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*

By ELY NAHAS,[†] HÉCTOR F. TERENCE[‡] AND ANTONIO ROSSI*

Departamento de Química, FFCLRP-USP, 14.100 Ribeirão Preto, SP, Brazil

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Growth conditions (carbon source and pH of the medium) affected the level and distribution of derepressed acid and alkaline phosphatases in *Neurospora crassa*. Regardless of the pH, the production of both acid and alkaline phosphatases was stimulated by sucrose. Irrespective of the carbon source used, at pH values higher than 7.4 the secretion of alkaline phosphatase was stimulated, while the production and secretion of acid phosphatase was restricted. The converse was true at pH values lower than 5.7. The secretion of one of these enzymes did not exclude the simultaneous secretion of the other at pH values of around 7.0. These results suggest that the pH of the medium may play an important role in the survival of the organism in phosphorus-limited environments.

INTRODUCTION

Neurospora crassa derepresses a number of phosphate-metabolizing enzymes during phosphate starvation, or mycelial growth on limiting concentrations of phosphate (Nyc *et al.*, 1966; Nyc, 1967; Kadner *et al.*, 1968; Lehman *et al.*, 1973; Hasunuma, 1973; Burton & Metzenberg, 1974; Hasunuma *et al.*, 1976). Under these conditions a wild-type strain secretes a significant fraction of the acid phosphatase produced into the growth medium, and retains most of the alkaline phosphatase in a cell-bound form (Kadner *et al.*, 1968; Lehman *et al.*, 1973; Hasunuma, 1973; Hasunuma & Ishikawa, 1977). The production of these two enzymes is reported to be regulated by the same derepression mechanism (Littlewood *et al.*, 1975; Metzenberg, 1979). However, the relative levels of these enzymes and their secretion into the growth medium could also be controlled by additional physiological factors. With this in mind, we investigated further the derepression and secretion of these two enzymes by *N. crassa* mycelium. Our results suggest that, besides the derepression by phosphate starvation, the carbon source used for growing the cells influences the level of phosphatase production and that, for a specified carbon source, the production and secretion of these two enzymes is dependent on the pH of the growth medium.

METHODS

Chemicals. 4-Nitrophenyl disodium orthophosphate (PNP-P) was purchased from Merck. All other chemicals were of analytical reagent grade.

Strains and maintenance of organisms. The following strains of *N. crassa* were used; wild-type strain St.L. 74A, the *nuc-1 A* mutant FGSC 1995, the *nuc-2 A* mutant FGSC 1996 and the *pho-2 A* mutant FGSC 3061. Stock cultures were maintained on slants of semi-solid Vogel's (1956) medium.

Growth conditions and preparation of cell extracts. Conidia from each strain were suspended in sterilized

[†] Present address: Departamento de Microbiologia, FCAVJ-UNESP, 14.780 Jaboticabal, SP, Brazil.

[‡] Present address: Departamento de Fisiologia, FMRP-USP, 14.100 Ribeirão Preto, SP, Brazil.

deionized water and filtered with a double layer of gauze to remove contaminating mycelia. Conidial suspensions were adjusted to 10^8 cells ml^{-1} . Unless otherwise mentioned, 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) prepared as previously described (Crocken & Nyc, 1963). After a period of growth the mycelium was harvested by filtration, washed with distilled water and pressed to remove excess liquid. It was then ground in a pre-cooled mortar with washed sand and 50 mM-sodium acetate buffer, pH 5.0 [40 ml buffer (g mycelium) $^{-1}$]. The mycelial extract was centrifuged at 20000 g for 20 min, at 4 °C, and the supernatant used for enzyme assays without further treatment.

Enzyme assays. The repressible acid phosphatase assay was carried out in 0.1 M-sodium acetate buffer (pH 5.4), using 2 ml 6 mM-PNP-P as substrate, at 37 °C. The reaction was stopped by the addition of 1 ml 1 M-NaOH, and the *p*-nitrophenoxide ion absorbance measured at 405 nm ($\epsilon = 17800 \text{ l mol}^{-1} \text{ cm}^{-1}$). The repressible alkaline phosphatase assay was carried out in 0.3 M-glycine buffer (pH 9.0) containing 1 mM-EDTA, using the same procedure as for acid phosphatase (Nyc *et al.*, 1966). Both enzyme activities were assayed in the culture filtrate and the cell extracts. One unit of phosphatase activity was defined as 1 μmol substrate hydrolysed h^{-1} . Specific activities were expressed as units (mg dry wt mycelium) $^{-1}$.

