM $CaCl_2$, four distinct protein bands were revealed by silver staining but only one, the fast-moving band, exhibited phosphohydrolytic activity. This activity band was coincident with the fast-moving band observed in the absence of 5 mM $CaCl_2$. Apparently, treatment with calcium ions induced the formation of low activity aggregates of conidial alkaline phosphatase molecules. Aggregation by calcium ions has been reported for other fungal enzymes [24–27]. The aggregation of enzyme molecules modulated by calcium ions might be of physiological relevance during the latency period of the spores. However, further work is required to clarify this point.

Effect of pH

The apparent optimum of pH for PNPP hydrolysis by conidial alkaline phosphatase varied with substrate concentration, becoming more acidic at lower concentrations. However, in the presence of 10 mM substrate and 2 mM $MgCl_2$ an optimum pH of *ca* 9.8 was determined. This value was similar to that reported for the Pi-repressible mycelial alkaline phosphatase, which is of 9.0–9.5 [3].

Substrate specificity studies

The hydrolysis of PNPP by the native enzyme was affected by the presence of $MgCl_2$. In the presence of magnesium ions the enzyme followed 'Michaelian' kinetics and showed a specific activity 3.5-fold higher than in the absence of $MgCl_2$ (Table 2). These results suggested that magnesium ions are allosteric effectors for the enzyme. Other phosphorylated compounds also served as substrates for the conidial enzyme (Table 3). However, they were hydrolysed at less than 20% of the rate of PNPP hydrolysis. Interestingly, the enzyme did not hydrolyse pyrophosphate. While the conidial enzyme hydrolysed PNPP 5.5-fold faster than β-glycerophosphate, the Pi-repressible enzyme exhibits the same relative rate of hydrolysis for both substrates [5]. On account of this property, the conidial enzyme was similar

Table 2. Kinetic parameters for the hydrolysis of PNPP by purified conidial alkaline phosphatase from *Neurospora crassa**

Parameters	Without $MgCl_2$	With 2 mM $MgCl_2$
V (U mg^{-1})	4.3	14.1
$K_{0.5}$(mM)	0.3	1.3
n	1.4	1.0
$V/K_{0.5}$	15.8	10.7

*The activity was determined in 50 mM AMPOL buffer, pH 9.4, containing PNPP varying from 0.1 to 10 mM, by using 1.0 μg protein.

Table 3. Substrate specificity of conidial alkaline phosphatase from *Neurospora crassa*

Substrate	Relative rate of hydrolysis (%)
PNPP	100
Pyrophosphate	0
Glucose-6-phosphate	6.8
Glucose-1-phosphate	10.8
Fructose-6-phosphate	2.3
β-Glycerophosphate	18.1
ATP	14.8
ADP	15.5
AMP	10.6
bis(*p*-Nitrophenyl)phosphate	0.3

*The activity was determined in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 10 mM substrate, at 37°, by using 1.0 μg protein for PNPP assay and 4.5 μg protein for all other substrates.

to the constitutive alkaline phosphatase, which hydrolyses PNPP at a higher rate in comparison with β-glycerophosphate [2]. However, the conidial enzyme did not hydrolyse inorganic pyrophosphate, as the Pi-repressible enzyme does [3]. Another significant difference was that the conidial enzyme hydrolysed glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) slowly, in contrast with the relatively fast hydrolysis rates of G6P (40%) by the constitutive enzyme [2], or of G6P (71%) and F6P (33%) by the Pi-repressible enzyme [2]. Altogether these results demonstrated significant differences between the conidial alkaline phosphatase and the alkaline phosphatases purified from mycelium [2, 3] and culture medium [9].

