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PROPERTIES OF A REPRESSIBLE ALKALINE PHOSPHATASE SECRETED BY THE WILD-TYPE STRAIN 74A of *NEUROSPORA CRASSA*

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Key Word Index—*Neurospora crassa*; fungi; enzyme secretion; alkaline phosphatase.

Abstract—A repressible extracellular alkaline phosphatase (with activity increasing steadily even up to pH 10.5) was purified from cultures of the wild-type strain 74A of *Neurospora crassa*, after growth on acetate and under limiting amounts of inorganic phosphate for 72 hr at 30°. The enzyme was homogeneous on polyacrylamide gel electrophoresis (PAGE) with or without sodium dodecyl sulphate (SDS). The MW was *ca* 172 000 and 82 000 as determined by Sephadex G-200 gel filtration and SDS-PAGE, respectively. The enzyme contained 23.6% neutral sugars, cations were not required for activity, and it was not inactivated by 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) at pH 8. Kinetic data showed Michaelian behaviour for the enzymatic hydrolysis of 4-nitrophenyl disodium orthophosphate (PNP-P) at pH 9 (the K_m value and Hill coefficient were 2.2×10^{-4} M and 0.95, respectively). It was also shown that, at pH 9, the apparent number of Pi bound per dimer molecule equalled one, with a K_i value of 7.0×10^{-4} M. The secreted enzyme showed half-lives of 23.5, 49.0 and 23.5 min at, pH 5.4, 7.4 and 9.0, respectively, after thermal inactivation at 60°. At pH 5.4, the half-life value was quite similar, while the others were respectively 2 and 4 times greater than those previously described for the repressible alkaline phosphatase retained by the mycelium at pH 5.6 or secreted by 'slime' cells.

INTRODUCTION

The synthesis of repressible alkaline phosphatase during growth of a wild-type strain of *Neurospora crassa* in media with limiting phosphate seems to be under the same control mechanism that regulates the synthesis of other repressible enzymes [1–4]. It was also shown that, besides the derepression by phosphate starvation, the production and secretion of repressible alkaline phosphatase were stimulated by an increase in the pH of the medium [5]. Furthermore, in the *pho*-2 mutant, which is considered to represent its structural locus [6, 7], the production and secretion of this enzyme were negligible [5]. From these results it appears likely that the repressible alkaline phosphatase retained by the mycelium at pH 5.6 or secreted into the growth medium at a pH greater than 8.0 is the product of the same gene [5]. A similar conclusion has been assumed for the alkaline phosphatase secreted by 'slime' cells, which differs from that retained by the mycelium in the degree of post-translational modifications [8].

In the present paper we describe the purification and some kinetic and structural properties of the repressible alkaline phosphatase (EC 3.1.3.1) secreted by the wild-type strain 74A of *Neurospora crassa* when it was grown on acetate and under limiting amounts of inorganic phosphate. Our results suggest that this secreted enzyme and that retained by the sucrose-grown mycelium seem to differ in the carbohydrate content attached to the enzyme molecule. Furthermore, its thermal stability is also higher than that obtained for the enzyme retained by the acetate-grown mycelium.

RESULTS AND DISCUSSION

The procedure described in this paper, and summarized in Table 1, provided optimal conditions for the purification of the alkaline phosphatase secreted by the wild-type strain 74A of *Neurospora crassa* when it was grown on acetate and under limiting amounts of phosphate. Overall, the enzyme was purified 51-fold with a yield of 15%. It can be observed that, after ammonium sulphate fractionation, the yield was *ca* 1.28-fold over the growth medium, which could be attributed to the elimination of enzyme inhibitors. All enzyme preparations appeared homogeneous on 7.5% PAGE at pH 8.3, with the protein band being superimposable on alkaline phosphatase activity.

The neutral sugar content of the purified enzyme was *ca* 23.6%, suggesting that it was a glycoprotein. This value is

Table 1. Purification of a repressible alkaline phosphatase secreted by the wild-type strain 74A of *Neurospora crassa*

Fraction	Volume (ml)	Total activity (units)	Specific activity (units/mg)
I. Culture medium	2190	111	0.84
II. $(NH_4)_2SO_4$	193	142	8.2
III. Sephadex G-200	35	54	9.0
IV. DEAE-cellulose	3	17	42.5

For details see Experimental.

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similar to that previously found for the alkaline phosphatase secreted by 'slime' cells [8]. The MWs were *ca* 172 000 and 82 000, as determined by exclusion chromatography or by SDS-PAGE, respectively, suggesting that the enzyme was a dimer. Similar MWs were also determined for the alkaline phosphatase secreted by 'slime' cells and for the derepressed intracellular enzyme when the wild-type strain was grown on sucrose [8].

