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Thermostable conidial and mycelial alkaline phosphatases from the thermophilic fungus *Scytalidium thermophilum*

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An extracellular (conidial) and an intracellular (mycelial) alkaline phosphatase from the thermophilic fungus *Scytalidium thermophilum* were purified by DEAE-cellulose and Concanavalin A-Sepharose chromatography. These enzymes showed allosteric behavior either in the presence or absence of $MgCl_2$, $BaCl_2$, $CuCl_2$, and $ZnCl_2$. All of these ions increased the maximal velocity of both enzymes. The molecular masses of the conidial and mycelial enzymes, estimated by gel filtration, were 162 and 132 kDa, respectively. Both proteins migrated on SDS-PAGE as a single polypeptide of 63 and 58.5 kDa, respectively, suggesting that these enzymes were dimers of identical subunits. The best substrate for the conidial and mycelial phosphatases was *p*-nitrophenylphosphate, but β-glycerophosphate and other phosphorylated compounds also served as substrates. The optimum pH for the conidial and mycelial alkaline phosphatases was 10.0 and 9.5 in the presence of AMPOL buffer, and their carbohydrate contents were about 54% and 63%, respectively. The optimum temperature was 70–75ºC for both activities. The enzymes were fully stable up to 1 h at 60ºC. These and other properties suggested that the alkaline phosphatases of *S. thermophilum* might be suitable for biotechnological applications. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 265–270.

Keywords: *Scytalidium thermophilum*; alkaline phosphatase; acid phosphatase; thermophilic fungi

Introduction

Phosphatases (orthophosphoric monoesterphosphohydrolases) are widely distributed in nature. These enzymes are classified according to optimum pH as alkaline phosphatases (EC 3.1.3.1), which have optimal activity at pH higher than 8.0, and acid phosphatases (EC 3.1.3.2), which act at pH lower than 6.0. These enzymes do not show high substrate specificity and are able to hydrolyze a wide variety of esters and anhydride phosphoric acids, releasing phosphate, and are also able to perform transphosphorylation reactions from phosphoesters of phenol, *p*-nitrophenylphosphate, etc. to various receptors, for example, glucose and pyridoxine [29].

There are not many studies on acid and alkaline phosphatase activities produced by thermophilic fungi, although these microorganisms have been considered as good sources of thermostable enzymes with high catalytic activity, greater resistance to denaturing agents, and lower incidence of contamination [15,20,23]. A conidial alkaline phosphatase from the thermophilic fungus *Humicola grisea* var. *thermoidea* was recently purified and characterized in our laboratory [3]. The remarkable thermostability of this enzyme may be of interest for practical applications. Commercial alkaline phosphatase, obtained from calf intestine, is being widely used due to its high specific activity. However, the usefulness of this enzyme is limited by its inherently low thermal stability and limited shelf life. Stable alkaline phosphatase from microorganisms may be used in cloning experiments [5]. These enzymes may also be used in enzyme-linked immunosorbent assays (ELISA) and in other applications [21].

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A significant level of extracellular alkaline phosphatase was detected in conidiospores of *Scytalidium thermophilum*. This activity was easily solubilized from intact conidia, as has been reported from *Myxococcus coralloides* [8], *Neurospora crassa* [22], *Bacillus subtilis* [28] and *H. grisea* var. *thermoidea* [3]. This activity, as well as the intracellular activity obtained from mycelial extracts from *S. thermophilum*, was studied. Here, we report the biochemical and kinetic characterization of the thermostable conidial and mycelial alkaline phosphatases produced by the thermophilic fungus *S. thermophilum*.

Materials and methods

Microorganism

S. thermophilum (strain 15.1, CBS 619.91) was isolated from mushroom compost soil [24]. The fungus was maintained at 45°C on slants of solid 4.0% oatmeal baby food (Quaker) medium [7].

Preparation of crude conidial enzyme

The mold was grown at 45°C for 10 days, on petri dishes containing 20 ml of solid culture medium, as described above. Ten milliliters of chilled water was added to each culture and the mycelial surface was gently scraped with a spatula to suspend the conidia. After 12 h of stirring at 4°C, the spore suspension was filtered through Whatman paper and the filtrate was used as the source of crude conidial enzyme.