Effect of different effectors on enzyme activity

Several divalent metal ions stimulated phosphohydrolytic activity of the conidial alkaline phosphatase showing site–site interactions (Table 4). Interestingly, magnesium was the only metal ion that stimulated the enzyme follwing a bimodal saturation curve. The high-affinity hydrolysing sites showed 'Michaelian' behaviour and a K_m of *ca* 3.3 μM. For low-affinity sites the K_m was of *ca* 2.5 mM and site–site interactions were observed. In the presence of EDTA the enzyme activity decreased to about 0.6 U mg^{-1}, but addition of magnesium ions restored the original activity (*ca* 14.1 U mg^{-1}), suggesting that *N. crassa* conidial alkaline phosphatase was a metalloenzyme. A similar property is also reported for the constitutive enzyme [2], but not for the Pi-repressible mycelial [3] and culture-filtrate enzymes [9] of the same organism. In addition, while the conidial alkaline phosphatase was inhibited by EDTA, the mycelial and culture-filtrate Pi-repressible enzymes were stimulated by such compounds [3, 9]. These results suggested that the

Table 4. Effect of several effectors of phosphohydrolytic activity of conidial alkaline phosphatase from *Neurospora crassa*

Compound	K_i(M)	$K_{0.5}$(M)	V (U mg^{-1})	n
None		3.0×10^{-4}	4.3	1.4
Cobalt		2.0×10^{-6}	8.1	1.8
Zinc		4.9×10^{-6}	7.4	1.8
Manganese		1.7×10^{-6}	5.3	2.0
Calcium		1.6×10^{-6}	3.8	1.7
Nickel		2.1×10^{-6}	9.3	1.3
Magnesium		3.3×10^{-6}	12.2	1.0
		2.5×10^{-3}	5.2	1.9
Vanadate	0.6×10^{-6}			
Arsenate	31.0×10^{-6}			
Phosphate	1.5×10^{-3}			
Phenylalanine	27.5×10^{-3}			

*The activity was determined in 50 mM AMPOL buffer, pH 9.4, at 37°, by using 1.0 μg protein. Substrate concentration varied from 0.1 to 10 mM and 2 mM $MgCl_2$ was added to the reaction medium, for all inhibitors assayed. The effect of metal ions was studied in 50 mM AMPOL buffer, pH 9.4 containing 10 mM PNPP and variable concentrations of metal ions.

enzyme obtained from *N. crassa* conidia differs from the constitutive alkaline phosphatase which is stimulated only 62% by magnesium ions [2] or Pi-repressible alkaline phosphatases, which are stimulated by magnesium and EDTA [3, 9]. Interestingly, calcium ions had remarkable effects on the conidial native alkaline phosphatase. At concentrations lower than 0.1 mM calcium ions had a slight stimulatory effect on the enzyme, while in excess (5 mM) they induced the formation of low-activity aggregates.

Phosphate ($K_i = 1.5$ mM), arsenate ($K_i = 31$ μM) and vanadate ($K_i = 0.6$ μM) were competitive inhibitors, while phenylalanine ($K_i = 27$ mM) was uncompetitive.

Thermal inactivation studies

Conidial alkaline phosphatase was stable after 6 hr of incubation at 45° in 5 mM Tris–HCl buffer, pH 7.5, but it was inactivated at higher temperatures with $t_{0.5}$ varying from 28.6 hr (50°) to 6.5 min (70°). Temperature inactivation followed bimodal kinetics indicating the existence of slow and fast inactivation processes. Arrhenius' plots indicated similar energy of activation for denaturation of 45 600 cal mol^{-1}, for fast and slow inactivation processes.

EXPERIMENTAL

Materials. Tris, 2-amino-2-methyl-1-propanol (AMPOL), Fast Blue RR salt, Phenyl-Sepharose CLB4, α-naphthyl phosphate, and *N*-d(2-hydroxyethyl) piperazine-*N'*-ethanesulphonic acid (HEPES) were from Sigma. *p*-Nitrophenyl phosphate (PNPP) disodium salt was from Merck. All other reagents used were of analytical grade.

Organism and growth conditions. Neurospora crassa wild type (FGSC 424) was a gift from the Fungal Genetics Stock Center (Kansas City, KS, USA). The organism was maintained on slants of solid minimal Vogel's medium [28] supplemented with 2% (w/v) sucrose. Conidia were produced in 300 ml conical flasks containing 50 ml of solid Vogel's medium, incubated at 30° in the dark for 4 days and then for 3 more days under light, to stimulate the production of macroconidia.

