Differential expression of thermophilic phosphatases in the wild type and auxotrophic mutant strains of *Thermoactinomyces vulgaris*

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Abstract In the wild type strain (stock no.1227) of *Thermoactinomyces vulgaris*, as reported earlier [Sinha and Singh (1980) Biochem. J. 190, 457–460], all phosphatase isoenzymes (three alkaline – AlpI, AlpII and AlpIII, and one acidic – Acp) are present. However, the auxotrophic mutants, the strains 1286 (*thi*⁻), 1279 (*nic*⁻, *ura*⁻) and 1278 (*thi*⁻, *ura*⁻) exhibited two alkaline phosphatase isoenzymes (AlpII and AlpIII), but AlpI was lacking. In the strain 1261 (*nic*⁻, *thi*⁻), only AlpIII was expressed, and AlpI and AlpII isoenzymes were missing. The results suggest that the strains, which require either thiamine (1286 and 1278) or nicotinamide (1279) for their growth, were *AlpI*⁻ mutants; and the strain (1261), which requires both thiamine and nicotinamide for its growth, was *AlpI*⁻/*AlpII*⁻ double mutant. There was no direct correlation between uracil auxotrophy and the expression of phosphatases. The uniform expression of AlpIII and Acp in all the strains, irrespective of their nutrient requirements, suggest that these constitutive phosphatases are species-specific. The specific activities of the thermophilic acid and alkaline phosphatases were maximum in the wild type strain (1227) of *T. vulgaris*. The next in phosphatase activity was the strain 1279 (an *AlpI*⁻ mutant), followed by their decrease, in order, in the strains 1286 and 1278 (which were also *AlpI*⁻ mutants); while least activity of these enzymes was observed in the obligate thermophile strain 1261 (*AlpI*⁻/*AlpII*⁻ double mutant).

Key words: auxotrophic mutants · obligate thermophile · phosphatase isoenzymes · phosphatase mutations · *Thermoactinomyces vulgaris*.

Introduction

Thermophilic enzymes are receiving considerable attention of biotechnologists due to their application in high temperature catalysis of various industrialized enzymatic processes^{1–3}. Among enzymes of microbial origin, phosphatases have been considered to be one of the most versatile groups of hydrolases, as they regulate the phosphate metabolism and maintain the phosphorus economy of the cell to fulfill its growth as well as bioenergetic requirements under different environmental extremes^{4,5}. The participation of inorganic phosphate in the regulated expression of repressible phosphatases has been recognized since the first description of *Escherichia coli* alkaline phosphatase (EC 3.1.3.1)⁶. However, nonspecific acid (EC 3.1.3.2) and alkaline phosphatases of *Mycobacterium bovis* BCG have been found to be constitutive⁷; and the strains of *Serratia marcescens* synthesise two different types of alkaline phosphatases, a constitutive and an inducible⁸.

Investigations on the thermophilic phosphatases have been carried out in a variety of thermophiles with respect to their thermostability^{9–11}, their nature of synthesis^{12,13} and their enhanced rates of catalysis in response to metal ions⁹. Also in our earlier studies, thermophilic acid and alkaline phosphatases of an obligate thermophile – *Thermoactinomyces vulgaris* were characterized in relation to these parameters^{14–16}. However, most of these investigations were confined to the characterization of phosphatases from the wild type organisms; and till to date there is no report which could account for the expression of phosphatases (in terms of appearance or disappearance of their isoenzymes) in any thermophilic microbe in response to the specific nutrient requirements of its auxotrophic mutants. The present investigation was, therefore, undertaken to study the thermophilic acid and alkaline

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phosphatases in the wild type strain (stock no.1227) of *T. vulgaris* as well as its four auxotrophic mutants (strains 1286, 1279, 1278 and 1261) so as to look for their differential expression by comparing their isoenzyme patterns as well as specific activities.

