Phosphate utilization and constitutive synthesis of phosphatases in *Thermoactinomyces vulgaris* Tsilinsky

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Thermoactinomyces vulgaris utilized both organic and inorganic phosphates with equal efficiency for its growth. The specific activities of the thermophilic acid and alkaline phosphatases were found to be maximum at 1 mM concentration of each phosphate source. All the phosphatase isoenzymes (three alkaline and one acidic) were observed irrespective of the substrate source and concentration, suggesting constitutive synthesis of the enzymes. During growth and differentiation, both acid and alkaline phosphatases exhibited uniformly stable patterns of isoenzymes with slight variations in their specific activities.

The participation of inorganic phosphate in the regulation of phosphatases has been recognized since the first description of *Escherichia coli* alkaline phosphatase (EC 3.1.3.1), whose activity exhibited an inverse relationship to the concentration of exogenous phosphate (Torriani, 1960). Mutants producing alkaline phosphatase constitutively were able to hydrolyse *sn*-glycero-2-phosphate to glycerol, which in turn could serve as a sole source of carbon and energy (Torriani & Rothman, 1961). Alkaline phosphatase was also found to be constitutive in the wild type strain of *Salmonella typhimurium* (Carlillo-Castaneda & Ortega, 1967) and one of its mutant strains TA 2365 (Kier *et al.*, 1977), in four out of five strains of marine bacteria tested (Hassan & Pratt, 1977) and in *Blastocladia emersonii* (Selitrennikoff & Sonneborn, 1977). The activity *in vivo* was sensitive to exogenous inorganic phosphate, except in the first case. The role of phosphatases in fulfilling the phosphate requirement for growth has been well documented in *Klebsiella aerogenes* (Bolton & Dean, 1972), *Anacystis nidulans* (Ihlenfeldt & Gibson, 1975), *E. coli* (King & Francis, 1975; Willsky & Malamy, 1976), *Saccharomyces cerevisiae* (Ueda *et al.*, 1975) and *Chlamydomonas reinhardtii* (Patni *et al.*, 1977).

Thermophilic phosphatases have been reported from a variety of micro-organisms, including *Bacillus stearothermophilus* (Amlunxen & Lins, 1968), *Bacillus licheniformis* (Hulett-Cowling & Campbell, 1971) and *Thermus aquaticus* (Yeh & Trella, 1976; Smile *et al.*, 1977). However, information concerning differential utilization of phosphate

sources for growth, and the synthesis and amounts of phosphatase isoenzymes during various phases of growth and differentiation is lacking. The present investigation was, therefore, undertaken to study growth pattern and phosphatase formation in *Thermoactinomyces vulgaris* in response to various physiological concentrations of different phosphate sources. The catalytic activities of both acid (EC 3.1.3.2) and alkaline phosphatases, together with isoenzyme synthesis at various phases of growth and differentiation have also been studied for an insight into their regulated synthesis in this thermophilic microbe.

Materials and methods

The wild type strain of *Thermoactinomyces vulgaris* Tsilinsky (stock no. 1227), which grows optimally at 50–52°C, was kindly supplied by Professor D. A. Hopwood, John Innes Institute, Norwich, U.K. The media recommended by Hopwood & Wright (1972) were used with certain modifications. Minimal medium with casaminoacid (MMC) was used for liquid shake cultures. Approximately 10⁹ spores/30 ml of the medium, in 250 ml flasks, were incubated in a gyrotory shaker (New Brunswick, model G25) at 50–52°C at 150 rev./min. The mycelium was harvested on Whatman no. 1 filter paper and homogenized in 0.025 M-veronal buffer (pH 8.2). The slurry was centrifuged at 15000g for 30 min at 4°C and the supernatant was used as the enzyme extract.

