Brazilian Journal of Medical and Biological Research (2000) 33: 905-912
ISSN 0100-879X

Properties of a constitutive alkaline phosphatase from strain 74A of the mold *Neurospora crassa*

A.C. Morales[1], S.R. Nozawa[1], G. Thedei Jr.[2], W. Maccheroni Jr.[3] and A. Rossi[1]

[1]Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil
[2]Instituto de Ciências da Saúde, Universidade de Uberaba, Uberaba, MG, Brasil
[3]Departamento de Genética, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, SP, Brasil

Correspondence
A. Rossi
Departamento de Química
FFCLRP-USP
Av. Bandeirantes, 3900
14040-901 Ribeirão Preto, SP
Brasil
Fax: +55-16-633-8151
E-mail: anrossi@usp.br

Research supported by FAPESP (No. 96/05834-0), CNPq and CAPES. W. Maccheroni Jr. was supported by a FAPESP postdoctoral fellowship.

Received October 13, 1999
Accepted March 22, 2000

Abstract

A constitutive alkaline phosphatase was purified to apparent homogeneity as determined by polyacrylamide gel electrophoresis from mycelia of the wild strain 74A of the mold *Neurospora crassa*, after growth on acetate and in the presence of saturating amounts of inorganic phosphate (Pi) for 72 h at 30°C. The molecular mass was 58 kDa and 56 kDa as determined by exclusion chromatography and SDS-PAGE, respectively. This monomeric enzyme shows an apparent optimum pH ranging from 9.5 to 10.5 and Michaelis kinetics for the hydrolysis of *p*-nitrophenyl phosphate (the K_m and Hill coefficient values were 0.35 mM and 1.01, respectively), α-naphthyl phosphate (the K_m and Hill coefficient values were 0.44 mM and 0.97, respectively), ß-glycerol phosphate (the K_m and Hill coefficient values were 2.46 mM and 1.01, respectively) and L-histidinol phosphate (the K_m and Hill coefficient values were 0.47 mM and 0.94, respectively) at pH 8.9. The purified enzyme is activated by Mg^{2+}, Zn^{2+} and Tris-HCl buffer, and is inhibited by Be^{2+}, histidine and EDTA. Also, 0.3 M Tris-HCl buffer protected the purified enzyme against heat inactivation at 70°C (half-life of 19.0 min, $k = 0.036$ min^{-1}) as compared to 0.3 M CHES (half-life of 2.3 min, $k = 0.392$ min^{-1}) in the same experiment.

Key words
- *Neurospora crassa*
- Fungi
- Alkaline phosphatase
- L-Histidinol-Pi phosphatase

Introduction

The mold *Neurospora crassa* synthesizes three mycelial alkaline phosphatases (APase) when grown at pH 5.4. While two of them are expressed irrespective of the concentration of inorganic phosphate (Pi) in the culture medium a third one is only expressed when the medium is growth-limiting in Pi (1-3). Only one gene encoding the Pi-repressible APase has been identified (*pho*-2), cloned and characterized at the molecular level (4). The *pho*-2 gene is a component of the phosphate acquisition system of *N. crassa* and is expressed irrespective of the growth pH, except that at alkaline pH the enzyme is largely secreted into the growth medium (5,6). Also, the molecular properties of the Pi-repressible APase expressed at both pH 5.4 and pH 7.8 have been extensively characterized (7-10). On the other hand, much remains to be clarified about the molecular properties and physiological role of these constitutive APases of *N. crassa* (1,11,12). Thus, in an attempt to further investigate its properties, the major APase synthesized by strain 74A

of *N. crassa* grown on high-Pi medium supplemented with acetate as the sole carbon source at pH 7.1 was purified to apparent homogeneity as determined by SDS-PAGE. Some properties of the purified enzyme such as molecular mass, relative heat stability and hydrolysis of L-histidinol-Pi, which is part of the pathway of histidine biosynthesis in *N. crassa* (13), were also determined.

Material and Methods

Material

Except where otherwise stated, all chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO, USA).