Mycelium growth conditions and preparation of crude extracts

Conidial suspensions of 10-day-old cultures were inoculated (final concentration of 10^5 spores/ml) into 125-ml Erlenmeyer flasks containing 25 ml of liquid medium [1], containing 2.0%

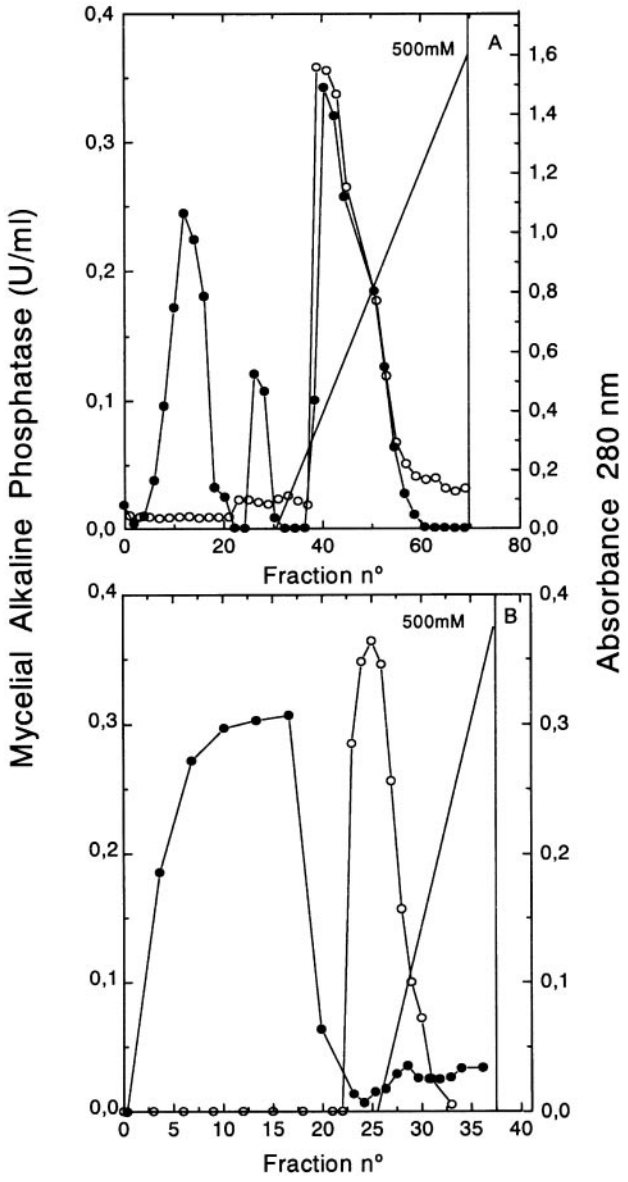

Figure 1 Distribution of protein (●) and alkaline phosphatase activities (○) of crude cell extracts after chromatography on DEAE-cellulose (A) and Concanavalin A-Sepharose (B). Mycelial alkaline phosphatase activity was eluted from DEAE-cellulose with a linear gradient of 0–500 mM NaCl in 10 mM Tris–HCl buffer, pH 7.5 at 100 ml/h and fractions of 5.0 ml were collected. The pool of alkaline phosphatase activities was applied in Concanavalin A-Sepharose equilibrated with 20 mM Tris–HCl buffer, pH 7.5, plus 500 mM NaCl, 0.5 mM $MnCl_2$ and 0.5 mM $CaCl_2$. Alkaline phosphatase was eluted as a single peak throughout of a linear gradient of methyl α-D-mannopyranoside (0–500 mM) in the same buffer in the flow rate at 36 ml/h. A similar profile for conidial alkaline phosphatase activity was obtained with the same procedure.

glucose, 0.2% yeast extract (Difco, Detroit, MI), 0.1% KH_2PO_4 and 0.05% $MgSO_4 \cdot 7H_2O$. The cultures (initial pH 6.0) were incubated for 48 h at 40°C, with agitation (100 rpm). The mycelium was harvested by vacuum filtration and blotted with filter paper. The filtrate was used for determination of the extracellular phosphatase activity. Mycelial pads were ground in a porcelain mortar with twice their weight of acid-washed sea sand at 4°C, extracted in 10 mM Tris–HCl buffer, pH 7.5, 4°C and centrifuged at 23,700×*g* for 15 min. The supernatant was the source of crude mycelial activity.