Determination of the pH activity profile of the secreted alkaline phosphatase showed no apparent optimum at 9.0–9.5 (Fig. 1), which was the optimum for the repressible enzyme retained by the mycelium grown on sucrose [8]. A similar profile was previously obtained for an alkaline phosphatase secreted by a sucrose-grown wild-type strain of *N. crassa* [9]. The secreted enzyme showed, at pH 9.0, hydrolytic activity towards various phosphomonoesters. *p*-Nitrophenylphosphate gave the highest activity, and phenylphosphate, *β*-glycerylphosphate and glucose-6-phosphate were significantly hydrolysed (*ca* 87, 36 and 45%, respectively, relative to that obtained with *p*-nitrophenylphosphate). No significant activity towards bis(*p*-nitrophenyl)phosphate was detected. Various divalent metal ions examined showed little or no inhibitory effect on *p*-nitrophenylphosphatase activity at pH 9.0 (5 mM $CuSO_4$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $ZnSO_4$ and 1 mM $CoCl_2$). This characteristic was similar to that shown by the repressible intracellular alkaline phosphatase, which was also unaffected by divalent cations [8]. Furthermore, it seemed clear that metals were not required for enzyme activity, since an activation of *ca* 100% was observed in the presence of 1–10 mM EDTA. This effect was also observed for the enzyme secreted by a wild-type strain grown on sucrose [9]. It was also observed that 25 μM $HgCl_2$ or DTNB (after incubation for 3 hr at 37°) had little or no effect on enzyme activity, suggesting that the enzyme was not SH-dependent [10].

Fig. 2 shows the double-reciprocal plots of initial velocities, at pH 9.0, as a function of *p*-nitrophenylphosphate concentration. It can be observed that, either in the absence or in the presence of phosphate (a competitive inhibitor), the double-reciprocal plots give straight lines and are characterized by a Hill coefficient equal to one (inset, Fig. 2), which is compatible with Michaelian

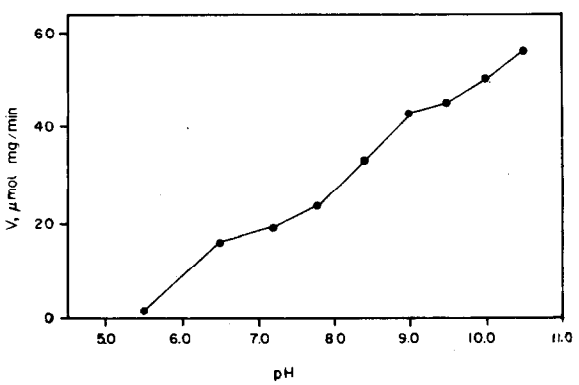

Fig. 1. pH activity profile (PNP-P as substrate) of repressible extracellular alkaline phosphatase purified from cultures of the wild-type strain 74A of *Neurospora crassa*. For details see Experimental.

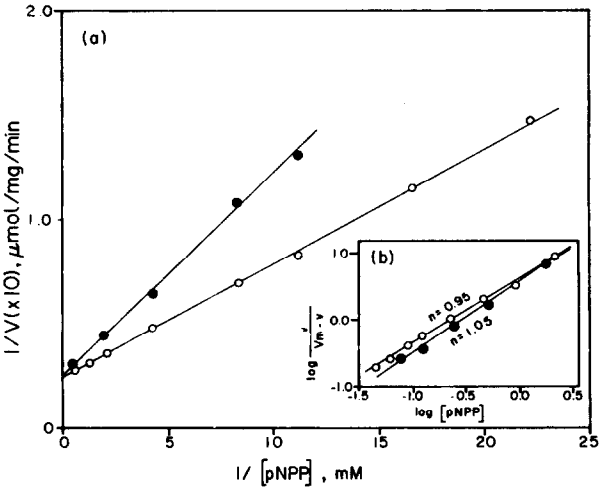

Fig. 2. (a) Double-reciprocal plots of velocity of purified extracellular enzyme as a function of PNP-P concentration at pH 9.0. ○, ● represent alkaline phosphatase velocity without and with 0.4 mM Pi, respectively. (b) Hill plot of log $V/V_{max} - V$ vs log PNP-P concentration, where V and V_{max} are the velocities of the enzyme-catalysed reaction at sub-optimal and optimal substrate concentrations, respectively. ○, ● represent the velocities measured, at pH 9, in the presence or absence of Pi, respectively.