Table 1 - Partial purification of the constitutive alkaline phosphatases synthesized by the wild 74A strain of N. crassa grown in high-Pi medium at 30ºC, supplemented with 44 mM acetate as the carbon source and buffered at pH 5.4 with 100 mM sodium maleate.

For details, see Material and Methods.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	42	161	225	1.4	100	1
Heat	32	67.2	170	2.5	75.6	1.8
$(NH_4)_2SO_4$ (40-60%)	10	28.0	54	1.9	24	1.4
DEAE-cellulose						
Fraction I	5	0.4	5.5	13.8	2.4	9.9
Fraction II	7	1.8	30	16.7	13.3	11.9

Table 2 - Partial purification of the constitutive alkaline phosphatases synthesized by the wild 74A strain of N. crassa grown in non-buffered high-Pi medium at 30ºC, supplemented with 44 mM sucrose as the carbon source and adjusted to pH 5.4.

For details, see Material and Methods.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	80	361.6	96	0.3	100	1
Heat	77	183.3	84.8	0.5	88	1.7
$(NH_4)_2SO_4$ (40-60%)	12	33.6	32.4	1.0	34	3.3
DEAE-cellulose						
Fraction I	3	1.8	3.0	1.7	3	5.7
Fraction II	4	6.4	5.2	0.8	5.4	2.7

Strain and growth conditions

The wild type strain St. L. 74A of *N. crassa* (Fungal Genetic Stock Center, University of Kansas Medical Center, Kansas City, KS, USA) was used in the present study. Stock cultures were maintained on slants of Vogel's medium (1.6% agar) (14). Conidial suspensions (2.5 ml containing about 10^8 cells/ml) were grown for 72 h at 30ºC in an orbital shaker (160 rpm) in Erlenmeyer flasks (500 ml) containing 100 ml of high (10 mM)-Pi medium, supplemented with 44 mM of the desired carbon source as follows: non-buffered sucrose adjusted to pH 5.4, acetate buffered at pH 5.4 with 100 mM sodium maleate, non-buffered acetate adjusted to pH 7.1 and sucrose buffered with 50 mM Tris-HCl and prepared as described previously (5,15).

Preparation of extracts and assays

The mycelium harvested by filtration was extracted with sand and 50 mM Tris-HCl buffer, pH 8.8 (10 ml buffer/g mycelium), containing 40 mM $MgSO_4$, 1 mM benzamidine and 1 mM PMSF (final concentrations). After shaking for 15 min at 4ºC the supernatant (crude extract) was collected by centrifugation (20 min at 17,000 *g*) and used for enzyme assays or enzyme purification. Except where otherwise stated, constitutive APase activity was determined as described previously (1) in 0.3 M Tris-HCl buffer, pH 8.9, containing 1 mM $MgSO_4$, using 6 mM *p*-nitrophenyl phosphate (PNP-P) as substrate at 37ºC. L-Histidinol-Pi, ß-glycerol-Pi and α-naphthyl-Pi hydrolysis was carried out in 0.3 M Tris-HCl buffer as described by Kuo and Blumenthal (1). The liberated Pi was measured by the method of Heinonen and Lahti (16). Incubations were carried out at 37ºC, and all enzyme activities were measured in duplicate for at least two time intervals. One unit of APase activity was defined as one μmol substrate hydrolyzed min^{-1}. Protein concentration was estimated by the

method of Folin as described by Hartree (17), with BSA (fraction V) as the standard. The protein content of fractions obtained by column chromatography was monitored by measuring absorbance at 220 nm.