Enzyme determination

The activity of the alkaline phosphatase was determined discontinuously using as substrate *p*-nitrophenylphosphate disodium salt (*p*-NPP) (final concentration of 2.7 mM), in 100 mM AMPOL (2-amino-2-methyl-1-propanol, Merck) buffer, pH 9.0. To determine acid phosphatase activity the buffer was 100 mM sodium acetate, pH 4.5. The reaction mixture was incubated at 70°C and the reaction was stopped at appropriate time intervals with a saturated solution of sodium tetraborate. The liberated *p*-nitrophenol was determined at 410 nm. Determinations were carried out in duplicate and the initial velocities were constant for at least 20 min, provided less than 5% of the substrate was hydrolyzed. β-Glycerophosphate and other phosphorylated substrates were used at 10 mM in 100 mM AMPOL, pH 9.0. At predetermined intervals, the reaction was stopped by addition of trichloroacetic acid to 20% final concentration. After centrifuging the samples, inorganic phosphate was assayed in the supernatants as described elsewhere [10]. An enzyme unit (U) was defined as the amount of enzyme that produces 1 μmol of *p*-nitrophenol or inorganic phosphate per minute. The specific activity was expressed as units per milligram of protein.

Purification of the alkaline phosphatases

All steps were carried out at 4°C. The crude conidial and mycelial alkaline phosphatases were purified using the same protocol: the crude enzyme extracts were applied to a DEAE-cellulose column (2.0×10.0 cm) equilibrated in 10 mM Tris–HCl buffer, pH 7.5. Elution was at a flow rate of 100 ml/h with a linear gradient of 0–0.5 M NaCl in the same buffer and 5.0-ml fractions were collected. The conidial and mycelial alkaline phosphatases were eluted with 178.5 and 140.8 mM NaCl, respectively. Elution of proteins was monitored by measurement of absorbance at 280 nm. Alkaline phosphatase activity fractions were pooled, dialyzed against 0.02 M Tris–HCl buffer, pH 7.5, plus 0.5 M NaCl, 0.5 mM $MnCl_2$, and 0.5 mM $CaCl_2$, and applied to a Concanavalin A-Sepharose affinity chromatographic column (1.2×9.0 cm), equilibrated and eluted with the same buffer. Both alkaline phosphatases were eluted as single peaks with a linear gradient of methyl α-D-mannopyranoside (0–0.5 M) in Tris–HCl buffer, at a flow rate of 36 ml/h. Conidial and mycelial alkaline phosphatases were eluted with 193.8 and 127.5 mM of this compound, respectively.

Table 1 Purification of conidial and mycelial alkaline phosphatases from *S. thermophilum*

Step	Protein (total mg)	Activity (total U)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
Conidial extract	34.05	43.50	1.27	100.00	1.00
DEAE-cellulose	9.16	20.44	2.23	46.98	1.75
Concanavalin A-Sepharose	0.56	13.22	23.60	30.39	18.58
Mycelial extract	107.10	31.27	0.29	100.00	1.00
DEAE-cellulose	6.15	11.92	1.94	38.13	6.64
Concanavalin A-Sepharose	0.28	8.65	30.89	27.68	106.52