behaviour [11]. The K_m value for *p*-nitrophenylphosphate (2.2×10^{-4} M) was comparable to that determined for the enzyme previously described [8, 9]. Figure 3 shows the plot of residual activity, at pH 9.0, at a fixed concentration of *p*-nitrophenylphosphate (2.7 mM) against phosphate concentration. From these data, a plot of log $(V_o - V_i)/V_i$ vs log Pi concentration (where V_o and V_i are, respectively, the reaction velocities without and with Pi) gives a straight line with a slope of 1.0 (inset, Fig. 3), which equals the apparent number of Pi bound per enzyme molecule [12]. The K_i value at pH 9.0 (7.0×10^{-4} M) was *ca* 6 times higher than those determined for the enzyme retained by the mycelium of the wild-type strain, or that secreted by 'slime' cells [8, 9].

Heat inactivation at 60°, and at pH 9.0, showed for the repressible intracellular alkaline phosphatase (partially purified from acetate-grown mycelium) a half-life of 7.5 min (Fig. 4). This value was quite similar to those found for the repressible enzyme retained by the mycelium at pH 5.6, or that secreted by 'slime' cells [8]. The half-life of 23.5 min, found at pH 5.4 (Fig. 4), was also quite similar to those found for these repressible enzymes. However, the half-lives found at pH 7.4 and 9.0, respectively, 49.0 and 23.5 min (Fig. 4), were *ca* 2 and 4 times greater than those found for the enzyme secreted by the mutant or retained by the sucrose-grown mycelium [8]. As already proposed [5, 8], it appears likely that these enzymes, which were derepressed under various growth conditions, were the product of the same gene. With this in mind, it seems possible that the differences observed in these inactivation velocities at alkaline pH, were due to qualitative or quantitative differences in the carbohydrate attached to the derepressed enzyme. Thus, interaction of pH and some membrane functions could be necessary for the secretion of appreciable amounts of the derepressed alkaline phosphatase by the wild-type strain.

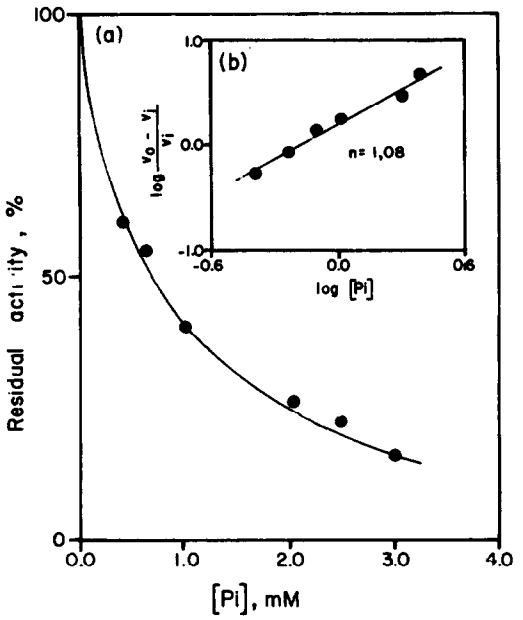

Fig. 3. (a) Plots of % residual velocity (PNP-P as substrate) of repressible extracellular enzyme vs Pi concentration at pH 9.0. (b) Hill plot of log $V_o - V_i/V_i$ vs log Pi concentration, where V_o and V_i are the velocities of the enzyme-catalysed reaction without and with inhibitor, respectively.

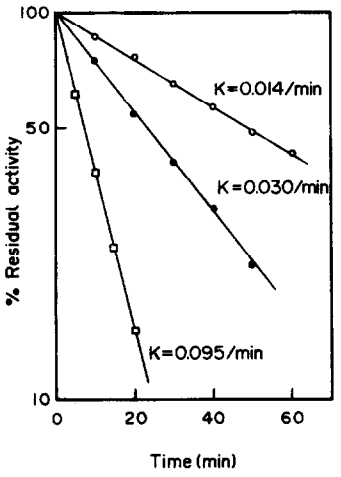

Fig. 4. Thermal inactivation of repressible extracellular and intracellular enzymes at 60°. ○ represents the purified extracellular enzyme assayed at pH 7.4; ● represents the purified enzyme assayed at pH 9.0 (an identical profile was obtained for the enzyme assayed at pH 5.4); □ represents the partially purified repressible intracellular enzyme assayed at pH 9.0. For details see Experimental.