Enzyme purification

The procedure used for the purification to apparent homogeneity as determined by PAGE of a constitutive alkaline APase synthesized by the mold *N. crassa* was a modification of previously described methods (1,3). Mycelium extract (crude extract) of the wild-type strain 74A grown on high-Pi medium, pH 7.1, was incubated at 50°C for 45 min and centrifuged at 17,000 *g* for 20 min at 4°C and the supernatant was fractionated by $(NH_4)_2SO_4$ precipitation. The APase activity recovered in the 40-60% salt saturation step was suspended in a small volume of 10 mM Tris-HCl buffer, pH 8.8, containing 30 mM $MgSO_4$, dialyzed for 24 h against 4 l of the same buffer (3 changes of buffer) and centrifuged if necessary (14,000 *g* for 10 min). The dialyzed enzyme was applied to a column (1.1 x 50 cm) of DEAE-cellulose previously equilibrated with the buffer used for dialysis. Non-absorbed proteins were eluted with about 200 ml of the same buffer and showed little phosphatase activity. Enzyme elution was performed with a non-continuous concentration gradient (12.5 and 50 mM KCl in 10 mM Tris-HCl buffer, pH 8.8, containing 40 mM $MgSO_4$) at a flow rate of about 150 ml/h (10-ml fractions). The tubes representing the enzyme peak were pooled and concentrated by ultrafiltration through Amicon (YM 10) membranes. This concentrate was applied to a column (1.5 x 130 cm) of Sephacryl S-200-HR previously equilibrated with 10 mM Tris-HCl buffer, pH 8.8, containing 30 mM $MgSO_4$ and 200 mM NaCl. Elution was performed with this buffer at a flow rate of 12 ml/h (2.5-ml fractions). The tubes representing the enzyme peak were pooled, concentrated by ultrafiltration through Amicon (YM 10) membranes and applied to a column (1.5 x 9.2 cm) of phenyl-Sepharose CL-4B previously equilibrated with 10 mM Tris-HCl buffer, pH 8.8, containing 10 mM $MgSO_4$ and 2 M NaCl (final concentrations). Enzyme elution was performed with a discontinuous concentration gradient (10 mM Tris-HCl buffer containing 10 mM $MgSO_4$, and 5 mM Tris-HCl buffer containing 5 mM $MgSO_4$ and 5 mM Tris-HCl buffer, pH 8.8, respectively) at a flow rate of 60 ml/h (1-ml fractions). The effluent containing the enzyme peak was pooled and concentrated by ultrafiltration through Amicon (YM 10) membranes and stored at 4°C.

Table 3 - Partial purification of a constitutive alkaline phosphatase synthesized by the wild 74A strain of N. crassa grown in high-Pi medium at 30°C, supplemented with 44 mM sucrose as the carbon source and buffered at pH 7.8 with 50 mM Tris-HCl.

n.d., Not detected. For details, see Material and Methods.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	72	319.7	115.2	0.4	100	1
Heat	67	174.9	76.4	0.4	66	1
$(NH_4)_2SO_4$ (40-60%)	12	40.9	38.4	0.9	33	2.2
DEAE-cellulose						
Fraction I	6	1.4	15	10.7	13	26.8
Fraction II	-	-	n.d.	-	-	-

Table 4 - Purification of the major constitutive alkaline phosphatase synthesized by the wild 74A strain of N. crassa grown in non-buffered high-Pi medium at 30°C, supplemented with 44 mM acetate as the carbon source and adjusted to pH 7.1.

For details, see Material and Methods.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	142	445.4	772.4	1.7	100	1
Heat	132	280.5	719.5	2.5	93.1	1.5
$(NH_4)_2SO_4$ (40-60%)	10.4	59.3	288.2	4.9	37.3	2.9
DEAE-cellulose						
Fraction I	3.5	3.7	138.1	37.2	17.9	21.9
Fraction II	3.9	1.9	15.6	8.1	2.0	4.8
Sephacryl S-200						
Fraction I	1.7	0.6	85.2	152.1	11.1	89.5
Phenyl-Sepharose CL-4B						
Fraction I	2.6	0.18	40.2	223.3	5.2	131.4

Characterization of the purified enzyme

Unless otherwise stated, the buffers used to cover the pH range required were 0.3 M PIPES/NaOH, pH 6.5-7.6, 0.3 M TAPS/NaOH, pH 7.6-8.5, 0.3 M CHES/NaOH, pH 8.5-10.0, and 0.3 M CAPES/NaOH, pH 10.0-11.0.

Relative heat stability was determined by incubating the enzyme in 0.03 M, 0.15 M and 0.30 M CHES or Tris-HCl buffers containing 1 mM $MgSO_4$ (final concentrations), pH 8.9, at 70°C, in the same experiment. At appropriate times, samples were removed to measure the remaining APase activity using the standard procedure.