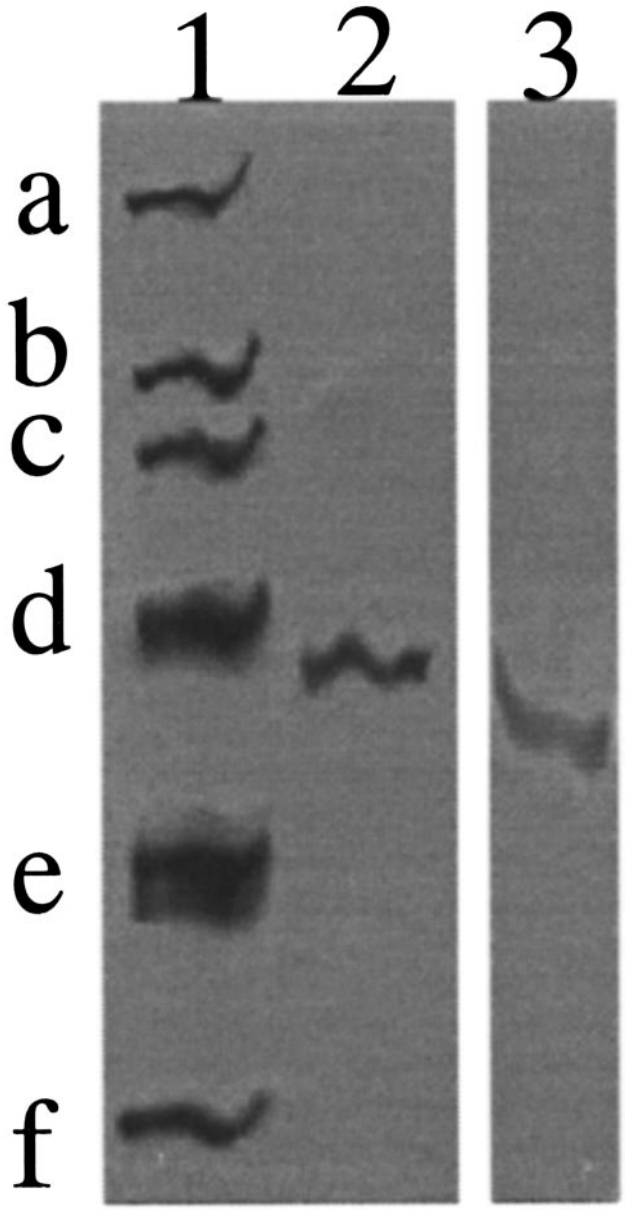

Figure 2 SDS-PAGE of the purified alkaline phosphatase. Lane 1: molecular mass markers: (a) myosin 205 kDa, (b) β-galactosidase 116 kDa, (c) phosphorylase b 97.4 kDa, (d) bovine albumin 66 kDa, (e) egg albumin 45 kDa and (f) carbonic anhydrase 29 kDa. Lane 2: conidial alkaline phosphatase. Lane 3: mycelial alkaline phosphatase.

Molecular mass estimation by gel filtration

The molecular mass of the purified enzymes was estimated using a Sepharose CL-6B column (2.0×90 cm) equilibrated and eluted with 50 mM Tris–HCl buffer, pH 7.5, containing 100 mM KCl. Molecular mass markers were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa).

Polyacrylamide gel electrophoresis

Electrophoresis of proteins was carried out in 6% acrylamide gels (PAGE), according to the method of Davis [4]. SDS-PAGE was performed by the method of Laemmli [14]. Proteins were stained with silver. Phosphatase activity was assayed on the gel in 100 mM

