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 Halobacterium 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 phosphatases, 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 their 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 Halobacterium requires manganese (6), and a few use calcium (7, 8).

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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 $\mu$M), and the results were analyzed by the method of Hanes (9). D10A had an apparent $K_m$ of 89 $\mu$M for PNPP, a $V_{max}$ value of 6 U/mg, and a catalytic efficiency ($K_{cat}/V_{max}$) of $2 \times 10^5$ s$^{-1}$. Inorganic phosphate acted as a competitive inhibitor of D10A phosphatase with a $K_i$ of 15 $\mu$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 $\mu$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 monophosphoesterase activity and a second with both mono- and diphosphoesterase activities. To
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 EcoRI, SmaI, and PstI 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.

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