Limiting velocities (V_{max}) and Michaelis constants (K_m) were determined as Lineweaver and Burk plots (18) by incubating the enzyme in 30 mM CHES buffer containing 1 mM $MgSO_4$, pH 8.9, at 37°C, using substrate concentrations in the range of 0.5 to 6.67 mM. The interaction constant for the substrate (n) was determined by the Hill procedure as described by Koshland Jr. (19). The kinetic constants reported here were obtained by linear-square analysis calculated from the data obtained in at least three independent experiments.

PAGE was carried out at pH 8.3 by the method of Davis as described by Han et al. (9) using 7.5% (w/v) polyacrylamide slab gels (10 x 10 x 0.1 cm). The phosphomonoesterase activity bands were developed by the method of Dorn as described by Maccheroni Jr. et al. (20) using sodium α-naphthyl-Pi as the substrate. SDS-PAGE was carried out as described previously (21) using polyacrylamide slab gels, and the protein bands were visualized with Coomassie blue. Prior to loading, all samples were incubated in the presence of 1% (w/v) SDS and 100 mM ß-mercaptoethanol for 3 min at 100°C. When necessary, the protein bands were stained with silver (22). The ratio of the distance covered by the enzyme bands to the distance covered by bromophenol blue (relative electrophoretic mobility, R_f) was measured.

The molecular mass was measured by exclusion chromatography under standard conditions (see above) and by SDS-PAGE (21), using appropriate protein markers.

Table 5 - Summary of kinetic constants for the enzymatic hydrolysis of some organic esters at 37°C, pH 8.9.

*Calculated from double-reciprocal plots. For details, see Material and Methods.

Substrate	K_m (mM)	n	V_{max}* (units/mg)
p-Nitrophenyl phosphate	0.35	1.01	241
α-Naphthyl phosphate	0.44	0.97	142
L-Histidinol phosphate	0.47	0.94	108
ß-Glycerol phosphate	2.46	1.01	428

Results and Discussion

Confirming earlier reports (3), chromatography of crude extracts on DEAE-cellulose, irrespective of the carbon source used, revealed the presence of two enzymatic fractions showing APase activity when the mold *N. crassa* was grown on high-Pi medium at pH 5.4 (Figures 1 and 2). It was also observed at pH 5.4 that fraction II was more abundant when the mold was grown on acetate (Table 1) and was poorly recovered when the mold was grown on sucrose (Table 2). Furthermore, fraction II was not detected or was poorly recovered at alkaline pH after growth on sucrose (Table 3) or acetate (Table 4), respectively. Although speculative, the above results (at least three independent experiments, done using each carbon source at both acid and alkaline pH, gave almost the same results) indicate that the expression of these two enzyme forms may be under the effect of the pH regulatory circuit (6,23,24).

The purification procedure described in this paper and summarized in Table 4 provided optimal conditions for the purification of the major constitutive APase (fraction I) synthesized by the wild-type strain 74A of the mold *N. crassa* grown in high-Pi medium supplemented with acetate at pH 7.1. As