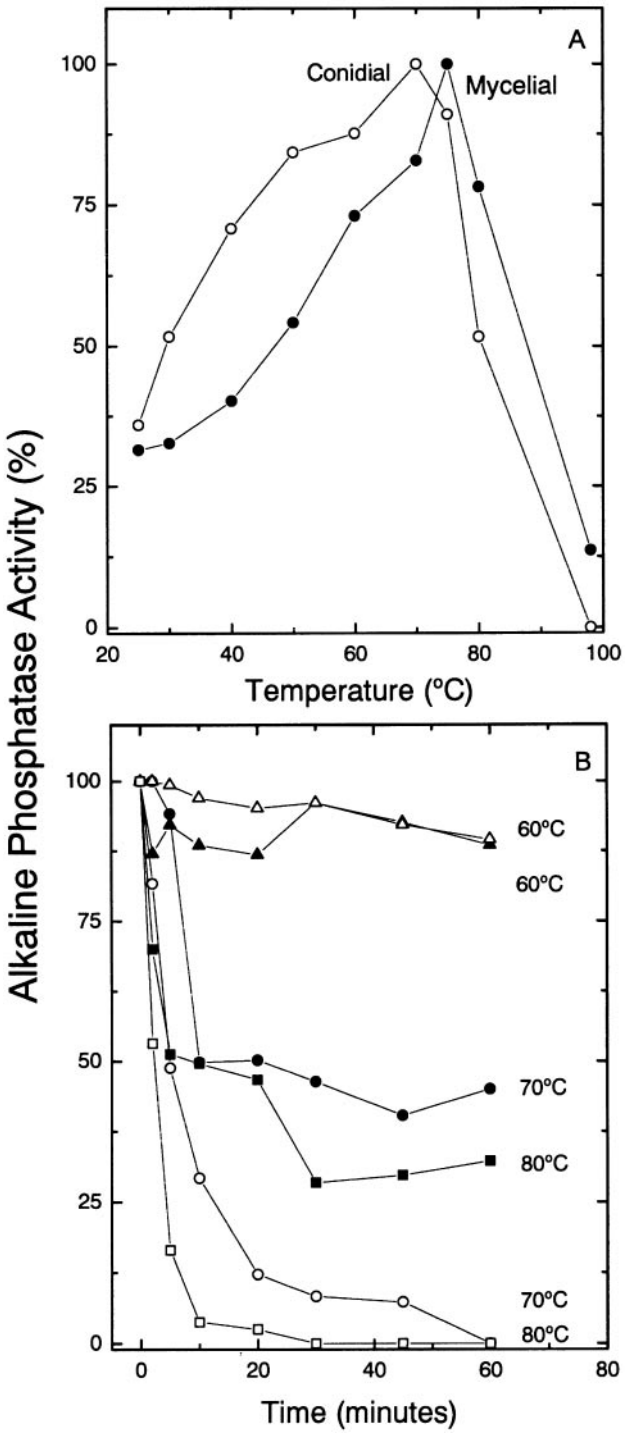

Figure 3 (A) Effect of temperature and (B) thermal inactivation of purified alkaline phosphatase activities. For determination of the effect of temperature, conidial and mycelial samples were incubated with the substrate, at a different temperature (25–97°C), for 10 min. For determination of thermal inactivation the enzymes were incubated at 60°C (△, ▲), 70°C (○, ●), 80°C (□, ■) in 100 mM Tris HCl buffer, pH 9.0 in the absence of the *p*-nitrophenylphosphate. After the treatment, phosphatase activities were determined as described in Materials and methods. Conidial alkaline phosphatase is represented by open symbols and mycelial alkaline phosphatase by closed symbols.

Table 2 Effect of various salts on the conidial and mycelial alkaline phosphatase activities

Salt	Concentration (mM)	Conidial phosphatase[a]	Mycelial phosphatase[a]
None	–	100	100
$BaCl_2$	1.0	132.36	111.11
	2.0	108.51	110.38
$CaCl_2$	1.0	73.43	111.40
	2.0	55.31	110.77
$CuCl_2$	1.0	44.68	72.72
	2.0	34.04	66.66
$HgCl_2$	1.0	2.94	46.46
$MgCl_2$	1.0	180.88	137.75
	2.0	170.21	135.71
$MgSO_4$	1.0	195.58	119.19
$MnCl_2$	1.0	35.29	87.54
NaCl	1.0	97.05	97.97
NH_4Cl	1.0	117.64	85.18
$ZnCl_2$	1.0	42.64	52.38
	2.0	17.02	58.36
EDTA	1.0	7.35	3.03

[a]Activity is expressed as a percentage of that of the control without additions.

Tris–HCl buffer, pH 8.0, containing 2 mM $MgCl_2$, 0.12% Fast Blue RR salt, and 0.12% β-naphthyl phosphate, at 70°C.

Determination of protein and carbohydrate

The proteins were determined according to Lowry *et al* [18] using bovine serum albumin as standard. Quantification of carbohydrate was carried out according to Dubois *et al* [6].