Materials and Methods

Organism, culture conditions and preparation of enzyme extracts: The wild type strain of *Thermoactinomyces vulgaris* (stock no. 1227) and its four auxotrophic mutant derivatives (strain nos. 1286, 1279, 1278 and 1261), which grow optimally at 50–52°C, were obtained from Professor Sir D.A. Hopwood, FRS, John Innes Centre, Norwich, U.K. The media recommended by Hopwood and Wright¹⁷ were used with certain modifications¹⁵. For growing wild type strain, minimal medium with casamino acid (DIFCO, Michigan, USA) (MMC) was used. Liquid media were prepared without adding agar. The media for culturing auxotrophic mutants consisted of MMC supplemented with specific growth requirements of the respective strains⁵. The nutrient requirements of mutant strains are as follows:

Strain no. (auxotrophic mutant)	Nutrient requirement (conc. in the medium)
1286 (<i>thi</i> ⁻)	Thiamine (1 g/ml)
1279 (<i>nic</i> ⁻ , <i>ura</i> ⁻)	Nicotinamide (1 g/ml), Uracil (10 g/ml)
1278 (<i>thi</i> ⁻ , <i>ura</i> ⁻)	Thiamine (1 g/ml), Uracil (10 g/ml)
1261 (<i>nic</i> ⁻ , <i>thi</i> ⁻)	Nicotinamide (1 g/ml), Thiamine (1 g/ml)

The methods for raising the cultures and assays of phosphatases along with their isoenzyme studies were described earlier¹⁵ except that, instead of disc gel electrophoresis, slab gel electrophoresis was employed in the present investigation for localizing acid and alkaline phosphatases in the polyacrylamide gels, using *p*-nitrophenyl phosphate as the substrate.

Assays of phosphatases: 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 (AR, Central Drug House, India). Incubation was done at 70°C in 0.05 M-Tris/HCl buffer (pH 9.5) for alkaline phosphatase and at 65°C in 0.25 M-sodium acetate buffer (pH 6.0) for acid phosphatase. Another set of assays contained 10 mM-MgCl₂, as it stimulates both the phosphatases of *T. vulgaris*¹⁴. The reaction rates remained constant for 45 min. both in the presence and absence of Mg²⁺. The reaction was terminated with 1 ml of 1 M-NaOH, and the absorbance was read at 410 nm on a UV spectrophotometer (UV-1700 Pharma Spec Shimadzu). Protein was estimated

by Lowry's method¹⁸, using bovine serum albumin (BSA) as standard. The specific activity was expressed as nmol of *p*-nitrophenol released/min per mg of protein.

Isoenzyme studies of phosphatases: Electrophoretic analysis of enzymes was done at 4°C by one dimensional native polyacrylamide slab gel electrophoresis, which was carried out in the modified discontinuous system of Laemmli¹⁹, using 8% separating gels and 5% stacking gels²⁰. After electrophoresis, the gels for both acid and alkaline phosphatases were incubated at their respective pH and temperature optima for 30 min in 20 mM-*p*-nitrophenyl phosphate with 20 mM-MgCl₂. Intense yellow bands of *p*-nitrophenol (released due to activities of acid and alkaline phosphatase isoenzymes) appeared in the gels, which were scanned with the help of HP Scanjet C7716A, immediately after stopping the reaction with 1 M-NaOH. The R_F values for isoenzymes of phosphatases were determined, using the following formula:

$$R_F = \text{Distance traveled by the isoenzyme band} / \text{Distance traveled by the tracking dye}$$

This is to emphasize that, in the present study, the same substrate (*p*-nitrophenyl phosphate) has been used both for assaying the activities of acid and alkaline phosphatases as well as for localizing their isoenzymes in the polyacrylamide gels, using slab gel electrophoresis, as enzymes are substrate specific.

Results and Discussion

All phosphatase isoenzymes (three alkaline and one acidic) were observed in the polyacrylamide gels, while using slab gel electrophoresis similar to the results of Sinha and Singh¹⁵. In the wild type strain (1227), the three alkaline phosphatase isoenzymes, having their R_F values of 0.41, 0.47 and 0.52 (Table 1), were designated as AlpI, AlpII and AlpIII, respectively (Figure 1A); and a single acid phosphatase isoenzyme, which had its R_F value same (0.47) as that of AlpII, was designated as Acp (Figure 1B). All the alkaline phosphatases were optimally active at pH 9.5 and at 70°C; whereas, the acid phosphatase exhibited its maximal activity at pH 6.0 and at 65°C in the presence of Mg²⁺, which stimulates these enzymes. As reported earlier also¹⁵, the high temperature optima of all the phosphatase isoenzymes indicated that they are thermophilic.

