A REPRESSIBLE ACID PHOSPHATASE IN NEUROSPORA CRASSA*

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This communication concerns some of the properties of a heretofore unknown orthophosphate-repressible acid phosphatase in Neurospora crassa. Previous work in this laboratory (Nyc et al., 1966) had disclosed the presence of an orthophosphate-repressible alkaline phosphatase. Both an acid phosphomonoesterase and an alkaline phosphomonoesterase are also known in this organism. Kuo and Blumenthal (1961 a,b,c) had shown that these two enzymes are not repressed by high levels of inorganic phosphate in the growth medium. In the present studies, the repressible acid phosphatase was recovered from both the tissues and the culture media of a normal strain of N. crassa grown on limiting amounts of phosphate.

METHODS - Normal strain 1A of N. crassa was grown on a basic phosphate-free medium (Crocken and Nyc, 1963) supplemented with variable amounts of potassium phosphate. The mycelium used for routine extraction of enzymes came from derepressed 4-liter aerated cultures containing 50 μmoles of P_i per liter. The studies concerning the effect of P_i on enzyme produced by the mold were done with 100-ml surface cultures grown in Roux flasks. All cultures were grown at 25° and harvested 60-72 hours after conidial inoculation.

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All procedures involving extraction and purification of enzymes were done at 2-4° and deionized distilled water was used. For routine extraction of the repressible acid phosphatase the mycelium was ground in a TenBroeck homogenizer with 15 parts of 0.05 M acetate buffer, pH 5.0. The homogenate was centrifuged for 15 min at 12,000 X g. The residue resulting after decanting the supernatant was resuspended in 5 parts water and reprecipitated by centrifugation as before. The combined extracts, containing over 90% of the measurable repressible acid phosphatase, were used to determine the extractable amounts of this enzyme in N. crassa. In the repression studies where an estimate of the total enzyme produced was desired the amount of enzyme in the culture medium was also determined.

The standard assay for the repressible acid phosphatase involved incubating the enzyme for 10 min at 37° in a 2.5 mM solution of the substrate p-nitrophenyl phosphate in 0.3 M acetate buffer at pH 5.6, the optimal pH. Hydrolysis was proportionate to enzyme concentration at the levels used (0.1 to 1.0 units per ml incubation) and remained linear with time for 20 min at pH 4.0 to 5.6. The repressible alkaline phosphatase was determined as previously described (Nyc et al., 1966) with p-nitrophenyl phosphate as substrate. The p-nitrophenol released by these enzymes was determined by the method of Bessey et al. (1946). A unit of enzyme activity was defined as the quantity of enzyme that catalyzes the release of 1 μmole of P_i per hour from p-nitrophenyl phosphate at these specified conditions and all data were converted to these units. Specific activity was defined as the enzyme units per mg of protein. With the exception of the repression studies (Table 1), all other determinations were made on acid phosphatase that had been separated by gel-filtration from other known phosphatases that interfere with its determination by the standard assay. Except for p-nitrophenyl phosphate and bis-(p-nitrophenyl) phosphate, all other substrates were assayed for 20 min under the prescribed conditions, and the P_i released was determined by the method of Fiske and Subbarow (1925). Protein was measured according to Lowry et al. (1951) with bovine albumin as the reference standard.

The enzyme content of tissues used for repression studies (Table 1) was determined with crude mycelial extracts since further enzyme purification might have affected the quantitative recovery. Here the standard assay for the repressible acid phosphatase was done at pH 4.0 to negate interference by the

TABLE 1. Repression of Phosphatases by P_i

P_i in medium	Alkaline phosphatase in mycelium[+]		Acid phosphatase			
			In mycelium[+]		In medium[*]	
	1[±]	2	1	2	1	2
50 μM	5.50	5.70	5.88	5.60	5.72	7.30
200 μM	4.80	4.68	5.10	5.50	4.75	4.86
500 μM	2.40	2.56	2.10	2.58	3.54	2.88
1 mM	0.09	0.07	<0.01	<0.01	0.72	0.90
5 mM	0.02	0.02	<0.01	<0.01	0.78	0.84

[+]Units per mg dry tissue
[*]Units recovered per mg dry tissue in culture
[±]Duplicate cultures

alkaline phosphatase which expresses some activity at pH 5.6. The acid phosphatase retains 80% of its optimal activity at pH 4.0 and values obtained at this pH were readily converted to the standard units. The nonrepressible acid phosphomonoesterase also utilizes p-nitrophenyl phosphate as a substrate at pH 4.0, and a correction for it was needed when the repressible acid phosphatase was assayed in crude preparations. The acid phosphomonoesterase is the only phosphatase in N. crassa extracts that utilizes β-glycerophosphate (20 mM) as a substrate at pH 4.0. The relative activities of this enzyme with respect to β-glycerophosphate and p-nitrophenyl phosphate, under conditions defined in these studies, were used to correct for it in the assays for the repressible acid phosphatase. The acid phosphatase found in culture media was readily determined since negligible amounts of the other phosphatases were excreted by fast-growing cultures. The assay for the repressible alkaline phosphatase is specific for this enzyme and unaffected by other phosphatases.