Estimation of kinetic parameters

Kinetic parameters obtained from substrate hydrolysis measurements were fitted on an IBM/PC microcomputer using SIGRAF [17]. V_{max}, K_d, and n, which appear in this paper as computed values, stand for maximal velocity, apparent dissociation constant, and Hill coefficient, respectively.

Results and discussion

Culture conditions and purification of alkaline phosphatases

Maximum mycelial alkaline phosphatase activity levels were obtained by culturing the fungus between pH 5.5 and 6.5 with 2% glucose, for 48 h at 40°C. This activity was totally recovered from mycelial extracts. Under these conditions alkaline phosphatase activity in crude extracts (2.02 U/mg protein) was fivefold higher than that of the acid phosphatase (not shown).

Conidial and mycelial alkaline phosphatases were purified by two chromatographic steps (Figure 1 and Table 1). After the second purification step, both enzyme preparations appeared homogeneous, each producing a single protein band stained with silver under nondenaturing PAGE. Alkaline phosphatases were also assayed for activity on the gel, after PAGE, and the activities were coincident with the protein band (not shown).

Determination of M_r

The molecular masses of the purified native conidial and mycelial enzymes estimated by Sepharose CL-6B gel sieving were 162 and 132 kDa, respectively. Both proteins migrated under SDS-PAGE as single polypeptides of 63 and 58.5 kDa, respectively, suggesting that these native enzymes were composed of dimers of identical subunits (Figure 2). Most of the alkaline phosphatases reported for a number of bacteria, fungi, invertebrates, fishes, and mammals are also dimers, except for the alkaline phosphatase produced by *Thermus* sp., which is a trimer with subunits of 160 kDa [9], or alkaline phosphatases of *N. crassa*, which are monomers [19] or tetramers [22].

Determination of carbohydrate and thermal stability

The carbohydrate contents of the conidial and mycelial alkaline phosphatases were 54.3% and 63.7%, respectively. Comparable neutral sugar content (67%) was reported for a nonspecific acid phosphatase from *Candida lipolytica* [26]. The activities were maximal at 70°C and 75°C, respectively (Figure 3A) at pH 9.0. The activities at pH 6.0 and pH 5.0 were 12.4% and 3.8%, respectively, of the activity at pH 9.0 (not shown). Both alkaline phosphatases were fully stable after 1 h at 60°C (Figure 3B). However, at 70°C and 80°C these enzymes were inactivated (t_{50} of 10 and 9.5 min for the mycelial activity, and 5.5 and 2.5 min for the conidial activity, respectively). The half-life of the alkaline phosphatases of *S. thermophilum* was somewhat lower than that of *Thermus* sp. (5 min at 85°C) [9], but higher than that of the alkaline phosphatase produced by *Echinococcus granulosus* (6.7% after 60 min at 65°C) and that of the host liver, which was totally inactivated under these conditions [16]. These properties suggested that the alkaline phosphatase activities from *S. thermophilum* might be suitable for practical applications.

Influence of metal ions on activities and kinetic constants

The presence of 1 mM EDTA produced strong inhibition of the enzyme activities; thus, the effect of several salts was tested (Table 2). Addition of Mg^{2+} restored the original activities (not

Table 3 Kinetic parameters for the hydrolysis of *p*-NPP by purified conidial and mycelial alkaline phosphatases

Compound (0.4 mM)	V_{max} (U/mg protein)		K_d (mM)		V_{max}/K_d		n	
	Conidial	Mycelial	Conidial	Mycelial	Conidial	Mycelial	Conidial	Mycelial
No additions	0.79	3.77	0.10	0.11	7.90	34.27	2.38	3.31
$MgCl_2$	0.97	4.81	0.08	0.12	12.12	40.08	2.51	3.09
$BaCl_2$	0.80	4.75	0.08	0.11	10.00	33.93	3.03	1.63
$CuCl_2$	0.20	4.73	0.06	0.11	3.33	43.00	1.96	2.42

