Purification and Characterization of Two Extracellular Alkaline Phosphatases from a Psychrophilic *Arthrobacter* Isolate

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Two extracellular, heat-labile alkaline phosphatases were purified from a psychrophilic *Arthrobacter* isolate, D10. The enzymes were active over different pH ranges, used distinct substrates, and had different kinetic properties. Each enzyme reacted specifically to its own antibody during immunoblot analysis. One had both monophosphatase and diesterase activities.

Phosphatases are classified as either phosphomonoesterases (EC 3.1.3) or phosphodiesterases (EC 3.1.4), with most enzymes limited to the monoesterase activity catalyzed through the formation of a phosphoseryl intermediate (13). Alkaline phosphatases vary in their sizes, metal requirements, and substrate specificities. For example, monomer sizes range from 15.5 kDa (6) to as large as 160 kDa (8), and although extracellular enzymes are generally monomers, an enzyme from a *Bacillus* sp. has two subunits (14) and one from *Thermus aquaticus* is a trimer (18). Zinc is often required; however, an enzyme from *Halobacter* requires manganese (6), and a few use calcium (7, 8).

Microorganisms need phosphatases to release phosphate from organic compounds when inorganic phosphate is limiting. One question that has not been examined is how microorganisms obtain phosphate in low-temperature environments. To gain insight into the types of enzymes produced at low temperatures, we screened our psychrophilic isolates (2, 12, 17) for phosphatase activities. One strain, D10, a member of the *Arthrobacter* genus (3), appeared to produce two different phosphatase activities, designated D10A and D10B. These enzymes differed in their ability to hydrolyze X-phos (5-bromo-4-chloro-3-indolylphosphate), in their pH ranges for activity, and in the requirement by D10B for the addition of calcium. In this study, we purified and characterized each enzyme and found that two distinct, heat-labile phosphatases were produced, and that D10B had both monophosphatase and diesterase activities.

Purification of enzymes. The two extracellular alkaline phosphatase activities were precipitated from spent medium with 70% ammonium sulfate, and the resulting pellet was resuspended in buffer containing 50 mM Tris-HCl (pH 8.6), 2 mM CaCl₂, and ammonium sulfate at 30% saturation. The D10A activity was eluted with 10% ammonium sulfate buffer from a phenyl-Sepharose column (Sigma Chemical Co., St. Louis, Mo.), and the fractions were desalted with a Pharmacia G-25 column. The D10B activity was eluted from the phenyl-Sepharose column with a 0% ammonium sulfate wash, loaded onto a Red-A agarose column (Reactive Red 120 agarose 3000-CL; Sigma), and removed with 500 mM sodium chloride buffer R (50 mM Tris-HCl [pH 8.6]–2 mM CaCl₂).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (11) of the purified samples revealed single polypeptides with molecular masses of 71 and 61 kDa for D10A and D10B, respectively. When each preparation was subjected to nondenaturing electrophoresis and activity was detected by staining for phosphate release from PNPP (*para*-nitrophenyl phosphate) in a zymogram (3, 15), each enzyme migrated differently and only one activity band was observed for each (data not shown). The molecular weights were determined to be 80,000 and 78,000 for D10A and D10B, respectively, by using a Sepharose 6B gel filtration column. Antibodies prepared by immunizing two New Zealand rabbits with purified protein were used in Western blot analyses (16). When equal amounts of both enzymes were used, each antibody was found to be specific to its corresponding protein (data not shown). These results are consistent with D10A and D10B being distinct monomeric proteins.

D10A and D10B metal requirements and thermostabilities. The purified preparations were treated with EDTA, and various metals were added to the reaction mixture (Fig. 1). One millimolar EDTA was required to eliminate D10A activity, and calcium restored only about 20% of the activity. In contrast to D10A, D10B required added calcium for activity even without being treated with EDTA (3), and only calcium restored phosphatase activity after treatment.

We examined the stabilities of the purified enzymes in incubations at different temperatures. The results (Fig. 2) showed that both D10A and D10B were stable at 50°C for at least 1 h, but that they lost more than 90% of their initial activity when incubated at 60°C for as little as 10 min.

Substrate utilization. The ability of the D10A and D10B phosphatases to dephosphorylate a variety of substrates was tested by measuring phosphate release in reaction mixtures containing 400 mM substrate and 200 mM sodium glycine buffer at pH 9.6 for D10A and 400 mM substrate and 200 mM sodium glycine buffer at pH 9.0 plus 2 mM CaCl₂ for D10B (1) (Table 1). D10B was inactive with the chromogen X-phos (3), GTP, and pyrophosphate, which D10A hydrolyzed, whereas D10B hydrolyzed bis-PNPP uniquely. Since enzymes with both monoester and diester activities are rare, we examined D10B for its ability to dephosphorylate additional substrates. A 3':5'-cyclic AMP phosphodiesterase was found for a *Vibrio fischeri* strain that can degrade cyclic AMP (4, 5). However, the D10B enzyme did not hydrolyze cyclic AMP (Table 1), nor was the strain able to grow when cyclic AMP was the carbon source. Because some phosphodiesterases have exonucleolytic activity, we tested RNA,

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FIG. 1. Effect of divalent cations on D10A and D10B alkaline phosphatase activities. Purified D10A (A) and D10B (B) alkaline phosphatases were incubated with 0.5 or 1 mM EDTA in 50 mM Tris-HCl (pH 8.6) for 2 h at 4°C. EDTA was removed with a desalting column before the divalent cations were added as chloride salts at a final concentration of 10 mM. Activity was assayed as described in the text, and the results are expressed as a percentage of the activity measured prior to EDTA treatment. The activity at 100% was 0.012 U for D10A and 0.02 U for D10B.

linearized plasmid DNA, and denatured salmon sperm DNA as substrates for the D10B activity. None was degraded by D10B, nor was closed circular plasmid DNA linearized by the enzyme (data not shown).