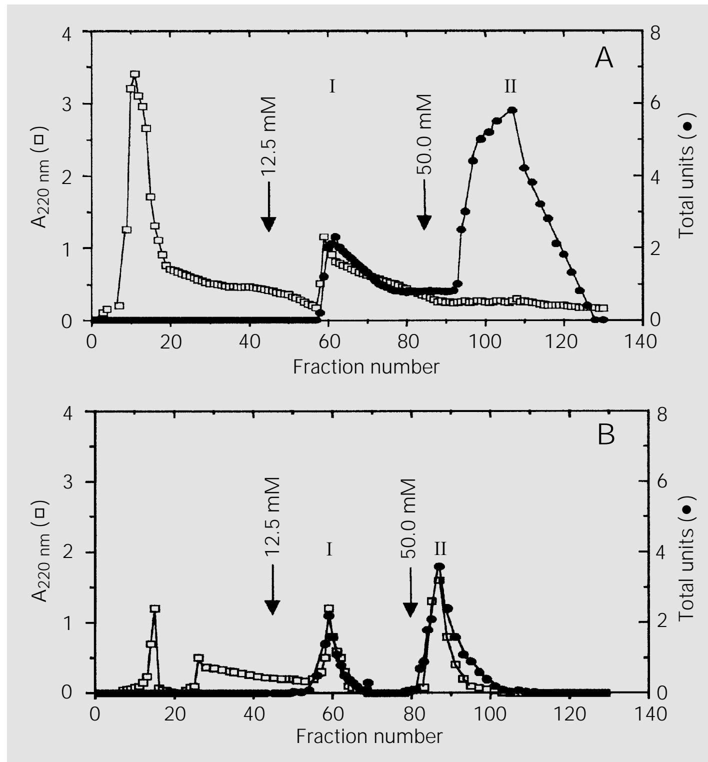

Figure 1 - Stepwise elution from DEAE-cellulose of constitutive alkaline phosphatases synthesized by the wild 74A strain of N. crassa grown in high-Pi medium at 30ºC, pH 5.4, and supplemented with 44 mM acetate (A) or 44 mM sucrose (B) as the carbon source. After elution of non-absorbed proteins, enzyme was eluted by the stepwise addition of 12.5 and 50.0 mM KCl in 10 mM Tris-HCl buffer, pH 8.8, containing 40 mM $MgSO_4$. For details, see Material and Methods.

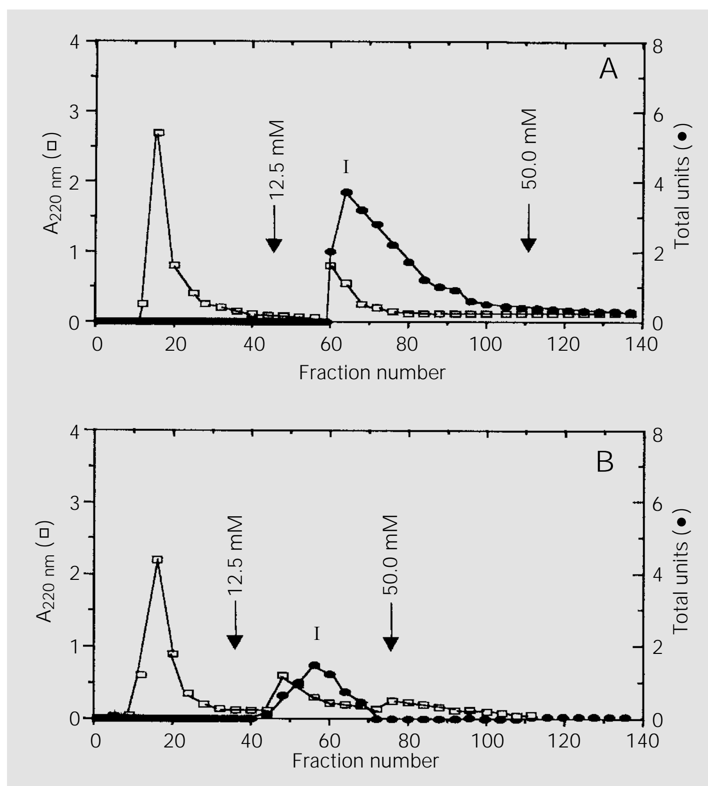

Figure 2 - Stepwise elution from DEAE-cellulose of constitutive alkaline phosphatases synthesized by the wild 74A strain of N. crassa grown in high-Pi medium at 30ºC, and supplemented with 44 mM acetate (A), pH 7.1, or 44 mM sucrose (B), pH 7.8, as the carbon source. After elution of non-absorbed proteins, enzyme elution was performed by the stepwise addition of 12.5 and 50.0 mM KCl in 10 mM Tris-HCl buffer, pH 8.8, containing 40 mM $MgSO_4$. For details, see Material and Methods.