Table 4 Substrate specificities of conidial and mycelial alkaline phosphatases

Substrates (10 mM)	Relative rate of hydrolysis (%)	
	Conidial	Mycelial
p-NPP	100.00	100.00
G6P	67.68	1.43
F6P	27.25	2.41
βGP	61.40	64.62
ATP	39.96	3.43
GTP	72.43	4.17
UTP	39.66	6.83
ITP	55.28	1.37
ADPG	74.73	1.97
UDPG	29.09	1.97
NADP	54.51	0.91

G6P=glucose-6-phosphate; F6P=fructose-6-phosphate; βGP=β-glycerophosphate.

shown), indicating that the *S. thermophilum* alkaline phosphatases were metalloenzymes. Both enzymes were inhibited by Zn^{2+}, Cu^{2+}, and Hg^{2+}, and were stimulated by Mg^{2+}. The conidial enzyme was more sensitive to inhibition by Hg^{2+}, and was somewhat inhibited also by Mn^{2+} ions, which had little effect on the mycelial phosphatase. Other microbial alkaline phosphatases, such as the enzymes produced by *M. coralloides* [8], *Lactococcus lactis* [30], *N. crassa* [22] and *H. grisea* [3] are also stimulated by divalent metal ions, showing site–site interactions.

The purified enzymes exhibited allosteric behavior in the absence or/and presence of $MgCl_2$, $BaCl_2$, and $CuCl_2$ (Table 3), when *p*-NPP was used as substrate. Mammalian alkaline phosphatases are also allosteric enzymes. These enzymes are dimeric metalloenzymes encoded by four genes, i.e., for the placental, germ cell, intestinal, and tissue-nonspecific alkaline phosphatase isoenzymes [11]. Two Zn^{2+} and one Mg^{2+} ions are essential in the active site for enzymatic activity and contribute substantially to the conformation of alkaline phosphatase monomer, and indirectly regulate subunit–subunit interactions.

Substrate specificity

The best substrate for the conidial and mycelial alkaline phosphatases was *p*-NPP, but other phosphorylated compounds also served as substrates (Table 4). Among natural substrates, only β-glycerophosphate (βGP) was utilized significantly. We found evidence for the hydrolysis of *p*-NPP and βGP at a common catalytic site, for the conidial and mycelial alkaline phosphatases. This possibility was examined by the mixed substrate kinetic method [25] using as substrates *p*-NPP, βGP or a mixture of both substances. The rate of hydrolysis of the substrate mixture was not additive in relation to the rate obtained when each substrate was tested separately (Figure 4A and B), suggesting the existence of a unique catalytic site for both substrates. These results are similar to those reported previously for *H. grisea* [3].

A significant difference between the two enzymes was observed when the conidial alkaline phosphatase was tested with the same substrates used with the mycelial enzyme (Table 4). The conidial alkaline phosphatase hydrolyzed nonspecifically (54–75%) all of the phosphorylated compounds tested: ADPG, GTP, G6P, βGP, ITP, and NADP. Alkaline phosphatases reported for other microorganisms exhibit a broad specificity for phosphate esters, e.g., an