RESULTS AND DISCUSSION

The production of acid and alkaline phosphatase by *N. crassa* when grown on sucrose is shown in Fig. 1. Confirming earlier reports (Nyc, 1967), the production of acid phosphatase was stimulated, with a significant fraction of this enzyme being secreted into the growth medium. It can also be seen that the production of alkaline phosphatase was stimulated and that most of its activity was present in the mycelial extract. When the organism was grown on acetate, the production of acid and alkaline phosphatase was, respectively, 17% and 160% of that obtained in sucrose-grown cultures (Table 1). Moreover, about 40% of the alkaline phosphatase produced by the fungus in acetate cultures was secreted into the growth medium. It was previously reported (Hasunuma & Ishikawa, 1977) that when a wild-type strain of *N. crassa* was grown on sucrose, a significant fraction of the alkaline phosphatase produced was secreted into the growth medium. Our results show that only about 5% of the alkaline phosphatase produced by the fungus on sucrose cultures was secreted into the growth medium (Fig. 1). However, after growth on acetate the secretion of this enzyme was enhanced about 10-fold (Table 1). When *N. crassa* was grown on glycerol the production of acid and alkaline phosphatase was only 25% of that found when it was grown on sucrose. It was also observed that the relationship between production and secretion of these enzymes was similar to that obtained for the sucrose-grown cultures (data not shown). The pH of the medium remained almost constant during mycelial growth on sucrose (inset Fig. 1) and glycerol (data not shown) while during growth on acetate the pH increased, reaching 8.8 after 72 h incubation (Table 1). When the fungus was grown on a mixture of acetate and variable concentrations of sucrose, the production and secretion of acid phosphatase was stimulated by sucrose (Fig. 2). Nevertheless, the production and secretion of this enzyme was lower than that obtained for cultures grown on sucrose alone (Fig. 1). The production of alkaline phosphatase was also stimulated by sucrose in combination with acetate (Fig. 2). However, this was only observed for the mycelial-bound enzyme. It can also be seen that, after growth for 72 h on acetate/sucrose (44 mM), the pH was about two units lower than after growth on acetate alone (inset Fig. 2). From the results presented above it might be supposed that the pattern of derepression of acid and alkaline phosphatase by mycelium is affected by (apart from phosphate starvation) variations in the pH and/or by the carbon source used for growing the cells.

For a further understanding of the effects of carbon source and pH on the production and secretion of acid and alkaline phosphatases, the wild-type strain of *N. crassa* was grown on sucrose and acetate, buffered with 50 mM-Tris/HCl at pH 7.5 and with 100 mM-sodium maleate at pH 5.4, respectively. (It was previously observed that neither of these buffers could be utilized by the fungus as carbon or nitrogen sources.) From the results in Table 1 it is clear that the production and secretion of alkaline phosphatase was stimulated by the increase in the pH of the sucrose cultures. The results also show that the secretion of acid

Fig. 1. Production of acid and alkaline phosphatases in cultures of *N. crassa* (St.L. 74A) grown on sucrose (44 mM) under limiting phosphate conditions (50 μ M), at 30 °C. ●, Mycelial-bound acid phosphatase; ○, extracellular acid phosphatase; ■, mycelial-bound alkaline phosphatase; □, extracellular alkaline phosphatase. Inset: Δ, dry weight of mycelial yield (mg per 50 ml medium); ▲, pH of medium. See Methods for details.