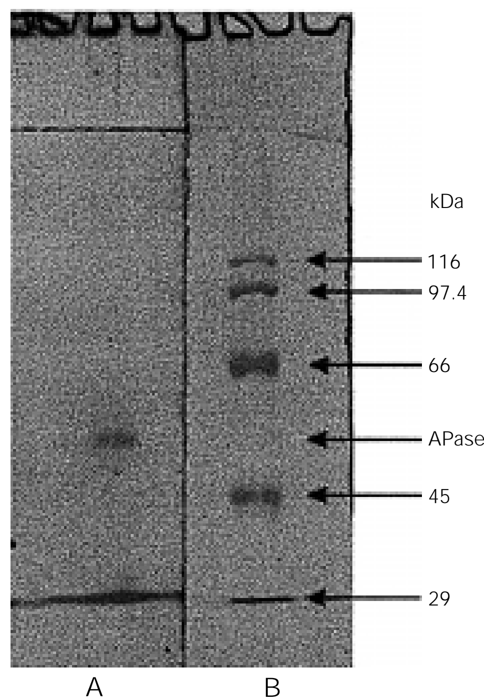

Figure 3 - SDS-PAGE of purified alkaline phosphatase synthesized by N. crassa mycelium grown for 72 h on high-Pi medium. About 10 µg of purified protein was loaded onto lane A and stained with silver after electrophoresis. About 1 µg of protein markers (lane B) was stained with Coomassie blue after electrophoresis.

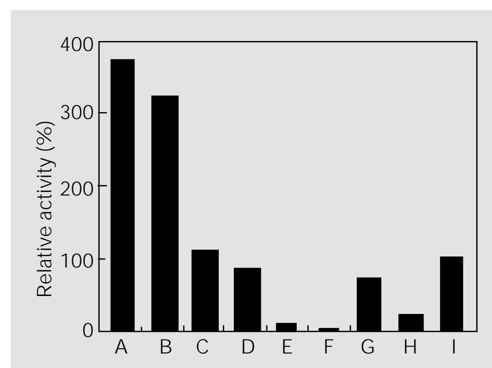

Figure 4 - Effect of various compounds on the p-nitrophenyl phosphatase activity, pH 8.9, of purified constitutive alkaline phosphatase synthesized by strain 74A of N. crassa. A-G represent 1 mM Mg^{2+}, Zn^{2+}, Cu^{2+}, F^-, Be^{2+}, EDTA and histidine, respectively. H and I represent 10 mM histidine and control, respectively.

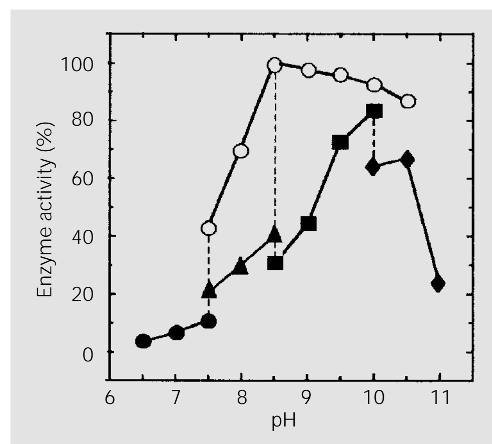

Figure 5 - pH activity profile of purified constitutive alkaline phosphatase synthesized by strain 74A of N. crassa grown in high-Pi medium at 30ºC, pH 7.1. Filled circles, triangles, squares and lozenges represent the enzyme activity in the presence of 0.3 M PIPES buffer, pH 6.5-7.5, 0.3 M TAPS buffer, pH 7.5-8.5, 0.3 M CHES buffer, pH 8.5-10.0, and 0.3 M CAPES buffer, pH 10.0-11.0, respectively. Open circles represent the enzyme activity in the presence of the mixture containing the proper buffer and 0.3 M Tris-HCl, pH 7.5-10.5. For details, see Material and Methods.

already described (5,15), the pH of the medium increased during growth on acetate, reaching a value of 8.7 after 72-h incubation. At least five independent preparations of the enzyme appeared homogeneous as determined by 7.5% PAGE at pH 8.3, with the protein band being superimposable on APase activity. All of these preparations also essentially showed the same electrophoretic mobility, the R_f value being 0.53. Overall, the constitutive APase was purified about 131-fold with a yield of 5.2% (specific activity of 223 units/mg). Furthermore, the presence of Mg^{2+} in all steps of the purification procedure was necessary for the maintenance of the enzyme activity, since activity was not restored when Mg^{2+} was added *a posteriori*.