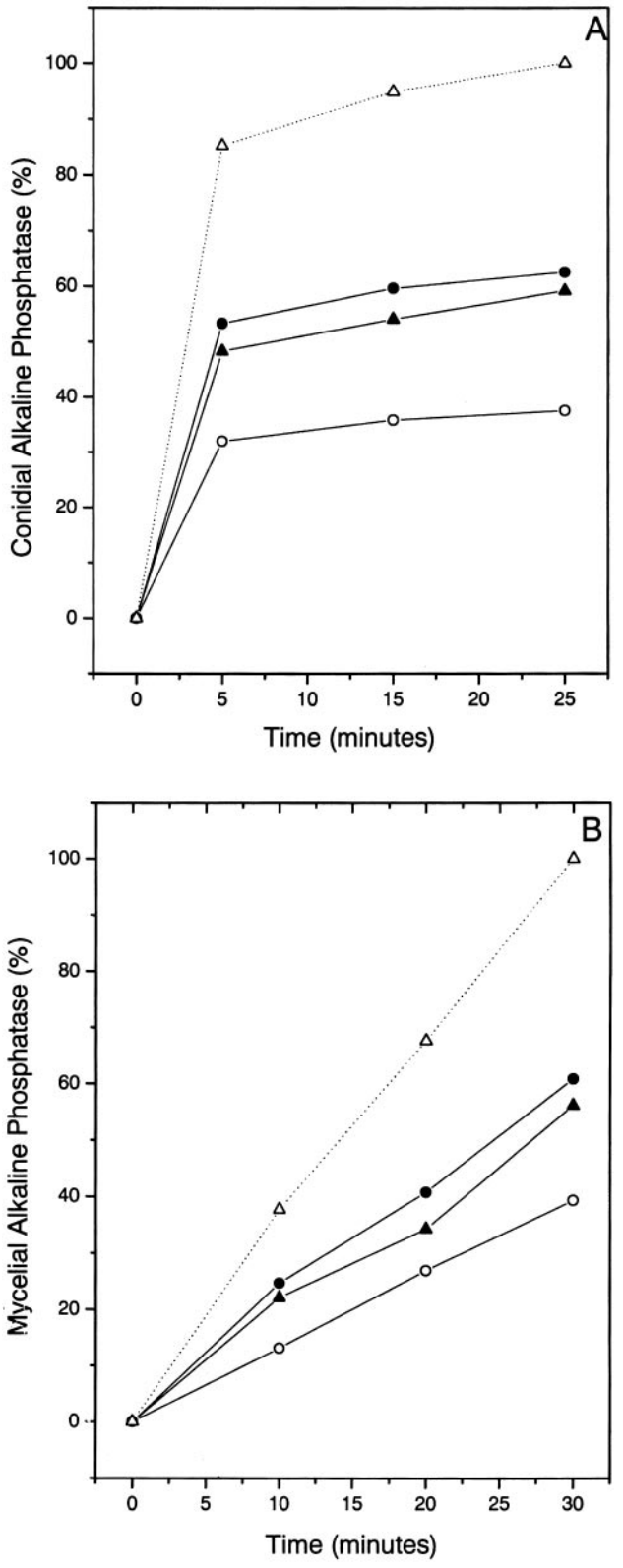

Figure 4 Hydrolysis of *p*-nitrophenylphosphate (●), β-glycerophosphate (○), and a mixture of both substrates at the same concentrations (▲), as a function of time. The broken line represents the theoretical rate assuming separate, noninteracting catalytic sites. (A) represents conidial phosphatase activity and (B) mycelial phosphatase activity.

unusually high alkaline phosphatase activity from *Thermus* sp. This enzyme hydrolyzes (expressed as percentages relative to *p*-NPP) ribose 5-phosphate (177%), α-naphthylphosphate (143%), βGP (127%), dAMP (116%), but the substrate hydrolysis rate was pH dependent [9]. A phosphate-irrepressible membrane-bound alkaline phosphatase from *Zymomonas mobilis* also exhibited broad substrate specificity for ATP (96%) and AMP (83%) [2].

In conclusion, the differences in subcellular localization, substrate specificity, molecular mass, carbohydrate content, thermal stability, reactivity to metal ions, and kinetic constants indicated that the conidial and mycelial alkaline phosphatases produced by *S. thermophilum* are distinct proteins and some questions regarding the specific function of each of these enzymes could be implicit. An unspecific conidial phosphatase might be important for assimilation of phosphate in the medium during conidial germination. The mycelial phosphatase might be associated with metabolic processes during vegetative growth. Studies on the pattern of phosphatase activities during the development of *Myxococcus xanthus* [27] and *M. corraloides* D [8] suggest that these activities can be used as reliable and easy markers during development. Various sporulating microorganisms, such as *B. subtilis* [12] and *Dictyostelium discoideum* [13] express higher levels of alkaline phosphatase in spores than in vegetative cells. Similar results were observed for *S. thermophilum* conidia (1.27 U/mg protein) compared to the mycelial enzymatic form (0.29 U/mg protein). The high levels of conidial alkaline phosphatase favor the view that this phosphatase has a nutritional role in spore cells, providing orthophosphate from phosphorylated substances present in the medium. Obviously, for a microorganism living in the soil such as *S. thermophilum*, the ability to produce these enzymes must provide an important ecological advantage.

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