With respect to the pH specificity, the present findings are in contrast to those reported for alkaline phosphatases from *Mycobacterium bovis* BCG⁷, which exhibited two different pH optima, one at pH 10.0 and other at pH 12.0. However, the pH optimum of acid phosphatase of this

obligate thermophile was exactly the same as that (pH 6.0) of *M. bovis*. The observation that isoenzymes AlpII (with optimal pH 9.5) and Acp (with optimal pH 6.0), which occupy the same position with a single R_F value of 0.47 in the polyacrylamide gel, represent their isoforms, similar to those observed for the hyperalkaline phosphatase of *Thermus thermophilus*²¹, which has two pH optima, one at pH 10.0 and other at pH 12.0. Parallel observations have also been made for three acid phosphatase isoenzymes from *Metarhizium anisopliae* which had same molecular weight and the ability to hydrolyze similar sugar phosphate substrate²².

The results of the present investigation indicate that out of the three constitutive alkaline phosphatases present in the wild type strain of *T. vulgaris*, only two alkaline phosphatase isoenzymes (AlpII and AlpIII) were present in the auxotrophic mutant strains 1286 (*thi*⁻), 1279 (*thi*⁻, *ura*⁻) and 1278 (*nic*⁻, *ura*⁻), showing the absence of AlpI; whereas only one alkaline phosphatase isoenzyme (AlpIII) was present in the strain 1261 (*nic*⁻, *thi*⁻) with the absence of the other two (i.e., AlpI and AlpII) (Figure 1A). Similar to AlpIII, a single acid phosphatase (Acp) was uniformly present in all

the strains (i.e., wild type as well as auxotrophic mutants) (Figure 1B), irrespective of their nutrient requirements.

These findings suggest that the acid and alkaline phosphatases behave differently, so far as their expression in the wild type as well as mutant strains is concerned. And, such differential expression of phosphatases in *T. vulgaris* is thought to be associated with the nutrient supplements added in their culture media which, in turn, are directly or indirectly associated with the phosphorus metabolism^{23,24}. Almost identical observations have been made with *E. coli* acid phosphatase mutants, which were expressed in *Pichia pastoris* to assess the effect of strategic mutations or deletion on the biochemical properties of this enzyme²⁵.

The observations with regard to the specific activities of acid and alkaline phosphatases in *T. vulgaris* strains clearly indicate that their activities in its wild type strain (1227) were found to be maximum, and their least activities were observed in a *nic*⁻, *thi*⁻ mutant strain (1261), in which two alkaline phosphatases isoenzymes (AlpI and AlpII) were missing (Table 2). On this basis, the strain 1261 was designated as *AlpI*⁻/*AlpII*⁻ double mutant with respect to phosphatase mutations. The specific activities of phosphatases in the other three auxotrophic mutant strains were found to be less than the wild type strain (1227), but higher than the mutant strain 1261, in the following order of the decrease in their activities: strain 1279 (*nic*⁻, *ura*⁻) > strain 1286 (*thi*⁻) > strain 1278 (*thi*⁻, *ura*⁻). These three auxotrophs (1279, 1286 and 1278) were lacking in one alkaline phosphatase isoenzyme (AlpI) and were, therefore, designated as *AlpI*⁻ mutants. The divalent cation, Mg²⁺ stimulated the activities of both acid and alkaline phosphatases in all the strains of *T. vulgaris* (Table 2). However, no *Acp* mutation was observed in any of the strains under investigation, as the single constitutive acid phosphatase isoenzyme (Acp) was

Table 1 R_F values of acid (Acp) and alkaline (AlpI, AlpII and AlpIII) phosphatase isoenzymes of wild type strain (1227) and auxotrophic mutants (strains 1286, 1279, 1278 and 1261) of *T. vulgaris*.