RESULTS - The data in Table 1 show the effect of variable P_i in the growth medium on the concentration of the repressible acid phosphatase in the mycelia and culture media of N. crassa harvested after 60 hours of growth. The relative effect of P_i repression on the repressible alkaline phosphatase, found only in the mycelium, is also shown for the same cultures in Table 1. The acid phosphatase in crude extracts of mycelium from derepressed cultures with a specific activity of about 12 was routinely fractionated by column chromatography on Sephadex G-150 to yield a 10-fold purified enzyme. The same purification procedure applied to the acid phosphatase recovered from dialyzed and lyophilized culture media yielded preparations with a specific activity of about 170. The data in Table 2 compare the elution peaks of three N. crassa phosphatases with marker proteins of known molecular weights in the eluate of a Sephadex G-150 column (2.5 X 80 cm) with 0.05 M acetate, pH 5.0, as the mobile phase. The nonrepressible alkaline phosphomonoesterase was not recovered in these studies since it is destroyed at pH 5.0 at which the acid phosphatase of primary interest here is very stable.

TABLE 2. Elution Peaks from Sephadex G-150 Column

Substance	Molecular weight	Elution peak (ml eluate)
Blue dextran 2000	2×10^6	190
Acid phosphomonoesterase		243
Alkaline phosphatase (R)*		250
Bovine serum albumin	6.9×10^4	308
Acid phosphatase (R)*		323
Ovalbumin	4.5×10^4	349
Myoglobin (horse)	1.78×10^4	402

*P_i-repressible

With the exception of the repression studies, all other properties of the acid phosphatase were determined with preparations recovered from the mold and fractionated by the gel-filtration procedure to specific activities above 100. Solutions of the enzyme at pH 5.0 were stored in a frozen state with very little loss of activity after two weeks.

The repressible acid phosphatase has optimal activity at pH 5.6 and retains 80, 91, 72 and 22% of this activity in the standard assay at pH 4.0, 5.0, 6.0 and 6.5, respectively. The enzyme retains most of its activity after dialysis for 16 hours at 4° against dilute acetate buffer at pH 5.0, and the presence of EDTA at levels up to 100 mM in the standard assay has no effect on its activity. This phosphatase is markedly inhibited by P_i. The kinetics of this inhibition with p-nitrophenyl phosphate is of the competitive type whereby P_i increases the K_m for this substrate without changing the maximal velocity. In the standard assay the K_m for p-nitrophenyl phosphate was calculated to be 5.0×10^{-4} M, and the K_i for P_i, 3.6×10^{-4} M. This acid phosphatase is more substrate-specific than either the repressible alkaline phosphatase or the two phosphomonoesterases in N. crassa. It expresses phosphodiesterase activity with bis-(p-nitrophenyl) phosphate (2.5 mM) as substrate in the standard assay with a relative hydrolysis rate of 50% that observed for p-nitrophenyl phosphate. The enzyme also uses phosphoenolpyruvate and phenylphosphate as substrates but does not release measurable P_i or nitrophenol from the following compounds tested in the standard assay at a 10 mM concentration: phosphocholine, 3-phospho-L-serine, O-phosphoethanolamine, α-D-glucose 1-phosphate, α-D-glucose 6 phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, ribose 5-phosphate, β-glycerophosphate, 2-phosphoglyceric acid, 3-phosphoglyceric acid, diphosphopyridine nucleotide, AMP, PP_i, trimetaphosphate, p-nitrophenyl acetate and p-nitrophenyl sulfate.

DISCUSSION - A P_i-repressible acid phosphatase was obtained from both the culture medium and the mycelium of N. crassa. It is a fairly specific acid phosphomonoesterase and in its present state of purification exhibits phosphodiester-

ase activity. The general term "repressible acid phosphatase" was used in this communication to distinguish it from the previously described nonrepressible acid phosphomonoesterase. These two acid esterases are readily separated by gel-filtration on Sephadex G-150. The esters serving as substrates for the repressible acid phosphatase have in common a conjugated double-bond system associated with the carbon involved in the phosphate linkage. This enzyme, like the repressible alkaline phosphatase in N. crassa, undoubtedly assumes some essential role in the cell when P_i becomes limiting.

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