The molecular mass of the purified constitutive APase was 58 kDa and 56 kDa as determined by exclusion chromatography and SDS-PAGE (Figure 3), respectively, demonstrating that the enzyme is a monomer. This enzyme also showed no deviation from Michaelis kinetics for the hydrolysis of PNPP, α-naphthyl-Pi, ß-glycerol-Pi and L-histidinol-Pi (Table 5). The purified enzyme is activated by Mg^{2+} and Zn^{2+} and is inhibited by histidine, EDTA and 1 mM Be^{2+} (Figure 4). Beryllium sensitivity was not observed for the hydrolysis of L-histidinol-Pi because of the very low concentration of Be^{2+} previously used in the assay (0.1 mM) (1), i.e., this concentration is about 9 times lower than the K_m value (0.91 mM) earlier determined for the hydrolysis of L-histidinol-Pi (1), which makes its effect almost experimentally undetectable.

Determination of the pH activity profile showed an apparent optimum ranging from 9.5 to 10.5 for the purified APase (Figure 5). Also, the hydrolytic activity of the enzyme was enhanced at pH ranging from 7.5 to 8.5 in the presence of Tris-HCl buffer (Figure 5). Furthermore, Tris-HCl buffer had a protective effect when the enzyme was incubated at 70ºC, pH 8.9, as compared to the

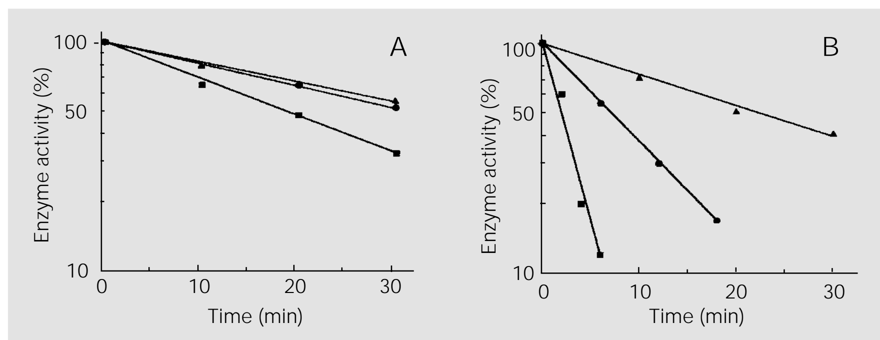

Figure 6 - Heat inactivation of purified constitutive alkaline phosphatase at 70ºC, pH 8.9, synthesized by strain 74A of N. crassa grown in high-Pi medium at 30ºC, pH 7.1. A, Triangles, circles, and squares represent the enzyme incubated in 0.03, 0.15 and 0.30 M Tris-HCl buffer, pH 8.9, respectively. B, Triangles, circles, and squares represent the enzyme incubated in 0.03, 0.15 and 0.30 M CHES buffer, pH 8.9, respectively. For details, see Material and Methods.

effect observed for the incubation in CHES buffer (Figure 6).

Taken together, the above results indicate that the expression of a constitutive APase by the mold *N. crassa*, which is also probably responsible for the hydrolysis of L-histidinol-Pi *in vivo* (Table 5), in spite of its beryllium sensitivity, is under the effect of the pH regulatory circuit in the mold *N. crassa*.

Acknowledgments

We thank Newton R. Alves for technical assistance.