Preparation of crude conidial alkaline phosphatase. Conidia were harvested in H_2O and the suspension was stirred during 30 min at 0°. The suspension was then filtered through Whatman number 1, and made 5 mM Tris–HCl, pH 7.5. Finally the filtrate was concd by ultrafiltration on an Amicon cell equipped with an YM-10 membrane.

Purification of conidial alkaline phosphatase. The conidial extract was centrifuged at 5000 *g* for 10 min, at 4°, the resulting supernatant was made 0.3 M in NaCl and applied to a phenyl-Sepharose CL-4B column (1.4 × 16 cm) previously equilibrated with 5 mM Tris–HCl buffer pH 7.5, containing 0.3 M NaCl. Elution was effected in 2 steps, first with 125 ml of the same buffer, and then with 175 ml of 5 mM Tris–HCl buffer, pH 7.5. Frs (5 ml) were collected at a flow rate of 30 ml hr^{-1} and the active ones were pooled and concd by ultrafiltration. After dialysis overnight at 4° against 5 mM Tris–HCl buffer, 7.5, 1 ml samples were frozen in liquid N_2 and stored at −20° for a year without appreciable loss of activity.

Enzymatic activity measurements. p-Nitrophenylphosphatase (PNPPase) activity was estimated discontinuously at 37°, by following the liberation of *p*-nitrophenolate ion (ε 1M, pH 13 = 17 600 $M^{-1}cm^{-1}$) at 410 nm. Standard assay conditions were 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$ and 10 mM PNPP in a final vol. of 1 ml. The reaction was started by the addition of the enzyme and stopped at appropriate time intervals with 1 ml of 1 M NaOH. Determinations were carried out in duplicate and the initial velocities were constant, for at least 90 min, provided that less than 5% of the substrate was hydrolysed. One enzyme unit (EU) was defined as the amount of enzyme hydrolysing 1 μmol of substrate min^{-1} at 37° and at pH 9.4.

Determination of protein. Protein concns were determined according to the method of Ref. [29] using bovine serum albumin (BSA) as standard.

Polyacrylamide gel electrophoresis. Electrophoresis was conducted in 7.5% gels as described in Ref. [30]. Protein was stained with $AgNO_3$ [31] and phosphohydrolytic activity on the gel was assayed in 50 mM AMPOL buffer, pH 9.4, containing 2 mM $MgCl_2$,. 0.12% Fast Blue RR salt and 0.12% α-naphthyl phosphate, at 37°. Electrophoresis was also carried out in the presence of $CaCl_2$. In that case, 5 mM $CaCl_2$ was added to the electrophoresis buffer. When necessary, the samples were concd by uing Centryflo-Amicon cones. SDS–PAGE was carried out in 10% gels according to Ref. [32], using $AgNO_3$ for protein staining [31].

Isoelectric focusing on polyacrylamide gel. Polyacrylamide gel rods (6%) containing 5% (v/v) Pharmacia Fine Chemicals ampholytes in the range of pH 2–5 were used as described in Ref. [33]. Samples containing 2.3 μg of purified enzyme were applied and the focusing was done at 500 V for 6 hr. After the run the gel was sliced (5 mm thick), slices were extracted with 1 ml 25 mM KCl and the pH was determined. Coomassie Blue R-250 was used for protein staining and enzyme activity on the gel was determined as above.

M_r *determination.* The M_r of the purified conidial alkaline phosphatase was estimated in the presence or absence of 2 mM $MgCl_2$ on a Sephacryl S-300 column (130 × 1.7 cm) equilibrated and eluted with 5 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl. M_r markers were myosin, β-galactosidase, phosphorylase b, BSA, egg albumin, carbonic anhydrase and myoglobin.

Carbohydrate. Carbohydrate was determined by the phenol-H_2SO_4 method [34].

Estimation of kinetic parameters. Kinetic parameters obtained from substrate hydrolysis were fitted on an IBM/PC microcomputer by using SIGRAF [35]. V, $K_{0.5}$ and n, which appear in this paper as computed values, stand for maximal velocity, apparent dissociation constant and Hill coefficient, respectively.

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