EXPERIMENTAL

Chemicals. The following reagents were purchased from the sources indicated: glucose-6-phosphate (monosodium salt), yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin, α-chymotrypsinogen A, cytochrome *c*, DEAE-cellulose, Sephadex G-200-120 and blue dextran were from Sigma; sodium β-glycerylphosphate, phenylphosphate, *p*-nitrophenylphosphate (PNP-P), bis(*p*-nitrophenyl)phosphate (bis-PNP-P) and DTNB were from Merck. All other chemicals were of analytical reagent grade.

Growth conditions and maintenance of the organism. Stock cultures of the wild-type strain St. L. 74A of *Neurospora crassa* were maintained on slants of semi-solid Vogel's medium [13]. Conidia of this strain were suspended in sterilized, deionized H_2O, filtered with a double layer of gauze to remove contaminating mycelia and adjusted to 10^8 cells/ml. Unless otherwise stored, 0.5 ml of this conidial suspension was inoculated into each Petri dish (14 cm diameter), containing 50 ml low-phosphate medium (adjusted to pH 5.6 and supplemented with 44 mM NaOAc as carbon source) prepared as described in ref. [14]. After growth for 72 hr at 30° the culture medium was harvested by filtration.

Assay procedures. Enzyme assays were as described in ref. [15], carried out in 0.3 M glycine buffer (pH 9), containing 1 mM EDTA, using 2 ml 6 mM PNP-P, bis-PNP-P or phenylphosphate as substrates, at 37°. The reaction was stopped by the addition of 1 ml M NaOH, and the *p*-nitrophenol or phenol was measured at 405 nm (E = 17800/mol/cm) or 278 nm (E = 2600/mol/cm), respectively. When glucose-6-phosphate or β-glycerylphosphate (1.5 ml, 5 mM) was the substrate, the Pi liberated was measured as described in ref. [16], with the exception that the solvent used was EtOAc. Incubation was carried out at 37° for 15–30 min and the reaction was terminated by adding 0.5 ml cold 20% (w/v) TCA. All enzyme activities were measured in duplicate, for at least two time points. One unit of phosphatase activity is defined as 1 μmol substrate hydrolysed/min.

The buffers used to cover the pH range required, all containing 1 mM EDTA, were 0.1 M HOAc–NaOH (pH 5.5), 0.1 M Na maleate (pH 5.5–7.2), 0.1 M Tris–HCl (pH 7.2–9.0) and 0.1 M glycine–NaOH (pH 9.0–10.5). Protein was measured by a modification of the Lowry method as described in ref. [17] using bovine serum albumin as standard. Neutral sugars were measured by the method described in ref. [18] with glucose as standard. MW was measured by gel filtration as described in ref. [19] using a Sephadex G-200 column (1.5 × 116 cm), equilibrated and eluted with 10 mM NaOAc buffer (pH 5.2) containing 0.1 M NaCl, at a flow rate of 11 ml/hr (2.5 ml fractions). Yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin and α-chymotrypsinogen A were used as protein standards.

Disc electrophoresis was carried out on 7.5% acrylamide gel in Tris–glycine buffer (0.05 and 0.38 M, respectively), pH 8.3 as described in ref. [20]. Protein samples (10–50 μg) were layered in 30 μl glycerol. Bromophenol blue was used as the tracker dye. A constant current of 3 mA per gel was applied for 2 hr at 4°. The protein bands were located by staining with Coomassie brilliant blue. MW was also determined by SDS-disc electrophoresis on 10% (w/v) acrylamide gel containing 0.1% SDS as described in ref. [21]. Bovine serum albumin, ovalbumin, α-chymotrypsinogen A and cytochrome *c* were used as protein standards. The enzyme (3.3 μg/ml) was incubated for 3 hr at 37°, with DTNB as described in ref. [22]. The reaction mixture contained in a final vol. of 3 ml, 0.1 Tris–HCl buffer, pH 8 and 1 mM DTNB. Aliquots (0.1 ml) were taken at appropriate times to measure the *p*-nitrophenylphosphatase activity.

Thermal inactivation. The alkaline phosphatase (15 μg/ml) was incubated at 60° in a final vol. of 3 ml of the following buffers: 0.2 M NaOAc, pH 5.4, or 0.2 M Tris–HCl, pH 7.4 or pH 9.0. At appropriate times, samples (0.1 ml) were taken, at pH 9.0, to measure the residual *p*-nitrophenylphosphatase activity.

Kinetic studies. Maximum velocity (V_{max}) and Michaelis constant (K_m) were determined by plotting initial velocities as

described in ref. [23]. The apparent inhibitor constant for Pi was determined as described in ref. [24]. Interaction constant (n) was determined by the Hill procedure as described in ref. [25]. All kinetic constants given in this paper were obtained from linear-square analysis.