References

1. Kuo M-H & Blumenthal HJ (1961). An alkaline phosphomonoesterase from Neurospora crassa. Biochimica et Biophysica Acta, 54: 101-109.
2. Nyc JF, Kadner RJ & Crocken BJ (1966). A repressible alkaline phosphatase in Neurospora crassa. Journal of Biological Chemistry, 241: 1468-1472.
3. Davis FW & Lees H (1969). Alkaline phosphatases of Neurospora crassa. Part I. Canadian Journal of Microbiology, 15: 455-459.
4. Grotelueschen J, Peleg Y, Glass NL & Metzenberg RL (1994). Cloning and characterization of pho-2+ gene encoding a repressible alkaline phosphatase. Gene, 144: 147-148.
5. Nahas E, Terenzi HF & Rossi A (1982). Effect of carbon source and pH on the production and secretion of acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) in Neurospora crassa. Journal of General Microbiology, 128: 2017-2021.
6. Maccheroni Jr W, Thedei Jr G & Rossi A (1995). Are the structural genes of Pi-repressible phosphatases regulated by multiple circuits in the filamentous mold Neurospora crassa? Revista Brasileira de Genética, 18: 135-137.
7. Thedei Jr G, Nozawa SR, Simões AL & Rossi A (1997). Gene pho-2 codes for the multiple active forms of Pi-repressible alkaline phosphatase in the mold Neurospora crassa. World Journal of Microbiology and Biotechnology, 13: 609-611.
8. Pereira M, Pereira Jr H, Thedei Jr G, Rossi A & Martinez-Rossi NM (1995). Purification of Neurospora crassa alkaline phosphatase without DNAse activity for use in molecular biology. World Journal of Microbiology and Biotechnology, 11: 505-507.
9. Han SW, Michelin MA, Barbosa JE & Rossi A (1994). Purification and constitutive excretion of acid phosphatase in Neurospora crassa. Phytochemistry, 35: 1131-1135.
10. Palma MS, Han SW & Rossi A (1989). Dissociation and catalytic activity of phosphate-repressible alkaline phosphatase from Neurospora crassa. Phytochemistry, 28: 3281-3284.
11. Davis FW & Lees H (1972). Alkaline phosphatases of Neurospora crassa. Part II. Product inhibition studies. Canadian Journal of Microbiology, 18: 407-421.
12. Davis FW & Lees H (1973). Alkaline phosphatases of Neurospora crassa. Part III. Effects of pH and mechanism of action. Canadian Journal of Microbiology, 19: 135-146.
13. Ames BN (1957). The biosynthesis of histidine: D-erythro-imidazole-glycerol phosphate dehydrase. Journal of Biological Chemistry, 228: 131-143.
14. Vogel HJ (1956). A convenient growth medium for Neurospora. Microbial Genetics Bulletin, 13: 42-43.
15. Han SW, Nahas E & Rossi A (1987). Regulation of synthesis and secretion of acid and alkaline phosphatases in Neurospora crassa. Current Genetics, 11: 521-527.
16. Heinonen JK & Lahti RJ (1981). A new and convenient colorimetric determination of inorganic orthophosphate and its application to assay inorganic pyrophosphatase. Analytical Biochemistry, 113: 313-317.
17. Hartree EF (1972). Determination of protein: a modification of Lowry method that gives a linear photometric response. Analytical Biochemistry, 48: 422-427.
18. Lineweaver H & Burk D (1934). The determinations of the enzyme dissociation constants. Journal of the American Chemical Society, 56: 658-666.
19. Koshland Jr DE (1970). The molecular basis for enzyme regulation. In: Boyer PD (Editor), The Enzymes. Academic Press, New York.
20. Maccheroni Jr W, Martinez-Rossi NM & Rossi A (1995). Does gene palB regulate the transcription or the post-translational modification of Pi-repressible phosphatases of Aspergillus nidulans. Brazilian Journal of Medical and Biological Research, 28: 31-38.
21. Thedei Jr G & Rossi A (1994). Is the sense

of Pi levels abolished in the preg^c strain of the mold Neurospora crassa? Plant and Cell Physiology, 35: 837-840.

22. Morrissay JH (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Analytical Biochemistry, 117: 307-310.
23. Tilburn J, Sarkar S, Widdick DA, Espeso EA, Orejas M, Mungroo J, Peñalva MA & Arst Jr HN (1995). The Aspergillus PacC zinc finger transcription factor mediates regulation of both acid and alkaline expressed genes by ambient pH. EMBO Journal, 14: 779-790.
24. Maccheroni Jr W, May GS, Martinez-Rossi NM & Rossi A (1997). The sequence of palF, an environmental pH response gene in Aspergillus nidulans. Gene, 194: 163-167.