The suitably diluted enzyme extract (0.2 ml, containing 25–75 µg of protein) was incubated for 30 min with 0.4 ml of 15 mM-*p*-nitrophenyl phosphate;

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for alkaline phosphatase incubation was at 70°C in 0.05 M-Tris/HCl buffer (pH 9.5) and for acid phosphatase at 65°C in 0.25 M-sodium acetate buffer (pH 6.0). Another set of assays contained 10 mM-MgCl₂, as it stimulates both the phosphatases of *T. vulgaris* (Sinha *et al.*, 1979). The reaction rates remained constant, both in the presence and absence of Mg²⁺, for 45 min. The reaction was terminated with 1 ml of 1 M-NaOH and the absorbance was read at 410 nm on a Bausch and Lomb Spectronic-20 spectrophotometer. Protein was estimated by Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as standard. The specific activity was expressed as nmol of *p*-nitrophenol released/min per mg of protein.

Electrophoretic analysis of enzymes was done at 4°C by the method of Davis (1964) in 15% (w/v) polyacrylamide gels with a current of 3 mA/gel tube. After electrophoresis the gels were incubated for approx. 30 min in 20 mM-*p*-nitrophenyl phosphate with 20 mM-MgCl₂ for both acid and alkaline phosphatases at their respective pH and temperature optima. Intense yellow bands of *p*-nitrophenol appeared in the gels, which were photographed immediately after stopping the reaction with 1 M-NaOH.

Results and discussion

The growth of *Thermoactinomyces vulgaris* continued to be exponential for 10 h. Growth (mycelial dry wt./30 ml of the medium), after 9 h, in the presence of *sn*-glycero-2-phosphate, KH₂PO₄ or combinations of the individual moieties of glycerol

phosphate, i.e. glycerol and phosphate, was almost the same provided that the concentration of substrate was 1 mM (Table 1). Glycerol alone had no effect, but KH₂PO₄ alone increased the dry weight by about 60%. KH₂PO₄ was not as effective at 5 mM as at 1 mM. Under these conditions, 50% of the spores germinated by 2 h and maximum germination of 70% was reached in 3 h. In *Chlamydomonas reinhardtii* ribose 5-phosphate (organic phosphate) has been found to cause approx. 44% more growth as compared with an equimolar concentration of inorganic phosphate (Patni *et al.*, 1977). In contrast with these findings, *T. vulgaris* appears to utilize both organic and inorganic phosphates with equal efficiency. Since the phosphate concentration in the medium influences the synthesis of phosphatases in various micro-organisms, such as efficient system of phosphate utilization prompted us to investigate the amounts of both acid and alkaline phosphatase isoenzymes in cultures grown with organic or inorganic phosphate. The cultures were grown for 9 h with varying concentrations (zero, 1.0 or 5.0 mM) of *sn*-glycero-2-phosphate, KH₂PO₄, glycerol, glycerol + KH₂PO₄ or glycerol + *sn*-glycero-2-phosphate, and were assayed for phosphatases in the absence as well as the presence (10 mM) of Mg²⁺. The specific activities of acid and alkaline phosphatases under these conditions suggested that a 1 mM concentration of these compounds resulted in optimum activities. At a higher concentration (5 mM), the specific activities decreased, but never below 50% (alkaline phosphatase) or 60% (acid phosphatase) of the activities in cultures grown in 1 mM-substrate. Glycerol was an exception, the

Table 1. Effect of various compounds on the dry weight of, and specific activities of acid and alkaline phosphatases in, *T. vulgaris*

Wild type strain 1227 was grown for 9 h with different concentrations of *sn*-glycero-2-phosphate, KH₂PO₄, glycerol, glycerol + KH₂PO₄ and glycerol + *sn*-glycero-2-phosphate. Enzyme extracts were prepared as described in the Materials and methods section. Mg²⁺ where indicated was present at 10 mM. Each result is the average of duplicate determinations.