Enzyme purification. All purification steps were carried out at 0–4°. *p*-Nitrophenylphosphatase activity was determined at each stage of purification. After growth for 72 hr at 30°, the harvested culture medium (2190 ml) was freeze-dried and then suspended in H_2O to 100 ml. This conc protein soln was brought to 35% saturation by the addition of solid $(NH_4)_2SO_4$ (168 mg/ml). The mixture was stirred for 15 min, left standing for 1 hr and then centrifuged 15 min at 20 000 *g*. The ppt. was discarded and the supernatant was dialysed for 24 hr against 12 l. H_2O (with 4 changes). The dialysed enzyme was freeze-dried, suspended to 6 ml in 10 mM Tris–HCl buffer, pH 7.8, containing 0.2 M $(NH_4)_2SO_4$, and then applied to a Sephadex G-200 column (1.5 × 116 cm), which had been previously equilibrated with this buffer. Elution was performed with the same buffer at a flow rate of 6.4 ml/hr (3 ml fractions). The tubes representing the enzyme peak were pooled and dialysed for 48 hr against 8 l. of 10 mM Tris–HCl buffer, pH 7.8 (with 4 changes of buffer). The dialysed fraction was applied to a column (2.2 × 19 cm) of DEAE-cellulose previously equilibrated with the buffer used for dialysis. Enzyme elution was performed with the same buffer at a flow rate of 70 ml/hr (7 ml fractions). The absorbed proteins were eluted with 10 mM Tris–HCl, pH 7.8, containing 0.5 M NaCl and showed little phosphatase activity. The fraction corresponding to alkaline phosphatase was concd by ultrafiltration, dialysed for 48 hr against 3 l. H_2O and stored at 4°.

The intracellular alkaline phosphatase, derepressed by acetate-grown mycelium, was extracted as described in ref. [5], and then brought to 65% saturation by the addition of solid $(NH_4)_2SO_4$ (406 mg/ml). The mixture was stirred 15 min, left standing for 30 min and then centrifuged 15 min at 20 000 *g*. The ppt. was discarded and the supernatant, which contained *ca* 90% of the enzyme activity, was dialysed 24 hr against 8 l. of 0.2 M Tris–HCl buffer, pH 9 (with 4 changes of buffer) and stored at 4°.

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REFERENCES

1. Littlewood, B. S., Chia, W. and Metzenberg, R. L. (1975) *Genetics* **79**, 419.
2. Metzenberg, R. L. (1979) *Microbiol. Rev.* **43**, 361.
3. Metzenberg, R. L. and Chia, W. (1979) *Genetics* **93**, 625.
4. Free, S. J. and Metzenberg, R. L. (1982) *Biochem. Genet.* **20**, 883.
5. Nahas, E., Terenzi, H. F. and Rossi, A. (1982) *J. Gen. Microbiol.* **128**, 2017.
6. Lehman, J. R. and Metzenberg, R. L. (1976) *Genetics* **84**, 175.
7. Perkins, D. D., Radford, A., Newmeyer, D. and Bjorkman, M. (1982) *Microbiol. Rev.* **46**, 426.
8. Burton, E. G. and Metzenberg, R. L. (1974) *J. Biol. Chem.* **249**, 4679.
9. Hasunuma, K. and Ishikawa, T. (1977) *Biochim. Biophys. Acta* **480**, 170.
10. Means, G. and Feeney, R. E. (1971) in *Chemical Modifications of Proteins*. Holden-Day, San Francisco.
11. Levitzki, A. and Koshland, D. E., Jr. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 1121.
12. Atkinson, D. E., Hathaway, J. A. and Smith, E. C. (1965) *J. Biol. Chem.* **240**, 2682.
13. Vogel, H. J. (1956) *Microbiol. Genet. Bull.* **13**, 42.
14. Crocken, B. and Nyc, J. F. (1963) *Can. J. Microbiol.* **9**, 689.
15. Nyc, J. F., Kadner, R. J. and Croken, B. J. (1966) *J. Biol. Chem.* **241**, 1468.
16. Sanui, H. (1974) *Analyt. Biochem.* **60**, 489.
17. Hartree, E. F. (1972) *Analyt. Biochem.* **48**, 422.
18. Dubois, B., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Analyt. Chem.* **28**, 350.
19. Andrews, P. (1964) *Biochem. J.* **91**, 222.
20. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.
21. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406.
22. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70.
23. Lineweaver, H. and Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658.
24. Dixon, M. (1953) *Biochem. J.* **55**, 170.
25. Koshland, D. E., Jr. (1970) in *The Enzymes* (Boyer, P. D., ed.), Vol. I. Academic Press, New York.