D10B pH optima. One difference between the D10A and D10B activities was their pH profile with PNPP as the substrate (3). Since the D10B enzyme had both monoesterase and diesterase activities, we compared its activity with either 1 mM PNPP or bis-PNPP as the substrate at different pH values (Fig. 3). The enzyme was active over a greater pH range (between 7.5 and 9.5) with the bis-PNPP than with the PNPP substrate.

Kinetic and biochemical properties. The K_m and V_{max} values for the D10A phosphatase were determined by varying the PNPP concentrations (25 to 800 μ M), and the results were analyzed by the method of Hanes (9). D10A had an apparent K_m of 89 μ M for PNPP, a V_{max} value of 6 U/mg, and a catalytic efficiency (K_{cat}) of 2×10^5 s⁻¹. Inorganic phosphate acted as a competitive inhibitor of D10A phosphatase with a K_i of 15 μ M.

Repeated experiments with D10B, however, failed to show typical Michaelis-Menten kinetics with increasing concentrations of PNPP. Concentrations as high as 100 mM failed to saturate the reaction, and the curve appeared to have a sigmoidal shape with an inflection in linearity (data not shown). To determine whether the results were specific to PNPP, AMP and bis-PNPP were individually substituted as substrates. No saturation was observed at concentrations up to 1 mM AMP or 5 mM bis-PNPP. Other changes, such as altering the buffer or metal ion concentrations did not yield typical saturation kinetics. No inhibition of the D10B activity by phosphate was detected up to 500 μ M (data not shown).

We observed that the D10B activity released *p*-nitrophenol linearly with PNPP as the substrate; however, the reaction with bis-PNPP appeared to be much slower and exhibited a lag period before the release of product (Fig. 4). One explanation for the different pH profiles and release kinetics with PNPP and bis-PNPP as substrates may be that D10B has two separate active sites, one with only monoesterase activity and a second with both mono- and diphosphoesterase activities. To

FIG. 2. Thermostability of D10A (A) and D10B (B) alkaline phosphatases. The enzymes were incubated in a 50 mM Tris-HCl (pH 8.6) buffer at three different temperatures. Samples were removed at different time points and assayed with PNPP as the substrate in 200 mM sodium glycine buffer (pH 9.6) for D10A and 200 mM sodium glycine buffer (pH 9.0) plus 2 mM CaCl₂ for D10B. Activities are expressed relative to the unincubated enzyme; 100% activity corresponded to 0.02 U for D10A and 0.025 U for D10B.

TABLE 1. Substrate specificity for D10A and D10B alkaline phosphatases

Substrate	Relative activity (%)	
	D10A ^a	D10B ^b
PNPP	100	100
bis-PNPP	4	33.4
AMP	99.8	128
ATP	94.4	25
GTP	89.4	<0.1
Cyclic AMP	<0.1	<0.1
Glycerol-P	98.8	108
Glucose-1-P	94	102
Glucose-6-P	97	83.3
Pyrophosphate	165	<0.1
NADP	<0.1	<0.1
X-phos ^c	+	—

^a 100% activity equals 0.02 U.^b 100% activity equals 0.025 U.^c + and — indicate the presence and absence of color in the reaction mixture, respectively, as determined on activity gels.

examine whether the PNPP and bis-PNPP competed for the same site, bis-PNPP was added to the PNPP reaction at concentrations of 0, 0.625, and 2.5 mM. A lag would have been expected if bis-PNPP interfered with PNPP hydrolysis; however, the curves were identical to that observed for PNPP alone, which is consistent with the possibility of D10B having two separate sites (data not shown).

Heat-labile phosphatases could be used to dephosphorylate DNA in recombinant DNA research (10). Because D10A was heat labile, was active over a wide range of pH values, and dephosphorylated both ATP and GTP, its ability to dephosphorylate pUC18 plasmids linearized with *Eco*RI, *Sma*I, and *Pst*I restriction endonucleases was tested. Linearized plasmids treated with D10A did not religate, but recombinant molecules were efficiently formed when phosphorylated DNA fragments were added and ligated with the plasmid (data not shown). The analysis of the D10A enzyme suggests that it could be a useful, heat-labile phosphatase for dephosphorylating nucleic acids and nucleotides.

FIG. 3. Effect of pH variation on D10B enzymatic activities. The following buffer systems were used at a final concentration of 200 mM: Tris-HCl (pH 7.0 to 8.5) and sodium glycine (pH 9.0 to 10). Enzymatic activities towards bis-PNPP (open symbols) and PNPP (filled symbols) are expressed as percentages of the maximal activities, which were 0.015 U for PNPP and 0.004 U for bis-PNPP.

FIG. 4. Time course study of D10B activities with PNPP and bis-PNPP. The amount of product released was determined at different time points in reactions with PNPP (A) and bis-PNPP (B) as the substrate. For PNPP, 0.015 U of the enzyme was used; for bis-PNPP, 0.02 U was used. The amount of product released is expressed in micromoles of *p*-nitrophenol formed.

We thank M. Tien and members of our laboratory for helpful discussions.

This work was partially supported by Department of Energy grant DE-FG02-93ER20117 from the Division of Energy Biosciences.

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