Phosphatase Isoenzymes	R_F values of phosphatase isoenzymes in <i>T. vulgaris</i> strains				
	1227	1286	1279	1278	1261
Alp I	0.41	Absent	Absent	Absent	Absent
Alp II	0.47	0.47	0.47	0.47	Absent
Alp III	0.52	0.52	0.52	0.52	0.52
Acp	0.47	0.47	0.47	0.47	0.47

Fig. 1 Isoenzyme patterns of phosphatases from the wild type strain (1227) and auxotrophic mutants (strains 1286, 1279, 1278 and 1261) of *T. vulgaris*. Preparation of enzyme extract and electrophoresis were done as described in the Materials and Methods.

Table 2 Specific activities of acid and alkaline phosphatases in the wild type strain (1227) and auxotrophic mutants (strains 1286, 1279, 1278 and 1261) of *T. vulgaris* in the absence as well as in the presence of Mg^{2+} . Each result is based on the mean of triplicate determinations.

	Specific activity (nmol of <i>p</i> -nitrophenol released/min per mg of protein)			
	Acid phosphatase*		Alkaline phosphatase*	
	Without Mg^{2+}	With Mg^{2+}	Without Mg^{2+}	With Mg^{2+}
1227	65.00 ± 0.89	261.11 ± 0.55	54.45 ± 0.82	135.50 ± 0.56
1286	59.45 ± 0.66	121.12 ± 0.11	44.50 ± 0.12	95.56 ± 0.13
1279	60.84 ± 0.54	216.67 ± 0.58	50.31 ± 0.95	108.43 ± 0.23
1278	52.23 ± 0.21	111.12 ± 0.78	33.35 ± 0.72	90.50 ± 0.32
1261	33.34 ± 0.54	94.67 ± 0.33	28.32 ± 0.33	76.12 ± 0.30

* Mean ± Standard Deviation.

uniformly present in the wild type as well as all the auxotrophic mutants of this thermophile. Thus, with respect to the expression of alkaline phosphatase isoenzymes in response to auxotrophy for specific nutrient requirements, our findings are in agreement with those obtained for thermophilic alkaline phosphatases from *Scytalidium thermophilum*⁹ and *Aspergillus caespitosus*¹⁰. But, with respect to the expression of acid phosphatase isoenzyme in *T. vulgaris*, our observations are in contrast to those obtained for different strains of *Salmonella*, in which the expression of nonspecific acid phosphatase gene (*phoP*) showed its differential expression²⁶.

From these findings, the following inferences could be drawn for *T. vulgaris* phosphatases:

- (i) The mutant strains, which were auxotrophic either to thiamine (strains 1286 and 1279) or to nicotinamide (strain 1278) were *AlpI*[−] mutants.
- (ii) The strain (1261), which was auxotrophic to both thiamine and nicotinamide, was *AlpI/AlpII* double mutant.
- (iii) The auxotrophy for uracil did not show any correlation with the expression of any of the alkaline phosphatase isoenzymes. This was evident from the fact that the two alkaline phosphatases, *AlpII* and *AlpIII* were observed in the strains 1286, 1279 and 1278, although the first one (strain 1286) did not require uracil for its growth, whereas the other two (strains 1279 and 1278) required this compound for their growth.
- (iv) The presence of the acid phosphatase isoenzyme (*Acp*) and one (*AlpIII*) of the three alkaline phosphatases in all the strains suggested that these isoenzymes followed their uniform and stable patterns, which were quite different from the other two alkaline phosphatases (*AlpI* and *AlpII*) that showed their differential expressions in the wild type and mutant strains of *T. vulgaris*.

However, the presence of uniform and stable patterns of *Acp* and *AlpIII* in all the strains, irrespective of their nutrient requirements, suggested that these constitutive phosphatase isoenzymes are species-specific. And, these phosphatase isoenzymes appear to have their taxonomic, evolutionary as well as adaptive significance in *T. vulgaris* so as to enable it to regulate its phosphorus metabolism and other associated enzymatic pathways, involved in the synthesis of vitamins or coenzymes (as evident from the present study) in fulfilling the nutrient requirement for its growth and survival at high temperature.

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