Table 1. Activity of acid and alkaline phosphatases in cultures of *N. crassa* (St.L. 74A) grown under different conditions for 72 h

Carbon source and medium conditions	Final pH of medium	Acid phosphatase*			Alkaline phosphatase*		
		Mycelial extract	Culture medium	Total	Mycelial extract	Culture medium	Total
Sucrose (non-buffered medium)	5.6	1.4	4.7	6.1	3.9	0.2	4.1
Sucrose (buffered medium)	7.5	1.0	1.0	2.0	10.5	5.3	15.8
Sucrose + acetate (non-buffered medium)	6.9	2.0	2.0	4.0	11.3	3.2	14.5
Acetate (non-buffered medium)	8.8	0.6	0.4	1.0	4.0	2.6	6.6
Acetate (buffered medium)	5.4	0.8	1.5	2.3	2.5	0.3	2.8

* Units (mg dry wt mycelium)⁻¹.

phosphatase was significantly reduced, with the cell-bound enzyme level remaining close to that obtained from the non-buffered sucrose cultures (Fig. 1). In contrast, when *N. crassa* was grown on buffered acetate media (pH 5.4), the secretion of alkaline phosphatase was significantly reduced, the intracellular enzyme level also being lowered (Table 1). Furthermore, the production of acid phosphatase was stimulated, and most of this enzyme activity was secreted into the growth medium. The same phenomena were observed when the mycelium harvested from cultures grown on high-phosphate supplemented media (3 d old),

Fig. 2. Final specific activity of acid and alkaline phosphatases in cultures of *N. crassa* (St.L. 74A) grown on acetate (44 mM) and mixtures of acetate (44 mM) and sucrose (11, 22 and 44 mM), under limiting phosphate conditions (50 μ M), at 30 °C for 72 h. ○, Mycelial-bound acid phosphatase; ●, extracellular acid phosphatase; ■, mycelial-bound alkaline phosphatase; □, extracellular alkaline phosphatase. Inset: △, dry weight of mycelial yield (mg per 50 ml medium); ▲, final pH of medium. See text for details.

was re-incubated in all conditions of phosphate starvation described in this paper (data not shown).

The secretion of alkaline phosphatase as a consequence of possible structural changes of the cell membrane during growth on acetate can be excluded, since this enzyme was not secreted by the fungus when grown on acetate buffered at pH 5.4 (Table 1). Cell lysis may also be ruled out, because the secretion of alkaline phosphatase was observed in the growing phase of mycelia.

The data presented in this report show that, regardless of the pH, the production of both acid and alkaline phosphatase was stimulated by sucrose. On the other hand, irrespective of the carbon source used, the pH seems to represent an important physiological factor which selectively affected the secretion of one of these two enzymes. Thus, at pH values lower than 5.7 (non-buffered sucrose and buffered acetate media) most of the derepressed acid phosphatase was secreted into the growth medium by the fungus. Under these conditions, only a small fraction of the alkaline phosphatase produced was secreted by the wild-type strain. At pH values close to 7.0 (buffered sucrose and non-buffered sucrose/acetate media) significant fractions of both enzymes were secreted by the fungus, the secretion of alkaline phosphatase being predominant. At pH values close to 9.0 (non-buffered acetate medium) the production and secretion of acid phosphatase was more restricted, and about 40% of the alkaline phosphatase produced was secreted into the growth medium. The production and secretion of alkaline phosphatase by mycelium grown on non-buffered acetate or buffered sucrose medium was investigated in the regulatory mutants *nuc-1* and *nuc-2*. These mutants do not derepress a number of enzymes associated with phosphorus assimilation, including acid and alkaline phosphatase (Littlewood *et al.*, 1975). These mutants produced less than 0.1 unit of alkaline phosphatase (mg dry weight)⁻¹ under all conditions specified in this paper. Similarly, in the *pho-2* mutant, which is considered to represent the structural locus of the repressible alkaline phosphatase (Lehman & Metzberg, 1976), the production of this enzyme was negligible. Thus, it appears that the effects of pH and the carbon source involved the participation of the regulatory circuit already proposed (Metzberg, 1979), and that the secreted alkaline phosphatase and that retained by the mycelium when the wild-type strain

was grown on acetate and sucrose, respectively, were the product of the *pho-2* gene. However, they may differ in the degree of post-translational modification, as already noted for the alkaline phosphatase secreted by 'slime' cells (Burton & Metzenberg, 1974).

From these results it is clear that the pattern of secretion of derepressed acid and alkaline phosphatase is affected by the pH of the medium. This property may be of physiological significance for the survival of the organism in a phosphorus-limited environment.

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