Compounds added	Concentration (mM)	Dry weight (mg/30 ml of medium)	Specific activity (nmol of <i>p</i> -nitrophenol released/min per mg of protein)			
			Acid phosphatase		Alkaline phosphatase	
			-Mg ²⁺	+Mg ²⁺	-Mg ²⁺	+Mg ²⁺
None	—	39.7	43.2	150.7	26.1	65.2
<i>sn</i> -Glycero-2-phosphate	1	62.8	76.2	256.7	43.4	107.2
	5	62.0	47.7	171.1	26.4	57.4
KH ₂ PO ₄	1	63.0	76.0	267.9	50.4	124.4
	5	56.3	53.9	192.5	25.6	81.5
Glycerol	1	39.7	25.7	104.5	11.0	34.8
	5	40.0	30.9	115.7	15.4	40.1
Glycerol + KH ₂ PO ₄	1 (each)	62.5	62.3	223.1	40.3	103.9
	5 (each)	59.5	44.3	160.4	27.5	72.6
Glycerol + <i>sn</i> -glycero-2-phosphate	1 (each)	60.5	65.7	236.8	42.8	114.5
	5 (each)	62.0	57.5	188.1	36.5	86.9

activities of both acid and alkaline phosphatases at 1 mM- and 5 mM-glycerol were more or less the same, though their amounts were low.

In contrast with the findings of Carlillo-Castaneda & Ortega (1967) on the alkaline phosphatase of *Salmonella typhimurium*, which does not appear to be repressible and whose amount *in vivo* is not sensitive to phosphate in the culture medium, our observations suggest that both acid and alkaline phosphatases of *T. vulgaris* are synthesized constitutively, and their amounts *in vivo* are only partially sensitive to phosphate in the culture medium. Electrophoretic analyses of enzyme samples prepared from cultures grown in zero, 1.0 or 5.0 mM organic or inorganic phosphate, and localized with *p*-nitrophenyl phosphate, revealed the existence of one acidic and three alkaline isoenzymes in all cases (Fig. 1). These observations are suggestive of a constitutive nature of all the isoenzymes of phosphatases within the limits of the phosphate concentrations tried. Our findings are similar to those of Willsky & Malamy (1976), Hassan & Pratt (1977), Kier *et al.* (1977) and Selitrennikoff & Sonneborn (1977). The acidic and

alkaline phosphatase II had the same mobility. It is not clear if these two activities are due to the same protein.

To study the pattern of phosphatase synthesis during growth and differentiation, *T. vulgaris* cultures were grown for 5 (since visible growth starts only around this time), 6, 9 and 12 h, using 1 mM-*sn*-glycero-2-phosphate as the sole phosphate source. The specific activities of both acid and alkaline phosphatases were found to be maximum at 5 h, followed by a slight decrease up to 6 h (Fig. 2). From 6–12 h of growth the specific activities remained constant. Spores had 6% of the acid phosphatase and 15% of the alkaline phosphatase activities found in 9 h old mycelia. Again, the activities were much higher if assayed in the presence of Mg^{2+} . All the phosphatase isoenzymes were also observed in these cultures. Our observations are similar to those made by Selitrennikoff & Sonneborn (1977) on the non-repressible alkaline phosphatase of *Blastocladiella emersonii*, where the change in

Fig. 1. Electrophoretic patterns of phosphatases from *T. vulgaris*

Preparation of enzyme extract from the wild type strain 1227 (grown for 9 h) and electrophoresis was carried out as described in the Materials and methods section. Protein (80 μ g) was loaded on each gel. The photograph shows the positions of alkaline (a) and acid (b) phosphatase isoenzymes.

Fig. 2. Specific activities of acid and alkaline phosphatases at different times of growth of *T. vulgaris*

The mycelia were harvested at different time intervals. Enzyme extracts were prepared as described in the Materials and methods section. Alkaline (●, ○) and acid (▲, △) phosphatases were assayed in the absence (○, △) or presence (●, ▲) of 10 mM- $MgCl_2$. Each result is the average of duplicate determinations.

activity through the life cycle (as from 5–6 h in our study) might, indeed, be developmentally regulated.

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